Organisation et intégrité des chromosomes parentaux à la fécondation chez la drosophile

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Résumé :

Organisation et intégrité des chromosomes parentaux à la fécondation chez la drosophile

La reproduction sexuée implique une différentiation extrême des gamètes qui s'accompagne de profonds remaniements des chromosomes parentaux. Au moment de la fécondation, ces chromosomes doivent être rendus compétents pour la formation du premier noyau zygotique. Au cours de ma thèse, j'ai étudié l'importance fonctionnelle de plusieurs voies moléculaires paternelles et maternelles participant à cette étape chez la drosophile.

Le complexe HIRA est impliqué dans l'assemblage de nucléosomes dans le pronoyau mâle à la fécondation. J'ai décrit le rôle de HIRA et de son partenaire Yemanucléine-α dans cette voie. J'ai caractérisé plus finement ce complexe en étudiant son rôle somatique dans l'assemblage des nucléosomes et son implication dans la stabilité de l'hétérochromatine, améliorant notre compréhension des besoins biologiques qui conditionnent sa conservation et son évolution.

Je me suis aussi intéressé à diverses situations affectant l'intégrité des chromosomes parentaux à la fécondation. (1) J'ai décrit les conséquences catastrophiques pour la méiose femelle de l'expression naturelle d'un transposon à travers l'étude d'un cas de dysgénésie hybride. (2) J'ai contribué à montrer que la protéine K81 est essentielle pour la protection des télomères dans les chromosomes paternels au cours de la spermatogénèse. (3) J'ai participé à caractériser les conséquences pour les chromosomes paternels de l'incompatibilité cytoplasmique induite par la bactérie Wolbachia.

Ensemble, ces travaux soulignent les particularités des chromosomes parentaux à la fécondation et aident à cerner l'importance des voies maternelles et paternelles dans leur intégration dans le premier noyau du zygote.

Summary:

Organization and integrity of parental chromosomes at fertilization in Drosophila

Sexual reproduction involves dramatic gamete differentiation and profound parental chromosomes remodelling. At fertilization, these chromosomes need to be rendered competent for the formation of the fist zygotic nucleus. I have studied the functional relevance of several paternal and maternal molecular pathways that participate during this process in Drosophila.

The HIRA complex is required for nucleosome assembly in the male pronucleus at fertilization. I have further described the rôle of HIRA and its obligatory partner Yemanuclein-α during this step. I have characterized the somatic roles of this complex during nucleosome assembly and its involvment in heterochromatin stability, which gives us a better understanding of the biological needs that drive its conservation and evolution.

I have also focused on several situations where parental chromosomes integrity at fertilization is compromised. (1) I have described a meiotic catastrophe associated with the natural expression of a transposon in the female germline during hybrid dysgenesis. (2) I have contributed to show that K81 is an essential protein for telomere protection in paternal chromosomes during spermiogenesis. (3) I have participated in the characterization of the chromosomal abnormalities associated with cytoplasmic incompatibility induced by Wolbachia.

Together, these results underscore the specificities of parental chromosomes at fertilization and shed light into the importance of maternal and paternal pathways for their integration in the first zygotic nucleus.

Abréviations

IR : Indépendant de la Réplication
CR : Couplé à la réplication
SF : Stérilité Femelle
IC : Incompatibilité cytoplasmique
MPT : Modification post-traductionnelle
piARN : ARN associé à PIWI
CenH3 : Histone H3 centromérique
ARNm : ARN messager
SNBP : Sperm nucleus basic protein
ADNr : ADN ribosomiques
ARNi : ARN interférence
piARN : ARN associé à PIWI
NHEJ : Non-homologous end joining

La nomenclature courante est utilisée pour les modifications des histones

Mots clés :

Fécondation ; Epigénétique ; Chromatine ; Drosophile ; HIRA ; H3.3 ; Yemanucléine ; Dysgénésie Hybride

Keywords :

Fertilization ; Epigenetics ; Chromatin ; Drosophila ; HIRA ; H3.3 ; Yemanuclein ; Hybrid dysgenesis

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Avant-propos

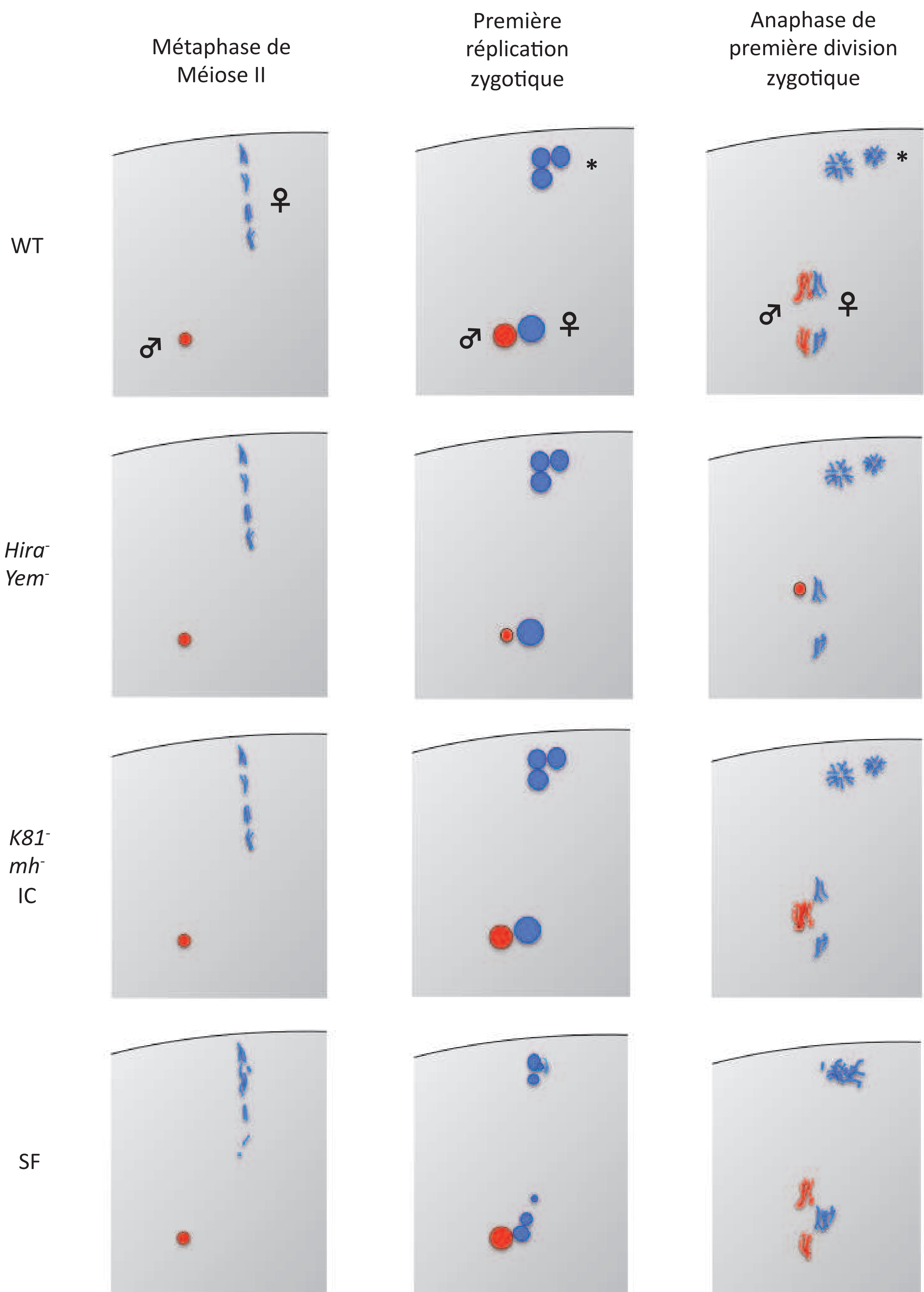
Between the conception and the creation
Between the potency and the existence
Between the essence and the descent
Falls the shadow

T.S. Eliot, *The hollow men*

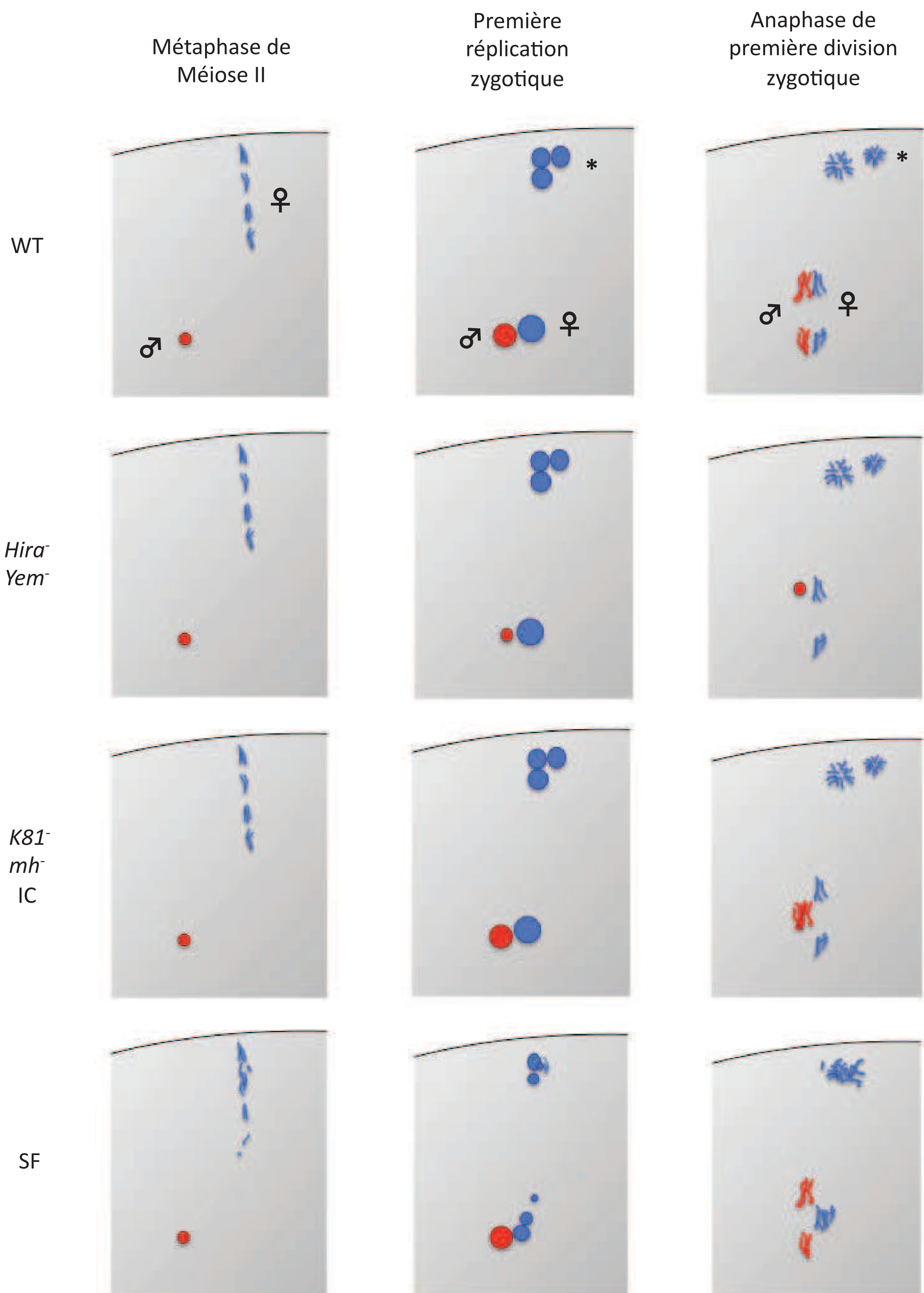
La reproduction sexuée a été retenue par la plupart des espèces animales et végétales comme le mécanisme de transmission génétique entre générations d'individus. Or, ce choix pose des contraintes extraordinairement complexes, car il doit conjuguer le brassage génétique avec la stabilité des génomes, et la spécialisation des gamètes des deux sexes avec la totipotence de la première cellule zygotique. En particulier, cette stratégie s'appuie sur un remodelage extrême de la chromatine dans les cellules germinales, qui impose la présence d'une batterie de mécanismes de mise en commun des chromosomes parentaux au moment de la fécondation. Les chromosomes d'origine paternelle et maternelle sont le résultat de deux histoires épigénétiques complètement différentes et ils ont une composition et des propriétés remarquablement spécifiques. Leur intégration dans le premier noyau du zygote est donc, de tout point de vue, un défi majeur pour l'œuf, et les processus mis en jeu à la fécondation sont d'autant plus indispensables que leur issue est critique. Or, c'est probablement à cause de cela que la reproduction sexuée représente aussi une barrière entre espèces, un terrain d'affrontement du conflit sexuel, et une opportunité stratégique pour des attaques parasitaires. La bataille qui se livre ainsi à la fécondation traduit la grande complexité des mécanismes qui assurent son succès.

Au cours de ma thèse, j'ai pris conscience de la portée de ce système comme modèle privilégié pour étudier l'importance biologique de mécanismes moléculaires fondamentaux affectant les chromosomes. Par l'étude de ces mécanismes chez la drosophile, je vais tenter d'illustrer en quoi la réussite de l'intégration des chromosomes parentaux à la fécondation est un évènement aussi précis que complexe, aussi essentiel qu'improbable.

La partie principale de ce manuscrit traitera du rôle du complexe HIRA dans l'assemblage de la chromatine à la fécondation, rôle essentiel pour assurer l'organisation des chromosomes paternels et permettre la formation du zygote. HIRA est un chaperon spécifique du variant



Métaphase de
Méiose II
Première
réplication
zygotique
Anaphase de
première division
zygotique
WT
♀
♂
*
♂
♀
*
♂
♀
Hira⁻
Yem⁻
K81⁻
mh⁻
IC
SF


Métaphase de
Méiose II
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Hira-
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K81-
mh-
IC
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d'histone H3.3, impliqué dans une grande variété de situations biologiques ; ceci m'amènera à commenter ensuite mes travaux sur les rôles somatiques du complexe. En introduction à ce sujet, je discuterai de l'extraordinaire variabilité du nucléosome et de la difficulté à décrire l'importance fonctionnelle de ses différents degrés de complexité. L'étude des variants d'histone constitue une opportunité précieuse pour comprendre certaines de ses fonctions. Cependant, alors que nous commençons à cerner l'étendue de cette famille de protéines, H3.3 apparaît comme un cas particulier, universellement représenté parmi les espèces eucaryotes. J'argumenterai sur la possibilité que ceci reflète le caractère universel de la fonction à laquelle le variant semble asservi au cours de la reproduction. Si cette introduction n'a pas vocation à présenter de façon exhaustive le contexte bibliographique, elle permet de poser des éléments d'analyse au cœur de la problématique de cette thèse et de notre équipe.

De façon plus générale, ces interrogations m'ont amené à l'étude d'une diversité de modèles où les chromosomes parentaux sont affectés au moment de la fécondation chez la drosophile, qui seront présentés tout au long de ce manuscrit (Figure 1). Ensemble, ces travaux soulignent la difficulté du passage par la fécondation pour la stabilité des chromosomes, dont l'intégrité est pourtant absolument critique pour la formation du zygote. A travers eux, j'essaierai de défendre l'idée que la réussite de la fécondation conditionne la nécessité et la conservation de diverses voies moléculaires.

Figure 1: Schéma représentant les différents phénotypes abordés dans cette thèse.
Les schémas représentent les premières étapes de la formation du zygote chez la Drosophile : la fin de la deuxième division de méiose femelle, la première réplication zygotique et la première anaphase du zygote (la situation sauvage est représentée dans la ligne 1). La partie antérieure de l'œuf est située à gauche. Les chromosomes paternels (en rouge) et maternels (en bleu) sont indiqués ainsi que les globules polaires (étoile).
(Ligne 2) dans les œufs issus de femelles mutantes pour Hira ou yem, le pronoyau mâle est incapable de se décondenser et les chromosomes paternels sont exclus dès la première division zygotique.
(Ligne 3) dans les situations mutantes K81, maternal hapoid ou dans le cas de l'incompatibilité cytoplasmique liée à Wolbachia (IC), les chromosomes paternels se décondensent, se répliquent mais sont partiellement ou totalement exclus au cours de la première division du zygote.
(Ligne 4) dans une situation de stérilité SF associée à la dysgénésie I-R (SF), tandis que les chromosomes paternels ne présentent pas de défauts, la méiose femelle est catastrophique et aboutit à la perte partielle ou totale de chromosomes maternels au cours du premier cycle du zygote.

La drosophile est un modèle animal privilégié pour étudier ces problèmes. Un siècle de travaux a permis d'établir chez cet organisme une énorme batterie d'outils génétiques et moléculaires, qui rendent le travail expérimental ludique et exaltant. L'étendue de ce qu'il est possible de faire n'est limitée que par l'imagination de l'expérimentateur, et il est facile de tomber éperdument amoureux de cet univers de possibilités. Ce manuscrit se veut aussi un hommage personnel à la beauté de ce modèle, et à tous ceux qui ont participé à cultiver ce champ extraordinairement fertile où l'on sème en permanence des graines d'idées nouvelles. Les travaux de thèse que j'y exposerai sont une partie infime des produits de ce terroir, mais, je l'espère, ils transpirent le plaisir que j'ai eu en les réalisant.

INTRODUCTION

I. La plasticité de la chromatine à l'échelle du nucléosome

Presque toutes les espèces eucaryotes étudiées jusqu'à ce jour possèdent en commun une unité de base de la chromatine sous la forme de nucléosomes, constitués de ce joyau de l'évolution que sont les histones. Les quatre histones « de cœur » sont parmi les protéines les plus conservées, et semblent être un élément clé, difficilement perfectible, pour organiser les chromosomes. Ce système doit cependant s'adapter à l'extraordinaire complexité des processus qui défient la chromatine, comme l'expression ou la répression des gènes, la réplication de l'ADN ou sa réparation, la recombinaison des chromosomes, leur compaction ou leur relaxation, et la détermination de territoires chromosomiques spécialisés… Face à ces contraintes, au nucléosome se superposent différents degrés de variabilité qui confèrent à la chromatine une plasticité adaptée à une grande variété de besoin biologiques. Cet ensemble constitue un socle robuste et dynamique pour le codage et la transmission d'information épigénétique.

La complexité inhérente au système rend cependant l'étude fonctionnelle de cette organisation très difficile. Par exemple, le vaste champ d'investigation des modifications post traductionnelles des histones (MPTs) contribue amplement à décortiquer les propriétés des nucléosomes mais se heurte souvent à la difficulté d'y associer des fonctions biologiques précises.

I.1. La complexité de l'étude fonctionnelle du nucléosome

Un insondable potentiel de variabilité

Au cœur de la variabilité du nucléosome, les MPTs représentent un potentiel combinatoire vertigineux (environ 1,19 milliards de combinaisons possibles seulement pour la queue N-terminale d'une histone H3) soumis, de plus, à des changements dynamiques. Deux grands types d'effets peuvent être attendus des MPTs : (1) une altération des propriétés des nucléosomes qui voient leur affinité pour l'ADN modifiée ; (2) leur capacité à recruter (ou

empêcher le recrutement) de protéines non-histones (Zhou *et al.*, 2010). De ces propriétés, des conséquences fonctionnelles peuvent être attendues pour tous les processus utilisant l'ADN comme substrat. De fait, ces dernières années, la littérature s'est accélérée en ce sens pour décrire la distribution des MPTs à l'échelle du génome (Barski *et al.*, 2007), au cours du développement ou de la différenciation cellulaire (Mikkelsen *et al.*, 2007) ainsi que dans des situations pathologiques (Chi *et al.*, 2010). Se dégagent de fortes corrélations conservées au cours de l'évolution entre certaines MPTs et certains effets biologiques, dont sans doute les plus étudiés sont la promotion et la répression de la transcription (comme en témoigne l'impressionnant nombre d'articles sur le sujet, évoqués notamment dans les revues suivantes (Huisinga *et al.*, 2006; Berger, 2007; Goldberg *et al.*, 2007; Loyola et Almouzni, 2007; Hublitz *et al.*, 2009). Les données massives apportées dans ce domaine ont même fait rentrer une série de MPTs « classiques » dans notre définition moléculaire de la chromatine active ou inactive. Les études visant à décrire les différents domaines de la chromatine ont en plus bénéficié d'une importante catalyse grâce au développement de techniques massives d'analyse à l'échelle du génome entier (Chi *et al.*, 2010; Roy *et al.*, 2010; Young *et al.*, 2010; Zhou *et al.*, 2010). Ces approches pangénomiques permettent de construire progressivement une image dynamique et territorialisée de la chromatine, où les MPTs semblent suivre une orchestration dévouée au fonctionnement du chromosome (Campos et Reinberg, 2009; Zhou *et al.*, 2010).

De la difficulté à lier les MPTs et les activités des chromosomes

L'ensemble de ces études nous conduit généralement à l'idée que chaque MPT est associée à une certaine fonction biologique. Cependant, il reste très difficile de démontrer un lien causal entre les MPTs et ces activités (Kouzarides, 2007). Certaines données fonctionnelles capitales proviennent de l'analyse du rôle des enzymes qui modifient les histones. Bien que ces études soient très informatives, de plus en plus de ces enzymes sont décrites comme ayant des cibles non-histones (Glozak *et al.*, 2005; Huang et Berger, 2008; Sims et Reinberg, 2008), et ceci empêche de conclure sur les conséquences directes de la MPT.

Les études à grande échelle ont aussi révélé leurs propres contradictions, en montrant la plurivalence des MPTs associés à des domaines de la chromatine fonctionnellement distincts.

Dans cette grande complexité, les régions considérées comme appartenant à l'hétérochromatine sont un exemple de cette difficulté. L'hétérochromatine a souvent été définie moléculairement par certains marqueurs historiques, comme les protéines HP1 et divers degrés de méthylation de l'histone H3 (Eissenberg *et al.*, 1992; Aagaard *et al.*, 1999; Bannister *et al.*, 2001; Nakayama *et al.*, 2001; Richards et Elgin, 2002; Barski *et al.*, 2007; Wen *et al.*, 2009). Cependant, cette notion est nuancée, en particulier parce que la littérature révèle de plus en plus de contrexemples (Huisinga *et al.*, 2006; Vermaak et Malik, 2009). En effet, les fleurons de l'arsenal hétérochromatique HP1 et H3K9Me sont également associés, dans certains contextes, à la chromatine transcriptionnellement active (Piacentini *et al.*, 2003; Vakoc *et al.*, 2005; Lin *et al.*, 2008). Ce type d'exemple montre qu'il est délicat de définir des territoires fonctionnels de la chromatine par la description d'une partie de ses composants. La complexité du système veut donc qu'il soit extrêmement difficile d'établir, au delà de tout doute raisonnable, un lien causal entre une MPT et un effet biologique. Le volume toujours grandissant de données à l'échelle de tout le génome doit être concilié avec des fonctions biologiques concrètes par le retour à l'étude fonctionnelle des variations du nucléosome.

Certains systèmes (comme *S.cerevisiae*) offrent cependant l'opportunité de faire des études fonctionnelles directes en mutant ponctuellement les différents résidus des histones (Grunstein, 1990; Smith, 1991). Ce type d'analyse permet de confronter directement l'altération des nucléosomes à une conséquence biologique et tirer des informations sur le rôle *in vivo* d'un certain acide aminé (à défaut de pouvoir conclure définitivement sur le rôle des MPTs associées). Ces approches ne sont cependant pas envisageables chez les modèles pluricellulaires courants qui possèdent un grand nombre de copies des gènes histones canoniques. Au contraire, il est possible de décortiquer les rôles biologiques du nucléosome à travers l'étude des variants d'histone chez ces mêmes modèles. Ces variants non-alléliques des histones canoniques, universellement représentées parmi les espèces, constituent un degré très important de variabilité du nucléosome (revu dans Malik et Henikoff, 2003; Kamakaka et Biggins, 2005; Banaszynski *et al.*, 2010; Elsaesser *et al.*, 2010; Talbert et Henikoff, 2010; Szenker *et al.*, 2011). L'étude des variants d'histone est possible du point de vue génétique, moléculaire et biochimique, et de plus en plus de travaux tentent d'élucider leurs rôles. De la même façon, l'analyse fonctionnelle des acteurs de l'assemblage de la chromatine est précieuse pour construire une image mécanistique de la régulation des chromosomes. L'étude

de ces facteurs constitue donc une opportunité expérimentale précieuse pour permettre d’associer certains paramètres de modulation des nucléosomes à des conséquences biologiques. Le paragraphe suivant traitera de l’univers en expansion des variants d’histone et me permettra d’introduire les fondements de l’importance biologique du variant H3.3 et de la voie d’assemblage HIRA, au cœur de mon travail de thèse.

I.2. La grande famille des variants d’histone

I.2.a Conservation et variabilité des variants d’histone

Il est communément admis que les histones canoniques structurent le plus gros du génome (ce qui est certainement vrai pour un grand nombre de types cellulaires), tandis que l’incorporation de variants répond à des besoins ponctuels et est donc minoritaire et hétérogène. Cependant, cette vision évolue au fil de découvertes sur la biologie de ces variants, qui révèlent leur grande importance fonctionnelle et leur abondance dans la chromatine.

La diversité des variants d’histone H2A/H2B et leurs rôles biologiques

L’histone H2A possède deux types de variant archétypiques: H2A.Z et H2A.X. Le variant H2A.Z semble avoir un rôle double, dont les fondements restent à élucider, alternativement dans l’activation ou la répression transcriptionnelle, tandis que H2A.X est connu pour son rôle dans la réponse aux dommages à l’ADN de même que dans certains mécanismes de répression de l’expression (revu dans Zlatanova et Thakar, 2008; Altaf *et al.*, 2009). Malgré leur très large représentation parmi les eucaryotes, il existe un seul le variant H2AvD chez la drosophile, qui possède des caractéristiques de séquence proches de H2A.X comme de H2A.Z, et est essentiel pour la survie (van Daal *et al.*, 1988; van Daal et Elgin, 1992).

Reflétant cette diversification, il existe aussi des variants seulement présents dans certains lignages : ceux-ci pourraient répondre à des besoins biologiques spécifiques à certaines espèces ou, alternativement, être facultatifs pour des besoins universels. Les variants macroH2A et H2A.Bbd sont présents notamment chez les vertébrés, certains protistes et des

échinodermes. Tandis que macroH2A semble jouer un rôle dans l'inactivation conditionnelle des gènes et, en particulier, du chromosome X inactivé, H2A.Bbd est associé au relâchement de la chromatine (Doyen *et al.*, 2006a; Doyen *et al.*, 2006b; Gonzalez-Romero *et al.*, 2008; Buschbeck *et al.*, 2009). Chez la souris et l'homme, au moins six variants de H2A et H2B sont spécifiquement exprimés dans la lignée germinale mâle, dont certains ont été fonctionnellement impliqués dans l'établissement de régions hétérochromatiques dans le sperme (Churikov *et al.*, 2004; Govin *et al.*, 2004; Govin *et al.*, 2007). Un autre exemple de cette adaptabilité évolutive est observé chez les rotifères bdelloïdes, un ordre d'espèces qui n'ont ni mâles ni méiose mais qui, adaptés à des contraintes environnementales, sont capables de réparer très efficacement leur ADN (Van Doninck *et al.*, 2009). De façon surprenante, les variants H2A.Z, H2A.X et l'histone canonique H2A n'existent pas chez ces espèces, et se substituent à elles trois histones d'un nouveau type (appelé H2Abd) qui pourraient être impliquées dans la résistance aux dommages à l'ADN. Au contraire, leurs cousines rotifères monogonontes, à reproduction sexuée facultative et faible résistance aux dommages, possèdent une histone H2A canonique et aucune H2Abd. Ces exemples illustrent le rôle des variants d'histone comme des « jokers adaptatifs » qui permettraient à la chromatine de répondre à des contraintes spécifiques à certaines espèces.

La famille grandissante des variants de H3

L'histone H3 canonique possède la séquence la plus conservée parmi les histones de cœur, à l'instar de son variant universel H3.3 (dont il sera question dans le prochain paragraphe) (Malik et Henikoff, 2003; Elsaesser *et al.*, 2010; Szenker *et al.*, 2011)(voir aussi Orsi *et al.*, 2009). Néanmoins, il existe aussi des variants de H3 plus divergents, spécifiques de certains lignages. *A.thaliana* possède 15 gènes pouvant coder pour des variants de l'histone H3, dont l'existence d'au moins cinq a été vérifiée expérimentalement (Okada *et al.*, 2005; Ingouff *et al.*, 2007; Ingouff et Berger, 2010; Ingouff *et al.*, 2010). Récemment, il a été montré que les primates expriment les variants H3.X et H3.Y dans certains types cellulaires (Wiedemann *et al.*, 2010). L'expression de H3.Y est dépendante des conditions de croissance des cellules et ce variant joue un rôle dans le contrôle de certains gènes liés au cycle cellulaire et à la structure de la chromatine. Ces variants sont donc des acteurs potentiels de la régulation du

transcriptome et vont probablement susciter un grand intérêt dans les années à venir. H3 possède aussi un variant spécifique de la lignée germinale mâle chez l'homme et chez la souris, H3t (Witt *et al.*, 1996; Churikov *et al.*, 2004; Govin *et al.*, 2007). Finalement, les hominidés possèdent H3.5, un variant exprimé dans les testicules qui pourrait avoir un rôle dans la croissance cellulaire (Schenk *et al.*, 2011). Ces découvertes laissent présager que l'univers des variants d'histone est encore en pleine expansion. Jusqu'à présent, notre raisonnement a été contraint par la difficulté à étudier un éventail large du spectre du vivant, mais l'ère de la post génomique apportera certainement de plus en plus de lumière sur la multiplicité des histones au cours de l'évolution, et sur son importance fonctionnelle.

Le variant H3 centromérique, un variant modulable pour une fonction universelle

Il existe cependant des variants d'histone qui, malgré une divergence de séquence relativement forte, sont asservis à des fonctions universelles comme l'histone centromérique CenH3. Les histones appartenant à cette catégorie possèdent 40 à 60% d'homologie avec l'histone H3 canonique et sont associées au centromère de façon très conservée (Malik et Henikoff, 2003; Malik et Henikoff, 2009). Ces histones sont fonctionnellement analogues en ceci qu'elles participent à l'identité épigénétique du centromère et sont nécessaires pour sa fonction (Collins *et al.*, 2005; Heun *et al.*, 2006; Dalal *et al.*, 2007). Cependant, bien que les centromères soient eux-mêmes fonctionnellement analogues entre les espèces, la séquence d'ADN aux centromères diffère grandement (Henikoff *et al.*, 2001; Malik et Henikoff, 2009). De plus, une diversité d'eucaryotes, dont *C.elegans*, possèdent des chromosomes holocentriques qui sont aussi déterminés par, notamment, des protéines de type CenH3. Contrairement à certains variants de H2A ou H3, qui pourraient s'adapter à des contraintes évolutives spécifiques, la nécessité pour toute cellule de fabriquer un centromère, ou un système alternatif de rattachement des kinétochores, semble conditionner l'évolution des protéines de type CenH3.

I.2.b Le cas unique du variant H3.3

Les promesses de H3.3 comme régulateur de la transcription

Le variant d'histone H3.3 est un exemple unique en ceci qu'il est aussi bien conservé que sa contrepartie canonique (sinon mieux), ne différant d'elle que par 4 à 5 acides aminés (Malik et Henikoff, 2003; Orsi *et al.*, 2009; Elsaesser *et al.*, 2010; Szenker *et al.*, 2011). L'importance fonctionnelle de cette protéine est certainement reflétée par ce niveau extraordinaire de conservation, comme par le fait qu'elle est probablement apparue au moins quatre fois au cours de l'évolution (Malik et Henikoff, 2003), et nous ne pouvons qu'imaginer que H3.3 joue un rôle universellement nécessaire chez les eucaryotes.

L'histone H3.3 et l'histone H3 canonique n'ont pas le même profil d'expression et les transcrits de H3.3 deviennent majoritaires dans des cellules qui sortent du cycle de réplication (Wu *et al.*, 1982). Dans les cellules à longue durée de vie, H3.3 devient majoritaire dans la chromatine, et il a été proposé que son incorporation pourrait faire partie d'un mécanisme passif de renouvellement des histones (Urban et Zweidler, 1983; Grove et Zweidler, 1984; Piña et Suau, 1987; Wunsch et Lough, 1987; Bosch et Suau, 1995). Ce rôle de structuration de la chromatine qui pourrait être une fonction essentielle à l'origine de sa nécessité biologique sera plus amplement discuté dans un article de revue dans la partie Résultats (Orsi *et al.*, 2009).

Cependant, parmi ses fonctions potentielles, celle qui a reçu le plus d'intérêt est son rôle dans l'assemblage de la chromatine suite à la transcription. Les histones H3 canonique et H3.3 définissent respectivement des voies d'assemblage de la chromatine couplée à la synthèse de l'ADN (que l'on appelle couramment couplée à la réplication, CR) et indépendante de celle-ci (IR)(Ahmad et Henikoff, 2002a). Continuellement, dans toute cellule se crée le besoin de réassembler des nucléosomes sur une multitude de sites actifs du génome par le mode IR. H3.3 est recrutée pour remplir ce besoin (qui pourrait être simplement structural), et se retrouve en conséquence systématiquement enrichie dans les régions transcrites (Mito *et al.*, 2005; Wirbelauer, 2005; Jin et Felsenfeld, 2006; Sutcliffe *et al.*, 2009; Goldberg *et al.*, 2010). Ce constat a amené à l'idée que cette histone pourrait jouer un rôle moteur dans l'activation (ou la répression) de la transcription, plutôt que d'être simplement une cicatrice du processus (Hake et Allis, 2006). Cette hypothèse a été nourrie au cours des dernières années par de

nombreux travaux : les arguments autour de cette question seront abordés dans la partie Résultats.

Rôles essentiels de H3.3 pour la reproduction

Néanmoins, les résultats surprenants des études fonctionnelles de H3.3 chez le protiste *Tetrahymena thermophila* ou chez la drosophile ont placé le curseur sur des rôles essentiels de H3.3 éloignés de son incidence sur l'expression des gènes. En effet, chez ces deux modèles, des mutations des gènes codant pour H3.3 ne résultent pas en une altération drastique de l'activité transcriptionnelle des cellules (Cui *et al.*, 2006; Hodl et Basler, 2009; Sakai *et al.*, 2009). Ainsi, bien que H3.3 puisse faciliter la transcription, son importance *in vivo* dans ce processus ne suffit pas à rendre compte de son extraordinaire degré de conservation. Au contraire, les mutations de H3.3 induisent chez toutes les espèces modèles étudiées jusqu'à présent un phénotype associé aux fonctions de reproduction.

La descendance de *T.thermophila* dépourvus de variants de type H3.3 présente des défauts de survie (Cui *et al.*, 2006). De façon similaire, une mutation sur un des gènes codant pour H3.3 chez la souris conduit à un phénotype de fertilité réduite des mâles (Couldrey *et al.*, 1999). Finalement, les mutants de H3.3 chez la drosophile sont viables mais présentent des phénotypes de stérilité mâle comme femelle (Hodl et Basler, 2009; Sakai *et al.*, 2009). Nos travaux sur l'implication de H3.3 dans la formation du zygote nous ont amenés à proposer que le rôle de H3.3 dans la reproduction constitue probablement une clé de sa conservation au cours de l'évolution (Orsi *et al.*, 2009). Cette idée gagne du terrain, comme en témoignent des publications récentes qui soulignent l'importance de cette histone dans ce processus quasi universel chez les eucaryotes (Ooi et Henikoff, 2007; Banaszynski *et al.*, 2010; Elsaesser *et al.*, 2010; Szenker *et al.*, 2011).

I.3. Les facteurs d'assemblage de la chromatine, artisans de l'organisation des nucléosomes

Les facteurs d'assemblage de la chromatine se spécialisent dans l'incorporation d'un type d'histone, voire d'un variant particulier, et peuvent donc être recrutés sélectivement en fonction du besoin biologique (Probst *et al.*, 2009; Banaszynski *et al.*, 2010; Campos et Reinberg, 2010; Ransom *et al.*, 2010). Les histones H2A-H2B sont associées à FACT, NAP-1 (Ito *et al.*, 1996; Belotserkovskaya *et al.*, 2003), et Chz1, spécifiquement associé au variant H2A.Z (Luk *et al.*, 2007). Le variant centromérique CenH3 possède des chaperons spécifiques : HJURP est nécessaire pour son incorporation chez l'homme et probablement chez le xénope et Scm3 joue un rôle analogue chez les levures (Camahort *et al.*, 2007; Dunleavy *et al.*, 2009; Foltz *et al.*, 2009; Shuaib *et al.*, 2010; Bernad *et al.*, 2011). De façon intéressante, seul le facteur universel RbAp48 a été trouvé en association à Cid chez la drosophile (Furuyama et Henikoff, 2006).

La première identification des facteurs d'assemblage de H3 et H3.3 provient d'élégantes expériences de co-immunoprécipitation qui ont montré que ces histones sont associées à des complexes spécifiques (Tagami *et al.*, 2004). Définis classiquement par la présence de CAF-1 ou HIRA, ces complexes ont été d'abord montrés comme capables d'assemblage CR ou IR in vitro (Tagami *et al.*, 2004). En plus de certains facteurs communs, dont ASF1, NASP et RbAp48, l'histone H3 canonique interagit spécifiquement avec CAF-1 tandis que les complexes HIRA/Ubinucléine/Cabine ou ATRX/DAXX et leur interacteur potentiel DEK prennent en charge H3.3 chez l'homme (Tagami *et al.*, 2004; Drane *et al.*, 2010; Lewis *et al.*, 2010; Sawatsubashi *et al.*, 2010). La nature exacte de l'interaction physique entre ces différents complexes est en cours de débat. Il a été suggéré que HIRA n'est pas stablement associé à H3.3, et que, même en absence du complexe ATRX/DAXX, ce variant est associé au complexe CAF-1 plutôt qu'à HIRA (Drane *et al.*, 2010). Néanmoins, les complexes HIRA et ATRX ont été tous les deux montrés comme fonctionnellement impliqués dans l'incorporation de H3.3 chez la souris (Goldberg *et al.*, 2010). Le premier lien entre HIRA et l'assemblage de la chromatine dans le contexte du développement a été décrit chez la drosophile, où ce facteur a un rôle essentiel dans l'incorporation du variant H3.3 dans le pronoyau mâle à la fécondation (Loppin *et al.*, 2005a). Ce rôle semble être conservé et est indispensable pour l'intégration des chromosomes paternels dans le premier noyau zygotique.

Les contraintes fonctionnelles qui conditionnent la nécessité de H3.3 et HIRA pour la reproduction sont liées à l'histoire épigénétique complexe des chromosomes parentaux. Dans le prochain paragraphe il sera question des profonds remaniements qui affectent ces chromosomes, des défis de réorganisation et de maintien de l'intégrité auxquels ils font face après la fécondation, et du rôle crucial dans ce processus de la voie d'assemblage de la chromatine HIRA/H3.3.

II. Dynamique de la chromatine et organisation des chromosomes à la fécondation

« The wren goes to't, and the small gilded fly
Does lecher in my sight.
Let copulation thrive… »

William Shakespeare, *King Lear*

II.1. L'histoire épigénétique des gamètes

Chez les organismes multicellulaires à reproduction sexuée, la sélection sexuelle favorise le dimorphisme et le développement de gamètes de tailles différentes (Bulmer et Parker, 2002; Charlesworth et Charlesworth, 2010). Il est même possible que cette divergence soit déterminante pour définir initialement les sexes dans une espèce donnée, puis pour contrôler l'évolution des chromosomes sexuels. L'œuf, ovule ou oosphère (le gamète le plus gros), accumule des transcrits, des protéines et des réserves énergétiques, alors que le gamète mâle est adapté aux contraintes de son déplacement et a souvent une taille réduite. Les contraintes pour la production des gamètes sont extrêmement différentes entre les deux sexes et le traitement des chromosomes parentaux n'y déroge pas.

II.1.a La réorganisation de la chromatine au cours de la spermatogénèse

Une caractéristique commune aux gamètes mâles est l'extinction de l'activité du noyau dans les dernières étapes de leur différenciation (Monesi, 1964; Monesi *et al.*, 1978; Zheng *et al.*, 2008). Les gènes nécessaires à leur différenciation sont majoritairement exprimés avant la méiose ou peu de temps après celle-ci et les ARNm ainsi produits font ultérieurement l'objet d'une régulation traductionnelle de laquelle dépend le timing de différenciation (Zakeri *et al.*, 1988; Steger, 1999). Or, ceci est lié au remodelage massif de ces chromosomes, un processus très largement conservé. L'organisation de la chromatine en nucléosomes paraît être un choix quasi exclusif chez les eucaryotes. Cependant, pour des raisons qui sont encore mal comprises, un grand nombre d'espèces animales à reproduction sexuée ont inventé des modes uniques de structuration des chromosomes paternels. La chromatine du gamète mâle apparaît souvent organisée avec des protéines fortement basiques, spécifiques de la lignée germinale mâle que l'on regroupe sous l'appellation Sperm Nucleus Basic Proteins (SNBPs) (Ausió, 1999). Cet acronyme cache une grande diversité qui accentue la singularité de ce type d'organisation.

Différents modes d'organisation de la chromatine du sperme

Il existe en effet plusieurs types de chromatine du sperme dont l'organisation peut être très différente même entre espèces proches (Lewis *et al.*, 2003a; Eirin-Lopez et Ausio, 2009). (1) Certaines espèces ont une chromatine du sperme majoritairement composée d'histones (comme dans le genre Rana) (Frehlick *et al.*, 2006). (2) Le xénope, possède des nucléosomes particuliers dont toutes les histones de type H2A-H2B sont remplacées dans le sperme par des protéines spécifiques, alors que les histones H3-H4 sont retenues (Frehlick *et al.*, 2006). (3) Nombre d'espèces, dont, notamment, la drosophile, mais aussi les crapauds du genre Bufo ou les bovidés, utilisent majoritairement des protéines type protamine (Lewis *et al.*, 2003b). Cette famille de protéines très hétérogène pourrait dériver des histones de liaison de type H1 (Ausió, 1999). (4) D'autres (dont certains mollusques) ont des protéines proches des protamines, appelées protamine-like, aux propriétés biochimiques similaires (Ausió, 1999). (5) Finalement, des espèces comme l'homme ou la souris sont caractérisées par une

chromatine du sperme généralement associée à des protamines à l'exception d'un certain nombre de loci qui retiennent des histones (en proportions variables selon l'espèce, calculée à 10-15% chez l'homme mais 50% chez certains marsupiaux) (Lewis *et al.*, 2003a; Chu *et al.*, 2006).

Possibles rôles des SNBPs

Cette grande diversité interspécifique contraste fortement avec la composition de la chromatine somatique fortement homogène entre ces mêmes espèces. Peu de travaux ont étudié l'importance fonctionnelle des différents types d'empaquetage. Ceux-ci pourraient conférer aux cellules une adaptation aux contraintes hydrodynamiques liées à la reproduction. Alternativement, il est suggéré que le fort degré de compaction conféré par ces protéines est adapté à la protection de l'ADN contre des agressions environnantes (Rathke *et al.*, 2010). Récemment, une étude fonctionnelle chez la drosophile va dans ce sens, montrant que des individus dépourvus de protamines sont plus sensibles aux dommages à l'ADN. De façon surprenante, ces auteurs montrent que les protamines ne sont pas essentielles pour la fertilité. Au contraire, les deux gènes codant pour des protamines sont requis pour la fertilité mâle chez la souris (Cho *et al.*, 2001). Les bases fonctionnelles et les possibles rôles du système mixte histones/protamines chez les mammifères ne sont par ailleurs pas connus. Il a été proposé que les histones pourraient constituer des points d'attache du génome à la matrice nucléaire, portant une information épigénétique sur l'architecture de noyau (Ward, 2010). D'autres auteurs proposent l'implication de cette organisation dans la transmission trans-générationnelle de l'état d'activité de certains gènes, une idée actuellement en débat (Boussouar *et al.*, 2008; Hammoud *et al.*, 2009; Banaszynski *et al.*, 2010; Kota et Feil, 2010; Miller *et al.*, 2010). En particulier, il a été montré que les histones paternelles transmises dans le sperme participent à la chromatine zygotique chez l'homme, posant des bases théoriques pour cette hypothèse (van der Heijden *et al.*, 2008).

Dans tous les cas, l'importance fonctionnelle des SNBPs et la nature des pressions de sélection qui ont conduit à l'apparition et la diversification de ces modes d'organisation restent pour l'heure largement mystérieuses.

II.1.b La transmission paternelle de territoires épigénétiques

Lors de la gamétogénèse mâle, un défi supplémentaire qui se présente est celui du maintien trans-générationnel de territoires de la chromatine dont la nature ou l'activité est définie épigénétiquement. Les premiers exemples historiques viennent de l'étude de la perte de chromosomes chez le diptère *Sciara coprophila*. Pendant le développement embryonnaire précoce, un ou deux chromosomes X paternels sont perdus (en fonction du sexe de l'individu). Ceci reflète un mécanisme épigénétique d'identification des chromosomes X parentaux, probablement lié à certaines MPTs des histones (de Saint Phalle et Sullivan, 1996; Greciano et Goday, 2006). Un autre exemple chez les insectes vient de l'étude des cochenilles du genre *Planococcus*, dont le génome paternel est entièrement hétérochromatinisé chez les descendants mâles de façon héritable, mais pas chez les femelle : ce mécanisme aussi est lié à un profil spécifique de MPTs (Bongiorni *et al.*, 2009).

L'empreinte est plus largement étudiée chez les mammifères : plus d'une centaine de gènes sont exprimés à partir d'un seul allèle, en fonction de l'origine parental du chromosome (revu dans Ooi et Henikoff, 2007; Reik, 2007; Schaefer *et al.*, 2007; Chow et Heard, 2009; Feil, 2009; Kota et Feil, 2010). Ces mécanismes dépendent d'un dialogue complexe entre les profils de méthylation de l'ADN, la transcription d'ARN non codants et la mise en place de MPTs. L'incorporation de variants d'histones (notamment par la voie HIRA) pourrait aussi contribuer à la reprogrammation épigénétique des cellules germinales primordiales chez la souris (Hajkova *et al.*, 2008). Il est inféré que H3.3 pourrait jouer un rôle dans ce processus, comme dans l'établissement de signatures épigénétiques dans le zygote, notamment dans le contexte du pronoyau mâle (Hajkova *et al.*, 2008; Santenard et Torres-Padilla, 2009). D'autres exemples qui ont reçu un grand intérêt pendant cette dernière décennie, comme l'inactivation sélective du chromosome X paternel, ont grandement contribué à éclairer les mécanismes en jeu dans la mise en place de l'empreinte épigénétique (Chow et Heard, 2009).

Chez la drosophile, la transmission transgénérationnelle d'information épigénétique sur l'expression des gènes n'a pas, pour l'heure, été montrée. En effet, aucun cas de gène soumis à l'empreinte n'est encore connu et l'expression zygotique n'est enclenchée que 12 à 14 cycles de division après la fécondation. De plus, le problème du chromosome X est réglé par compensation de dose chez le mâle, plutôt que par inactivation chez la femelle (Gelbart et

Kuroda, 2009). Finalement, les sites concernés par la méthylation de l'ADN, la fenêtre de temps pendant laquelle celle-ci est présente et le rôle des ADN methyltransférases sont actuellement des sujets de débat. (Lyko *et al.*, 2000; Phalke *et al.*, 2009; Schaefer et Lyko, 2010). Néanmoins, la drosophile est confrontée à la transmission de territoires du chromosome définis épigénétiquement, comme les télomères et centromères que j'aborderai en détail dans la partie Résultats.

II.1.c Organisation des chromosomes maternels

A l'instar des chromosomes paternels, les chromosomes maternels sont verrouillés de façon relativement précoce au cours de l'ovogenèse. Cependant, le mécanisme retenu diffère grandement de celui du remplacement des protéines chromosomiques, employé dans la lignée germinale mâle. De façon conservée, les chromosomes maternels adoptent, peu après la recombinaison méiotique, une structure compacte associée à des MPTs généralement trouvées à la mitose et entrent en division de méiose (Ivanovska, 2005; Sasaki et Matsui, 2008; Kota et Feil, 2010). Le degré de protection des chromosomes, et leur inactivité ainsi obtenus pourraient donc partager des aspects fonctionnels avec la stratégie employée par les chromosomes paternels, bien que sur des bases moléculaires très différentes.

Reflétant peut être cette caractéristique, les chromosomes maternels sont soumis à un remodelage de la chromatine au moment de la fécondation qui implique l'incorporation du variant H3.3 chez *C.elegans* et *A.thaliana* (Ooi *et al.*, 2006; Ingouff *et al.*, 2007). Le degré de conservation de ce mécanisme n'est pas connu mais il est possible que l'activité de ces chromosomes dépende d'un enlèvement programmé des marques de la chromatine inactive, passant par l'assemblage de nucléosomes de novo. L'importance fonctionnelle de ce processus n'est néanmoins pas élucidée.

II.1.d La reproduction : talon d'Achille des espèces

Le verrouillage des chromosomes parentaux traduit bien l'importance du maintien de leur intégrité pour assurer le succès de la fécondation, une condition indispensable pour la survie

d'une espèce. Il est essentiel pour tout organisme de disposer des mécanismes nécessaires pour la protection contre toute attaque à ces étapes. Or, c'est exactement pour cela que la reproduction sexuée représente une fenêtre d'opportunité pour l'installation et la prévalence d'agents à comportement parasitaire. Quelle que soit la nature de l'attaque, des moyens sont mis en œuvre pour assurer la descendance, même quand cela implique l'installation durable de l'envahisseur. Au cours de ma thèse, j'ai abordé deux exemples assimilables à du parasitisme dans les lignées germinales chez la drosophile: le potentiel invasif des transposons et la manipulation de la fécondation par des bactéries du genre *Wolbachia*. Dans les deux cas, l'intégrité des chromosomes parentaux est compromise et ceci a des conséquences pour la réussite de la première division et la viabilité du zygote. Ces situations seront abordées en détail dans la deuxième partie de la section Résultats.

II.2 La formation du zygote

II.2.a Des contraintes spécifiques pour les chromosomes paternels après la fécondation

L'organisation unique de la chromatine du sperme est incompatible avec, notamment, la réplication de l'ADN et la transcription des gènes, ce qui impose une réorganisation *ad hoc* de la chromatine paternelle sous la forme de nucléosomes au moment de la fécondation (Poccia *et al.*, 1984; Nonchev et Tsanev, 1990; McLay et Clarke, 2003). Dans un premier temps, les SNBPs doivent être enlevées par des mécanismes actifs dont la nature est mal connue (voir prochain paragraphe). Dans un deuxième temps, des facteurs maternels d'assemblage de la chromatine doivent être recrutés pour assembler et organiser des nucléosomes dans le génome paternel, constituant alors le pronoyau mâle en cours de maturation (Frehlick *et al.*, 2006; Orsi *et al.*, 2009). Le processus est complété avant l'initiation de la première réplication zygotique et constitue donc un exemple unique d'assemblage de la chromatine IR à l'échelle de tout un génome (Poccia et Collas, 1996). De plus, ce processus est complété parfois de façon extrêmement rapide (moins de 15 minutes chez la drosophile), ce qui suppose une grande efficacité de la machinerie d'assemblage en jeu (Sonnenblick, 1950). La fécondation pose donc aux chromosomes paternels un défi majeur, où le moindre faux pas peut conduire à l'arrêt immédiat du développement ou compromettre sévèrement la viabilité de l'individu.

A ce stade, l'œuf n'est plus un œuf mais n'est pas tout à fait encore un embryon, et les chromosomes paternels et maternels cessent progressivement de suivre leur propre programme autonome pour entrer dans un cycle commun au sein du premier noyau du zygote. Dès l'instant où la chromatine paternelle est constituée de nucléosomes dans l'oeuf, le pronoyau mâle a un potentiel d'activité de transcription. Cependant, le moment où celle-ci devient effectivement active dépend de l'espèce. Chez la souris, la transcription zygotique commence au stade une cellule dans les deux pronoyaux, l'activité transcriptionnelle globale du pronoyau mâle étant plus forte que celle du pronoyau femelle (Aoki *et al.*, 1997). Chez *A.thaliana*, cette activité transcriptionnelle commence, au plus tard, quelques heures après la fécondation (à 2-3 cycles de division zygotique) (Aw *et al.*, 2010). Au contraire, chez la drosophile, la transcription zygotique ne commence que 12 à 14 cycles de division nucléaire après la fécondation (Pritchard et Schubiger, 1996). L'organisation du génome paternel de la souris et celui de la drosophile dès la fécondation doivent donc s'adapter à l'initiation très précoce, ou celle plutôt tardive, de l'activité transcriptionnelle. Il serait en ce sens possible que les histones présents dans de la chromatine du sperme de souris facilitent le démarrage rapide de la transcription zygotique (Banaszynski *et al.*, 2010; Kota et Feil, 2010). Cependant, des données fonctionnelles manquent pour défendre cette idée. La drosophile, au contraire, peut théoriquement s'accommoder d'une chromatine parentale transcriptionnellement silencieuse pendant plusieurs cycles de division zygotique et n'a pas de niveaux détectables d'histones transportés dans le sperme. Il est probable que cette différence fondamentale soit à l'origine de mécanismes spécifiques de remodelage de la chromatine paternelle chez l'un et l'autre organisme. Le paragraphe suivant traite de nos connaissances sur ces mécanismes et du rôle essentiel et conservé de la voie HIRA/H3.3.

II.2.b Rôle essentiel de la voie HIRA/H3.3 dans l'assemblage de la chromatine dans le pronoyau mâle.

Les mécanismes responsables de l'enlèvement des SNBPs dans le pronoyau mâle n'ont pas été clairement identifiés. Chez l'oursin, ce mécanisme pourrait passer par la phosphorylation des histones présentes dans le spermatozoïde, et être dépendant d'une cystéine protéase (Green et Poccia, 1985; Imschenetzky *et al.*, 1997). Parmi les facteurs potentiellement

impliqués, des études montrent que la nucléoplasmine de xénope pourrait servir d'échangeur entre des SNBPs et les histones H2A-H2B (Philpott *et al.*, 1991; Philpott et Leno, 1992). En effet, cette protéine fortement basique serait capable de remodeler in vitro la chromatine du sperme de xénope, mais aussi de drosophile (dont la composition est pourtant très différente). Bien que la drosophile possède un orthologue de la nucléoplasmine, sa fonction *in vivo* n'a pas été testée. Chez les mammifères, l'enlèvement de protamines pourrait dépendre de la réduction de ponts disulfure par la présence de glutathion (Perreault *et al.*, 1988).

L'ADN paternel est ensuite pris en charge par une batterie de protéines d'assemblage de la chromatine par un mode IR. La nucléoplasmine pourrait participer à l'assemblage de dimères H2A/H2B au cours de cette étape (Frehlick *et al.*, 2006). Chez de nombreuses espèces, dont la drosophile, la souris, *C.elegans*, *A.thaliana* et la carpe, l'assemblage de la chromatine IR dépend du variant d'histone H3.3 (Loppin *et al.*, 2005a; Ooi *et al.*, 2006; Torres-Padilla *et al.*, 2006; Ingouff *et al.*, 2007; Ingouff *et al.*, 2010; Zhao *et al.*, 2011). De plus, des travaux qui ont véritablement fondé mon équipe d'accueil, ont révélé que, chez la drosophile, la voie d'assemblage de la chromatine HIRA/H3.3 est essentielle dans ce processus (Loppin *et al.*, 2005a).

Les produits des gènes *hir1* et *hir2*, orthologues *Hira* chez la levure *S.cerevisiae*, ont été initialement décrites comme étant responsables de la répression transcriptionnelle des gènes histones de façon dépendante du cycle cellulaire (Sherwood *et al.*, 1993; Spector *et al.*, 1997). Chez l'homme, le gène *Hira* est codé dans un locus du chromosome 22 en cause dans le syndrome vélo-cardio-facial de DiGeorge, et c'est en ce sens que les premières études sur ce facteur ont été conduites (Lamour *et al.*, 1995). Sa fonction en tant que facteur de chromatine chez les animaux a été rapidement proposée en lien avec sa capacité à s'associer aux histones (Lorain *et al.*, 1998). La première caractérisation fonctionnelle de HIRA a été réalisée chez la souris où elle a été décrite comme une protéine essentielle au développement embryonnaire précoce (Roberts *et al.*, 2002).

Le rôle de *Hira* dans la formation du pronoyau mâle a été mis à jour fortuitement, s'intégrant dans une démarche de génétique classique portant sur l'étude d'une mutation à effet maternel chez la drosophile. La protéine HIRA se caractérise par un domaine conservé formant une hélice béta à sept répétitions Tryptophane-Aspartate (WD)(Lorain *et al.*, 1998), affecté par la

mutation ponctuelle *Hira*^{ssm}. Le phénotype de stérilité femelle associé s'explique par l'incapacité de la chromatine paternelle à être assemblés pendant la fécondation dans des œufs issus de femelles mutantes (Loppin *et al.*, 2000; Loppin *et al.*, 2001; Loppin *et al.*, 2005a). Ces études ont cristallisé le concept de différences fondamentales entre les chromosomes parentaux à la fécondation, et ont démontré l'implication essentielle de la voie HIRA/H3.3 dans l'assemblage massif de la chromatine paternelle. Le rôle crucial de HIRA dans ce processus pose la question des fonctions *in vivo* de ce facteur et du rôle que la pression de sélection liée à la reproduction a joué dans sa conservation.

Un de mes intérêts majeurs au cours de ma thèse a été de mieux comprendre cette voie, et les enjeux de ce mode d'assemblage *in vivo*. Je consacrerai la première partie de la section Résultats à retracer l'histoire récente de la littérature sur cette voie, en mettant en avant comment mes travaux de thèse ont été rythmés par elle.

RESULTATS
ET
DISCUSSIONS

PREMIERE PARTIE

Rôles du complexe HIRA chez la drosophile

I. La fonction essentielle de HIRA dans la formation du pronoyau mâle

La létalité associée à une mutation nulle de *Hira* chez la souris et l'expression ubiquitaire du gène *Hira* chez la drosophile, suggéraient un rôle général pour ce facteur dans les cellules somatiques (Kirov *et al.*, 1998; Llevadot *et al.*, 1998; Roberts *et al.*, 2002). De plus, les travaux sur le rôle *in vivo* de HIRA s'inscrivaient dans un contexte bibliographique qui argumentait pour une fonction centrale de ce facteur dans l'organisation des chromosomes. Nous avons voulu tester l'hypothèse que des mutations plus sévères affectant *Hira* soient associées à des phénotypes plus drastiques. Nous nous attendions en réalité à ce que *Hira* soit un gène essentiel, et qu'il ait un rôle important dans la régulation de l'expression de tous les gènes.

Or, de façon surprenante, comme décrit dans l'article ci-après, les individus adultes nuls pour *Hira* sont viables, et présentent une fertilité mâle normale. Seul le phénotype de stérilité femelle totale, déjà révélé par la mutation ponctuelle $Hira^{ssm}$, est évident. De plus, les individus mutants, de même que les œufs issus de femelles mutantes sont capables de réaliser un assemblage de nucléosomes apparemment normal (à l'échelle de la microscopie) de l'histone H3.3 dans l'ensemble des noyaux, à l'exception notable et spectaculaire du pronoyau mâle. A l'époque de la publication de notre article, HIRA était le seul facteur d'assemblage de la chromatine connu comme étant spécifiquement associé à H3.3, ce qui posait des questions sur l'importance de la voie IR d'assemblage de la chromatine elle même, ou à de possibles redondances entre HIRA et des facteurs de nature inconnue. L'article qui suit décrit ces travaux et a marqué le départ de mon parcours de thèse.

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The Essential Role of *Drosophila* HIRA for De Novo Assembly of Paternal Chromatin at Fertilization

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In many animal species, the sperm DNA is packaged with male germ line–specific chromosomal proteins, including protamines. At fertilization, these non-histone proteins are removed from the decondensing sperm nucleus and replaced with maternally provided histones to form the DNA replication competent male pronucleus. By studying a point mutant allele of the *Drosophila Hira* gene, we previously showed that HIRA, a conserved replication-independent chromatin assembly factor, was essential for the assembly of paternal chromatin at fertilization. HIRA permits the specific assembly of nucleosomes containing the histone H3.3 variant on the decondensing male pronucleus. We report here the analysis of a new mutant allele of *Drosophila Hira* that was generated by homologous recombination. Surprisingly, phenotypic analysis of this loss of function allele revealed that the only essential function of HIRA is the assembly of paternal chromatin during male pronucleus formation. This HIRA-dependent assembly of H3.3 nucleosomes on paternal DNA does not require the histone chaperone ASF1. Moreover, analysis of this mutant established that protamines are correctly removed at fertilization in the absence of HIRA, thus demonstrating that protamine removal and histone deposition are two functionally distinct processes. Finally, we showed that H3.3 deposition is apparently not affected in *Hira* mutant embryos and adults, suggesting that different chromatin assembly machineries could deposit this histone variant.

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Introduction

The assembly of nucleosome particles on nuclear DNA is the initial step for the formation of chromatin. Nucleosome assembly initiates with the formation of a H3-H4 histone tetramer on DNA followed by the addition of two H2A-H2B dimers to form the octameric particle [1,2]. Although this organisation of genomic DNA is remarkably conserved in eukaryotes, sperm cells of many species are characterized by a very different type of chromatin architecture involving non-histone proteins such as protamines [3]. The replacement of histones with protamines or other sperm nuclear basic proteins (SNBPs) during the differentiation of post-meiotic spermatids is generally associated with a high level of nuclear condensation, a general shutdown of transcriptional activity, and a state of chromatin that is incompatible with DNA replication [3–5]. Although the precise advantages of acquiring a specialized type of chromatin for the sperm cell are poorly known, the protamine type of chromatin could protect the paternal DNA from damaging agents or allow the resetting of epigenetic marks carried by histones [6–8]. In any case, once entered in the egg cytoplasm, the fertilizing sperm nucleus must replace its SNBPs with maternally provided histones that are stored in the egg cytoplasm. This process, called sperm chromatin remodelling (SCR), allows the paternal DNA to recover a nucleosomal chromatin and thus guarantees the ability of the male pronucleus to replicate its DNA in coordination with its female counterpart [3–5]. SCR can be separated into two key processes. The first process is the removal of SNBPs from the paternal DNA once the sperm nucleus is released in the egg cytoplasm. The second is the assembly of nucleosomes from maternal components before the first round of DNA replication. SCR has been almost exclusively studied in animal models that produce large quantities of eggs, such as amphibians or sea urchins, thereby facilitating the biochemical characterization of factors capable of remodelling sperm nuclei in vitro [3]. *Drosophila* embryonic extracts have also been used as a source of sperm chromatin decondensation factors [9–12], but none of the identified molecules has been demonstrated so far to have a function in SCR in vivo. In *Drosophila*, the sperm DNA is packaged with two protamines, whereas core histones are not detectable in male gamete nuclei [13,14]. In this sense, *Drosophila* represents a good model for the functional study of SCR in vivo. In previous publications, we characterized *sésame (ssm)*, a *Drosophila* maternal effect mutation that specifically prevented male pronucleus formation [15] and SCR [16]. This

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Abbreviations: HR1, homologous recombination 1; RC, replication coupled; RI, replication independent; SCR, sperm chromatin remodelling; SNBP, sperm nuclear basic protein; *snky, sneaky; sra, sarah; ssm, sésame;* TC, trancription coupled

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Author Summary

Chromatin is composed of basic units called nucleosomes, in which DNA wraps around a core of histone proteins. HIRA is a histone chaperone that is specifically involved in the assembly of nucleosomes containing H3.3, a universally conserved type of histone 3. To understand the function of HIRA in vivo, the authors generated mutant fruit flies with a non-functional Hira gene. Surprisingly, mutant flies were viable, but females were completely sterile. By analysing the female fruit flies' eggs, the authors found that in the absence of HIRA protein, the sperm nucleus was unable to participate in the formation of the zygote. In *Drosophila,* as in many animals, the condensed sperm chromatin contains protamines instead of histones. The authors found that the only crucial role of HIRA in flies was to assemble nucleosomes containing H3.3 in the male pronucleus, after the removal of protamines. This fundamental process, which is presumably also controlled by HIRA in vertebrates, allows the paternal DNA to reconstitute its chromatin and participate in the development of the embryo.

mutation was subsequently shown to cause a single amino acid substitution (R225K) in the *Hira* gene [17].

HIRA is a conserved chromatin assembly factor that allows the replication-independent (RI) deposition of core histones on DNA, in contrast to the CAF-1 complex whose replication-coupled (RC) nucleosome assembly activity is strictly linked to DNA synthesis [18]. Accordingly, it has been established in vitro that HIRA specifically deposits H3-H4 dimers that contain the histone H3 variant H3.3, which is expressed throughout the cell cycle, whereas CAF-1 deposits H3-H4 dimers that contain the replicative histone H3.1 [19]. Our functional analysis of the *Drosophila Hira* gene allowed us to demonstrate in vivo that HIRA was indeed involved in the RI deposition of H3.3 [17]. In addition, we observed that maternal HIRA localized in the decondensing sperm nucleus where it deposited H3.3-H4 histones before the first zygotic S phase, thus establishing the essential role of HIRA in SCR. Recently, the $Hira^{ssm}$ allele was found to enhance the variegation of a *white* reporter transgene, indicating that HIRA could help counteract the spread of heterochromatin by mediating histone replacement at specific sites [20]. However, because of the subtle nature of the $Hira^{ssm}$ mutation and the absence of obvious phenotype in mutant adults, it was not clear whether HIRA could have important functions during development or in adult flies. In this paper, we report the characterization of a loss of function *Hira* allele that we have generated by homologous recombination. Surprisingly, we show that paternal chromatin assembly at fertilization is the only developmental process that absolutely requires HIRA. We also demonstrate that protamine removal does not depend on HIRA and is thus functionally distinct from the paternal nucleosome assembly process. Finally, we show that H3.3 is deposited in the chromatin of mutant embryos and adults, suggesting that other factors are implicated in the assembly of H3.3 nucleosomes.

Results

Targeting the *Hira* Gene by Homologous Recombination

The original ssm^{185b} allele (referred to as $Hira^{ssm}$) is a point mutation that replaces an evolutionary conserved arginine with a lysine (R225K) in the N-terminus region of HIRA [17]. This region is characterized in all HIRA proteins by the presence of a well-conserved domain containing seven WD-repeats. WD-repeats assemble into a structure called beta-propeller [21]. The $Hira^{ssm}$ mutation does not affect the normal recruitment of HIRA in the male nucleus at fertilization [17]. Nevertheless, it completely prevents the deposition of histones on paternal DNA [16,17], suggesting that the beta-propeller domain is important for the nucleosome assembly activity of HIRA. To gain insight into other possible functions of *Hira* not evident from the subtle $Hira^{ssm}$ mutation, we generated a new mutant allele using ends-out homologous recombination [22]. The targeting construct was designed to delete a 319 bp DNA fragment encompassing the complete predicted 5′ UTR, the first exon, the first intron, and the 5′ part of the second exon of *Hira*. In addition, the recombination arms used in this construct did not overlap any other predicted coding sequence, thus minimizing the risk of damaging adjacent genes. Finally, in the recombined allele, the 319 bp deletion was replaced with a 4778 bp sequence from the pW25 vector [23], containing the *white* marker gene flanked with stop codons in the six reading frames (Figure 1A). We recovered 59 independent recombination events on the X chromosome that did not complement the 100% female sterility associated with the $Hira^{ssm}$ mutation (Table 1). Surprisingly, all these lines produced viable and fertile mutant males. In all the lines that were further examined ($n = 7$), homozygous mutant females were also viable but produced embryos that never hatched (unpublished data). One line, named $Hira^{HR1}$ (homologous recombination 1), was arbitrarily chosen to conduct the rest of the analysis. The nature of the molecular lesion at the $Hira^{HR1}$ locus was verified by PCR analysis and sequencing of genomic DNA, and the expected recombination event was found, with no other detectable alteration (Figure 1B and unpublished data). We verified that the maternal effect phenotype associated with $Hira^{HR1}$ remained unchanged in hemizygous $Hira^{HR1}$/*Df(1)ct4b1* females, *Df(1)ct4b1* being a large X chromosome deficiency that covers the *Hira* region [15]. In addition, the $Hira^{HR1}$ phenotype was fully rescued by a single copy of a wild-type *Hira* transgene [17], demonstrating that no other important gene was affected by the $Hira^{HR1}$ recombination event (unpublished data).

The $Hira^{HR1}$ mutation was expected to destroy the normal transcriptional regulation of *Hira*. However, transcriptional activity was detected by RT-PCR analysis at the junction between the pW25 vector and the beginning of the *Hira* sequence (unpublished data), suggesting that the $Hira^{HR1}$ allele could be transcribed from the hsp70 promoter associated with the w^{hs} marker gene or from another promoter in or upstream from the pW25 vector. To check for the translation of any truncated HIRA protein from the $Hira^{HR1}$ allele, we first established transgenic lines containing a pW25-$Hira^{HR1}$-Flag transgene (Figure 1A). This construct is identical to the donor transgene used for the homologous recombination with the exception of a 3X-Flag tag fused in frame to the C-terminus of HIRA. RT-PCR analysis of two independent pW25-$Hira^{HR1}$-Flag lines confirmed that the *Hira* sequence in these transgenes is also transcribed (unpublished data). However, western-blot analysis of embryo extracts from both lines did not detect any HIRA-FLAG protein (Figure 1C).

We then directly tested the presence of HIRA in eggs from

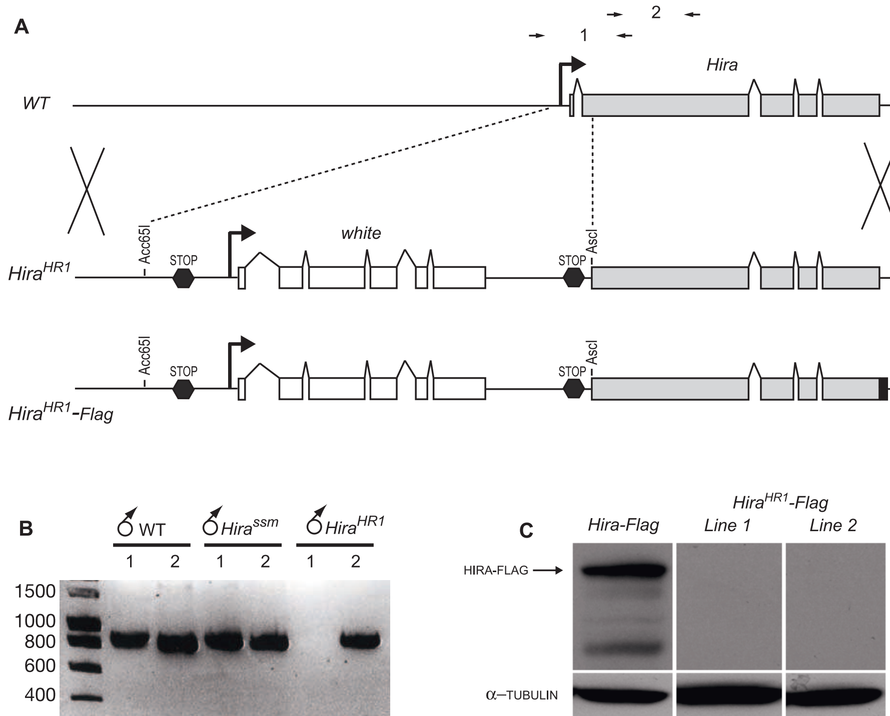

Figure 1. Targeting the *Hira* Gene by Homologous Recombination

(A) Schematic representation of the wild-type (WT) *Hira* locus, the $Hira^{HR1}$ recombined allele, and the *pW25*-$Hira^{HR1}$-*Flag* reporter transgene. The dotted lines indicate the region that is replaced by the *pW25* vector sequence in $Hira^{HR1}$. The gray and white boxes indicate the *Hira* and *white* exons, respectively, and the black box is the *3X-Flag* tag at the 3′ end of the *pW25*-$Hira^{HR1}$-*Flag* transgene. The dark gray hexagons represent termination codons in the six reading frames. The positions of the primer pairs used in (B) are shown (arrows).

(B) Example of a genomic PCR with the primer pairs shown in (A). Note that the primer pair #1 does not amplify the large *pW25* insertion in the $Hira^{HR1}$ allele. The tested male genotypes are indicated.

(C) Anti-FLAG and anti-tubulin western blot analysis of embryo extracts from *Hira-Flag* and $Hira^{HR1}$-*Flag* transgenic lines. The arrow indicates the HIRA-FLAG protein. Other smaller bands are interpreted as HIRA-FLAG degradation products.

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$Hira^{HR1}$ females using two independent HIRA polyclonal antibodies. The first antibody was raised against a mix of two synthetic HIRA oligopeptides [17] whose cognate DNA coding sequences are intact in the $Hira^{HR1}$ allele. The second antibody was raised against a recombinant protein containing residues 381–935 of HIRA (see Methods). Both sera readily detect maternal HIRA in wild-type and $Hira^{ssm}$ fixed eggs, as the protein specifically accumulates in the male pronucleus (Figure 2A, 2C, and 2D). As reported before [17], at the pronuclear apposition stage in $Hira^{ssm}$ eggs, the male pronucleus appeared much more condensed and smaller than the female pronucleus and brightly stained with anti-HIRA antibodies (Figure 2D). In $Hira^{HR1}$ eggs at the same stage, the male pronucleus looked identical to that in $Hira^{ssm}$ eggs, but did not contain any detectable HIRA protein (Figure 2B and 2E).

Considering the fact that maternal HIRA protein is immediately available at fertilization to assemble paternal chromatin, we speculated that the protein must accumulate in growing oocytes during oogenesis. Indeed, wild-type ovaries stained with anti-HIRA antibodies revealed a specific signal in the oocyte nucleus (also called germinal vesicle) that was well visible from stage 10 of egg chamber formation (Figure 3A). The same staining of the oocyte nucleus was obtained with transgenic *Hira-Flag* ovaries stained with anti-FLAG antibodies (Figure 3C). Strikingly, the germinal vesicle staining was absent in $Hira^{HR1}$ ovaries and $Hira^{HR1}$-*Flag* ovaries stained with anti-HIRA or anti-FLAG antibodies, respectively (Figure 3B and 3D). Altogether, these results strongly support the hypothesis that no HIRA protein is produced from the $Hira^{HR1}$ mutant allele.

The $Hira^{HR1}$ and $Hira^{ssm}$ Phenotypes at Fertilization Are Indistinguishable

Previous studies of the $Hira^{ssm}$ allele had revealed that the male nucleus in mutant eggs was unable to undergo SCR [16]. Despite the fact that the mutant HIRA protein normally accumulates in the male nucleus in $Hira^{ssm}$ eggs ([17] and Figure 2D), it is unable to assemble chromatin. Consequently, the male nucleus does not achieve its decondensation and does not replicate its DNA.

At the cytological level, fertilized eggs from $Hira^{HR1}$ females appeared phenotypically identical to $Hira^{ssm}$ eggs. In all cases observed ($n > 100$), the male pronucleus remained abnormally small and condensed after pronuclear apposition (Figure 2E) and was unable to participate in the formation of the zygote (see Figure 4). As a consequence of this early defect, embryos from $Hira^{HR1}$ females were haploid, with only the maternal chromosome set.

To check for any RI nucleosome assembly in $Hira^{HR1}$ eggs, we used an anti-acetylated histone H4 antibody that brightly and specifically stains the decondensing male nucleus in wild-type eggs [17]. As expected, the massive RI nucleosome assembly that normally occurs during male pronucleus

Table 1. *Hira* Ends-Out Targeting

Donor Line	Chromosome	Phenotype	Number of Female Germlines[a]	Number of w+ Progeny[b]	Number of HR Events[c]	Percent[d]
DL1	3	WT	3122	106	30	0.96
DL2	2	Lethal	995	24	5	0.50
DL3	2	WT	2015	62	15	0.74
DL4	3	Fs	166	7	1	0.60
DL5	3	WT	714	17	6	0.84
DL6	2	Lethal	145	5	2	1.38
Total	—	—	7157	221	59	0.82

Results of the targeting experiments are given for each independent *pW25-Hira* transgenic donor line.
Chromosome, chromosome insertion of the donor line; Phenotype, Phenotype of the homozygous donor insertion; WT, viable and fertile; fs, female sterile; HR, homologous recombination.
[a]Number of screened F1 virgin females with the *pW25-Hira* donor transgene and the *P{70FLP}11 P{70I-SceI}2B, Sco* Chromosome with *white* or mosaic eyes.
[b]Number of vials of four females crossed with *P{70FLP}10* males that gave *white+* progenies.
[c]Number of independent *white+* chromosomes that did not complement the $Hira^{ssm}$ phenotype.
[d]c/a × 100. Note that the actual rate of homologous recombination is possibly slightly underestimated because females were screened in vials of four individuals.
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formation was not detected in $Hira^{HR1}$ eggs (Figure 4A and 4B). In contrast, RC deposition of acetylated H4 was normally detected in maternal nuclei (Figure 4C). Thus both $Hira^{ssm}$ and $Hira^{HR1}$ mutant alleles specifically prevent assembly of paternal chromatin and do not affect maternal nuclei.

HIRA Is Not Involved in the Removal of Protamines from the Fertilizing Sperm Nucleus

In *Drosophila*, during spermiogenesis, post-meiotic spermatid nuclei progressively elongate and condense to eventually reach the typical needle-shape of mature sperm nuclei [24]. This complex process is also characterized by the replacement of histones with SNBPs, including two closely related protamines, ProtA and ProtB [13,14]. At fertilization, protamines are removed from the paternal chromatin, and nucleosomes are assembled in an RI process before the onset of the first zygotic S phase. The incapacity of the male nucleus to form in $Hira^{ssm}$ eggs led us to hypothesize that this phenotype could result from a defect in protamine removal [16]. Indeed, we would expect the persistence of protamines on paternal DNA to prevent nucleosome assembly and male nucleus decondensation. However, the presence of the HIRA protein in the male nucleus in $Hira^{ssm}$ eggs precluded drawing any conclusion about its role in protamine removal [13]. In contrast, the $Hira^{HR1}$ allele allowed us to address this point because in this case the protein is absent from the male nucleus. To document the dynamics of protamine removal at fertilization, we used transgenic males expressing ProtA-GFP or ProtB-GFP in their germ line [13]. These males are fertile and their testes contain groups of spermatid nuclei that achieve maximum fluorescence toward the end of the condensation process (Figure 5A, left panel). To verify that protamine-GFP can be detected in eggs, we crossed wild-type females with *ProtA-GFP* males homozygous for *sneaky* (*snky*), a paternal effect mutation that prevents sperm plasma membrane breakdown at fertilization and sperm activation [25]. We found that fertilizing sperm nuclei from *ProtA-GFP ; snky* males were brightly fluorescent in all cases observed (Figure 5B). We then looked at wild-type and $Hira^{HR1}$ eggs fertilized with *ProtA-GFP* or *ProtB-GFP* sperm. Even in the earliest eggs we observed, we never detected any trace of Prot-GFP in the decondensing male nucleus (Figure 5C and 5D). We thus concluded that the removal of protamines from the fertilizing sperm nucleus is a fugacious, HIRA-independent process that must occur immediately after sperm plasma membrane breakdown and before the onset of the second meiotic division.

RI Paternal Chromatin Assembly Does Not Depend on Egg Activation

In *Drosophila*, mature oocytes are arrested in metaphase of the first meiotic division until egg ovulation and activation. In contrast to many animals, egg activation in flies is not dependent on fertilization. Instead, eggs are reactivated during ovulation and immediately resume meiosis [26]. *Drosophila* females with a mutated *sarah* (*sra*) gene lay eggs that are defective in several aspects of egg activation, including a meiotic block in anaphase of the first division [27]. Interestingly, these authors observed that the male pronucleus in fertilized *sra* eggs remained abnormally condensed and did not replicate its DNA. This aspect of the sra phenotype presents striking similarities with the Hira mutant phenotype, raising the possibility that HIRA activity could depend on egg activation. In their paper, Horner et al. observed that the male nucleus and maternal chromosomes stained, although rather diffusely, with an anti-histone H1 antibody. They concluded that paternal chromatin remodelling was not impaired in *sra* eggs. However, it has been previously reported that early *Drosophila* embryos lack histone H1 [28], opening the possibility that anti-H1 antibodies could cross-react with a non-H1 epitope. To directly analyse paternal chromatin assembly in *sra* eggs, we used anti-acetylated-H4 antibodies. In all cases, the condensed male nucleus, but not the maternal chromosomes, brightly stained with the anti-acetylated-H4 antibody, confirming that paternal chromatin assembly is not dependent on egg activation (Figure 6A). In addition, we verified that ProtA-GFP was not detected from the male nucleus in *sra* eggs fertilized with *ProtA-GFP* males (unpublished data).

In *sra* eggs blocked in anaphase of the first meiotic division, the male nucleus frequently presented a rather irregular shape (Figure 6A) and an apparent level of DNA condensa-

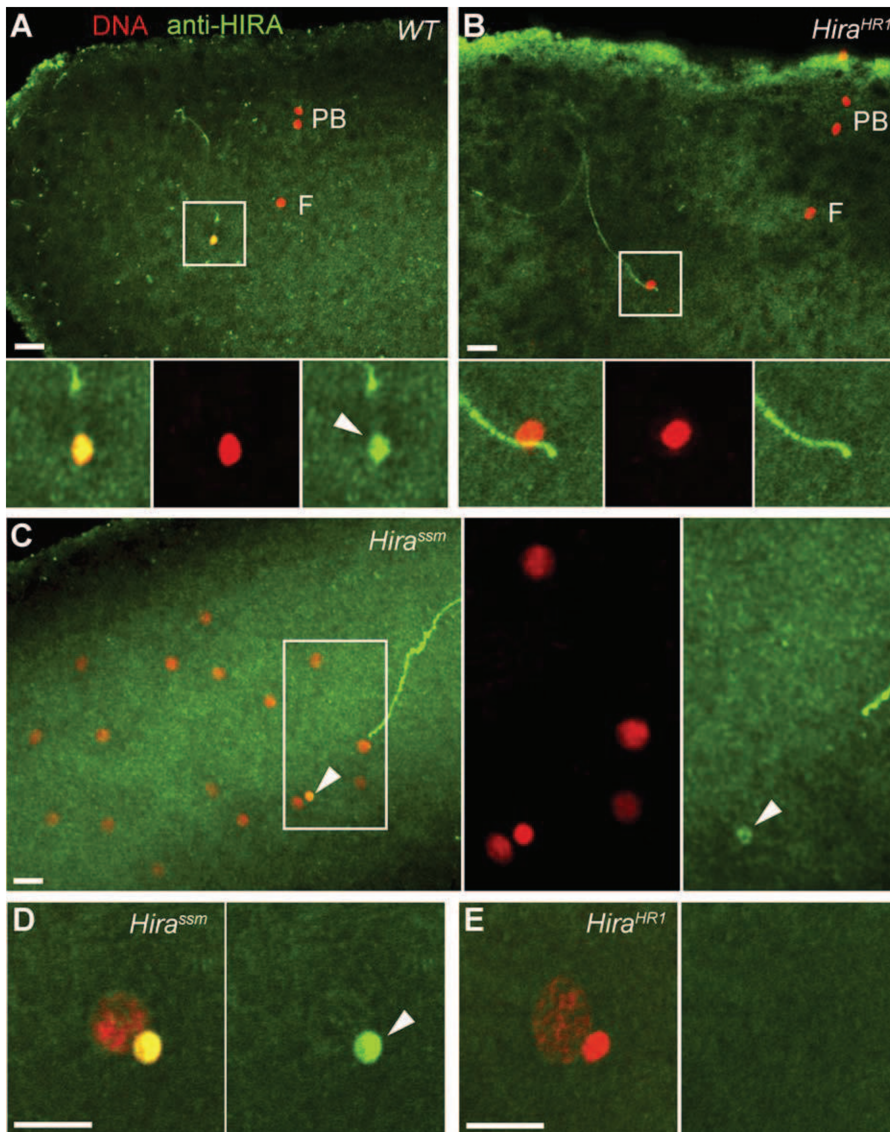

Figure 2. HIRA Is Not Detected in *Hira*^{HR1} Eggs

Confocal sections of eggs or embryos stained for DNA (red) and anti-HIRA antibodies (green).
(A) In wild-type (WT) fertilized eggs, HIRA is specifically detected in the male nucleus (arrowhead in the inset).
(B) In eggs from *Hira*^{HR1} females, HIRA is not detected in the male nucleus (inset). Note that the HIRA antibody 830 non-specifically binds the sperm tail (elongated structure visible in the green channel) [17].
(C) A Cycle 5 haploid embryo from a *Hira*^{ssm} female stained with antibody 830. The only stained nucleus is the condensed male nucleus (arrowheads).
(D) Apposed pronuclei in a *Hira*^{ssm} egg stained with HIRA antibody PG1 showing a strong signal in the male nucleus (arrowhead).
(E) A *Hira*^{HR1} egg at the same stage stained with the same antibody. F: Female pronucleus. PB: Polar Bodies. Bars: 10 μm.
doi:10.1371/journal.pgen.0030182.g002

tion that was comparable with the highly condensed maternal chromosomes blocked in anaphase I of the first meiotic division. Hence, the high level of cyclin B in *sra* eggs that causes the meiotic block [27] could also affect the male nucleus and force it to recondense its unreplicated chromatin. In comparison to *sra*, the male nucleus in *Hira*^{ssm} mutant eggs is a uniformly round nucleus that systematically adopts its definitive shape by the end of female meiosis II [17]. To see if the *Hira*^{ssm} male nucleus could recondense in *sra* eggs, we constructed double mutant *Hira*^{ssm}/*Hira*^{ssm} ; *sra*^{A108}/*Df(3R)sbd45* females. In fertilized eggs from these double mutant females, we observed that the male nucleus did not stain with anti-acetylated-H4 antibodies and looked identical in shape and size to the male nucleus in *Hira*^{ssm} eggs (Figure 6B). Thus, in the absence of an assembled chromatin, the male nucleus is unable to recondense in response to the meiotic block of *sra* eggs.

The ASF1 Histone Chaperone Is Not Involved in the RI Assembly of Paternal Chromatin

SCR provides a unique opportunity to study de novo nucleosome assembly in vivo at the scale of a whole nucleus and in the absence of DNA synthesis or transcription. A striking feature of this process is the very specific use of the H3.3 histone variant to assemble paternal nucleosomes, despite the presence of large quantities of canonical H3

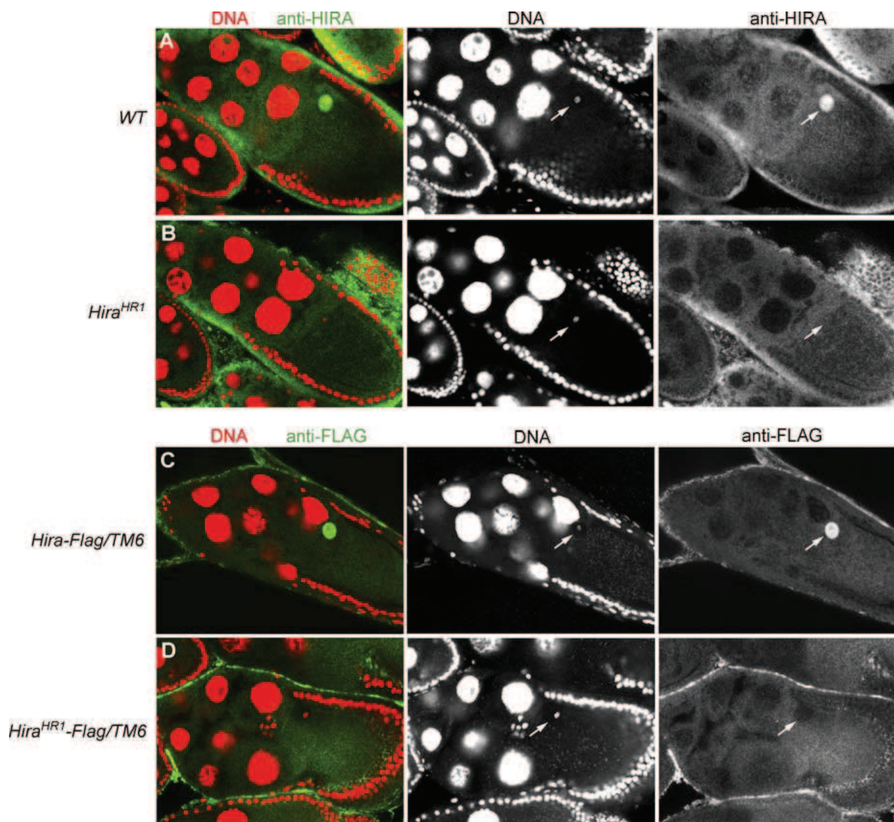

Figure 3. HIRA Accumulates in the Germinal Vesicle in Wild-Type but Not in *Hira*^{HR1} Oocytes

Stage 10 egg chambers stained for DNA (red) and anti-HIRA PG1 or anti-FLAG antibodies (green).

(A) In wild-type egg chambers, HIRA is specifically detected in the germinal vesicle where it occupies the whole nuclear volume. The karyosome, the compact structure containing the maternal chromosomes, is visible in the DNA channel (arrow).

(B) In *Hira*^{HR1} egg chambers, the antibody does not detect HIRA in the germinal vesicle (arrow).

(C) In transgenic *Hira-Flag* egg chambers, HIRA-FLAG protein is found in the germline vesicle (arrows) like the endogenous protein.

(D) No HIRA-FLAG protein is detected in the oocyte nucleus in *Hira*^{HR1}*-Flag* transgenic egg chambers.

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stored in the egg cytoplasm. ASF1 is a conserved histone chaperone involved in the assembly of chromatin during DNA replication (reviewed in [29]). Recent studies have shown that ASF1 specifically interacts with H3-H4 dimers [30,31] and with HIR proteins [32,33], and could play a key role in presenting dimers containing specific H3 variants to their corresponding chaperones, such as H3 to CAF-1 and H3.3 to HIRA [29,31,33]. Accordingly, ASF1 proteins are found in both H3.1 and H3.3 complexes in human cells [19]. To investigate this possibility in our model, we stained fertilized eggs with an antibody against the unique *Drosophila* ASF1 protein [34]. We observed that ASF1 was systematically detected in replicating nuclei, including the pronuclei (Figure 7C). However, ASF1 was not found on the decondensing male nucleus in wild-type eggs or in the male nucleus in *Hira* mutant eggs (Figure 7A, 7B, 7D, and 7E). Thus, ASF1 does not directly cooperate with HIRA during the RI assembly of paternal chromatin. This is consistent with a recent report showing that ASF1 is dispensable for direct de novo histone deposition in Xenopus egg extracts [35]. So far, HIRA is the only H3-H4 chaperone involved in SCR in vivo.

H3.3 Deposition Is Not Globally Affected in *Hira*^{HR1} Mutant Embryos and Adults

The analysis of the *Hira*^{HR1} allele confirmed the essential role of maternal HIRA for the RI chromatin assembly in the male pronucleus. In *Drosophila*, early development is under maternal control and zygotic transcription essentially begins at the blastoderm stage [26]. In embryos, HIRA antibodies did not produce any detectable staining, suggesting that the protein, if it plays any role, does not accumulate at high levels in embryo nuclei like in the male pronucleus (unpublished data). Haploid embryos laid by *Hira*^{HR1} females (named *Hira*^{HR1} embryos for simplicity) arrest their development just before hatching. We used this situation to study H3.3 deposition in wild-type and *Hira*^{HR1} early embryos. We used a previously described transgenic line expressing H3.3-FLAG under the regulatory sequences of the *Drosophila His3.3A* gene [17]. Maternally expressed H3.3-FLAG was then revealed using anti-FLAG antibodies. Zygotically expressed H3.3-FLAG becomes detectable in chromatin only at the gastrula stage (Figure 8I and 8J) and was thus not detected in our experiments on early embryos. As reported before [17], in wild-type eggs, H3.3-FLAG is first detected in the decondensing male nucleus shortly after fertilization (Figure 8A). As expected, the male nucleus does not contain any H3.3-FLAG in *Hira*^{HR1} eggs, confirming the absence of chromatin assembly in the male nucleus (Figure 8B). At the pronuclear apposition stage in wild-type eggs, after the first round of DNA replication, H3.3-FLAG is still abundant in the male nucleus, but a faint staining is also visible in the female

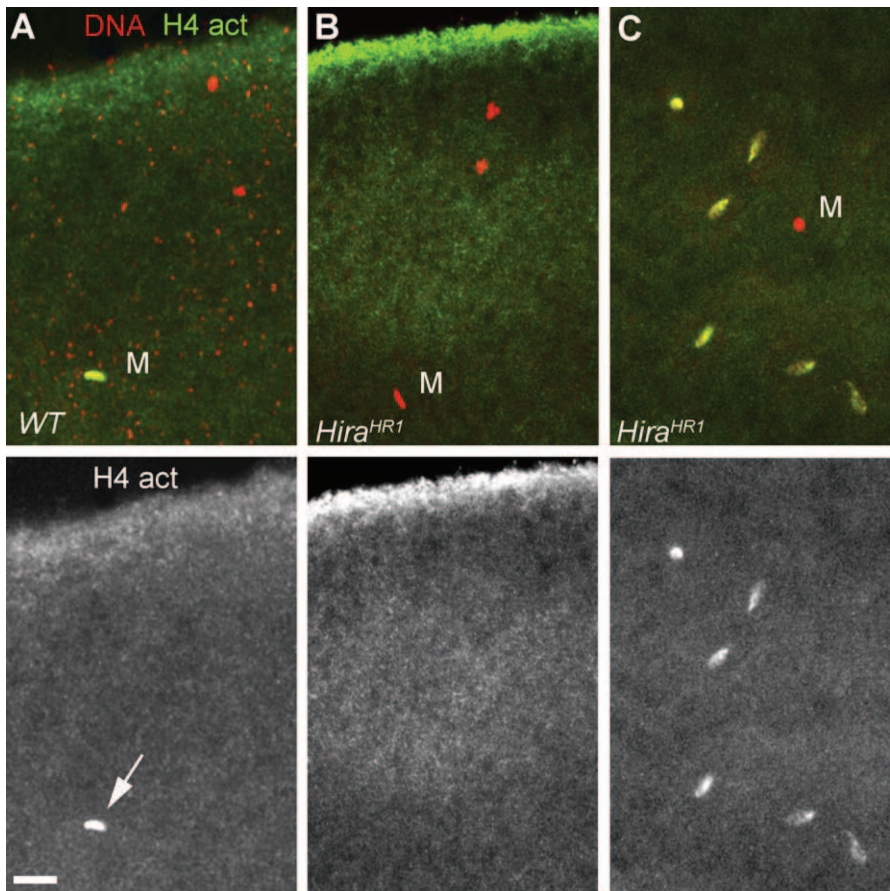

Figure 4. *Hira*^{HR1} Eggs Are Unable to Assemble Paternal Chromatin at Fertilization

Confocal sections of eggs and embryos stained for DNA (red) and anti-acetylated histone H4 antibody (green).

(A) A wild-type egg in meiosis II with the elongated fertilizing male nucleus (M) that brightly stains for acetylated-H4 (arrow).

(B) A *Hira*^{HR1} egg at the same stage with no acetylated-H4 detected in the male nucleus.

(C) A cycle 3 haploid embryo from a *Hira*^{HR1} mother. The maternal nuclei, but not the male nucleus, stain for acetylated-H4. Bar: 10 μm.

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pronucleus (Figure 8C) and polar bodies (unpublished data). Interestingly, this H3.3-FLAG staining in the female pronucleus is also detected in *Hira*^{HR1} eggs at the same stage (Figure 8D). H3.3 can be deposited on DNA through a transcription-coupled (TC) assembly mechanism, suggesting that the passage of the RNA polymerase complex displaces nucleosomes and creates a need for RI assembly [36]. In the absence of transcription in early *Drosophila* embryos, the observed H3.3-FLAG must occur through a transcription-independent process, presumably during DNA replication. In wild-type embryos, we observed that the initial enrichment of H3.3-FLAG on paternal chromosomes was still detectable during the first 3 or 4 nuclear cycles (Figure 8E). In *Hira*^{HR1} early embryos, only a faint H3.3-FLAG staining was detected on the sole maternally derived set of chromosomes (Figure 8F). The paternal H3.3 mark in wild-type embryos was no longer detectable in later embryos (unpublished data) suggesting a rapid dilution by the massive RC deposition of H3 that occurs at each S phase. To verify this point, we used a transgenic line that expresses H3-FLAG with the regulatory sequences of *His3.3A* [17]. Both H3-Flag and H3.3-Flag transgenes produce equivalent levels of tagged histones in embryos [17] and allow a direct comparison of their respective deposition during early development. During the earliest mitoses, the H3-FLAG staining on chromosomes was much stronger than the H3.3-FLAG staining (Figure 8K, compare with Figure 8E), confirming that H3 is much more efficiently incorporated in chromatin than H3.3 at this stage. The difference between H3.3-FLAG and H3-FLAG chromosome staining was also visible in blastoderm embryos (Figure 8G and 8L). At the blastoderm stage, H3.3-FLAG clearly marked the chromatin of all nuclei in both WT and *Hira*^{HR1} (Figure 8G and 8H). In conclusion, although H3 is preferentially deposited during the early nuclear cycles, our results demonstrate that H3.3 is also deposited at this stage, through a HIRA-independent assembly pathway. Further work will be required to determine whether this HIRA-independent H3.3 deposition occurs during or independently of DNA replication.

The migration of nuclei at the embryo periphery correlates with the onset of zygotic transcription, with the notable exception of germ line pole cells that are kept silent until stage 9/10 of embryo development [37]. Interestingly, we observed that H3.3-FLAG is deposited at equivalent levels in somatic and in pole cell nuclei in both wild-type and *Hira*^{HR1} embryos (Figure 9). Thus, TC assembly does not seem to contribute substantially to the observed level of H3.3-FLAG in chromatin at this stage. The activation of the zygotic genome in blastoderm embryos correlates well with the

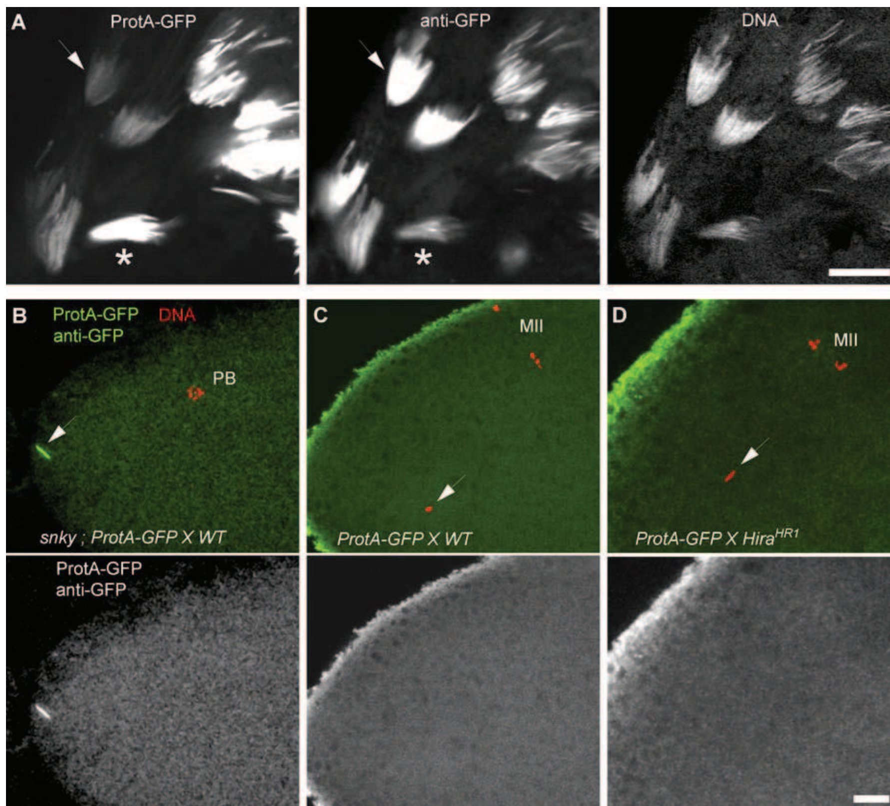

Figure 5. HIRA Is Not Required for Protamine Removal from the Decondensing Sperm Nucleus

(A) Left panel: in a fixed *ProtA-GFP* transgenic testis, the GFP fluorescence is very strong in the most condensed spermatid nuclei (asterisk), whereas less condensed nuclei are much less bright (arrow). Middle panel: the same testis stained with an anti-GFP antibody considerably enhances the GFP detection in less condensed nuclei (arrow, compare with left panel), whereas highly condensed nuclei are comparatively less stained. Right panel: the same testis stained with the DNA dye TO-PRO3.

(B) In wild-type (WT) eggs fertilized with sperm from *snky*^{1} ; *ProtA-GFP* males, the sperm nucleus is not activated (arrow), remains at the egg periphery, and its protamines are not removed.

(C) In wild-type eggs fertilized with *ProtA-GFP* sperm and fixed before the end of meiosis II (MII), ProtA-GFP is never detected in the decondensing male nucleus (arrows).

(D) The same result is obtained for *Hira*^{HR1} eggs. Eggs in (B–D) were stained with an anti-GFP antibody revealed with a green secondary antibody to cumulate the GFP and secondary antibody respective fluorescence in the green channel of the confocal microscope. Identical results were obtained with ProtB-GFP transgenic males (unpublished data). PB: Polar Body. Bar: 10 μm.

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apparition of histone post-translational modifications associated with transcriptionally active chromatin, such as the methylation of histone H3 at lysine 4 [38]. Figure 9 shows that this active mark is normally detected in *Hira*^{HR1} embryos, suggesting that HIRA is not required for the remodelling of chromatin associated with the onset of zygotic transcription. Accordingly, *Hira*^{HR1} embryos develop without obvious problems until late embryogenesis and eventually arrest development with a phenotype typical of haploid embryos produced by other mutants ([39,40] and unpublished data).

That *Hira*^{HR1} flies are viable offered us the possibility to evaluate the impact of the mutation on H3.3-FLAG distribution in adult tissues. We chose to focus on the testis, an organ where H3.3 distribution had been characterized already [41]. In wild-type transgenic adult testis, we observed a strong nuclear staining of H3.3-FLAG in all somatic and germline nuclei with the exception of late spermatid and sperm nuclei, similar to previous reports [41]. In *Hira*^{HR1} testis we found no detectable alteration of the distribution of H3.3-FLAG in both somatic and germ line nuclei (Figure 10). We then looked at other adult tissues including ovaries, malpighian tubules, and gut; again, we found no difference between control and mutant (unpublished data). We conclude that, with the sole exception of the male pronucleus, HIRA does not seem to play any crucial role for the assembly of H3.3 nucleosomes during *Drosophila* development.

Discussion

HIRA and SCR

The analysis of maternal effect mutations in the *Drosophila Hira* gene has revealed that SCR at fertilization involves at least two functionally distinct steps. The first step is a HIRA-independent process that allows the rapid removal of protamines from the activated sperm nucleus. The second step is the RI nucleosome assembly on paternal DNA and requires maternal HIRA. That the male pronucleus seems to be the only nucleus where H3.3 deposition is critically dependent on HIRA (see below) indicates a peculiar case of RI assembly. This could reflect specific features of the sperm nucleus itself or constraints inherent to the tightly time-controlled, whole paternal genome assembly at fertilization. At least we know that this specific requirement of HIRA for SCR is not directly linked to the removal of protamines.

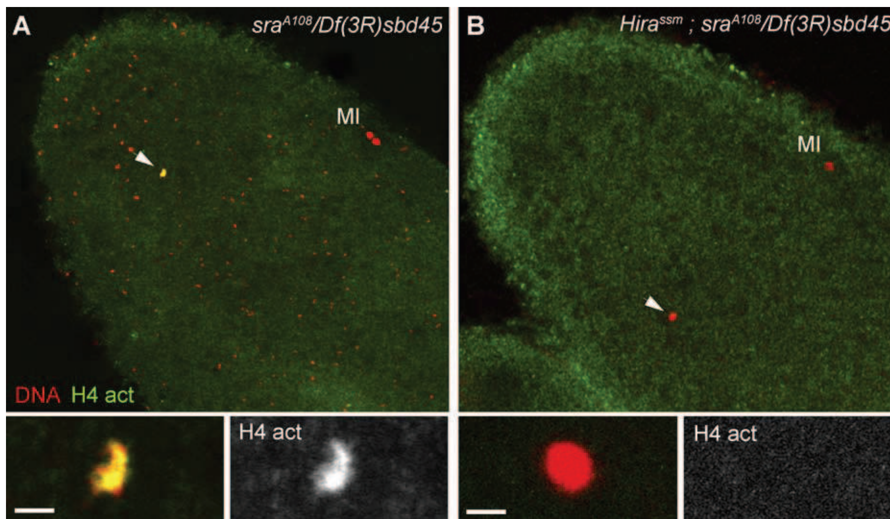

Figure 6. The Male Nucleus Does Not Recondense in *Hira* ; *sra* Double Mutant Eggs

(A) In *sra^{A108}/Df(3R)sbd45* mutant eggs, the female meiosis arrests in anaphase of the first meiotic division (MI). The male nucleus (arrowhead and bottom panels) appears condensed but irregular in shape and stains with anti-acetylated histone H4 antibodies (bottom right panel). Note that the DNA positive dots that are visible in this egg are *Wolbachia* bacteria that naturally infect the stock.

(B) In *$Hira^{ssm}$; sra^{A108}/Df(3R)sbd45* Double Mutant Eggs, the Male Nucleus Is Round and Does Not Stain with Anti-Histone Antibodies. Bars: 2 μm.

doi:10.1371/journal.pgen.0030182.g006

Our finding that SNBP removal activity is functionally uncoupled to nucleosome assembly in *Drosophila* does not apply to all known cases of SCR in animals. In fact, in the classical example of SCR in *Xenopus laevis*, it was demonstrated through in vitro experiments that a unique histone chaperone, nucleoplasmin, was necessary and sufficient to perform both SNBP removal and histone deposition [42,43]. Nucleoplasmin is a small, acidic protein that is highly abundant in amphibian oocytes and forms pentameric complexes that associate with core histones [2,44,45]. It is important to consider, however, that the protein composition of *Xenopus* sperm chromatin is rather peculiar since it essentially retains H3-H4 tetramers on paternal DNA, whereas H2A and H2B are replaced with protamine-like proteins named SPs [43,46]. In vitro, nucleoplasmin allows the replacement of SPs with H2A and H2B and reconstitute nucleosomes [43,44]. There is apparently no need for a H3-H4 assembly factor such as HIRA for *Xenopus* SCR. A nucleoplasmin-like protein exists in *Drosophila*, but studies of its ability to decondense demembranated *Xenopus* sperm nuclei in vitro have led to contradictory results [11,12]. The actual function of *Drosophila* nucleoplasmin remains to be determined. In addition, other *Drosophila* embryonic nuclear factors are known to decondense *Xenopus* sperm in vitro, such as DF31 [10] and NAP-1 [11], but their protamine removal activity has not been confirmed in vivo. In mouse, as in *Drosophila*, sperm chromatin is essentially packaged with protamines [47]. Interestingly, the knock-out of *NPM2*, the mouse ortholog of *Xenopus* nucleoplasmin, does not affect SCR [48]. In contrast, HIRA is very likely involved in the assembly of paternal chromatin in the mouse zygote. Indeed, in this species, HIRA is detected in the decondensing male nucleus [49] and H3.3 is specifically deposited on paternal DNA in an RI manner [49,50]. We thus expect HIRA to be generally involved in the assembly of paternal chromatin in animal species in which histones H3 and H4 are totally or partially replaced with SNBPs in the mature sperm. As an H3.3-H4 deposition factor, HIRA itself is not expected to mediate the deposition of H2A-H2B required for the completion of nucleosome assembly on paternal DNA. It will be interesting to identify this H2A-H2B chaperone and see if it is dedicated to RI assembly or involved in both RI and RC assembly pathways.

In *Hira* mutant eggs, the male nucleus is a small, round nucleus that appears homogeneously condensed when stained with a DNA dye. How the paternal DNA is organised in this nucleus is not known. That it is surrounded by a de novo assembled nuclear lamina [16] probably participates in the maintenance of its round shape. Also, it is established that the four centromeric regions are the only regions that are organized with histones, most likely because centromeric chromatin is not replaced with protamines in the sperm nucleus [16]. In this paper, we have demonstrated that the male nucleus in *Hira* mutant eggs is also devoid of protamines, strongly suggesting that most paternal DNA is free of chromosomal proteins. A similar situation was reported in decondensation assays using sperm from *Bufo japonicus*, a toad species whose sperm chromatin only contains protamines [51]. In the presence of nucleoplasmin, protamines are efficiently removed but nucleosomes are not assembled. Consequently, *B. japonicus* sperm nuclei decondensed with egg extracts containing the protamine removal activity possess neither protamines nor core histones, and are very fragile [51]. Similarly, in *Hira* mutant eggs, the removal of protamines from the male nucleus permits its partial decondensation as the sperm nuclear volume increases when the nucleus loses its specific needle shape and becomes round. However, in the absence of a nucleosomal organisation, the male nucleus cannot achieve its decondensation and does not replicate its DNA. This unique, inert state of the male nucleus in *Hira* mutant eggs is also well illustrated by its incapacity to recondense in blocked *sra* mutant eggs.

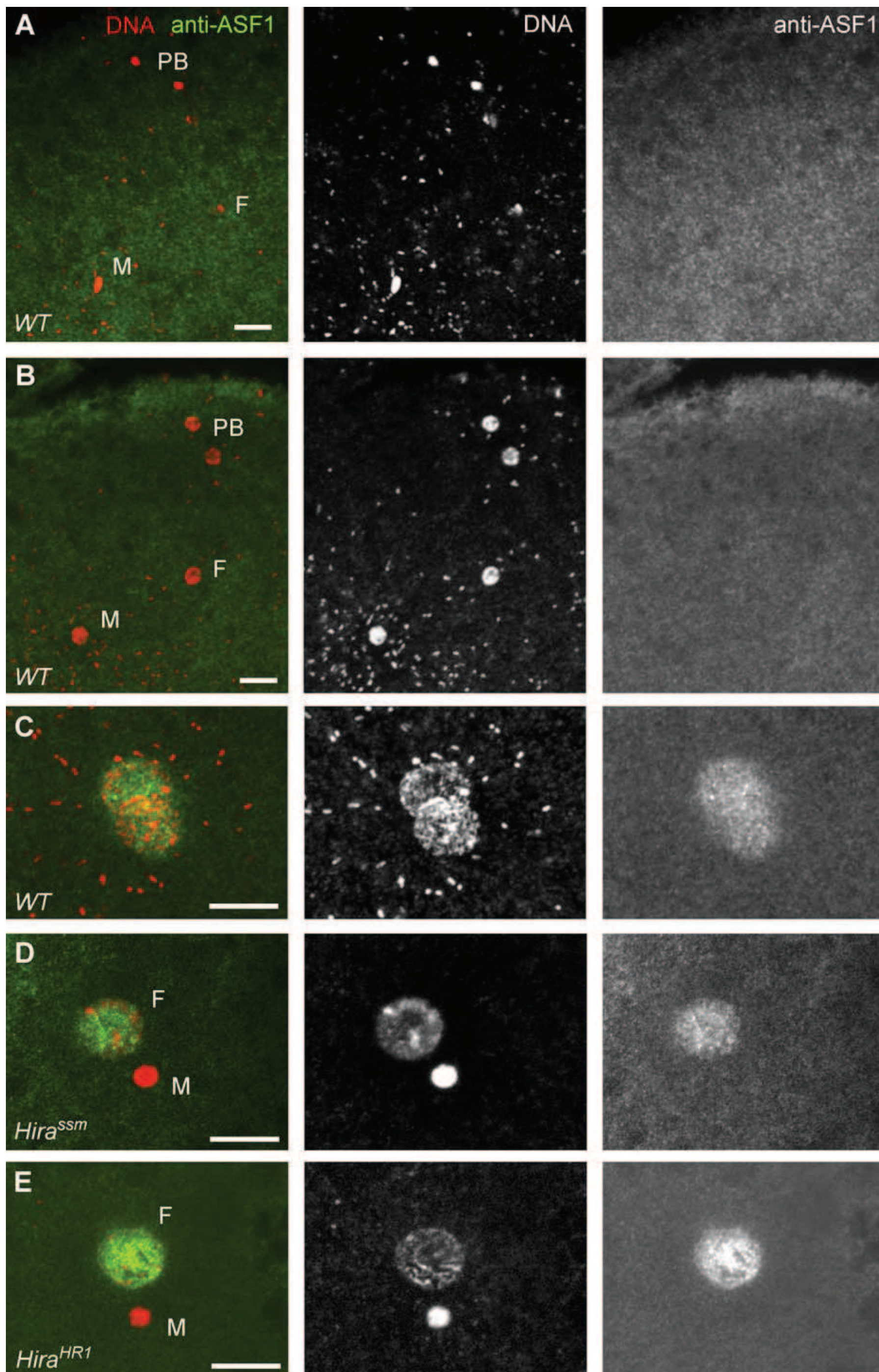

Figure 7. ASF1 Is Not Directly Involved in the RI Paternal Chromatin Assembly

Confocal sections of eggs stained for DNA (red) and anti-ASF1 antibody (green).

(A) In wild-type fertilized eggs, ASF1 is not detected in the male nucleus or in maternal nuclei during the decondensation phase.

(B) ASF1 is not detected in the male nucleus during pronuclear migration.

(C) ASF1 stains both pronuclei in a wild-type egg during the first S phase.

(D) ASF1 is not detected in the male nucleus in $Hira^{ssm}$ eggs.

(D) The same result was obtained with the $Hira^{HR1}$ allele. F: Female pronucleus, M: Male pronucleus, PB: Polar Bodies. Bar: 10 μm.

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The Function of HIRA during *Drosophila* Development

A surprising aspect of this study is the viability of $Hira^{HR1}$ homozygous flies. This was unexpected, because in mouse the *Hira* knock-out is embryonically lethal [52]. From a genetic point of view, both $Hira^{ssm}$ and $Hira^{HR1}$ alleles behave as null alleles with respect to the *Df(1)ct4b1* deficiency. In addition, several lines of evidence indicate that no HIRA protein is translated in $Hira^{HR1}$ flies, including the absence of detection of HIRA in the germinal vesicle and the male pronucleus, and the absence of HIRA-FLAG protein expressed from the

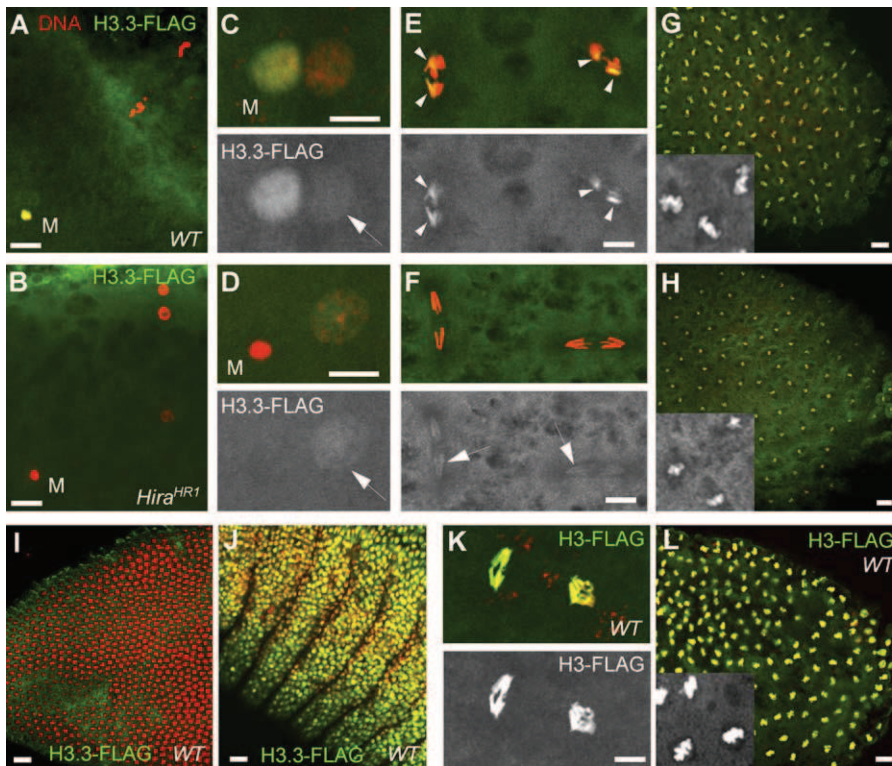

Figure 8. Dynamics of H3.3 Deposition in Wild-Type and *Hira*^{HR1} Early Embryos

Confocal sections of eggs/embryos stained with propidium iodide (red) and anti-FLAG antibody (green).
(A) In wild-type (WT) eggs, RI deposition of maternal H3.3-FLAG is observed in the decondensing male nucleus (M) before the first zygotic S phase.
(B) H3.3-FLAG is not detected in the male nucleus in *Hira*^{HR1} eggs.
(C) At pronuclear apposition, during the first S phase, limited RC deposition of H3.3-FLAG is detected in the female pronucleus (arrow) in WT eggs.
(D). The same, faint H3.3-FLAG staining of the female pronucleus is observed in *Hira*^{HR1} eggs (arrow).
(E) A WT embryo in anaphase of the third nuclear division. At this early stage, the stronger H3.3-FLAG staining of the paternally derived chromosomes (arrowheads) is still detectable (note that paternal and maternal chromosomes tend to remain separated during the early syncytial mitoses).
(F) A *Hira*^{HR1} haploid embryo in its fourth mitosis showing a weak H3.3-FLAG staining on maternally derived chromosomes (arrows).
(G) A wild-type, diploid blastoderm embryo in metaphase showing a strong H3.3-FLAG chromosomal staining on all nuclei.
(H) H3.3-FLAG is also detected on the chromosomes of *Hira*^{HR1} haploid blastoderm embryos.
(I) Embryos from wild-type mothers crossed with *H3.3-Flag/CyO* males showing no detection of zygotic H3.3-FLAG at this stage.
(J) Zygotic H3.3-FLAG appears in the chromatin of gastrula embryos.
(K) A wild-type, cycle 3 embryo in anaphase showing a strong H3-FLAG staining on all chromosomes.
(L) A blastoderm embryo with a strong maternal H3-FLAG staining. Gray panels or insets show the H3.3-FLAG or H3-FLAG staining for a representative group of nuclei. Bar: 10 μm.
doi:10.1371/journal.pgen.0030182.g008

pW25-Hira^{HR1}*-Flag* reporter transgene. In the alternative possibility that some truncated HIRA protein would be translated from this allele and escaped our detection, the first possible translation initiation codon downstream from the deleted region in *Hira*^{HR1} is at position 61, after the second WD repeat. Such a truncated HIRA would thus be expected to have, at best, a destabilized beta-propeller domain, which represents the most evolutionarily conserved part of HIRA proteins [53,54]. The fact that both *Hira*^{ssm} and *Hira*^{HR1} alleles display identical mutant phenotypes also highlights the very important role of the arginine 225 mutated in *Hira*^{ssm}, and by extension, the important role of the beta-propeller domain for the assembly of paternal chromatin. A recent study implicated *Drosophila* HIRA and the GAGA factor–FACT complex in a histone replacement mechanism that prevents the spreading of heterochromatin into a *white* reporter transgene inserted near centromeric heterochromatin [20]. Nakayama et al. observed that silencing of this variegating transgene was enhanced in *Hira*^{ssm} males, and concluded that the mutation affected H3.3 replacement at a site near the *white* gene. Their work suggests that *Drosophila* HIRA could indeed function in RI assembly in other situations and is consistent with the fact that *Hira* is expressed throughout development, in addition to its strong maternal expression [17,53,54]. Nevertheless, the fact that *Hira*^{HR1} mutant adults are viable indicates that this function is dispensable.

H3.3 Deposition without HIRA

Another important aspect of this study lies in the fact that the *Hira*^{HR1} mutation does not have detectable effect on the deposition of H3.3-FLAG in embryos or adult cells. First, it clearly establishes that H3.3 nucleosomes can be efficiently assembled in the absence of functional HIRA in vivo. So far HIRA is the only chaperone known to deposit the H3.3 variant. This study demonstrates the existence of at least one alternative assembly pathway for H3.3 nucleosomes, although the nature of the histone chaperone(s) involved is unknown. A simple hypothesis is the deposition of H3.3 by the CAF-1 complex. In fact, we have shown that in early embryos, the bulk of H3.3 is deposited independently of transcription,

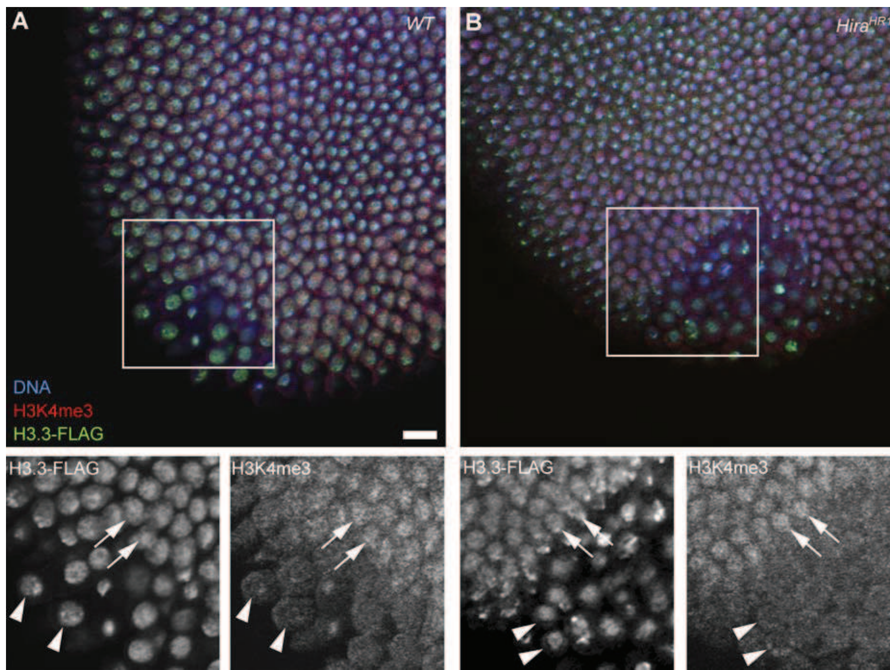

Figure 9. H3.3-FLAG Is Deposited in the Germ Line Chromatin in Blastoderm Embryos

Confocal sections of blastoderm embryos stained for DNA (blue), H3.3-FLAG (green), and H3K4me3 (red).
(A) H3.3-FLAG (left inset) is deposited at equivalent levels in somatic (arrows) and germ line (arrowheads) nuclei in wild-type embryos. H3K4me3 is enriched in somatic nuclei (right inset).
(B) An identical situation is observed in $Hira^{HR1}$ embryos. Bar: 10 μm.
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presumably at each S phase of the early nuclear cycles. Indeed, these cycles consist on a very rapid succession of S and M phases and lack gap phases [26]. The S phase deposition of H3.3 is consistent with a previous report showing that overexpressed H3.3-GFP was deposited during DNA replication in *Drosophila* Kc cells [55]. In human cells, only the small subunit of CAF-1 was found in the H3.3 complex, whereas all three subunits of the complex were copurified with the replicative histone H3.1 [19]. In early cycles, H3 is preferentially deposited compared with H3.3. However, a peculiarity of *Drosophila* embryos is the storage of large maternal pools of both H3 and H3.3, a situation that could favour a competition of these histones for their interaction with CAF-1. In contrast, in differentiated cells, the massive expression of S phase histones at the onset of DNA replication could strongly reduce the use of H3.3-H4 dimers by the CAF-1 complex. The early *Drosophila* embryo should represent a good model to address this point.

A study of *Hira* –/– mouse ES showed that these cells undergo early differentiation, suggesting that core histone deposition during this process could use HIRA-independent pathways [56]. Although it is well established that H3.3 deposition correlates with active chromatin in many instances, there is yet no link between HIRA and transcription in higher eukaryotes [57]. In budding yeast, nucleosome reassembly at the PHO5 promoter absolutely requires the histone H3-H4 chaperone Spt6 [58], whereas Hir1 is not absolutely required [59,60]. In *Drosophila*, Spt6 is clearly involved in transcription elongation [61,62] and thus represents an interesting candidate for TC deposition of H3.3 [57]. The biochemical analysis of H3.3 complex in $Hira^{HR1}$ mutant could help identify alternative H3.3 chaperone(s).

Our results support the hypothesis that multiple and possibly redundant pathways are involved in the assembly of H3.3 nucleosomes in multicellular organisms. Besides, it is now established that H3.3 nucleosomes can be assembled independently of RC and TC assembly pathways. For example, nucleosome replacement mechanisms at cis-regulatory elements implicating the deposition of H3.3 have been recently reported in *Drosophila* [20,63]. The ability of cells to assemble chromatin independently of DNA replication is apparently common to all eukaryotes. In fact, some organisms such as yeasts have only one type of histone H3, which is related to H3.3 and is deposited throughout the cell cycle [64]. The coexistence of RC and RI histone H3s in most other eukaryotes indicates that these distinct modes of chromatin assembly fulfil important complementary functions. Interestingly and surprisingly, the deletion of all RI H3 histone genes in the protist *Tetrahymena thermophila* does not compromise survival and, in particular, does not affect nucleosome density at highly transcribed regions [65]. However, RI H3 genes in *T. thermophila* appear to be critical for the production of viable sexual progeny and for the function of germline micronuclei [65], suggesting that sexual reproduction and/or developmental processes could have played an important role in the evolution of the RI mode of nucleosome assembly. RI H3.3 replacement at fertilization is apparently a conserved mechanism in nematodes, insects, vertebrates, and plants [17,49,50,66,67]. That the paternal chromatin assembly is the only essential function of *Drosophila* HIRA suggests that this factor acquired new important roles during the evolution of vertebrates. So far, in mammals, the implication of the HIRA/H3.3 complex has been shown or at least suspected in various remodelling processes, including heterochromatin repair

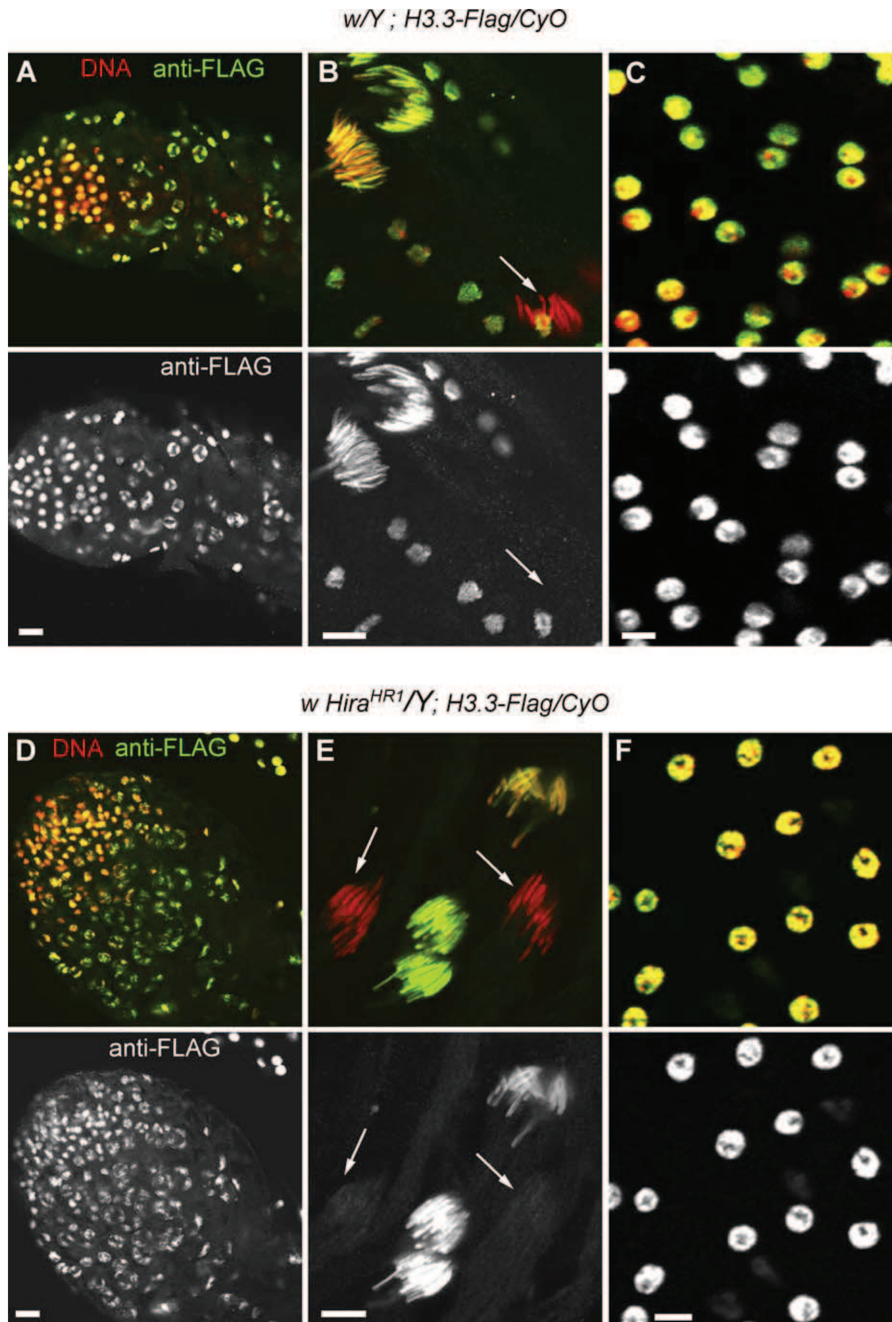

Figure 10. *Hira*^{HR1} Does Not Affect the Distribution of H3.3-FLAG in Adult Testis

Testis and accessory glands from *H3.3-Flag/CyO* transgenic adult males with a wild-type (A–C) or *Hira*^{HR1} (D–F) X chromosome, stained with anti-FLAG antibody and propidium iodide.

(A) Apical tip of a wild-type testis.

(B) A group of elongating spermatids in a wild-type testis showing a bright H3.3-FLAG nuclear staining that disappears in late condensing spermatid nuclei (arrow).

(C) Nuclei from a wild-type accessory gland.

(D) Apical tip of a *Hira*^{HR1} testis.

(E) Spermatid nuclei in a *Hira*^{HR1} testis. H3.3-FLAG is not detected in late spermatid nuclei (arrows).

(F) Nuclei from a *Hira*^{HR1} accessory gland. Bars: 10 µm.

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[68], mammalian meiotic sex chromosome inactivation [69], fertilization [49,50], and possibly, formation of senescence-associated heterochromatin foci [70] and histone exchange during spermiogenesis [71]. More functional studies should reveal if all these processes strictly rely on HIRA, in the context of the developing organism.

Note: After the preparation of our manuscript, a paper by A. Konev et al. [72] was published that reported the implication of the motor protein CHD1 in the deposition of histone H3.3 in *Drosophila*. This finding supports our own conclusions about the existence of Hira-independent H3.3 deposition pathways.

Materials and Methods

Flies. The w^{1118} ssm^{185b}/FM7c stock was described before [15]. The ProtamineA/B-GFP stocks [13] are a gift from S. Jayaramaiah Raja and R. Renkawitz-Pohl. The sra^{A108} allele [27] is a gift from V. Horner and M. Wolfner. Df(3R)sbd45 is a deficiency that covers the *sra* locus. The H3.3-Flag, H3-Flag, and *Hira*-Flag stocks have been described before [17]. The *y* w^{67c} and w^{1118} stocks were used as wild-type controls. All the other stocks or chromosomes used in this paper were obtained from the Bloomington *Drosophila* stock center.

***Hira* targeting by homologous recombination.** The *Hira* gene was targeted by ends-out homologous recombination as described in [22,23]. Two DNA fragments from the *Hira* locus were PCR-amplified from the cosmid genomic DNA clone 107B5 (European *Drosophila* Genome Project) using the following primers: 5′-ATGAAAT-GAGTGCCAGCAGC-3′ and 5′-GGTACCTATCGGTAACGATGCC-CATC-3′ for the *Hira* upstream arm (4209 bp) and 5′-GGCGCGCCGTGGTCATCTGGAATCTGCT-3′ and 5′-CGTACGA-TATTGGTTCCCGGTACCAG-3′ for the *Hira* downstream arm (3530 bp). These fragments were ligated in the pW25 vector [23] using the following restriction sites: Sac II and Acc65I for the upstream arm and AscI and BsiWI for the downstream arm. The final construct, named pW25-Hira, was verified by PCR and restriction analysis (unpublished data).

Six independent autosomal pW25-Hira transgenic lines were established in a *y* w^{67c} background. Batches of 15–20 virgin *y w; P{70FLP}11 P{70I-SceI}2B, Sco/CyO* females were crossed with approximately 10 males from a given donor line in plastic vials. Vials containing 24-h egg collections from these crosses were heat shocked for 90 min at 37 °C in a water bath on days 3, 4, and 5 after egg laying. *pW25-Hira /P{70FLP}11 P{70I-SceI}2B, Sco* virgin F1 females with white or mosaic eyes were collected and crossed with *w ; P{70FLP}10* males. Non-mosaic, coloured-eyed progenies were then crossed again with *w ; P{70FLP}10* to establish individual lines. Each line with a $white^{+}$ chromosome resistant to constitutive Flipase activity was tested for its complementation with the *w* $Hira^{ssm}$ chromosome. Chromosomes that did not complement the maternal effect embryonic lethality associated with $Hira^{ssm}$ were selected, outcrossed with w^{1118} for five generations, and balanced with the FM7c Chromosome.

$pW25\text{-}Hira^{HR1}$-Flag transgenes. The $pW25\text{-}Hira^{HR1}$-*Flag* transgene was constructed by replacing an AgeI-BsiWI restriction fragment in the 3' *Hira* arm from the *pW25-Hira* vector with a 729 bp fragment excised from the *pW8-Hira-Flag* transgene [17] to introduce the 3X-Flag tag at the 3' end of *Hira*. The final construct was verified by sequencing, and transgenic lines were established.

RT-PCR. Total RNA was extracted by the Trizol method (Invitrogen) and first-strand cDNAs were synthesized with the Superscript II reverse transcriptase (Invitrogen) and oligo-dT primers. The primer sequences used for PCR amplification of the cDNAs or genomic DNA are available on request.

Antibodies for immunofluorescence. Anti-Flag M2 mouse monoclonal antibody (F-3165, Sigma Aldrich) was used at 1:2000, rabbit anti-acetylated histone H4 polyclonal antibody (06–598, Upstate) at 1:500, rabbit anti-H3K4me3 polyclonal antibody (ab8580, Abcam) at 1:250, and mouse monoclonal anti-GFP antibody (Roche 1814460, clones 7.1 and 13.1) at 1:500 (IF). The anti-*Drosophila* ASF1 antibody [34] is a gift from F. Karch and was used at a 1:1000 dilution. The HIRA 830 anti-peptide antibody was described before and used at 1:500 [17].

For the production of the PG1 anti-HIRA polyclonal antibody, a plasmid *PW8-Hira-Flag* [17] was used as a template to amplify a 1943-pb fragment from 1241 to 3183 (amino acids 381–935) by PCR using primers 5′-ACATATGGTGAACGGTCTGGGAAAGTC-3′ and 5'TGGATCCGTACCCGTTGTCACAGCCAT-3′. The fragment was cloned into the NdeI and BamHI of pET15b vector (Novagen) in frame with the His•Tag® at the N-terminus end of the recombinant protein. The recombinant plasmid was transformed into Escherichia coli BL21-CodonPlus® (DE3)-RIL competent cells (Stratagene) and expression of the recombinant protein was induced by IPTG (isopropyl β-D-thiogalactoside) as described by the manufacturer and analysed by SDS-PAGE. Two rabbits were immunized with the purified HIRA-HIS-TAG protein purified on a Nickel column. Crude sera were purified on a Proteine-G column (Proteogenix) and were used at 1:1000.

Immunofluorescence. Eggs and embryos were collected, fixed in methanol, and immunostained as described [16]. For each experiment, we observed a minimum of 25 eggs/embryos at the desired stage. Testes and ovaries were dissected in PBS-Triton 0.1%, fixed in 4% paraformaldehyde for 20 min (testis) or 30 min (ovaries) at room temperature, rinsed in TBST (0.1% Triton), and stained as for embryos. DNA was stained either with propidium iodide as described [16] or with TO-PRO-3 (Molecular Probes) used at a 1:10,000 dilution. Preparations were observed under a Zeiss LSM Meta confocal microscope. Images were processed with the LSM and Photoshop (Adobe) softwares.

Western blot. WT and transgenic O/N embryos were collected, washed, dechorionated, and homogenized in Laemmli 2X sample buffer (125 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 100mM DTE, 1% bromophenol blue) with an Eppendorf fitting pestle-homogenizer using the bio-vortexer™ mixer (Roth). Protein samples were centrifuged 5 min at 5000 rpm, boiled for 10 min at 95 °C, and subjected to electrophoresis on an 10% SDS-PAGE gel. Immunoblotting was performed using a tank transfer system (Mini Trans-Blot Cell, Bio-Rad) and Hybond-C Extra nitrocellulose membranes (Amersham Biosciences) in transfer buffer (25 mM Tris, 20 mM glycine, 20% ethanol, 0.05% SDS). Antibodies incubation was in TBST (20 mM Tris-HCl [pH 7.5], 130 mM NaCl, 0.1% Tween 20) supplemented with 1% (w/v) nonfat dry milk as blocking agent. Detection was performed using the ECL western blotting detection system (Amersham Pharmacia). Anti-FLAG M2 (F-3165, Sigma Aldrich) and anti-α-tubulin Dm1A (T-9026, Sigma Aldrich) mouse monoclonal antibodies were used at a 1:20,000 dilution. Goat anti-mouse horseradish peroxidase–conjugated antibody (170-5047, Bio-Rad) was used at 1:15,000 dilution. Note that our HIRA antisera did not work on western blots using the extraction and detection procedures that worked very well with the HIRA-FLAG recombinant protein detected with the anti-FLAG antibody.

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Author contributions. All authors conceived and designed the experiments and analysed the data. EB, GAO, and BL performed the experiments. BL wrote the paper.

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En plus d'améliorer notre compréhension de la biologie de HIRA et H3.3 chez la drosophile, cette étude nous a permis d'étudier des aspects fondamentaux autour du processus de maturation du pronoyau mâle et de renforcer l'idée que la fécondation mobilise un ensemble de facteurs dont la conservation est conditionnée par son succès.

Un autre fruit intéressant de ce travail concerne ASF1, considéré comme une pierre angulaire de l'assemblage de la chromatine IR. Nous avons formalisé l'hypothèse qu'il pourrait exister un cas (particulier) d'assemblage de la chromatine IR indépendant de ASF1 *in vivo*. Cette idée avait été proposée à partir d'une étude travaillant sur un système *in vitro* (Ray-Gallet *et al.*, 2007). Chez la drosophile, les mutations affectant *asf1* sont létales et, de ce fait, les rôles de ASF1 dans le contexte du développement restent mal connus (Moshkin *et al.*, 2002; Moshkin *et al.*, 2009). Cependant, l'absence de recrutement de ce facteur au pronoyau mâle nous a permis de proposer que ce facteur n'est pas actif dans l'assemblage de la chromatine paternelle à la fécondation *in vivo*. ASF1 est considéré comme un donneur d'histones et cette fonction est importante pour la régulation de la réplication (Groth *et al.*, 2007). Son absence de recrutement dans le pronoyau mâle pose la question des mécanismes par lesquels l'histone H3.3 est mise à disposition du complexe HIRA, et souligne les particularités de ce noyau. Je discuterai plus de cette idée à travers une étude que nous avons mené plus récement (voir Orsi *et al.*, en préparation).

L'apparente nécessité de HIRA spécifiquement à la fécondation pouvait s'expliquer par quatre hypothèses non exclusives.

(1) HIRA pourrait être un facteur spécialisé dans la fécondation, d'autres facteurs pouvant prendre en charge H3.3 en son absence dans les tissus somatiques. Récemment, la voie ATRX/DAXX a été impliquée dans l'incorporation de H3.3 dans la chromatine de façon indépendante de HIRA, rendant possible un certain degré de redondance fonctionnelle entre ces complexes (Goldberg *et al.*, 2010). Je présenterai plus tard des résultats récents montrant que cette voie est conservée chez la drosophile, et qu'elle est partiellement redondante avec la voie HIRA dans les cellules somatiques (voir Schneiderman *et al.*, en préparation).

(2) Le phénotype associé à nos mutants pouvait aussi suggérer que l'assemblage de la chromatine avec l'histone H3.3, n'est, lui même, que strictement nécessaire au moment de la fécondation. Chez certaines espèces dont les cellules différentiées les plus âgées peuvent

atteindre plusieurs années de vie, le remplacement IR avec le variant H3.3 devient si étendu qu'il finit par concerner la majorité de la chromatine (Urban et Zweidler, 1983; Piña et Suau, 1987; Wunsch et Lough, 1987; Bosch et Suau, 1995). Dans ce contexte, il est difficile d'imaginer que l'assemblage IR ne soit pas essentiel. Cependant, la première étude fonctionnelle du rôle des variants IR (non-centromériques) de H3 avait été menée chez le protiste Tetrahymena thermophila et montrait que ces variants n'étaient pas remplaçables mais pas indispensables à la survie ni à l'activité globale de transcription non plus (Yu et Gorovsky, 1997; Cui *et al.*, 2006). La drosophile est un métazoaire complexe, mais à durée de vie relativement courte, et la possibilité que l'assemblage IR ne soit pas absolument critique ne pouvait pas être totalement écartée.

(3) Par ailleurs, la nécessité absolue du facteur HIRA dans ce processus aurait pu être un reflet du caractère massif et rapide de cet évènement d'assemblage de la chromatine. Nous avons imaginé que le succès de ce cas particulier d'assemblage IR requiert une efficacité maximale de tous les acteurs de l'assemblage, alors que d'autres processus biologiques pourraient fonctionner malgré des défauts dans la machinerie. Cette idée serait renforcée par plusieurs travaux au cours de ma thèse (voir en particulier Schneiderman *et al.*, en préparation et Résultats supplémentaires).

(4) Finalement, au cours de ces travaux, nous avons peut être sous-estimé l'importance potentielle de l'apport maternel en ARNm et protéine HIRA. En effet, il est concevable chez la drosophile que des individus génétiquement mutants se développent exclusivement avec les apports sauvages maternels. Ainsi, l'apport maternel en HIRA pourrait masquer des phénotypes chez les individus génétiquement mutants, biaisant partiellement nos conclusions.

II. Un rôle purement structural et un rôle en tant que marque épigénétique pour le variant d'histone H3.3

La démonstration que l'incorporation de H3.3 est couplée à la transcription (Ahmad et Henikoff, 2002b; Janicki *et al.*, 2004; Schwabish et Struhl, 2004; Schwartz et Ahmad, 2005), sa localisation préférentielle dans les régions promotrices et transcrites (Chow *et al.*, 2005; Mito *et al.*, 2005; Wirbelauer, 2005), et sa corrélation avec les marques classiques de la

chromatine active (McKittrick *et al.*, 2004; Hake, 2005) ont placé les fonctions épigénétiques de H3.3 au centre du débat. Le contexte bibliographique aurait supposé que la voie HIRA/H3.3 fasse partie d'un mécanisme de mise en place de la chromatine active si les seules données fonctionnelles disponibles sur ce complexe *in vivo* n'aguillaient pas dans la direction inverse. En effet, les seuls effets décrits des mutations de l'histone H3 de remplacement (chez *T.thermophila* et chez la souris) renvoyaient à un rôle dans l'activité de reproduction sexuée (Couldrey *et al.*, 1999; Cui *et al.*, 2006), rappelant le cas de HIRA chez la drosophile.

Dans l'article de revue ci-après, nous avons récapitulé les données selon lesquelles HIRA et H3.3, auraient un rôle central dans les activités liées à la reproduction sexuée. Ceci nous a permis d'argumenter sur l'importance de ce rôle pour la conservation et l'évolution de ces protéines. Finalement, nous avons ouvert la discussion sur nos résultats concernant la fonction de HIRA chez la drosophile.

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Epigenetic and replacement roles of histone variant H3.3 in reproduction and development

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ABSTRACT The nucleosomal organization of eukaryotic chromatin is generally established during DNA replication by the deposition of canonical histones synthesized in S phase. However, cells also use a Replication Independent (RI) nucleosome assembly pathway that allows the incorporation of non-canonical histone variants in the chromatin. H3.3 is a conserved histone variant that is structurally very close to its canonical counterpart but nevertheless possesses specific properties. In this review, we discuss the dual role of H3.3 which functions as a neutral replacement histone, but also participates in the epigenetic transmission of active chromatin states. These properties of H3.3 are also explored in the light of recent studies that implicate this histone and its associated chromatin assembly factors in large scale, replication-independent chromatin remodeling events. In particular, H3.3 appears as a critical player in the transmission of the paternal genome, from sperm to zygote.

KEY WORDS: *H3.3, epigenetic, sperm chromatin remodeling, histone chaperone*

Introduction

The organization of chromatin in eukaryotic cells is remarkably conserved. The basic unit of chromatin, the nucleosome, is constituted by a hetero-octamer of histones that are wrapped with about 146bp of DNA. The structural properties of nucleosomes can be modulated by a large variety of post-translational modifications (PTMs) of histone proteins. The combinatorial complexity of these modifications is at the origin of the "histone code" hypothesis, which proposes that histone PTMs participate, along with other epigenetic marks such as DNA methylation, in the functional organization of the genome (Jenuwein and Allis, 2001). Nucleosomes can also be modulated by the incorporation of histone variants that differ from the major, canonical histones synthesized during S phase. Histone variants differ from their canonical counterpart at the level of the primary sequence. These differences can range from a few amino-acid positions (e.g. H3.1 vs H3.3) to large protein domains (e.g. H2A vs macroH2A) and usually confer specific properties to nucleosomes. In contrast to canonical histones that are devoted to Replication Coupled (RC) chromatin assembly, histone variants are expressed throughout the cell cycle and are thus available, at least theoretically, in nucleosome assembly pathways that occur in a Replication-Independent (RI) manner (Henikoff and Ahmad, 2005; Sarma and Reinberg, 2005). For this reason, histone variants are also called "replacement" histones. Finally, certain variants are preferentially or specifically expressed in certain tissues, such as the testis specific histone H3 variant, for instance (Witt *et al.*, 1996).

The combination of PTMs and histone variant creates a wide diversity of nucleosomes. This variability is important to determine the properties of the chromatin fiber at a local and regional level, with respect to essential aspects of DNA metabolism, such as replication, transcription, heterochromatin formation, repair, condensation or kinetochore formation.

In this article, we focus on the function of the histone H3.3 variant during development and reproduction. A main feature of H3.3 is its association with transcriptionally active chromatin and its potential role in the epigenetic transmission of active chromatin states. These properties are at the origin of a growing interest for H3.3 over the past few years. However, recent studies in various model organisms have revealed unexpected roles for this variant, particularly during sexual reproduction. We will discuss the respective importance of replacement and epigenetic roles of this histone, in the context of its diversity and evolutionary history, and in the light of its interactions with nucleosome assembly machineries.

Abbreviations used in this paper: MSCI, meiotic sex chromosome inactivation; MSUC, meiotic silencing of unsynapsed chromatin; PTM, post-translational modification; RC, replication coupled; RI, replication independent; SCR: sperm chromatin remodeling; SNBP, sperm nuclear basic protein.

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The histone H3.3 family of proteins

Genes encoding canonical histones are usually organized in tandem, multi-copy clusters and have no introns. Replicative histone mRNAs are not polyadenylated. Instead, translation is tightly regulated by the binding of SLBP (Stem Loop Binding Protein) and U7 snRNP to the 3' end of the histone RNAs (Jaeger *et al.*, 2005). This peculiar genomic organization and transcriptional regulation allows a massive production of canonical histones at the beginning of the S phase and ensures the synthesis of stoichiometric quantities of each protein. On the contrary, histone variant genes are regular genes that are represented by a single or a few copies and are scattered throughout the genome. In addition, they often possess introns and their polyadenylated mRNAs are expressed throughout the cell cycle (Fig. 1).

In mouse, thirteen canonical *H3* genes are present in the genome, encoding two versions of canonical H3: H3.1 and H3.2. They differ by a single amino acid in position 96 (Graves *et al.*, 1985). The functional relevance of having two different replicative H3s is unclear (Hake and Allis, 2006). In *Drosophila*, the histone gene cluster on the left arm of chromosome 2 contains twenty-three copies of each *H1*, *H2A*, *H2B*, *H3* and *H4* genes (Fig. 1). All the *Drosophila* histone *H3* genes encode the same H3 protein, identical to mammalian H3.2.

Histone H3 variant types include centromeric H3 variants (CenH3s), H3.3 and testis specific H3 in mammals (Fig.1). CenH3s form a highly divergent family of histone H3 variants that are characterized by an H3-like histone fold domain and a variable N-terminus tail (reviewed in Ahmad and Henikoff, 2002a; Dalal *et al.*, 2007). In mouse, human and *Drosophila*, two *H3.3* genes (*H3.3A* and *H3.3B*) encode the same conserved protein, but the transcripts have distinct untranslated regions (Akhmanova *et al.*, 1995; Frank *et al.*, 2003; Krimer *et al.*, 1993).

H3.3 is one of the most conserved proteins and appears to be present in all eukaryotes (Malik and Henikoff, 2003). H3.3 differs from H3.2 (mouse) or H3 (*Drosophila*) by only four amino acids at positions 31, 87, 89 and 90 (Fig. 2). The residue at position 31 sits in the N-terminal tail of the protein while positions 87, 89 and 90 are located in the α2 helix of the histone fold domain (Fig. 2). In spite of the great sequence similarity between H3.3 and H3, it has been proposed that these residues could account for specific properties of H3.3 proteins. In vertebrates, the serine in position 31 (H3.3S31p) can be phosphorylated and this PTM is detected on metaphase chromosomes, at specific sites bordering centromeres, unveiling a possible role of this mark during cell division (Hake *et al.*, 2005). H3.3S31p also exists in the urochordate *Oikopleura dioica* and is detected during mitosis and oogenic meiosis (Schulmeister *et al.*, 2007). In addition, a potentially phosphorylable threonine residue is found in position 31 in *C. elegans* and *A. thaliana* H3.3, respectively, but this is not the case for other members of the family (Fig. 2).

The residues in positions 87, 89 and 90 are necessary and sufficient to exclude canonical H3 from RI assembly pathways in *Drosophila* (Ahmad and Henikoff, 2002b), suggesting that they could directly or indirectly mediate the interaction of H3 and H3.3 with their specific histone chaperone. In vertebrates and *Drosophila*, the residues at positions 87, 89 and 90 are S, V and M in H3, and A, I and G in H3.3, respectively. Interestingly however, the identities of the residues found at these positions in H3 and H3.3 vary between species but distinguish H3 from H3.3 (Fig. 2) (Malik and Henikoff, 2003). It has been proposed that these three residues could participate in histone-histone interaction stability: nucleosomes assembled with H3.3 may have different intrinsic stability properties than those assembled with canonical H3 (Hake and Allis, 2006).

In the nematode *C.elegans* at least two different H3.3 proteins are encoded (namely, His71 and His72) (Ooi *et al.*, 2006). *His71* and *His72* individually mutated animals are viable, suggesting that these genes are functionally redundant. In the protist *Tetrahymena thermophila*, two H3.3 proteins have also been characterized (H3.3 and H3.4) (Cui *et al.*, 2006). These two versions present differences to *Tetrahymena* canonical H3 on three of the four characteristic positions but, in addition, present 8 non-conserved amino acid differences. Moreover, these proteins

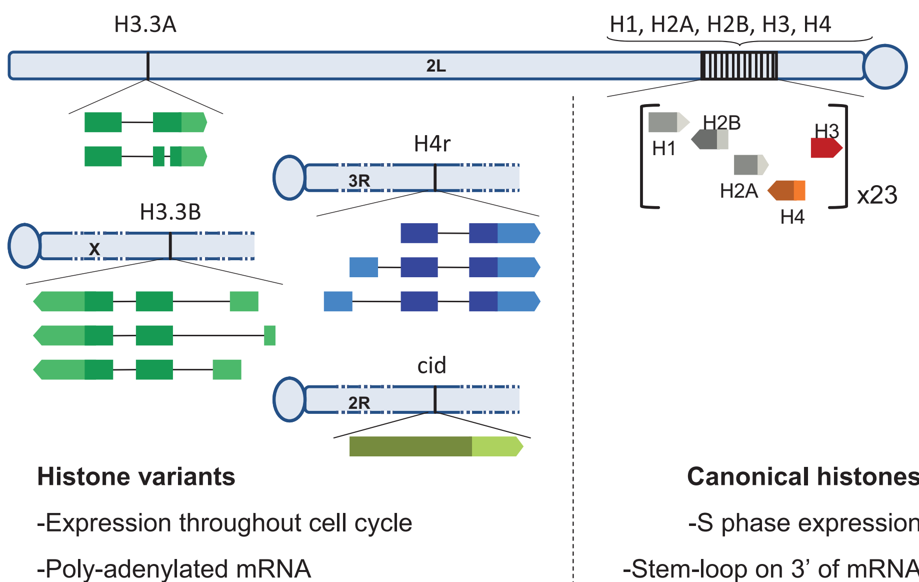

Fig. 1. Genomic organisation of histone *H3* and *H4* genes in *Drosophila*. Drosophila melanogaster *chromosomes are represented and histone gene locations are showed.* H3.3A, H3.3B, H4r *and* Cid *are single-copy genes and their corresponding transcripts are shown. Known or putative introns are represented as thin lines. Coding regions are shown in darker colors. Lighter color boxes represent untranslated regions. Canonical histones H1, H2B, H2A, H3 and H4 are encoded by multi-copy genes in the histone gene cluster of chromosome 2L. Note that Flybase predicts a H3.3A transcript that would result in a shorter protein. Although supported by independent EST sequences, the signification of this transcript remains to be investigated. Gene annotations are from Flybase (http://flybase.bio.indiana.edu).*

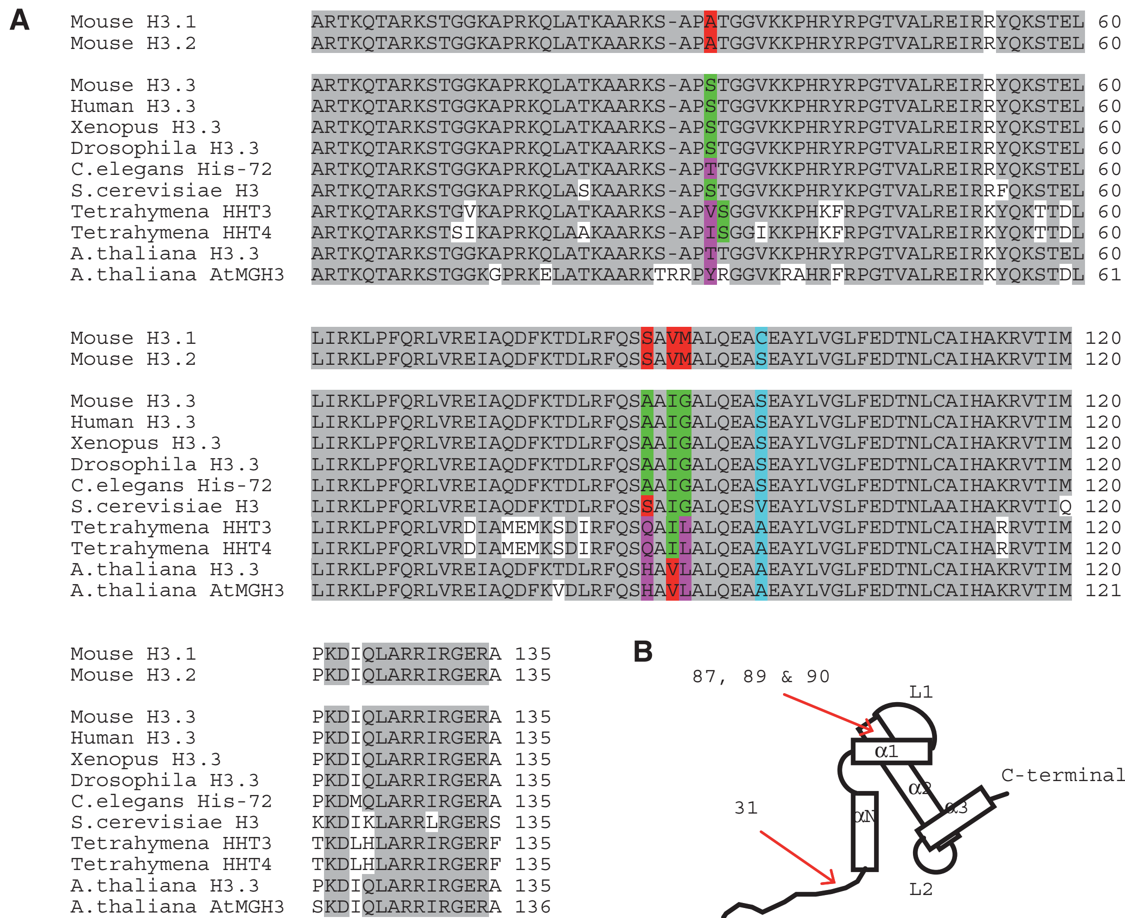

Fig. 2. Sequence alignment of H3.3 proteins. (A) *CLUSTAL alignement of H3 and H3.3 histones. Accession numbers are from GenBank:* M. musculus *H3.3A (NP_032236.1),* H. sapiens *H3.3A (NP_002098.1),* X. tropicalis *H3.3A (NP_001091902),* D. melanogaster *H3.3A (NP_523479.1),* C. elegans *His-72 (NP_499608.1),* S. cerevisiae *H3 (NP_009564.1),* T. thermophila *HHT3 (XP_001008397.1),* T. thermophila *HHT4 (XP_001008400.1) and* A. thaliana *H3.3 (NP_195713.1),* M. musculus *H3.1 (NP_038578.2) and H3.2 (NP_473386.1), and* A. thaliana *AtMGH3 (NP_173418.1). Alignments were performed with EMBL-EBI ClustalW2 software. Significantly conserved amino-acid residues are shaded in grey. In positions 31, 87, 89 and 90, amino-acids from canonical H3s are shaded in red, those from H3.3 family are shaded in green and those not fitting these categories are shaded in purple. The single residue that differentiates mouse H3.1 and H3.2 and differs among the H3.3 family is shaded in cyan.* **(B)** *Residues 31, 87, 89 and 90 are positioned on a schematic representation of nucleosomal H3 protein.*

differ from vertebrate H3.3 by twenty-two and twenty-five amino acids respectively, which accounts for the diversity of the H3.3 family across evolutionary divergent species. In plants, the diversification of H3.3 proteins seems to be even more accentuated: for instance, *Arabidopsis thaliana* has eight non-centromeric RI H3 variants (Okada *et al.*, 2005). The greater diversity of H3.3 proteins in certain groups, such as plants, for instance, opens the possibility that new H3.3 functions have emerged during evolution. Indeed, an evolutionary scenario proposes that H3.3 has independently arisen at least four times in plants, animals, ciliates and apicomplexans (Malik and Henikoff, 2003). Interestingly, a single version of H3 exists in ascomycetes (yeasts) and it is of the RI type (see Fig. 2). Since both RI and RC H3 versions are present in basidiomycetes, it has been proposed that canonical H3 genes have been lost in ascomycetes (Malik and Henikoff, 2003).

Epigenetic and replacement roles of H3.3 in somatic cells

Replacement roles of H3.3

The fact that expression of *H3.3* genes is not linked to S phase has been known for decades (Wu *et al.*, 1982). Because of this property, a simple expected function of H3.3 is to replace H3 whenever nucleosome assembly takes place independently of DNA synthesis, hence the term "replacement" variant. In an alternative view to this neutral replacement role of H3.3, the deposition of H3.3 can confer specific properties to the nucleosomes that are functionally important for the establishment of epigenetic marks (see the next section). A simple example of neutral replacement is provided by differentiated cells, after their exit from the cell cycle. In the absence of DNA replication and S

phase histone gene expression, differentiated cells are expected to rely on replacement histones for the assembly of new nucleosomes. Indeed, during the differentiation of various cell types, *H3.3* transcripts are abundant whereas replication dependent *H3* transcripts are no longer detected (Brown *et al.*, 1985; Krimer *et al.*, 1993; Pantazis and Bonner, 1984). The replacement of replicative H3 with H3.3 has been also observed at the protein level during the course of cell differentiation in vertebrates (Bosch and Suau, 1995; Pina and Suau, 1987; Urban and Zweidler, 1983; Wunsch and Lough, 1987). The underlying mechanism responsible for this H3.3 enrichment in chromatin of differentiated cells is not clear. It has been proposed that a general mechanism of nucleosome turnover allows the slow incorporation of histone variants in the chromatin in absence of DNA replication (Grove and Zweidler, 1984). This process is probably critical for the maintenance of a normal nucleosome density in long-lived cells as H3.3 nucleosomes compensate for the loss of old H3 nucleosomes.

Another example of H3.3 deposition that fits well into this type of neutral replacement is a recently described "repair" mechanims of heterochromatin in human cells after treatment with histone deacetylase inhibitors (Zhang *et al.*, 2007b). Exposure to these drugs triggers the recruitment of Heterochromatin Protein 1 (HP1) to sites of altered pericentric heterochromatin. This recruitment occurs independently of DNA replication and is mediated by the deposition of H3.3 by the histone chaperone HIRA at these sites (Zhang *et al.*, 2007b). The authors proposed that this mechanism could participate in the maintenance of centromere integrity and kinetochore formation. Interestingly, HIRA is also involved in the formation of SAHF (Senescence Associated Heterochromatin Foci) in human cells (Zhang *et al.*, 2007a; Zhang *et al.*, 2005). These cytological markers of cellular senescence are condensed domains of facultative heterochromatin that notably contain the macroH2A histone variant and HP1 proteins (Adams, 2007). The implication of HIRA in this process strongly suggests that H3.3 is also involved, although this remains to be formally demonstrated (Adams, 2007; Zhang *et al.*, 2007a). If it is the case, it could establish H3.3 as a key actor for the remodeling of heterochromatin in different biological situations, a property that is clearly not related to its role as a mark of active chromatin.

Although the need for H3.3 in non-dividing cells is expected, it is less clear whether H3.3 can actually replace H3 in cycling cells. A recent study in the protist *Tetrahymena thermophila* addressed this point through elegant genetic analyses (Cui *et al.*, 2006). In this organism, replacement or "minor" H3s are represented by two similar RI histone genes, called *H3.3* and *H3.4*, that are probably the result of a recent duplication event (Cui *et al.*, 2006) (Fig. 2). When both canonical *H3* genes were knocked-out, the expression of *H3.3* with a *H3* gene promoter was able to partially rescue the growth defect associated with the loss of RC H3 histones. This result indicates that the growth phenotype is mainly the consequence of an inadequate amount of histone protein rather than a specific absence of H3. However, H3.3 cannot fully replace H3 as rescued cells display a slight growth reduction and a small micronuclei phenotype. Thus, in *Tetrahymena*, H3 must have some intrinsic properties not shared with H3.3 (Cui *et al.*, 2006). These same authors also directly tested the function of minor H3s by generating cells with both *H3.3* and *H3.4* genes deleted. Surprisingly, minor H3s appear not essential for cell growth but only for the production of viable conjugation progeny. In addition, in the absence of minor H3s, Transcription-Coupled (TC) nucleosome assembly is apparently abolished without causing any obvious growth problem. These surprising phenotypes indicate that minor H3s seem to contribute to still unknown functions related to sexual reproduction.

Availability of H4 for RI nucleosome assembly?

Nucleosome assembly is initiated by the deposition of H3 along with H4 on DNA to form a (H3-H4)2 tetramer. This implies that H4 is made available at stoichiometric levels with H3.3 throughout the cell cycle, in order to be deposited through RI chromatin assembly pathways. Surprisingly, the problem of the source of H4 for RI assembly has received little attention. Interestingly, RI H4 genes encoding a H4 identical to canonical H4 have

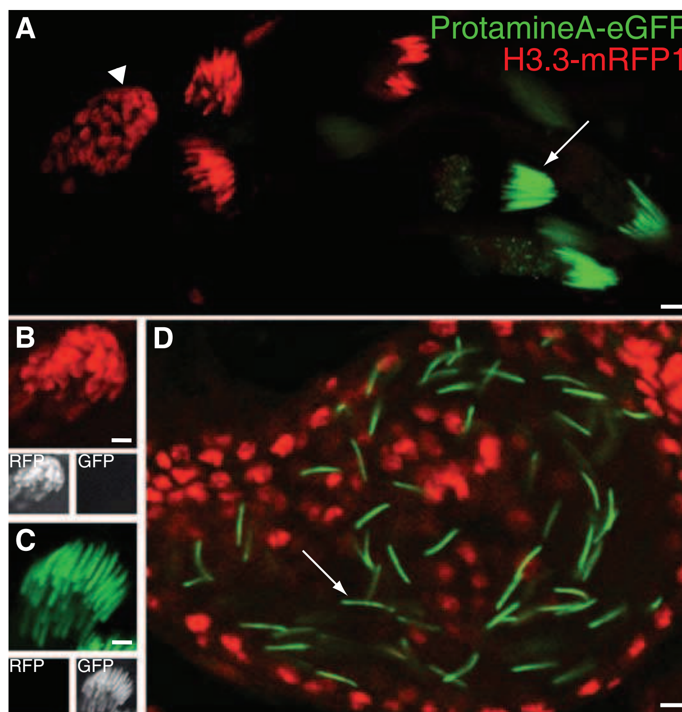

Fig. 3. H3.3 was not detected in *Drosophila* sperm. *Confocal images of fixed testes from males expressing both* H3.3-mRFP1 *and* ProtamineA-EGFP *transgenes. H3.3-mRFP1 is expressed from the* Drosophila H3.3A *gene promoter.* **(A)** *In this testis, groups of spermatid nuclei at different stages of differentiation (from left to right) are visible. Arrowhead points a group of spermatids with round nuclei and strong H3.3-mRFP1 fluorescence. Arrow indicates a group of elongated spermatid with strong ProtamineA-GFP fluorescence.* **(B)** *Close-up of a group of round spermatid nuclei.* **(C)** *Close-up of a group of elongated spermatid nuclei. In (B,C), small panels show the same nuclei with only RFP (left) or GFP (right) respective fluorescence.* **(D)** *A seminal vesicle containing mature gametes with strong ProtamineA-EGFP fluorescence (arrow). All bars, 5 μm. The mRFP1 (monomeric Red Fluorescent Protein1) protein is described in Campbell* et al.*, 2002. The* ProtamineA-EGFP *transgene is described in Jayaramaiah Raja and Renkawitz-Pohl (2005).*

been reported in *Drosophila*, nematodes and mammals (Akhmanova *et al.*, 1996; Gendron *et al.*, 1998; Poirier *et al.*, 2006). In *Drosophila*, the single copy *His4r* gene contains two introns, its mRNA is polyadenylated and it is expressed independently of DNA synthesis. In addition, it is preferentially expressed in adult, non-dividing cells, like *H3.3* genes (Akhmanova *et al.*, 1996). *H4r* might thus serve as a source of H4 for RI assembly processes. Once assembled, however, this protein is expected to behave identically to its RC counterpart. The role of *H4r* can thus only be explained by its RI expression profile. Another possible way of providing H4 to RI assembly pathways could be by recycling already assembled histones, by storing H4 expressed during S phase as pre-deposition complexes, or by allowing a certain level of transcription outside S phase. In this regard, it has been reported that the replicative histone H3.1 is deposited at sites of DNA repair, indicating that deposition of canonical histones is not absolutely coupled to S phase, at least for H3.1 (Polo *et al.*, 2006). Similarly, in *Tetrahymena*, H3 is specifically used for nucleosome assembly at sites of DNA synthesis associated with meiotic recombination (Cui *et al.*, 2006). The functional analysis of RI H4 genes should help distinguish between these possibilities.

H3.3 as an epigenetic mark of active chromatin

Opposed to the neutral replacement of H3 with H3.3 is the observation that H3.3 deposition does not occur homogeneously in the genome but instead correlates with regions of high transcriptional activity (Ahmad and Henikoff, 2002b; Chow *et al.*, 2005; Cui *et al.*, 2006; Janicki *et al.*, 2004; Mito *et al.*, 2005; Schwartz and Ahmad, 2005; Wirbelauer *et al.*, 2005).

It has been proposed that the passage of the RNA polymerase complex displaces nucleosomes, a situation that potentially creates a need for deposition of histones in a RI manner (Li *et al.*, 2007; Schwabish and Struhl, 2004). This Transcription Coupled deposition of H3.3 has been directly observed *in vivo* on *Drosophila* polytene chromosomes, throughout large transcription units such as the induced HSP70 genes, indicating that H3.3 deposition is associated with transcriptional elongation (Schwartz and Ahmad, 2005). Other studies have led to a similar conclusion based on the analysis of the distribution of H3.3 nucleosomes at high resolution by chromatin immunoprecipitation (ChIP) (Mito *et al.*, 2005; Wirbelauer *et al.*, 2005). However, this methodology also revealed an enrichment of H3.3 at the promoters of active genes, suggesting that chromatin remodeling associated with transcriptional initiation is also responsible for H3.3 deposition (Chow *et al.*, 2005). Finally, some studies found an enrichment of H3.3 at regulatory sites of active but also silent genes, such as the beta-globin locus control region in chicken or Polycomb Response Elements in *Drosophila* (Jin and Felsenfeld, 2006; Mito *et al.*, 2007; Nakayama *et al.*, 2007). These observations point to the possible existence of two distinct roles of H3.3 linked with gene activity. A first role for H3.3 in TC deposition is to compensate for the eviction of nucleosomes by the RNA polymerase complex in the body of highly transcribed genes (Schwartz and Ahmad, 2006). Another role links H3.3 to a continuous process of histone turnover that maintains accessibility of regulatory elements to their cognate factors (Henikoff, 2008).

In addition to its preferential incorporation at sites of active chromatin, H3.3 is enriched with PTMs typically associated with gene activity, such as methylation of lysine 4 among other marks (Hake *et al.*, 2006; McKittrick *et al.*, 2004; Mito *et al.*, 2005). How these PTMs are established on H3.3 and their importance in conferring an epigenetic role to this variant are crucial questions (Loyola and Almouzni, 2007). A recent study proposed that non-nucleosomal H3 and H3.3 carry a distinct set of modifications before their deposition, which in turn determine their final PTMs in nucleosomes (Loyola *et al.*, 2006).

The potential role of H3.3 in the epigenetic memory of active gene states has been recently studied in nuclear transfer experiments of *Xenopus* oocytes (Ng and Gurdon, 2008). Inheritance of active gene states of donor somatic nuclei is observed in embryos after nuclear transfer. For instance, about half of the embryos obtained after the transfer of a donor somite cell nucleus expressing the muscle-specific gene *MyoD* still express this marker in animal and vegetal regions, which do not differentiate into muscle (Ng and Gurdon, 2005). The authors found that this epigenetic memory of an active gene state correlates with the presence of H3.3 in its promoter. Importantly, this epigenetic memory can persist through 24 cell divisions in the absence of transcription (Ng and Gurdon, 2008). This finding supports a model where the H3.3 epigenetic mark is faithfully transmitted during DNA replication rather than through a mechanism involving the reactivation of transcription at each cycle. However, it is also compatible with the dynamic replacement model proposed by S. Henikoff (2008). Importantly, Ng and Gurdon found that the lysine 4 of H3.3 was required for the epigenetic memory, suggesting that the sole presence of the histone variant on promoter is not sufficient for the inheritance of the active gene state, but also requires the presence of specific PTMs. Functional studies, including formal genetic analyses of *H3.3* genes, are now required to progress on these fascinating aspects of chromatin function.

Functions of H3.3 in sexual reproduction

Besides its general replacement and epigenetic roles in somatic cells, several recent studies have highlighted the implication of H3.3 in chromatin remodeling mechanisms unique to the germline (Ooi and Henikoff, 2007). Although different in nature and function, these processes all require extensive RI nucleosome disassembly/reassembly at the genome or chromosome level.

Meiotic sex chromosome inactivation (MSCI)

The pachythene phase of the first meiotic prophase in mammalian males is characterized by the formation of synapses between chromosome pairs in preparation of recombination. Only the non-homologous X and Y chromosomes partially escape this process and are separated in a specific chromatin domain, the “XY body”. In this domain, the sex chromosomes are transcriptionally silenced in a process called MSCI (Turner, 2007). Unsynapsed autosomal chromatin is also silenced in a similar mechanism called MSUC (Meiotic Silencing of Unsynapsed Chromatin). A recent study discovered that both MSCI and MSUC depend on an extensive nucleosome replacement mechanism involving the deposition of the H3.3 variant (van der Heijden *et al.*, 2007). To which extent H3.3, and its chaperone HIRA, are critical for this process is yet unknown, but it is interesting to note that male mice with an impaired *H3.3A* gene have reduced fertility (Couldrey *et*

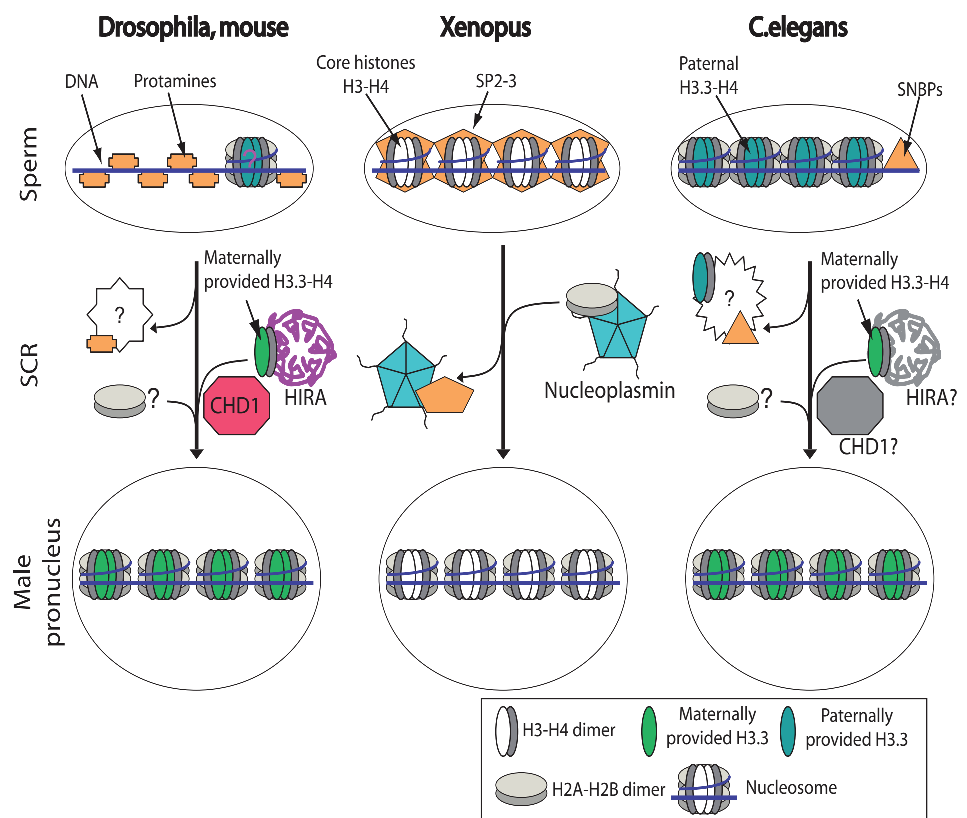

Fig. 4. Comparison of SCR in different animal models. *Schematic illustrations of sperm chromatin, SCR and male pronucleus chromatin in* Drosophila, *mouse,* Xenopus *and* C. elegans. Drosophila *and mouse present a protamine-based sperm chromatin structure although small levels of core histones could remain associated to DNA.* Xenopus *sperm chromatin is organized in nucleosome-like structures where core histones H3 and H4 are associated with sperm specific nuclear basic proteins SP2-3. Whether these core H3s are canonical H3 or H3.3 variants is not known.* C. elegans *sperm chromatin is probably organized with nucleosomes containing H3.3 although sperm-specific Small Nuclear Basic Proteins (SNBPs) are present as well. During SCR, a yet unknown factor removes protamines in* Drosophila *and mouse, and histone chaperone HIRA deposits maternally provided H3.3 and H4. In* Xenopus, *nucleoplasmin exchanges SP2-3 for H2A-H2B thereby reconstituting nucleosomes in male pronucleus. In* C. elegans, *unknown factors participate in the exchange of paternally provided H3.3 and SNBPs with maternally provided H3.3.*

al., 1999). MSCI represents a case of chromosome wide, RI chromatin remodeling that is involved in silencing. Along with the implication of H3.3 in sperm chromatin remodeling at fertilization (see below), this developmental process indicates that H3.3 can be deposited at large genomic regions that are depleted in nucleosomes. In *C. elegans*, a mechanism presenting similarities with MSCI is responsible for the silencing of the X chromosome during male meiosis (reviewed in Ooi and Henikoff, 2007). In the absence of a homologous counterpart, the X chromosome is silenced. Similar to the situation in mouse, it correlates with enrichment in silent PTMs such as H3K9me2 (Reuben and Lin, 2002). However, in the nematode, H3.3 is surprisingly depleted from the silent X chromosome, suggesting that, in contrast to MSCI, silencing does not involve chromosome wide RI nucleosome assembly (Ooi and Henikoff, 2007; Ooi *et al*., 2006).

Spermiogenesis

After the completion of meiosis, spermatids undergo a complex differentiation process called spermiogenesis, which results in the production of mature gametes. Marking features of this maturation include the formation of a motile flagellum, the elimination of excess cytoplasmic materials and the dramatic rearrangement of the nuclear architecture. In many species, spermiogenesis is in fact the only differentiation process where nuclei loose, in a reversible manner, their nucleosome-based chromatin to a totally different structure. Indeed, histones are first replaced with transition proteins and then with Sperm Nuclear Basic Proteins (SNBPs) during the condensation phase of spermatid nuclei. SNBPs include testis specific histone variants but also non-histone proteins such as protamine-like proteins and protamines (Caron *et al*., 2005; Govin *et al*., 2004; Lewis *et al*., 2003; Poccia and Collas, 1996). The sperm chromatin structure is highly diverse in animals, even between species of the same animal group (Frehlick *et al*., 2006). In general, sperm chromatin is highly condensed and thus not compatible with DNA replication or transcription (Poccia and Collas, 1996). Like other core histones, H3.3 is expressed in the male germ line. In *Drosophila*, only the histone *H3.3A* gene is strongly expressed in testis (Akhmanova *et al*., 1995) and the protein is detected in nuclei at all stages of spermatogenesis, with the exception of late spermatid and mature sperm nuclei (Akhmanova *et al*., 1997; Bonnefoy *et al*., 2007). Because these studies relied on immunofluorescence techniques, the possibility remained that H3.3 epitopes were not accessible in highly condensed spermatid and sperm nuclei. However, the use of a *H3.3-mRFP1* expressing transgene confirms that H3.3 is eliminated from the spermatid nuclei, just before the deposition of protamines (Fig. 3). This situation is in clear contrast with the case of the nematode *C. elegans* that retains H3.3 in mature sperm nuclei (Ooi *et al*., 2006), illustrating the diversity of sperm chromatin architecture and composition in animals. Although the bulk of sperm chromatin in *Drosophila*, mouse or humans is packaged with protamines, it also retains a variable proportion of histones (Caron *et al*., 2005; Dorus *et al*., 2006; Poccia and Collas, 1996; Raja and Renkawitz-Pohl, 2005). Consequently, it has been proposed that histones, and, possibly, H3.3, could play a role in transmitting epigenetic information

through the male gamete (Ooi and Henikoff, 2007). In mammals, similarly, it has also been proposed that paternal imprinting control regions could escape the histone/protamine exchange and would remain organized in nucleosomes in mature sperm (Delaval *et al*., 2007). The emergence of global ChIP approaches should help determining the putative role of H3.3 in the chromatin landscape of the male gamete.

In *Drosophila*, almost all the transcription required for spermiogenesis occurs in primary spermatocytes (Fuller, 1993). Thus, the abundance of H3.3 in the male germline also probably reflects the high level of transcription that takes place in these cells. Another possible role for H3.3 in spermatid nuclei could be related to its "nucleosome destabilizing" property. Indeed, nucleosomes containing H3.3, alone or in synergy with the H2A.Z variant, are more prone to loose H2A/H2B dimers in salt-disruption experiments, than regular nucleosomes (Jin and Felsenfeld, 2007). Similarly, assembly and disassembly of nucleosomes containing the mammalian variant H2A.Bbd occur more efficiently in association with H3.3 than with H3 (Okuwaki *et al*., 2005). If this were true in the context of *in vivo* chromatin, it would be interesting to see if it has any role in facilitating the replacement of nucleosomes with transition proteins and protamines during spermatid differentiation.

In flowering plants, the structure of the male gamete chromatin is poorly known. Recently however, a pollen specific *H3* gene called *AtMGH3* has been identified in *Arabidopsis*, along with eight *H3.3* genes (Okada *et al*., 2005). Although AtMGH3 is quite distantly related to animal H3.3 (see Fig. 2), this histone has the same amino-acid substitutions at position 87, 89 and 90 than those found in plant *H3.3* genes. Moreover, this gene was found to exhibit RI expression in male gametic cells (Okada *et al*., 2005). AtMGH3 is present in the chromatin of the male gamete and, similarly to the situation found in *C. elegans*, this H3 variant is removed from the zygote nucleus in a RI manner (Ingouff *et al*., 2007). Interestingly, *AtMGH3* mutants do not seem to display any phenotype, probably indicating a redundant role with other H3 variants (Okada *et al*., 2005).

Male pronucleus formation

The formation of the male pronucleus at fertilization implies the removal of SNBPs followed by *de novo* assembly of paternal nucleosomes, a process called SCR (Sperm Chromatin Remodeling) (Fig. 4). An essential, although largely overlooked aspect of SCR, is the fact that paternal chromatin assembly takes place independently of DNA synthesis (Nonchev and Tsanev, 1990; Poccia *et al*., 1984). The recent discovery that H3.3 was specifically deposited in the decondensing sperm nucleus in *Drosophila* and mouse confirmed the RI nature of this conserved process (Loppin *et al*., 2005; Torres-Padilla *et al*., 2006; van der Heijden *et al*., 2005). In these two model species, the sperm chromatin is essentially packaged with protamines (see previous section). Thus, the RI reassembly of H3.3 containing nucleosomes on paternal DNA is a genome wide process. The male pronucleus is in fact the only nucleus to undergo whole genome RI chromatin assembly during development. The specific use of the H3.3 variant in SCR is remarkable, in particular for those species where large pools of maternally expressed histones, including H3 and H3.3, are stored in the egg. In *Drosophila*, for instance, early development is under strict maternal control and zygotic transcription begins when several thousands nuclei have already assembled their chromatin (Foe, 1993). By analyzing transgenic fly lines expressing tagged versions of H3 or H3.3, we have shown that H3.3, and not H3, is deposited during SCR (Loppin *et al*., 2005). SCR is thus under the control of a specific nucleosome assembly machinery that specifically uses H3.3, despite the availability of both histone types in large quantities. Thus, SCR is clearly a process where H3.3 deposition is not determined by the simple unavailability of H3, but by its proper nucleosome assembly pathway.

In *C. elegans* and *Arabidopsis*, H3.3 histones are present at apparently high levels in the male gamete, in contrast to mouse or *Drosophila*. Surprisingly however, these paternal histones are also removed at fertilization, before the first zygotic DNA replication (Ingouff *et al*., 2007; Ooi *et al*., 2006). In *C. elegans* (this is not known for *Arabidopsis*), a RI deposition of maternally expressed H3.3 is observed during SCR, as in *Drosophila* or mouse. The functional signification of this apparent replacement is not clear. Mass spectrometry analysis of *C. elegans* sperm has revealed the presence of SNBPs similar to invertebrate protamines (Chu *et al*., 2006), suggesting that maternal H3.3 replaces the removed SNBPs. In this case, paternal H3.3 would be removed along with SNBPs before global deposition of maternal H3.3. It is thus difficult to imagine any epigenetic role for paternal H3.3, at least for the bulk of it. More probably, the persistence of high levels of H3.3 in sperm could only reflect the vast diversity of sperm chromatin types in animals (Poccia and Collas, 1996). In their recent finding that H3.3 was the support for the epigenetic memory of active gene states in nuclear transfer experiments, Ng and Gurdon (2008) pointed the importance of H3.3 lysine 4 in this phenomenon. Indeed, a mutant form of H3.3 with a glutamic acid in position 4 interfered with the epigenetic inheritance. It is interesting to note that maternal H3.3 incorporated during *Drosophila* or mouse SCR is not methylated on lysine 4 (Loppin *et al*., 2005; van der Heijden *et al*., 2005), thus reinforcing the view that SCR is essentially a neutral replacement process. Accordingly, in *Drosophila*, the paternal H3.3 enrichment is lost after a few nuclear cycles as the chromatin accumulates H3 nucleosomes at each S phase (Bonnefoy *et al*., 2007). In this case, the perpetuation of a putative H3.3 "barcode", as proposed by Hake and Allis (2006), is not observed. Whether the distinction between methylated and non-methylated forms of H3.3 is involved here remains to be established.

Roles of nucleosome assembly machineries in the deposition of H3.3

The HIRA nucleosome assembly pathway

Although the implication of the CAF-1 complex in RC chromatin assembly was established long ago (Smith and Stillman, 1989), the identification of assembly factors able to deposit histones in the absence of DNA synthesis received attention only recently. HIRA belongs to the HIR family of proteins whose funding members are the budding yeast Hir1p and Hir2p proteins (Spector *et al*., 1997). These two proteins are orthologs to the N- and C-terminus of HIRA proteins, respectively (Lamour *et al*., 1995). HIRA proteins are characterized by the presence of seven WD-repeats known to assemble into a secondary structure called a Beta propeller (Smith *et al*., 1999). In mouse, *Hira* is an essential

gene and knocked-out embryos die early in development with a complex phenotype that has been interpreted as resulting from precocious cell differentiation (Meshorer *et al.*, 2006; Roberts *et al.*, 2002). It is the finding that HIRA had histone binding properties that fuelled its functional characterization *in vitro* (Lorain *et al.*, 1998). The nucleosome assembly activity of HIRA was initially characterized from *Xenopus* egg extracts, and found to be specific for a DNA synthesis-independent assembly pathway (Ray-Gallet *et al.*, 2002). The subsequent purification and characterization of proteins interacting with H3.1 and H3.3 in human cells established a first link between H3.3 and HIRA (Tagami *et al.*, 2004). HIRA and the two largest CAF-1 subunits were specifically found in the H3.3 and H3.1 complexes, respectively, hence confirming the existence of distinct assembly pathways defined by their dependence on DNA synthesis, assembly factors and preferential histone H3 type.

SCR: a challenging task for RI nucleosome assembly machineries

The *in vivo* function of *Hira* received an unexpected highlight from the characterization of *sésame* (*ssm*), its first mutant allele in *Drosophila*. Embryos produced by homozygous mutant *ssm* females are haploid and develop with the sole, maternally derived, chromosome set. The loss of paternal chromosomes occurs at the first embryonic mitosis and is the consequence of a defect in male pronucleus formation (Loppin *et al.*, 2000). In *Drosophila*, SCR classically involves the rapid replacement of two closely related protamines with maternally provided histones (Bonnefoy *et al.*, 2007; Raja and Renkawitz-Pohl, 2005; Rathke *et al.*, 2007). Moreover, *Drosophila* SCR is a RI process that specifically involves the H3.3 variant (Loppin *et al.*, 2005). In *ssm* eggs, SCR is defective: although protamines are normally removed, the sperm derived nucleus does not incorporate histones. As a consequence, the male pronucleus does not fully decondense and does not replicate its DNA (Bonnefoy *et al.*, 2007; Loppin *et al.*, 2001; Loppin *et al.*, 2005). In *Drosophila*, HIRA is thus critical for the RI chromatin assembly of the whole paternal genome and specifically assembles H3.3 containing nucleosomes (Loppin *et al.*, 2005). In addition, *Drosophila* HIRA has also been implicated in H3.3 deposition at a regulatory site near a variegating *white* transgene inserted near centromeric heterochromatin (Nakayama *et al.*, 2007). Histone exchange at this site is dependent on the binding of the GAGA factor-FACT complex. In *ssm* flies, the silencing of this *white* transgene is enhanced, indicating that HIRA is involved in counteracting the spreading of heterochromatin in this locus (Nakayama *et al.*, 2007). Surprisingly, homozygous flies with a null allele of *Hira* are viable and female sterility is the only associated phenotype (Bonnefoy *et al.*, 2007). This surprising result indicates that any function of HIRA not related to SCR is dispensable in *Drosophila*. In mouse, the zygotic lethality of *Hira* knocked-out embryos does not allow testing the requirement of maternal HIRA for SCR. However, considering that mouse HIRA actually localizes to the decondensing male pronucleus and that SCR involves the massive deposition of H3.3 in this species (Torres-Padilla *et al.*, 2006; van der Heijden *et al.*, 2005), this critical function of HIRA is most probably conserved. In fact, HIRA is expected at least to play this role in a majority of species whose sperm contains non-nucleosomal chromatin. Some species, like the frog *Rana catesbeiana*, for instance, do not seem to contain protamines or protamine-like SNBPs but instead retain core histones in the sperm chromatin (Frehlick *et al.*, 2006). It is thus possible that HIRA is not required for SCR in these species. Similarly, *Xenopus* sperm chromatin retains H3 and H4 whereas H2A and H2B are replaced with protamin-like proteins. Since nucleoplasmin, a histone chaperone for H2A and H2B is necessary and sufficient for *Xenopus* SCR *in vitro* (Philpott and Leno, 1992; Philpott *et al.*, 1991), it suggests that this process does not actually require a H3/H4 RI assembly factor such as HIRA (Fig. 4).

The specific *Hira* mutant phenotype observed in *Drosophila* could result from a function of HIRA related to some peculiar features of SCR, rather than from a general RI nucleosome assembly defect. At least, we know that the removal of protamines itself does not seem to depend on HIRA because these SNBPs are normally removed in *Hira* mutant eggs (Bonnefoy *et al.*, 2007). The recent discovery that another nucleosome assembly factor, CHD1, was important for male pronucleus formation in *Drosophila* shed a new light on this process (Konev *et al.*, 2007). CHD1 (Chromo-ATPase/Helicase-DNA-binding protein 1) is an ATP-dependent nucleosome remodeling factor of the SNF2-like family of proteins, which is characterized by the presence of two chromodomains (Brown *et al.*, 2007; Hall and Georgel, 2007; Marfella and Imbalzano, 2007; Woodage *et al.*, 1997). *In vitro*, CHD1 facilitates the transfer of histones from the NAP-1 histone chaperone to DNA and allows the assembly of regularly spaced nucleosomes (Lusser *et al.*, 2005). *Drosophila* adults with no functional CHD1 survive but are sterile. In females, the sterility results from a 100% maternal effect embryonic lethality. As in embryos produced by *Hira* mutant females, the male nucleus in *chd1* mutant eggs is unable to participate in the formation of the zygote (Konev *et al.*, 2007). In contrast to *Hira* mutant eggs, where the male nucleus is always spherical and devoid of histones, the male nucleus in *chd1* mutant eggs adopts various shapes and histones are detected (Konev *et al.*, 2007) (G.A.O and B.L unpublished observations). Notably, H3.3 is detected in the paternal chromatin of *chd1* mutant eggs (Fig. 5), indicating that at least some HIRA-dependent histone deposition occurs in the absence of this motor protein. Thus, CHD1 could synergize with HIRA for the very rapid and massive RI nucleosome assembly activity required for SCR or could participate in the regular spacing of nucleosomes on paternal DNA.

Although the exact function of CHD1 at fertilization remains to be determined, it is remarkable that SCR, a process that occurs once in the life cycle and in a single nucleus, represents a critical task for at least two different nucleosome assembly factors. Understanding how these proteins are orchestrated *in vivo* for RI assembly over a whole genome is a fascinating question for future research.

Multiple assembly pathways involved in H3.3 deposition?

Although the functional characterization of H3.3 in metazoans awaits formal genetic analysis, it is now clear that this histone variant is involved in a variety of chromatin remodeling mechanisms. Whether these mechanisms rely on different nucleosome assembly pathways largely remains to be investigated. The fact that H3.3 is deposited independently of DNA synthesis is a major property that distinguishes it from H3, although at least one exception has been reported in the *Xenopus* oocyte where H3 seems to be RI deposited by a dynamic histone exchange process (Stewart *et al.*,

2006). Several lines of evidence indicate that H3.3 is deposited during S phase. In *Drosophila* cultured cells, overexpressed H3.3 is deposited at sites of DNA replication (Ahmad and Henikoff, 2002b). In early *Drosophila* embryos, during the rapid nuclear cleavages and before the onset of zygotic transcription, we have observed a relatively weak and uniform deposition of H3.3 in the chromatin of all nuclei that we interpret as S phase deposition (Bonnefoy *et al.*, 2007). Interestingly, this H3.3 deposition does not depend on the presence of the HIRA protein, opening the possibility that the CAF-1 complex could be responsible for the bulk of H3.3 nucleosome assembly during early *Drosophila* development. In this peculiar developmental context, where both H3 and H3.3 are stored in the egg and are thus available in large quantities, the RC assembly machinery seems to allow some deposition of the RI variant despite the fact that H3 is preferentially deposited (Bonnefoy *et al.*, 2007). More generally, the different models accounting for the propagation of epigenetic states through cell divisions also imply the deposition of H3.3 at DNA replication forks (Eitoku *et al.*, 2007; Hake and Allis, 2006; Henikoff *et al.*, 2004; Polo and Almouzni, 2006). However, the simple hypothesis that HIRA could participate in this task is challenged by our observations in fly embryos and thus deserves a real investigation.

Asf1 (Anti Silencing Factor 1) is a conserved histone chaperone involved in both RC and RI assembly pathways (reviewed in (De Koning *et al.*, 2007; Eitoku *et al.*, 2007; Mousson *et al.*, 2007). Several recent studies have showed that Asf1 interacts with a single H3-H4 dimer (Agez *et al.*, 2007; Antczak *et al.*, 2006; English *et al.*, 2005; Mousson *et al.*, 2005) suggesting that Asf1 could function in distributing H3-H4 or H3.3-H4 dimers to CAF-1 and HIRA, respectively. In addition, Asf1 plays a critical role for the unwinding of DNA replication forks by disrupting (H3-H4)2 tetramers (Natsume *et al.*, 2007) and by interacting with the putative replicative helicase MCM2-7 (Groth *et al.*, 2007). However, Asf1 is not directly involved in *de novo* RI or RC histone deposition in *Xenopus* egg extracts (Ray-Gallet *et al.*, 2007). Similarly, Asf1 is not detected in the decondensing male nucleus during *Drosophila* SCR (Bonnefoy *et al.*, 2007).

Interestingly, while TC assembly of H3.3 nucleosomes is well established, the histone chaperone responsible for this deposition remains elusive. In *Drosophila*, adults devoid of HIRA are viable suggesting that HIRA is not critical for TC assembly (Bonnefoy *et al.*, 2007). In addition, the absence of HIRA only causes a slightly delayed growth in chicken cells (Ahmad *et al.*, 2005). Spt6 and FACT are histone binding proteins that are involved in the reassembly of nucleosomes after the passage of the RNA polymerase II and thus represents interesting candidates for TC H3.3 deposition (Adkins and Tyler, 2006; Andrulis *et al.*, 2000; Belotserkovskaya *et al.*, 2004; Kaplan *et al.*, 2000). In addition to its role in SCR mentioned above, the CHD1 assembly factor has also been shown to affect H3.3 deposition in *Drosophila* blastoderm embryos suggesting its participation in TC assembly (Konev *et al.*, 2007).

The diversity of RI chromatin assembly processes should thus be reflected by the implication of various assembly factors, depending both on the model species as well as on the developmental or cellular processes considered. Understanding how these different factors

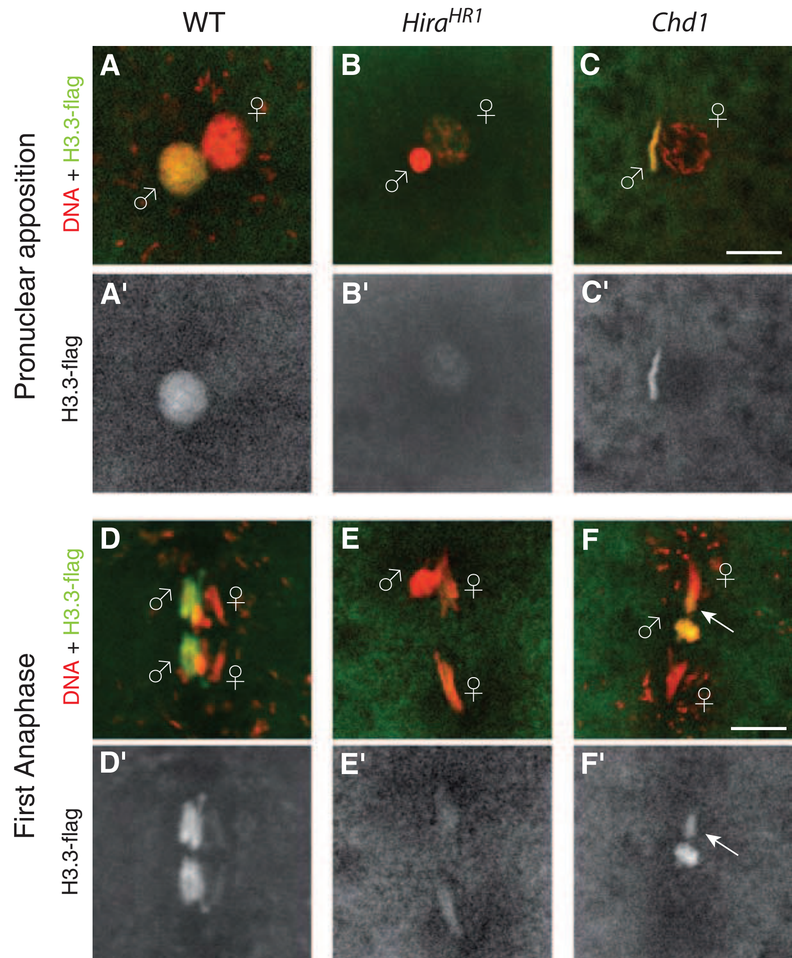

Fig. 5. HIRA and CHD1 are involved in *Drosophila* SCR. *Confocal images of eggs at the pronuclear apposition stage* **(A,B,C,A′,B′,C′)** *or embryos at the first zygotic anaphase* **(D,E,F,D′,E′,F′)** *stained for DNA (red) and with an anti-FLAG peptide antibody to detect maternally expressed H3.3-FLAG (green or gray).* **(A,A′)** *In eggs from wild type females, H3.3-FLAG is detected in the male pronucleus.* **(B,B′)** *In eggs laid by mutant* $Hira^{HR1}/Hira^{HR1}$ *females, H3.3-FLAG is not detected in the abnormally condensed male nucleus.* **(C,C′)** *In eggs laid by* chd1[1]/Df(2L)Exel7014 *females (with no functional CHD1 protein), the male nucleus is aberrant in shape but stains for H3.3-FLAG (see also Konev* et al.*, 2007).* **(D,D′)** *During the first zygotic anaphase, paternal chromosomes still contain high levels of H3.3-FLAG.* **(E,E′)** *In* $Hira^{HR1}$ *mutant eggs, the male nucleus is excluded from the first spindle that contains only maternal chromosomes.* **(F,F′)** *In* chd1 *mutants eggs, the male nucleus is occasionally incorporated in the first mitosis but paternal chromosomes (stained with H3.3-FLAG) segregation is defective (arrow). Wild-type males were used to fertilize females of indicated genotypes. All females used in these crosses contained a copy of a* H3.3-FLAG *transgene (Loppin* et al.*, 2005). DNA positive dots visible in (A,D,F) are* Wolbachia *endosymbiotic bacteria.*

cooperate and interact on the nucleosome assembly line will certainly need the forces of both biochemical and *in vivo* approaches.

Conclusion

Two levels of complexity challenge the dynamic nature of eukaryotic chromatin. The first level is common to most cells and includes the invariable remodeling events associated with the cell cycle, from DNA replication to cellular senescence. The diversity of remodeling processes that occur during development represents a second level of complexity, which is best illustrated by the dramatic reorganization of chromatin associated with the transmission of paternal DNA from one generation to another. The universal ability of eukaryotic cells to assemble nucleosomes independently of DNA replication drives this versatility. The H3.3 histone variant is at the heart of RI nucleosome assembly mechanisms. Being very close to its RC counterparts at the primary sequence level, H3.3 fulfills a neutral replacement role supported by its constitutive expression. In addition, the biochemical characterization of the H3.3 deposition pathway, the association of this variant with active PTMs, as well as its dynamic distribution over the genome have paved the road to establish a role in the epigenetic transmission of active chromatin states. Finally, developmental and genetic studies have unveiled unexpected roles for H3.3 or associated assembly factors in chromatin remodeling events essential for sexual reproduction. In this regard, the evolution of new functions for RI nucleosome assembly factors could be the key for the diversification of H3.3 roles. These different aspects of H3.3 biology must be considered to understand the evolutionary forces that shaped this histone and perpetuated it as one of the most conserved proteins in life.

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Le cœur du débat exposé dans cet article réside dans l'idée que nous pouvons conceptuellement distinguer un rôle de H3.3 dans l'établissement et/ou le maintien de l'information épigénétique à proprement parler (celle qui détermine directement l'activité des gènes), d'un rôle purement structural où H3.3 ne ferait que remplacer de façon neutre (sans conséquence pour l'activité) la protéine H3 canonique.

En plus des arguments exposés dans cet article, un éclaircissement récent est venu de deux études biochimiques qui ont montré que les propriétés intrinsèques des nucléosomes contenant H3.3 *in vivo* étaient de nature à rendre les nucléosomes plus solubles, donc théoriquement rendant l'ADN associé plus facile à relaxer et plus accessible aux facteurs de régulation (Jin et Felsenfeld, 2007; Henikoff *et al.*, 2008; Henikoff, 2009; Jin *et al.*, 2009; Thakar *et al.*, 2009). Ainsi, nous savons aujourd'hui que la présence d'une histone H3.3 en lieu et place d'une histone H3 canonique n'est très probablement pas neutre pour les propriétés locales, et donc l'activité de la chromatine. Cependant, ces études ne traitent pas la question de l'importance fonctionnelle d'un tel aiguillage épigénétique.

Au contraire, la construction et la caractérisation des premiers mutants de H3.3 chez la drosophile par les laboratoires de Konrad Basler et de Kami Ahmad ont montré que le programme transcriptionnel des individus mutants n'est globalement pas affecté (Hodl et Basler, 2009; Sakai *et al.*, 2009). Ces résultats ont été un coup de tonnerre qui a fait basculer notre vision sur le rôle épigénétique de H3.3. Ces études montrent, en plus, que H3.3, bien que non indispensable pour la viabilité, est crucial pour la reproduction, ayant un rôle dans la méiose mâle et un dans la fertilité femelle. Le rôle de H3.3 dans les processus liés à la reproduction sexuée que nous proposions a bien été confirmé par des travaux plus récents.

Un point d'ombre reste le rôle de H3.3 au cours de la maturation du pronoyau mâle. En effet, bien qu'il soit maintenant établi qu'une mutation sur H3.3 est à l'origine d'un phénotype de stérilité femelle chez la drosophile, les bases cytologiques et moléculaires de cette stérilité sont pour l'heure inconnues. De plus, de façon surprenante et inattendue, l'histone H3 canonique est capable d'être incorporée dans la chromatine de façon IR en l'absence de H3.3, au moins dans certains loci à forte activité transcriptionnelle (Sakai *et al.*, 2009). Nous ne connaissons pas les mécanismes mis en jeu dans cet assemblage. Il serait intéressant de savoir

si l'histone H3 peut se substituer à l'histone H3.3 dans les œufs issus de femelles mutantes pour H3.3, le suppléant dans l'assemblage du pronoyau mâle.

Dans le pronoyau mâle a lieu un cas extrême d'assemblage de la chromatine IR et c'est à ce titre que ce modèle est pertinent pour explorer le rôle *in vivo* des facteurs associés à H3.3 et impliqués dans son dépôt. Bien que le facteur HIRA semble être absolument critique pour accomplir cet assemblage, l'étude fonctionnelle d'autres facteurs candidats met en avant le caractère unique de ce rôle. Par exemple, les facteurs DAXX et ATRX font partie d'une voie IR d'assemblage de la chromatine indépendante de HIRA chez la souris et chez l'homme (que j'aborderai en détail plus loin)(Drane *et al.*, 2010; Goldberg *et al.*, 2010; Lewis *et al.*, 2010; Wong *et al.*, 2010). Cependant, l'étude fonctionnelle de XNP, orthologue de ATRX chez la drosophile, montre que ce facteur n'est pas essentiel à la fertilité, suggérant fortement qu'il n'est pas requis pour l'assemblage de la chromatine paternelle à la fécondation, contrairement à HIRA (Bassett *et al.*, 2008; Schneiderman *et al.*, 2009).

La voie HIRA semble donc avoir en exclusivité la charge des premières étapes de l'assemblage de la chromatine dans ce noyau. L'étude biochimique des facteurs potentiellement impliqués dans cette voie chez l'humain, la souris, les levures *S.cerevisiae* et *S.pombe* et la drosophile montrent qu'un groupe de trois facteurs conservés pourraient faire partie du complexe. Ces protéines sont HIRA, bien sûr (Tagami *et al.*, 2004)(Hir1 et Hir2 chez *S.cerevisiae* et Slm9 et Hip1 chez *S.pombe*)(Xu *et al.*, 1992; Kanoh et Russell, 2000; Blackwell *et al.*, 2004; Green *et al.*, 2005; Prochasson *et al.*, 2005), Cabine (Hir3 chez *S.cerevisiae* et Hip3 chez *S.pombe*)(Greenall *et al.*, 2006), et l'Ubinucléine (UBN1 et UBN2 chez l'humain et la souris, Hpc2 chez *S.cerevisiae*, Hip4 chez *S.pombe* (Anderson *et al.*, 2010) et Yemanucléine-α chez la drosophile (Moshkin *et al.*, 2009)). Nous n'avons identifié aucun orthologue potentiel de Cabine chez la drosophile. En revanche, la Yemanucléine est un partenaire de HIRA auquel je consacrerai la prochaine partie.

III. Yemanucléine-α, un partenaire obligatoire de HIRA pour la formation du pronoyau mâle

Mon étude de la Yemanucléine-α (Yem) a commencé par la description d'un allèle ponctuel, *yem*[1] (entrainant un changement d'acide aminé V478E dans un domaine peu conservé), associé à un phénotype de létalité embryonnaire à effet maternel (Meyer *et al.*, 2010). Cet allèle a été isolé par notre collaboratrice sur ce projet, Ounissa Aït-Ahmed, mais son intérêt était porté sur le rôle de *yem* dans la régulation de la méiose femelle.

Nous avons voulu explorer le rôle potentiel de Yem dans la formation du pronoyau mâle. Nous avons découvert que dans les œufs issus de femelles *yem*[1], les chromosomes paternels étaient incapables d'être incorporés dans le premier noyau du zygote. Ce phénotype remarquablement similaire à celui associé aux mutations affectant *Hira* est cent pourcent pénétrant et est donc suffisant pour expliquer la stérilité des femelles mutantes. De plus, nous avons construit un allèle nul de *yem*, *yem*[2], qui est, lui aussi, viable et mâle fertile. L'analyse de l'assemblage de la chromatine dans le pronoyau de ces œufs mutants révèle que l'assemblage des nucléosomes y est fortement perturbé. Nous avons en somme montré que HIRA et Yem sont étroitement associées dans le processus de remodelage de la chromatine paternelle à la fécondation.

Les conclusions que nous avions tirées en raisonnant sur le phénotype associé aux mutants Hira s'élargissaient donc au complexe fonctionnel HIRA/Yem. Il s'agit ici de la première caractérisation *in vivo* du rôle de Yem (ou ses orthologues) dans l'assemblage de la chromatine. Ce travail montre, en effet, que Yem fait partie des facteurs d'assemblage de novo de la chromatine par le mode IR. L'ensemble de ces résultats est présenté et discuté dans l'article qui suit.

Drosophila HIRA Cooperates with Yemanuclein-α during Male Pronucleus Chromatin Assembly

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Running head: Function of Yemanuclein-α in *Drosophila*

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Abstract

During spermiogenesis, post-meiotic spermatids undergo a global chromatin remodeling whereby histones are replaced with sperm specific chromosomal proteins such as protamines. At fertilization, reconstitution of a nucleosome-based paternal chromatin requires the deposition of maternally provided histones before the first round of DNA replication. This critical process exclusively uses the histone H3 variant H3.3 and constitutes a unique example of genome-wide replication-independent (RI) *de novo* chromatin assembly. We had previously shown that the H3.3 chaperone HIRA plays a central role for paternal chromatin assembly in *Drosophila*. Although several conserved HIRA-interacting proteins have been identified from yeast to human, their actual implication in RI nucleosome assembly *in vivo* has remained unclear. Here, we show that Yemanuclein-α (Yem), the *Drosophila* member of the Hpc2/Ubinuclein family of HIRA-interacting proteins is essential for RI H3.3 assembly in the male pronucleus. We further show that *yem* mutations affect male pronucleus formation in a way remarkably similar to HIRA and we demonstrate the specific interdependence of these proteins for their targeting to the decondensing male pronucleus. Our work thus establishes that HIRA and Yem cooperate *in vivo* for paternal chromatin assembly, suggesting an ancient functional partnership for these two protein families in RI nucleosome assembly.

Author summary

Chromosome organization relies on a basic functional unit called nucleosome, in which DNA is wrapped around a core of histones proteins. However, during male gamete formation, histones are entirely replaced by sperm-specific proteins that are adapted to sexual reproduction but incompatible with the formation of the first zygotic nucleus. These proteins must therefore be conversely replaced by histones upon fertilization, during a chromatin assembly process that is crucial for zygote formation and that requires the histone deposition

factor HIRA. In this study, we identified the conserved protein Yemanuclein-α (Yem) as a new partner of HIRA at fertilization. To test Yem function, we generated mutations affecting the *yem* gene and found that adult mutant females were completely sterile. We showed that sterility was caused by a defect in eggs laid by these mutant females, as histone assembly failed in paternal chromosomes, which excluded them from the first zygotic nucleus, and subsequently caused the death of the embryos. Finally, we found that Yem and HIRA are mutually dependent to perform chromatin assembly at fertilization, demonstrating that they tightly cooperate *in vivo*. In conclusion, our results shed new light into critical mechanisms controlling paternal chromosomes formation at fertilization.

Introduction

The basic unit of chromatin is the nucleosome, which consists of DNA wrapped around an octamer of histones [1]. Nucleosome assembly is a stepwise process where deposition of a histone H3-H4 heterotetramer precedes incorporation of two H2A-H2B dimers. While the bulk of chromatin *de novo* assembly occurs during genome replication and mainly involves canonical histone H3, alternative, replication-independent (RI) chromatin assembly exclusively uses the conserved histone variant H3.3 [2]. Notably, RI chromatin assembly occurs during transcription, resulting in an enrichment of H3.3 in actively transcribed regions, and opening the possibility that this histone variant could constitute an epigenetic mark for active chromatin [3-7]. However, this idea has been challenged as the specific requirement for the RI histone variant has been addressed *in vivo* in the protist *Tetrahymena thermophila* and, more recently, in *Drosophila*. Surprisingly, H3.3 is not absolutely required for survival or to ensure the bulk of adequate gene expression in either of these systems but, instead, the RI histone variant is essential to generate viable sexual progeny in *Tetrahymena* and for male and

female fertility in *Drosophila* [8-10]. These results thus suggested that H3.3 has critical roles in sexual reproduction that could account for its wide conservation (see[11,12]).

In many sexually reproducing animals, sperm chromatin organization dramatically differs from somatic cells. Indeed, during spermiogenesis, histones are progressively replaced with sperm-specific proteins, such as protamines, which leads to a chromatin configuration adapted to the constraints of sexual reproduction but incompatible with DNA replication or transcription [13]. As a consequence, massive RI chromatin assembly occurs during male pronucleus formation at fertilization, when protamines are entirely replaced with maternally provided histones before the onset of the first zygotic replication [14-16]. We have previously shown that, in *Drosophila,* this process specifically uses histone variant H3.3 and requires its conserved chaperone HIRA [14]. Indeed, mutations in *Hira* are viable but H3.3 assembly in the male pronucleus is completely abolished in eggs laid by mutant females, resulting in the loss of the paternal set of chromosomes and embryonic death [14,17]. Therefore, male pronucleus chromatin assembly is a crucial step for zygote formation, specifically requiring the activity of a HIRA/H3.3 pathway. In addition, this constitutes a unique genome-wide, transcription-independent, *de novo* chromatin assembly process that represents an opportunity to study the different actors involved in RI H3.3 deposition. However, although several conserved members of the HIRA and H3.3 complexes have been identified, their role *in vivo* and, in particular, during male pronucleus formation, has remained largely unexplored.

In *S.cerevisiae*, the Hir chromatin assembly complex includes the HIRA-related proteins Hir1 and Hir2 as well as Hir3/Cabin, Asf1 and Hpc2 [18,19]. Recently, human HIRA-interacting Ubinuclein 1 and Ubinuclein 2 (UBN1 and 2) were found to be the orthologs of yeast Hpc2 [20-22]. Interestingly, the homology is restricted to a ~50 amino-acid domain called HRD or HUN domain, which directly interacts with the WD repeats of HIRA and Hir proteins [20,21]. In *Drosophila*, Yemanuclein-α (Yem)[23] is the only HRD containing protein [20].

Accordingly, Yem has been shown to co-purify with HIRA in *Drosophila*, thus establishing Yem as a conserved member of the HIRA complex [24]. In this paper, we report the functional characterization of Yem as an essential partner of HIRA for H3.3 deposition in the male pronucleus. We have generated different *yem* mutations and found that their associated phenotype is remarkably similar to *Hira* mutants. We show that Yem is essential for nucleosome assembly in paternal chromosomes at fertilization, thus demonstrating a direct role in RI chromatin assembly for any member of the Hpc2/UBN/Yem family. We further show that HIRA and Yem are inter-dependent for their recruitment to the forming male pronucleus and, hence, for their chromatin assembly activity, underlining the requirement of a functional HIRA/Yem cooperation *in vivo*.

Results

Yem is a maternally expressed protein essential for female fertility.

In human and mouse, UBN1 is found in a large variety of cell types and tissues, and its localization is mainly nuclear [21,25,26]. In *Drosophila*, *yem* trancripts are found in both males and females, at all stages of development, and are very abundant in ovaries (Figure 1B and not shown)[23,27,28]. In addition, in the female germline, Yem protein specifically accumulates in the oocyte nucleus (called Germinal Vesicle, GV) [23], suggesting that it could play a role in female fertility. The original *yem*^{1} mutant allele was isolated in a EMS mutagenesis that was designed to identify female sterile mutations that did not complement *Df(3R)3450*, a large deficiency covering the *yem* locus (Meyer *et al.*, in preparation)(Table 1). The *yem*^{1} point mutation causes a single amino-acid replacement in Yem protein (V478E; see Figure 1A). *yem*^{1}/*Df(3R)3450* mutant females showed no change in *yem* mRNA levels or Yem protein accumulation in the GV (Figure 1B, C). The female sterile phenotype associated with the *yem*^{1} chromosome is fully rescued by a transgene expressing a Yem-Flag tagged

protein (*yem-flag*^{HPF16}), thus demonstrating that the phenotype is specifically caused by the *yem*^{1} mutation (Table 1). In addition, we obtained a second allele, *yem*^{2}, by P-element imperfect excision (Figure 1A). *yem*^{2} is a 3180bp deletion uncovering the 5' UTR and most of the coding region of the *yem* locus, suggesting that it could be a null or at least a strong loss of function allele. Accordingly, *yem*^{1}/*yem*^{2} and *yem*^{2}/*Df(3R)3450* females were completely sterile (Table 1). In addition, *yem*^{2} causes a strong reduction in *yem* transcript levels and Yem protein was no longer detected in the GV (Figure 1 B,C).

We had previously reported the remarkable and specific accumulation of HIRA in the GV throughout oogenesis, in a way identical to Yem [17,23]. In fact, both proteins filled the whole nuclear volume of the GV with the exception of the karyosome, a compact sub-nuclear structure that contains the oocyte chromosomes (Figure 2A). Interestingly, it has been proposed that in human cells, formation of senescence-associated heterochromatin foci by HIRA requires its prior localization to promyelocytic leukemia nuclear bodies, suggesting that these structures could participate in the formation of the HIRA complex before its translocation to chromatin [21,29]. We thus wondered whether the intriguing accumulation of both HIRA and Yem in the GV nucleoplasm could similarly favor the assembly of the HIRA complex in oocytes. Strikingly, we observed that HIRA accumulation in the GV was completely abolished in *yem*^{2}/*Df(3R)3450* mutant ovaries, suggesting that Yem is required for the localization or stabilization of HIRA in this nucleus (Figure 2B). Conversely, we found that Yem was frequently undetected in null *Hira*^{HR1} mutant background (Figure 2B). In human cells, the knockdown of *Hira* or *ubn1* results in a strong reduction of UBN1 and HIRA protein levels, suggesting that the presence of one partner is necessary for the stability of the other [21]. In *Drosophila* however, both HIRA and Yem protein were readily detected in ovaries of *yem*^{2}/*Df(3R)3450* or homozygous *Hira*^{HR1} females, respectively (Figure 2C). Thus, loss of one protein either affects the recruitment or the stability of its partner specifically in

the GV. In any case, these results underline the potential role of the GV as a staging ground for HIRA/Yem complex assembly in maturing oocytes and, importantly, are a strong indication that HIRA and Yem are functional partners *in vivo*.

Yem is required for paternal chromatin remodeling at fertilization

While *yem*^{1}*/Df(3R)3450* or *yem*^{2}*/Df(3R)3450* mutant males appeared normally fertile (not shown), mutant females were sterile (Table 1) as a result of a complete maternal effect embryonic lethality phenotype. Eggs laid by *yem* mutant females were readily fertilized (Figure 3A) but never hatched and turned brown after death (Table 1 and Figure 3B). These features are strongly reminiscent of the maternal effect embryonic lethality phenotype of *Hira* mutants, where gynogenetic haploid embryos die before hatching, regardless of the paternal genotype [30]. This phenotype is a consequence of a defect in *de novo* chromatin assembly in the male pronucleus, which results in the loss of paternal chromosomes in the zygote [14,17,31]. Nucleosome assembly at this stage is readily detectable by pan-acetylation of histone H4 staining, a mark of newly assembled chromatin (Figure 4 and [14]). Strikingly, we observed that acetylated H4 essentially failed to be incorporated in the male pronucleus in eggs from *yem*^{1}*/Df(3R)3450* or *yem*^{2}*/Df(3R)3450* females (referred to as *yem*^{1} or *yem*^{2} eggs for simplicity)(Figure 4). At pronuclear apposition, male pronuclei in *yem* mutant eggs always appeared round and un-decondensed, in a way identical to *Hira* mutants (Figure 5A). Paternal chromosomes subsequently failed to integrate the first zygotic division in *yem* mutant eggs, systematically resulting in gynogenetic haploid development (Figure 4).

While the *yem-flag*^{HPF16} transgene efficiently rescued *yem* female sterility, another insertion of the same construct (*yem-flag*^{HPF1}) only restored fertility to very low levels, likely because of its weak expression (see Table 1). Interestingly, in eggs laid by *yem*^{1}*/Df(3R)3450; yem-flag*^{HPF1} females, male pronuclei still appeared round and un-decondensed but consistently

incorporated significant levels of acetylated histone H4 (Figure 5A). This indicates that Yem protein is limiting for both nucleosome assembly and male pronucleus decondensation.

We have previously shown that HIRA-dependent nucleosome assembly in the male pronucleus exclusively uses the histone H3 variant H3.3 [12,14]. As expected, H3.3 was not incorporated in paternal chromatin of yem^{1} eggs, as in *Hira* mutants (Figure 5B). However, the female pronucleus still incorporated low levels of H3.3 during the first round of DNA replication, arguing that, like HIRA, Yem does not participate to the limited Replication-Coupled (RC) assembly of H3.3 which occurs in the female pronucleus and in cleavage nuclei (Figure 5B and not shown) [14]. Thus, Yem and HIRA are both specifically required for RI nucleosome assembly during male pronucleus formation.

Although mutant yem^{1}*/Df(3R)3450* and yem^{2}*/Df(3R)3450* adults were viable, survival rates were mildly reduced for yem^{2}*/Df(3R)3450* individuals (Supplementary Table 1). Together with the ubiquitous expression of *yem* and *Hira* [14], this indicates that these factors have nevertheless somatic roles. Interestingly, however, the partial lethality of yem^{2} mutant individuals was not aggravated when combined with $Hira^{HR1}$ and, notably, double null males were fertile (not shown). This thus suggests that HIRA and Yem do not have redundant functions but, instead, are obligate partners for male pronucleus chromatin assembly.

HIRA and Yem are interdependent for their localization to the male pronucleus.

Consistent with its critical role in paternal chromatin assembly, maternally expressed HIRA is recruited to the male nucleus shortly after fertilization in both *Drosophila* and mouse [14,16]. Surprisingly, while robust HIRA-Flag staining is observed in the decondensing male nucleus in control eggs, HIRA-Flag was never detected in eggs from yem^{1} and yem^{2} females (Figure 6A). Thus, Yem is required for the recruitment or for the stabilization of HIRA in the male nucleus. Accordingly, in control eggs, we specifically detected maternally expressed Yem-

Flag protein in the decondensing male nucleus, before pronuclear apposition (Figure 6C). However, in contrast to the apparently strong and homogeneous HIRA distribution in the male nucleus, Yem-Flag was more weakly detected and appeared enriched in a nuclear focus of unknown nature in about half of the observed male nuclei. Nevertheless, we found that Yem-Flag failed to be properly recruited in a majority of *Hira*^{ssm} eggs, suggesting that the conserved arginine residue mutated in *Hira*^{ssm} (R225K) is important for the HIRA/Yem interaction in the male pronucleus (Figure 6C). Interestingly, this result is consistent with the fact that the corresponding arginine in Human HIRA (R227) is involved in HIRA/UBN1 interaction [21]. We conclude that HIRA and Yem are interdependent for male pronucleus localization and, hence, for their chromatin assembly activity within this nucleus. Notice that, in contrast, point mutations do not affect HIRA/Yem localization in the GV (see Figure 2B), suggesting that the mechanisms controlling HIRA/Yem recruitment to the GV or to the male pronucleus are distinct. This could reflect the fact that the HIRA/Yem complex is active in the male pronucleus where these proteins are in a chromatin environment in contrast to their nucleoplasm distribution in the GV.

The H3-H4 histone chaperone ASF1 is a conserved member of Hir/HIRA complexes [18,19,22,24], which has been shown to directly interact with the conserved B domain of HIRA [22,32]. This interaction could allow ASF1 to transmit H3.3-H4 dimers to the HIRA complex before their deposition on DNA [32]. Accordingly, in human cells, specific deletion of the B domain prevents HIRA to induce the formation of senescence-associated heterochromatin foci [29]. Banumathy et al. have proposed that ASF1, HIRA and UBN1 form a tripartite complex where HIRA would be a scaffold for the UBN1 and ASF1 proteins [21]. The lethality of *Drosophila asf1* mutant alleles [33] prevented us to directly evaluate the influence of ASF1 on HIRA/Yem activity in the male pronucleus. Instead, we generated transgenic flies expressing a HIRA-Flag protein deleted for the ASF1-interacting B domain

(HIRA$^{\Delta B}$-Flag). Surprisingly, *Hira*$^{\Delta B}$*-Flag* fully rescued the sterility of *Hira*^{HR1} mutant females (Supplementary Figure 1). Moreover, HIRA$^{\Delta B}$-Flag properly localized in the male pronucleus (Figure 6B). These results indicate that an ASF1/HIRA interaction is not required for RI paternal chromatin assembly, consistent with our previous finding that ASF1 does not localize in the decondensing male pronucleus [17].

Discussion

Studying male pronucleus formation offers a unique opportunity to identify and functionally characterize factors required for *de novo* RI H3.3 assembly *in vivo*. The identical features of *Hira* and *yem* mutant phenotypes and their interdependence for recruitment in the male nucleus show that they intimately cooperate for RI nucleosome assembly after the removal of protamines. In clear contrast, we show that nucleosome assembly on paternal DNA is an ASF1-independent process. Interestingly, ASF1 has been shown to be dispensable for HIRA-mediated *in vitro* RI chromatin assembly using xenopus egg extracts, [34]. Taken together, these data suggest that the HIRA complex could use alternative sources of H3.3-H4 dimers in animal eggs. Finally, along with Hpc2/UBN/Yem and ASF1, Cabin/Hir3/Hip3 are conserved members of the various HIRA/HIR or H3.3 complexes [4,18,19,22,35]. Intriguingly, however, we failed to identify orthologs of Cabin/Hir3/Hip3 in *Drosophila*, despite the wide conservation of this protein family.

The *Drosophila* SNF2-like chromatin remodeling factor Chd1 has been found associated to HIRA in embryo extracts [36]. Interestingly, mutations in *chd1* affect the formation of the male pronucleus, which shows abnormal morphology in eggs laid by mutant females [36]. However, in comparison to *Hira* or *yem* mutants, we observed that mutations in *chd1* do not drastically affect H3.3 incorporation in paternal chromatin (Figure 5C and [12]). We thus favor a model in which Chd1 organizes the ordered spacing of nucleosomes through its

ATPase motor activity [37], following histone deposition by the HIRA/Yem complex. Absence of Chd1 activity would then lead to defective nucleosome spacing at a genome-wide scale. This defect could cause decreased processivity of assembly and, ultimately, prevent proper male pronucleus decondensation.

Interestingly, recent studies in human and mouse have unveiled the existence of an alternative H3.3 deposition pathway, which is dependent on the histone chaperone DAXX (Death-associated factor) and the chromatin remodeling factor ATRX [4,38-41]. Implication of the ATRX *Drosophila* ortholog, XNP, in RI chromatin remodeling suggests that this pathway is conserved [42]. Moreover, the oncogene DEK has been shown to assemble H3.3 *in vitro* and at ecdysone-induced loci in *Drosophila* salivary gland cells [43]. These recent findings give a more complex picture of H3.3 assembly pathways than previously appreciated. Future work should evaluate the implication of these factors in RI paternal chromatin assembly, which will give a more complete view of the interplay between these different pathways.

In contrast to the situation in *Drosophila*, HIRA is essential for viability in mammals [44]. Accordingly, HIRA has been implicated in RI H3.3 assembly in mouse embryonic stem cells, where its activity seems to be mainly restricted to active genomic loci. Indeed, the exclusive use of histone H3.3 for RI, transcription-coupled chromatin assembly has long led to the hypothesis that this histone variant could constitute an epigenetic mark for active chromatin regions [3,6]. However, this view has recently been challenged by studies in *Drosophila*, showing that H3.3 itself is not essential for either viability or for global gene expression but, instead, has critical functions in male and female fertility [9,10]. Viability of *His3.3A; His3.3B* double null mutants can be at least partly explained by the fact that, in the absence of H3.3, canonical H3 can be assembled in a RI manner [10]. However, this substitution mechanism does not operate in chromatin assembly during male meiosis, where H3.3 is

absolutely required [10]. Future work should aim at determining if H3.3 is also specifically required for paternal chromatin assembly at fertilization.

Materials and Methods

Flies

Flies were grown in standard conditions at 25°C. The $Hira^{ssm}$ and $Hira^{HR1}$ alleles and the *Hira-flag* and $Hira^{ssm}$*-flag* transgenic constructs have been described before [14,17,30]. The *Dj-gfp* transgenic line was kindly provided by R. Renkawitz-Pohl [45]. The chd^{1} allele is a gift from A. Lusser, and mutant flies were built by combining this chromosome with Exelixis deficiency *Df(2L)Exel7014,* as described [36]. The *Df(3R)3450* deficiency and the *P{EPgy2}EY23024* insertion were obtained from the Bloomington Drosophila Stock Center.

Generation of *yem* mutants

The yem^{1} mutation is a 2356T>A substitution (position 24946242 in the genome sequence) falling in the 5th exon of *yem*. The yem^{2} mutation was isolated after standard remobilization of the P{EPgy2}EY23024 element and selected for its non-complementation of the yem^{1} chromosome. DNA sequencing revealed that yem^{2} is a 3180bp deletion from position +2 in the 5'UTR (positions 24945416 to 24948596 in the genome), uncovering the first 5 exons (including the HUN domain) and part of the 6th of *yem*.

RT-PCR

Total RNAs were extracted with the Trizol method (Invitrogen) from at least 50 whole adults, ovaries or carcasses. Reverse transcription was performed using oligo(dT) primers and the SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen). For the *yem* and *RP49* PCR reactions, the following primers were used YEMAPRIMER15/YEMAPRIMER16 and RP49FWD/RP49REV (see primers section).

Transgenic constructs

The *yem* Eco RI genomic fragment in bluescript vector (described in [23]) was digested with NheI and XbaI. This fragment was replaced by a PCR amplification product with primers OA37 and OA38, bearing the Flag tag sequence in 3' of *yem* (verified by sequencing). Next, the resulting vector was digested with EcoRI and XbaI and the *yem-flag* fragment was inserted into the Casper vector. Finally, SV40 polyadenylation signals were added to the previous construct as a XbaI-PstI fragment from the pCasper{AUG-βgal} plasmid (Thummel). The resulting transgenesis vector is called HPF (for holoprotein flanked with FLAG). HPF1 and HPF16 are two independent insertions of HPF.

To build *PW8-Hira*$^{\Delta B}$*-3xflag*, the *PW8-Hira-3xflag* plasmid [14] was used as a template to amplify a 2440-pb fragment from position -400 before ATG to +2040 by PCR using primers HIRABGL2 and HIRAPEPT13’. The PCR fragment was cloned into PGEM-T vector (Promega) and verified by sequencing. The resulting plasmid was used as a template to generate a deletion of the B domain with the QuikChange II site directed mutagenesis kit (Stratagene). Two mutagenic primers DELETBFWD and DELETBREV were used to delete 48pb from positions 246 to 293 (aa 453 to 468). The construct was verified by sequencing, digested by BglII and cloned into the plasmid *PW8-Hira-Flag*. This vector was used for establishment of transgenic lines with random insertions.

Primers:

YEMAPRIMER2: TGCGAAAACCGCGACCAGTG
YEMAPRIMER9: GGGCAGTTGTTGCGTGGATG
YEMAPRIMER15: GGATCCCATTCCTCCGCTTG
YEMAPRIMER16: CTCAGGCAGCAGCACTCAAT
RP49FWD: AAGATCGTGAAGAAGCGCAC
RP49REV: ACTCGTTCTCTTGAGAACGC
OA37: ACGTCCAAGCAGCTAGCTGCCA
OA38: GAATCTAGACTTGTCATCGTCGTCCTTGTAGTCTTGGCGCGTGGGCGTACT
HIRABLG2: CGCCCGCGGAAAGATCTATTCTTATATG
HIRAPEPT13’: TGGATCCGCGCAATGCACTGCAGAACT
DELETBFWD:AGCGACCCATTAGTAAACAAACGGAAACGCACGAAGATGGACCCA CATCGCTGA
DELETBREV:TCAGCGATGTGGGTCCATCTTCGTGCGTTTCCGTTTGTTTACTAATG GGTCGCT

Immunofluorescence

Eggs were collected, dechorionated, devitelinized and fixed in methanol as described [30]. Before staining, eggs were rehydrated in TBS-Triton 0,15% and incubated with primary and secondary antibodies at the indicated dilution, as in [14]. For anti-Yem AS2 antibody staining, ovaries were dissected in PBS-Triton 0,1% and were immediately incubated with the antiserum without fixation, as described [46]. Ovaries for all other stainings were dissected in PBS-Triton 0,1% and fixed at room temperature in 4% PFA in PBS for 25 minutes. DNA was stained with DAPI or propidium iodide; samples were mounted in medium (Dako) and observed under an LSM 510 META confocal microscope (Zeiss). Images were treated with LSM image browser, ImageJ or Photoshop CS2.

We used the following antibodies: AS2 anti-Yem antibody (1/100; [23,46]), M2 monoclonal anti-Flag antibody (1:500 in ovaries, 1:1000 in embryos; Sigma) and anti-Polyacetylated histone H4 (1:200; Millipore 06-589). Secondary antibodies were Alexa488 goat anti-mouse or goat anti-rabbit (1:1000, Invitrogen) and Cy3 donkey anti-rabbit (1:800, Millipore).

Western Blots

50µl of ovaries (Figure 2) were homogenized in lysis buffer (15mM Hepes (pH 7.6); 10mM KCl; 5mM MgCl2; 0.5mM EDTA; 0.5mM EGTA; 350mM Sucrose; 1mM DTT) with protease inhibitors (Halt Protease Inhibitor Single Use Cocktail, Thermo Scientific; 1mM PMSF). The protein extract was centrifuged, isolated from debris and stocked in half volume of glycerol at -80°C if necessary. SDS-Page electrophoresis was carried out on 8% acrylamide gels and western blot was performed using standard procedures using Pierce ECL Western Blotting Substrate (Thermo Scientific). The following antibodies were used: M2 anti-Flag (1:1000; Sigma), anti-Tubulin (1/1000; Sigma), peroxydase-coupled goat anti-mouse (1:10000; Beckman).

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Figure Legends

Figure 1. Mutations affecting the *yem* gene.

(A) Schematic representation of the *yem* gene and mutant alleles. The yem^1 mutation (V478E) falls outside of the conserved HUN domain of this protein (in blue). The yem^2 deletion was obtained by mobilizing the P-element insertion *P{EPgy2}EY23024*. Coding sequence is in purple and untranslated regions are in yellow. Primers used for RT-PCR analysis are shown in green.

(B) RT-PCR analysis of *yem* expression in the indicated tissues and genotypes. Notice that in yem^2 females, transcription of the remaining *yem* sequence is strongly reduced. RT-PCR amplification used the primer pair shown in (A).

(C) Yem accumulates in the Germinal Vesicle (GV, arrowhead) in wild type and yem^1/ *Df(3R)3450*, but not in yem^2/*Df(3R)3450* mutants. Confocal images of wild type or mutant ovaries stained for DNA (blue) and with the anti-Yem AS2 antibody (red) [23,46]. Bar: 20µm.

Figure 2. Yem and HIRA are interdependent for their localization in the Germinal Vesicle.

(A) Yem and HIRA colocalize in the GV (arrowhead) throughout oogenesis. Wild type ovaries bearing a Hira-Flag transgene were stained with anti-Flag (green) and anti-Yem (red) antibodies, and DAPI (blue).

(B) Yem and HIRA proteins are interdependent for their localization in the GV. Ovaries from wild type, yem^1/*Df(3R)3450*, or yem^2/*Df(3R)3450* females bearing a HIRA-Flag transgene were stained to visualize DNA (blue) and Flag (green). HIRA accumulation in the GV (arrowheads) is not affected in yem^1/*Df(3R)3450* but is abolished in yem^2/*Df(3R)3450* ovaries. Conversely, Yem-Flag GV localization was not significantly affected in $Hira^{ssm}$ ovaries, but appeared highly variable in $Hira^{HR1}$ ovaries. Percentages indicate the number of egg chambers

with positive staining in the GV, equal or superior to the background fluorescence. At least 60 egg chambers were observed for each experiment.

(C) Western-blot analysis of HIRA-Flag (left) and Yem-Flag (right) from protein extracts of the indicated genotypes. α-Tubulin was used as a loading control. Bars: 20µm.

Figure 3. Maternal effect embryonic lethality phenotype associated to *yem* mutations.

(A) An embryo from a *yem*^{1}/*Df(3R)3450* female crossed with males expressing Dj-GFP, which marks the sperm tail [45], indicating successful fertilization. Bar: 20µm.

(B) Late maternal effect embryonic lethality of *yem* mutants. Embryos from *yem*^{1}/*Df(3R)3450*, *yem*^{2}/*Df(3R)3450* and *Hira*^{HR1} females have brownish appearance as a sign of late embryonic death. A wild type, unfertilized egg is shown for comparison on the left.

Figure 4. Yem is essential for chromatin assembly in the male pronucleus at fertilization.

Left: Fertilized eggs in telophase of meiosis II stained for DNA (red) and acetylated histone H4 (H4act, green). The male pronucleus (arrows) does not incorporate H4act in *yem*^{1} mutant eggs. Middle: Metaphase (top) and telophase (bottom) in embryos laid by females of the indicated genotype. In *yem*^{1}/*Df(3R)3450* mutant eggs, the male nucleus (arrowheads) is excluded from the first mitosis. Right: Blastoderm embryos from *yem*^{1}/*Df(3R)3450* females are haploid. The male nucleus (red) is still detected (arrowhead) among the haploid nuclei containing chromosomes of maternal origin.

Figure 5. Paternal chromatin assembly defects associated with *yem* mutations.

(A) Fertilized eggs laid by females of the indicated genotypes at the pronuclear apposition stage. The male pronucleus (arrowheads) does not incorporate H4act and does not decondense

in *yem* mutants. Note that paternal chromatin assembly is partially restored in eggs laid by weakly fertile $yem^{1}/Df(3R)3450$; $yem\text{-}flag^{HPF1}$ females (see also Table 1).

(B) At pronuclear apposition in wild-type eggs, the male nucleus (arrowhead) contains high levels of maternally expressed H3.3-Flag whereas the female pronucleus incorporates very low levels of H3.3-Flag, presumably through a RC pathway. In $yem^{1}/Df(3R)3450$ mutant eggs, only the weak incorporation of H3.3-Flag in the female pronucleus is detected.

(C) Cycle 1 embryos laid by H3.3-Flag females of the indicated genotype. The male nucleus is indicated with an arrowhead. The male nucleus phenotype is identical in *yem* and *Hira* mutants eggs. In contrast, in $chd1^{1}/Df(2L)Exel7014$ mutant eggs, the male nucleus appears variable in shape and does incorporate H3.3-Flag. Bars: 10µm.

Figure 6. HIRA and Yem are interdependent for recruitment to the male pronucleus.

(A) Confocal sections of male pronuclei in eggs laid by *Hira-Flag* females of the indicated genotype (HIRA-Flag is shown in green, DNA in red) (n>20). In wild-type eggs, HIRA-Flag accumulates in the decondensing male nucleus. In eggs from *yem* mutant females however, HIRA-Flag is no longer detected.

(B) $HIRA^{\Delta B}$-Flag expressed in $Hira^{HR1}$ background readily localizes in the male nucleus ($HIRA^{\Delta B}$-Flag is shown in green, DNA in red).

(C) Yem-Flag is shown in green, DNA in red. In addition to its faint localization throughout the nuclear volume, Yem-Flag also accumulates in a single focus of unknown identity in about half of the observed eggs (n=12/23). In eggs from $Hira^{ssm}$ mutants females, Yem-Flag fails to properly localize in male pronuclei (percentages of positive staining are shown)(n>20). Bars: 5µm.

Table 1 Female sterility associated to *yem* mutations

Genotype of females	No. of eggs	No. of larvae	Hatch. rate (%)
w ; yem^{EY23024}*/ Df(3R)3450*	885	758	85.6
w ; yem^{1}*/ Df(3R)3450*	984	0	0
w ; yem^{2}*/ Df(3R)3450*	948	0	0
w ; yem^{1}*/ yem*^{2}	1109	0	0
w yem-flag^{HPF1}*/w ; yem*^{1}*/ Df(3R)3450*	1444	8	0.5
w yem-flag^{HPF1}*/w ; yem*^{2}*/ Df(3R)3450*	1337	0	0
w yem-flag^{HPF1}*/w yem-flag*^{HPF1} *; yem*^{1}*/ Df(3R)3450*	513	362	70.5
w yem-flag^{HPF1}*/w Yem-flag*^{HPF1} *; yem*^{2}*/ Df(3R)3450*	577	256	44.4
w ; yem-flag^{HPF16}*/+ ; yem*^{2}*/ Df(3R)3450*	638	507	79.5
w ; yem-flag^{HPF16}*/yem-flag*^{HPF16} *; yem*^{2}*/ Df(3R)3450*	667	660	98.9

All *yem* alleles used are described in Figure 1. Both *yem*^{1} and *yem*^{2} alleles are associated to complete female sterility. This phenotype can be saved by a YEM-Flag transgene: two insertions, *yem-flag*^{HPF1} and *yem-flag*^{HPF16} were used in this study. Better rescue was obtained with *yem-flag*^{HPF16} (used in two copies for experiments on Figure 6) and only weak rescue was obtained with single copy *yem-flag*^{HPF1} (used in one copy for Figure 5).

Figure 1. Orsi et al.

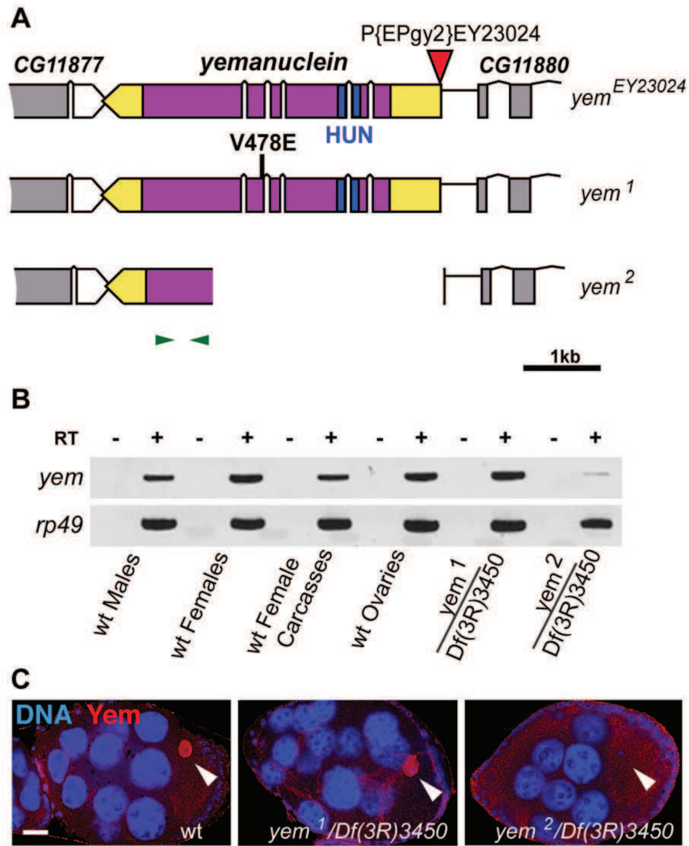
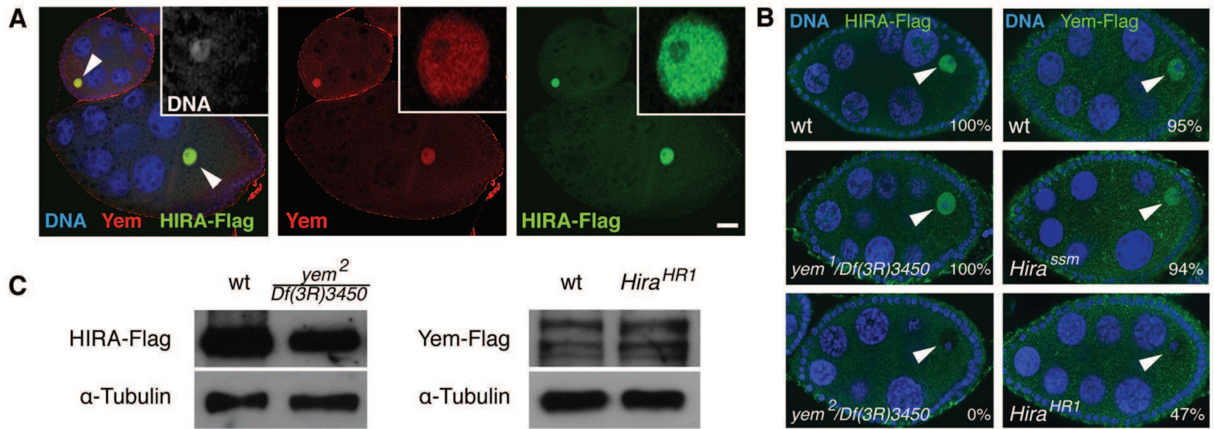


Figure 2. Orsi et al.



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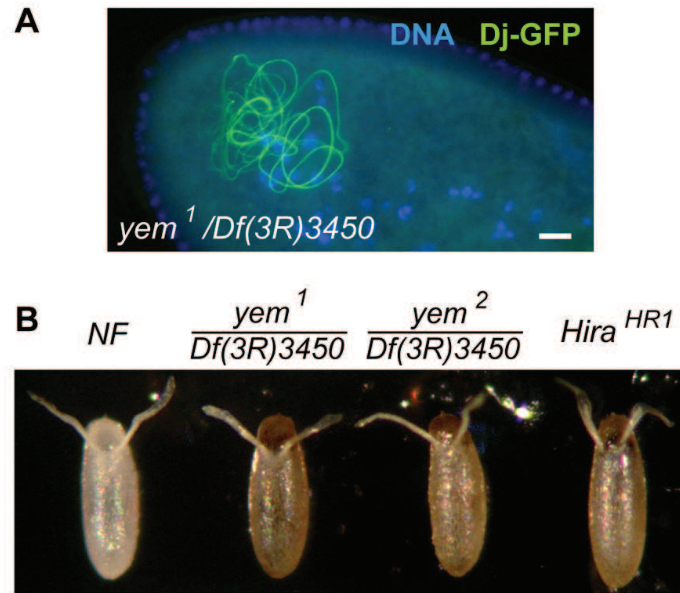


Figure 4, Orsi et al.

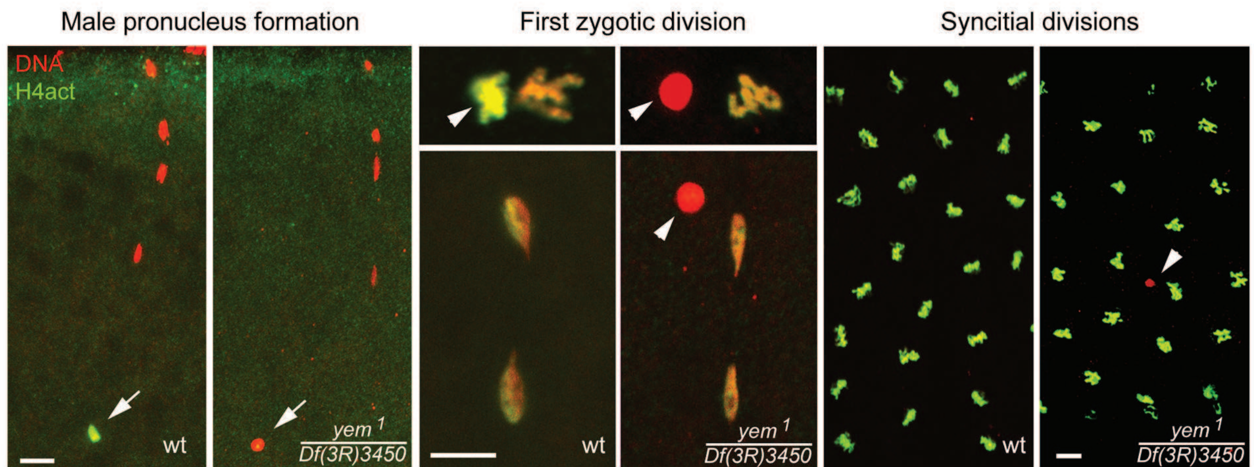


Figure 5, Orsi et al.

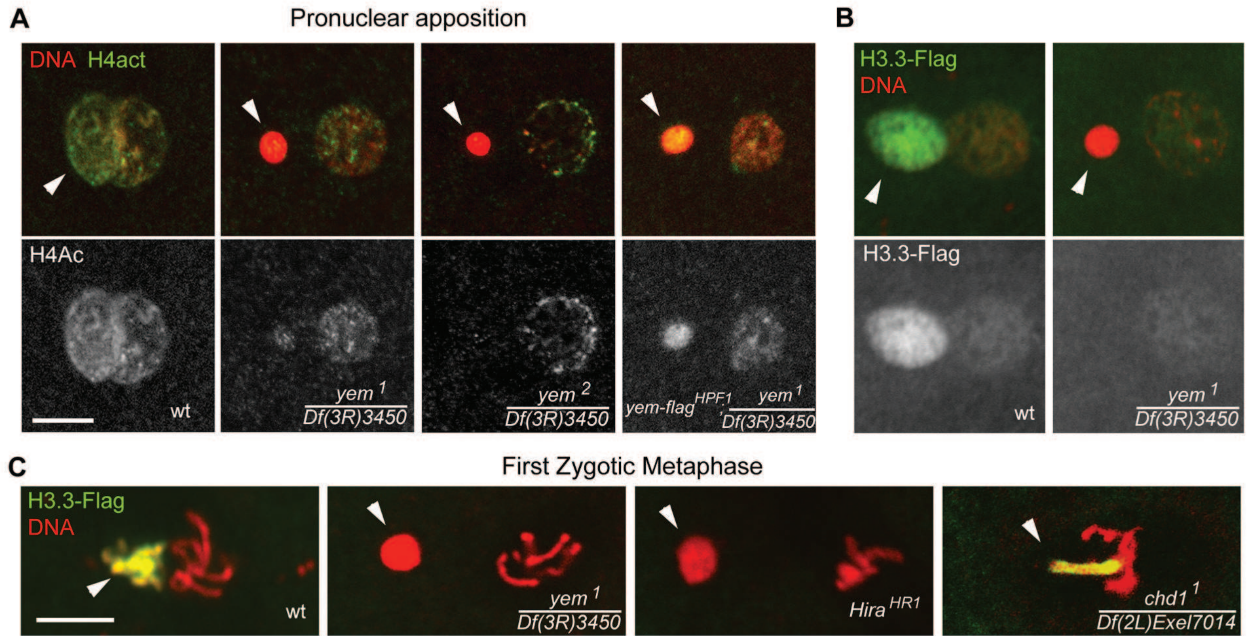
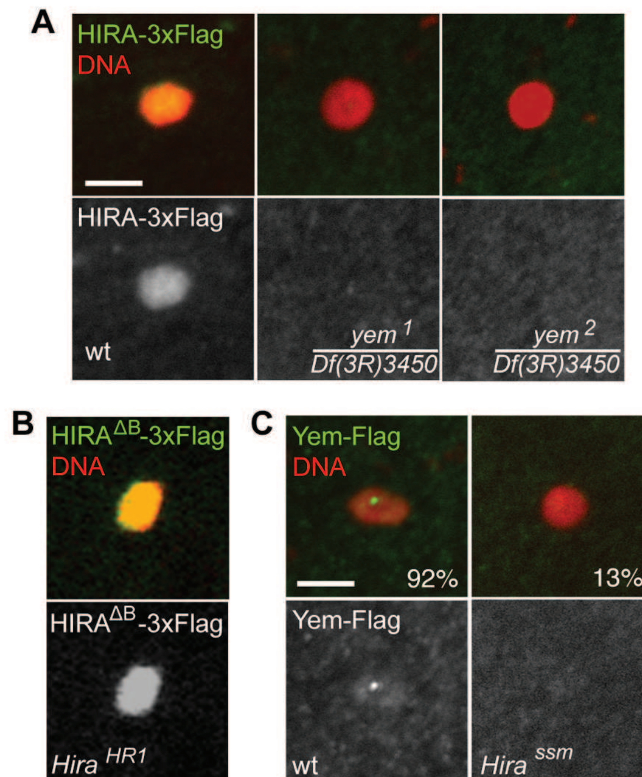
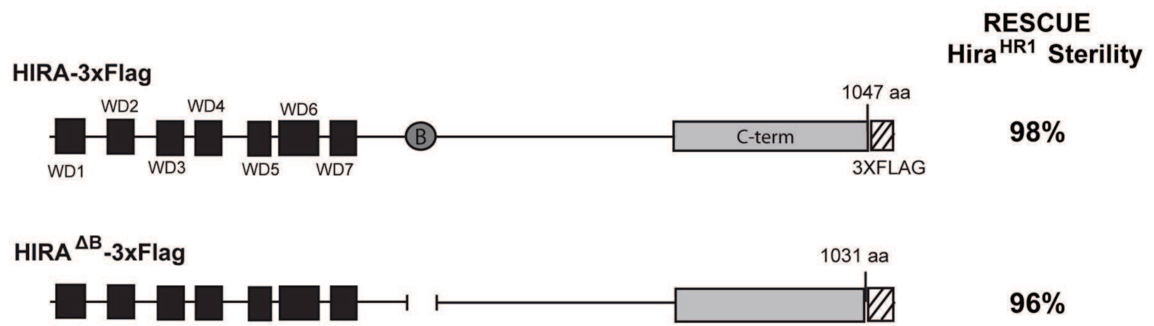


Figure 6, Orsi et al.



Supplementary Figure 1. Orsi et al.



Supplementary Table 1. Viability of the *yem* mutant alleles

Crosses	No. of progeny	[Hu+] progeny (%)
♀♀ *w ; yem^{EY23024}/TM6* X ♂♂ *w/Y ; yem^{EY23024}/TM6*	265	101 (38.1)
♀♀ *w ; yem^{2}/TM6* X ♂♂ *w/Y ; yem^{2}/TM6*	144	8 (5.6)
♀♀ *w ; yem^{2}/TM6* X ♂♂ *w/Y ; yem^{1}/TM6*	369	152 (41.2)
♀♀ *w ; yem^{1}/TM6* X ♂♂ *w/Y ; yem^{2}/TM6*	313	133 (42.5)
♀♀ *w ; Df(3R)3450/TM6* X ♂♂ *w/Y ; e yem^{1}/TM6*	985	412 (41.8)
♀♀ *w ; Df(3R)3450/TM6* X ♂♂ *w/Y ; yem^{EY23024}/TM6*	724	341 (47.1)
♀♀ *w ; Df(3R)3450/TM6* X ♂♂ *w/Y ; yem^{2}/TM6 (#1)*[¥]	627	120 (19.1)
♀♀ *w ; Df(3R)3450/TM6* X ♂♂ *w/Y ; yem^{2}/TM6 (#2)* [¥]	137	28 (19.7)
♀♀ *w yem-flag^{HPF1}/w ; Df(3R)3450/TM6* X ♂♂ *w/Y ; yem^{2}/TM6*	201*	75* (37.3)
♀♀ *w ; yem-flag^{HPF16}/+ ; Df(3R)3450/TM6* X ♂♂ *w/Y ; yem^{2}/TM6*	61*	26* (42.6)

* Only w^{+} progeny (that received the rescue transgene) were considered in these crosses.
¥ These are two independent repeats of the same experiment.

All the indicated crosses were carried under standard conditions, at 25°C in several non-crowded vials. All the progeny from each cross was considered, [Hu^{+}] progeny (not carrying a balancer chromosome) was counted separately and their rate to total population was calculated (in every cross, [Hu^{+}] progeny is expected to be 33% of total). For most of the crosses, this percentage exceeds 33%, showing perfect viability of the *yem^{EY23024}* insertion allele and of the *yem^{1}* allele. The *yem^{2}* allele is viable but shows lower survival rate than *yem^{1}*. This sub-viability can be rescued with two *yem-flag* insertions, showing that it is indeed a specific effect of the *yem* mutation.

L'hypothèse que Yem puisse être un partenaire fonctionnel de HIRA lors de l'assemblage de la chromatine paternelle découle du contexte bibliographique. Cependant, un aspect surprenant de nos résultats est l'extrême similitude entre les phénotypes associés aux mutations affectant ces facteurs. Ceci nous permet d'émettre l'idée que ces facteurs travaillent ensemble dans une étape très précoce du processus d'assemblage. L'absence de rôle de ASF1 au sein de ce complexe, et l'absence d'orthologue de Cabine chez la drosophile, soulignent davantage le rôle cœur de HIRA/Yem. Comme mentionné précédemment, les mutations affectant H3.3 sont associées à un phénotype de stérilité dont les bases cytologiques sont inconnues. Il serait intéressant de comprendre si ce variant est absolument nécessaire à la formation du pronoyau mâle, de façon analogue à Yem et HIRA.

Par ailleurs, la distribution dans le pronoyau mâle de la protéine Yem apparaît différente de celle de HIRA, présentant des foyers d'accumulation qui pourraient révéler des sites particuliers d'assemblage de la chromatine. J'ai tenté d'identifier la nature de ces foyers en comparant la distribution de Yem à celle des protéines centromériques Cid, celle des potéines télomériques K81 (voir Dubruille *et al.*, 2010) et celle du marqueur de dommages à l'ADN γH2AvD. Aucun de ces marqueurs n'est colocalisé avec les foyers marqués par le transgène Yem-Flag, suggérant que Yem ne joue pas un rôle particulier dans ces territoires (Figure 2).

La viabilité associée aux allèles nuls de yem pose la question des fonctions somatiques de Yem, et par extension du complexe HIRA. L'allèle yem^2, notamment, est associé à une létalité partielle, suggérant que Yem pourrait avoir des rôles dans la biologie de la chromatine somatique (des résultats concernant cette hypothèse seront présentés plus tard). Par ailleurs, nous sommes une fois de plus confrontés au problème de l'apport maternel en ARNm et protéine Yem. Il est donc difficile de conclure, à ce stade, sur l'importance d'autres rôles de *yem* après la fécondation.

Si Yem et HIRA font partie d'un même complexe, la question des sous-fonctions précises de chacune de ces protéines reste ouverte. Une hypothèse intéressante émane de la proposition que la protéine Yem pourrait directement lier l'ADN (Aït-Ahmed *et al.*, 1992). Ainsi, nous pouvons imaginer qu'au sein du complexe Yem/HIRA, Yem reconnaîtrait l'ADN non-nucléosomé et HIRA pourrait effectuer une étape précoce de l'assemblage (en acheminant par

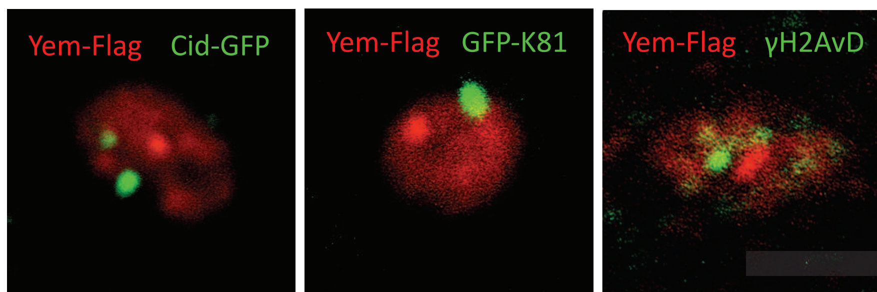


Figure 2. Les foyers d'accumulation de Yem ne correspondent pas aux centromères, aux télomères, ou aux sites de réparation de l'ADN.
Images confocales montrant des pronoyaux mâles dans des oeufs issus de femelles exprimant la protéine Yem-Flag. Le marquage révèle Yem-Flag (en rouge) et la protéine centromérique Cid-GFP, la protéine télomérique GFP-K81 ou le marqueur γ H2AvD (vert). Les foyers Yem-Flag ne coïncident avec la localisation d'aucune des protéines testées. Barre: 5µm.

exemple un dimère H3.3-H4 au site d'assemblage ou en catalysant la formation du nucléosome).

Un tel modèle pourrait, par ailleurs, rendre compte de l'évolution à deux vitesses de ces deux facteurs. En effet, alors que la séquence de la protéine HIRA est très bien conservée (notamment sur le large domaine en hélice béta à répétitions WD), la séquence de Yem est, elle, beaucoup plus divergente, l'homologie n'étant restreinte qu'à un segment de 45-55 acides aminés (le domaine HRD ou HUN)(Banumathy *et al.*, 2008; Balaji *et al.*, 2009; Anderson *et al.*, 2010). Ce domaine HUN a été montré comme étant nécessaire et suffisant à l'intéraction physique entre Yem et HIRA chez l'homme (Banumathy *et al.*, 2008). Au contraire, la forte divergence des autres domaines de Yem pourrait s'expliquer par la nécessité de reconnaître l'ADN de tout le génome. La protéine HIRA pourrait être le médiateur entre l'histone H3.3 (et ses protéines associées) et la protéine Yem : il paraît intuitif que la séquence de HIRA soit dans ces conditions mieux conservée. Des études biochimiques pourraient aider à décortiquer le fonctionnement de ce complexe au cours de l'assemblage d'un nucléosome.

Le pronoyau mâle est un bon modèle pour étudier la séquence d'évènements au cours de l'assemblage de la chromatine. Cependant, il ne représente jamais qu'un cas unique, et le fonctionnement de la machinerie d'assemblage n'est certainement pas généralisable. Au contraire, des progrès récents ont été réalisés dans ce domaine grâce à l'étude du rôle des voies HIRA et XNP dans les cellules somatiques. J'ai participé à ce projet piloté par le laboratoire de Kami Ahmad.

IV. HIRA et XNP gèrent ensemble l'incorporation de H3.3 dans les cellules somatiques

Récemment, une voie alternative à HIRA participant à l'assemblage de la chromatine de façon IR et promouvant l'incorporation de l'histone H3.3 a été mise à jour (Drane *et al.*, 2010; Goldberg *et al.*, 2010; Lewis *et al.*, 2010). Cette voie implique la protéine à domaine death DAXX et le facteur ATRX appartenant à la sous-famille RAD54 des facteurs de remodelage de type SNF2. La protéine ATRX possède un domaine ATPase de type SWI/SNF

et un domaine HeliC, caractéristiques cohérentes avec un rôle da ns le remodelage de la chromatine (McDowell *et al.*, 1999). Les premières études s'intéressaient en réalité au rôle d'ATRX dans le syndrome alpha-thalassémique de retard mental lié au chromosome X, qui associe des mutations affectant ATRX à un déficit systématique d'expression de l'alpha-globine (un gène sub-télomérique). Chez l'homme, ATRX a été montré comme se localisant aux bras courts des cinq chromosomes acrocentriques, qui codent notamment pour les ARN ribosomiques, et ses mutations sont associées à des défauts de méthylation de l'ADN sur ces régions (McDowell *et al.*, 1999; Gibbons *et al.*, 2000). Chez la souris, ATRX est responsable de l'intégrité des régions péri-centromériques au cours de la méiose femelle et le développement embryonnaire précoce (Baumann *et al.*, 2010). Par ailleurs, cette protéine a un rôle essentiel dans l'inactivation du chromosome X soumis à l'empreinte, mais les bases moléculaires de cette fonction restent inconnues (Garrick *et al.*, 2006; Baumann et De La Fuente, 2009). Finalement, ATRX participe à la protection des télomères dans les cellules souches embryonnaires, un processus qui implique aussi le recrutement massif d'histones H3.3 (Wong *et al.*, 2008; Wong *et al.*, 2010).

ATRX a été montré comme étant capable de lier l'ADN avec une spécificité particulière pour les régions en répétition en tandem, jetant des bases moléculaires pour diverses fonctions impliquant ce type de séquence (Law *et al.*, 2010). Notamment, ATRX a une affinité particulière pour les G-quadruplex, des structures ternaires qu'adoptent certaines régions d'ADN riches en guanine et qui ont un rôle sur, notamment, la transcription (Huppert, 2010). Par ailleurs, les G-quadruplex sont stables dans les régions non-nucléosomales (Wong et Huppert, 2009). Le modèle proposé est donc que ATRX pourrait détecter les régions de ce type et y adresser l'assemblage de nucléosomes utilisant le variant H3.3 pour les réorganiser (Gibbons et Higgs, 2010). Le rôle, par exemple, du recrutement de ATRX et H3.3 aux télomères des cellules souches embryonnaires serait cohérent avec la nécessité à ce stade (et uniquement à ce stade) de répliquer les extrémités télomériques. La voie ATRX est donc impliquée dans l'assemblage de H3.3, comme la voie HIRA, mais possède des spécificités fonctionnelles chez les mammifères.

Chez la drosophile, contrairement aux mutants affectant *Hira*, les mutations du gène *xnp*, l'orthologue de ATRX, n'ont aucun effet important sur la fertilité des femelles (Bassett *et al.*, 2008; Schneiderman *et al.*, 2009; Emelyanov *et al.*, 2010). Ces mutations se comportent

comme des forts suppresseurs de variégation et la protéine ATRX a été montrée comme interagissant avec HP1a. L'exploration en détail de la distribution de XNP a amené le groupe de Kami Ahmad à montrer que cette protéine est recrutée massivement sur une séquence répétée satellite (TAGA), renvoyant à ses propriétés chez les mammifères. De plus, la dérégulation transcriptionnelle de ce satellite a, elle même, des conséquences sur la stabilité de l'hétérochromatine à échelle globale. Cependant, le rôle d'ATRX comme facteur d'assemblage de la chromatine chez la drosophile restait inconnu. Des études fonctionnelles de HIRA et ATRX dans des cellules souches embryonnaires de souris ont montré que ces facteurs sont tous les deux nécessaires pour incorporer l'histone H3.3 (Goldberg *et al.*, 2007). Cet élégant travail a, pour la première fois, souligné un lien fonctionnel global entre HIRA et l'assemblage de H3.3 lié à l'activité de transcription. Cependant, ATRX semble agir de façon indépendante de HIRA sur des loci spécifiques, et inversement. En particulier, HIRA n'a aucun rôle dans la chromatine télomérique, tandis que ATRX ne semble pas intervenir dans l'assemblage sur les gènes et promoteurs. Or, la nature des interactions entre ces complexes et le degré de redondance de leurs rôles *in vivo*, dans le contexte du développement, ne sont, pour l'heure, pas connus.

Dans le travail qui suit, j'exposerai notre participation à une analyse fonctionnelle plus poussée du rôle de XNP, et de son interaction fonctionnelle avec la voie HIRA chez la drosophile, menée par le groupe de Kami Ahmad. Ces travaux décrivent que XNP et HIRA colocalisent sur certains sites dans des cellules somatiques, mais ont aussi certains sites cibles spécifiques (notamment, HIRA est le seul facteur capable d'être recruté dans le nucléole). Par ailleurs, les deux facteurs sont recrutés sur certaines séquences activement transcrites, avec un temps relativement court de résidence. Nous avons conjointement montré que XNP et HIRA sont nécessaires pour l'assemblage somatique de H3.3. De façon surprenante, le temps de résidence de ces facteurs sur les sites d'assemblage est largement augmenté en absence de H3.3, leur substrat dans ce processus. Le modèle qui est proposé est que XNP et HIRA reconnaissent de façon partiellement redondante l'ADN nu et y jouent un rôle dans l'initiation du processus de recrutement de H3.3. Ces travaux sont exposés dans le manuscrit qui suit.

Precocious recruitment of assembly factors promotes nucleosome replacement

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Abstract

Diverse processes displace nucleosomes in eukaryotic chromatin. Replication-Independent (RI) deposition using the H3.3 histone variant fills these gaps. However, how H3.3 histones are recruited to chromatin gaps remains mysterious. Here we show that the Xnp remodeler and the Hira histone chaperone bind chromatin disrupted by transcription or by anti-nucleosomal sequences before H3.3 is delivered to a site. We propose that Xnp and Hira form a binding platform for the efficient recruitment of H3.3 pre-deposition complexes to disrupted chromatin. The Xnp and Hira assembly factors are redundant for RI deposition, but double mutants are synthetic lethal and abrogate H3.3 deposition. Our results imply that RI deposition is essential to maintain chromatin structure in active regions of genomes.

Introduction

DNA in the eukaryotic nucleus is associated with histone proteins to form nucleosomes, the fundamental units of chromatin. Organisms also have histone variants that form specialized nucleosomes at specific sites in genomes. While most nucleosomes are assembled using the canonical H3 histone during DNA replication in S-phase of the cell cycle, nucleosomes containing the H3.3 histone variant are assembled throughout the cell cycle in a Replication-Independent (RI) process (Ahmad and Henikoff 2002; Tagami et al. 2004). RI assembly occurs repeatedly at sites where DNA-based processes destabilize and displace nucleosomes, including transcription, chromatin remodeling, and anti-nucleosomal sequence features. Together, these processes result in the enrichment of the H3.3 histone variant in active chromatin regions (Schwartz and Ahmad 2005; Mito et al. 2005; Goldberg et al. 2010). Both the inclusion of histone variants and

the dynamics of displacement and re-assembly contribute to structural properties of nucleosomes in active chromatin (Henikoff et al. 2008; Jin and Felsenfeld 2009). Biochemical isolation of pre-deposition complexes has identified shared and distinctive assembly factors that associate with the H3 and H3.3 histones, and these mediate the Replication-coupled or RI assembly of nucleosomes. H3 histones are uniquely complexed with the CAF-1 histone chaperone complex (Tagami et al. 2004). CAF-1 binds the processivity factor PCNA at replication forks, thereby efficiently delivering new H3 histone to new DNA (Shibahara and Stillman 1999). The H3.3 histone is complexed with the Hira and DAXX histone chaperones, and the ATRX/XNP chromatin remodeler (Tagami et al. 2004; Drane et al. 2010; Goldberg et al. 2010). These factors discriminate soluble H3.3 from H3 before deposition, and contribute to the targeting of H3.3 to distinct sites in chromatin.

Histone chaperones and chromatin remodelers are important for new nucleosome assembly to mediate the wrapping of DNA around histones, and the formation of new DNA-histone contacts. However, how H3.3 pre-deposition factors guide the variant to active chromatin has not been defined. Furthermore, mutants in some of these factors have surprisingly limited phenotypes. In mammalian cells the Hira chaperone is required only for H3.3 deposition at genes (Goldberg et al. 2010) but in Drosophila Hira is not required in somatic cells, and only mediates H3.3 deposition on sperm chromatin during fertilization (Loppin et al. 2005; Bonnefoy et al. 2007). In Drosophila the ATRX/XNP remodeler homolog Xnp co-localizes with H3.3 in somatic cells, but is not essential (Schneiderman et al. 2009). In mammals, ATRX/XNP is important for H3.3 deposition only at telomeres, where the DAXX chaperone is also required (Wong et al. 2010; Goldberg et al. 2010). These results have raised the possibilities that H3.3 assembly factors are redundant or that additional factors exist.

In this study we provide evidence that H3.3 pre-deposition factors mediate two separable steps in RI nucleosome assembly. We show that the Xnp and Hira factors bind genomic sites as nucleosomes are disassembled, thereby marking sites and poising chromatin for RI assembly when new H3.3 is delivered. Our results further reveal that RI nucleosome replacement is essential for chromatin structure and viability, and uncovers a cellular system that surveys chromatin for defects and promotes its repair.

Results

The Xnp and Hira assembly factors have overlapping and distinct targets

To study the function of the Xnp and Hira assembly factors in Drosophila, we first examined their localization on polytene chromosomes of larval salivary glands. We previously reported that the Xnp remodeler is found at active genes and sites of dynamic chromatin, where H3.3 is deposited (Schneiderman et al. 2009; Figure 1A). We found that the Hira chaperone is detectable at many sites throughout euchromatin. Like Xnp, Hira co-localizes with elongating RNA polymerase II (ePolII) and H3.3 at active genes (Figure 1B,C). However, the two assembly factors do not coincide at other sites. The bulk of Hira is localized at the repeated ribosomal DNA (rDNA) within the nucleolus, where Xnp is absent (Figure 1A,B). A band near the base of the 3R chromosome arm is also a strong site of Hira binding that lacks Xnp. Finally, the TAGA satellite block at the base of the *X* chromosome is a major site of Xnp binding, but lacks Hira (Figure 1A,B). Overall, there is a close correspondence between the intensity of Xnp signals and the amount of H3.3 at sites across the genome, consistent with this remodeler being involved in RI nucleosome replacement. In contrast, the rDNA and the base of 3R show moderate amounts of H3.3 staining, even though Hira is abundant at these sites (Figure 1C). This suggests that Hira has additional roles at some loci that are independent of

Xnp and H3.3.

To test if the localization of Xnp and Hira are interdependent at active genes where they co-localize, we examined the localization of each assembly factor in mutants. Xnp patterns across the chromosomes are unchanged in a *Hira* null mutant, and Hira patterns are unchanged in an *xnp* null mutant (Supplemental Figure S1). We conclude that Xnp and Hira co-localize independently at active genes.

Induced genes recruit assembly factors

To further explore the relationship between Xnp, Hira, and RI nucleosome replacement, we used the inducible *Hsp70* genes as a controlled system. Heat-shock activates transcription of *Hsp70*, disrupting nucleosomes and stimulating high levels of nucleosome replacement (Schwartz and Ahmad 2005). Two copies of *Hsp70* are located at the cytological position 87A in polytene chromosomes, and three more copies are interspersed with repetitive elements at 87C (Leigh Brown and Ish-Horowicz 1981). Both loci de-compact and expand as puffs within seconds after induction, and are heavily transcribed by RNA polymerase II (Kramer et al. 1980, Figure 2A). These puffs regress and RNA polymerase leaves after heat-shock within 30-60 minutes. Xnp is not enriched at the *Hsp70* loci before induction, but is rapidly recruited under heat-shock conditions (Figure 2B). Xnp persists while the genes are transcribed, but then leaves the sites within 60 minutes after heat-shock. These dynamics are consistent with the idea that Xnp is involved in co-transcriptional nucleosome replacement.

Like Xnp, Hira is not enriched at *Hsp70* genes before induction. At the 87A *Hsp70* locus upon heat-shock, Hira is rapidly recruited, and persists while the genes are transcribed (Figure 2C). Hira then leaves the 87A locus within 60 minutes of recovery. This indicates that the Hira chaperone is involved in co-transcriptional nucleosome

replacement. However, the behavior of Hira at the 87C locus is more complex, because Hira persists at this site during recovery (Figure 2C). This prolonged retention must be due to sequence features that distinguish the 87C locus from 87A, and argues that Hira has additional roles at certain sites.

Assembly factor recruitment precedes RI nucleosome assembly

Previous studies have reported that pre-deposition H3.3 is complexed with Xnp and Hira in soluble nuclear extracts (Tagami et al. 2004; Drane et al. 2010; Goldberg et al. 2010). To test whether the binding of Xnp and Hira to chromosomes was dependent on interactions with H3.3, we examined their localization in cells deficient for the variant histone. We produced a hairpin RNA homologous to H3.3 transcripts specifically in salivary glands using a GAL4-inducible construct, which gives efficient knock-down of H3.3 (Methods; Supplemental Figure S2). Surprisingly, we observed greatly enhanced chromatin signals for both Xnp and Hira in these glands (Figure 3A,B *versus* 3G,H). A similar increase in chromatin binding for both factors was observed in H3.3 null mutant animals (Supplemental Figure S3). These results demonstrate that the H3.3 histone is not required for recruitment of these assembly factors to target sites.

To examine the relationship between transcriptional disruption of nucleosomes and the binding of Xnp and Hira to chromatin, we again used the inducible *Hsp70* genes. We first assessed changes in the chromatin packaging of *Hsp70* during induction and repression. Heat-shock activates transcription, and *Hsp70* DNA becomes hypersensitive to micrococcal nuclease (MNase) due to nucleosome loss (Figure 4A,B; Petesch and Lis 2008). After heat-shock transcription ceases and DNA protection is restored as new nucleosomes are assembled (Figure 4B). Protection of *Hsp70* sequences before induction is indistinguishable between wild-type and H3.3 knock-down salivary glands, and in both genotypes induced *Hsp70* genes become hypersensitive to similar degrees.

However, nuclease protection is not restored during recovery in H3.3 knock-down glands (Figure 4B), demonstrating that nucleosomes are not rebuilt at *Hsp70*. While some new RI nucleosome assembly may still occur in H3.3 knock-down glands using the canonical H3 histone (Sakai et al. 2009), the hypersensitivity of *Hsp70* sequences after heat-shock confirms that chromatin structure is not restored.

We then asked if the Xnp and Hira factors are recruited to induced *Hsp70* genes when RI nucleosome replacement is crippled. Strikingly, both Xnp and Hira are rapidly recruited to transcribing *Hsp70* genes in H3.3 knock-down glands, as they were in wild-type cells (Figure 5). Thus, these factors must bind chromatin before new histones are delivered, as their recruitment is independent of both H3.3 and of RI nucleosome assembly.

Because nucleosome depletion of chromatin might affect transcriptional regulation, we examined the induction of *Hsp70* using Northern blots (Figure 4C). The basal expression of *Hsp70* and its kinetics of induction and repression were similar between wild-type and H3.3 knock-down salivary glands. Thus, RI nucleosome assembly is not required for the repression of *Hsp70* genes after a heat-shock. In contrast, there is a moderate reduction in the amount of mRNA during the heat-shock (Figure 4C). Cytological observations are consistent with this moderate change in *Hsp70* expression. Histone H3 S10-phosphorylation (H3S10P) is correlated with transcriptional activity (Nowak and Corces 2000; Ivaldi et al. 2007), and we observed reduced levels of both H3S10pho and ePolII at *Hsp70* loci during heat-shock (Figure 4D,E). These results reveal a positive role for nucleosome replacement in *Hsp70* expression.

We then examined *Hsp70* genes during recovery after heat-shock in H3.3-deficient cells. While Xnp and Hira leave the repressed *Hsp70* genes within 60 minutes after heat-shock ceases in wild-type animals, both factors persist at these loci in H3.3

knock-down glands (Figure 2; Figure 5A,B). This argues that Xnp and Hira are normally displaced from chromatin when new H3.3-containing nucleosomes are assembled. While transcription ceases after heat-shock (Figure 4C) and RNA polymerase II leaves the *Hsp70* loci, chromosome banding of these sites often appears smeared and stringy, supporting the idea that chromatin is disrupted (Figure 5). We conclude that Xnp and Hira specifically bind nucleosome-depleted chromatin before RI nucleosome assembly occurs, and must be displaced as new nucleosomes are assembled.

Xnp and Hira recognize chromatin defects

The retention of Xnp and Hira at nucleosome-depleted chromatin at repressed *Hsp70* genes explains why these assembly factors show enhanced binding throughout the chromosomes in H3.3 knock-down glands (Figure 3G,H), as all active regions probably become nucleosome-depleted. However, new binding sites for Xnp and Hira also appear after H3.3 knock-down. First, Xnp does not normally bind rDNA within the nucleolus, but strongly labels DAPI-stained rDNA chromatin in H3.3 knock-down glands (Figure 3C). Hira signals appear enhanced at this site, consistent with the idea that rDNA genes are depleted of nucleosomes and retain these factors. Second, Hira is recruited to the TAGA satellite block in H3.3 knock-down glands, and Xnp signal at this site is elevated (Figure 3F). Strikingly, the volume of TAGA satellite block is expanded in H3.3 knock-down glands, implying that ongoing nucleosome replacement is required for compaction of the region (Figure 3E,F). Both the re-localization and increased binding of Xnp and Hira to all sites of dynamic chromatin after H3.3 knock-down support the idea that these assembly factors are recruited to aberrant, nucleosome-depleted chromatin.

Xnp and Hira are important for H3.3 deposition

Our results show that Xnp and Hira are recruited to chromatin before nucleosomes are re-assembled. To test if these factors promote nucleosome replacement at these sites,

we assayed deposition of GFP-tagged truncated H3.3 histone into chromatin in wild-type and mutant genotypes. The H3.3core-GFP protein can only be incorporated by RI nucleosome assembly, and strongly labels active genes (Ahmad and Henikoff 2002; Schwartz and Ahmad 2005). We therefore produced a pulse of H3.3core-GFP in salivary glands and prepared chromosome spreads 2 hours later to assess the efficiency of RI nucleosome assembly (Figure 6). In wild-type cells H3.3core-GFP strongly labels chromosome arms and active genes (Figure 6A). In contrast, the H3.3core-GFP protein is efficiently produced in *xnp* null mutant cells, but most protein does not deposit onto chromosomes; instead, most of the protein accumulates in a non-chromatin-bound form within the nucleolus (Figure 6B). *Hira* mutants have a similar defect in H3.3 deposition: H3.3core-GFP protein is produced, but accumulates in the nucleolus with reduced chromosomal signals (Figure 6C). These results demonstrate that the rate of RI nucleosome assembly is reduced in either *xnp* or *Hira* mutants, although assembly can still occur. Thus, both Xnp and Hira contribute to the efficiency of H3.3 replacement.

The *Hira* and *xnp* genes are not essential in Drosophila, and bulk H3.3 levels appear normal in null mutants for these factors (Bonnefoy et al. 2007; Schneiderman et al. 2009). This implies that moderate delays in nucleosome replacement do not adversely affect steady-state chromatin structure or function. To test if Xnp and Hira have redundant roles in replacement, we generated *Hira ; xnp* double-mutants animals. Single mutants are fully viable, but we found that double-mutant larvae grow significantly slower than wild-type siblings and die during larval development, presumably surviving on maternally-provided Hira or Xnp proteins. Some double-mutant larvae survive to late stages, and we examined the deposition of H3.3core-GFP in these survivors. Strikingly, high levels of H3.3core-GFP could be produced in *Hira ; xnp* double-mutants, but virtually all of the protein accumulates in the nucleolus with little or no staining of chromosomes

(Figure 6D). Thus, both Hira and Xnp can contribute to the efficiency of H3.3 replacement, but this fails when both factors are depleted.

We wondered if the Hira and Xnp assembly factors are also important for H3.3 replacement at rDNA genes. The rDNA genes are repressed in late-stage salivary glands, and thus a pulse of epitope-tagged H3.3 does not label DAPI-stained chromatin within the nucleolus (data not shown). Therefore, we assayed deposition of H3.3 in the somatic follicle cells of ovaries, where rDNA is highly transcribed. In this cell type, Xnp localizes broadly in the nucleus but not within the nucleolus, and Hira is predominantly localized in punctate spots within the nucleolus (Figure 6E,F). A pulse of epitope-tagged H3.3 produced in follicle cells broadly labels the nucleus and punctate spots within the nucleolus, due to efficient RI deposition at transcribed sites in this cell type (Figure 6G). Follicle cells from *xnp* mutant ovaries also show nuclear staining and nucleolar spots (Figure 6H). In contrast, a pulse of epitope-tagged H3.3 in *Hira* mutant follicle cells shows no spots of deposition within the nucleolus, indicating that rDNA chromatin uniquely relies on the Hira chaperone for H3.3 deposition (Figure 6I). Thus, while Hira and Xnp are redundant at sites where they co-localize, some sites in the genome rely predominantly on individual factors for replacement nucleosome assembly.

Discussion

The H3.3 variant associates with a number of distinct pre-deposition factors (Tagami et al. 2004; Drane et al. 2010; Goldberg et al. 2010). These factors must escort the histone into the nucleus, deliver it to selected target sites, and assemble it in a replication-independent (RI) manner into nucleosomes. We show here that two of these factors – Xnp and Hira – are recruited to chromatin before new H3.3 arrives, and are displaced as nucleosomes assemble. This implies that H3.3 pre-deposition factors can be separated

into those that deliver the new histone, and those that only complex with H3.3 when it arrives at its target sites. We suggest that Xnp and Hira identify and poise sites for new nucleosome assembly through a stepwise process. In the first step, Xnp and Hira bind chromatin where a nucleosome has been disassembled. In the second step, new histones are delivered to the site, perhaps as delivery factors bind to Xnp and Hira. In the final step, a new nucleosome is assembled, and pre-deposition factors are released from chromatin. This model can account for the loose and sub-stoichiometric association of Hira and Xnp in purified H3.3 pre-deposition complexes (Drane et al. 2010; Goldberg et al. 2010).

Our results show that both Hira and Xnp are important for H3.3 deposition. Hira is a histone chaperone that will reduce the affinity of histones for DNA, and Xnp is a chromatin remodeler that destabilizes DNA-histone contacts (Lorain et al.1998; Picketts et al. 1996; Xue et al. 2003). These activities may affect the efficiency of histone transfer from delivery complexes to DNA, and pre-localizing these factors will poise sites for replacement. Xnp and Hira can clearly function independently, as each factor has unique sites in the Drosophila genome. Hira has a broad role in H3.3 replacement in both mammals and in Drosophila, but Hira may also have additional unrelated functions. Previous experiments have implicated it in gene regulation and cellular senescence, and a fraction of Hira protein is associated with transcriptional repressors (Sherwood et al. 1993; Zhang et al. 2007; Moshkin et al. 2009). These additional functions may account for its retention at select loci in the Drosophila genome.

The ATRX/XNP remodelers have also been implicated in transcriptional regulation (Gibbons et al., 2000; Bassett et al. 2008; Schneiderman et al. 2009). Nucleosome replacement mediated by this remodeler may account for some of these transcriptional functions, athough the mammalian protein has only been implicated in

H3.3 deposition at telomeres (Wong et al. 2010; Goldberg et al. 2010). Xnp in Drosophila appears to have a more general role, corresponding to its localization at all active sites except the rDNA genes. We note that the interpretation of function for one factor may be complicated by changes induced in the localization of other assembly factors. As the depletion of nucleosomes causes the recruitment and retention of assembly factors, they may serve as useful readouts of nucleosome density, and report changes in the activity of underlying chromatin regions in different cell types (Berube et al. 2000; Wong et al. 2010; Law et al. 2010; Zhang et al. 2007).

How do Xnp and Hira detect and bind nucleosome-depleted chromatin? Diverse processes in the nucleus can disrupt nucleosomes. Xnp and Hira may be recruited by structural features that are common to all depleted chromatin. Furthermore, the retention of these factors in H3.3-deficient cells suggests that Xnp and Hira compete with H3.3 for the same substrate. Perhaps these assembly factors simply bind exposed DNA. The ATRX/XNP remodelers contain an ADD domain that may bind DNA or histone tails (Cardoso et al. 2000; Argentaro et al. 2007; Otani et al. 2009). Xnp, Hira, or associated factors may directly bind DNA in chromatin gaps after a nucleosome has been disassembled, or bind histone tails that are exposed in partially assembled regions. Indeed, ATRX is recruited to the genomes of DNA viruses as they enter the nucleus (Lukashchuk et al. 2008). The intrinsic antiviral properties of ATRX may be due to its detection of exposed DNA and its subsequent promotion of nucleosome assembly to repress a viral genome.

Regardless of how Xnp and Hira sense gaps in chromatin, their recruitment allows any non-nucleosomal DNA to be targeted for new nucleosome assembly. It is striking that cells can thereby prevent the exposure of any DNA within the nucleus. Nucleosome-free regions are critical features of promoters and enhancers to allow factor

binding for gene regulation, but even such regions are only transiently exposed (Mito et al. 2007). The rapid recruitment of assembly factors like Xnp and Hira to chromatin gaps suggests that maintaining the nucleosomal structure of chromatin is important. Perhaps persistent exposure of DNA cripples transcriptional regulatory mechanisms, or leads to DNA damage. Thus, assembly factors that survey chromatin and ensure rapid nucleosome assembly may be critical for genome stability and function.

Materials and methods

Fly stocks and crosses

All stocks and crosses were grown at 25°C. Heat-shock induction was conducted at 37°C water-bath as previously described (Schwartz and Ahmad 2005). Null alleles for *xnp* (*xnp403* and *Df(3R)Exel6202*) were previously described in Schneiderman et al. 2009. The null *HiraHR1* allele was previously described by Bonnefoy et al. 2007. The unmarked *Hira1* null allele was derived from *HiraHR1* by *Cre* recombinase-mediated excision of the *mini-w+* marker (Gong and Golic 2004). The H3.3 null alleles *His3.3B0* and *His3.3A2X1* were described by Sakai et al. 2009. The GAL4- and heat-shock-inducible *P*[*H3.3-GFP*]B6 and truncated *P*[*H3.3core-GFP*]G5A lines were described in (Schwartz and Ahmad 2005). The GAL4-inducible knockdown line 5825R-3 (Umemori et al. 2009) was obtained from the National Institute of Genetics Fly Stock Center (Japan) and was expressed using the *P*[*SGS3-GAL4*]*TP1* driver line (Bloomington Drosophila Stock Center).

For construction of the *Hira-GFP* fusion gene, the eGFP coding sequence was inserted between the *Hira* and FLAG tag sequences of PW8-Hira-3xFLAG (Loppin et al. 2005), and the construct was transformed into flies by standard procedures (Rubin and Spradling 1983).

Polytene chromosome cytology

Salivary glands from male larvae were fixed and spread and slides were treated as described in (Schneiderman et al. 2009) except using 1% BSA in PBST as a blocking solution. Antibodies used for Xnp (1:2000) and Hira (PG1, 1:100) have been previously described (Bonnefoy et al. 2007; Schneiderman et al. 2009). Other antibodies used were against elongating RNA Polymerase II (Convance, H5), GFP (Clontech, JL8; Abcam, ab6556), and H3S10-P (Millipore, 06-570). Fluorescently labeled secondary antibodies (Jackson ImmunoResearch) were used at a 1:200 dilution. Polytene spreads were imaged using a 60X or 100X objective on a Nikon 80i Microscope using UV-2E/C, FITC HQ and TRITC HQ filter sets. Images were collected with a Hamamatsu ORCA-R2 camera using the MetaMorph image software. Image processing was performed with IPLab imaging software, and assembled in Adobe Photoshop and Adobe Illustrator.

Ovarian follicle cell cytology

2-4 day old adult females were heat shocked for 1 hour at 37°C in a water bath to induce expression from *P*[*H3.3-GFP*]B6 or *P*[*H3.3core-GFP*]G5A constructs, and then allowed to recover for 30 minutes at 25˚ before dissection in PBST. Ovaries were fixed in 4% Paraformaldehyde/PBT for 25 minutes, and washed 3 times for 10 minutes with PBST before blocking with 10% milk in PBT or 1% BSA in PBST. Antibodies to Xnp (Schneiderman et al. 2009) or Fibrillarin (Abcam, ab582) were applied overnight at 4˚, and detected with secondary antibodies (Molecular Probes, or Jackson ImmunoResearch). FITC filter sets were used to detect fluorescence from H3.3-GFP and Hira-GFP. Images were collected from stage 10 egg chambers by confocal or wide-field microscopy.

Nuclease protection assays

Micrococcal nuclease digests were performed as described by Corona et al. 2007, with

the following modifications: 4-5 gland pairs were isolated from each sample in cold M-buffer supplemented with 1mM ZnCl2 to inhibit endogenous nucleases, and incubated in the same buffer on ice. Glands were permeabilized with 0.5% NP-40 for 10 minutes on ice, centrifuged at 2100*g* for 5 minutes at 4°C, and resuspended in 200 μl of M-buffer supplemented with 2mM CaCl2 and 400U of MNase (USB, 70196Y). Digests were carried out at 25°C for 40 minutes with occasional agitation. The reactions were then stopped by adjusting samples to final concentrations of 20 mM EDTA and 0.5% SDS before Protease K treatment and DNA purification. This treatment produced predominantly mono-nucleosomal DNA fragments (Supplemental Figure S4). Real-time PCR measurements were performed in triplicate using the 7500 Fast Real-Time PCR System (Applied Biosystems) in 96-well plates. Primers for *Hsp70* were *hsp70 +334F/+423R* (“A”), *hsp70 +645F/+718R* (“B”), *hsp70 +872F/+1019R* (“C”), and *hsp70 +1649F/+1754R* (“D”) described in (Boehm et al. 2003). Ct values for each digest were normalized to background reads (“BackF” 5-TTGCACTCACCGTGATTGGAATG-3; “BackR” 5- GTCACAATGCTAACATCTCCTTAT-3) to give ΔCt values. Two biological replicates were performed for each experiment.

RNA analysis

RNA for northern blotting was extracted from 5 pairs of salivary glands using Trizol (Invitrogen) and precipitated with glycogen, yielding ~1.25 μg RNA per sample. RNA was resuspended in 1XMOPS/formamide/formaldehyde buffer containing 20 ng ethidium bromide according to standard protocols, and run on a 1.2% formaldehyde gel. The gel was imaged with UV and capillary-transferred overnight to a positively charged nylon membrane (Roche). DIG-labeled probe for *Hsp70* mRNA was made using a DNA template generated with primers *hsp70 +334F/+1490* (Boehm et al. 2003) and detected using the DIG DNA Labeling and Detection Kit (Roche) and CSPD ready-to-use

substrate (Roche). Membrane was exposed to autoradiography film, and integrated band intensities were calculated using the ImageJ software.

Acknowledgments

We thank Welcome Bender for comments, and Joe Geisberg for assistance with real-time PCR. Microscopy data for this study were acquired in the Nikon Imaging Center at Harvard Medical School. We appreciate fly lines provided by the Bloomington Stock Center (University of Indiana, Bloomington). Work in the laboratory of B. Loppin was supported by the CNRS, the French Ministry of Research, and the ANR (ANR-08-BLAN-0139-01).

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Figure legends

Figure 1. Overlapping localization of Xnp and Hira on polytene chromosomes. The cytological positions of the TAGA satellite block (orange arrow), the repetitive region 81F near the base of chromosome 3R (blue arrowhead), and the nucleolus (dotted outline)

are indicated in each image. (**A**) Chromosome spreads stained for elongating RNA polymerase II (red) and the Xnp chromatin remodeler (green). Xnp is enriched at actively transcribed genes and at the non-transcribed TAGA satellite block, but not in nucleolar chromatin or at 81F. (**B**) Chromosome spreads stained for the Hira chaperone (green) and elongating RNA polymerase II (red). Hira co-localizes with RNA polymerase at active genes, and is heavily enriched at nucleolar chromatin. Hira often stains the 81F region. (**C**) Chromosomes stained for constitutively expressed H3.3core-GFP (red) that marks sites of nucleosome replacement and Hira (green). Hira co-localizes H3.3 along the chromosome arms and at nucleolar chromatin.

Figure 2. The Xnp and Hira assembly factors are recruited to induced *Hsp70* genes. Polytene chromosomes were prepared from larvae heat-shocked at 37˚ for the indicated time (minutes) with or without recovery at 25˚. The location of the *Hsp70* genes at cytological positions 87A and 87C are indicated (white lines). DAPI is shown in red. (**A**) Heat-shock causes the rapid recruitment of elongating RNA polymerase II (blue) to the *Hsp70* loci. RNA polymerase leaves within 60 minutes of recovery as the genes are repressed. (**B**) Xnp staining (green) of the same chromosomes in (A) show that Xnp is rapidly recruited to induced *Hsp70* genes, and leaves during recovery. (**C**) Hira (green) is also recruited to induced *Hsp70* genes. Hira leaves the 87A *Hsp70* locus within 60 minutes of recovery, but persists at the 87C locus.

Figure 3. The Xnp and Hira assembly factors bind chromatin in the absence of the H3.3 histone. The cytological positions of the TAGA satellite block (orange arrow) and the nucleolus (dotted outline) are indicated in each image. Chromosome spreads from wildtype animals are on the left, and from RNAi H3.3 knock-down salivary glands on the

right. (**A,G**) Xnp staining in H3.3 knock-down glands is broadly enhanced on chromosome arms, and new sites appear within the nucleolus. (**B,H**) Hira staining in H3.3 knock-down glands is broadly enhanced on chromosome arms. (**C,I**) Zooms of the nucleolus. Xnp is not normally enriched on nucleolar chromatin, but is strongly enriched in H3.3 knock-down glands. (**D,J**) Zooms of the nucleolus show that Hira enrichment at nucleolar chromatin is increased in H3.3 knock-down glands. (**E,K**) Zooms of the TAGA satellite block show that the Xnp-staining region appears to expand in H3.3 knock-down glands. (**F,L**) Zooms of the TAGA satellite block. Hira is not normally enriched at the satellite, but is strongly enriched in H3.3 knock-down cells.

Figure 4. Nucleosomes are not restored to repressed *Hsp70* genes in H3.3-deficient cells. (**A**) Schematic depiction of the nucleosomal *Hsp70* gene. The location of PCR amplicons and the Northern probe are shown. (**B**) DNA survival after MNase digestion. Chromatin was purified from salivary glands before (NoHS), during heat-shock (HS), or 1 hour into recovery after heat-shock (HS+60'). Blue, DNA survival in wildtype controls; red, DNA survival in H3.3 knock-down glands. PCR Cycle-to-threshold (Ct) values were normalized to a background intergenic amplicon and to NoHS values. DNA survival is presented in a log2 scale. Dotted lines show results for biological repeats of experiments. (**C**) Northern detection of *Hsp70* transcripts in control and H3.3 knock-down salivary glands. The amount of the *Hsp70* signal was calculated relative to the *18S* signal (stained with ethidium bromide) after subtraction of background. (**D,E**) Signals for elongating RNA polymerase II (blue) and histone H3 S10 phosphorylation (H3S10P, green) at the *Hsp70* loci during heat-shock in control (**D**) and H3.3 knock-down (**E**) polytene chromosome spreads. Signals for H3S10P are standardized to a neighboring band (arrowhead). H3S10P appears noticeably reduced in knock-down glands.

Figure 5. The Xnp and Hira assembly factors persist at repressed *Hsp70* genes in H3.3-deficient cells. Polytene chromosomes from H3.3 knock-down salivary glands heat-shocked at 37° for the indicated time (minutes) with or without recovery. The *Hsp70* genes at 87A and 87C are indicated (white lines). DAPI is in red. (**A**) Elongating RNA polymerase II (blue) labels the induced *Hsp70* loci, and leaves within 60' of recovery. (**B**) Xnp staining (green) on the same chromosomes in (A) show that Xnp is recruited to induced *Hsp70* genes, but remains bound after recovery. Xnp strongly stains the 87A and 87C loci after 120' in two bands that are not observed in wildtype chromosomes (Figure 2). (**C**) Hira (green) is also recruited to the *Hsp70* genes during induction, and persists after recovery.

Figure 6. Xnp and Hira are required for H3.3 nucleosome replacement. (**A-D**) Deposition of H3.3core-GFP (green) in polytene chromosomes after recovery from heat-shock. DAPI is in red, and the nucleolus is outlined with a dotted line. (**A**) H3.3core-GFP is efficiently deposited along chromosome arms in wildtype, and the nucleolus is devoid of soluble tagged protein. (**B**) *xnp403/Df(3R)Exel6202* and (**C**) *HiraHR1* mutants show reduced H3.3core-GFP signals along chromosome arms, and increased accumulation of the tagged protein within nucleoli. (**D**) Deposition of H3.3core-GFP is completely blocked in *HiraHR1 ; xnp403/Df(3R)Exel6202* double mutants and the tagged protein accumulates within the nucleolus. (**E-I**) Assembly factor localization and H3.3 deposition in Stage 10 somatic follicular epithelia of adult ovaries. DNA is in white. The nucleolus is a clear area within each nucleus, with 1-6 DNA-rich foci. (**E**) Hira-GFP (green) broadly stains chromatin and foci within the nucleolus. (**F**) Xnp (green) broadly stains chromatin but does not localize within the nucleolus. (**G-I**) Hira is required for H3.3 deposition (green)

at active rDNA genes. (**G**) A pulse of H3.3-GFP accumulates in nucleolar foci. The nucleolus is marked by anti-fibrillarin staining (red). (**H**) A pulse of H3.3core-GFP deposits within the nucleolus in *xnp403/Df(3R)Exel6202* adults. (**I**) H3.3-GFP does not deposit within the nucleolus in *Hira1* adults.

Supplementary Figure Legends

Figure S1. Xnp and Hira chromatin binding are not interdependent. Polytene chromosome spreads from *HiraHR1* and *xnp403* mutant larvae. The site of the TAGA satellite block is marked with an orange arrow, and the nucleolus is outlined with a dotted line. Signals from elongating RNA polymerase II are in blue. (**A**) Xnp (green) stains normally in *Hira* null mutants, and (**B**) Hira (green) localizes normally in *xnp* mutants.

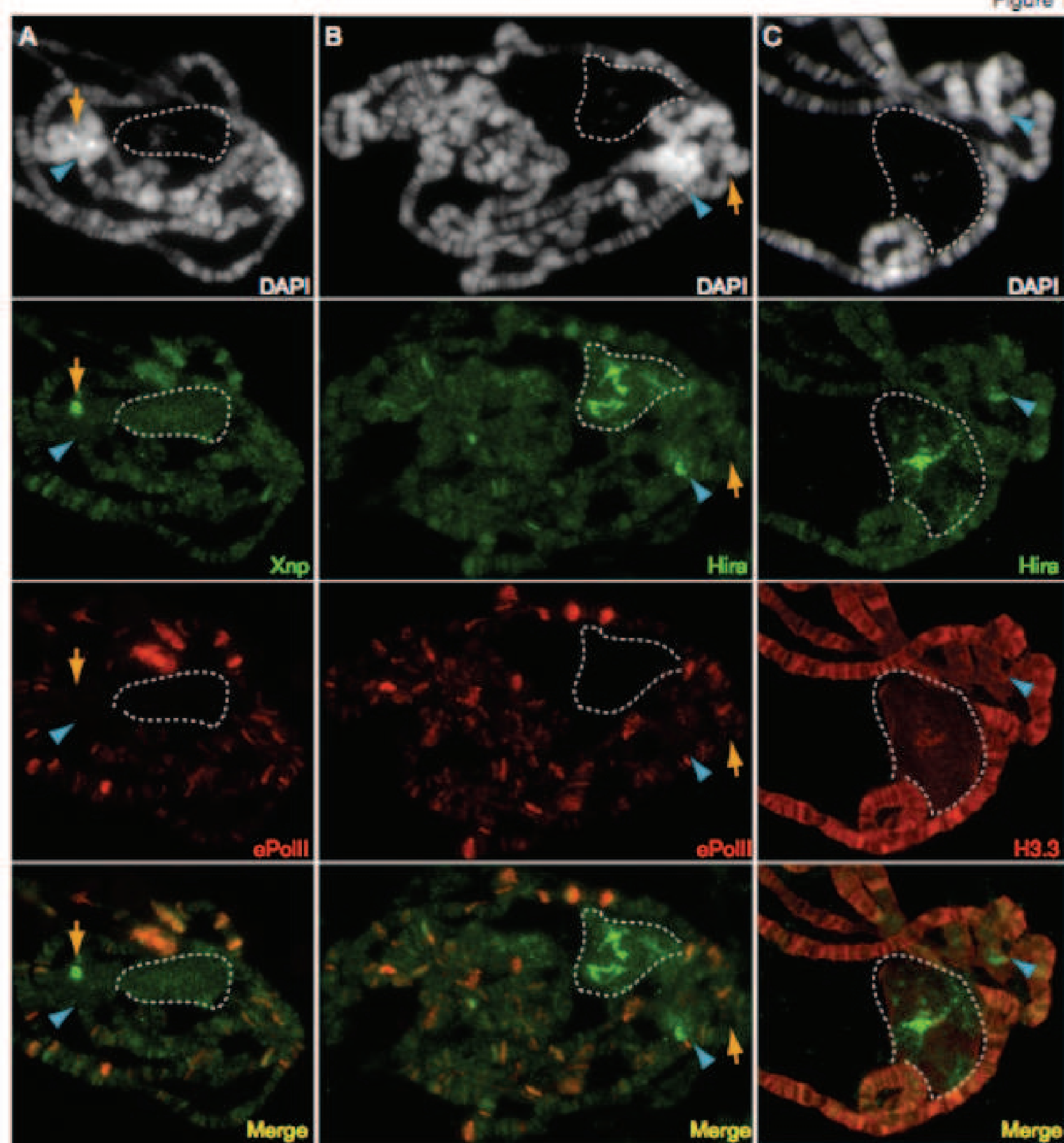
Figure S2. H3.3 is efficiently knocked down by RNAi in larval salivary glands. Western detection of H3.3-GFP expressed in salivary glands by the *SGS-GAL4* driver, with (dsH3.3A) or without (–) co-induction of a *UAS-H3.3A* hairpin construct. Membranes were stained with Ponceau to confirm similar loading.

Figure S3. Assembly factor binding in H3.3 null mutants. Polytene chromosome spreads from *His3.3B0 ; His3.3A2X1* H3.3 null mutant larvae were stained with antibodies to assembly factors. (**A**) Xnp staining (green) is increased throughout the chromosome arms and at the TAGA satellite block. (**B**) Hira staining (green) is increased throughout chromosome arms and on chromatin in the nucleolus.

Figure S4. MNase digestion of salivary gland chromatin produces

mononucleosomes. Ethidium-stained gel of a time course of MNase digestion with 5 dissected salivary glands in each lane. 40 minute digests were used for qPCR assays.

Figure 1



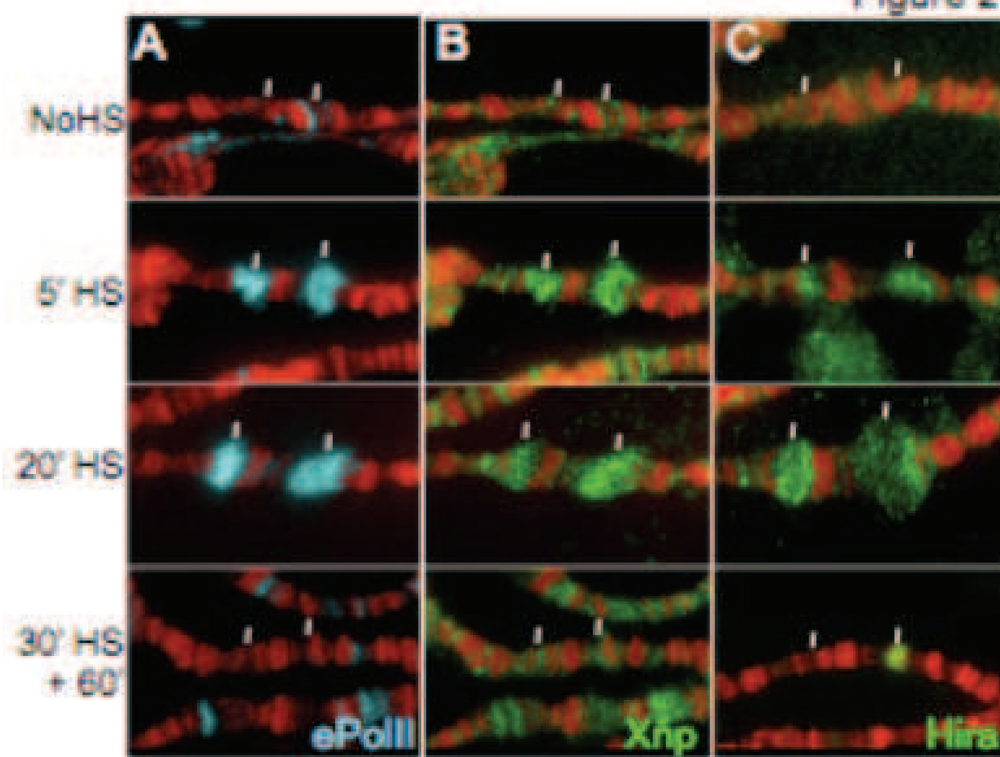
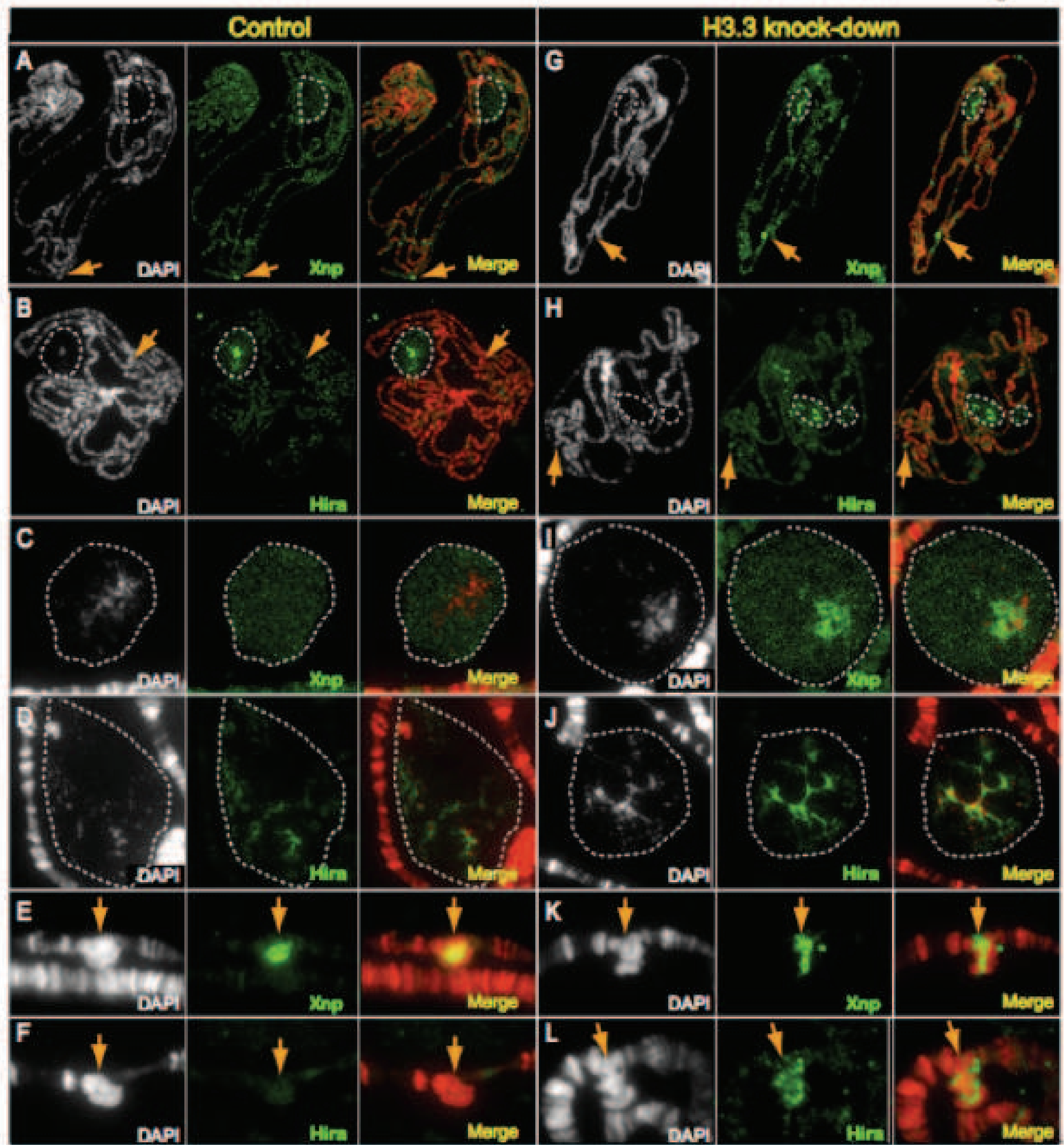


Figure 2
A
B
C
NoHS
5' HS
20' HS
30' HS
+ 60'
ePolII
Xnp
Hira

Figure 3



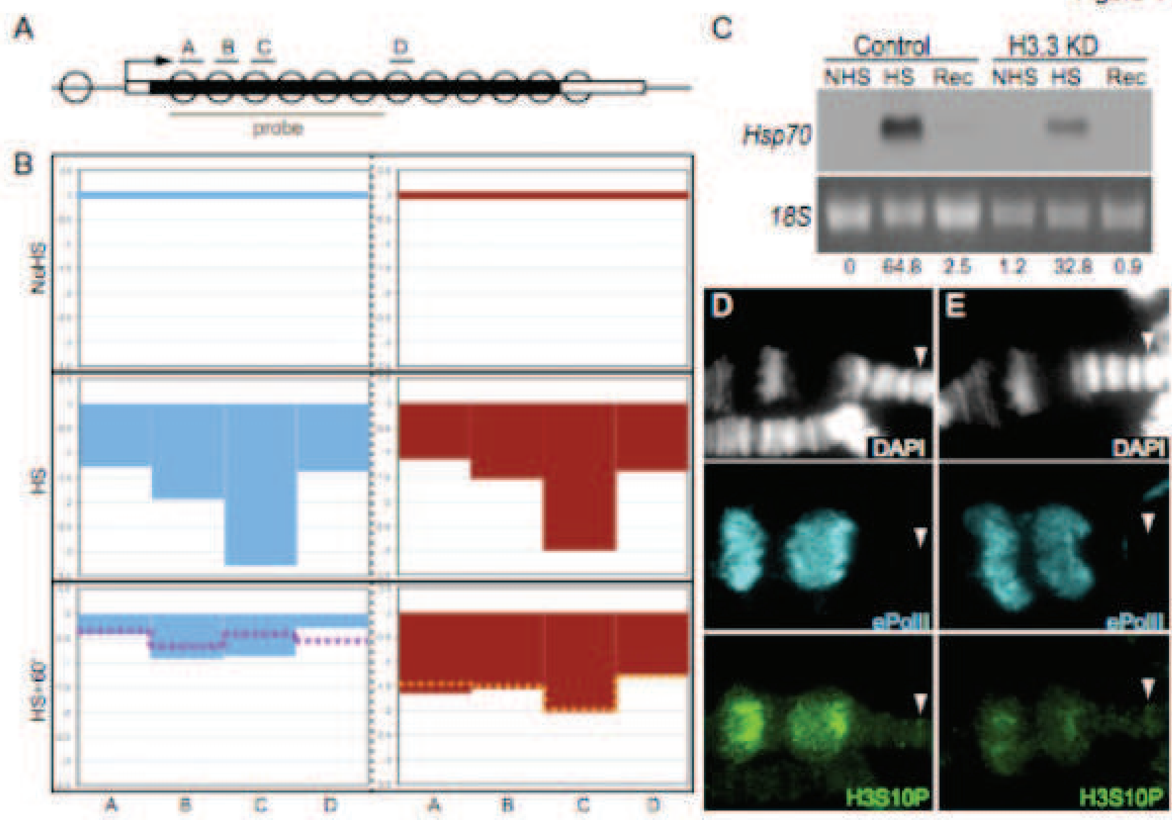


Figure 4
A
A B C D
probe
B
NoHS
HS
HS+60'
A B C D A B C D
C
Control H3.3 KD
NHS HS Rec NHS HS Rec
Hsp70
18S
0 64.8 2.5 1.2 32.8 0.9
D
E
DAPI
ePolII
H3S10P

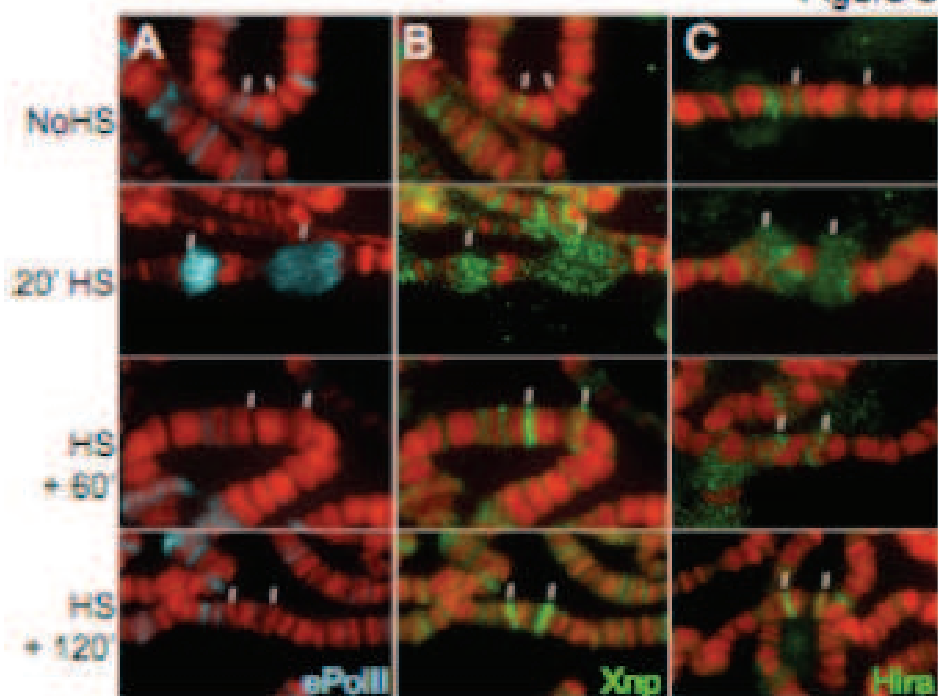
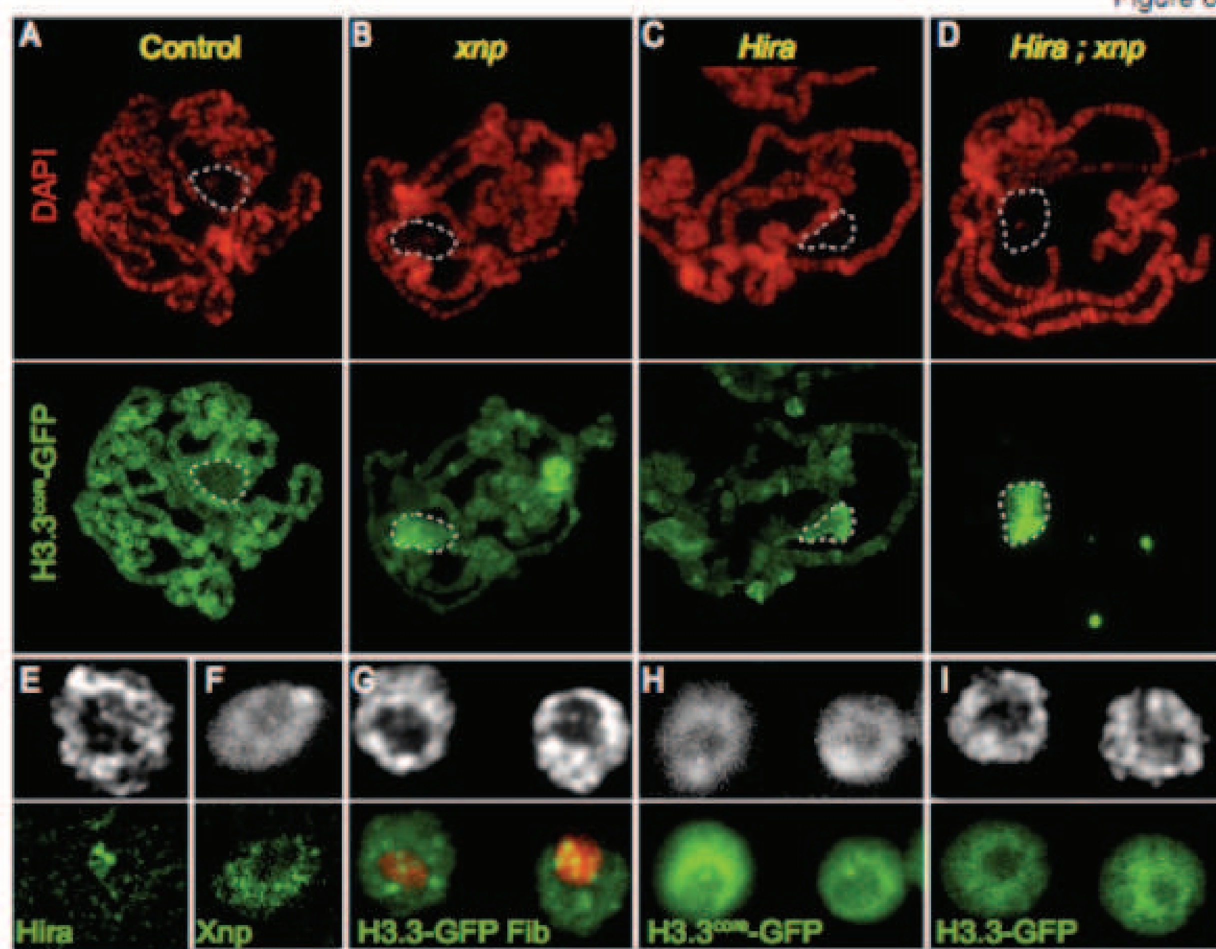
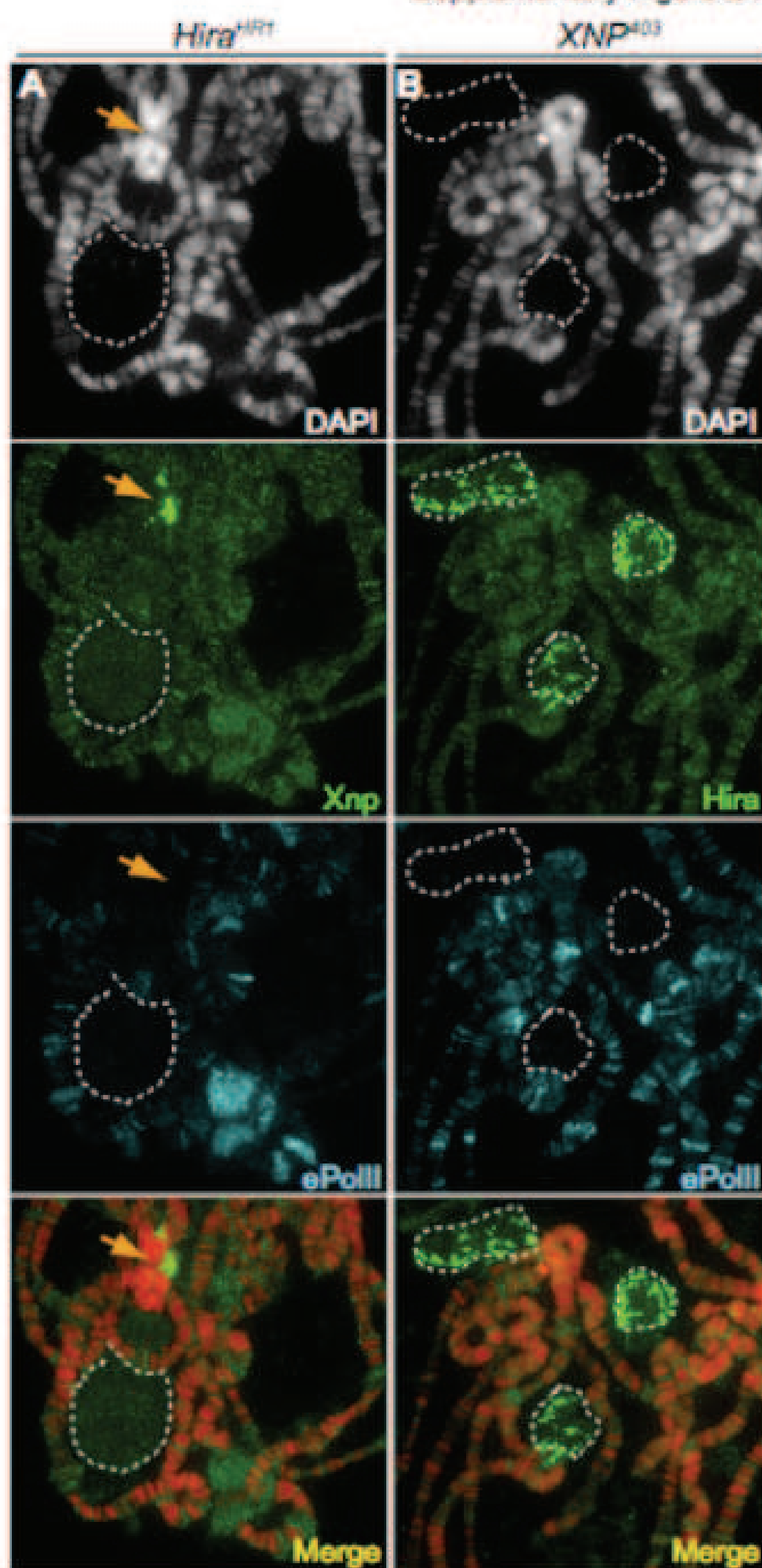


Figure 5
A B C
NoHS
20' HS
HS + 60'
HS + 120'
ePolII
Xnp
Hira

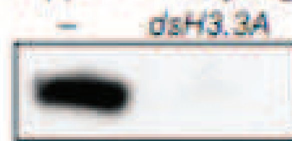
Figure 6



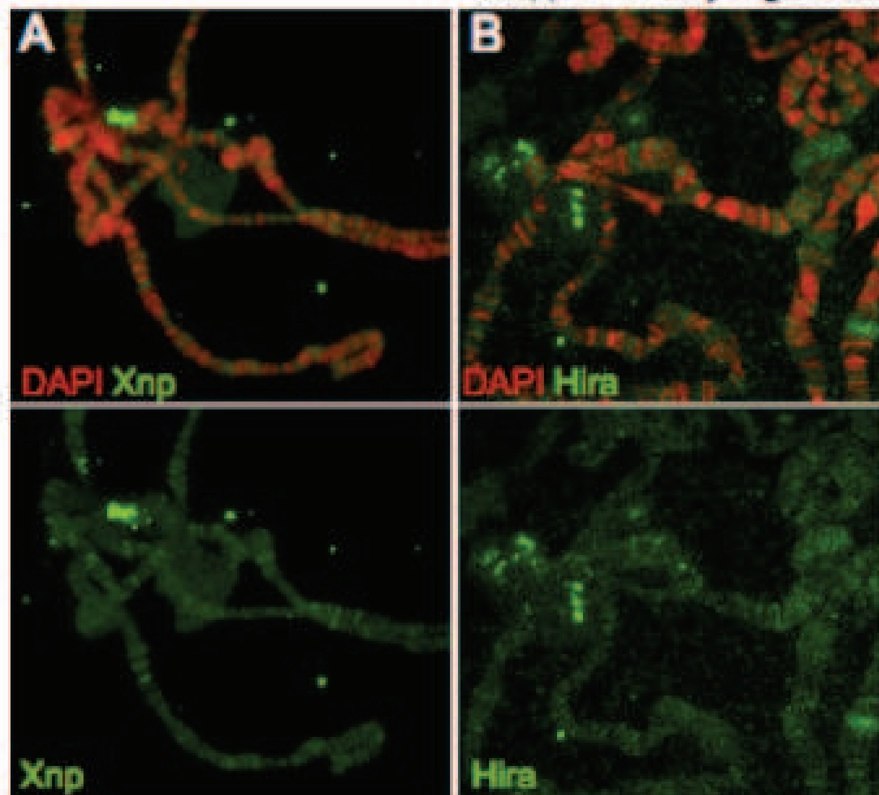
Supplementary Figure S1



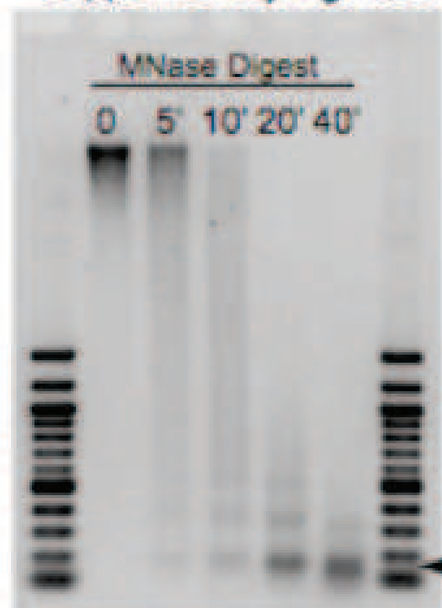
Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4



La question posée par ce manuscrit, bien que fondamentale, a reçu relativement peu d'attention dans la littérature. Quels sont les mécanismes qui identifient l'ADN nu pour promouvoir son assemblage sous la forme de nucléosomes? La question semble résolue en ce qui concerne la réplication du génome. Le complexe d'assemblage de la chromatine CAF-1 interagit avec le facteur PCNA, un acteur essentiel à la réplication de l'ADN présent aux fourches de réplication (Shibahara et Stillman, 1999; Moggs *et al.*, 2000), ce qui suffit à diriger CAF-1 aux sites d'assemblage CR. Cependant, la question est plus complexe en ce qui concerne l'assemblage IR.

Dans le cas du centromère chez la drosophile, le recrutement de CenH3 semble être le résultat d'un processus dépendant finement de son timing d'expression et de sa dégradation spécifique en dehors du centromère (Heun *et al.*, 2006; Moreno-Moreno *et al.*, 2006; Schuh *et al.*, 2007).

Dans le cas de l'histone H3.3, les facteurs impliqués dans son assemblage pourraient interagir avec une large variété de facteurs impliqués de fait dans les divers processus biologiques concernés. Alternativement, l'ADN non nucléosomique pourrait émettre un signal qui permette le recrutement de facteurs. Finalement, il pourrait exister des protéines qui sondent activement la chromatine pour y détecter des besoins d'assemblage. Ces facteurs devraient directement reconnaître l'ADN : dans ce sens, d'après des travaux récents, ATRX pourrait être recruté de façon spécifique sur les régions répétées et/ou riches en G-quadriplex (Law *et al.*, 2010). De même, il est possible que le complexe HIRA, éventuellement par le truchement de Yem, soit capable de reconnaître l'ADN lui même plutôt que des facteurs associés aux processus nécessitant un assemblage.

Dans le but d'élargir la question au complexe HIRA/Yem, j'ai voulu tester directement le rôle de Yem dans l'assemblage des nucléosomes dans les cellules somatiques. De façon analogue aux expériences décrites dans le manuscrit ci-dessus, l'induction transitoire de l'expression en contexte mutant permet d'évaluer le rôle des différents facteurs dans l'assemblage IR. Dans des glandes salivaires, l'incorporation de H3.3-GFP est sévèrement perturbée en absence de HIRA, de façon cohérente avec les résultats précédents. De façon intéressante, l'absence de Yem bloque aussi l'incorporation de H3.3 dans la chromatine (Figure 3). De même, comme dans le cas de *Hira*, un défaut d'incorporation de H3.3-GFP dans le nucléole peut être observé dans des cellules folliculaires de femelles adultes mutantes *yem*^{1} ou *yem*^{2} (Figure 3). Yem

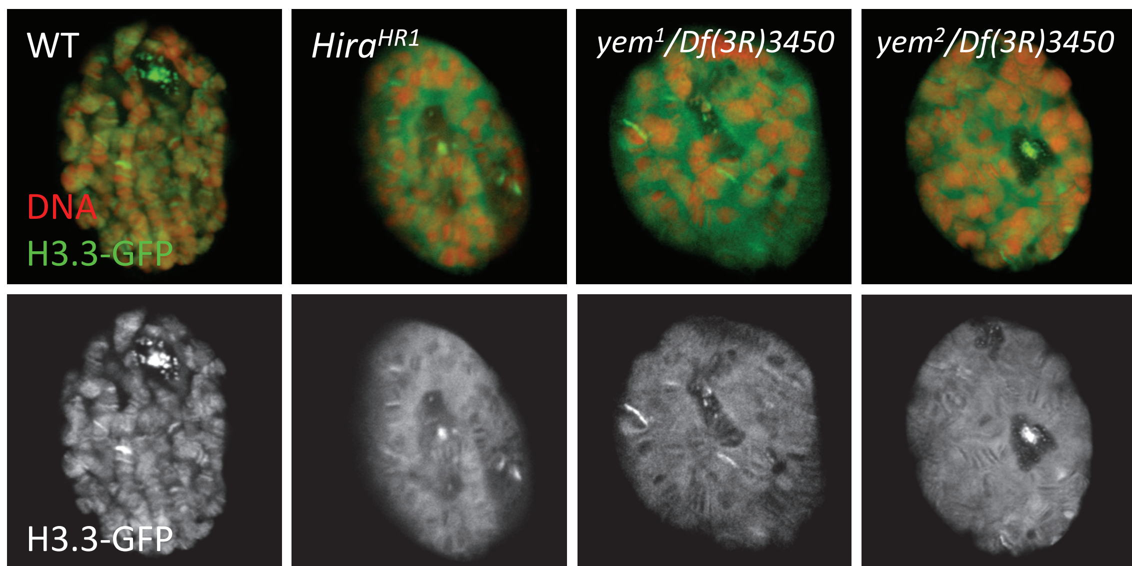
semble donc avoir un rôle essentiel pour l'assemblage de H3.3 dans ces cellules somatiques. Yem et HIRA collaborent donc probablement au sein d'un complexe fonctionnel intervenant aussi bien dans l'assemblage du pronoyau mâle que l'assemblage somatique.

Le modèle fonctionnel proposé dans cet article suggère que les complexes ATRX ou HIRA attendent leur substrat pour réaliser l'assemblage. L'histone pourrait être fournie par d'autres facteurs. ASF1 serait un candidat privilégié dans le cas du complexe HIRA (Tagami *et al.*, 2004; Tang *et al.*, 2006; Moshkin *et al.*, 2009), et pourrait agir comme donneur d'histones dans les régions gérées par HIRA. Par ailleurs, DAXX a été proposé comme étant un chaperon d'histones H3.3 chez l'homme, et un fournisseur possible d'histones pour ATRX (Drane *et al.*, 2010; Lewis *et al.*, 2010). Le rôle *in vivo* de DAXX dans l'assemblage de la chromatine, ainsi que les fonctions de l'orthologue de DAXX, Daxx Like Protein (DLP) chez la drosophile, restent à évaluer.

De façon intéressante, la situation semble être très différente dans le pronoyau mâle. Premièrement, contrairement aux mutations affectant *Hira* ou *yem*, celles affectant *xnp* n'ont pas de conséquences pour la fertilité suggérant que la voie XNP n'est pas impliquée dans l'assemblage dans ce noyau. Deuxièmement, nous avons vu que ASF1 ne participe probablement pas à cet assemblage non plus. Les données fonctionnelles actuelles laissent supposer qu'à lieu dans le pronoyau mâle un mécanisme d'assemblage recrutant un minimum de facteurs pour compléter le processus, dont HIRA et Yem, s'agissant peut être d'un mécanisme unique, en place exclusivement pendant la fécondation.

Le rôle de HIRA dans l'assemblage somatique des nucléosomes contenant H3.3 que nous avons montré nous oblige à revoir notre conception de ses rôles *in vivo*. Bien que ce facteur semble avoir un rôle absolument critique pour l'assemblage IR à la fécondation, cette fonction est compatible avec des rôles plus subtils de la machinerie HIRA dans le contrôle de la chromatine somatique. J'ai donc mené l'exploration de nouveaux rôles somatiques du complexe HIRA, en particulier dans la formation ou le maintien de l'hétérochromatine.

A.



B.

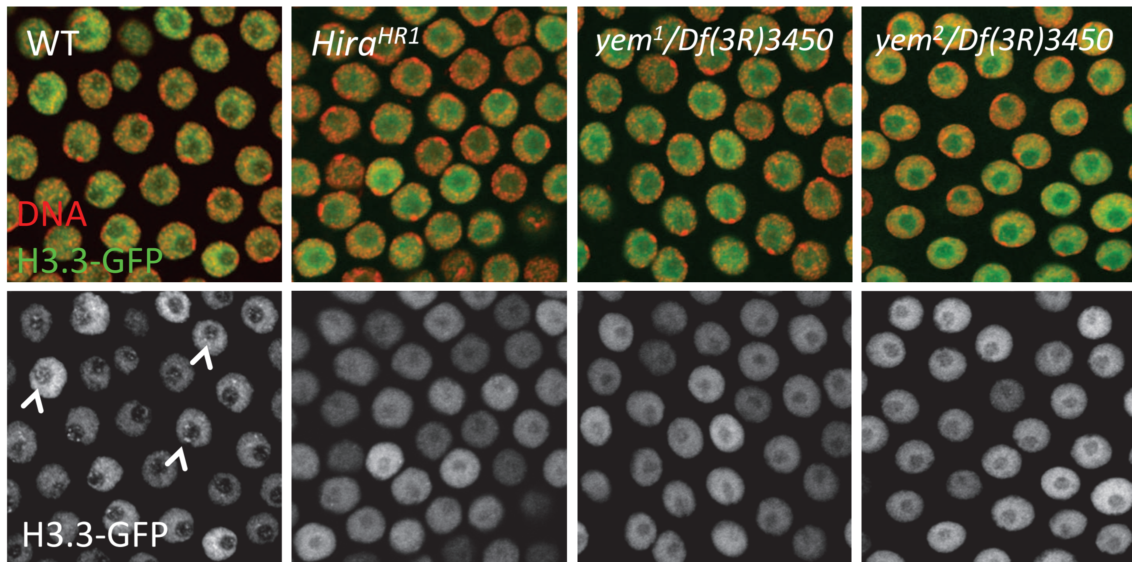


Figure 3. Yem est requis pour l'assemblage de H3.3 dans les cellules somatiques. L'expression de la protéine H3.3-GFP a été induite en contexte sauvage ou mutant pour Hira ou yem, dans des larves de stade 3 (A) ou des femelles adultes (B) par choc thermique. Un temps de chasse de 4 heures (A) ou 30 minutes (B) a été laissé avant dissection. Les images sont des photographies confocales de glandes salivaires (A) ou ovaires (B) marqués pour révèler l'ADN (rouge) et H3.3-GFP (vert). (A) L'incorporation de H3.3-GFP sur tous les chromosomes est fortement affectée en contexte mutant pour Hira comme pour yem. (B) De façon similaire, l'incorporation de H3.3-GFP dans le nucléole(flèches) est abolie chez ces mêmes mutants. Barres: 10µm.

V. Rôle somatique de HIRA dans la régulation fine de la chromatine

La viabilité des mutants affectant HIRA chez la drosophile permet de poser la question de son rôle dans la dynamique de la chromatine chez un organisme multicellulaire au cours du développement. Les premières pistes venaient de l'étude de l'expression d'un transgène comportant un promoteur fort, suivi d'un insulateur et d'un gène rapporteur, inséré dans un locus soumis à la variégation (Nakayama *et al.*, 2007). Les auteurs ont décrit que l'expression du rapporteur est atténuée en contexte mutant pour *Hira*, suggérant que HIRA joue un rôle positif dans son expression. Le modèle envisagé est que le renouvellement de nucléosomes sur l'élément insulateur, impliquant un assemblage de H3.3 par HIRA, constitue une barrière à l'étalement de l'hétérochromatine.

Or, cette fonction contredit plusieurs données dans la littérature. Chez les levures *S.cerevisiae* et *S.pombe*, le complexe HIRA joue un rôle de répression de l'activité transcriptionnelle (notamment des gènes codant pour les histones)(Xu *et al.*, 1992; Blackwell *et al.*, 2004) et un rôle analogue a été suggéré chez le poulet (Ahmad *et al.*, 2005). De plus, chez *S.pombe*, le complexe HIRA participe à la répression des régions péricentromériques, sub-télomériques, le locus mating type et plusieurs types de transcrits cryptiques, dont notamment des transposons de type LTR (Anderson *et al.*, 2009). Finalement, HIRA a récemment été impliqué dans un mécanisme de désacétylation des histones sur des régions hétérochromatiques chez cette levure (Yamane *et al.*, 2011). A partir de ces travaux, l'hypothèse est faite que HIRA participe à la formation ou le maintien de l'hétérochromatine, bien que ce lien reste indirect.

Chez l'homme, HIRA est un composant essentiel des foyers d'hétérochromatine associés à la sénescence dans des modèles cellulaires (Zhang *et al.*, 2005; Zhang *et al.*, 2007a). Le lien entre HIRA, H3.3 et des facteurs classiquement associés à l'hétérochromatine est par ailleurs illustré par son interaction avec des protéines HP1 dans une voie de « réparation de l'hétérochromatine perturbée » (Zhang *et al.*, 2007b). Il s'agit de la reconstitution de chromatine (notamment péricentromérique) après sa déstabilisation par traitement avec des drogues. Ce travail jette les bases d'un lien potentiel entre l'assemblage IR par la voie HIRA/H3.3, la formation d'hétérochromatine, et la protection de l'intégrité de la chromatine. Finalement, les mutations sur *Hip* sont associées à une sensibilité à différents stress

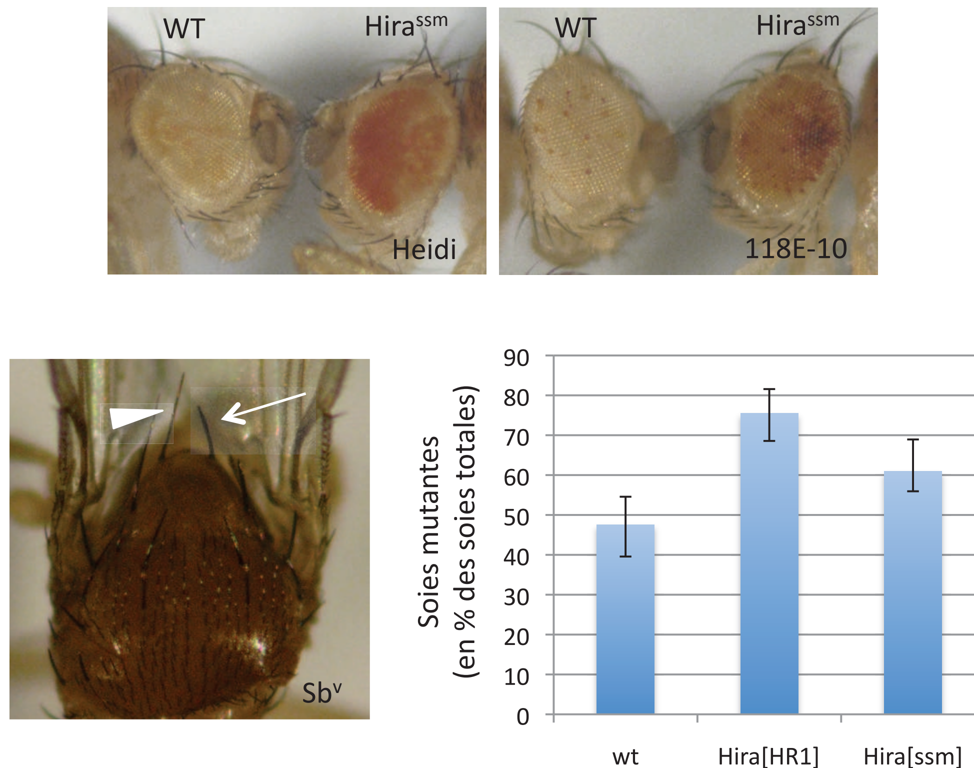


Figure 4. Les mutations de Hira sont des suppresseurs de variégation.
L'aberration Sb^{v} et les insertions Heidi et 118E-10, qui présentent un profil d'expression soumis à la variégation ont été combinés avec les mutations Hira^{ssm} ou Hira^{HR1}. L'effet des mutants sur la variégation a été évalué chez des mâles hémizygotes. La variégation de l'allèle Sb a été mesurée en comparant les soies mutantes (flèches) et sauvages (tête de flèche): les résultats sont présentés dans le graphe.

génotoxiques chez *S.pombe*, suggérant que cette voie pourrait être conservée (Anderson *et al.*, 2009). Toutefois, le rôle de HIRA/H3.3 dans l'hétérochromatine n'a pour l'heure pas été directement évalué *in vivo*, dans le contexte du développement.

Les mutations sur Hira se comportent comme des suppresseurs de variégation

J'ai voulu tester le rôle de HIRA dans la propagation de l'hétérochromatine dans des systèmes plus classiques de variégation chez la drosophile. De façon intéressante, les mutations de *Hira* se comportent comme des suppresseurs de variégation dans les trois systèmes testés, suggérant que HIRA joue un rôle positif dans la formation et/ou le maintien de l'hétérochromatine (Figure 4). Néanmoins, l'effet Su(var) associé aux mutations sur *Hira* apparaît comme faible, et la réduction de moitié de dose de HIRA chez les individus hémizygotes ne semble pas avoir un effet sur la variégation. Ceci suggère que HIRA n'est pas un élément crucial pour la formation de l'hétérochromatine, mais joue probablement un rôle dans sa modulation.

Intéraction génétique entre *Hira* et la voie JAK/STAT (non canonique)

Il est possible d'imaginer que, comme dans d'autres modèles, HIRA participe à la stabilité de la chromatine, son rôle n'étant nécessaire qu'en situation de stress. Chez la drosophile, une de ces situations est générée par la suractivation du facteur de transcription STAT dans le contexte d'un allèle hyperactif de JAK, appelé $hopscotch^{Tumoral\text{-}l}$ ($hop^{Tum\text{-}l}$) (Harrison *et al.*, 1995; Luo *et al.*, 1995). En effet, la suractivation constitutive de JAK est associée à un fort effet de suppression de variégation (Shi *et al.*, 2006; Shi *et al.*, 2008). Les individus exprimant cet allèle sont viables et fertiles mais développent de masses mélaniques (souvent assimilées à des tumeurs de la lignée hématopoïétique). Le modèle proposé attribue à STAT un rôle « non-canonique » en tant qu'interacteur de HP1, sa phosphorylation par JAK entraînant la perte de cette interaction, et la déstabilisation de l'hétérochromatine. J'ai voulu savoir si les mutations sur *Hira* aggravaient le phénotype associé à $hop^{Tum\text{-}l}$. De façon intéressante, le double mutant n'arrive jamais à l'âge adulte, démontrant une interaction synthétique létale entre ces voies (Figure 5). HIRA pourrait donc jouer un rôle protecteur de l'intégrité de l'hétérochromatine,

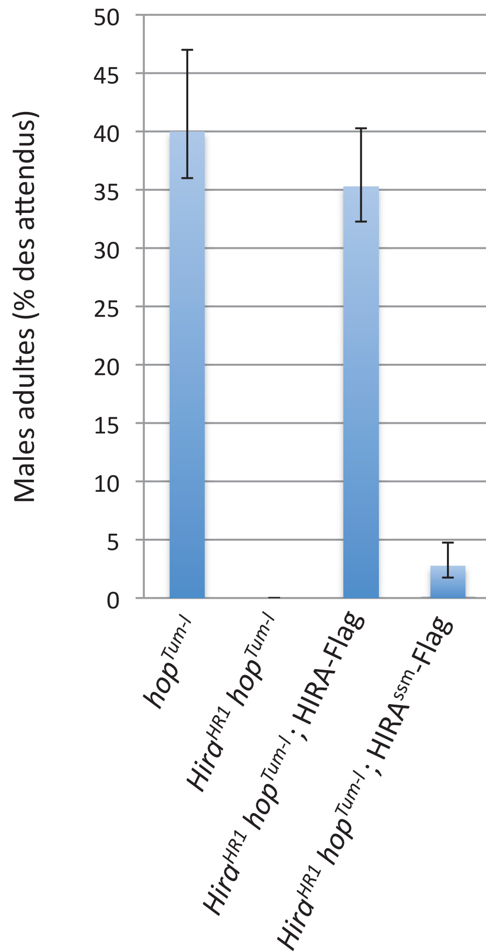


Figure 5. Intéraction létale entre les mutations affectant *Hira* et *hopscotch*$^{\textit{Tumoral-l}}$ (*hop*$^{\textit{Tum-}\mathrm{l}}$).

Des femelles possédant à l'état hétérozygote les chromosomes *hop*$^{Tum\text{-}l}$ ou *Hira*^{HR1} *hop*$^{Tum\text{-}\mathrm{l}}$ ont été croisées par des mâles sauvages (colonnes 1 et 2), ou portant des transgènes HIRA-Flag (colonne 3) ou *Hira*^{ssm}-Flag (colonne 4). Les mâles adultes survivants des génotypes indiqués ont été comptés. La double mutation *Hira*^{HR1} *hop*$^{Tum\text{-}\mathrm{l}}$ est synthétique létale. Cet effet est sauvé par un transgène HIRA-Flag, mais ne l'est que faiblement par un transgène HIRA$^{\mathrm{ssm\text{-}}}$Flag, démontrant qu'il dépend de HIRA.

compromise en contexte *hop*$^{Tum\text{-}l}$ (bien que la nature de cette interaction fonctionnelle ne soit pas claire).

HIRA participe à la protection des ADN ribosomiques

Comme il a été exposé précédemment (voir Schneiderman *et al.*, en préparation), HIRA est important pour l'assemblage processif de nucléosomes dans le nucléole. Or, ce territoire nucléaire abrite environ deux cents répétitions en tandem des ADN ribosomiques (ADNr) hautement actifs est en proie à un risque important de dommages à l'ADN et de recombinaisons ectopiques (Tsang et Carr, 2008). Les ADNr sont, par ailleurs, enclavés dans des régions hétérochromatiques dont la dynamique participe à leur régulation fine et à leur stabilité (Bryk *et al.*, 2002; Santoro *et al.*, 2002; Peng et Karpen, 2007, 2008; Peng *et al.*, 2009; Plata *et al.*, 2009). Le renouvellement d'histones par HIRA aux ADNr pourrait contribuer à leur protection.

Pour tester directement ce rôle protecteur, j'ai utilisé un système qui permet d'induire des cassures double brin de l'ADN spécifiquement aux ADNr. La méganucléase I-CreI reconnaît une séquence qui n'est présente chez la drosophile que dans la bibliothèque d'ADNr (Maggert, 2005; Paredes et Maggert, 2009a). Son induction transitoire chez des larves amène souvent à la réparation aberrante par recombinaison homologue des ADNr, ce qui entraine la réduction du nombre de copies de la bibliothèque, associée à des phénotypes variables allant de phénotypes classiques visibles, à la létalité (Paredes et Maggert, 2009a). Dans mes conditions, l'induction de cette enzyme est associée à une survie de près de 50% des individus jusqu'à l'âge adulte (Figure 6). Cependant, quand l'expression de I-CreI est induite chez des individus mutants pour *Hira*, le taux d'adultes survivants devient proche de 0%. Les dommages aux ADNr ne sont plus tolérés en contexte mutant pour *Hira* faisant provoquant des défauts globaux. Ce résultat est en faveur d'un rôle protecteur de HIRA.

De façon intéressante, plusieurs études montrent que les loci des ADNr est un régulateur global de l'état de la chromatine chez la drosophile (Hilliker et Appels, 1982; Paredes et Maggert, 2009b; Plata *et al.*, 2009). En particulier, les petites bibliothèques d'ADNr agissent comme des suppresseurs de variégation (Paredes et Maggert, 2009b). L'interprétation est

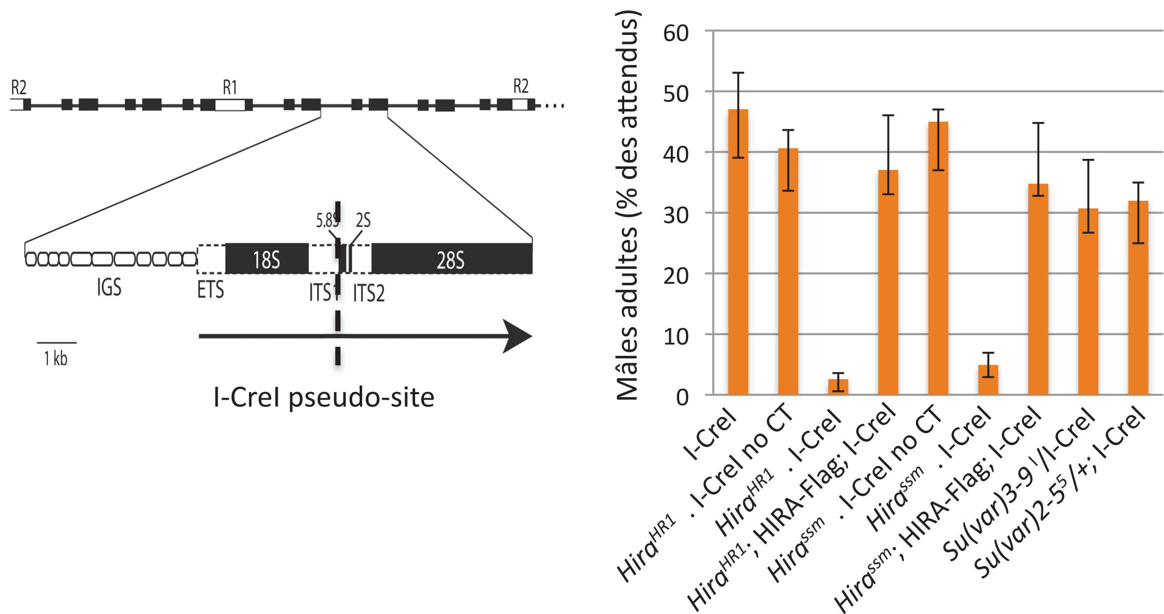


Figure 6. HIRA participe au maintien de l'intégrité du nucléole.
Test de survie après induction de la méganucléase I-CreI en contexte mutant pour *Hira*. I-CreI reconnaît un site de coupure dans chaque copie d'ADN ribosomique (schéma). L'expression de I-CreI a été induite par choc thermique (sauf pour les pistes no choc thermique, "no CT") dans des larves de stade 3 mutantes ou non pour $Hira^{HR1}$, $Hira^{ssm}$ (sauvées ou non par un transgène HIRA-Flag) ou hétérozygotes pour $Su(var)3\text{-}9^{1}$ ou $Su(var)205^{5}$. Les mâles adultes survivants ont été comptés.

qu'en situation limitante pour la production ARNr, des mécanismes compensatoires favorisent la transcription à l'échelle de tout le noyau, en particulier en défavorisant la formation d'hétérochromatine. Réciproquement, l'état général de l'hétérochromatine a une influence sur la biologie du nucléole. En particulier, Su(var)3-9 et HP1a semblent être critiques pour maintenir la structure du nucléole chez la drosophile (Peng *et al.*, 2009). Ce dialogue pourrait contribuer à maintenir un équilibre d'expression des gènes à l'échelle du génome entier. Notre modèle implique que, par l'assemblage de nucléosomes de façon IR dans les ADNr, HIRA contribue à maintenir cet équilibre. Les conséquences directes de l'absence de HIRA sur la taille de la bibliothèque des ADNr et l'expression des ARNr restent à évaluer.

Alternativement, HIRA pourrait participer à la protection de toute la chromatine. Nous pouvons dans ce cas attendre que HIRA participe au réassemblage de l'hétérochromatine comme à celui de la chromatine fortement active dans les ADNr. La nécessité de l'un ou l'autre de ces rôles dépendrait du type cellulaire et des stress environnants, ce qui expliquerait la diversité de situations affectées par l'absence de HIRA.

Discussion générale autour des rôles du complexe HIRA

HIRA a un rôle conservé dans la répression de la transcription

Les orthologues de HIRA ont été impliqués dans la répression transcriptionnelle de façon conservée (chez *S.cerevisiae, S.pombe, A.thaliana* et le poulet) (Xu *et al.*, 1992; Ahmad *et al.*, 2005; Phelps-Durr, 2005; Anderson *et al.*, 2009). Chez *S.cerevisiae* et *S.pombe*, cette répression semble affecter un panel large de cibles transcriptionnelles dont la plupart fait partie de régions constitutivement silencieuses. Cependant, les complexes Hir et Hip ont été initialement décrits comme essentiels pour réprimer l'expression des gènes histones en dehors de la phase S (Xu *et al.*, 1992; Blackwell *et al.*, 2004). Ces facteurs joueraient donc un rôle dans le contrôle du cycle cellulaire, en bloquant le passage en phase proliférative. Ce rôle pourrait être conservé chez le poulet (Ahmad *et al.*, 2005). Chez A.thaliana (et probablement

chez le maïs), le produit de l'orthologue de *Hira*, *Asymetric Leaves 2*, participe à la répression du gène knox, et par ce biais contrôle la détermination cellulaire des feuilles (Phelps-Durr, 2005). Ce gène joue un rôle dans le maintien des divisions cellulaires et d'un état indifférencié, impliquant HIRA dans l'engagement dans une voie de différenciation, ou de sortie de phase proliférative. Bien que les rôles montrés chez ces espèces ne soient pas tout à fait comparables (du fait même de la biologie des systèmes modèles), HIRA semble contrecarrer la phase proliférative du cycle par la répression de certains gènes. De façon étonnante, chez la souris, HIRA pourrait jouer un rôle contraire. En effet, la mutation du gène *Hira* dans des cellules souches embryonnaires entraine l'apparition accélérée de colonies présentant des caractéristiques de la différenciation (Meshorer *et al.*, 2006). J'ai montré que HIRA a chez la drosophile un rôle dans le maintien de l'équilibre de l'hétérochromatine à l'échelle du noyau. Il sera intéressant d'étudier si ceci contribue à la régulation fine de gènes cibles, en particulier en lien avec le cycle cellulaire et le programme de différenciation.

De façon intéressante, le facteur de remodelage Chd1 a, lui aussi, un rôle dans la différenciation des cellules souches embryonnaires qui semble contraire à celui de HIRA. L'absence de ce facteur n'induit pas la différenciation, mais réduit le potentiel de différenciation de ces cellules (Gaspar-Maia *et al.*, 2009). De plus, Chd1 y semble requis pour empêcher la formation de foyers d'hétérochromatine, et semble donc de fait associé au maintien d'un état ouvert de la chromatine. Or, si la nature de l'interaction fonctionnelle entre HIRA et Chd1 n'est pas encore élucidée, l'altération du profil d'expression des gènes ne semble pas être en cause dans les phénotypes liés à la différenciation (Persson et Ekwall, 2010; Gaspar-Maia *et al.*, 2011). En effet, l'absence de Chd1 ou de HIRA n'affecte pas de façon globale le profil de transcription associé à l'état indifférencié dans les cellules souches embryonnaires (Gaspar-Maia *et al.*, 2009; Goldberg *et al.*, 2010). De plus, HIRA contrôle l'assemblage de H3.3 dans un grand nombre de gènes dans les cellules souches embryonnaires de souris, mais ceux-ci peuvent être aussi bien actifs que réprimés. Ainsi, l'assemblage de H3.3 par HIRA n'est pas nécessairement lié à une activation de l'expression.

HIRA, H3.3, l'établissement de l'hétérochromatine et la reproduction chez les mammifères

L'établissement de l'hétérochromatine semble être une fonction importante de la voie HIRA/H3.3 au cours de la reproduction. J'ai parlé précédemment du rôle de ce duo dans l'inactivation du chromosome sexuel en préparation à la méiose chez la souris (Orsi *et al.*, 2009). Ce processus n'a pas d'équivalent connu chez la drosophile, mais H3.3 joue un rôle critique au cours de la méiose mâle dans ce modèle, dont les bases ne sont pas connues et pourraient être analogues.

Un autre lien remarquable entre H3.3 et l'organisation des chromosomes après la fécondation a été mis à jour récemment. Le pronoyau mâle est, chez la souris, actif et doit en conséquence établir adéquatement des profils de transcription (activation et répression) rapidement après la fécondation (Aoki *et al.*, 1997). Or, certaines voies qui jouent ce rôle dans le pronoyau femelle, ne semblent pas être capables de réprimer la chromatine paternelle (Arney *et al.*, 2002; Santos *et al.*, 2005; Van Der Heijden *et al.*, 2005). Par exemple, le pronoyau femelle accumule H3K9Me mais pas le pronoyau mâle. Ceci est peut être lié au fort enrichissement en H3.3 du pronoyau mâle, et le faible niveau de corrélation entre cette histone et les marques répressives (Loyola *et al.*, 2006). Quel que soit le cas, le pronoyau mâle doit gérer par des voies alternatives le problèmes crucial de la mise en place de territoires dans la chromatine, comme l'hétérochromatine péricentromérique.

Récemment, un mécanisme a été mis à jour par lequel une forte transcription séquentielle de transcrits sens, puis anti-sens à partir de ces territoires est essentielle au développement (Probst *et al.*, 2010). Ces transcrits semblent être importants pour la mise en place de ce type d'hétérochromatine. De façon remarquable, l'expression d'une version mutée de H3.3 sur la lysine 27 est capable d'empoisonner ce processus en dérégulant l'expression de ces transcrits, ce qui conduit à l'établissement de MPTs aberrantes et à l'arrêt du développement (Santenard *et al.*, 2010). Par ailleurs, il a été montré que la répression de ces transcrits dépend de l'adressage aux péricentromères du complexe PRC1, appartenant à la famille polycomb de répresseurs (Puschendorf *et al.*, 2008). Or, de façon conservée, ce complexe est recruté sur la chromatine par son interaction avec les protéines de type Polycomb, elles-mêmes recrutées à travers la méthylation de la lysine 27 de H3 (Bernstein *et al.*, 2006; Boyer *et al.*, 2006). Ainsi, nous pouvons imaginer un modèle par lequel l'histone H3.3 interagisse, par la méthylation de sa lysine 27 et des protéines Polycomb, avec le complexe PRC1 pour établir un mécanisme de répression des transcrits péricentriques paternels, se substituant à la methylation de la lysine 9

de H3. Cette hypothèse porte, par ailleurs, l'écho d'une possible interaction fonctionnelle entre H3.3 et les protéines du groupe Polycomb.

Chez la drosophile, des études remarquablement résolutives sur la dynamique de substitution de H3.3 ont montré que son renouvellement est particulièrement rapide aux éléments de réponse aux protéines Polycomb (Mito *et al.*, 2007; Deal *et al.*, 2010). Les auteurs ont proposé que ce renouvellement n'est pas simplement une conséquence de la régulation de ces éléments mais qu'il y participe proprement. Cette idée est par ailleurs étayée par le rôle de HIRA dans la régulation d'un élément insulateur sous contrôle du facteur GAGA (appartenant à la famille trithorax) évoqué plus tôt (Nakayama *et al.*, 2007). Dans ce modèle, HIRA et H3.3 facilitent l'activation d'un gène contrôlé par GAGA *in vivo* dans un système transgénique. La drosophile amène la possibilité unique d'étudier fonctionnellement *in vivo* l'interaction entre la voie HIRA/H3.3 et la voie Polycomb/Trithorax.

L'association de HIRA/H3.3 à la chromatine inactive qui dégage de cette section est cependant largement contredite par un nombre de données dans la littérature. Dans le prochain paragraphe, je traiterai du lien entre HIRA/H3.3 et l'activation de la transcription.

Données fonctionnelles impliquant HIRA et H3.3 dans la promotion de la transcription

Plusieurs études ont montré ces dernières années que, de façon conservée, H3.3 est enrichi en MPTs habituellement associées à la chromatine active (Hake et Allis, 2006). De plus, ces MPTs décorent l'histone avant même que le nucléosome soit assemblé, ce qui suppose que la machinerie responsable de l'assemblage de H3.3 participe indirectement mais de fait à la facilitation de la transcription (Loyola *et al.*, 2006; Loyola et Almouzni, 2007). Par ailleurs, certains marqueurs de l'hétérochromatine, comme H3K9Me ou HP1 sont appauvris sur les nucléosomes contenant H3.3, reflétant que, au sein de la chromatine, H3.3 répond à une territorialisation de la chromatine active (Loyola *et al.*, 2006). Cette idée a été reprise et renforcée par plusieurs études récentes montrant que les propriétés intrinsèques des nucléosomes assemblés avec H3.3 *in vivo* sont de nature à rendre la chromatine accessible. En effet, la présence de H3.3 est anti-corrélée à la présence de l'histone de liaison H1, qui participe à la compaction des chromosomes, suggérant que H3.3 se localise globalement dans

des régions « relâchées » (Braunschweig *et al.*, 2009). De plus, des nucléosomes contenant H3.3 présentent une résistance à l'extraction par sels relativement faible, suggérant que leur association à l'ADN *in vivo* est labile (Jin et Felsenfeld, 2007; Henikoff *et al.*, 2008; Henikoff, 2009; Jin *et al.*, 2009; Thakar *et al.*, 2009). Ensemble, ces études ouvrent la possibilité théorique que H3.3 soit un facteur favorisant la transcription.

Ce rôle pourrait passer par la dynamique même d'échange des nucléosomes. Le renouvellement rapide de nucléosomes par le mode IR pourrait favoriser l'accessibilité à des facteurs de régulation sur certains loci. Cette dynamique semble conservée et a été décrite chez la drosophile et la levure (Mito *et al.*, 2005; Dion *et al.*, 2007; Mito *et al.*, 2007; Deal *et al.*, 2010). Aussi, un renouvellement important de H3.3 a lieu sur certains insulateurs, et ce avant même que la transcription ne soit effective chez le poulet (Jin et Felsenfeld, 2006). Dans ce contexte, H3.3 pourrait préparer la chromatine à une activité d'expression ultérieure au cours du développement.

Chez la drosophile et chez *T.thermophila* le rôle de H3.3 lui même dans la régulation de la transcription paraît être mineur (Cui *et al.*, 2006; Hodl et Basler, 2009; Sakai *et al.*, 2009). Au contraire, une étude a récemment montré que H3.3 est nécessaire pour l'activation des gènes contrôlés par la voie interféron en modèle humain, ouvrant la possibilité de ses rôles *in vivo* dans la transcription (Tamura *et al.*, 2009). Dans certains systèmes, HIRA jouerait aussi ce rôle. Par exemple, une étude a montré que HIRA intéragit avec le facteur de transcription Pax3, bien que les bases fonctionnelles de cette interaction ne soient pas connues (Magnaghi *et al.*, 1998). Plus récemment, il a été montré que lors de l'angiogénèse, la différenciation de cellules endothéliales est accompagnée de l'incorporation d'histones H3K56Ac sur certains gènes clés, mécanisme qui dépend de HIRA (Dutta *et al.*, 2010). De façon similaire, l'invalidation de *Hira* dans des myoblastes empêche l'assemblage de H3.3 sur les régions régulatrices et géniques du gène maître MyoD et fait échouer la différenciation en muscle squelettique (Yang *et al.*, 2011). Ces exemples montrent un rôle pour HIRA/H3.3 dans l'activation de gènes dans des lignées cellulaires, mais aussi, par ce biais, dans la promotion de la différenciation, ce qui semble contraire aux résultats observés dans les cellules souches embryonnaires (Meshorer *et al.*, 2006). Cette contradiction apparente révèle probablement la complexité des situations biologiques faisant appel à la voie HIRA/H3.3.

L'exemple du gène MyoD est particulièrement intéressant car H3.3 a été impliqué dans son activité mais aussi dans sa capacité à conserver une « mémoire épigénétique » en absence de toute transcription (Ng et Gurdon, 2008). Lors de la transplantation nucléaire dans l'embryon de xénope, la conservation aléatoire de la capacité du gène MyoD à être transcrit 24 cycles de division plus tard corrèle avec la présence de H3.3 à son promoteur. De plus, la surexpression ou une mutation de H3.3 augmente ou réduit cette capacité de MyoD à conserver une mémoire. Cette étude pose des questions intéressantes sur l'existence même et la plasticité du système de mémoire épigénétique. En particulier, elle renvoie à la question capitale de la pérennité d'un profil de distribution du variant d'histone H3.3 au fil des divisions cellulaires, question pour laquelle les approches *in vivo* manquent cruellement.

H3.3 : marqueur épigénétique ?

Car le nœud de la question est bien là. Est ce qu'un système actif pour pérenniser la marque épigénétique H3.3 est mis en place ? Si la cellule investit pour copier le « code barre de H3.3» (Hake et Allis, 2006), nous ne pouvons qu'imaginer que ceci jouera un rôle, sinon essentiel, au moins amplement bénéfique dans la régulation des profils de transcription.

Les attentes dans le domaine des variants d'histone ont été exacerbés par la possibilité théorique que ces marques puissent être transmises entre générations de cellules (Probst *et al.*, 2009; Kaufman et Rando, 2010). Lors de la réplication de l'ADN, le mode de distribution des nucléosomes sur les deux chromosomes résultants n'est encore pas élucidé. Les modèles les plus courants sont (1) la répartition au hasard des nucléosomes parmi les chromatides filles (aléatoire), (2) la préférence de tous les nucléosomes pour l'une des chromatides filles (asymétrique ou conservatif), (3) la répartition de chaque nucléosome par division des tétramères H3-H4 (semi-conservatif) ou (4) une combinaison des précédents, territoire par territoire. Une remarquable étude récente a mis en évidence qu'un certain pourcentage des nucléosomes contenant H3.3 (mais pas H3 canonique) pourraient être scindés au moment de la réplication, chaque chromatide fille héritant d'un dimère de H3.3-H4, portant une information épigénétique sous la forme de variants d'histone et de MPTs (Xu *et al.*, 2010). Si ce demi tétramère était complété par un nouveau dimère, les marques codées par le variant lui même et ses MPTs pourraientt être « copiées » dans la nouvelle moitié du nucléosome, ou sur

des nucléosomes avoisinants, permettant ainsi de restaurer dans les deux cellules filles le profil local de variants et MPTs de la cellule mère (au moins en ce qui est de H3-H4) (Ray-Gallet et Almouzni, 2010). Des preuves manquent pour rendre compte de ce modèle *in vivo*, mais il est tentant d'imaginer que les les variants d'histone, et H3.3 en particulier, puissent être des supports majeur d'information héritable.

Cependant, les expériences fonctionnelles tendent à montrer que H3.3 est critique pour d'autres fonctions biologiques ponctuelles (revu dans Orsi *et al.*, 2009; Elsaesser *et al.*, 2010; Szenker *et al.*, 2011). Le débat entre des rôles épigénétiques et structuraux pour l'histone H3.3 ne fait probablement que commencer. Pour l'heure, la fonction cruciale de H3.3 dans la reproduction remet le projecteur sur son importance (parfois négligée) et sur son rôle en tant que contrainte évolutive pour la conservation de ce variant et ses facteurs associés. La posture à laquelle m'amènent nos travaux est d'imaginer le variant H3.3 avant tout comme une histone structurante, essentielle pour l'organisation et la stabilité de la chromatine.

De même, l'implication de HIRA dans des voies ayant parfois des conséquences inverses pour le destin cellulaire traduit la grande complexité des ses rôles dans différentes espèces modèles. Ces fonctions, apparemment contradictoires, pourraient être réconciliées si nous considérons HIRA comme un simple ouvrier de l'assemblage IR de la chromatine. Je vois HIRA comme un pion dans la machinerie d'assemblage utilisant le substrat H3.3 dans une diversité de situations, plutôt que comme ayant un rôle direct dans les décisions épigénétiques.

Suite des travaux

Notre compréhension sur les rôles *in vivo* des mécanismes d'assemblage de la chromatine impliquant H3.3 ont été largement bouleversés au cours de cette thèse. Des travaux devront à l'avenir évaluer la portée réelle de ses fonctions liées à la transcription, qui devraient passer par une modulation fine au carrefour d'un grand nombre de voies de régulation. Des modèles comme la drosophile ou T.thermophila qui admettent un certain degré de survie des individus dépourvus de variants de type H3.3 sont des terrains de chasse privilégiés pour la recherche d'interacteurs fonctionnels.

Le rôle hypothétique de H3.3 dans la modulation de la transcription n'est vraisemblablement pas indispensable pour la survie de toutes les cellules, mais pourrait avoir une incidence sur le fitness des individus. Ceci pourrait expliquer que les espèces à développement plus lent et à nombre de cellules plus élevés (où, de plus, l'apport maternel en H3.3 devient rapidement négligeable) souffrent d'avantage de son absence. Ainsi, une piste prometteuse pour comprendre les fonctions de ce variant est de tester en son absence la résistance du système à différents stress. Cette approche pourrait tester directement la plasticité des chromosomes et de l'intégrité du matériel génétique, mais aussi la sensibilité de l'individu à toute autre sorte de challenges. Par exemple, il serait intéressant de déterminer si la voie H3.3 a un rôle dans la résistance à la température ou au vieillissement, ou d'évaluer si elle a une incidence sur le comportement. L'importance fonctionnelle de l'utilisation de H3.3 dans d'autres processus que ceux liés à la reproduction est au cœur du problème.

Si la question des rôles de H3.3 est mystérieuse, celle des rôles des différents facteurs impliqués dans son assemblage, asservis à des fonctions biologiques spécifiques, est tout aussi excitante. En effet, nous réalisons à peine que deux machineries d'assemblage différentes sont capables d'intégrer le même variant d'histone en fonction de contextes spécifiques. Ceci est une première en matière de variants d'histone et ouvre une perspective intéressante sur l'assujettissement de chacune de ces voies à un sous-groupe des fonctions de H3.3. Derrière cette intrigue se cachent en plus les fondements de l'évolution (et la conservation) de ces deux machineries, dont le dialogue fonctionnel sera passionnant à explorer. Ainsi, définir les degrés de redondance et complémentarité des voies ATRX et HIRA dans différentes situations biologiques est un défi important pour la suite.

Du point de vue moléculaire, les travaux sur ATRX et HIRA posent aussi des questions de fond sur le mode de reconnaissance des régions à assembler. Comment et quand ces complexes reconnaissent la cible ADN de l'assemblage pour le diriger ? Mon hypothèse est que le complexe HIRA, prenant en charge de façon pangénomique l'assemblage dans le pronoyau mâle, reconnaît un motif très fréquent, voire la molécule d'ADN nue en elle même. Aucune preuve ne permet pour l'heure d'étayer ces idées qui doivent être testées par des approches biochimiques, notamment. Cependant, le cas du noyau mâle mène à penser qu'un des membres du complexe HIRA pourrait lier l'ADN *in vivo* avec une très faible, voire nulle, spécificité de séquence.

Finalement, ma proposition sur le lien circulaire entre l'organisation de la chromatine nucléolaire et la stabilité de l'hétérochromatine à l'échelle du noyau s'inscrit dans un contexte dans lequel la chromatine est de plus en plus considérée comme le produit d'une modulation dynamique générale. Des perturbations (ou simplement des variations temporelles) de certaines structures entrainent un rééquilibrage globale qui remobilise toute la batterie de facteurs impliqués dans la structure des chromosomes. Le rôle de H3.3 lui même dans ce genre d'équilibre est encore une voie de recherches intéressante pour cerner l'étendue de ses fonctions. Par exemple, cette balance s'appuie sur la distribution tridimensionnelle au sein du noyau des différents territoires fonctionnels. H3.3, ou les complexes HIRA ou ATRX pourraient avoir un rôle dans cet équilibre spatial mais celui-ci reste pour l'heure totalement inconnu. De façon plus générale, un défi conceptuel majeur est de cerner comment H3.3, substrat d'une pléthore de processus au cœur de la dynamique de la chromatine, répond à tous les appels et joue sur tous les fronts des rôles apparemment si disparates.

DEUXIEME PARTIE

Organisation et intégrité de la chromatine parentale à la fécondation

Elle était infiniment stérile. Ça faisait partie de l'extase.

Emil Cioran. *Le mauvais démiurge*

Chez les espèces à reproduction sexuée, les chromosomes dans les gamètes subissent des remaniements intenses mais doivent s'adapter à cette contrainte pour assurer la réussite de la formation du zygote. De plus, le passage obligatoire par la fécondation et par le stade une cellule expose ces chromosomes à l'effet d'attaques à comportement parasitaire. Ces problématiques, au cœur de l'activité de notre laboratoire, m'ont amené à étudier une diversité de situations où l'intégrité des génomes parentaux est compromise, entrainant des conséquences catastrophiques à la fécondation. Dans ce paragraphe je parlerai de ces modèles qui sont extrêmement informatifs sur la complexité de l'intégration des chromosomes parentaux lors de la formation du zygote.

I. L'incompatibilité cytoplasmique liée à *Wolbachia* est liée à un défaut d'assemblage IR dans le pronoyau mâle

Nos travaux sur les protéines HIRA et Yem sont d'autant plus surprenants qu'il n'existe qu'un nombre limité d'aberrations qui affectent exclusivement les chromosomes paternels à la fécondation (et épargnent les chromosomes maternels). La bactérie intracytoplasmique *Wolbachia* est capable de manipuler ces chromosomes en contexte d'incompatibilité cytoplasmique (IC). Ces bactéries sont très largement répandues parmi les arthropodes (environ 60% des espèces d'insectes) et les nématodes, et sont toujours intra-cytoplasmiques. Elles sont capables d'infecter le cytoplasme de l'œuf, et c'est par ce moyen qu'elles assurent leur transmission maternelle à une nouvelle génération d'individus hôtes. En plus de cette stratégie, *Wolbachia* a développé une diversité de mécanismes qui lui permettent de manipuler la reproduction de son hôte pour favoriser la colonisation d'une population (revu dans Werren *et al.*, 2008). Elle est chez certaines espèces, capable d'éliminer les mâles ou de

les féminiser, ce qui fait augmenter le nombre de femelles contaminées dans une population. De plus, elle peut conduire les femelles de certaines espèces à pratiquer la reproduction par parthénogénèse. Finalement, elle peut contre-sélectionner les œufs non-infectés à travers l'IC.

Du point de vue cytologique, l'IC est un défaut qui affecte exclusivement les chromosomes paternels au moment de la fécondation et entraine leur exclusion des noyaux du zygote. Il paraissait donc pertinent de supposer que les rares voies exclusivement dévouées à la biologie des chromosomes paternels, comme HIRA/H3.3, pourraient être des cibles privilégiées de la manipulation par *Wolbachia*. Bien que le phénomène de l'IC soit connu depuis des décennies, et que ce modèle soit extensivement étudié du point de vue de la biologie des populations, les mécanismes cytologiques en jeu ont reçu relativement peu d'attention (Serbus *et al.*, 2008). Nous nous sommes interrogés sur la possibilté que l'assemblage de la chromatine paternelle par HIRA et H3.3 soit perturbé en contexte incompatible.

D'autres espèces de drosophile se prêtent mieux que D.melanogaster à l'étude de l'IC, essentiellement parce que Wolbachia y génère un taux d'incompatibilité plus élevé ; mais c'est chez D.melanogaster que sont disponibles la plupart des outils moléculaires. Le modèle choisi a donc été de générer un croisement hybride entre des mâles de l'espèce D.simulans et des femelles de l'espèce D.melanogaster. Ce croisement génère une quantité d'œufs fécondés beaucoup plus faible que celle qu'on attend pour un croisement intraspécifique, mais ce modèle répond de façon fiable aux lois de l'incompatibilité cytoplasmique (Ferree et Sullivan, 2006). Nos collaborateurs ont mis en évidence que lors de ce type de croisement, l'histone H3.3 présentait des défauts de distribution dans le pronoyau mâle en contexte incompatible. Nous avons montré que ce problème est indépendant de l'enlèvement des protamines dans ce noyau. Le modèle est donc que l'assemblage IR de la chromatine est affecté en contexte incompatible, retardant (ou empêchant partiellement) la réplication des chromosomes paternels et causant in fine leur exclusion de la première division zygotique. Ces résultats constituent une avancée dans notre compréhension des mécanismes de l'IC et une piste intéressante pour comprendre comment la bactérie Wolbachia manipule les gamètes mâles. L'article qui suit détaille ces résultats.

Wolbachia-Mediated Cytoplasmic Incompatibility Is Associated with Impaired Histone Deposition in the Male Pronucleus

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Abstract

Wolbachia is a bacteria endosymbiont that rapidly infects insect populations through a mechanism known as cytoplasmic incompatibility (CI). In CI, crosses between *Wolbachia*-infected males and uninfected females produce severe cell cycle defects in the male pronucleus resulting in early embryonic lethality. In contrast, viable progeny are produced when both parents are infected (the Rescue cross). An important consequence of CI–Rescue is that infected females have a selective advantage over uninfected females facilitating the rapid spread of *Wolbachia* through insect populations. CI disrupts a number of prophase and metaphase events in the male pronucleus, including Cdk1 activation, chromosome condensation, and segregation. Here, we demonstrate that CI disrupts earlier interphase cell cycle events. Specifically, CI delays the H3.3 and H4 deposition that occurs immediately after protamine removal from the male pronucleus. In addition, we find prolonged retention of the replication factor PCNA in the male pronucleus into metaphase, indicating progression into mitosis with incompletely replicated DNA. We propose that these CI-induced interphase defects in *de novo* nucleosome assembly and replication are the cause of the observed mitotic condensation and segregation defects. In addition, these interphase chromosome defects likely activate S-phase checkpoints, accounting for the previously described delays in Cdk1 activation. These results have important implications for the mechanism of Rescue and other *Wolbachia*-induced phenotypes.

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Introduction

Wolbachia are intracellular bacteria that infect some 65% of all insect species [1]. Their success is in large part due to their efficient maternal transmission and their ability to alter host reproduction such that infected females produce more offspring than uninfected females [2]. The most common form of altered reproduction is known as cytoplasmic incompatibility (CI), a form of conditional sterility resulting from crosses of *Wolbachia*-infected males to uninfected females [3]. These crosses produce defects in the first zygotic mitosis resulting in inviable embryos. Significantly, if both the female and the male are infected, no defects are observed and viable embryos are produced. This phenomenon is known as Rescue [4]. Consequently in *Wolbachia*-infected populations, infected females produce viable progeny whether they mate to infected or uninfected males. In contrast, uninfected females produce viable progeny only when mated to uninfected males. Thus infected females enjoy a tremendous selective advantage over uninfected females resulting in the rapid spread of *Wolbachia* via the maternal lineage [5]. The success of this strategy is underscored by the fact that CI has been documented in every insect order [3].

CI crosses produce embryos in which the paternal chromosomes are improperly condensed when aligned at the metaphase plate of the first mitotic division following fertilization [6–8]. It should be noted that the first mitotic division is unique in many insects, including Drosophila, because the paternal and maternal chromosomes reside on separate regions of the metaphase plate and are independently regulated with respect to entry into anaphase [7,9]. As the embryo progresses into anaphase, paternal sister chromatids either fail to segregate, or exhibit extensive bridging and fragmentation during segregation, a hallmark of damaged or incompletely replicated chromosomes [9]. It is thought that strong CI elicits chromosome condensation defects severe enough to activate the spindle assembly checkpoint and prevent segregation while weak CI results in more mild defects in which the checkpoint fails to activate, allowing improper segregation [8]. Defects earlier in the cell cycle at the prophase/metaphase transition have also been reported. These include a delay in Cdk1 activation and nuclear envelope breakdown in the male pronucleus relative to the female pronucleus [10].

These observations leave unresolved the cause and effect relationship between the chromosome condensation and Cdk1 activation defects in CI embryos. It is well established that defects in DNA replication and chromosome condensation lead to cell cycle checkpoint induced delays in Cdk1 activation [11]. However Cdk1 activation is required to drive chromosome condensation and failed Cdk1 activation results in failed chromosome

Author Summary

Wolbachia are among the most successful of all intracellular bacteria, infecting an estimated 65% of insect species. *Wolbachia* are also present in filarial nematodes and are the cause of African river blindness. *Wolbachia*'s success is due in part to its ability to induce a conditional form of sterility known as cytoplasmic incompatibility (CI), endowing infected females with a tremendous selective advantage. CI results in the severe reduction in progeny from crosses between uninfected females and *Wolbachia*-infected males. However, *Wolbachia*-infected females can mate with either infected or uninfected males with no reduction in progeny. CI may drive speciation and is intensively being pursued as a means to control insect-borne human disease. In spite of its biological and medical significance, the molecular basis of CI is not understood. We take advantage of newly generated chromatin reagents to demonstrate that prior to the well-documented defects in chromosome condensation and segregation, CI produces a delay in recruiting the replication-independent histone H3.3/H4 complex to the male pronucleus. There is great interest in histone H3.3 because of its general role in transcription and in remodeling of the sperm chromatin following fertilization. In addition, these findings may provide insight into other *Wolbachia*–host interactions such as CI–Rescue and male-killing.

condensation [12]. To identify the proximal defects in CI embryos, we sought to determine whether CI-induced chromatin defects occur prior to Cdk1 activation during the interphase/prophase transition. Identification of earlier chromatin defects, during the sperm to male pronucleus transformation, would strongly argue that these are proximal to and the cause of the delayed Cdk1 activation and chromosome condensation/segregation defects observed during prophase and metaphase.

Based on this reasoning, the work presented here focuses on sperm formation and sperm transformation into the male pronucleus in normal and CI crosses. To facilitate a compact configuration, the sperm chromatin is packaged with specialized small basic proteins known as protamines [13]. Another unique property of the *Drosophila* sperm is that the nuclear envelope lacks lamins and nuclear pores [14]. Immediately following fertilization, the nuclear envelope, the plasma membrane and the protamines are removed, and *de novo* nucleosome assembly is initiated using maternally supplied core histones [15]. This nucleosome assembly occurs prior to DNA replication, and is executed by a replication-independent pathway that uses histone variant H3.3 and its specific chaperone HIRA [15]. In addition, the formation of the male pronucleus requires the ATP-dependent chromatin remodeling enzyme CHD1 [16]. After these remodeling events, the nucleus acquires a conventional nuclear envelope containing lamins and nuclear pores. As the egg completes meiosis, the newly formed male and female pronuclei initiate DNA replication while migrating towards one another. Once the replication is complete, Cdk1 activation triggers mitotic entry in the closely apposed pronuclei [17].

The studies presented here demonstrate CI- specific defects in H3.3/H4 deposition and prolonged retention of PCNA in the male pronucleus. These results suggests that in CI crosses, the male pronucleus enters mitosis with improperly condensed chromatin and incompletely replicated DNA. Significantly remodeling of the sperm chromatin including protamine removal and H3.3/H4 deposition occurs during interphase, well before Cdk1 activation and entry into mitosis. Thus our results suggest a model in which the initial defects in chromatin assembly in the male pronucleus activate cell cycle checkpoints delaying Cdk1 activation and mitotic entry. These chromatin remodeling defects also explain previous findings of defects during metaphase and anaphase in chromatin condensation and segregation. Because H3.3 deposition plays a key role in the transcriptional regulation throughout development, our results may provide insight into other effects *Wolbachia* has on its host.

Results

CI–Induced Defects Are Limited to Paternal Chromosomes

To confirm that the CI-induced segregation and condensation defects are limited to the paternal chromosomes, we used an antibody directed against acetylated histone H4 that preferentially labels the *de novo* assembled paternal chromatin after protamine removal in *Drosophila* eggs (Figure 1, [15]). We used *D. simulans* rather than *D. melanogaster*, since CI is very robust in the former species only. In CI embryos, the maternal chromosomes segregate normally at anaphase while the paternal chromosomes lag on the metaphase plate. At late telophase, bridges are observed between separating paternal sister chromosome complements (Figure 1, [7]). This results in severe nuclear division failures and accounts for the pre-cellular embryonic lethality in CI crosses. In stronger CI cases, severe disruption of paternal chromosome segregation results in their exclusion from both daughter nuclei. In haplo-diplo species this pattern of segregation produces viable haploid males [8]. The detection of acetylated histone H4 also demonstrates that sperm chromatin remodeling is initiated in CI crosses and this led us to examine protamine removal and histone deposition during this period.

Protamine Removal Appears Normal in CI Embryos

During spermatogenesis in many higher eukaryotes, including *Drosophila*, core histones in the sperm nuclei are replaced by protamines, sperm-specific chromosomal proteins that allow a greater chromatin compaction [18]. To assay protamine deposition and removal in CI embryos, we created a transgenic *D. simulans* stock expressing *D. simulans* protamine fused to GFP under the control of its endogenous promoter. In non-infected and infected testis, the fusion protein was incorporated into spermatids and present in mature sperm in seminal vesicles. (Figure 2A, 2B, and 2C). In both, control and CI fertilized embryos, Protamine-GFP was removed immediately after sperm entry, before completion of the female meiotic division (Figure 2, $n = 22$ for CI (D–H), $n > 20$ for control (J)). To verify that Protamine-GFP can be visualized in early *D. simulans* embryos, we took advantage of rare double fertilization events (Figure 2I, asterisk). In this case Protamine-GFP was visible in the additional, non-activated sperm DNA while absent from the male chromosomes lagging on the metaphase plate (arrow). Thus, at the cytological level, no obvious differences in protamine removal and deposition are observed in CI embryos.

CI Affects Histone Deposition in the Male Pronucleus

Immediately following the removal of protamines from the male pronucleus, paternal nucleosomes are assembled using maternally supplied histones. This replication-independent nucleosome assembly specifically involves the H3.3 histone variant, which is deposited along with H4, followed by H2A and H2B [19]. H3.3 is thus specifically deposited in the male pronucleus before the completion of the female meiosis and remains enriched in paternal chromosomes throughout the first mitotic division. The paternal

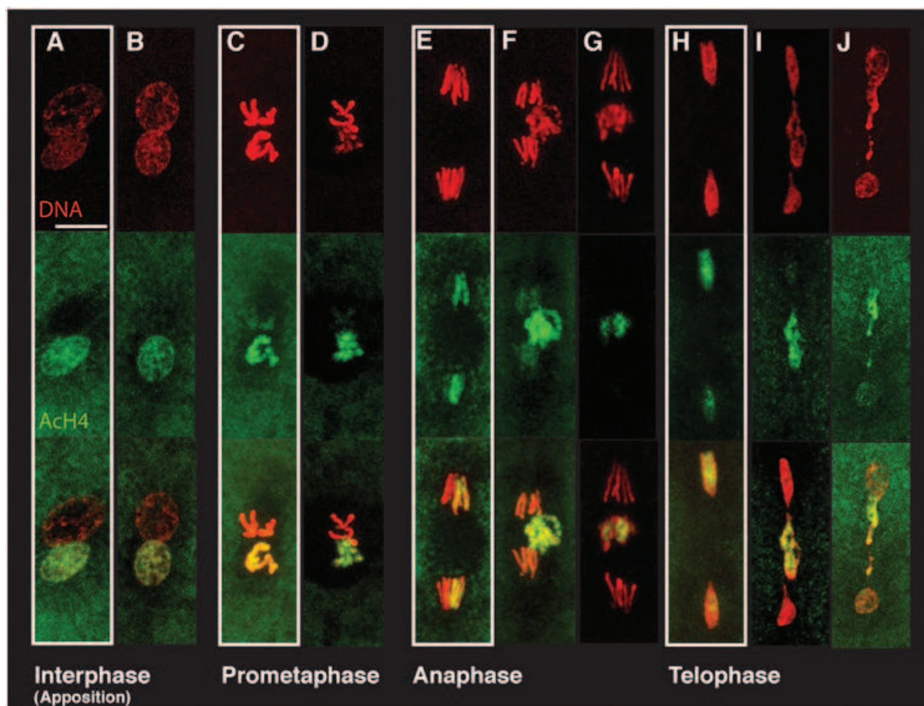

Figure 1. In *D. simulans* embryos from incompatible crosses (CI), paternal chromosomes fail to condense and improperly segregate during the first mitosis. (A,C,E,H) are uninfected controls in white boxes. (B,D,F,G,I,J) are CI embryos. Paternal, but not maternal chromosomes incorporate acetylated histone H4 during *de novo* nucleosome assembly (green). DNA is detected with propidium iodide (red). (A,B) pronuclear apposition. (C,D) prometaphase. (E,F,G) anaphase A (F) or B (E,G). (H,I) telophase. (J) late telophase/second S phase. Scale bar is 5 µm.
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chromosomes lose H3.3 by incorporation of canonical histone H3 with each new round of replication [20].

In order to take advantage of both the strong CI of *D. simulans* and of transgenic markers only available in *D. melanogaster*, we performed hybrid crosses between *D. simulans* males and *D. melanogaster* females. Previous studies demonstrated that this hybrid cross exhibits a robust CI and Rescue and is an appropriate system for studying CI [21]. Infected or non-infected *D. simulans* males were crossed with non-infected transgenic *D. melanogaster* females expressing a tagged H3.3-FLAG histone (CI and control crosses, respectively). In all embryos examined from the above control hybrid cross (n = 51), a robust H3.3 deposition was observed in the male pronucleus prior to completion of female meiosis, similar to the H3.3 deposition observed in single species *D. melanogaster* control crosses (not shown). All exhibited normal H3.3 deposition in the male pronucleus before the completion of female meiosis (n = 30, Figure 3A). However in hybrid CI crosses, 22% of the embryos exhibited an abnormal H3.3 accumulation at the periphery of the male pronucleus before the completion of female meiosis (n = 63, Figure 3A). In all nuclei with an abnormal accumulation at the periphery, no H3.3 staining was observed inside the nucleus suggesting a failure or an altered pattern of early H3.3 deposition. No lamin is detected at this stage (Figure S1), which suggests that nucleosome assembly occurs prior to the formation of the pronuclear envelope, ruling out a general nuclear import defect. Double immunostaining experiments showed that histone H4 colocalized with H3.3 in peripheral rings in CI embryos (Figure 3B). These abnormal rings of H3.3 and H4 are never observed during pronuclei apposition (Figure 3A′, n>30 for control and CI crosses). This suggests that CI results in a delayed, but not complete inhibition of H3.3/H4 nuclear deposition.

CI Affects Male Pronuclear DNA Replication

Once the paternal chromatin is assembled with maternally supplied core histones including H3.3 and H4, the DNA must replicate prior to mitotic entry in both pronuclei. We examined replication timing of pronuclei in control and CI embryos using an antibody directed against the *Drosophila* Proliferating Cell Nuclear Antigen (PCNA). PCNA is a conserved core component of the replication fork [22] and only present in S-phase nuclei [23]. To confirm this specificity in *Drosophila*, we examined PCNA localization in early embryos where the S-phase is well characterized with respect to chromosome and spindle morphology [24] (Figure S2). These studies demonstrate that PCNA is nuclear only during S-phase, confirming previous results. Early *D. simulans* embryos from uninfected and CI crosses were examined from the time of pronuclear migration to pronuclear apposition. In the uninfected crosses, both the male and female pronuclei exhibit robust PCNA staining during their migration, indicating that the S-phase is initiated during the early stages of pronuclei migration (Figure 4A, n>30). We always observed synchronous PCNA staining in both nuclei, indicating simultaneous S-phase initiation in the male and female pronuclei. During pronuclei apposition in the uninfected crosses, we either observe that both pronuclei possess (Figure 4A, "apposition I") or lack PCNA staining (Figure 4A, "apposition II"). S phase was completed during pronuclear apposition and not earlier. S phase was completed synchronously between male and female pronuclei in 88% of embryos (n = 26, Figure 3A and 3B). We performed the same analysis in embryos derived from the Rescue cross. The results for both pronuclear migration and apposition were very similar to the control cross (n = 27, Figure 4A and 4B).

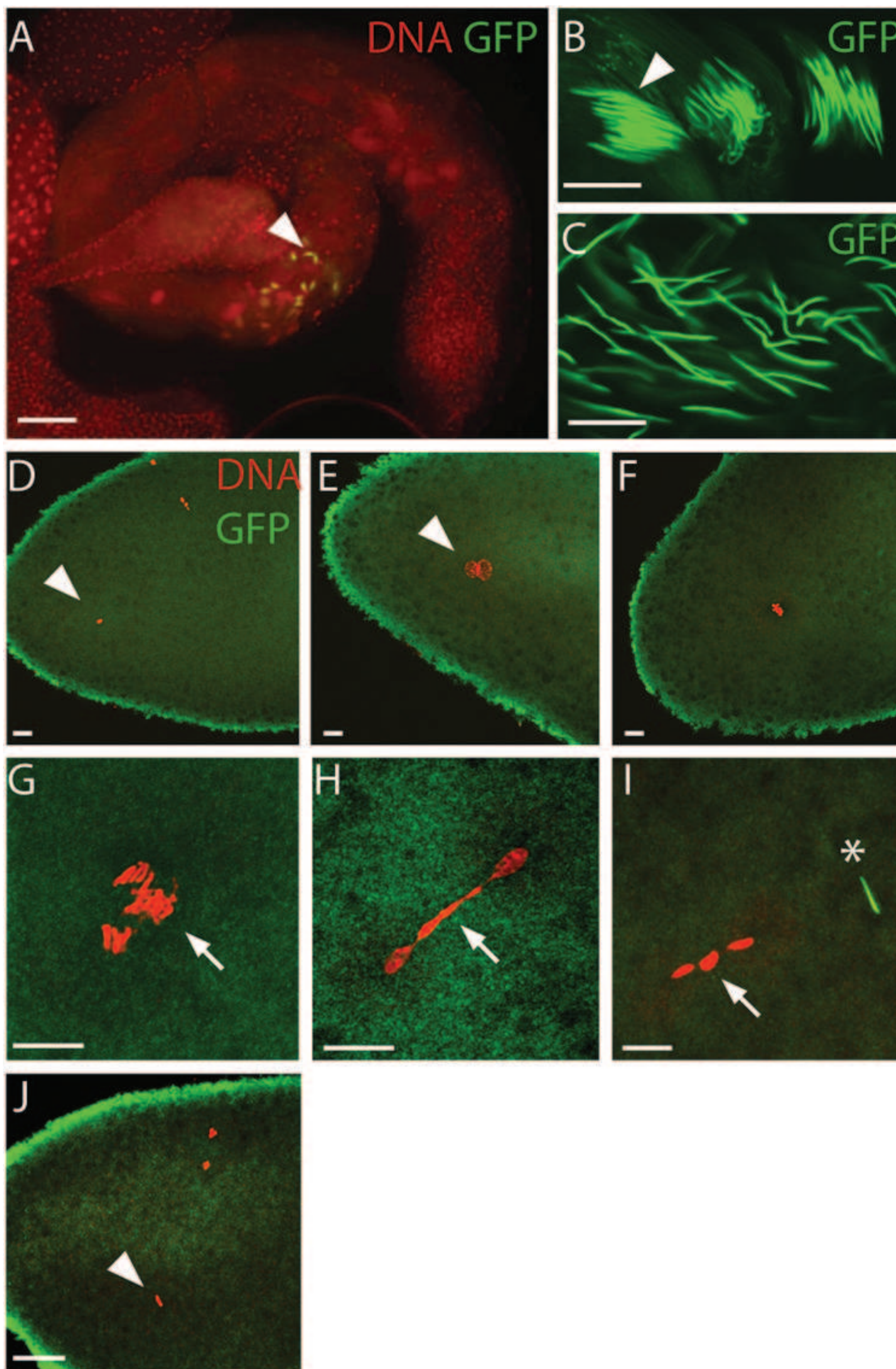

Figure 2. Protamine incorporation and removal appear normal in *D. simulans* CI crosses. (A,B,C) In infected *D.simulans* transgenic male testis, Protamine-GFP is detected in groups of late spermatid nuclei (arrowheads in A and B) and in sperm nuclei in seminal vesicles (C). (D,E,F,G,H) Confocal sections of embryos from non-infected females crossed with infected, transgenic males. Protamine-GFP is never detected in the male nucleus (arrowhead) as early as the second female meiotic division (D) or at the pronuclear apposition stage (E). (F,G,H,I) Cycle 1 embryos in metaphase (F), anaphase (G) or telophase (H,I). The embryos in G–I display an obvious CI phenotype with lagging paternal chromatids or chromatin bridges (arrows). No Protamine-GFP is detected in the late paternal chromatin. (I) embryo containing a second, non-activated sperm nucleus (asterisk) whose Protamine-GFP has not been removed serving as internal control for Protamine-GFP detection in embryos. (J) Embryo from non-infected females crossed with non-infected transgenic males. Protamine-GFP is never detected in the male nucleus (arrowhead) in this control. DNA is stained with propidium iodide (red) in all panels except B and C. GFP is detected either directly (A,B,C) or with the use of an anti-GFP antibody (green) (D,E,F,G,H,I,J). Scale bar is 50 µm in A and 10 µm in all other panels.
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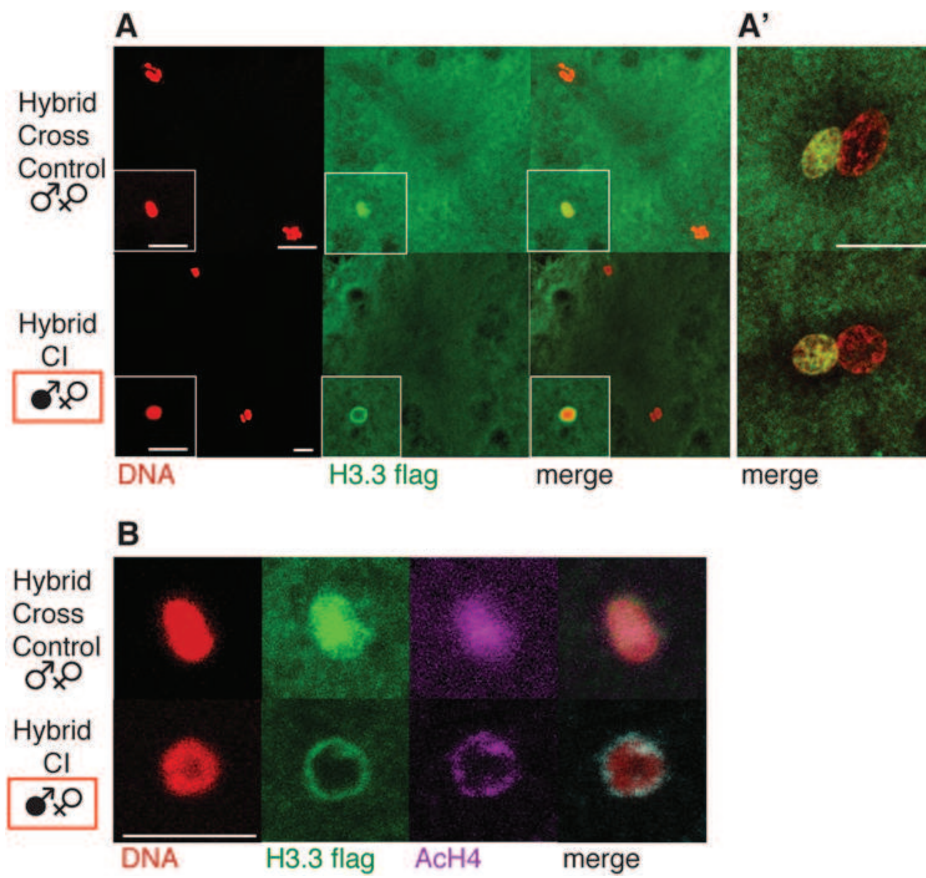

Figure 3. Histone variant H3.3 deposition is abnormal in CI *D. melanogaster* / *D. simulans* hybrid crosses. (A) Embryos from hybrid control (uninfected *D. melanogaster* females x uninfected *D. simulans* males) or CI (uninfected *D. melanogaster* females x infected *D. simulans* males) crosses were stained to reveal a tagged H3.3 (green) and DNA (propidium iodide in red), after sperm entry. The two female meiotic products are still in metaphase II, indicating that sperm entry just occurred (in white frame). (A′) H3.3 deposition is undistinguishable between embryos from hybrid control or CI crosses during pronuclear apposition. Note that the male pronucleus is always slightly smaller then the female pronucleus. (B) Acetylated histone H4 colocalizes with H3.3 in perinuclear rings in CI. Magnification of male pronuclei from hybrid crosses, acetylated H4 in purple. Scale bar is 10 µm.
doi:10.1371/journal.ppat.1000343.g003

Next, we analyzed PCNA staining in embryos derived from the CI cross. As with the control cross, both pronuclei stained positive for PCNA throughout migration (Figure 4A, n>30). Thus, like the control cross, S-phase is initiated simultaneously in the male and female pronuclei during the initial stages of pronuclear migration. Unlike the control crosses, however, we observed 43% of embryos (n = 36) with differential staining during apposition (Figure 4A and 4B). These results indicate that CI delays completion of replication in the male pronucleus. Because the timing of replication initiation does not appear to be altered in CI embryos, it is likely that the replication is slowed down or blocked in the male pronucleus of CI embryos relative to control embryos. Alternate interpretations include delayed release of PCNA or extra DNA replication in CI embryos. However delayed Cdk1 activation in the male pronucleus, presumably due to activation of cell cycle checkpoints, favors a model in which of disrupted replication in the male pronucleus of CI embryos.

CI Embryos Enter the First Zygotic Mitosis with Replication-Associated Defects in the Paternal Chromosomes

We also examined PCNA staining in control and CI *D. simulans* embryos that had progressed into prophase as evidenced by condensed DNA, spindle formation, and NEB. In control embryos, PCNA was never localized in the pronuclear DNA after NEB (n = 40, Figure 4C). In CI embryos however, 11% of pronuclei pairs observed after NEB showed a PCNA staining associated with the poorly and unevenly condensed male pronuclear DNA (n = 37, Figure 4C and 4D). Once the male pronuclei of CI embryos progress into metaphase, we no longer observe such PCNA staining.

It has been reported that PCNA is associated with damaged as well as replicating DNA (for a review see [25]). We favor a replication defect to explain CI rather than DNA breaks, given that chromatin remodeling defects are strongly associated with replication defects [26]. In addition, chromosome bridging during the first telophase but not free chromosome fragments is well documented in CI embryos. This is more consistent with DNA replication rather than damage defects. Taken together, our data suggest that in CI embryos DNA replication is slowed down or blocked in the male pronucleus.

Discussion

Genetic and cellular analyses indicate that CI specifically disrupts paternal chromosome condensation, congression and

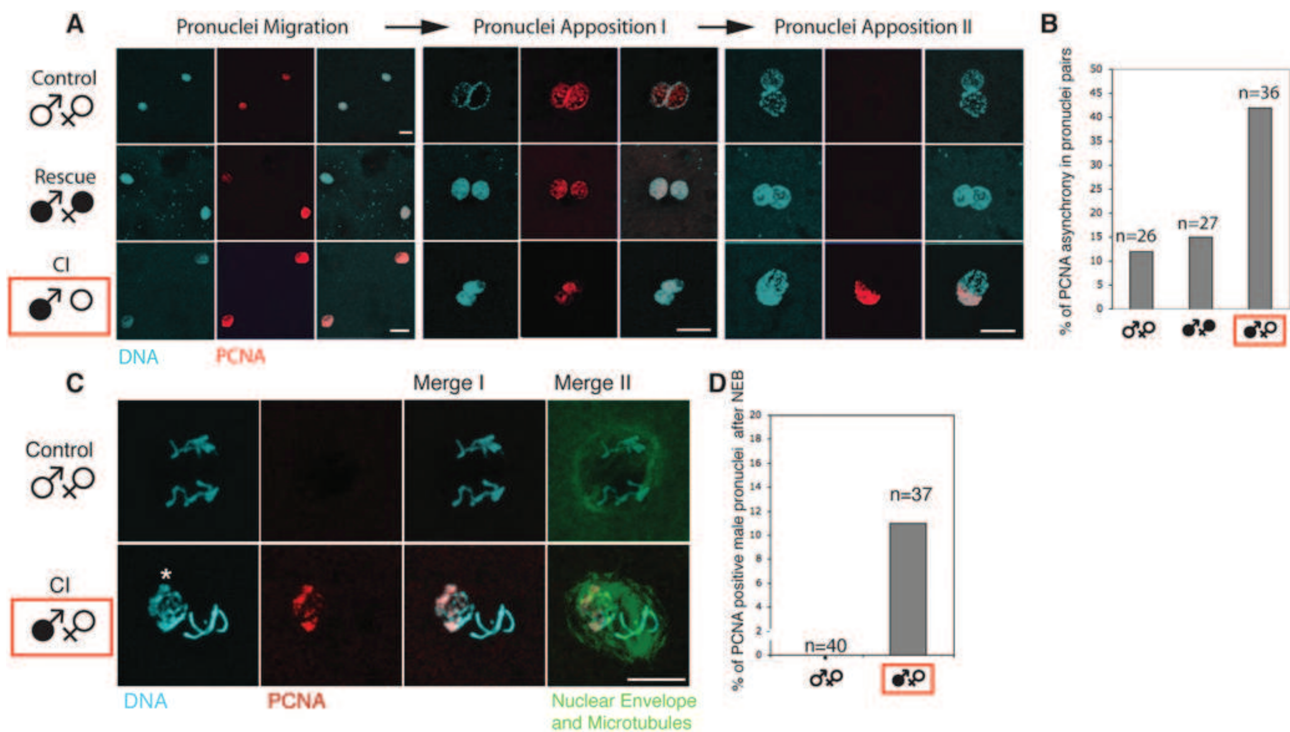

Figure 4. In *D. simulans*, replication of the male pronucleus is prolonged in CI embryo. (A) Embryos from control, rescue, or CI crosses were fixed and stained for PCNA (red), and DNA (propidium iodide, cyan). Scale bars are 10 µm. (B) Synchrony was scored when both apposed pronuclei were PCNA negative. Conversely, asynchrony was established when a pronucleus was PCNA positive whereas its counterpart was negative. (C) In CI embryo, PCNA is present in male pronuclear chromatin after pronuclear envelopes breakdown and spindle assembly. Embryos from control and CI crosses were fixed and stained for PCNA, and with two monoclonal antibodies, the anti-lamin ADL84 and an anti-tubulin to reveal the presence of the pronuclear envelopes and the spindle set up respectively (in green). The asterisk marks the uncondensed male pronucleus. Scale bar is 10 µm. Male pronuclei can be identified according to their smaller size compare to female pronuclei during apposition (A), or because of the chromosome condensation defects in CI (C). (D) % of PCNA positive male pronuclei after NEB in control crosses and CI crosses.
doi:10.1371/journal.ppat.1000343.g004

segregation [9,27]. Here we take advantage of anti-acetylated H4 histone antibodies that specifically stain the paternal chromosomes due to nucleosome assembly in the male pronucleus. This enabled us to directly demonstrate the effects of CI are limited to the paternal chromosomes. This implies that CI targets processes specific to the paternal chromosomes necessary for progression through mitosis.

To identify these processes, we focused on the chromosome remodeling events that are specific to sperm formation and transform the sperm into a male pronucleus. Our cytological examination of protamine deposition and removal did not reveal obvious abnormalities in CI embryos. This of course does not rule out more subtle defects. Protamines are normally removed immediately following fertilization and replaced with the replication-independent variant histone H3.3 and canonical H4, H2A/H2B histones. In CI embryos, a significant fraction of embryos exhibit delays in H3.3 incorporation before completion of the female meiosis. This results in an abnormal ring of H3.3 encompassing the male pronucleus. There is no nuclear envelope present at this early stage, indicating the H3.3 ring phenotype is not due to defects in nuclear import. More likely it is due to a delay in loading H3.3 onto the paternal chromosomes.

These CI-induced defects in H3.3 deposition are strikingly similar to those reported for mutants in the chromatin remodeling protein CHD1. Male pronuclei from *chd1* mutants also exhibit an improper accumulation of H3.3 around the male pronucleus. Like the CI-induced defects, chromosome condensation is severely disrupted presumably due to defects in H3.3-based chromatin remodeling [16]. Mutations affecting HIRA, the H3.3 chaperone, also prevent the formation of condensed paternal chromosomes [15]. These replication-independent histone deposition defects can explain the chromosome condensation and segregation defects observed in CI embryos since H3.3 and H3 share a conserved N terminal tail, whose phosphorylation is crucial for chromosome condensation [28]. Defects in histone deposition can also explain the delayed progression through S phase, as proper nucleosome assembly is required for DNA replication [29]. Both replication dependent and independent nucleosome assembly machineries share common interactors, like the histone chaperone ASF1 [19]. ASF1 siRNA knock down experiments and mutants clearly show DNA replication defects [26]. Late DNA replication in ORC2 (Origin Recognition Complex 2) mutants also provoke chromosome condensation defects and reveals that proper replication timing is crucial for the chromatin to be fully competent to condense [30]. However it should be pointed out that chromosome condensation defects alone can produce segregation defects [31].

In addition to playing a role in paternal chromatin remodeling, H3.3 plays a more general role in transcription regulation. The replication-independent deposition of H3.3 is correlated with active chromatin states [32]. This raises the intriguing possibility that *Wolbachia* may influence the transcription state of its host nuclei by altering H3.3 deposition. It has been shown that *Wolbachia* do not influence the *in vivo* expression level of

antimicrobial peptides specifically [33], but microarray data from *Drosophila* cell culture suggest that *Wolbachia* has some influence on host transcript levels [34]. Another alteration of the host reproduction caused by *Wolbachia* is a phenomenon called male killing (MK) [35]. In male killing, *Wolbachia* infection results in death of the male but not the female progeny. The resulting increase in the proportion of female progeny is beneficial to the maternally transmitted *Wolbachia*. Moving a specific *Wolbachia* strain from one *Drosophila* species to another results in an instantaneous transition from CI to MK, indicating that these *Wolbachia*-induced phenotypes share a common molecular mechanism [36]. Studies in *Drosophila* demonstrate that disruptions in some chromatin remodelers have a much greater impact on organization of the X chromosomes in males than females [37]. This raises the possibility that CI and MK evolved from *Wolbachia* having a more general effect on the transcriptional state of its host cell by regulating H3.3 deposition.

To determine whether CI influences replication we monitored for the presence of PCNA, an indicator of replicating DNA, in the male and female pronuclei. This analysis demonstrates that in normal embryos, both initiation and completion of DNA replication occur simultaneously in the two pronuclei. In CI embryos while we find replication is initiated simultaneously, completion of replication is significantly delayed in the male pronucleus. In fact we observe instances of PCNA positive paternal chromosomes during metaphase of the first zygotic division. It is likely that the chromatin remodeling defects described above are responsible for the replication delays of the male pronucleus (see Figure 5). These delays readily account for the extensive chromosome bridging observed during anaphase: segregation of unreplicated chromosomes creates bridges [38,39].

Delayed completion of replication of the paternal chromosomes provided an opportunity to more precisely determine the timing of CI rescue. Previous studies demonstrated that in the Rescue cross, the chromosome condensation defects at metaphase and segregation defects at anaphase are no longer observed [27]. Additional studies demonstrated that in CI crosses, activation of Cdk1, a highly conserved kinase that drives cells into mitosis [40] in the male pronucleus, is delayed relative to its activation in the female pronucleus [10]. These studies also demonstrated that in Rescue crosses, Cdk1 activation in the male and female pronuclei is synchronous. These studies raise the possibility that Rescue is

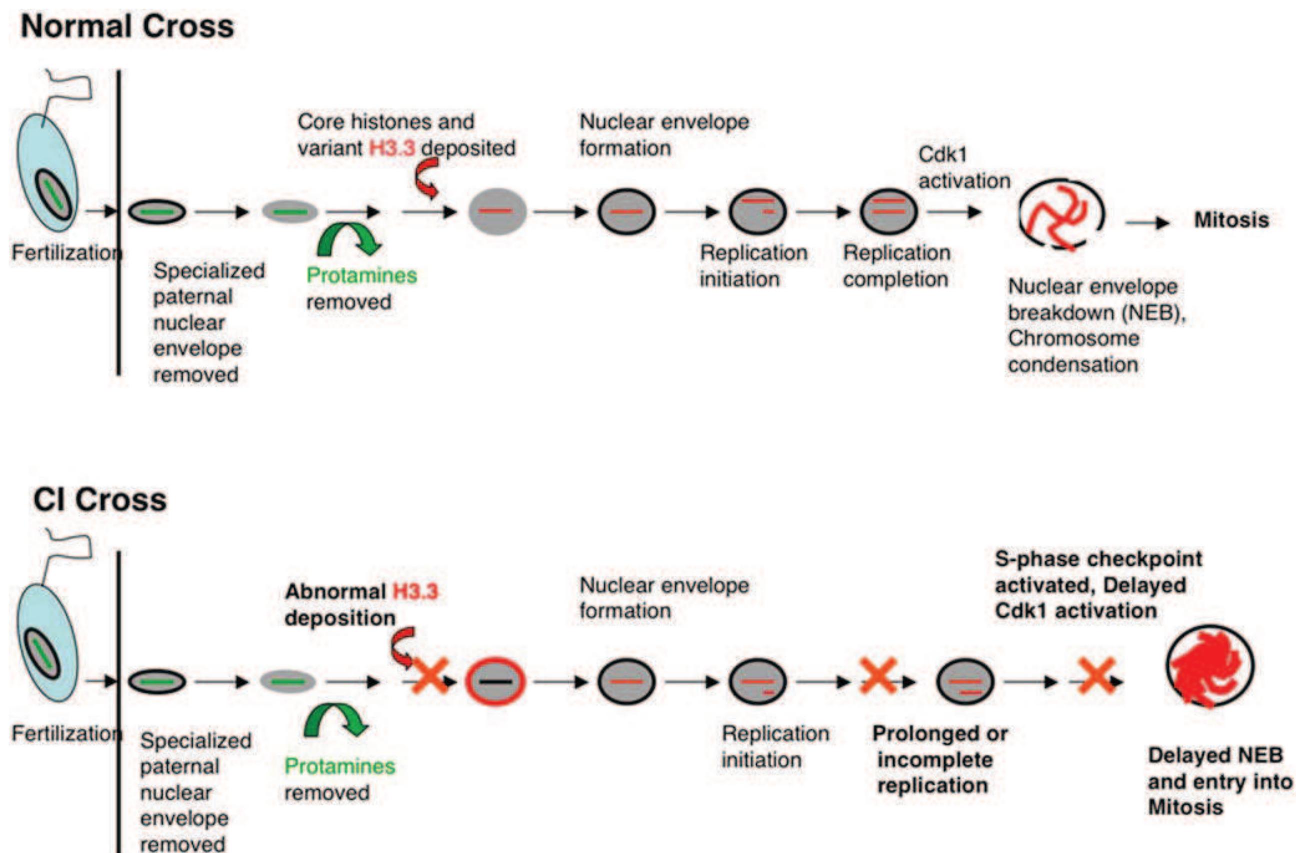

Figure 5. A schematic of key events in the transformation of sperm to male pronucleus in embryos from normal and CI crosses. Normal cross: Immediately following fertilization, the specialized nuclear envelope (lacking nuclear pores) of the male pronucleus is removed. Next, the protamines are removed and replaced by maternally supplied histones, including the replication-independent histone H3.3. This event is followed by lamin deposition and formation of a conventional nuclear envelope containing nuclear pores. Next, S-phase is initiated and upon completion, Cdk1 is activated driving nuclear envelope breakdown, chromosome condensation, and spindle assembly. CI cross: At the cytological level, removal of the sperm nuclear envelope and protamines appear normal. Often however, histone H3.3 deposition is abnormal, resulting in a ring of histone H3.3 encompassing the paternal pronucleus. This is the earliest documented CI phenotype in embryos and is similar to that observed for mutants in the chromatin remodeling protein Chd1. Imaging PCNA, a marker for replicating chromosomes, indicates that replication initiates normally in CI embryos, but is prolonged or incomplete. This may be a direct result of the earlier defects in H3.3 deposition. Replication delays activate S-phase checkpoints and thus are likely the cause of the previously described delays in Cdk1 activation and nuclear envelope breakdown.
doi:10.1371/journal.ppat.1000343.g005

achieved through correction of cell cycle defects in the male pronucleus. Alternatively, synchrony may be restored by a compensatory slowing of the female pronucleus cell cycle. Our data demonstrate that in Rescue crosses, we no longer observe a discordance in the state of PCNA staining in the male and female pronuclei, indicating the events mediating Rescue occur during interphase prior to Cdk1 activation during prophase. However, these studies do not resolve whether it is due to normalization of the interphase events in the male pronucleus or compensating delay in the female pronucleus. Evidence for the former alternative comes from our observation that unlike CI crosses, in Rescue crosses we never observe PCNA positive chromosomes after entry into metaphase in CI embryos.

Materials and Methods

Immunofluorescence and Microscopy

Embryos were collected every 15 minutes and immersed in a pure bleach solution for few seconds to remove the chorion. Next they were washed in distilled water and fixed by vigorous shaking in a 1:1 heptane/methanol mix. RNAse A (Sigma) treatment was performed for 3 hours at 37°C (10 mg/mL). Primary and secondary antibodies were diluted in PBS+ 0.2% Tween+ 2% BSA. Embryos were incubated overnight at 4°C with primary antibodies. For secondary antibodies, the embryos were incubated at 37°C for three hours.

The following antibodies were used: Polyclonal anti-*Drosophila* PCNA (1:300), polyclonal (1:1000) and monoclonal (ADL84, 1:50) anti- *Drosophila* Lamin (all kindly provided by Paul Fisher), monoclonal anti-alpha tubulin (1:500, Molecular Probes), polyclonal anti-GFP (1:500, Chemicon), monoclonal anti-FLAG M2 antibody from Sigma was used to detect flagged H3.3 at 1:2000, polyclonal anti-acetylated H4 (1:300, Upstate). Cy5 goat anti-rabbit IgG and Alexa Fluor 488 goat anti–mouse IgG antibodies were used at 1:150 (Invitrogen). DNA was detected with propidium iodide (Molecular Probes, 1.0 mg/mL solution) after a 20 minute incubation in PBS (1:50) and a 5 minute wash. To better observe pronuclei deep within the cytoplasm, embryos were cleared and mounted in a (2:1) benzyl benzoate and benzyl alcohol solution.

Confocal microscope images were captured on an inverted photoscope (DMIRB; Leitz) equipped with a laser confocal imaging system (TCS SP2; Leica) using an HCX PL APO 1.4 NA 63 oil objective (Leica) at room temperature.

Fly Stocks

D. simulans stocks were used as *Wolbachia riverside*-infected or cured. *D. melanogaster* stocks were used as cured. The *Wolbachia* infection status of the stocks was established by both PCR [41] and Propidium iodide staining of fixed reproductive tissues.

Transgenic Lines

We used the previously described PW8-His3.3-Flag [15]. To construct the PW8-ProtSim-GFP transgene, a *D. simulans* protamine gene was amplified from genomic DNA using the following pair of primers:

Primer Protamine simulans 1: GGGAATTCATGCAAATGC-CACACCTCCTCAGTC

Primer Protamine simulans 2: TTGGATCCTTGTTGCAA-CAAACCCGTCGGCGCT

This PCR fragment was cloned in the PW8 vector in frame with EGFP at the 3′ end of the protamine coding sequence. A homozygous viable and fertile transgenic *PW8-ProtSim-GFP* stock was obtained by P-mediated germline transformation of a *D. simulans white* stock (a gift from Elgion Loreto).

Supporting Information

Figure S1 Histone H3.3 deposition occurs prior to nuclear envelope formation. Male pronuclei from compatible or CI crosses were scored for the presence of lamin to time Histone H3.3 deposition with respect to nuclear envelop formation. In control crosses we observe H3.3 deposition prior to the association of lamins with the nuclear envelope indicating H3.3 deposition occurs prior to nuclear envelop formation. The same experiment performed in CI crosses reveals that in every instance that we observe an abnormal ring of H3.3 staining the lamins are not present. This suggests that a nuclear envelope has not been formed and that the CI induced defect in H3.3 deposition are not likely due to defects in nuclear import. The lamin becomes clearly visible when the male and female pronuclei are migrating towards each other (data not shown). In CI crosses, one third of the male pronuclei showed a peripheral H3.3 accumulation, and none of them showed cortical lamin (n = 16). Scale bar is 1 μm.
Found at: doi:10.1371/journal.ppat.1000343.s001 (1.20 MB TIF)

Figure S2 PCNA is only detected in interphase nuclei at cycle 10. Embryos at cycle 10 were stained with the anti drosophila PCNA (red), anti-lamin and anti-tubulin (green) were used to follow the nuclear envelope and the microtubule spindle respectively. DNA (blue) was revealed with propidium iodide. (S) S phase, (Pro) prophase, (Meta) metaphase, (Ana) anaphase, (Telo) telophase.
Found at: doi:10.1371/journal.ppat.1000343.s002 (2.34 MB TIF)

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Author Contributions

Conceived and designed the experiments: FL BL WS. Performed the experiments: FL GAO BL. Analyzed the data: FL GAO BL WS. Contributed reagents/materials/analysis tools: FL GAO BL. Wrote the paper: FL BL WS.

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Cet article pose la question des mécanismes par lesquels la bactérie Wolbachia pourrait verrouiller la chromatine paternelle lors des étapes précoces de la spermatogénèse, générant ce retard d'assemblage à la fécondation. Dans ce sens, deux hypothèses distinctes sont possibles. (1) La manipulation par Wolbachia pourrait concerner l'histone H3.3 elle même. Le processus d'assemblage de H3.3 au cours de la spermatogénèse serait perturbé chez les mâles infectés, laissant une trace épigénétique (de nature inconnue) qui doit être adéquatement détectée et inversée par la bactérie dans l'œuf. (2) Il serait possible que la bactérie présente dans la lignée germinale mâle laisse une trace épigénétique sur les chromosomes indépendante de H3.3, qui doit être détectée et enlevée par la même bactérie à la fécondation. Dans cette hypothèse, le phénotype de défaut d'assemblage des nucléosomes observé ne serait que symptomatique d'un défaut indépendant dans la chromatine, ou d'un retard engendré par un élément anormal du chromosome. Malheureusement, les mécanismes en jeu manquent cruellement de données fonctionnelles, essentiellement à cause de l'extrême difficulté à cultiver (et manipuler génétiquement) cette bactérie intra-cytoplasmique (Serbus *et al.*, 2008).

Le phénotype associé à l'incompatibilité cytoplasmique (du point de vue cytologique) est lui même source d'interrogations. La séparation aberrante des chromosomes paternels, et la formation de ponts de chromatine pourraient refléter un défaut global ou affectant spécifiquement certaines régions. C'est pour cette raison que nous attendons que la lumière sur les mécanismes en jeu émane de l'étude des rares mutants disponibles qui miment le phénotype du point de vue cytologique. Mes travaux de thèse m'ont amené à m'intéresser à deux d'entre eux : le mutant à effet maternel *maternal haploid, mh* (présenté plus tard) et le gène à effet paternel *K81*.

II. K81 et la protection des télomères dans la lignée germinale mâle

Les mutations affectant *K81* sont un cas unique d'un défaut affectant la formation des chromosomes paternels à la fécondation dans des œufs issus de pères mutants. Cette mutation été décrite par Yoshiaki Fuyama en 1984, mais la fonction du gène affecté restait inconnue. Les premières études ont montré que ce mutant générait une stérilité mâle totale, associée à un

phénotype de létalité embryonnaire à effet paternel. Le phénotype des mutants K81 se caractérise par l'exclusion des chromosomes paternels à la première division du zygote, extrêmement similaire à celui observé dans le contexte de l'IC (Yasuda *et al.*, 1995; Loppin *et al.*, 2005b). Le gène *K81* a été cartographié : il s'agit d'un gène issu d'un événement de rétroposition impliquant le gène *hiphop*, ayant acquis une expression spécifique dans la lignée germinale mâle, tandis que hiphop perdait cette expression. Ces gènes subissent cependant une évolution très rapide, n'étant présents que chez les drosophiles, et leur fonction n'était, jusqu'à récemment, pas connue (Loppin *et al.*, 2005b).

Au cours de ma thèse j'ai généré des outils pour décrire l'étonnante distribution de la protéine K81. Celle-ci s'accumule en un faible nombre de foyers dans les spermatides en cours de maturation et reste associée aux chromosomes paternels jusqu'au moment de la fécondation et pendant la première division du zygote. Cependant, la nature de ces foyers n'était pas immédiatement identifiable et, par choix d'autres priorités, je n'ai pas continué à caractériser cette protéine.

De nouvelles expériences, cependant, ont montré qu'une protéine fusion mRFP-K81, exprimée de façon ectopique en tissus somatiques, se localise de façon très spécifique aux télomères. Au cours de cette étude, le rôle de Hiphop, sœur de K81, dans la protection des télomères de toutes les cellules chez la drosophile (Gao *et al.*, 2010). Les télomères protègent l'intégrité du génome en empêchant la corrosion des extrémités terminales des chromosomes, mais doivent à leur tour être protégés par des protéines de coiffe qui empêchent leur fusion qui serait délétère (Palm et de Lange, 2008; O'Sullivan et Karlseder, 2010). Hiphop fait partie d'un complexe de coiffe des télomères impliquant aussi ses partenaires HOAP (qui a un rôle spécifique dans la protection des télomères) et HP1 (qui a en plus une multitude d'autres rôles chez la drosophile)(Cenci *et al.*, 2003; Perrini *et al.*, 2004). Ce complexe est essentiel pour empêcher les fusions télomériques dans des cellules en culture. Combinées, ces données suggéraient fortement que K81 jouait un rôle dans la protection des télomères dans la lignée germinale mâle.

Au sein de l'équipe, cette fonction a finalement été démontrée pour K81 et décrite dans l'article ci-après. Notre modèle est qu'en absence de coiffe les télomère paternels subissent des fusions qui empêchent leur division lors de la première division du zygote. De façon

remarquable, des analyses de complémentation réciproque ont permis de montrer que K81 et Hiphop ne sont pas interchangeables. Nous avons proposé un modèle par lequel la protéine K81 serait spécialisée dans la protection des télomères dans le contexte très particulier de la chromatine de type protamines dans la lignée germinale mâle. En plus de leurs implications fonctionnelles pour la biologie des télomères, ces travaux décrivent un scénario original pour l'évolution de cette famille de protéines, montrant la particularité des contraintes de sélection qui agissent sur le maintien épigénétique des télomères au fil des générations.

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Article

Specialization of a *Drosophila* Capping Protein Essential for the Protection of Sperm Telomeres

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Summary

Background: A critical function of telomeres is to prevent fusion of chromosome ends by the DNA repair machinery. In *Drosophila* somatic cells, assembly of the protecting capping complex at telomeres notably involves the recruitment of HOAP, HP1, and their recently identified partner, HipHop. We previously showed that the *hiphop* gene was duplicated before the radiation of the *melanogaster* subgroup of species, giving birth to *K81*, a unique paternal effect gene specifically expressed in the male germline.

Results: Here we show that K81 specifically associates with telomeres during spermiogenesis, along with HOAP and HP1, and is retained on paternal chromosomes until zygote formation. In *K81* mutant testes, capping proteins are not maintained at telomeres in differentiating spermatids, resulting in the transmission of uncapped paternal chromosomes that fail to properly divide during the first zygotic mitosis. Despite the apparent similar capping roles of K81 and HipHop in their respective domain of expression, we demonstrate by in vivo reciprocal complementation analyses that they are not interchangeable. Strikingly, HipHop appeared to be unable to maintain capping proteins at telomeres during the global chromatin remodeling of spermatid nuclei.

Conclusions: Our data demonstrate that K81 is essential for the maintenance of capping proteins at telomeres in postmeiotic male germ cells. In species of the *melanogaster* subgroup, HipHop and K81 have not only acquired complementary expression domains, they have also functionally diverged following the gene duplication event. We propose that K81 specialized in the maintenance of telomere protection in the highly peculiar chromatin environment of differentiating male gametes.

Introduction

Telomeres are specialized nucleoprotein structures at the extremities of eukaryote chromosomes [1–4]. They have at least two essential roles for the maintenance of chromosome integrity. First, they protect chromosome ends from long-term genetic erosion through the addition of repeated sequences. Second, telomere-associated capping protein complexes prevent DNA extremities from being processed as double-strand breaks by the DNA repair machinery. In most eukaryotes, telomerase, a specialized reverse transcriptase, adds short DNA repeats at the end of chromosomes in a highly regulated manner [1–3]. This activity counteracts the incomplete replication of linear DNA extremities occurring at each cell cycle. Telomere repeats are specifically recognized by DNA binding proteins that participate in the formation of a protective capping complex. For instance, telomeric repeat-binding factors 1 and 2 (TRF1 and TRF2) bind TTAGGG repeats in human telomeres and recruit other capping proteins to form the shelterin complex [2–4]. Thus, the end-replication and capping functions of telomeres are connected in species that have telomerase.

Although telomeres fulfill the same functions in *Drosophila*, their organization is rather unusual. Like other Dipterans, *Drosophila* lacks telomerase activity [5]. Indeed, telomere elongation in *Drosophila* is dependent on the transposition of three related non-long terminal repeat retrotransposons called *HeT-A*, *TART*, and *TAHRE* [6, 7]. *Drosophila* chromosome ends are thus devoid of the short telomere repeats found in other organisms and lack the battery of proteins that specifically binds these sequences. Instead, *Drosophila* telomeres are capped by a set of proteins that associate with chromosome ends independently of the DNA sequence [8–10]. This property is best illustrated by the fact that de novo telomere formation can occur at chromosomes bearing viable terminal deletions in the absence of telomere-specific transposon sequences [11–14]. It has been proposed that this epigenetic protection of *Drosophila* telomeres requires the initial recognition of chromosome extremities by proteins of the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) DNA damage response pathways, which then recruit telomere capping proteins [15]. Their presence at telomeres is, in turn, critical to prevent the ligation of chromosome ends by the DNA repair machinery [15].

Well-characterized *Drosophila* capping proteins include heterochromatin protein 1 (HP1, also known as HP1A) and its partner HP1/ORC-associated protein (HOAP) [11, 12, 16, 17]. HP1 is a nonhistone chromosomal protein notably involved in pericentric heterochromatin formation. On polytene chromosomes, HP1 is enriched in the chromocenter but is also detected on many euchromatic bands and at all telomeres [11, 12, 18]. Its essential capping function was revealed by the analysis of *Su(var)205* (encoding HP1) mutant larvae that exhibit chromosome end-to-end fusions in dividing cells such as neuroblasts or imaginal discs [11]. This phenotype is also observed in larvae bearing a mutation in the *caravaggio* (*cav*) gene, which encodes HOAP [17]. HOAP is predominantly enriched at telomeres and is required for the recruitment of other telomere proteins such as Modigliani (Moi) and Verrocchio (Ver) [19, 20], underlining its central role in telomere capping.

Despite their essential role in chromosome protection, several capping proteins, including the recently characterized HOAP-interacting protein, HipHop, have been shown to evolve rapidly [14]. In somatic cells, HipHop is specifically enriched at telomeres, where it directly interacts with HOAP and HP1. Moreover, RNA interference knockdown of *hiphop* in S2 cultured cells results in chromosome fusions at high frequency, thus functionally implicating HipHop in telomere protection [14].

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Interestingly, we had previously shown that the duplication of *hiphop* in the *melanogaster* subgroup of species, presumably through a retroposition mechanism, gave rise to *ms(3) K81* (*K81*), a gene specifically expressed in the male germline [21]. The original *K81* male sterile mutant, which was isolated from a natural population in Japan, is associated with a unique paternal effect embryonic lethal phenotype [22]. Indeed, although homozygous *K81* mutant males produce fertilization-competent sperm, their progeny die as aneuploids or gynogenetic haploid embryos after the loss of paternal chromosomes at the first zygotic division [21–24].

In this study, using a combination of genetic and imaging approaches, we demonstrate that K81 is a male germline-specific capping protein. We show that K81 is necessary for the association of HOAP and HP1 with telomeres in postmeiotic spermatid nuclei. In the absence of K81, mutant gametes transmit uncapped paternal chromosomes to the zygote, with catastrophic consequences at the first mitosis. Finally, despite the apparent similar function of K81 and HipHop in telomere capping, we demonstrate that these sister proteins functionally diverged, suggesting that K81 specialized in the epigenetic protection of telomeres in differentiating spermatid nuclei.

Results

K81 Localizes at Telomeres in Spermatids

To analyze the distribution of K81 during spermatogenesis, we generated transgenic flies expressing a GFP::K81 fusion protein under the *K81* regulatory region (*5′K81-GFP::K81*). This transgene fully rescued the sterility of homozygous *K81* mutant males (Table 1), hence validating this tool to study K81 function in vivo. For the rest of the experiments, we used *5′K81-GFP::K81*; $K81^2$ males that only expressed GFP::K81, and not the endogeneous protein. We stained adult testes with an anti-GFP antibody to determine the distribution of the recombinant protein. In *Drosophila*, cysts of 16 interconnected primary spermatocytes undergo meiosis to produce groups of 64 haploid spermatids. The differentiation of spermatids, a process known as spermiogenesis, results in the production of mature, individualized male gametes [25]. We observed that GFP::K81 accumulated in a small number of discrete foci in spermatid nuclei (Figures 1A and 1B). To gain insight into the nature of these foci, we ectopically expressed an mRFP1::K81 protein in larval salivary glands using the UAS/GAL4 system to determine its distribution on polytene chromosomes. Strikingly, mRFP1::K81 appeared to be enriched at all chromosome extremities, suggesting that K81 might also associate with telomeres in spermatid nuclei (Figure 1C). HOAP is a well-characterized telomere marker in *Drosophila* somatic cells [16, 17]. The robust expression of the HOAP encoding gene, *cav*, in adult testes [26] suggested that it could also be involved in the capping of telomeres in male germ cells. Indeed, using a specific antibody, we detected HOAP in discrete foci in spermatid nuclei. Moreover, these foci perfectly colocalized with GFP::K81 (Figure 1D). Taken together, these results strongly suggested that K81 was specifically associated with spermatid telomeres.

Typically, spermatid nuclei contained one or two large foci and zero to three smaller foci of GFP::K81 (Figure 1B), suggesting that the eight expected telomeres of these haploid nuclei gather in a smaller number of clusters over the course of spermatid differentiation.

Table 1. Complementation Analysis of *K81* Paternal Effect Embryonic Lethality

Genotype of Males	Genotype of Females	Number of Eggs	Number of Larvae	Hatching Rate (%)
w/Y ; $K81^2$/TM3	*y w*	426	297	69.7
w/Y ; $K81^1$/$K81^2$	*y w*	359	0	0
w/Y ; $K81^2$/$K81^2$	*y w*	311	0	0
w/Y ; 5′K81-GFP::K81/ 5′K81-GFP::K81 ; $K81^2$/$K81^2$	*w*	265	246	93
w/Y ; 5′K81-GFP::K81/ 5′K81-GFP::K81 ; $K81^2$/$K81^2$	*y w*	351	342	97.4
w/Y ; 5′K81-GFP::K81/+ ; $K81^1$/$K81^1$	*y w*	480	456	95
w/Y ; 5′K81-GFP::K81/+ ; $K81^2$/$K81^2$	*y w*	518	477	92
w/Y; 5′K81-GFP::hiphop[#2]/ TM3	*y w*	315	310	98.4
w/Y; 5′K81-GFP::hiphop[#2] $K81^2$/$K81^2$	*y w*	377	0	0
w/Y; 5′K81-GFP::hiphop[#2] $K81^2$/5′K81-GFP::hiphop[#2] $K81^2$	*y w*	430	0	0
w/Y; 5′K81-GFP::hiphop[#3]/ 5′K81-GFP::hiphop[#3]	*y w*	512	221	43.2
w/Y; 5′K81-GFP::hiphop[#3] $K81^2$/$K81^2$	*y w*	256	0	0
w/Y; 5′K81-GFP::hiphop[#3] $K81^2$/5′K81-GFP::hiphop[#3] $K81^2$	*y w*	363	0	0
w/Y ; 5′hiphop-GFP::K81 $K81^2$/5′hiphop-GFP::K81 $K81^2$	*y w*	433	2	0.4

Males and females of the indicated genotypes were crossed, and embryo hatching rates were calculated as described in the Experimental Procedures. *5′K81-GFP::hiphop[#2]* and *5′K81-GFP::hiphop[#3]* are two independent insertions of the same transgene.

K81 Remains Associated with Paternal Telomeres until Zygote Formation

In mature gametes, GFP::K81 foci were no longer detected, most likely as a consequence of the extreme compaction of sperm nuclei, which are not accessible to antibodies (data not shown and [27]). To determine whether GFP::K81 was still associated with paternal telomeres after fertilization, we crossed *5′K81-GFP::K81*; $K81^2$ males with wild-type females. Eggs laid by these females were stained with an anti-GFP antibody to detect GFP::K81 after fertilization. Strikingly, GFP::K81 was systematically detected in the decondensing male pronucleus (n = 16), often in one or two foci (Figure 1E). GFP::K81 was still detected in the male nucleus at pronuclear apposition and during the first zygotic mitosis (Figures 1F–1H). In anaphase of the first nuclear cycle, GFP::K81 was observed at the extremities of separating paternal sister chromatids, thus confirming the telomere localization of K81 (Figure 1H). A faint staining of paternally transmitted GFP::K81 was occasionally detected on paternal chromosomes during the second nuclear division, but never beyond this stage (data not shown).

Because GFP::K81 rapidly vanished from paternal chromosomes after zygote formation, we wondered whether K81 was replaced by its sister protein, HipHop, in early embryos. Interestingly, the *hiphop* gene shows a strong female-biased expression, and transcripts are very abundant in adult ovaries [26, 28]. We generated transgenic flies expressing a GFP::HipHop fusion protein under the regulatory region of *hiphop* (*5′hiphop-GFP::hiphop*; Figure 2A). As expected, the transgene was maternally expressed, and GFP::HipHop specifically

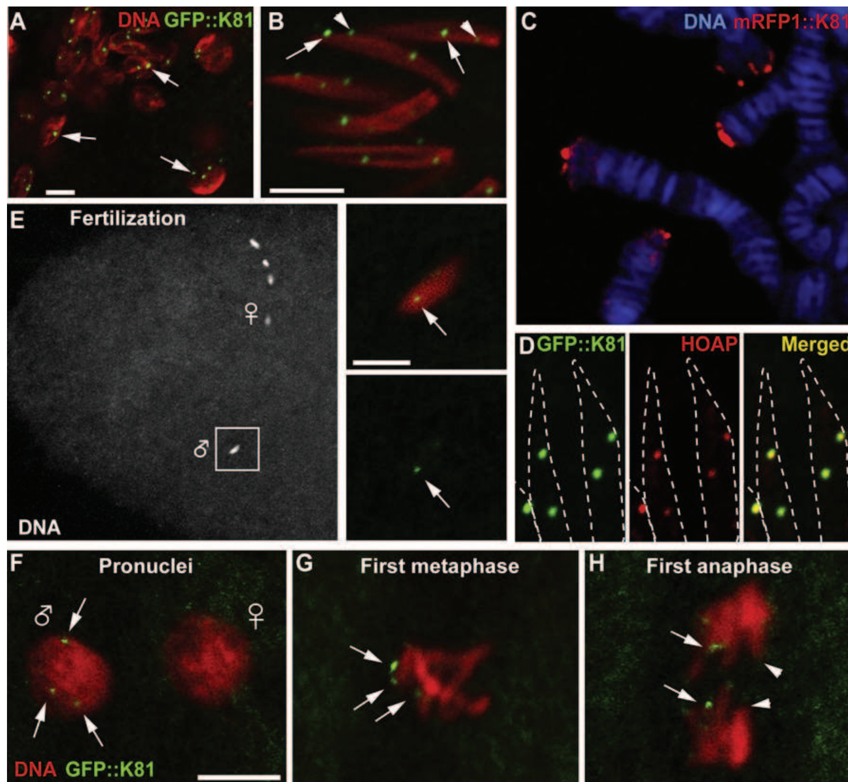

Figure 1. K81 Associates with Paternal Telomeres

(A and B) Confocal images of spermatids from a *5′K81-GFP::K81*; *K81²* adult testis stained for DNA (red) and GFP (green).

(A) In early, round spermatids, GFP::K81 accumulates in a small number of dots (arrows).

(B) In elongating spermatid nuclei, GFP::K81 is detected in large (arrows) or small (arrowheads) foci.

(C) Polytene chromosomes from *UAS-mRFP1::K81; Sgs3-Gal4* larval salivary glands. mRFP1::K81 (red) specifically localizes at all chromosome extremities.

(D) In spermatid nuclei, GFP::K81 foci (green), detected with an anti-GFP antibody, colocalize with the telomere marker HOAP (red). Spermatid nuclei are outlined.

(E–H) Eggs and early embryos from wild-type females mated with *5′K81-GFP::K81*; *K81²* rescued males.

(E) A fertilized egg with the four maternal meiotic products visible at the top. The still-elongated male nucleus (inset) contains at least one GFP::K81 focus (arrow).

(F) Male and female pronuclei shortly before apposition. Only the male pronucleus (left) contains GFP::K81 foci (arrows).

(G) First zygotic metaphase: GFP::K81 foci are still associated with paternal chromosomes (arrows).

(H) First zygotic anaphase: the telomere localization of GFP::K81 is visible (arrows). Arrowheads show unlabeled telomeres, presumably from maternal chromatids. Scale bars represent 5 μm.

associated with telomeres, for instance on polar body chromosomes (Figure 2B). Shortly after fertilization, GFP::HipHop was detected in female meiotic products, but not in the decondensing male pronucleus (Figures 2C and 2D). After the first round of DNA replication, however, GFP::HipHop was associated with both maternal and paternal telomeres (Figure 2E) and then on all embryonic telomeres throughout syncytial development (data not shown).

Taken together, our observations indicate that after fertilization, newly synthesized paternal telomeres are capped with maternally expressed HipHop, whereas paternally transmitted K81 is rapidly diluted during the first embryonic S phases.

K81 Is Necessary for the Association of HP1 and HOAP at Spermatid Telomeres

In *Drosophila* S2 cultured cells, HipHop and HOAP are interdependent for their stability and for their recruitment at telomeres [14]. The presence of HOAP at spermatid telomeres enabled the possibility that its distribution might be similarly dependent on K81. In wild-type testes, HOAP foci were detected in spermatid nuclei throughout spermiogenesis (Figure 3A). In *K81²* mutant testes, HOAP foci were detected in early, round spermatids (data not shown), but, strikingly, became undetectable in elongating nuclei (Figure 3A). Thus, like HipHop in somatic cells, K81 is required for the maintenance of HOAP at telomeres in differentiating spermatids.

We then aimed to determine whether HP1 was also involved in the capping of spermatid telomeres. In contrast to other capping proteins, HP1 is a multifunctional protein with a complex nuclear distribution in somatic cells, notably including a strong enrichment in pericentric heterochromatin [11, 18, 29]. In secondary spermatocytes, HP1 was detected throughout the nucleus and was also enriched at telomeres, as revealed by its colocalization with HOAP (Figure 3B;

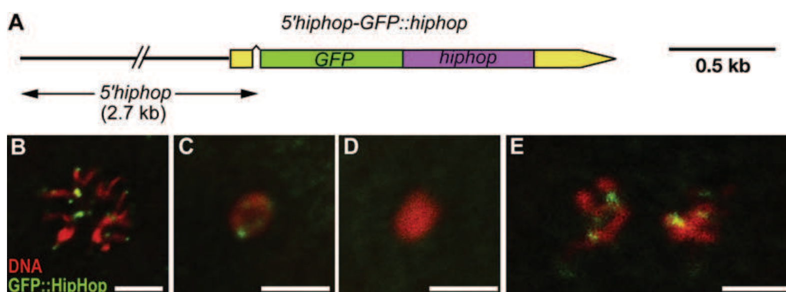

Figure 2. HipHop Distribution in Eggs and Early Embryos

(A) Representation of the *5′hiphop-GFP::hiphop* transgene. This construct contains a 2.7 kb fragment of genomic DNA (*5′hiphop*) immediately upstream of the *hiphop* coding sequence. A single copy of this transgene fully rescues the lethality associated with *hiphop* mutant alleles (see Figure 4).

(B–E) Confocal images of early embryos stained for DNA (red) and GFP::HipHop (green).

(B) Polar body.

(C) Migrating female pronucleus.

(D) Male pronucleus from the same egg.

(E) First metaphase.

HipHop is absent from the male pronucleus but is detected on both paternal and maternal telomeres at the first metaphase. Scale bars represent 5 μm.

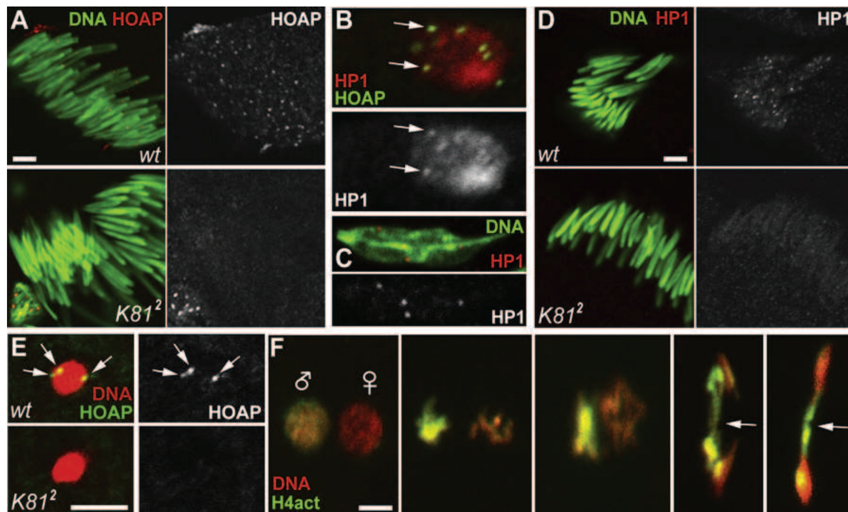

Figure 3. K81 Maintains Capping Proteins at Sperm Telomeres

(A) Confocal images of testes from wild-type or $K81^2$ mutant males stained for HOAP (red) and DNA (green). HOAP is detected at telomeres in wild-type (wt) but not $K81^2$ mutant elongating spermatid nuclei. Note that the HOAP foci visible in the $K81^2$ panels are from a somatic cell.

(B) In secondary spermatocyte nuclei, HP1 (red) has a broad nuclear distribution but appears to be enriched at telomeres (arrows), where it colocalizes with HOAP (green).

(C) In elongating spermatid nuclei, HP1 is only restricted to telomeres (see also Figure S1).

(D) Like HOAP, HP1 is not maintained at spermatid telomeres in $K81^2$ mutant testes.

(E) In eggs fertilized with wild-type sperm, HOAP is detected at telomeres in the decondensing male pronucleus (arrows; n = 27), whereas in eggs fertilized with sperm from $K81^2$ mutant males, HOAP is never detected in the male pronucleus (n = 17).

(F) Paternal chromosomes fail to divide normally in eggs fertilized with *K81* sperm. From left to right: pronuclear apposition, first metaphase, early anaphase, late anaphase, and telophase. The paternal chromatin is stained with an anti-acetylated H4 histone antibody (green) [27]. Paternal chromatin bridges are visible in late anaphase and telophase (arrows). Scale bars represent 5 μm.

see also Figure S1 available online). Surprisingly, however, after meiosis, HP1 distribution in spermatid nuclei became restricted to a few foci that colocalized with GFP::K81 (Figure 3C and data not shown). In addition, we verified that none of the four centromeres of these haploid nuclei colocalized with HP1 or GFP::K81 (Figure S1). Thus, HP1 is specifically retained at telomeres in spermatids, whereas it is completely removed from other genomic regions during spermiogenesis, including pericentric heterochromatin. In $K81^2$ mutant testes, however, HP1 foci were no longer detected in elongating spermatids, similar to HOAP (Figure 3D). Therefore, K81 is necessary for the association of HP1 at telomeres, thus underlining the similar roles of HipHop and K81 in capping complex formation in somatic cells and spermatids, respectively.

The presence of HOAP and HP1 at spermatid telomeres suggested that these capping proteins, like K81, were transmitted to paternal chromosomes at fertilization. Although the diffuse distribution of HP1 in the male pronucleus did not permit us to draw conclusions about its association with telomeres at this stage (data not shown), HOAP foci were clearly detected in decondensing male pronuclei (Figure 3E). As expected, we never observed HOAP foci in the male pronuclei transmitted by $K81^2$ mutant fathers (Figure 3E). Together, these data demonstrate that K81 is required for the maintenance of the HP1 and HOAP capping proteins at paternal telomeres before and after fertilization.

In the absence of HOAP and HP1 in somatic cells, unprotected telomeres frequently fuse, resulting in bridges of chromatin in anaphase [11, 17]. Interestingly, in eggs fertilized by sperm from $K81^2$ mutant males, paternal chromosomes fail to separate in anaphase [21, 24] and form a chromatin bridge that ultimately connects the dividing nuclei in telophase (Figure 3F). We thus propose that the *K81* paternal effect phenotype results from the fusion of uncapped paternal chromosomes prior to the first zygotic mitosis.

HipHop and K81 Are Not Functionally Equivalent

HipHop and K81 proteins display 53% amino acid identity (Figure S2) and are both involved in the maintenance of capping proteins at telomeres in their respective expression domain. We thus addressed the question of whether the two proteins have remained interchangeable or have functionally diverged since the gene duplication. We therefore tested the ability of HipHop and K81 to complement one another in vivo. As expected from its molecular function, *hiphop* is an essential gene. Indeed, all animals transheterozygous for two noncomplementing *P* element insertions in *hiphop* ($hiphop^1$/$hiphop^{EY07584}$) died before the second larval stage (Figure 4A and data not shown). A copy of the *5′hiphop-GFP::hiphop* transgene fully restored the viability of this allelic combination, thus confirming that the observed lethality was caused by *hiphop* loss of function (Figure 4B). We then replaced *hiphop* with the *K81* coding region in the same construct to generate *5′hiphop-GFP::K81* transgenic flies. Importantly, this construct was inserted at the same genomic position as the *5′hiphop-GFP::hiphop* transgene to achieve identical expression levels (see Experimental Procedures). We verified that, in larval salivary glands, both GFP::HipHop and GFP::K81 similarly localized at telomeres on polytene chromosomes (Figure 4B). Strikingly, however, the *5′hiphop-GFP::K81* transgene had no effect on *hiphop* mutant lethality (Figure 4B), thus demonstrating that K81 cannot functionally replace HipHop.

In a mirror experiment, GFP::HipHop was expressed under the *K81* regulatory region (*5′K81-GFP::hiphop*). Three independent insertions of this construct drove robust expression of GFP::HipHop in the male germline (data not shown). In testes from these transgenic animals, GFP::HipHop was detected as nuclear foci in spermatids in a way that was identical to GFP::K81 (Figure 4C). However, none of these transgenic insertions rescued *K81* male sterility (Table 1 and Figure 4C). We verified that the *5′K81-GFP::hiphop*, $K81^2$ males induced a typical *K81* paternal effect embryonic phenotype, characterized by a systematic bridging of paternal chromatin during the first mitosis (data not shown). Furthermore, although GFP::K81 was systematically detected in the decondensing male pronucleus, paternally expressed GFP::HipHop, in clear contrast, was not retained on paternal chromatin after fertilization (Figure 4D). Hence, we conclude that HipHop cannot replace K81 for the protection of paternal telomeres in the male germline.

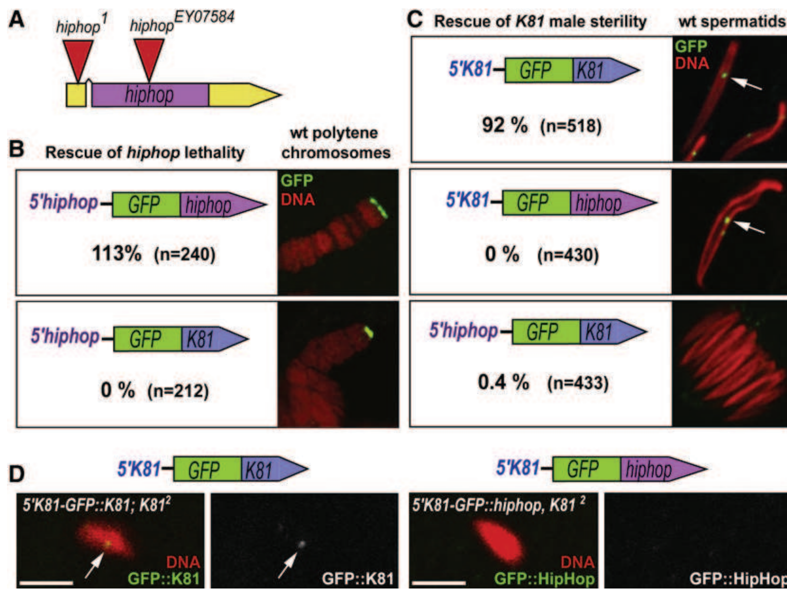

Figure 4. HipHop and K81 Have Functionally Diverged

(A) Representation of *hiphop*^{1} and *hiphop*^{EY07584} lethal *P* element insertion alleles (red triangles) used in (B). Untranslated and coding regions are shown in yellow and purple, respectively.

(B) Rescue experiments of *hiphop* lethality with the indicated transgenes. The percentages indicate the fraction of rescued transgenic *hiphop*^{1}/*hiphop*^{EY07584} adults over the expected 1/3 Mendelian ratio for this genotype (see Experimental Procedures). The localization of GFP fusion proteins (green) on polytene chromosomes (red) is shown for each transgene. n denotes total number of adult progeny obtained from the cross.

(C) Rescue experiments of *K81* male sterility with the indicated transgenes. Hatching rates of embryos from *K81*^{2} homozygous mutant males with two copies of the indicated transgene are shown (see Experimental Procedures). For each transgene, the localization of GFP fusion proteins on spermatid nuclei is shown. n denotes total number of embryos.

(D) GFP::K81 is detected in the male pronucleus (arrows) in eggs fertilized with sperm from *5′K81-GFP::K81*; *K81*^{2} rescued males (n = 16), whereas GFP::HipHop is never detected in eggs fertilized with sperm from *5′K81-GFP::hiphop*; *K81*^{2} males (n = 15). Scale bars represent 5 μm.

HipHop Fails to Maintain Capping Proteins at Telomeres during the Histone-to-Protamine Transition

To better understand the basis of the functional divergence of K81 and HipHop, we studied the distribution of HOAP and HP1 during the course of spermatid differentiation in *5′K81-GFP::hiphop, K81*^{2} males. During spermiogenesis, the canoe stage is characterized by the massive replacement of histones with sperm-specific chromosomal proteins, such as protamines and Mst77F [30]. To study the distribution of capping proteins during this process, we costained testes for HOAP or HP1 and Mst77F, which is deposited in spermatid nuclei at the onset of histone removal [31, 32]. In control testes, HP1 and HOAP foci were detected in early and in late canoe stage spermatid nuclei that had already begun to incorporate Mst77F (Figures 5A and 5B, left panels). In *K81*^{2} mutant testes expressing GFP::HipHop, HOAP and HP1 foci were only

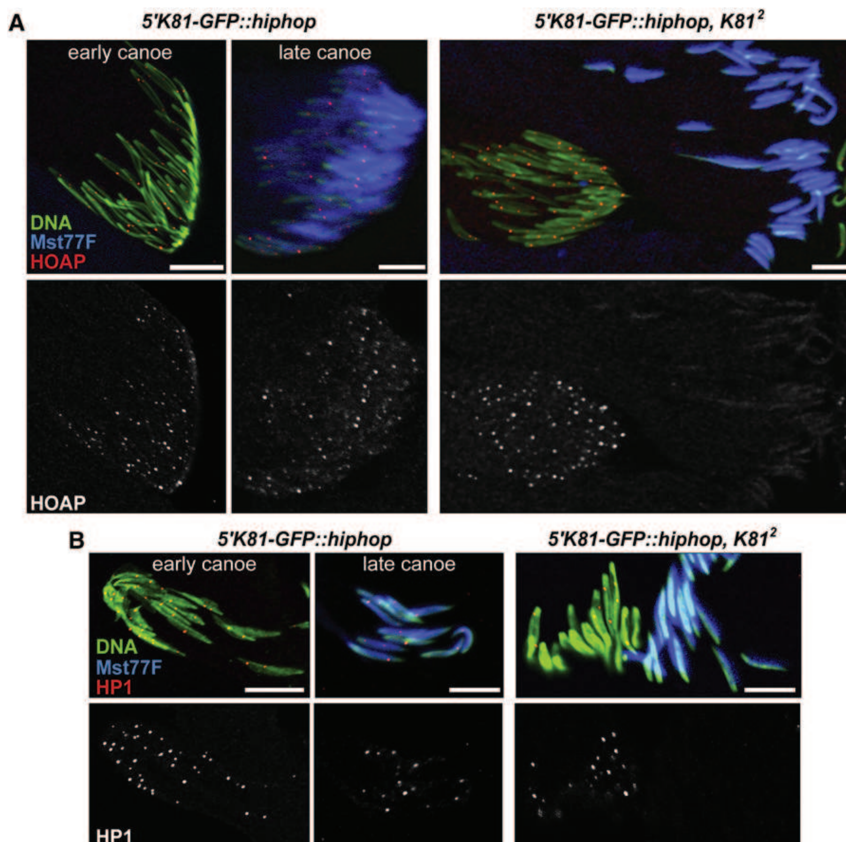

Figure 5. HipHop Cannot Maintain HOAP and HP1 at Telomeres during Sperm Chromatin Remodeling

(A) In *5′K81-GFP::hiphop* control testes, HOAP (red) is detected in early and in late canoe spermatid nuclei that have already incorporated the Mst77F (blue) sperm chromatin protein (left panels). In *5′K81-GFP::hiphop*; *K81*^{2} testes, HOAP foci are only detected in spermatid nuclei that have not yet incorporated Mst77F (right panels). Note that the Mst77F staining is used as an internal control for antibody accessibility in spermatid nuclei.

(B) Similarly, HP1 is detected only in early canoe spermatid nuclei of *5′K81-GFP::hiphop, K81*^{2} testes (right panels). Scale bars represent 10 μm.

observed in early canoe spermatid nuclei that were negative for Mst77F (Figures 5A and 5B, right panels). Indeed, the loss of these foci was correlated with the onset of Mst77F deposition in condensing spermatid nuclei.

Hence, although GFP::HipHop is able to initially recruit HOAP and HP1 at spermatid telomeres in the absence of K81, the capping complex is not maintained during the global chromatin remodeling of spermatid nuclei. It thus suggests that K81 specialized to cap telomeres in the highly peculiar chromatin environment of maturing male gametes.

Discussion

K81 and the Epigenetic Protection of Telomeres in the Male Germline

We have shown that K81 encodes a new telomere capping protein required for the transmission of functional paternal chromosomes to the diploid zygote. This finding elucidates the origin of the unique paternal effect lethal phenotype associated with *K81*. To our knowledge, K81 is the first identified *Drosophila* telomere protein specifically expressed in the male germline. In fact, the structure and organization of telomeres in *Drosophila* male germ cells have remained largely unexplored. We show in this study that during spermiogenesis, K81 accumulates in a small number of foci, where it is systematically associated with the HOAP and HP1 capping proteins. In contrast to HOAP, which is essentially a telomere-specific protein, HP1 is mainly enriched in pericentric heterochromatin in somatic nuclei. In addition, HP1 is also detected at telomeres and at numerous euchromatic sites on polytene chromosomes [11, 18]. In this regard, it is remarkable that HP1 is only retained at telomeric regions in spermatid nuclei, suggesting that its sole function in differentiating male germ cells is in capping telomeres. The lethality associated with *cav* (encoding HOAP) and *Su(var)205* (encoding HP1) loss-of-function mutant alleles prevents us from directly testing their respective roles during spermiogenesis. Our study shows, however, that both HOAP and HP1 are lost from spermatid telomeres in *K81* mutant testes. This loss of telomere capping proteins does not interfere with male gamete differentiation and maturation. Instead, the *K81* mutant phenotype manifests itself only after fertilization and results in the incapacity of paternal chromosomes to segregate during the first zygotic mitosis. This initial defect leads to the formation of aneuploid embryos, which arrest development after a few abnormal nuclear divisions, or to the occasional escaping of haploid gynogenetic embryos that die shortly before hatching [21, 22, 24]. The systematic and specific bridging of paternal chromatin during the first anaphase most likely results from the presence of chromosome end-to-end fusions. Although telomere fusions can be easily observed in cultured cells or in squashed preparations of larval brains, where they form chains of connected chromosomes [9], these defects appeared to be very difficult to observe in detail in *Drosophila* zygotes. Nonetheless, chromatin bridges associated with telomere dysfunction have been reported in syncytial embryos from mothers bearing hypomorphic alleles of *mre11* or *nbs* [33], thus indicating that the DNA repair machinery presumably responsible for the fusion of uncapped telomeres is already active during early cleavage divisions.

The distribution of telomere capping protein foci in spermatid nuclei indicates that telomeres tend to associate within clusters during spermiogenesis. Interestingly, telomere clustering seems to be a conserved feature of animal spermiogenesis, such as in mammals, in which telomeres from the same chromosome are frequently associated in pairs [34, 35]. In *Drosophila*, telomere clustering is apparently the rule in late spermatids, as well as in the decondensing male pronucleus, because we frequently observed a single major focus of capping proteins in these nuclei. It is likely that this spectacular gathering of telomeres in a limited nuclear volume could favor the occurrence of paternal chromosome end-to-end fusions in *K81* mutants.

Diversification of the *hiphop/K81* Gene Family

Despite their critical role in chromosome protection, telomere proteins are rapidly evolving from yeasts to mammals [2, 36–38]. This tendency is observed in *Drosophila*, where important capping proteins such as HOAP, Verrocchio, Modigliani, and HipHop are encoded by fast-evolving genes [14, 20, 39]. We had previously shown that *K81* is a relatively young gene that is restricted to the nine species comprising the *melanogaster* subgroup [21]. *K81* originated after the duplication of its paralog, *hiphop* (originally known as *CG6874/l(3)neo26*), presumably through a retroposition mechanism. The predicted *K81* transcription start site is only about 100 bp from the 5′ end of the *Rb97D* gene, which is expressed in primary spermatocytes and is required for male fertility [40, 41]. The selection of both *hiphop* and *K81* genes was thus likely favored by the immediate acquisition of male germline-specific expression of the duplicated copy, after its landing close to *Rb97D*, followed by loss of *hiphop* expression in this lineage [21]. In a less parsimonious, alternative scenario, an ancestral male germline-specific *hiphop* gene could have evolved a somatic and female germline expression following the duplication. However, this possibility does not fit with the expected requirement of HipHop for telomere protection in somatic cells. Interestingly, with a single exception (see below), all *Drosophila* sequenced species outside the *melanogaster* subgroup have a single member of the *hiphop/K81* gene family (Figure 6). For instance, *D. ananassae*, *D. pseudoobscura*, and *D. persimilis* have *hiphop* with the same conserved synteny as in *melanogaster* species but lack *K81* (Figure 6). In these three species, *hiphop* is thus expected to protect telomeres in all cells, including male germ cells. Most interestingly, phylogenetic analysis reveals the existence of a second, independent duplication of *hiphop* in the lineage leading to *D. willistoni* (Figure 6). Moreover, this *D. willistoni hiphop* duplicate presents a male-biased expression (Figure S3), allowing the possibility that it could be required in the male germline, like *K81* in *D. melanogaster*. Although functional studies are not currently feasible in non-*melanogaster* species, developmental in situ expression analysis of members of this gene family may support these predictions.

Functional Divergence of HipHop and K81

In their respective cellular environments, HipHop and K81 are both specifically localized at telomeres, and they are required for the maintenance of the HOAP and HP1 capping proteins at chromosome ends. However, and despite the apparently identical molecular functions of K81 and HipHop, our experiments demonstrate that they cannot replace one another in vivo. When ectopically expressed in the male germline, GFP::HipHop is able to transiently restore the localization of HOAP and HP1 at spermatid telomeres in a *K81* mutant background. In this genetic context, telomeres remained capped until the global replacement of histones with sperm-specific nuclear proteins. What actually triggers the loss of HipHop, HP1, and

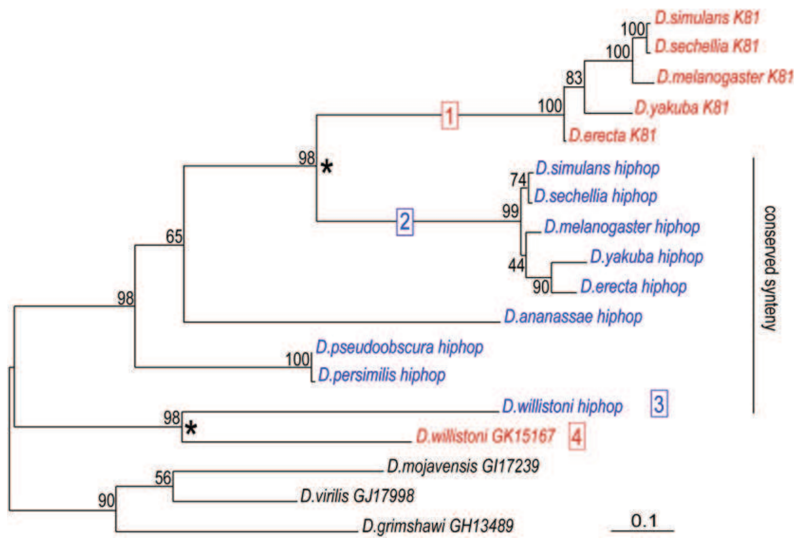

Figure 6. Phylogenetic Tree of the *hiphop/K81* Gene Family

This tree was obtained from a Gblocked alignment of the *hiphop/K81* CDS using the PhyML phylogenetic program (GTR model with a gamma distribution and four site categories) in Seaview (see Experimental Procedures). Results of nonparametric bootstrap (100 replicates) are shown. This tree indicates the occurrence of two *hiphop* independent duplication events (asterisks). All the *hiphop* genes in blue have conserved synteny. *hiphop* paralogs are shown in red. Numbers in boxes indicate the focal lineages in the d_N/d_S analysis (see Table S1). Gene annotation symbols not shown in the tree: *Drosophila melanogaster K81*: CG14251; *D. simulans K81:* GD21311 *D. sechellia K81: GM10349*; *D. yakuba K81:* GE23697; *D. erecta K81: GG11507*; *D. melanogaster hiphop: CG6874*; *D. simulans hiphop:* GD14769 *D. sechellia hiphop: GM14992*; *D. yakuba hiphop:* GE19974; *D. erecta hiphop: GG13678*; *D. ananassae hiphop:* GF10272; *D. pseudoobscura hiphop: GA19922*; *D. persimilis hiphop: GL24882*; *D. willistoni hiphop: GK12110*. Bar denotes number of substitution per nucleotide (see also Figure S3, Figure S4, and Table S1).

HOAP in these spermatids is not known. The fact that these proteins disappear concomitantly with the onset of global spermatid chromatin remodeling suggests a causal link, although this remains to be established. In mammals, although telomere integrity in male gametes is essential for zygote formation [42], little is known about the organization of telomeres in germ cells. However, a few studies point to the peculiar composition of telomere complexes in human sperm [35, 43], suggesting that the unique organization of sperm chromatin imposes constraints on the structure and function of telomeres. Similarly, our study suggests that K81 specialized in the epigenetic maintenance of telomere identity in the highly peculiar chromatin environment of male gametes. This scenario also implies that HipHop lost its ability to protect sperm telomeres after the emergence of *K81* function. Phylogenetic analysis of the *hiphop* and *K81* coding sequences actually supports this subfunctionalization scenario. First, *hiphop* and *K81* genes show a symmetrical acceleration of evolution in the *melanogaster* subgroup of species (Figure 6 and Table S1). Second, synonymous and nonsynonymous nucleotide substitution analysis of the coding sequences indicates that *hiphop* and *K81* evolved under purifying selection (Figure S4 and Table S1). Finally, *K81* expression in somatic cells does not rescue the zygotic lethality of *hiphop* mutants, thus confirming the functional divergence of both proteins.

The maternal expression of *hiphop* is apparently sufficient to protect telomeres during embryo development, as observed with mutations in other telomere capping genes [10]. Accordingly, we have shown that maternally expressed GFP::HipHop decorates both paternal and maternal telomeres as soon as the diploid zygote is formed. However, the early larval zygotic lethality of *hiphop* mutants prevented a more detailed in vivo phenotypic analysis using third instar larvae polytene chromosomes or neuroblast mitotic chromosomes. Although both mRFP1::K81 and GFP::K81 are fully able to associate with somatic telomeres, these experiments could only be carried out in a wild-type *hiphop* genetic background, for the reasons mentioned above. We thus do not currently know whether K81 associates with somatic telomeres autonomously or through its association with other capping proteins, such as HOAP and/or HP1, in a HipHop-dependent manner.

As discussed above, the functional divergence of HipHop and K81 could reflect their adaptation to different chromatin environments. However, as new *Drosophila* telomere proteins are regularly discovered, it is also reasonable to consider the possibility that K81 and HipHop require one or more yet-unknown protein partners to function properly. For instance, K81 could not protect telomeres in somatic cells if its capping activity requires another factor only expressed in spermatids. Interestingly, the HP1-related protein Umbrea/HP6 [29, 44], which has been recently proposed to function in telomere protection [45], is mainly expressed in the adult testis [26]. Future studies should thus aim at determining whether other capping proteins are specialized in the protection of telomeres in germ cells, like K81.

In conclusion, our study demonstrates that HipHop and K81 diverged not only in their domain of expression, but also in their ability to protect telomeres in their respective cellular environments. A challenge will be to understand the nature of the evolutionary pressure that ultimately shaped the diversification of the *hiphop/K81* gene family in the genus *Drosophila*.

Experimental Procedures

Drosophila Strains

w^{1118} and y^{1} w^{67c} strains were used as controls. Unless otherwise noted, we used the $K81^{2}$ (or *Df(3R)ms(3)K81-2*) allele, a small deficiency that completely deletes the *K81* gene [24]. The original $K81^{1}$ allele has been previously characterized [21]. The $hiphop^{1}$ allele (*P{hsneo}hiphop*^{1}) [46] was obtained from the Bloomington Drosophila Stock Center. The $hiphop^{EY07584}$ (*P{EPgy2}hiphop*^{EY07584}) allele is a *P* element insertion at genomic position 18814719 and was kindly provided by Hugo Bellen [47]. The *gcid-EGFP::cid* line is a gift from Stefan Heidmann [48]. The *5′K81-GFP::K81* and *5′K81-GFP::hiphop* transgenic stocks were obtained by standard *P* element-mediated germline transformation. The *5′hiphop-GFP::hiphop*, *5′hiphop-GFP::K81*, and *UAS-mRFP1::K81* constructs were inserted into the PBAc{y[+]-attP-3B}VK00031 platform (62E1) using the φ*C31*-mediated integration system [49].

Fertility Tests

Males and virgin females were allowed to mate for 2 days before overnight egg collection. Eggs were counted and allowed to develop at 25°C for 48 hr. Hatched larvae were then counted to determine hatching rates.

hiphop Complementation Tests

5′hiphop-GFP::hiphop, hiphop[1]/TM3, Sb or *5′hiphop-GFP::K81, hiphop[1]/TM3, Sb* flies were crossed to *hiphop^{EY07584}/TM3, Sb* flies. The number of flies for each expected genotype was counted in the progeny.

Immunofluorescence and Imaging

Polytenes Chromosomes

Samples were prepared as previously described [50]. Primary antibodies were rabbit polyclonal anti-RFP (Clontech) or mouse monoclonal anti-GFP (Roche), diluted at a 1:250 and 1:15 dilution, respectively. Alexa Fluor (Molecular Probes) or DyLight (Jackson Immunoresearch) conjugated secondary antibodies were used at a 1:300 dilution.

Testes

All testes were squashed for immunostaining except for the anti-Cid staining (Figure S1). In this case, whole-mount testes were stained as previously described [27] with an anti-Cid antibody (Abcam) at a 1:2000 dilution. The protocol for squashing testes was adapted from [51] with a few modifications. Briefly, five to six testes per genotype were fixed in 4% paraformaldehyde for 30 min and then in 50% acetic acid for 2–3 min before squashing. Immunostainings were done as described above for polytene chromosome preparations, except that blocking was done in 1% bovine serum albumin in phosphate-buffered saline. We used mouse anti-GFP (Roche) (1:100), guinea pig anti-HOAP [14] (1:200), mouse anti-HP1 (Developmental Studies Hybridoma Bank) (1:100), and rabbit anti-Mst77F (1:1000) [30] primary antibodies. Secondary antibodies were used at a 1:300 dilution.

Samples were mounted in mounting medium (Dako) containing 5 μg/ml propidium iodide (Sigma) or 1 mM YO-PRO-1 (Molecular Probes).

Eggs

Eggs were collected every 30 min, fixed, and stained as previously described [52]. Rabbit anti-GFP (Invitrogen), rabbit anti-acetylated histone H4 (Upstate), or guinea pig anti-HOAP primary antibodies were used at a 1:200 dilution, and corresponding secondary antibodies were used at 1:500.

All confocal images were obtained using an LSM 510 confocal microscope (Zeiss) and were processed using Photoshop (Adobe).

Plasmid Constructs

We used pW8 and pUASP vectors and modified them to obtain a pW8-attB and a pUASP-attB vector, allowing targeted insertion in the 62E1 platform located on chromosome 3L. Plasmid constructs were then done as follows.

UAS-mRFP1::K81

The pUASP-*mRFP1::K81* construction was obtained by polymerase chain reaction (PCR) amplification of the *K81* and *mRFP1* full-length coding sequences and cloning into the pUASP-attB vector. The following primers containing, respectively, the *Not*I and *Bam*HI restriction sites were used to amplify *K81:* 5′-CTAGCGGCCGCCATGTCGGATTCGC-3′and 5′-TGGATCCACATTATCCCCCAGTAGTTCC-3′. The primers 5′-CTAGCGGCCGCACCATGGCCTCCTCCGAGGACG-3′ and 5′-CATTTAGGCGGCCGCGCCGGTGGAGTGGCGG-3′, both containing *Not*I, were used to amplify *mRFP1*.

GFP::K81 and GFP::hiphop Transgenes

The *GFP* coding sequence was excised from a previously engineered construct (*pGEM-T-GFP*) using the *Sac*II and *Not*I restriction sites and was cloned into the pW8 (for the *5′K81-GFP::K81* and *5′K81-GFP::hiphop* constructs) or the pW8-attB (for the *5′hiphop-GFP::hiphop* and *5′hiphop-GFP::K81* constructs) vector. Then cloning of the *hiphop* and *K81* upstream regions and coding sequences was done as follows.

5′ K81-GFP::K81

A 555 bp and 674 bp fragment covering, respectively, the complete *K81* coding sequence (CDS) and upstream sequences (666 bp) were amplified from *yw* genomic DNA and subcloned into the pGEM-T vector. The following primers, 5′-CTAGCGGCCGCCATGTCGGATTCGC-3′ and 5′-TGGATCCACATTATCCCCCAGTAGTTCC-3′ for the *K81* CDS, were designed to introduce *Not*I and *Bam*HI restriction sites, whereas each of the *K81* promoter primers, 5′-CCGCGGGATAACATCGACCACCTTGCCCC-3′ and 5′-CCGCGGCCATTAGAACTTAAGTTGAATACTC-3′, contains a *Sac*II restriction site.

5′ hiphop-GFP::hiphop

A 2729 bp fragment covering the regulating sequence of the *hiphop* gene, including the 5′ untranslated region and the first intron, was amplified by PCR from *yw* genomic DNA and subcloned into a pGEM-T vector (Promega) using the following primers that both contain the *Sac*II restriction site: 5′-TCTTATCCGCGGACTCAGTAGAATGTTAAGG-3′ and 5′-ATGTTACCGCGGCTGGAATAGATCATGCACC-3′. Similarly, a 1519 fragment containing the coding sequence of *hiphop* was amplified and subcloned into a pGEM-T vector. Primers used were 5′-CTAGCGGCCGCCATGGCCTCCATTGACGAGG-3′ and 5′-TGGATCCAGTCAATCAACTGATTGGAAGC-3′, which introduce *Not*I and BamHI restriction sites, respectively. Both inserts were then excised and cloned into the pW8-attB-*GFP* construct.

5′ K81-GFP::hiphop and 5′ hiphop-GFP::K81

The coding sequences of the *hiphop* and *K81* genes from the *5′hiphop-GFP::hiphop* and *5′K81-GFP::K81* constructs described above were exchanged using the *Not*I and *Bam*HI restriction sites.

Reverse Transcription Analysis

Total RNAs from adult *Drosophila willistoni* males or females were extracted using the Trizol method (Invitrogen). cDNAs were synthetized using oligo-dT primers and the Superscript II reverse transcriptase (Invitrogen). The following sets of primers were used to amplify *hiphop* (gene annotation symbol GK12110) and its paralog (gene annotation symbol GK15167), respectively: 5′-CTGTATTTGATACATTTTCC-3′ and 5′-AACTTTCGTTGATTTAGC-3′, 5′-CGAACAAATTGAGAAATGC-3′ and 5′-CTGTATATTTGGTAGTCGC-3′. Primers designed to amplify the *D. melanogaster Rp49* gene (5′-AAGATCGTGAAGAAGCGCAC-3′ and 5′-ACTCGTTCTCTTGAGAACGC-3′) were used to amplify the *willistoni Rp49* gene as a control.

Alignment and Phylogenetic Trees

The *hiphop/K81* CDSs were aligned using the MUSCLE algorithm in Seaview [53]. The alignment was then cleaned using Gblock in the permissive mode (see http://molevol.cmima.csic.es/castresana/Gblocks.html) [54]. A maximum-likelihood tree was built using the PhyML algorithm in Seaview with the general time-reversible (GTR) model, a gamma distribution (four site categories), an estimated alpha parameter, an estimated percentage of invariants, an estimated transition/transversion rate, and a nonparametric bootstrapping [53].

Evolutionary Rates Analysis

Site Model Analysis

We ran Datamonkey (one of the HyPhy modules, see http://www.datamonkey.org/) on the *hiphop/K81* raw alignment and retrieved the d_N-d_S output for each site of the alignment [55].

Branch Model Analysis

We ran codeml on the *hiphop/K81* Gblocked alignment. The phylogenetic tree of the *hiphop/K81* gene family was slightly modified to be fully consistent with the 12 *Drosophila* Species tree [56]. We tried various nested models (with up to four d_N/d_S ratios) and compared these models using the likelihood ratio test approach.

Branch-Site Analysis

We ran fitmodel on the *hiphop/K81* raw alignment and the same phylogenetic tree as for the branch model analysis [57] with the M2a model (three site categories) and with the possibility of switching from one category to another during evolution (model S1). The wbest output file was analyzed, and we counted the number of sites with evidence for switching in relevant lineages.

Supplemental Information

Supplemental Information includes four figures and one table and can be found with this article online at doi:10.1016/j.cub.2010.11.013.

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La spécialisation du gène K81 pour la coiffe des télomères spécifiquement dans la spermatide mature constitue un des intérêts majeurs de ce travail. Nous pouvons nous interroger sur la nature de la pression de sélection qui a conduit à ce scénario. Bien que cette question reste ouverte, l'incapacité de K81 à suppléer Hiphop dans les cellules somatiques (et vice-versa) permet de poser deux hypothèses non-exclusives: (1) un autre acteur du complexe de coiffe (pour l'heure inconnu) distinguerait la coiffe de type « spermatide », interagissant avec K81, de la coiffe de type « somatique », interagissant avec Hiphop. (2) la nécessité de K81 pourrait refléter celle de la coiffe à s'adapter à l'environnement particulier de la chromatine des spermatides, tandis que Hiphop serait spécialisé dans la stabilisation de la coiffe en contexte nucléosomique. K81 et Hiphop seraient des adaptateurs de la coiffe aux protéines constituant le chromosome. Explorer ces deux modèles est un objectif majeur pour la suite des travaux.

Un autre intérêt de ce travail est de mettre en lumière la nécessité fondamentale de protéger les télomères paternels dans la lignée germinale mâle. Cette marque épigénétique de l'identité télomérique paternelle est enlevée (de façon probablement passive) des chromosomes dès la première réplication du zygote, démontrant que ce mécanisme est véritablement dédié au gamète mâle. Ceci permet de supposer que le télomère constitue une région particulièrement sensible dans le chromosome paternel. Dans ce sens, le télomère pourrait être une cible privilégiée de modifications par Wolbachia. En effet, la grande similitude entre le phénotype mutant K81 et l'incompatibilité cytoplasmique interpelle sur la possibilité que cette bactérie « modifie » le télomère au moment de la spermatogénèse.

Nos connaissances sur la détermination épigénétique de territoires chromosomiques dans le sperme chez la drosophile sont entrain d'évoluer. Le centromère constitue un territoire à identité purement épigénétique, ce qui amène à l'idée que des marques centromériques pourraient être transmises paternellement. La protéine CENP-A est, en effet, retenue dans le sperme mature chez les bovidés, (bien que sa transmission jusqu'au zygote n'a pas été décrite)(Palmer *et al.*, 1990). J'ai voulu tester la capacité de la protéine CenH3 chez la drosophile (Cid) à rester associée à la chromatine après la transition histones-protamines et jusqu'au moment de la fécondation. J'ai montré que dans des œufs issus d'un croisement entre des pères exprimant une protéine fusion Cid-GFP et des mères non transgéniques, la protéine étiquetée était détectable dans le noyau mâle (Figure 8). Ceci confirme que la protéine Cid est transmise paternellement à l'œuf, comme cela avait été suggéré (Loppin *et*

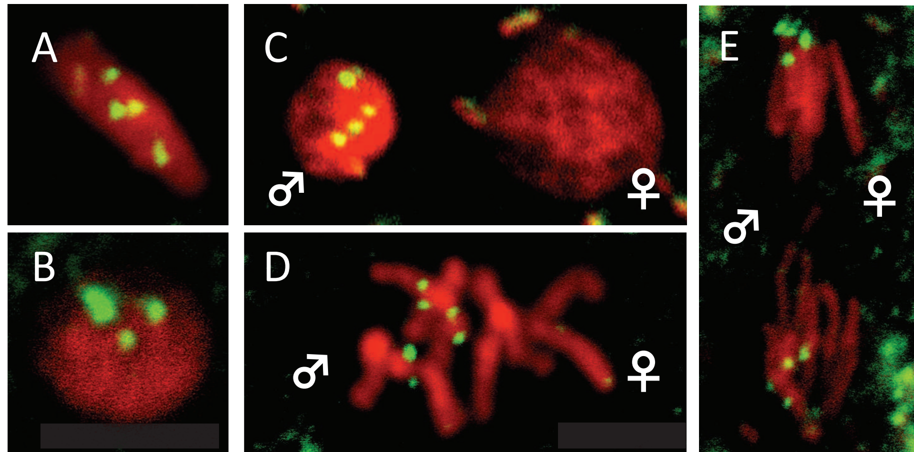

Figure 8. Transmission paternelle de Cid-GFP.
Images confocales d'oeufs issus de femelles sauvages croisées par des mâles exprimant Cid-GFP. L'ADN est en rouge, la GFP en vert. La protéine Cid-GFP est transmise paternellement et retrouvée dans le pronoyau mâle allongé (A) et en cours de décondensation (B) où les quatre centromères sont visibles. La protéine reste associée aux chromosomes patenrels pendant la première réplication zygotique et l'apposition des pronoyaux (C), la première métaphase (D) et anaphase (E) et est progressivement dilué au cours des premières divisions zygotiques (non montré). Barres: 10µm.

al., 2001). De plus, Cid-GFP était détectable dans les chromosomes d'origine paternelle après leur première réplication, et pendant au moins trois cycles de division. Il serait important de tester l'importance fonctionnelle de la rétention de Cid dans la chromatine du sperme : il est probable que ceci joue un rôle dans la participation des chromosomes paternels à la formation du zygote. Dans tous les cas, il s'agit du premier exemple connu d'un territoire chromosomique épargné par le remplacement massif d'histones par des protamines chez la drosophile.

Ainsi, la composition de la chromatine du sperme avant et pendant les premières étapes après la fécondation semble être plus complexe que ce que nous avons évalué précédemment. Ceci pose un défi supplémentaire à la batterie d'agents d'assemblage de la chromatine, qui doit respecter cette organisation pré-existante. L'étude fonctionnelle des mécanismes qui ont lieu à cette étape est difficile, dans la mesure où les mutations affectant spécifiquement ces processus sont rares. J'ai participé au cours de ma thèse à la caractérisation d'un autre gène à effet maternel qui pourrait avoir un rôle à ces étapes mais dont la fonction moléculaire est en passe d'être étudiée: *maternal haploid* (*mh*).

Etude de la mutation à effet maternel *mh*

La mutation *mh* a été générée par mutagénèse aléatoire dans les années '70 (Gans *et al.*, 1975; Santamaria et Gans, 1980). Cette mutation n'a pas de conséquences pour la survie des individus, mais est à l'origine d'un phénotype de stérilité femelle totale, associé à une létalité embryonnaire à effet maternel. Les embryons issus de femelles mutantes présentent un développement haploïde et ne sont pas viables (Santamaria, 1983). Il y a dix ans, le phénotype cytologique associé a été décrit : le défaut se manifeste dès la première division de l'œuf comme l'incapacité des chromosomes paternels à intégrer la première division du zygote, de façon extrêmement semblable aux situations mutante K81 ou d'IC (Loppin, 2001). Cependant, la nature du gène affecté par cette mutation était, jusqu'à récemment, inconnue, bloquant des études fonctionnelles.

J'ai entrepris la cartographie de ce gène, mais des problèmes d'ordre technique et l'absence d'outils disponibles m'ont contraint au choix de mettre en pause ce projet. L'étude a

récemment été reprise grâce à la mise à disposition récente de nouveaux outils génétiques. Le gène affecté par *mh* est *CG9203*, un gène à forte expression maternelle. *CG9203* code pour une protéine présentant des similitudes avec la famille RAD18, impliquées dans la réponse aux dommages à l'ADN et dans la recombinaison (Prakash, 1981; Tateishi *et al.*, 2003; Szuts *et al.*, 2006; Ting *et al.*, 2010). De nouvelles délétions générées sur le locus ne complémentent pas la mutation *mh* mais sont viables et présentent à l'état homozygote le même phénotype de stérilité femelle. Le gène *mh* semble donc avoir une fonction critique spécifiquement au moment de la fécondation. Un anticorps a été généré contre cette protéine, et des expériences préliminaires m'ont permis de détecter la protéine MH spécifiquement dans le pronoyau mâle en cours de décondensation, en accord avec le phénotype des mutants. Bien qu'à ses débuts, ce projet promet de mettre à jour un mécanisme unique nécessaire à l'intégration des chromosomes paternels dans le premier noyau du zygote.

Foyers de γH2AvD dans le pronoyau mâle

La fonction de MH pourrait être liée à une voie de réparation de l'ADN dans le pronoyau mâle. Dans d'autres modèles animaux, le pronoyau mâle présente spécifiquement des dommages à l'ADN (Derijck *et al.*, 2008; Hajkova *et al.*, 2010). Ces dommages pourraient résulter de l'exposition prolongée des chromosomes du sperme à des agressions naturelles par leur environnement, être une conséquence directe du processus de remplacement protamines-histones, ou, chez certaines espèces, contribuer à la reprogrammation épigénétique. Dans tous les cas, le pronoyau haploïde devrait avoir recours à des mécanismes indépendants de l'homologie pour réparer efficacement ces cassures tout en préservant son précieux contenu génétique. Pour poser les bases à cette idée chez la drosophile, j'ai montré que, de façon analogue à d'autres espèces, de nombreux foyers de localisation de l'histone H2AvD phosphorylée sont présents dans le pronoyau mâle, révélant probablement de nombreuses cassures de l'ADN (Figure 9). De plus, ces foyers disparaissent lors de la migration des pronoyaux, étape qui coïncide avec la première réplication zygotique, ce qui indique le caractère fugace de cette accumulation. Il est donc possible d'imaginer qu'un mécanisme de réparation soit spécifiquement recruté dans ce noyau, conditionnant la viabilité des chromosomes.

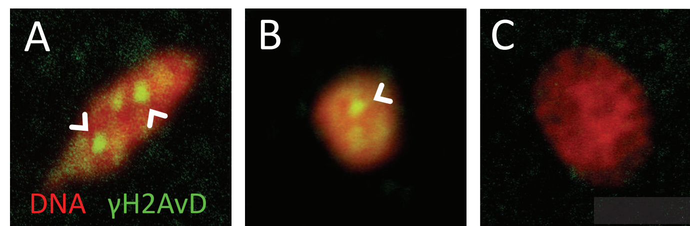

Figure 9. Accumulation de foyers H2AvD phosphorylés (γH2AvD) dans le pronoyau mâle.
Coupes confocales d'oeufs issus de femelles et mâles sauvages. Les oeufs ont été marqués pour révéler l'ADN (rouge) et l'histone γH2AvD (vert). L'histone γH2AvD est accumulée dans des foyers dans le pronoyau mâle allongé (A). Ce marquage est affaibli dans les pronoyaux arrondis plus matures (B) et disparaît dans les pronoyaux en cours de réplication (C), réflétant le caractère transitoire de ces foyers. Barre: 5µm.

Le génome maternel est enrichi en diverses méthylations des histones

Aucun dommage n'est plus critique pour la survie que ceux infligés aux gamètes, dont les conséquences en termes de mutations se propageront à toutes les cellules du nouvel individu. Il est donc crucial d'aligner une armada de résistance contre toutes les agressions que peuvent subir les génomes germinaux. Chez de nombreuses espèces, l'assemblage sous la forme de protamines du génome paternel pourrait répondre à ce besoin (Aoki *et al.*, 2005; Rathke *et al.*, 2010). Les chromosomes maternels ne bénéficient pas d'une organisation avec des protéines spécifiques, mais, en contre partie, présentent de façon conservée un jeu de MPTs des histones généralement associées à la chromatine compacte (Arney *et al.*, 2002; Ivanovska, 2005; Loppin *et al.*, 2005a). J'ai renforcé cette observation par la description de la distribution de H3K27Me2, H3K27Me3 et H3K9Me3 entre les chromosomes parentaux chez la drosophile (Figure 10). En somme, les chromosomes maternels sont spécifiquement enrichis en méthylations des lysines 4, 9 et 27. Bien que des études fonctionnelles manquent pour le tester, ceci reflète vraisemblablement une stratégie de protection du génome maternel. Dans le paragraphe qui suit, j'exposerai mes travaux sur la menace que représentent les éléments transposables pour l'intégrité de ce génome, à travers l'exemple des conséquences de la remobilisation naturelle de l'un d'entre eux.

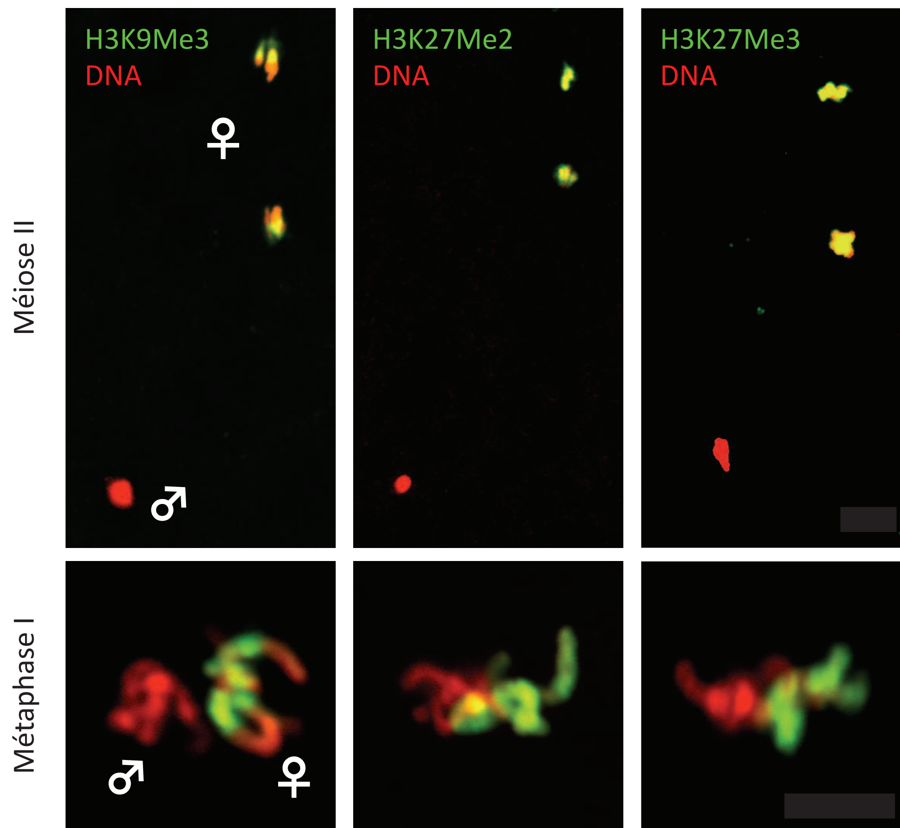

Figure 10. Distribution assymétrique de différentes méthylations de l'histone H3 entre les chromosomes paternels et maternels.
Images confocales d'oeufs pondus par des femelles sauvages et marqués pour révéler l'ADN en rouge et différentes méthylations de l'histone H3 (en vert). Les marques H3K9Me3, H3K27Me2 et H3K27Me3 marquent fortement les chromosomes maternels en méiose et pas pronoyaux mâles (Meiose II). Cette assymétrie persiste après la réplcation, pendant le premier cycle zygotique (Métaphase I) et 2 cycles plus tard au moins (non montré). Barres: 10µm.

III. Catastrophe méiotique associée à la remobilisation naturelle d'un transposon dans la lignée germinale femelle

Les éléments transposables sont souvent considérés comme des éléments génétiques égoïstes, qui assurent leur prolifération au détriment de leur génome hôte. Bien qu'il soit généralement accepté que les éléments transposables ont contribué positivement à l'évolution des espèces en façonnant ces mêmes génomes (Kazazian, 2004; Goodier et Kazazian, 2008), ils restent une menace pour leur intégrité (van der Heijden et Bortvin, 2009; Khurana et Theurkauf, 2010; Senti et Brennecke, 2010b). Ce danger est géré dans toutes les cellules par des mécanismes qui contrôlent leur expression, en évitant leur remobilisation massive qui serait délétère (Saito et Siomi, 2010). Ce contrôle cellulaire est constitué de voies moléculaires largement conservées au cours de l'évolution, ce qui reflète le caractère ancestral de cette « course aux armements » (revu dans Aravin *et al.*, 2007; Czech *et al.*, 2008; Hutvagner et Simard, 2008; Kawamura *et al.*, 2008; Bourc'his et Voinnet, 2010; Saito et Siomi, 2010). Pour se prémunir contre l'attaque par des éléments transposables, la cellule dispose d'un mécanisme de sauvegarde majeur mettant en jeu leur répression transcriptionnelle ou post-transcriptionnelle par des petits ARNs interférents. Plusieurs voies conservées participent à la répression des transposons. En particulier, une machinerie spécifique (impliquant des facteurs de type Argonaute 3, Aubergine et PIWI) est requise pour la prise en charge d'une famille de petits ARNi présents uniquement dans la lignée germinale, appelés PIWI-associated-RNAs (piARN) (Bourc'his et Voinnet, 2010; Khurana et Theurkauf, 2010; Senti et Brennecke, 2010a).

Le contrôle des transposons par cette voie suit un cycle qui assure une protection transmissible de génération en génération. Les mécanismes sont légèrement divergents entre les espèces mais il peut être conceptuellement divisé en trois étapes principales. Premièrement, des petits ARN d'origine maternelle et/ou paternelle (selon les espèces) retrouvés dans l'œuf sont comparés au contenu en transcrits du génome zygotique (c'est la confrontation). Quand des petits ARN sont associés à un transcrit cible dans l'œuf, une machinerie de dégradation est capable de digérer ces transcrits, ce qui aboutit in fine à la répression post-transcriptionnelle mais aussi à la génération de nouveaux petits ARN dirigés contre les mêmes cibles (c'est la répression). Progressivement, la présence d'abondants petits ARN correspondant à des cibles génomiques guidera l'établissement et/ou le maintien d'une

conformation hétérochromatique locale, contribuant à une répression renforcée et durable (c'est l'étape de consolidation) (Aravin *et al.*, 2007; Brennecke *et al.*, 2008; Bourc'his et Voinnet, 2010). Cependant, dans la lignée germinale (probablement suite à la reprogrammation de diverses marques épigénétiques), les transposons ont une nouvelle fenêtre d'opportunité pour être exprimés. Cette nouvelle menace est contrée par la pré-existence de piARN spécifiques qui, à leur tour, pourront entrainer une amplification du signal de répression. Finalement, les piARN produits pourront être transmis à la descendance, et le cycle sera pérenne.

Les mutations affectant les facteurs impliqués dans cette voie entraînent en effet chez la souris ou chez la drosophile une expression massive d'un grand nombre de familles de transposons dans la lignée germinale et résultent en un phénotype de stérilité mâle comme femelle (Vagin, 2006; Lim et Kai, 2007; Soper *et al.*, 2008). Le syndrome associé à cette stérilité est cependant très complexe, la voie des piARN intervenant probablement dans une diversité de fonctions dans de nombreux types cellulaires. La caractérisation des conséquences de l'expression massive d'un transposon en absence de toute mutation est principalement fondée sur l'étude de la dysgénésie hybride chez la drosophile (Bregliano *et al.*, 1980). Il s'agit d'une situation naturelle où un transposon présent dans le génome paternel (mais absent du maternel) n'est pas reconnu par des petits ARNs lors de la confrontation et ne rentre pas dans la boucle de contrôle (Brennecke *et al.*, 2008). Ceci a pour conséquence l'expression massive de l'élément dans la lignée germinale de la descendance, conduisant à un phénotype de stérilité femelle. Or, ce phénotype s'atténue avec l'âge et se stabilise finalement aboutissant à une descendance viable porteuse de nouvelles copies du transposon (Picard et L'Héritier, 1971; Picard *et al.*, 1978; Bucheton, 1979; Bucheton *et al.*, 1984).

La dysgénésie hybride de type *I-R* constitue en ce sens un exemple remarquable basé sur la transposition massive du facteur *I*, un rétrotransposon de type LINE-like, qui est capable de s'installer durablement dans un génome. En effet, face à la transposition massive du facteur *I*, l'hôte doit s'accommoder de l'installation de nouvelles copies contrôlées du transposon (qui constitueront à l'avenir une protection innée contre ce même type d'éléments), ou subir la perte de sa descendance. Le facteur *I* a d'ailleurs colonisé la quasi-totalité des populations sauvages de D.melanogaster au cours du XXème siècle, les rares souches dépourvues de cet élément étant celles qui ont été isolées en laboratoire avant les années 1950. Les transposons

se nichent dans le génome hôte qui sera transmis à la descendance : l'importance capitale de former à tout prix un noyau zygotique avec celui-ci permet à ce type d'éléments d'assurer la conquête de toute la population au fil des générations.

Le premier mutant affectant *Hira* a été isolé dans un crible de mutagénèse qui visait à identifier des régulateurs d'un transposon de type non-LTR LINE-like, appelé le facteur *I* (Tatout *et al.*, 1994). Dans une lignée portant un transgène rapporteur de l'activité de l'élément *I*, la mutation $Hira^{ssm}$ était associée à une régulation négative de cette construction dans la lignée germinale femelle. Nos résultats sur le rôle de HIRA dans l'hétérochromatine nous ont amené à nous interroger sur son possible rôle en tant que régulateur de l'activité des transposons. Cependant, pour explorer le rôle de HIRA dans l'activation de *I*, il aurait fallu disposer d'une lignée associant une mutation affectant *Hira* à un contexte Réactif. Malheureusement, nos souches portant des mutations sur *Hira*, comme la plupart des souches sauvages et de laboratoire, est envahie par le transposon *I* prohibant tout test sur la stérilité SF. Néanmoins, lors de nos expériences préliminaires, nous avons été amenés à observer les œufs issus de femelles SF, et leur phénotype a immédiatement attiré notre attention.

Ces embryons présentaient alternativement des caractéristiques de mort précoce ou tardive (comme celles qui sont typiques chez les embryons issus de femelles mutantes pour *mh* de mâles mutants pour *K81*). Nous avons donc voulu observer le phénotype de ces œufs à la fécondation. Nous avons décrit un phénomène remarquable de catastrophe méiotique qui rend les chromosomes d'origine maternel incompétents pour la formation du zygote. Le complément maternel étant partielle ou totalement absent à la première division du zygote, les embryons qui en découlent sont haploïdes ou aneuploïdes. L'échec de la méiose dans ces œufs suffit donc à expliquer la stérilité SF. Nous avons de plus montré que les noyaux des ovocytes présentent un phénotype de désorganisation chez les femelles SF, ce qui pourrait être en lien causal avec l'échec de la méiose. Cependant, nous avons montré que ce phénomène ne dépend probablement pas d'un degré important de cassures dans l'ADN, et n'est probablement pas en lien avec l'activation d'un checkpoint de réponse aux dommages à l'ADN. L'article qui suit détaille ces découvertes et leurs implications.

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Drosophila I-R hybrid dysgenesis is associated with catastrophic meiosis and abnormal zygote formation

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Summary

The *Drosophila I-R* type of hybrid dysgenesis is a sterility syndrome (SF sterility) associated with the mobilization of the *I* retrotransposon in female germ cells. SF sterility results from a maternal-effect embryonic lethality whose origin has remained unclear since its discovery about 40 years ago. Here, we show that meiotic divisions in SF oocytes are catastrophic and systematically fail to produce a functional female pronucleus at fertilization. As a consequence, most embryos from SF females rapidly arrest their development with aneuploid or damaged nuclei, whereas others develop as non-viable, androgenetic haploid embryos. Finally, we show that, in contrast to mutants affecting the biogenesis of piRNAs, SF egg chambers do not accumulate persistent DNA double-strand breaks, suggesting that I-element activity might perturb the functional organization of meiotic chromosomes without triggering an early DNA damage response.

Key words: Hybrid dysgenesis, I element, Meiotic catastrophe, Haploid embryos, Meiotic DNA damage checkpoint

Introduction

Transposable elements (TEs) are essential structural and regulatory components of genomes. Their ability to transpose provides a fundamental source of genetic variation but also represents a potential threat for genome integrity. Genomes have deployed a diversity of epigenetic defensive mechanisms against TEs and their concerted action results in the global, efficient and heritable repression of mobile elements throughout generations (Aravin et al., 2007; Siomi et al., 2008; Slotkin et al., 2007). In *Drosophila*, epigenetic control of TEs depends on histone modifications, chromatin structure, small RNA-based transcriptional silencing and DNA methylation (Aravin et al., 2007; Josse et al., 2007; Klenov et al., 2007; Dramard et al., 2007; Phalke et al., 2009; Klattenhoff et al., 2007; Klattenhoff et al., 2009; Li et al., 2009; Malone et al., 2009). Recent literature has abundantly described the mechanisms of Piwi-interacting small RNAs (piRNAs) biogenesis, as well as their essential role for the repression of TEs in germ cells (Brennecke et al., 2007; Vagin et al., 2006; Saito et al., 2006; Aravin et al., 2007; Siomi et al., 2008; Klattenhoff and Theurkauf, 2008). Accordingly, several families of TEs are derepressed in the germline of mutants affecting the piRNA pathway (Vagin et al., 2006; Chambeyron et al., 2008; Pane et al., 2007; Lim and Kai, 2007; Chen et al., 2007; Klattenhoff et al., 2007; Klattenhoff et al., 2009; Vagin et al., 2004). Remarkably, all these mutants are viable but induce female sterility associated with a complex phenotype including defects in germline stem cell maintenance, accumulation of germline DNA damage and aberrant egg axial patterning (Klattenhoff and Theurkauf, 2008). However, it is difficult to determine the actual contribution of TE activity to their complex sterility phenotype (Klattenhoff et al., 2007; Thomson and Lin, 2009).

In *Drosophila*, massive and deleterious TE germline mobilization is also observed in the progeny of certain intraspecific crosses. This phenomenon, known as hybrid dysgenesis, has long been recognized as a powerful experimental model for the study of TE regulation in a wild-type background (Bregliano et al., 1980). *Drosophila* hybrid dysgenesis systems are usually characterized by a severe gonadal atrophy in both sexes, resulting in sterility. These include the *D. melanogaster P-M* (P element) and *H-E* (hobo element) systems, as well as a hybrid dysgenesis in *D. virilis*, which involves several families of TEs (Kidwell and Novy, 1979; Blackman et al., 1987; Yannopoulos et al., 1987; Petrov et al., 1995; Blumenstiel and Hartl, 2005). The *I-R* type of hybrid dysgenesis is unique as it only occurs in females and does not result from a defective ovarian development. Instead, dysgenic females lay a normal amount of eggs but the resulting embryos fail to hatch (Picard and L'Héritier, 1971). The causative factor of this non-Mendelian female sterility is the *I* element, a 5.4 kb, non-LTR retrotransposon of the LINE (long interspersed nucleotidic element) superfamily of transposable elements (Bucheton et al., 1984). Most *D. melanogaster* strains are so-called *Inducer* (*I*) strains and contain about 10 transposition-competent but transcriptionally silenced *I* elements. Such functional *I* elements are absent from *Reactive* (*R*) strains that were established before the recent worldwide invasion of this retrotransposon in natural populations (Bucheton et al., 2002). Maternal transmission of piRNAs has been proposed to underlie the epigenetic repression of TEs revealed by *Drosophila* hybrid dysgenesis systems (Blumenstiel and Hartl, 2005; Brennecke et al., 2008; Chambeyron et al., 2008). In the case of the *I-R* system, maternal epigenetic protection is largely reduced in *R* strains, resulting in the expression of paternally transmitted *I* elements in the naive germline of dysgenic females (Brennecke et al., 2008; Chambeyron et al., 2008).

I-R hybrid dysgenesis occurs when *I* males are crossed with *R* females. The female progeny of this dysgenic cross, called SF (stérilité femelle) females, usually display a strong sterility phenotype associated with derepression of *I* elements. In addition, the *I-R* syndrome is characterized by a high mutation rate as well

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as chromosomal non-disjunctions and rearrangements (Bucheton et al., 2002). In contrast to SF females, the genetically identical RSF females obtained from the reverse cross (*R* males with *I* females) show much lower expression of *I* elements and are fully fertile (Picard and L'Héritier, 1971; Bucheton et al., 2002). From the early work of Picard et al. (Picard et al., 1977) and Lavige (Lavige, 1986), it was established that embryos produced by SF females died through a strict maternal effect and frequently presented abnormal syncytial divisions. However, despite extensive research on this system, the nature of SF sterility has remained enigmatic since its discovery (Picard and L'Héritier, 1971).

In this paper, we have undertaken a detailed cytological study of *I-R* hybrid dysgenesis to determine the origin of SF sterility. Our study revealed that meiotic divisions are catastrophic in SF oocytes and eggs. This highly penetrant phenotype prevents the integration of the full set of maternal chromosomes in the zygote, resulting in non-viable embryos. We also show that, in contrast to mutants affecting the biogenesis of piRNAs, SF germ cells do not accumulate massive DNA damage during early oogenesis, suggesting that *I* activity perturbs the functional organization of meiotic chromosomes without activating the early germline DNA damage response.

Results

Meiotic catastrophe in eggs of SF females

We performed a cytological study of SF eggs and embryos to understand the nature of SF maternal-effect embryonic lethality. We used SF females that were not older than a week as SF sterility decreases progressively with age (see below). Consistent with early cytological studies (Lavige, 1986), we observed that a majority of syncytial SF embryos contained catastrophic mitotic figures with isolated or broken chromosomes and asynchronously dividing nuclei of various sizes (Fig. 1). In addition, in SF embryos, we observed that the polar body did not form the typical triploid rosette and contained many fragmented chromosomes (Fig. 1D–F). This last aspect of the phenotype suggested that meiosis was defective in SF eggs. We then turned to late oocytes to observe the first meiotic division. In *Drosophila*, the mature stage-14 oocyte is arrested in metaphase of meiosis I (King, 1970). To visualize the organization of meiotic chromosomes and the first meiotic spindle, we used control and SF females expressing the fluorescent centromeric protein EGFP-Cid (Schuh et al., 2007) or the microtubule-associated Jupiter-GFP (Buszczak et al., 2007), respectively. In fixed control stage-14 oocytes (n=30), meiotic chromosomes appeared as a slightly elongated mass of chromatin with non-exchange chromosomes occasionally separated towards the spindle poles (Fig. 2) (Theurkauf and Hawley, 1992). In about 80% of SF oocytes (22/28), the chromatin appeared fragmented and/or abnormally distributed into several small masses (Fig. 2). Some of these masses of chromatin were associated with an EGFP-Cid spot, whereas others were not, thus suggesting the presence of fragmented chromosomes. These isolated or fragmented chromosomes formed miniature spindle-like structures as revealed with the Jupiter-GFP marker (Fig. 2). In the rest of the SF oocytes (6/28), the first meiotic division was apparently normal, although the low resolution of meiosis I chromosomes did not allow the detection of possible more subtle defects.

We then analyzed very early SF eggs to observe the second meiotic division and pronuclear formation. Strikingly, meiosis II in SF eggs was almost systematically abnormal with bridges of chromatin connecting the separating chromatids in anaphase and telophase (Fig. 3C,D; Table 1). This defective separation of chromatids was followed by chromosome fragmentation and unequal segregation of meiotic products. Notably, the loss of genetic material in the female pronucleus was obvious at the pronuclear apposition stage. In control RSF eggs, apposed pronuclei appeared identical in size (Fig. 3E). In SF eggs, however, the female pronucleus was either small, fragmented in several smaller nuclei or, in some instances, did not form at all (Fig. 3F,G; data not shown). We thus concluded that, in eggs from SF females, defective meiotic divisions compromised the formation of a normal female pronucleus.

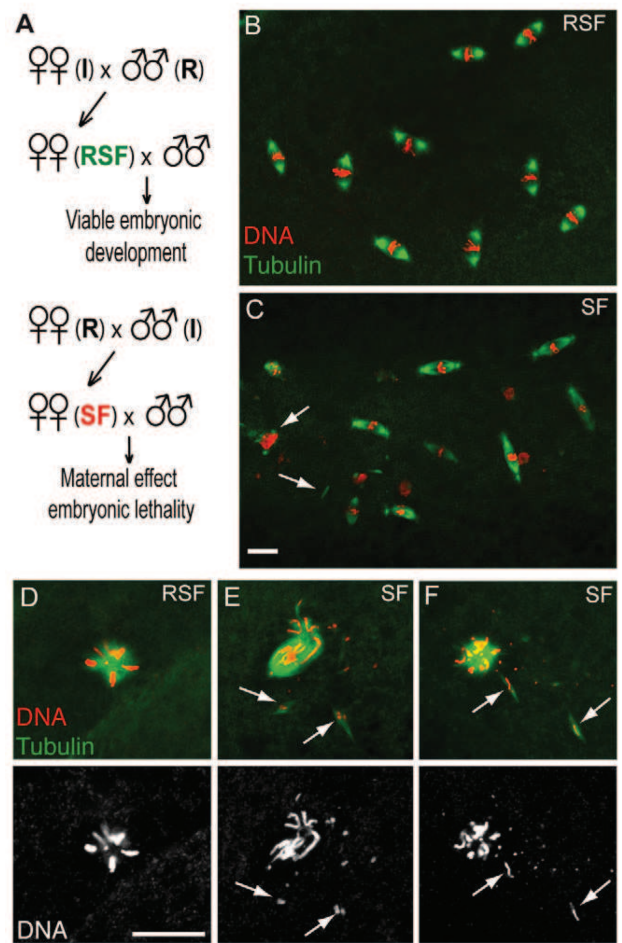

Fig. 1. The maternal-effect embryonic lethality associated with *I-R* hybrid dysgenesis. (**A**) Crossing scheme to obtain RSF (upper) and SF (lower) females. SF females lay eggs but the resulting embryos die before hatching. Genetically identical RSF females are fully fertile. (**B**,**C**) Confocal images of early syncytial embryos from RSF (B) or SF (C) females stained for Tubulin (green) and DNA (red). In contrast to the normal nuclear divisions observed in RSF embryos, SF embryos contain asynchronously dividing nuclei of various sizes and fragmented chromosomes (arrows). (**D**–**F**) In RSF embryos (D), fused polar bodies form a typical rosette of condensed chromosomes. In SF embryos (E,F), polar body organization is abnormal and many chromosomes are lost or fragmented (arrows). Scale bars: 15 μm.

Embryos from SF females develop with paternal chromosomes

In *Drosophila* fertilized eggs, pronuclei do not fuse but instead remain apposed during the first zygotic S phase and the paternal

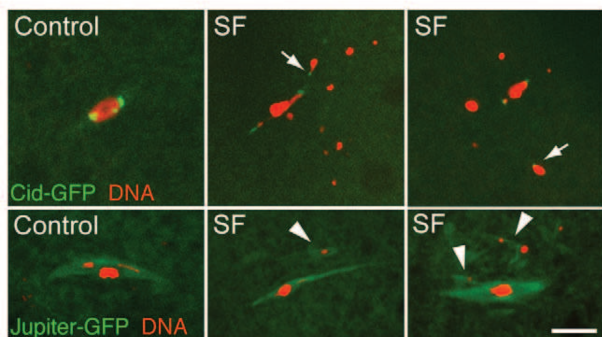

Fig. 2. Meiosis I is catastrophic in oocytes from SF females. Confocal images of meiosis I in stage-14 oocytes from females expressing the indicated marker. Control females are from the transgenic *EGFP-Cid* line and *Jupiter-GFP* line. SF females were obtained by crossing inducer *EGFP-Cid* or *Jupiter-GFP* males with *Charolles* females at 25°C. At day 5 of adult life, ovaries were dissected, fixed and stained for DNA (red). Scale bar: 10 µm. (**Top**) EGFP-tagged centromeric histone Cid (EGFP-Cid) marks centromeres in meiosis I chromosomes. In control oocytes, chromosomes show aligned centromeres in prometaphase. In SF oocytes, chromosomes appear fragmented or mislocalized. Centromeres are indicated with arrows. (**Bottom**) The control is an anastral first meiotic spindle in prometaphase marked with microtubule-associated Jupiter-GFP. In SF oocytes, mini-spindles organize around mislocalized or fragmented chromosomes (arrowheads).

and maternal sets of chromosomes enter mitosis as separate entities within a common mitotic spindle (Sonnenblick, 1950). In a majority of SF embryos at first mitosis, we observed that the spindle did not contain the full complement of chromosomes compared with RSF zygotes (Fig. 3H,I; Table 1). In other cases, some chromosomes were excluded from the spindle or lagged behind in anaphase of the first division (Fig. 3J,K; Table 1). To determine the identity of these absent or abnormal chromosomes, we stained SF eggs with an antibody directed against acetylated forms of histone H4 that preferentially marks paternal chromatin (Loppin et al., 2005a). We observed that, in SF eggs, from the pronuclear apposition until the end of the first zygotic division, the damaged or late chromosomes were systematically less-intensely stained than the unaffected chromosomes (Fig. 4A-F). In some cases, a single haploid set of strongly stained chromosomes was present at the first mitosis (Fig. 4E). We confirmed these observations by analyzing the progeny of transgenic SF females expressing the recombinant histone variant H3.3-Flag, a specific marker of paternal chromosomes at fertilization (Bonnefoy et al., 2007; Orsi et al., 2009). This experiment clearly confirmed the specific defective integration of maternal chromosomes in SF zygotes (supplementary material Fig. S1).

As previously reported by Picard et al. (Picard et al., 1977) and Lavige (Lavige, 1986), we observed that approximately 7% (*n*=1134) of SF embryos died at a late developmental stage as revealed by the fact that they turned brown after death and showed signs of organogenesis and cuticle deposition. By contrast, the rest of the unhatched eggs remained whitish, suggesting that they arrested development before cellularization (Fig. 4K). In the *Drosophila* mutant *maternal haploid* (*mh*), paternal chromosomes are unable to divide in anaphase of the first mitosis and form a chromatin bridge (Santamaria and Gans, 1980; Loppin et al., 2001). This frequently results in catastrophic early mitoses and most embryos die after a few rounds of nuclear divisions. However, a fraction of embryos escape this early arrest and

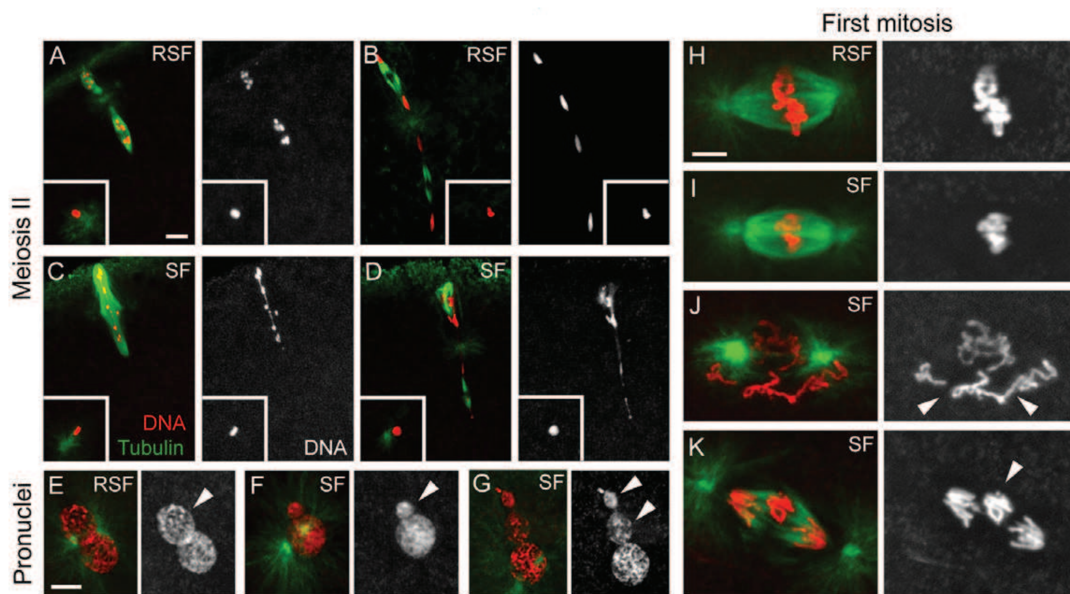

Fig. 3. Catastrophic meiosis and abnormal zygote formation in eggs from SF females. Confocal images of eggs and early embryos stained for Tubulin (green) and DNA (red). (**A–D**) Meiosis figures are shown with dorsal egg periphery at the top and the anterior end to the left. The corresponding male pronuclei are shown in insets. (A,B) RSF eggs in anaphase (A) or telophase (B) of the second meiotic division. (C,D) Meiosis II in SF eggs is catastrophic. Note the chromatin bridges in anaphase (C) and the unequal chromosome segregation in telophase (D). Loss of genetic material in the two innermost meiotic products is obvious in D. (**E**) Pronuclear apposition in an RSF egg. (**F**,**G**) In SF eggs, the female pronucleus looks abnormally small (F) or fragmented into several smaller nuclei (G). The female pronuclei are indicated with arrowheads. (**H–K**) First zygotic division. Metaphase of the first zygotic division in an RSF egg (H) containing the paternal and maternal chromosomes. First mitosis in an SF egg with either a reduced number of chromosomes (I), with chromosomes that appear excluded from the spindle (arrowheads in J) or with lagging chromosomes in anaphase (arrowheads in K). Scale bars: 10 µm.

Table 1. Phenotype quantification of SF eggs and embryos

	Meiosis II		First zygotic division		Cycle 2 –7 embryos		
	n	Abnormal (%)	*n*	Abnormal (%)	*n*	Aneuploid (%)	Haploid (%)
SF	40	97.5	56	96.4	90	82.2	11.1
RSF	41	2.4	25	0	50	0	0

SF or RSF females grown at 25°C were allowed to lay eggs between days 4 and 6 after emergence (day 1). Eggs at 0–1 hours were collected, fixed and stained for DNA. Phenotypes of SF eggs (meiosis II and zygote) and early embryos are described in the Results. *n*, the total number of eggs and/or embryos analyzed.

develop as non-viable, haploid gynogenetic embryos (Loppin et al., 2001). At the cytological level, early development of SF embryos appeared similar to *mh* embryos, with catastrophic syncytial divisions forming chromatin bridges (Fig. 1C; supplementary material Fig. S2). In addition, a minority of SF embryos developed beyond the blastoderm stage and contained normal mitotic figures but the nuclei were about half the size of control diploid nuclei (Fig. 4G-J). To demonstrate that these escaper embryos were actually haploid androgenetic embryos, we crossed SF females with males homozygous for the *K81* paternal effect mutation, which prevents the formation of functional paternal chromosomes in the progeny (Fuyama, 1984; Yasuda et al., 1995; Loppin et al., 2005b). As expected, these SF females failed to produce any brown embryos during their first week, confirming that late embryos from SF females developed with paternal chromosomes (Fig. 4L,M; supplementary material Table S1). In conclusion, our results demonstrated that most embryos from SF females die early with catastrophic mitoses, whereas a minority escape this early arrest as haploid androgenetic embryos.

Defective karyosome formation in SF oocytes

The meiotic phenotype observed in SF females prompted us to analyze the structure of the oocyte nucleus during SF oogenesis. In *Drosophila*, female meiosis initiates in region 2A of the germarium, at the anterior tip of each ovariole. After meiotic recombination, in later egg chambers, the oocyte nucleus enlarges while the condensed maternal chromosomes in prophase I of meiosis remain packaged within a subnuclear structure known as the karyosome (Spradling, 1993). In stage 6–9 control oocytes stained for DNA, the karyosome appeared as a round condensed structure within the unstained oocyte nucleus (Fig. 5A). By striking contrast, we observed that the karyosome was disorganized in a majority of SF oocytes (Fig. 5A; supplementary material Fig. S2). Typically, the SF karyosomes were fragmented and stretched along the inner side of the oocyte nuclear envelope. A remarkable and well-described feature of SF sterility is its modulation by age and temperature. Indeed, SF sterility is highest in young females but their fertility is progressively restored as they age (see supplementary material Table S1) (Picard and L'Héritier, 1971). In addition, SF sterility is strongest and lasts longer at relatively cooler temperatures and fertility can be transiently restored after a heat treatment (Bucheton, 1979). Interestingly, we observed that the penetrance and severity of the karyosome phenotype decreased with the age of SF females. In addition, most karyosomes were severely affected when SF females where placed at 18°C for 36 hours before dissection, whereas a heat treatment at 30°C dramatically suppressed the phenotype (Fig. 5B,C). Taken together, these observations suggest that defective karyosome formation in SF oocytes results in abnormal meiotic divisions.

SF germ cells do not accumulate unrepaired DNA double-strand breaks

In *Drosophila* female germ cells, the accumulation of unrepaired DNA double-strand breaks (DSBs) can trigger the activation of a well-characterized ATR-Chk2 (Mei-41-Lok) DNA damage response (Ghabrial and Schupbach, 1999; Abdu et al., 2002). In mutants that affect the repair of meiotic DNA DSBs, activation of the Chk2 checkpoint leads to a complex cellular response. This includes a specific disorganization of the karyosome and a strong egg ventralization phenotype that results from defective accumulation of the signaling protein Gurken in the oocyte (Ghabrial and Schupbach, 1999; Abdu et al., 2002). Interestingly, the Chk2 checkpoint is activated in the female germline of piRNA pathway mutants (Chen et al., 2007; Klattenhoff et al., 2007). In addition, these mutants are associated with egg patterning defects and defective karyosome formation (supplementary material Fig. S3) (Chen et al., 2007; Klattenhoff et al., 2007). Genetic analyses have demonstrated that, in these mutants, the checkpoint is not activated by meiotic DSBs, thus opening the possibility that these DNA damages could be induced by the activity of derepressed TEs (Chen et al., 2007; Klattenhoff et al., 2007; Klattenhoff et al., 2009).

As mutant stocks are generally not available in a reactive background, the activation of the checkpoint in SF germ cells could not be genetically tested. We thus examined the dorsal patterning of SF eggs to check for indications of DNA damage response. We observed that a fraction of SF eggs displayed a weak ventralization phenotype. In fact, fusion of egg dorsal appendages was only observed with very young SF females not older than 3 days (Table 2; Fig. 4K, arrow). Importantly, after a few days, SF females that were still fully sterile produced almost 100% of eggs with wild-type appendages. By clear contrast, *aub* or *armi* mutant females produced a majority of severely ventralized eggs throughout their life (Table 2). Interestingly, Van De Bor et al. (Van De Bor et al., 2005) have shown that *I* and *gurken* (*grk*) transcripts compete for the same RNA localization machinery in SF egg chambers, resulting in defective dorsoventral axis specification. This mechanism could indeed account for the ventralization of eggs produced by young SF females, where strong *I* transcription is expected to efficiently perturb *grk* mRNA localization. In conclusion, the egg patterning analysis did not support the hypothesis of early Chk2 checkpoint activation in SF germ cells. However, we wished to directly evaluate the impact of *I*-element activity on DNA integrity during early oogenesis. We thus stained SF and control ovaries with antibodies against the phosphorylated form of histone H2Av (γ-His2Av), which associates with DNA DSBs (Mehrotra and McKim, 2006). In wild-type or RSF ovaries, γ-His2Av foci were observed in oocytes of germarium regions 2A and 2B but were no longer detected in late-pachytene oocytes in their region 3 egg chambers (Fig. 6). In region 3 oocytes from *aub* mutant females, late-pachytene nuclei accumulated numerous γ-His2Av foci, as previously reported (Klattenhoff et al., 2007). By clear contrast,

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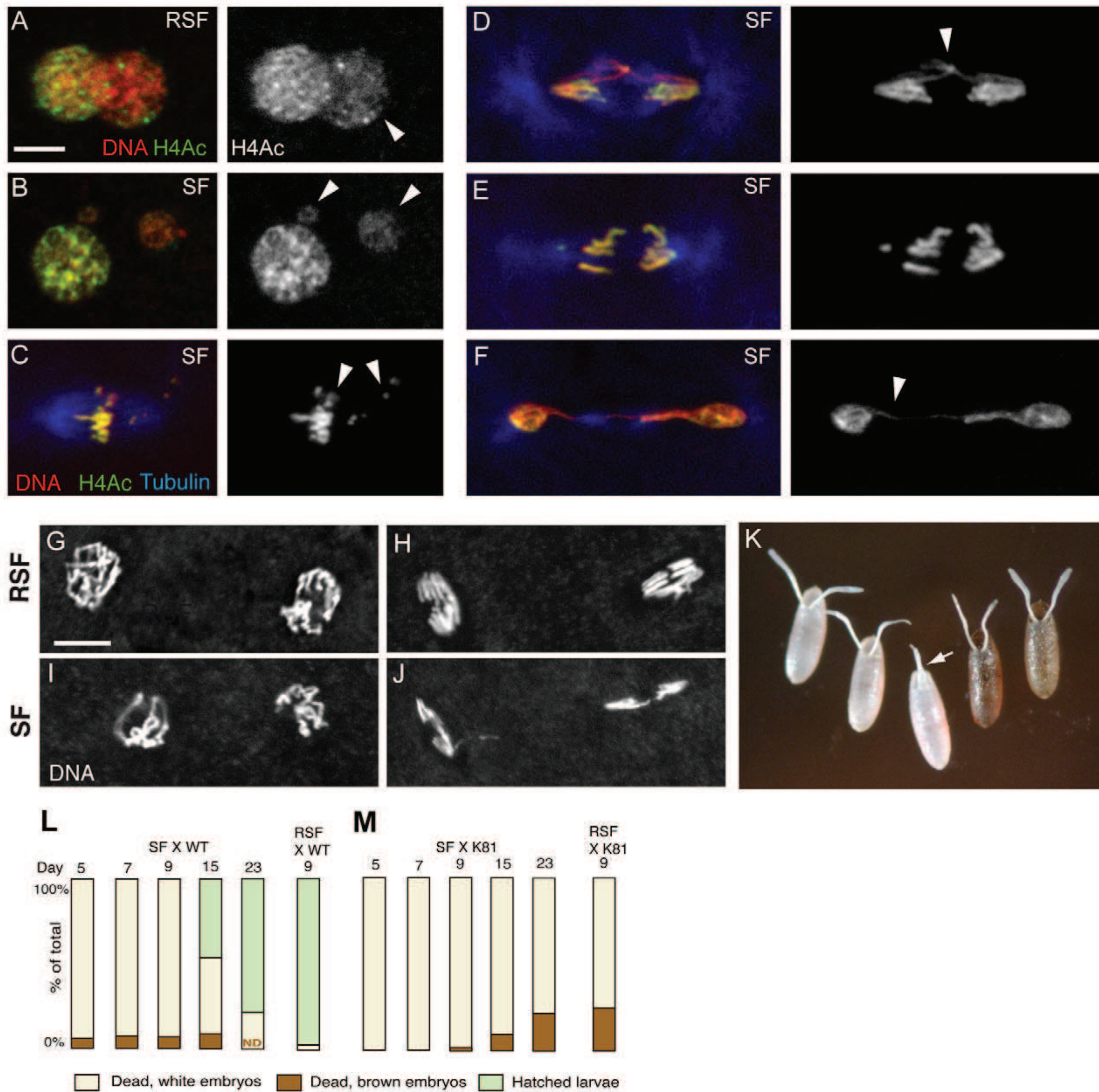

Fig. 4. Early loss of maternal chromosomes in SF embryos. (**A–J**) Confocal images of eggs and embryos stained with the indicated markers. (A,B) RSF (A) and SF (B) pronuclei stained with an anti-acetylated H4 antibody (green or white) that preferentially marks paternal chromatin (Loppin et al., 2005a). The female pronuclei are indicated with arrowheads. (C–F) First mitosis in SF eggs stained for acetylated H4 (green or white), DNA (red) and Tubulin (blue). Maternal chromosomes appear red and paternal chromosomes are yellow. Maternal chromosomes are abnormally positioned in the spindle or fragmented (arrowheads in C), lagging behind in anaphase or telophase (arrowheads in D and F) or absent (E). (G–J) Diploid nuclei from blastoderm RSF embryos in prophase (G) and anaphase (H). SF embryos that reach the blastoderm stage contain haploid nuclei (I, prophase; J, anaphase). Nuclei were stained with Propidium Iodide. (**K**) Unhatched eggs from SF females appear either whitish, indicative of early developmental arrest (the three eggs on the left), or brown, indicative of haploid development (the two eggs on the right). The arrow points to a weakly ventralized egg with the dorsal appendages fused at their base. (**L**) Diagrams showing the color phenotype of unhatched embryos produced from the same batch of SF females at the indicated days (day 1 is the day of emergence). Note that SF females progressively recover fertility as they age. ND, not determined. (**M**) When SF females are crossed with *K81* mutant males, brown embryos are not produced during the first week of life. Note that RSF females as well as aging SF females crossed with *K81* males produce an expected fraction of haploid gynogenetic embryos that turn brown after death. Scale bars: 10 μm. Numbers of examined embryos are in supplementary material Table S1.

such an accumulation of DNA DSBs was not observed in SF region 3 oocytes (n=10). In fact, half of region 3 SF oocytes were devoid of γ-His2Av foci, as in RSF controls. Interestingly, however, a few (1–3) γ-His2Av foci were observed in the other half of the late-pachytene SF oocytes but they never persisted beyond that stage. Thus, *I* activity either occasionally delays the repair of meiotic DSBs or, alternatively, generates a small number of non-persistent DSBs unrelated to meiotic recombination.

BicD aggregates are not observed in SF egg chambers

In wild-type inducer ovaries, endogenously expressed *I* transcripts are essentially sequestered in nurse cell nuclear foci in a piRNA-

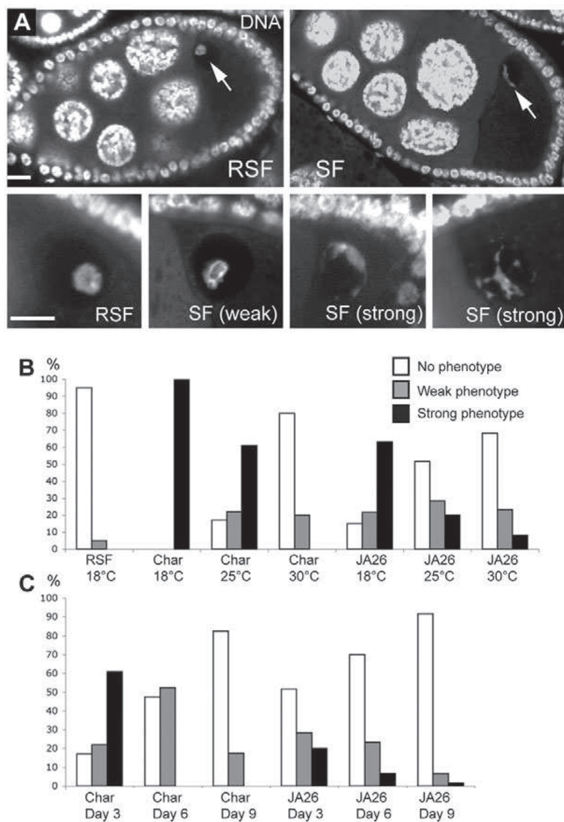

Fig. 5. Defective karyosome formation in SF oocytes. (**A**) Confocal images of stage 7–8 egg chambers dissected from RSF or SF females and stained for DNA (top). Egg chamber stages are from King (King, 1970). The oocyte is on the right, the karyosome is indicated with an arrow. (Bottom) In oocytes from RSF females, the karyosome appears spheric and condensed within the unstained oocyte nucleus. In SF oocytes, the karyosome is frequently abnormal, being slightly heterogeneous or elongated in aspect (weak phenotype) or displaying a severe distortion, fragmentation or attachment to the nuclear envelope (strong phenotype). (**B**) Effect of temperature on SF karyosome phenotype. Two-day-old RSF or SF females obtained at 25°C using the *Charolles* or *JA26* reactive stocks were placed at the indicated temperature for 36 hours before ovary dissection and DNA staining. For each condition, a minimum of 40 karyosomes from stage 6–9 oocytes were observed and classified according to the phenotypic classes described in A. Results are shown as a percentage of all observed karyosomes. (**C**) Effect of age on SF karyosome phenotype. SF females obtained at 25°C using the *Charolles* or *JA26* reactive stocks were dissected at the indicated age and ovaries were stained for DNA. Karyosome phenotype was analyzed as in B. Note that the same 25°C, 3-day-old SF female data is shown in B and C. Scale bars: 20 μm.

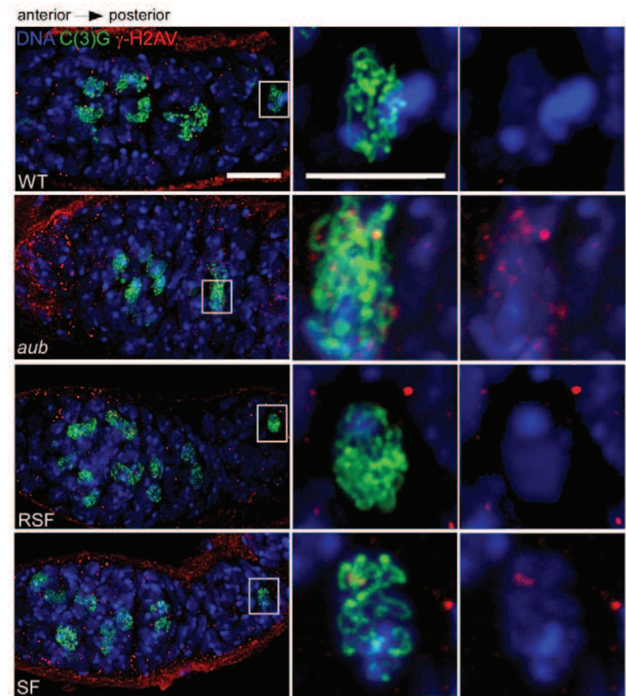

Fig. 6. γ-His2AvD distribution in the SF germline. Confocal images of wild-type (WT), aub^{QC42} or aub^{HN} (*aub*), RSF and SF germaria stained to visualize DNA (blue), C(3)G (green) and γ-His2AvD (red). Full views of germaria with their anterior tip on the left are shown in the left panels. Increased magnifications of late-pachytene oocytes (insets) are on the right. In WT and RSF germaria, γ-H2AvD foci are not detected in late-pachytene oocytes [identified by the C(3)G staining], indicating that meiotic DNA double-strand breaks are repaired at this stage. In generally disorganized *aub* mutant germaria, where oocyte determination is delayed, >10 γ-His2AvD foci accumulate in late-pachytene oocyte nuclei, shown here in an early region 3 oocyte. In SF germaria, 0–3 γ-His2AvD foci are observed in late-pachytene oocytes. A total of 10 late-pachytene oocytes were examined for each genotype. Scale bars: 5 μm.

dependent manner (Chambeyron et al., 2008), whereas overexpressed GFP-labeled *I* transcripts have been shown to accumulate in cytoplasmic particles called pi-bodies that localize around nurse cell nuclei (Lim et al., 2009). In SF egg chambers, *I* transcripts are essentially transported in the oocyte (Seleme et al., 2005; Chambeyron et al., 2008). Recently, it has been shown that large ribonucleoprotein (RNP) aggregates of the dynein-motor machinery form in egg chambers of piRNA biogenesis mutants (Navarro et al., 2009). Interestingly, injected *I* transcripts accumulate in these aggregates, suggesting that they could serve as degradation sites for retrotransposon products, in the absence of piRNA biogenesis (Navarro et al., 2009). Furthermore, these authors have also shown that formation of these dynein aggregates was largely dependent upon the activation of the Chk2 checkpoint.

To investigate the possibility that these structures could form in SF egg chambers, we stained ovaries with anti-BicD or anti-Orb antibodies that were shown to accumulate in dynein aggregates (Navarro et al., 2009). We indeed observed aggregates in a large majority of *aub* or *armi* mutant egg chambers. By clear contrast, however, Orb or BicD aggregates were only rarely observed in SF and RSF egg chambers (Fig. 7A,B; data not shown). We conclude that *I*-element activity is not sufficient to trigger the formation of these aggregates in dysgenic ovaries. In the course of these experiments, we observed that the oocyte marker BicD was abnormally distributed in the germinal vesicle of a majority of *aub* and *armi* mutant stage 6–9 egg chambers (Fig. 7A,C). This phenotype was fully rescued in *aub mnk* double-mutant females, indicating that it was dependent on checkpoint activation (Fig. 7A,C). Importantly, we observed that, in SF and RSF oocytes, BicD was normally excluded from the germinal vesicle. Taken together, these results reinforce the conclusion that SF sterility is independent of Chk2 checkpoint activation and downstream cellular responses.

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Table 2. Egg patterning of SF eggs

Maternal genotype	Dorsal appendage phenotype (%) Wild-type	Fused	Absent	Hatch rate (%)	*n*
aub^{HN}/*aub*^{QC42}	23.1	52.4	24.5	0	481
aub^{HN} *mnk*^{P6}/*aub*^{QC42} *mnk*^{P6}	99.2	0.8	0	0	354
armi^{1}/*armi*$^{72.1}$	0.2	11.7	88.1	0	463
mnk^{P6}/*mnk*^{P6}; *armi*^{1}/*armi*$^{72.1}$	76.6	16.1	7.3	0	137
RSF	100.0	0	0	96.2	498
SF (days 1–3)	69.3	30.4	0.3	0	743
SF (days 4–5)	99.2	0.8	0	0.2	651

Egg ventralization phenotypes are described in Staeva-Vieira et al. (Staeva-Vieira et al., 2003). SF females that emerged on day 1 were crossed with wild-type males and eggs from the same females were collected and analyzed after day 3 (days 1–3) and day 5 (days 4–5). The phenotype of eggs from other females remained unchanged over the same period of 5 days (data not shown).

Discussion

Extensive research on *Drosophila* hybrid dysgenesis systems has brought an essential contribution to the paradigm of TE epigenetic silencing. However, these models have comparatively received limited attention regarding the actual effect of TE activity in germ cells. In this context, the maternal-effect embryonic lethality associated with SF sterility appeared particularly difficult to link with *I* activity during oogenesis. In this study, we have shown that embryo lethality is a consequence of catastrophic meiosis in SF eggs. The loss or fragmentation of meiotic chromosomes leads to abnormal female pronucleus formation and prevents the subsequent development of viable diploid embryos. Instead, embryos from SF females initiate development with missing or damaged maternal chromosomes or with only the set of intact paternal chromosomes.

In contrast to the dramatic phenotype observed in eggs and embryos, SF oogenesis appeared relatively undisturbed by *I*-element activity. Our observation of meiosis prophase I progression in SF germaria has revealed the presence of a small number of non-persistent γ-His2Av foci in late-pachytene oocytes. These foci, supposedly associated with unrepaired DSBs, are thus the earliest phenotypic manifestation of *I* activity in SF germ cells that we were able to detect. Meiotic DSBs are normally repaired before the end of prophase and γ-His2Av foci are only exceptionally observed in wild-type region 3 oocytes (Mehrotra and McKim, 2006). The foci observed in SF oocytes could possibly result from a delay in the repair of DSBs induced by meiotic recombination, implying that *I* activity might disturb or slow down the normal repair process of meiotic DSBs. Alternatively, these DSBs could be directly generated by *I* retrotransposition. Indeed, in mammalian cells, retrotransposition of the *I*-related LINE 1 (L1) elements generates DNA DSBs associated with γ-His2AX foci (Bourc'his and Bestor, 2004; Belgnaoui et al., 2006; Gasior et al., 2006; Soper et al., 2008).

Whatever the origin of this DNA damage in early SF germ cells, they do not appear sufficient to trigger the activation of the Chk2-dependent checkpoint, at least as it is described for mutants affecting the repair of meiotic DSBs (Ghabrial and Schupbach, 1999; Abdu et al., 2002). For comparison, in certain hypomorphic alleles of meiotic DSB repair genes, the meiotic checkpoint is not activated despite the presence of about 7–10 persistent γ-His2Av foci (E.F.J. and K.S.M., unpublished data). The egg patterning analysis of SF eggs also supported the apparent absence of meiotic checkpoint activation in SF germ cells. Indeed, the weak ventralization phenotype observed with very young females disappeared after a few days despite the fact that dysgenic females remained fully sterile.

By contrast, SF egg chambers displayed a clear karyosome phenotype that was highly correlated with sterility. The morphology defect of SF karyosomes was reminiscent of the karyosomes in piRNA mutants. In these mutants, activation of the Chk2 checkpoint

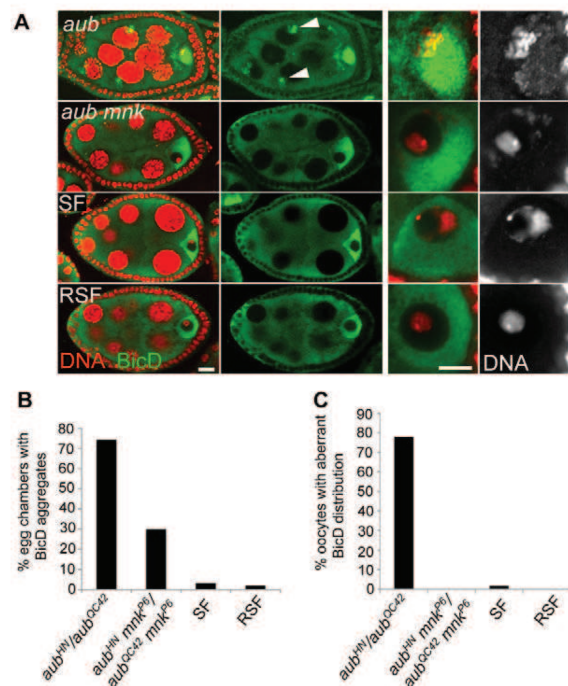

Fig. 7. BicD distribution is not affected in SF ovaries. (**A**) Confocal images of stage-9 egg chambers from *aub*^{HN}/*aub*^{QC42} (*aub*), *aub*^{HN} *mnk*^{P6}/*aub*^{QC42} *mnk*^{P6} (*aub mnk*), SF and RSF females raised at 25°C. At days 3–5 of adult life, ovaries were dissected, fixed and stained for BicD (green) and DNA (red). BicD aggregates accumulate in egg chambers from control *aub* mutants (arrowheads) but not from double *aub mnk* mutants, SF or RSF females (left panels). Magnification of germinal vesicles of the same stage and genotype are shown in the right panels. Note that BicD is abnormally distributed within the germinal vesicle in *aub* mutant oocytes, whereas it is normally excluded from the oocyte nucleus in *aub mnk*, SF and RSF egg chambers. (**B**) Quantification of BicD aggregates. For each type of ovary, a minimum of 70 egg chambers at stages 6–9 were evaluated for presence or absence of BicD aggregates. (**C**) Quantification of aberrant BicD distribution. For each type of ovary, a minimum of 60 oocytes at stages 6–9 were analyzed. Note that karyosomes show a strong phenotype in *aub* mutants but appear normally shaped in *aub mnk* oocytes. Scale bars: 10 μm.

is at least partially responsible for this phenotype, in a way similar to mutants affecting the repair of meiotic breaks (Ghabrial and Schupbach, 1999; Abdu et al., 2002; Staeva-Vieira et al., 2003). Indeed, we have observed that 97% (*n*=62) and 26% (*n*=94) of *aub mnk* and *mnk armi* karyosomes had a morphology rescued to wild-type, respectively (Fig. 7; data not shown).

Interestingly, DNA damage accumulation and karyosome defects in the absence of strong dorsoventral patterning defects have been described for mutants that affect both meiotic DNA damage repair and checkpoint signaling, such as *hus1* and *brca2* (Abdu et al., 2007; Klovstad et al., 2008). Similarly, germline derepression of TEs in the *tejas* mutant does not affect egg polarity (Patil and Kai, 2010). We thus cannot exclude that the karyosome defect in SF oocytes could reflect a partial or late DNA damage response, which would not trigger other known hallmarks of checkpoint activation, including egg ventralization. Indeed, in SF ovaries, *I* transcripts and ORF1 protein are first detected in germarium region 2A but they reach their highest levels in later-stage oocytes, where they presumably accumulate as RNPs (Seleme et al., 1999; Seleme et al., 2005). At these stages, however, any accumulation of DNA DSBs might go undetected with γ-His2Av antibodies. In this model, the DNA damage response could still cause the observed karyosome defect but would occur too late to significantly disturb Grk protein oocyte accumulation and dorsoventral axis specification.

In the alternative possibility, accumulation of *I* RNPs in the oocyte could directly affect karyosome formation without inducing any DNA damage response. However, and surprisingly, *I* products accumulate in the perinuclear cytoplasm of SF oocytes and do not appear to enter the nuclear compartment at cytologically detectable levels (Seleme et al., 1999; Seleme et al., 2005). Accordingly, GFP-tagged ORF1p remains cytoplasmic when transiently expressed in *Drosophila* cultured cells (Rashkova et al., 2002). Thus, only a minor fraction of *I* RNPs is expected to enter the oocyte nucleus in order to complete the retrotransposition process. This situation contrasts with the clear nuclear accumulation of L1 RNPs in $mael^{-/-}$ mutant mouse spermatocytes associated with DNA damage and chromosome asynapsis (Soper et al., 2008). In SF ovaries, we did not detect any gross defect in the distribution of the SC protein C(3)G in oocytes (supplementary material Fig. S4). However, the low resolution obtained with this kind of analysis (compared with mouse spermatocytes, for instance) cannot rule out the presence of undetected chromosome synapsis defects.

The modest effect of *I* activity on DNA integrity during early SF oogenesis contrasted with the situation observed in piRNA mutants where many TEs, including *I*, are derepressed. However, the origin of DNA damage in piRNA pathway mutants is not clear and the involvment of TEs in generating these breaks remains to be established (Chen et al., 2007; Klattenhoff et al., 2007; Klattenhoff and Theurkauf, 2008; Thomson and Lin, 2009). Genetic inactivation of the checkpoint does not restore the fertility of piRNA pathway mutant females (Table 2) (Klattenhoff et al., 2007). It thus indicates that additional, checkpoint-independent defects cause the female sterility in these mutants. Interestingly, embryos from *aub mnk* females display a specific and severe disorganization of cleavage nuclei that could explain the observed maternal-effect lethality (Blumenstiel et al., 2008). Furthermore, in *aub* and *spn-E* mutants, the *HeT-A* and *TART* retroelements involved in telomere maintenance are upregulated in the female germline and their retrotransposition to broken chromosome termini is increased, with potential consequences on chromosome stability (Savitsky et al., 2006). Finally, Piwi-family proteins are also involved in the biogenesis or processing of piRNAs directed against the 3′ UTR of a broad set of cellular transcripts, with possible regulatory functions (Robine et al., 2009). The overall phenotype of piRNA pathway mutants is thus expected to reflect this functional complexity, in contrast to *I-R* hybrid dysgenesis, where a single type of element is activated.

The meiotic defects we observed in SF oocytes and eggs are probably related to the chromosome rearrangements and non-disjunctions associated with *I-R* hybrid dysgenesis. Rearrangements are probably generated after illegitimate homologous recombination events between integrating *I* elements (Busseau et al., 1989; Prudhommeau and Proust, 1990; Proust et al., 1992). Considering the fact that these chromosomal aberrations were obtained in viable progeny from SF females, we suppose that more detrimental and frequent rearrangements are produced when SF females are still fully sterile. The accumulation of chromosomal rearrangements in oocyte nuclei could probably affect meiotic divisions by notably inducing non-disjunction and chromosome fragmentation events. In this model, the progressive *I* repression established in aging SF females would reduce the probability of these events occurring until oocyte chromosome architecture becomes compatible with normal meiosis.

Materials and Methods

Drosophila stocks

The w^{1118} standard inducer stock and the strong reactive wild-type stock *Charolles* were used to set up control or dysgenic crosses, unless otherwise specified. The *JA26 y w* reactive stock was provided by Alain Pelisson (Institute de Génétique Humaine, Montpellier, France). The *EGFP-Cid* stock (Schuh et al., 2007) and the *Jupiter-GFP* insertion (Buszczak et al., 2007) were obtained from Stefan Heidmann (University of Bayreuth, Bayreuth, Germany) and from the Carnegie Protein Trap Stock Collection (http://flytrap.med.yale.edu/), respectively. The mnk^{P6} stock was a gift from Tin Tin Su (Brodsky et al., 2004). The following alleles were obtained from the Bloomington *Drosophila* Stock Center at Indiana University (http://flystocks.bio.indiana.edu/): $armi^{1}$ and $armi^{72.1}$ (Cook et al., 2004), aub^{HN} and aub^{QC42} (Schupbach and Wieschaus, 1991), $krimp^{f06583}$ (Lim and Kai, 2007) and $mael^{r20}$ (Clegg et al., 1997). The following heterozygous or hemizygous females were used in this study: $armi^{1}$ $armi^{72.1}$ (*armi*), aub^{HN} aub^{QC42} (*aub*), $krimp^{f06583}$ *Df(2R)Exel6063* (*krimp*) and $mael^{r20}$ *Df(3L)ED230* (*mael*). The $K81^{2}$ paternal-effect embryonic lethal mutant is a small, viable deficiency that completely removes the *ms(3)K81* gene (Yasuda et al., 1995). *aub mnk* or *mnk armi* double-mutant females were obtained by standard crossing techniques and meiotic recombination.

Crosses and egg phenotype analysis

Control and dysgenic crosses were set up at the appropriate temperature using equal numbers of freshly emerged virgin males and females that were kept together throughout the experiment. Eggs were collected on agar plates, counted and, if necessary, the dorsal appendage phenotype was examined by direct observation under a stereomicroscope. Embryos were then allowed to develop for 3 days at 25°C before hatching rate and brown/white phenotype determination.

Egg collection, ovary dissection and immunofluorescence

Females that were no older than 1 week were allowed to lay eggs on agar plates in the presence of males at 25°C. Eggs were dechorionated in bleach and fixed as described (Loppin et al., 2001). Ovaries were dissected in TBST (TBS-0.15%, Triton X-100), fixed in a 1:1 mixture of heptane: 4% paraformaldehyde in PBS, rinsed in TBST and were immediately incubated with the primary antibodies as previously described (Bonnefoy et al., 2007). Antibodies and dilutions used were: anti-α-Tubulin (Sigma, T9026, 1/500); anti-H4Ac (Chemicon International, AB3062, 1/200); anti-Flag (Sigma, F3165, 1/1000); anti-H3K14Ac (Millipore, 06-911, 1/500); anti-C(3)G (kindly provided by R. S. Hawley, 1/500) (Page and Hawley, 2001); anti-γ-His2AvD (1/500) (Mehrotra and McKim, 2006); and anti-BicD (Developmental Studies Hybridoma Bank, 1B11-s, 1/200). DNA was stained with Propidium Iodide or Hoechst. Confocal images were acquired using either a LSM510 microscope (Carl Zeiss) or a Leica SP2 (for Fig. 5) and were processed with Adobe Photoshop software.

Karyosome defect assay

To analyze the effect of temperature on karyosome phenotype, 1-day-old SF females were kept at 25°C for 2 days and were then placed at 18°C, 25°C or 30°C for 36 hours before ovary dissection. To analyze the effect of age, SF females that were obtained at 25°C were aged for 3, 6 or 9 days before dissection. Stage 6–9 oocytes

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stained with Propidium Iodide and H3K14Ac were observed under a confocal microscope and karyosomes were classified into three phenotypical categories as described in Fig. 4. For each condition, a minimum of 40 karyosomes was observed.

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Table S1. Phenotype of SF eggs and embryos

		Day 5	Day 6	Day 7	Day 8	Day 9	Total, Days 5–9	Day 13	Day 15	Day 23
SF ɜ w^{1118}	*n*	228	108	559	29	210	1134	445	310	529
	Ventr. (%)	5 (2.2)	0 (0)	4 (0.7)	0 (0)	0 (0)	9 (0.8)	2 (0.45)	ND	ND
	Brown (%)	13 (5.7)	13 (12.0)	41 (7.3)	3 (10.3)	14 (6.7)	84 (7.4)	38 (8.5)	28 (9.0)	ND
	Larvae (%)	0 (0)	0 (0)	4 (0.7)	1 (3.4)	5 (2.4)	10 (0.9)	68 (15.3)	143 (46.1)	417 (78.8)
SF ɜ $K81^2$	*n*	650	382	413	92	213	1750	381	186	629
	Ventr. (%)	20 (3.1)	5 (1.3)	1 (0.2)	2 (2.2)	0 (0)	28 (1.6)	1 (0.3)	ND	ND
	Brown (%)	0 (0)	0 (0)	1 (0.2)	0 (0)	4 (1.9)	5 (0.3)	10 (2.6)	18 (9.7)	138 (21.9)
	Larvae (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
RSF ɜ w^{1118}	*n*	ND	ND	322	43	133	498	393	ND	ND
	Ventr. (%)	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	5 (1.3)	ND	ND
	Brown (%)	ND	ND	1 (0.3)	0 (0)	1 (0.75)	2 (0.4)	3 (0.8)	ND	ND
	Larvae (%)	ND	ND	317 (98.4)	42 (97.7)	129 (97.0)	488 (98.0)	384 (97.7)	ND	ND
RSF ɜ $K81^2$	*n*	350	357	522	115	227	1571	513	146	ND
	Ventr. (%)	1 (0.3)	0 (0)	1 (0.2)	1 (0.9)	0 (0)	3 (0.2)	4 (0.8)	ND	ND
	Brown (%)	81 (23.1)	96 (26.9)	131 (25.1)	27 (23.5)	59 (26.0)	394 (25.1)	138 (26.9)	32 (21.9)	ND
	Larvae (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND

Virgin SF or RSF females collected on day 1 were crossed with the same number of w^{1118}/Y or w^{1118}/Y ; $K81^2/K81^2$ 1-day-old males. Eggs were collected on plates everyday from day 5 until day 9 and then at days 13, 15 and 23, and the total number of eggs (*n*), as well as the number of ventralized eggs (eggs with fused or missing appendages), was determined. Eggs were then allowed to develop for 5 days at 18°C before determining the number of hatched larvae and the number of unhatched brown embryos (see Fig. 4K). Note that the brown unhatched embryos obtained in the RSF ɜ $K81^2$ cross are haploid gynogenetic embryos. Also note that aging SF females crossed with *K81* males produced an increasing proportion of late-arrested, haploid gynogenetic embryos. *n*, total number of eggs; Ventr., number of eggs with absent or fused dorsal appendages; Brown, number of brown unhatched eggs; Larvae, number of hatched larvae; ND, not determined.

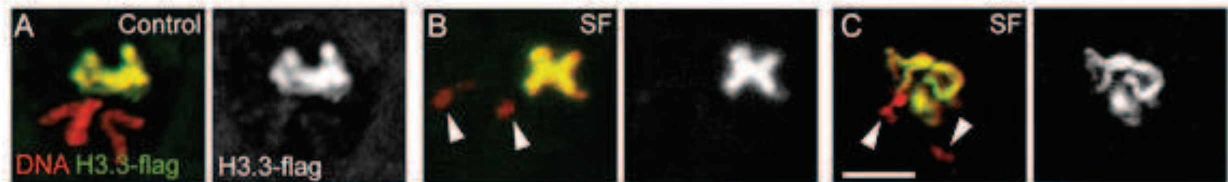

Fig. S1. Distribution of the paternal chromosome marker H3.3-Flag in SF zygotes. (A) Control zygote stained with anti-Flag (green or white) and DNA (red). Maternal chromosomes appear red. (B,C) H3.3-Flag distribution confirms the maternal identity of fragmented chromosomes (arrowheads) in SF early embryos. Scale bar: 10 µm.

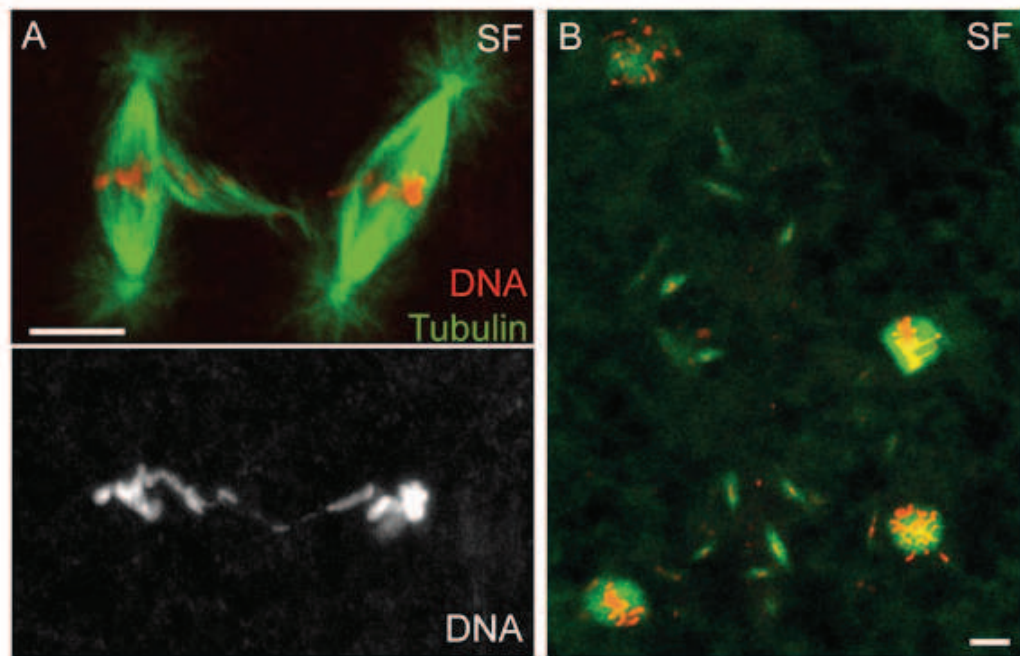

Fig. S2. Catastrophic mitoses in early SF embryos. (A) Second mitotic division in an SF embryo with a chromatin bridge, indicative of catastrophic first mitosis. (B) Early catastrophic mitoses lead to aneuploid development and early arrest of SF embryos. Tubulin is in green and DNA in red or white. Scale bars: 10µm.

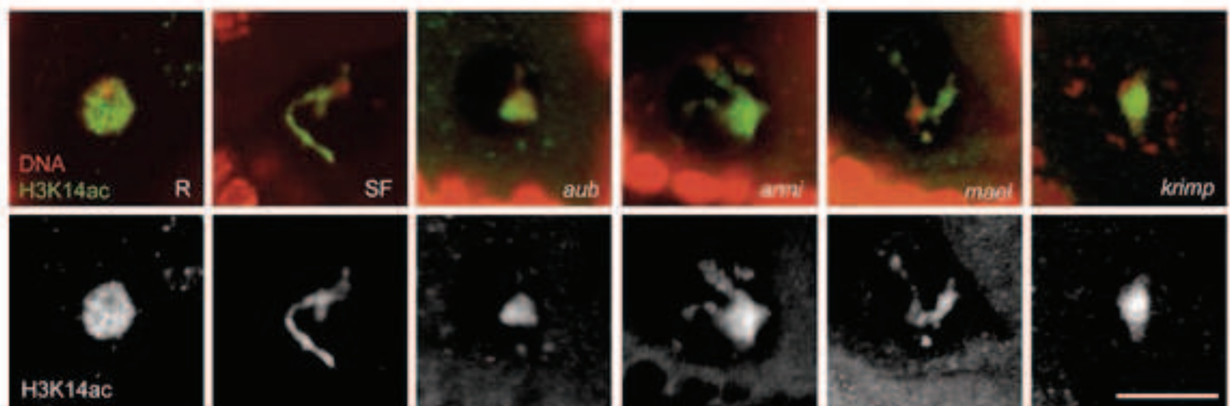

Fig. S3. Defective karyosome formation in SF and piRNA pathway mutants. Stage-9 oocytes from Reactive (R), SF, aub, armi, mael and krimp ovaries were stained for DNA (red) and the karyosome marker H3K14Ac (Histone H3 acetylated on lysine 14) (Ivanovska et al., 2005). Scale bar: 20 µm.

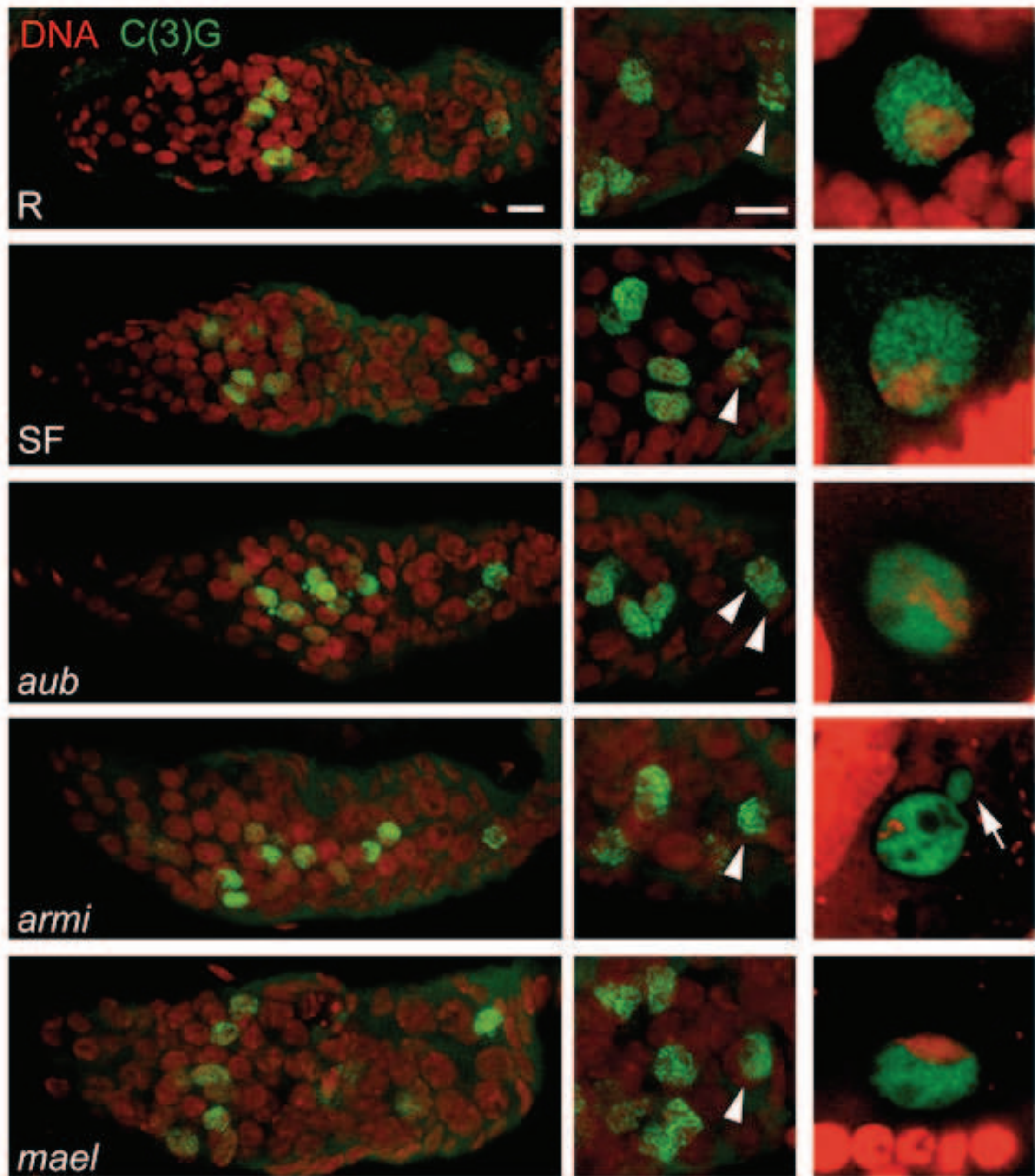

Fig. S4. Meiosis initiation and oocyte specification in the SF germline. R, SF, aub^{QC42}/aub^{HN} (aub), $armi^{1}/armi^{72.1}$(armi) and $mael^{r20}$/Df(3L)ED230 (mael) ovaries stained for DNA (red) and C(3)G (green). (Left) Germarium with anterior tip on the left. (Middle) Close-up of germarium region 3. (Right) Germinal vesicles from stage-9 oocytes. Synaptonemal complex formation revealed by C(3)G localization appears normal in R, SF, armi and mael germaria (left). Oocyte specification is indicated by the restriction of C(3)G to a single nucleus in germarium region 3 (arrowheads). Oocyte specification is delayed in aub mutant ovaries as revealed by the presence of two C(3)G-positive nuclei. C(3)G is detected in the germinal vesicles (right) but is not associated with chromatin. Note that germinal vesicles in armi mutants appeared frequently fragmented (arrow). Scale bars: 10 µm.

Cet article a permis de montrer, pour la première fois, que l'expression massive d'un transposon, en absence de toute mutation, a des conséquences dramatiques pour la méiose femelle. De façon remarquable, une mutation sur l'ADN méthyltransférase Dnmt3L est associée à une dérépression des transposons et une catastrophe méiotique chez la souris mâle, illustrant que la gestion des transposons est nécessaire de façon conservée pour la sauvegarde de la gamétogénèse. Récemment, il a été montré que les mutants affectant la voie des piARN chez la drosophile causent aussi des catastrophes méiotiques, comme l'avait prédit notre travail (Khurana *et al.*, 2010). De façon intéressante, ces auteurs montrent que la perturbation de la voie des piARN est associée à des défauts de coiffe des télomères dans les ovaires, et que des mutations sur la ligase IV (qui participe à la voie de réparation Non Homologous End Joining, NHEJ) suppriment le phénotype à la méiose. Ces résultats vont dans le sens que la surexpression des transposons dans ce contexte perturbe l'intégrité de la coiffe des télomères et induit des fusions qui résultent dramatiques. Le facteur I n'est pas trouvé dans des régions télomériques en contexte stabilisé, mais il serait intéressant de tester si sa surexpression est capable de perturber la coiffe.

Ces travaux se raccordent aux précédents de plusieurs façons. Le phénotype, du point de vue cytologique, est une image en miroir du phénotype observé en contexte mutant *mh*, *K81* ou dans le cas de l'IC. De façon intéressante, ils renforcent l'idée sous-jacente que la fécondation est la rencontre de deux jeux de chromosomes qui ont une histoire épigénétique très différente, et qui doivent donc gérer des contraintes disparates. Il est en particulier notable que la dysgénésie hybride n'affecte pas la fertilité des mâles issus du même croisement que les femelles SF. Les particularités de l'ovogénèse exposent les chromosomes maternels à des dangers spécifiques.

Les transposons façonnent les génomes et constituent un facteur essentiel pour la robustesse génétique des espèces. Cependant, ils représentent un danger permanent pour l'intégrité de ces mêmes génomes qu'ils enrichissent. Les cellules germinales doivent donc se prémunir contre leur attaque, pour assurer la protection du patrimoine génétique de génération en génération, mais aussi la transmission des éléments transposables eux mêmes. L'œuf doit gérer de nombreux paradoxes comme celui-ci au moment de la fécondation. La reproduction sexuée requiert le passage par le stade une cellule, et ceci impose aux gamètes de lourdes transitions d'état de la chromatine. La diversité des stratégies d'adaptation de chaque espèce à

ces défis révèle le délicat équilibre entre la stabilité d’un système qui doit assurer une fécondation efficace, et la plasticité de mécanismes à l’origine du brassage génétique et de la spéciation.

Conclusion

A aucun moment au cours de la vie la protection du matériel génétique n'est plus cruciale qu'à la fécondation, où seule une copie de chaque chromosome existe dans l'individu. Mais cette étape obligatoire de la reproduction sexuée impose l'existence de voies moléculaires complexes qui participent à son succès. Celles qui ont été présentées dans cette thèse ont en commun qu'elles sont critiques pour l'organisation et l'intégrité des chromosomes parentaux. Le mystère reste entier sur la nécessité des remaniements qui ont lieu au cours de la gamétogénèse et à la fécondation, mais ces travaux participent à montrer que le traitement du génome des gamètes est unique et mobilise des mécanismes très singuliers.

Au cours de cette thèse j'ai discuté de l'importance fonctionnelle de l'assemblage de la chromatine par le mode IR. Important régulateur de la dynamique de la chromatine, ce mécanisme est crucial lors de la formation du pronoyau mâle. Bien que ses multiples rôles soient progressivement mis à jour, et ses fonctions essentielles dans les cellules somatiques apparaissent évidentes chez des organismes à longe durée de vie, la reproduction sexuée a certainement joué un rôle crucial dans son évolution. L'étude fonctionnelle du variant H3.3, exceptionnellement bien conservé, mais toutefois remplaçable pour la plupart de ses rôles, excepté celui lié à la reproduction, surligne les contours de ce paradigme. Ma thèse apporte des éléments nouveaux sur les rôles *in vivo* de la voie HIRA dans le contexte du développement d'un modèle métazoaire, et renforce d'avantage cette idée.

A cause de leur complexité et de leur importance capitale, la fécondation et la gamétogénèse sont aussi des fenêtres d'opportunité pour diverses formes de parasitisme. Toutefois, ce dialogue avec l'environnement et les agents extérieurs sont aussi la source d'une grande variabilité : la reproduction est en ce sens à la fois une zone à risques pour les espèces, et un moyen puissant pour leur évolution et leur diversification.

Au cours de cette thèse, j'ai tenté de mieux cerner le fonctionnement de certaines situations affectant spécifiquement les chromosomes paternels ou maternels. Les facteurs maternels HIRA et Yem contribuent spécifiquement à l'organisation des chromosomes paternels, ces mêmes chromosomes qui sont spécifiquement protégés par les protéines K81 et qui sont aussi

la cible des bactéries Wolbachia. Au contraire, la remobilisation d'un transposon lors de la dysgénésie hybride I-R affecte exclusivement les chromosomes maternels. Ces études ont la force de matérialiser des spécificités majeures dans l'histoire épigénétique de chaque jeu de chromosomes parentaux. En effet, à la fécondation se joue la rencontre de deux génomes haploïdes organisés et structurés de façon très différente qui doivent être harmonisés pour constituer un noyau diploïde totipotent, intact, et fonctionnel du point de vue de la chromatine. Nous commençons seulement à comprendre comment les voies moléculaires qui participent à ce processus sont conditionnées par la nécessité absolue de sa réussite.

Commentaire de fin

« ¿Por qué di en agregar a la infinita
serie un símbolo más? ¿Por qué a la vana
madeja que en lo eterno se devana,
di otra causa, otro efecto y otra cuita? »

« Pourquoi voulu-je ajouter à l'infinie série un symbole de plus ?
Pourquoi au vain écheveau qui est bobiné dans l'éternité,
ai-je donné une nouvelle cause, un nouvel effet, et une nouvelle peine ? »

Jorge Luis Borges. *El Golem*.

Dans le poème de J.L. Borges « Le golem », le rabbin Juda Leon anime par magie kabbaliste un golem : il résulte être une œuvre chérie, attendrissante, imparfaite et finalement décevante. Borges s'interroge en réalité sur la création : le golem est au rabbin ce que le rabbin est à son dieu ; et il est aussi ce que le poème est au poète (et la thèse au thésard). La thèse est un légat qui contribue de façon à peine perceptible à la Science, mais elle constitue une démarche de création. La mienne a été alimentée par l'ouverture sur toujours de nouveaux problèmes et des nouvelles questions. Je veux retenir comme interrogation principale celle des toutes premières étapes de la formation du zygote, cette boîte noire qui ne cesse de surprendre. Comme tout apprenti généticien, c'est à travers l'étude de ses dysfonctionnements que j'ai entrevu ses fondements, et j'ai participé à dégager quelques aspects d'un processus critique et encore mal connu.

Si ma contribution à augmenter les connaissances dans ce domaine est très modeste, sa contribution à me construire en tant que personne scientifique ne l'est pas. Rien de tel que l'étude des premières étapes de la vie, résultat d'un dialogue extraordinairement complexe entre le hasard et la nécessité, pour apprécier les mécanismes du vivant à l'œuvre. La force heuristique de ce modèle est sans appel et m'a introduit pendant plusieurs années à des problèmes de fond de la biologie.

En fin de compte, je répondrais ceci à Judas Leon : la création n'est pas tant l'invention du nouveau qu'un miroir nécessaire à la construction de soi.

Matériel et Methodes

Cytologie

Les marquages sur embryons et sur ovaires ont été réalisés comme décrit précédemment (voir Bonnefoy *et al.*, 2007). Des mâles portant Cid-GFP (Henikoff *et al.*, 2000) ou GFP-K81 (voir Dubruille *et al.*, 2010) ont été croisés par des femelles portant Yem-Flag (voir Orsi *et al.*, en préparation) pour observer ces deux marqueurs. Pour observer l'assemblage de H3.3-GFP dans les tissus somatiques, le transgène H3.3-GFP B6 (Schwartz et Ahmad, 2005) a été combiné avec les chromosomes $Hira^{HR1}$, yem^{1} ou yem^{2}. L'induction de l'expression a été réalisée en plongeant des tubes contenant des mouches adultes ou des larves dans un bain marie à 37°C pendant 1 heure, puis en laissant les individus à 25°C pendant 30 minutes ou 4 heures (respectivement). Les dissections d'ovaires ou de glandes salivaires ont été réalisées dans du milieu PBS-Triton 0,15%, et les tissus ont été fixés en PBS-T suppléé de 4% de Paraformaldéhyde pendant 25 minutes à température ambiante. Le reste du marquage et l'observation a été réalisé comme décrit précédemment (voir Bonnefoy *et al.*, 2007).

Les anticorps utilisés sont : anti-GFP dans les œufs et dans les clones somatiques (Roche, 1/100 ou Invitrogen A11122, 1/500)(la GFP native a été observée dans le cas de l'expression de H3.3-GFP), anti-Flag (Sigma M2 F3165, 1/1000), anti-γH2avD (Rockland 600-404-914, 1/1000), anti-Fibrilarine (Millipore 5821, 1/200), anti-HP1 (DSHB, 1/100), anti-H3K4Me2 (Millipore, 1/500), anti-H3K9Me3 (Millipore 07-442, 1/100), anti-H3K27Me2 (Millipore 07-452, 1/500) et anti-H3K27Me3 (Millipore 07-449 1/1000).

Tests de variégation

Les transgènes Heidi et 118E-10 (offerts par Marion Delattre) portent un gène rapporteur $white^{+}$ soumis à une variégation d'expression et sont insérés à proximité des péricentromères des chromosomes 2 et 4 (respectivement). L'aberration Sb^{v} (stock Bloomington 878) place une mutation dominante du gène *Sb* en région péricentromérique du chromosome 2. Des femelles mutantes pour *Hira* ont été croisées avec des males portant ces constructions et la variégation a été évaluée chez les mâles descendants. Les images sont des photographies de males représentatifs. La variégation *Sb* a été évaluée en coptant les soies mutantes dans quatre paires de macrochaetes. Au moins 80 mouches pour chaque situation ont été observées.

Intéraction génétique Hira/hop

Les allèles *hop*$^{Tum\text{-}l}$ et *Hira*^{HR1} ont été combinés : les chromosomes recombinants ont été criblés grâce au phénotype de masses mélanotiques associés à l'allèle *hop*$^{Tum\text{-}l}$ et au phénotype *white*$^{+}$ associé à l'allèle *Hira*^{HR1} pour établir des lignées. Deux lignées différentes ont été utilisées pour tester les interactions génétiques. Les transgènes HIRA-Flag et HIRA^{ssm}-Flag utilisés pour le sauvetage ont été décrits précédemment (Loppin *et al.*, 2005a).

Construction de tissus mosaïques

Les chromosomes *Hira*^{ssm} ou *Hira*^{HR1} P{w[+mW.hs]=FRT(w[hs])}9-2 ont été construits par recombinaison méiotique et combinés à un chromosome P{w[+mC]=Ubi-GFP.nls}X1 P{w[+mW.hs]=FRT(w[hs])}9-2 en présence d'un chromosome P{w[+mW.hs]=GawB}T155 P{w[+mC]=UAS-FLP1.D}JD2 pour générer des clones somatiques sous contrôle d'un promoteur *Engrailed*. Les femelles adultes d'entre 1 et 5 jours ont été disséquées et les chambres d'œuf en stades 9-10 ont été photographiées.

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Epigenetic and replacement roles of histone variant H3.3 in reproduction and development

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ABSTRACT The nucleosomal organization of eukaryotic chromatin is generally established during DNA replication by the deposition of canonical histones synthesized in S phase. However, cells also use a Replication Independent (RI) nucleosome assembly pathway that allows the incorporation of non-canonical histone variants in the chromatin. H3.3 is a conserved histone variant that is structurally very close to its canonical counterpart but nevertheless possesses specific properties. In this review, we discuss the dual role of H3.3 which functions as a neutral replacement histone, but also participates in the epigenetic transmission of active chromatin states. These properties of H3.3 are also explored in the light of recent studies that implicate this histone and its associated chromatin assembly factors in large scale, replication-independent chromatin remodeling events. In particular, H3.3 appears as a critical player in the transmission of the paternal genome, from sperm to zygote.

KEY WORDS: *H3.3, epigenetic, sperm chromatin remodeling, histone chaperone*

Introduction

The organization of chromatin in eukaryotic cells is remarkably conserved. The basic unit of chromatin, the nucleosome, is constituted by a hetero-octamer of histones that are wrapped with about 146bp of DNA. The structural properties of nucleosomes can be modulated by a large variety of post-translational modifications (PTMs) of histone proteins. The combinatorial complexity of these modifications is at the origin of the "histone code" hypothesis, which proposes that histone PTMs participate, along with other epigenetic marks such as DNA methylation, in the functional organization of the genome (Jenuwein and Allis, 2001). Nucleosomes can also be modulated by the incorporation of histone variants that differ from the major, canonical histones synthesized during S phase. Histone variants differ from their canonical counterpart at the level of the primary sequence. These differences can range from a few amino-acid positions (e.g. H3.1 vs H3.3) to large protein domains (e.g. H2A vs macroH2A) and usually confer specific properties to nucleosomes. In contrast to canonical histones that are devoted to Replication Coupled (RC) chromatin assembly, histone variants are expressed throughout the cell cycle and are thus available, at least theoretically, in nucleosome assembly pathways that occur in a Replication-Independent (RI) manner (Henikoff and Ahmad, 2005; Sarma and Reinberg, 2005). For this reason, histone variants are also called "replacement" histones. Finally, certain variants are preferentially or specifically expressed in certain tissues, such as the testis specific histone H3 variant, for instance (Witt *et al.*, 1996).

The combination of PTMs and histone variant creates a wide diversity of nucleosomes. This variability is important to determine the properties of the chromatin fiber at a local and regional level, with respect to essential aspects of DNA metabolism, such as replication, transcription, heterochromatin formation, repair, condensation or kinetochore formation.

In this article, we focus on the function of the histone H3.3 variant during development and reproduction. A main feature of H3.3 is its association with transcriptionally active chromatin and its potential role in the epigenetic transmission of active chromatin states. These properties are at the origin of a growing interest for H3.3 over the past few years. However, recent studies in various model organisms have revealed unexpected roles for this variant, particularly during sexual reproduction. We will discuss the respective importance of replacement and epigenetic roles of this histone, in the context of its diversity and evolutionary history, and in the light of its interactions with nucleosome assembly machineries.

Abbreviations used in this paper: MSCI, meiotic sex chromosome inactivation; MSUC, meiotic silencing of unsynapsed chromatin; PTM, post-translational modification; RC, replication coupled; RI, replication independent; SCR: sperm chromatin remodeling; SNBP, sperm nuclear basic protein.

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The histone H3.3 family of proteins

Genes encoding canonical histones are usually organized in tandem, multi-copy clusters and have no introns. Replicative histone mRNAs are not polyadenylated. Instead, translation is tightly regulated by the binding of SLBP (Stem Loop Binding Protein) and U7 snRNP to the 3' end of the histone RNAs (Jaeger *et al.*, 2005). This peculiar genomic organization and transcriptional regulation allows a massive production of canonical histones at the beginning of the S phase and ensures the synthesis of stoichiometric quantities of each protein. On the contrary, histone variant genes are regular genes that are represented by a single or a few copies and are scattered throughout the genome. In addition, they often possess introns and their polyadenylated mRNAs are expressed throughout the cell cycle (Fig. 1).

In mouse, thirteen canonical *H3* genes are present in the genome, encoding two versions of canonical H3: H3.1 and H3.2. They differ by a single amino acid in position 96 (Graves *et al.*, 1985). The functional relevance of having two different replicative H3s is unclear (Hake and Allis, 2006). In *Drosophila*, the histone gene cluster on the left arm of chromosome 2 contains twenty-three copies of each *H1*, *H2A*, *H2B*, *H3* and *H4* genes (Fig. 1). All the *Drosophila* histone *H3* genes encode the same H3 protein, identical to mammalian H3.2.

Histone H3 variant types include centromeric H3 variants (CenH3s), H3.3 and testis specific H3 in mammals (Fig.1). CenH3s form a highly divergent family of histone H3 variants that are characterized by an H3-like histone fold domain and a variable N-terminus tail (reviewed in Ahmad and Henikoff, 2002a; Dalal *et al.*, 2007). In mouse, human and *Drosophila*, two *H3.3* genes (*H3.3A* and *H3.3B*) encode the same conserved protein, but the transcripts have distinct untranslated regions (Akhmanova *et al.*, 1995; Frank *et al.*, 2003; Krimer *et al.*, 1993).

H3.3 is one of the most conserved proteins and appears to be present in all eukaryotes (Malik and Henikoff, 2003). H3.3 differs from H3.2 (mouse) or H3 (*Drosophila*) by only four amino acids at positions 31, 87, 89 and 90 (Fig. 2). The residue at position 31 sits in the N-terminal tail of the protein while positions 87, 89 and 90 are located in the α2 helix of the histone fold domain (Fig. 2). In spite of the great sequence similarity between H3.3 and H3, it has been proposed that these residues could account for specific properties of H3.3 proteins. In vertebrates, the serine in position 31 (H3.3S31p) can be phosphorylated and this PTM is detected on metaphase chromosomes, at specific sites bordering centromeres, unveiling a possible role of this mark during cell division (Hake *et al.*, 2005). H3.3S31p also exists in the urochordate *Oikopleura dioica* and is detected during mitosis and oogenic meiosis (Schulmeister *et al.*, 2007). In addition, a potentially phosphorylable threonine residue is found in position 31 in *C. elegans* and *A. thaliana* H3.3, respectively, but this is not the case for other members of the family (Fig. 2).

The residues in positions 87, 89 and 90 are necessary and sufficient to exclude canonical H3 from RI assembly pathways in *Drosophila* (Ahmad and Henikoff, 2002b), suggesting that they could directly or indirectly mediate the interaction of H3 and H3.3 with their specific histone chaperone. In vertebrates and *Drosophila*, the residues at positions 87, 89 and 90 are S, V and M in H3, and A, I and G in H3.3, respectively. Interestingly however, the identities of the residues found at these positions in H3 and H3.3 vary between species but distinguish H3 from H3.3 (Fig. 2) (Malik and Henikoff, 2003). It has been proposed that these three residues could participate in histone-histone interaction stability: nucleosomes assembled with H3.3 may have different intrinsic stability properties than those assembled with canonical H3 (Hake and Allis, 2006).

In the nematode *C.elegans* at least two different H3.3 proteins are encoded (namely, His71 and His72) (Ooi *et al.*, 2006). *His71* and *His72* individually mutated animals are viable, suggesting that these genes are functionally redundant. In the protist *Tetrahymena thermophila*, two H3.3 proteins have also been characterized (H3.3 and H3.4) (Cui *et al.*, 2006). These two versions present differences to *Tetrahymena* canonical H3 on three of the four characteristic positions but, in addition, present 8 non-conserved amino acid differences. Moreover, these proteins

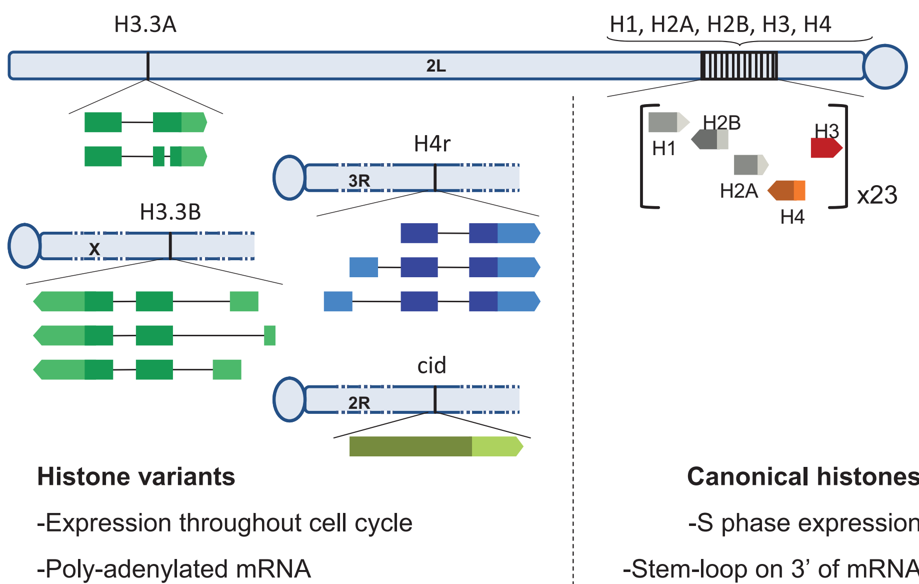

Fig. 1. Genomic organisation of histone *H3* and *H4* genes in *Drosophila*. Drosophila melanogaster *chromosomes are represented and histone gene locations are showed.* H3.3A, H3.3B, H4r *and* Cid *are single-copy genes and their corresponding transcripts are shown. Known or putative introns are represented as thin lines. Coding regions are shown in darker colors. Lighter color boxes represent untranslated regions. Canonical histones H1, H2B, H2A, H3 and H4 are encoded by multi-copy genes in the histone gene cluster of chromosome 2L. Note that Flybase predicts a* H3.3A *transcript that would result in a shorter protein. Although supported by independent EST sequences, the signification of this transcript remains to be investigated. Gene annotations are from Flybase (http://flybase.bio.indiana.edu).*

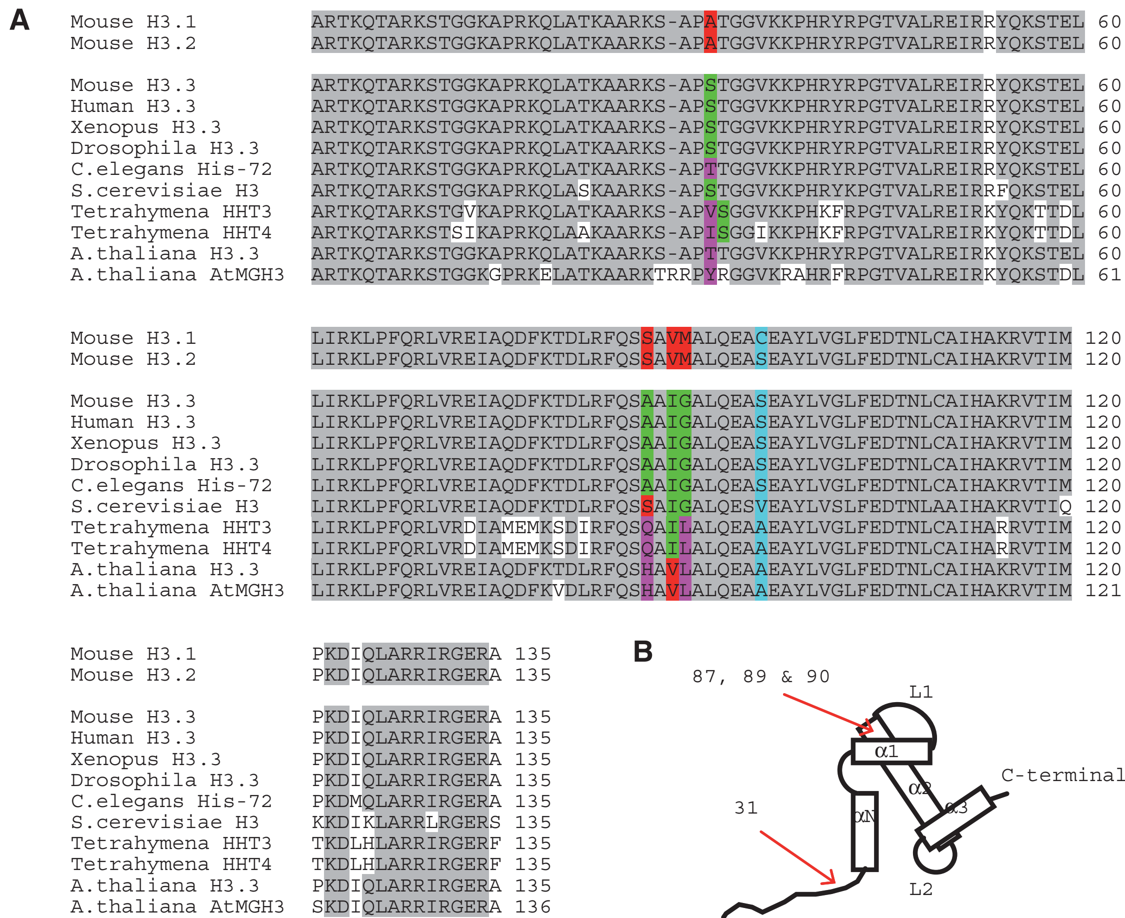

Fig. 2. Sequence alignment of H3.3 proteins. (A) *CLUSTAL alignement of H3 and H3.3 histones. Accession numbers are from GenBank:* M. musculus *H3.3A (NP_032236.1),* H. sapiens *H3.3A (NP_002098.1),* X. tropicalis *H3.3A (NP_001091902),* D. melanogaster *H3.3A (NP_523479.1),* C. elegans *His-72 (NP_499608.1),* S. cerevisiae *H3 (NP_009564.1),* T. thermophila *HHT3 (XP_001008397.1),* T. thermophila *HHT4 (XP_001008400.1) and* A. thaliana *H3.3 (NP_195713.1),* M. musculus *H3.1 (NP_038578.2) and H3.2 (NP_473386.1), and* A. thaliana *AtMGH3 (NP_173418.1). Alignments were performed with EMBL-EBI ClustalW2 software. Significantly conserved amino-acid residues are shaded in grey. In positions 31, 87, 89 and 90, amino-acids from canonical H3s are shaded in red, those from H3.3 family are shaded in green and those not fitting these categories are shaded in purple. The single residue that differentiates mouse H3.1 and H3.2 and differs among the H3.3 family is shaded in cyan.* **(B)** *Residues 31, 87, 89 and 90 are positioned on a schematic representation of nucleosomal H3 protein.*

differ from vertebrate H3.3 by twenty-two and twenty-five amino acids respectively, which accounts for the diversity of the H3.3 family across evolutionary divergent species. In plants, the diversification of H3.3 proteins seems to be even more accentuated: for instance, *Arabidopsis thaliana* has eight non-centromeric RI H3 variants (Okada *et al.*, 2005). The greater diversity of H3.3 proteins in certain groups, such as plants, for instance, opens the possibility that new H3.3 functions have emerged during evolution. Indeed, an evolutionary scenario proposes that H3.3 has independently arisen at least four times in plants, animals, ciliates and apicomplexans (Malik and Henikoff, 2003). Interestingly, a single version of H3 exists in ascomycetes (yeasts) and it is of the RI type (see Fig. 2). Since both RI and RC H3 versions are present in basidiomycetes, it has been proposed that canonical H3 genes have been lost in ascomycetes (Malik and Henikoff, 2003).

Epigenetic and replacement roles of H3.3 in somatic cells

Replacement roles of H3.3

The fact that expression of *H3.3* genes is not linked to S phase has been known for decades (Wu *et al.*, 1982). Because of this property, a simple expected function of H3.3 is to replace H3 whenever nucleosome assembly takes place independently of DNA synthesis, hence the term "replacement" variant. In an alternative view to this neutral replacement role of H3.3, the deposition of H3.3 can confer specific properties to the nucleosomes that are functionally important for the establishment of epigenetic marks (see the next section). A simple example of neutral replacement is provided by differentiated cells, after their exit from the cell cycle. In the absence of DNA replication and S

phase histone gene expression, differentiated cells are expected to rely on replacement histones for the assembly of new nucleosomes. Indeed, during the differentiation of various cell types, *H3.3* transcripts are abundant whereas replication dependent *H3* transcripts are no longer detected (Brown *et al*., 1985; Krimer *et al*., 1993; Pantazis and Bonner, 1984). The replacement of replicative H3 with H3.3 has been also observed at the protein level during the course of cell differentiation in vertebrates (Bosch and Suau, 1995; Pina and Suau, 1987; Urban and Zweidler, 1983; Wunsch and Lough, 1987). The underlying mechanism responsible for this H3.3 enrichment in chromatin of differentiated cells is not clear. It has been proposed that a general mechanism of nucleosome turnover allows the slow incorporation of histone variants in the chromatin in absence of DNA replication (Grove and Zweidler, 1984). This process is probably critical for the maintenance of a normal nucleosome density in long-lived cells as H3.3 nucleosomes compensate for the loss of old H3 nucleosomes.

Another example of H3.3 deposition that fits well into this type of neutral replacement is a recently described "repair" mechanims of heterochromatin in human cells after treatment with histone deacetylase inhibitors (Zhang *et al*., 2007b). Exposure to these drugs triggers the recruitment of Heterochromatin Protein 1 (HP1) to sites of altered pericentric heterochromatin. This recruitment occurs independently of DNA replication and is mediated by the deposition of H3.3 by the histone chaperone HIRA at these sites (Zhang *et al*., 2007b). The authors proposed that this mechanism could participate in the maintenance of centromere integrity and kinetochore formation. Interestingly, HIRA is also involved in the formation of SAHF (Senescence Associated Heterochromatin Foci) in human cells (Zhang *et al*., 2007a; Zhang *et al*., 2005). These cytological markers of cellular senescence are condensed domains of facultative heterochromatin that notably contain the macroH2A histone variant and HP1 proteins (Adams, 2007). The implication of HIRA in this process strongly suggests that H3.3 is also involved, although this remains to be formally demonstrated (Adams, 2007; Zhang *et al*., 2007a). If it is the case, it could establish H3.3 as a key actor for the remodeling of heterochromatin in different biological situations, a property that is clearly not related to its role as a mark of active chromatin.

Although the need for H3.3 in non-dividing cells is expected, it is less clear whether H3.3 can actually replace H3 in cycling cells. A recent study in the protist *Tetrahymena thermophila* addressed this point through elegant genetic analyses (Cui *et al*., 2006). In this organism, replacement or "minor" H3s are represented by two similar RI histone genes, called *H3.3* and *H3.4*, that are probably the result of a recent duplication event (Cui *et al*., 2006) (Fig. 2). When both canonical *H3* genes were knocked-out, the expression of *H3.3* with a *H3* gene promoter was able to partially rescue the growth defect associated with the loss of RC H3 histones. This result indicates that the growth phenotype is mainly the consequence of an inadequate amount of histone protein rather than a specific absence of H3. However, H3.3 cannot fully replace H3 as rescued cells display a slight growth reduction and a small micronuclei phenotype. Thus, in *Tetrahymena*, H3 must have some intrinsic properties not shared with H3.3 (Cui *et al*., 2006). These same authors also directly tested the function of minor H3s by generating cells with both *H3.3* and *H3.4* genes deleted. Surprisingly, minor H3s appear not essential for cell growth but only for the production of viable conjugation progeny. In addition, in the absence of minor H3s, Transcription-Coupled (TC) nucleosome assembly is apparently abolished without causing any obvious growth problem. These surprising phenotypes indicate that minor H3s seem to contribute to still unknown functions related to sexual reproduction.

Availability of H4 for RI nucleosome assembly?

Nucleosome assembly is initiated by the deposition of H3 along with H4 on DNA to form a (H3-H4)2 tetramer. This implies that H4 is made available at stoichiometric levels with H3.3 throughout the cell cycle, in order to be deposited through RI chromatin assembly pathways. Surprisingly, the problem of the source of H4 for RI assembly has received little attention. Interestingly, RI H4 genes encoding a H4 identical to canonical H4 have

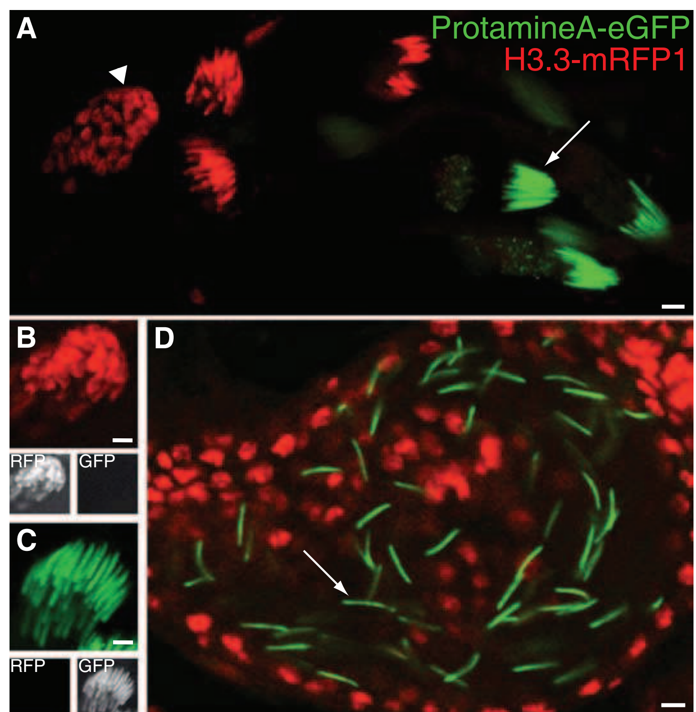

Fig. 3. H3.3 was not detected in *Drosophila* sperm. *Confocal images of fixed testes from males expressing both* H3.3-mRFP1 *and* ProtamineA-EGFP *transgenes. H3.3-mRFP1 is expressed from the* Drosophila H3.3A *gene promoter.* **(A)** *In this testis, groups of spermatid nuclei at different stages of differentiation (from left to right) are visible. Arrowhead points a group of spermatids with round nuclei and strong H3.3-mRFP1 fluorescence. Arrow indicates a group of elongated spermatid with strong ProtamineA-GFP fluorescence.* **(B)** *Close-up of a group of round spermatid nuclei.* **(C)** *Close-up of a group of elongated spermatid nuclei. In (B,C), small panels show the same nuclei with only RFP (left) or GFP (right) respective fluorescence.* **(D)** *A seminal vesicle containing mature gametes with strong ProtamineA-EGFP fluorescence (arrow). All bars, 5 µm. The mRFP1 (monomeric Red Fluorescent Protein1) protein is described in Campbell* et al.*, 2002. The* ProtamineA-EGFP *transgene is described in Jayaramaiah Raja and Renkawitz-Pohl (2005).*

been reported in *Drosophila*, nematodes and mammals (Akhmanova *et al.*, 1996; Gendron *et al.*, 1998; Poirier *et al.*, 2006). In *Drosophila*, the single copy *His4r* gene contains two introns, its mRNA is polyadenylated and it is expressed independently of DNA synthesis. In addition, it is preferentially expressed in adult, non-dividing cells, like *H3.3* genes (Akhmanova *et al.*, 1996). *H4r* might thus serve as a source of H4 for RI assembly processes. Once assembled, however, this protein is expected to behave identically to its RC counterpart. The role of *H4r* can thus only be explained by its RI expression profile. Another possible way of providing H4 to RI assembly pathways could be by recycling already assembled histones, by storing H4 expressed during S phase as pre-deposition complexes, or by allowing a certain level of transcription outside S phase. In this regard, it has been reported that the replicative histone H3.1 is deposited at sites of DNA repair, indicating that deposition of canonical histones is not absolutely coupled to S phase, at least for H3.1 (Polo *et al.*, 2006). Similarly, in *Tetrahymena*, H3 is specifically used for nucleosome assembly at sites of DNA synthesis associated with meiotic recombination (Cui *et al.*, 2006). The functional analysis of RI H4 genes should help distinguish between these possibilities.

H3.3 as an epigenetic mark of active chromatin

Opposed to the neutral replacement of H3 with H3.3 is the observation that H3.3 deposition does not occur homogeneously in the genome but instead correlates with regions of high transcriptional activity (Ahmad and Henikoff, 2002b; Chow *et al.*, 2005; Cui *et al.*, 2006; Janicki *et al.*, 2004; Mito *et al.*, 2005; Schwartz and Ahmad, 2005; Wirbelauer *et al.*, 2005).

It has been proposed that the passage of the RNA polymerase complex displaces nucleosomes, a situation that potentially creates a need for deposition of histones in a RI manner (Li *et al.*, 2007; Schwabish and Struhl, 2004). This Transcription Coupled deposition of H3.3 has been directly observed *in vivo* on *Drosophila* polytene chromosomes, throughout large transcription units such as the induced HSP70 genes, indicating that H3.3 deposition is associated with transcriptional elongation (Schwartz and Ahmad, 2005). Other studies have led to a similar conclusion based on the analysis of the distribution of H3.3 nucleosomes at high resolution by chromatin immunoprecipitation (ChIP) (Mito *et al.*, 2005; Wirbelauer *et al.*, 2005). However, this methodology also revealed an enrichment of H3.3 at the promoters of active genes, suggesting that chromatin remodeling associated with transcriptional initiation is also responsible for H3.3 deposition (Chow *et al.*, 2005). Finally, some studies found an enrichment of H3.3 at regulatory sites of active but also silent genes, such as the beta-globin locus control region in chicken or Polycomb Response Elements in *Drosophila* (Jin and Felsenfeld, 2006; Mito *et al.*, 2007; Nakayama *et al.*, 2007). These observations point to the possible existence of two distinct roles of H3.3 linked with gene activity. A first role for H3.3 in TC deposition is to compensate for the eviction of nucleosomes by the RNA polymerase complex in the body of highly transcribed genes (Schwartz and Ahmad, 2006). Another role links H3.3 to a continuous process of histone turnover that maintains accessibility of regulatory elements to their cognate factors (Henikoff, 2008).

In addition to its preferential incorporation at sites of active chromatin, H3.3 is enriched with PTMs typically associated with gene activity, such as methylation of lysine 4 among other marks (Hake *et al.*, 2006; McKittrick *et al.*, 2004; Mito *et al.*, 2005). How these PTMs are established on H3.3 and their importance in conferring an epigenetic role to this variant are crucial questions (Loyola and Almouzni, 2007). A recent study proposed that non-nucleosomal H3 and H3.3 carry a distinct set of modifications before their deposition, which in turn determine their final PTMs in nucleosomes (Loyola *et al.*, 2006).

The potential role of H3.3 in the epigenetic memory of active gene states has been recently studied in nuclear transfer experiments of *Xenopus* oocytes (Ng and Gurdon, 2008). Inheritance of active gene states of donor somatic nuclei is observed in embryos after nuclear transfer. For instance, about half of the embryos obtained after the transfer of a donor somite cell nucleus expressing the muscle-specific gene *MyoD* still express this marker in animal and vegetal regions, which do not differentiate into muscle (Ng and Gurdon, 2005). The authors found that this epigenetic memory of an active gene state correlates with the presence of H3.3 in its promoter. Importantly, this epigenetic memory can persist through 24 cell divisions in the absence of transcription (Ng and Gurdon, 2008). This finding supports a model where the H3.3 epigenetic mark is faithfully transmitted during DNA replication rather than through a mechanism involving the reactivation of transcription at each cycle. However, it is also compatible with the dynamic replacement model proposed by S. Henikoff (2008). Importantly, Ng and Gurdon found that the lysine 4 of H3.3 was required for the epigenetic memory, suggesting that the sole presence of the histone variant on promoter is not sufficient for the inheritance of the active gene state, but also requires the presence of specific PTMs. Functional studies, including formal genetic analyses of *H3.3* genes, are now required to progress on these fascinating aspects of chromatin function.

Functions of H3.3 in sexual reproduction

Besides its general replacement and epigenetic roles in somatic cells, several recent studies have highlighted the implication of H3.3 in chromatin remodeling mechanisms unique to the germline (Ooi and Henikoff, 2007). Although different in nature and function, these processes all require extensive RI nucleosome disassembly/reassembly at the genome or chromosome level.

Meiotic sex chromosome inactivation (MSCI)

The pachythene phase of the first meiotic prophase in mammalian males is characterized by the formation of synapses between chromosome pairs in preparation of recombination. Only the non-homologous X and Y chromosomes partially escape this process and are separated in a specific chromatin domain, the “XY body”. In this domain, the sex chromosomes are transcriptionally silenced in a process called MSCI (Turner, 2007). Unsynapsed autosomal chromatin is also silenced in a similar mechanism called MSUC (Meiotic Silencing of Unsynapsed Chromatin). A recent study discovered that both MSCI and MSUC depend on an extensive nucleosome replacement mechanism involving the deposition of the H3.3 variant (van der Heijden *et al.*, 2007). To which extent H3.3, and its chaperone HIRA, are critical for this process is yet unknown, but it is interesting to note that male mice with an impaired *H3.3A* gene have reduced fertility (Couldrey *et*

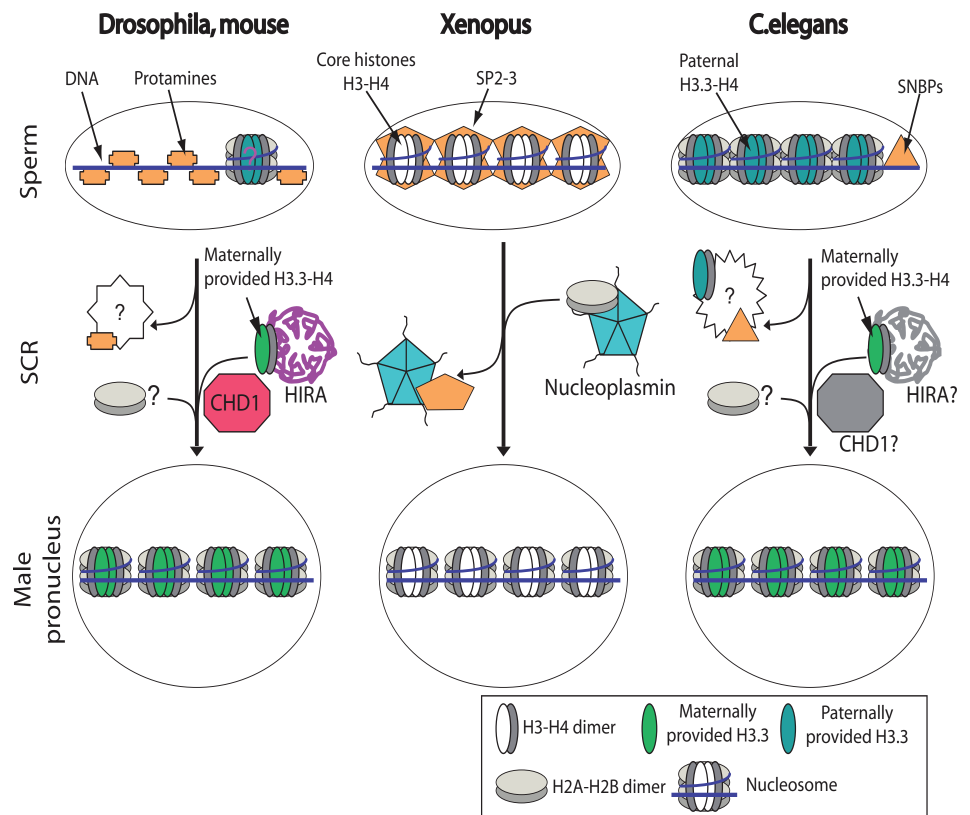

Fig. 4. Comparison of SCR in different animal models. *Schematic illustrations of sperm chromatin, SCR and male pronucleus chromatin in* Drosophila, *mouse,* Xenopus *and* C. elegans. Drosophila *and mouse present a protamine-based sperm chromatin structure although small levels of core histones could remain associated to DNA.* Xenopus *sperm chromatin is organized in nucleosome-like structures where core histones H3 and H4 are associated with sperm specific nuclear basic proteins SP2-3. Whether these core H3s are canonical H3 or H3.3 variants is not known.* C. elegans *sperm chromatin is probably organized with nucleosomes containing H3.3 although sperm-specific Small Nuclear Basic Proteins (SNBPs) are present as well. During SCR, a yet unknown factor removes protamines in* Drosophila *and mouse, and histone chaperone HIRA deposits maternally provided H3.3 and H4. In* Xenopus, *nucleoplasmin exchanges SP2-3 for H2A-H2B thereby reconstituting nucleosomes in male pronucleus. In* C. elegans, *unknown factors participate in the exchange of paternally provided H3.3 and SNBPs with maternally provided H3.3.*

al., 1999). MSCI represents a case of chromosome wide, RI chromatin remodeling that is involved in silencing. Along with the implication of H3.3 in sperm chromatin remodeling at fertilization (see below), this developmental process indicates that H3.3 can be deposited at large genomic regions that are depleted in nucleosomes. In *C. elegans*, a mechanism presenting similarities with MSCI is responsible for the silencing of the X chromosome during male meiosis (reviewed in Ooi and Henikoff, 2007). In the absence of a homologous counterpart, the X chromosome is silenced. Similar to the situation in mouse, it correlates with enrichment in silent PTMs such as H3K9me2 (Reuben and Lin, 2002). However, in the nematode, H3.3 is surprisingly depleted from the silent X chromosome, suggesting that, in contrast to MSCI, silencing does not involve chromosome wide RI nucleosome assembly (Ooi and Henikoff, 2007; Ooi *et al.*, 2006).

Spermiogenesis

After the completion of meiosis, spermatids undergo a complex differentiation process called spermiogenesis, which results in the production of mature gametes. Marking features of this maturation include the formation of a motile flagellum, the elimination of excess cytoplasmic materials and the dramatic rearrangement of the nuclear architecture. In many species, spermiogenesis is in fact the only differentiation process where nuclei loose, in a reversible manner, their nucleosome-based chromatin to a totally different structure. Indeed, histones are first replaced with transition proteins and then with Sperm Nuclear Basic Proteins (SNBPs) during the condensation phase of spermatid nuclei. SNBPs include testis specific histone variants but also non-histone proteins such as protamine-like proteins and protamines (Caron *et al.*, 2005; Govin *et al.*, 2004; Lewis *et al.*, 2003; Poccia and Collas, 1996). The sperm chromatin structure is highly diverse in animals, even between species of the same animal group (Frehlick *et al.*, 2006). In general, sperm chromatin is highly condensed and thus not compatible with DNA replication or transcription (Poccia and Collas, 1996). Like other core histones, H3.3 is expressed in the male germ line. In *Drosophila*, only the histone *H3.3A* gene is strongly expressed in testis (Akhmanova *et al.*, 1995) and the protein is detected in nuclei at all stages of spermatogenesis, with the exception of late spermatid and mature sperm nuclei (Akhmanova *et al.*, 1997; Bonnefoy *et al.*, 2007). Because these studies relied on immunofluorescence techniques, the possibility remained that H3.3 epitopes were not accessible in highly condensed spermatid and sperm nuclei. However, the use of a *H3.3-mRFP1* expressing transgene confirms that H3.3 is eliminated from the spermatid nuclei, just before the deposition of protamines (Fig. 3). This situation is in clear contrast with the case of the nematode *C. elegans* that retains H3.3 in mature sperm nuclei (Ooi *et al.*, 2006), illustrating the diversity of sperm chromatin architecture and composition in animals. Although the bulk of sperm chromatin in *Drosophila*, mouse or humans is packaged with protamines, it also retains a variable proportion of histones (Caron *et al.*, 2005; Dorus *et al.*, 2006; Poccia and Collas, 1996; Raja and Renkawitz-Pohl, 2005). Consequently, it has been proposed that histones, and, possibly, H3.3, could play a role in transmitting epigenetic information

through the male gamete (Ooi and Henikoff, 2007). In mammals, similarly, it has also been proposed that paternal imprinting control regions could escape the histone/protamine exchange and would remain organized in nucleosomes in mature sperm (Delaval *et al.*, 2007). The emergence of global ChIP approaches should help determining the putative role of H3.3 in the chromatin landscape of the male gamete.

In *Drosophila*, almost all the transcription required for spermiogenesis occurs in primary spermatocytes (Fuller, 1993). Thus, the abundance of H3.3 in the male germline also probably reflects the high level of transcription that takes place in these cells. Another possible role for H3.3 in spermatid nuclei could be related to its "nucleosome destabilizing" property. Indeed, nucleosomes containing H3.3, alone or in synergy with the H2A.Z variant, are more prone to loose H2A/H2B dimers in salt-disruption experiments, than regular nucleosomes (Jin and Felsenfeld, 2007). Similarly, assembly and disassembly of nucleosomes containing the mammalian variant H2A.Bbd occur more efficiently in association with H3.3 than with H3 (Okuwaki *et al.*, 2005). If this were true in the context of *in vivo* chromatin, it would be interesting to see if it has any role in facilitating the replacement of nucleosomes with transition proteins and protamines during spermatid differentiation.

In flowering plants, the structure of the male gamete chromatin is poorly known. Recently however, a pollen specific *H3* gene called *AtMGH3* has been identified in *Arabidopsis*, along with eight *H3.3* genes (Okada *et al.*, 2005). Although AtMGH3 is quite distantly related to animal H3.3 (see Fig. 2), this histone has the same amino-acid substitutions at position 87, 89 and 90 than those found in plant *H3.3* genes. Moreover, this gene was found to exhibit RI expression in male gametic cells (Okada *et al.*, 2005). AtMGH3 is present in the chromatin of the male gamete and, similarly to the situation found in *C. elegans*, this H3 variant is removed from the zygote nucleus in a RI manner (Ingouff *et al.*, 2007). Interestingly, *AtMGH3* mutants do not seem to display any phenotype, probably indicating a redundant role with other H3 variants (Okada *et al.*, 2005).

Male pronucleus formation

The formation of the male pronucleus at fertilization implies the removal of SNBPs followed by *de novo* assembly of paternal nucleosomes, a process called SCR (Sperm Chromatin Remodeling) (Fig. 4). An essential, although largely overlooked aspect of SCR, is the fact that paternal chromatin assembly takes place independently of DNA synthesis (Nonchev and Tsanev, 1990; Poccia *et al.*, 1984). The recent discovery that H3.3 was specifically deposited in the decondensing sperm nucleus in *Drosophila* and mouse confirmed the RI nature of this conserved process (Loppin *et al.*, 2005; Torres-Padilla *et al.*, 2006; van der Heijden *et al.*, 2005). In these two model species, the sperm chromatin is essentially packaged with protamines (see previous section). Thus, the RI reassembly of H3.3 containing nucleosomes on paternal DNA is a genome wide process. The male pronucleus is in fact the only nucleus to undergo whole genome RI chromatin assembly during development. The specific use of the H3.3 variant in SCR is remarkable, in particular for those species where large pools of maternally expressed histones, including H3 and H3.3, are stored in the egg. In *Drosophila*, for instance, early development is under strict maternal control and zygotic transcription begins when several thousands nuclei have already assembled their chromatin (Foe, 1993). By analyzing transgenic fly lines expressing tagged versions of H3 or H3.3, we have shown that H3.3, and not H3, is deposited during SCR (Loppin *et al.*, 2005). SCR is thus under the control of a specific nucleosome assembly machinery that specifically uses H3.3, despite the availability of both histone types in large quantities. Thus, SCR is clearly a process where H3.3 deposition is not determined by the simple unavailability of H3, but by its proper nucleosome assembly pathway.

In *C. elegans* and *Arabidopsis*, H3.3 histones are present at apparently high levels in the male gamete, in contrast to mouse or *Drosophila*. Surprisingly however, these paternal histones are also removed at fertilization, before the first zygotic DNA replication (Ingouff *et al.*, 2007; Ooi *et al.*, 2006). In *C. elegans* (this is not known for *Arabidopsis*), a RI deposition of maternally expressed H3.3 is observed during SCR, as in *Drosophila* or mouse. The functional signification of this apparent replacement is not clear. Mass spectrometry analysis of *C. elegans* sperm has revealed the presence of SNBPs similar to invertebrate protamines (Chu *et al.*, 2006), suggesting that maternal H3.3 replaces the removed SNBPs. In this case, paternal H3.3 would be removed along with SNBPs before global deposition of maternal H3.3. It is thus difficult to imagine any epigenetic role for paternal H3.3, at least for the bulk of it. More probably, the persistence of high levels of H3.3 in sperm could only reflect the vast diversity of sperm chromatin types in animals (Poccia and Collas, 1996). In their recent finding that H3.3 was the support for the epigenetic memory of active gene states in nuclear transfer experiments, Ng and Gurdon (2008) pointed the importance of H3.3 lysine 4 in this phenomenon. Indeed, a mutant form of H3.3 with a glutamic acid in position 4 interfered with the epigenetic inheritance. It is interesting to note that maternal H3.3 incorporated during *Drosophila* or mouse SCR is not methylated on lysine 4 (Loppin *et al.*, 2005; van der Heijden *et al.*, 2005), thus reinforcing the view that SCR is essentially a neutral replacement process. Accordingly, in *Drosophila*, the paternal H3.3 enrichment is lost after a few nuclear cycles as the chromatin accumulates H3 nucleosomes at each S phase (Bonnefoy *et al.*, 2007). In this case, the perpetuation of a putative H3.3 "barcode", as proposed by Hake and Allis (2006), is not observed. Whether the distinction between methylated and non-methylated forms of H3.3 is involved here remains to be established.

Roles of nucleosome assembly machineries in the deposition of H3.3

The HIRA nucleosome assembly pathway

Although the implication of the CAF-1 complex in RC chromatin assembly was established long ago (Smith and Stillman, 1989), the identification of assembly factors able to deposit histones in the absence of DNA synthesis received attention only recently. HIRA belongs to the HIR family of proteins whose funding members are the budding yeast Hir1p and Hir2p proteins (Spector *et al.*, 1997). These two proteins are orthologs to the N- and C-terminus of HIRA proteins, respectively (Lamour *et al.*, 1995). HIRA proteins are characterized by the presence of seven WD-repeats known to assemble into a secondary structure called a Beta propeller (Smith *et al.*, 1999). In mouse, *Hira* is an essential

gene and knocked-out embryos die early in development with a complex phenotype that has been interpreted as resulting from precocious cell differentiation (Meshorer *et al.*, 2006; Roberts *et al.*, 2002). It is the finding that HIRA had histone binding properties that fuelled its functional characterization *in vitro* (Lorain *et al.*, 1998). The nucleosome assembly activity of HIRA was initially characterized from *Xenopus* egg extracts, and found to be specific for a DNA synthesis-independent assembly pathway (Ray-Gallet *et al.*, 2002). The subsequent purification and characterization of proteins interacting with H3.1 and H3.3 in human cells established a first link between H3.3 and HIRA (Tagami *et al.*, 2004). HIRA and the two largest CAF-1 subunits were specifically found in the H3.3 and H3.1 complexes, respectively, hence confirming the existence of distinct assembly pathways defined by their dependence on DNA synthesis, assembly factors and preferential histone H3 type.

SCR: a challenging task for RI nucleosome assembly machineries

The *in vivo* function of *Hira* received an unexpected highlight from the characterization of *sésame* (*ssm*), its first mutant allele in *Drosophila*. Embryos produced by homozygous mutant *ssm* females are haploid and develop with the sole, maternally derived, chromosome set. The loss of paternal chromosomes occurs at the first embryonic mitosis and is the consequence of a defect in male pronucleus formation (Loppin *et al.*, 2000). In *Drosophila*, SCR classically involves the rapid replacement of two closely related protamines with maternally provided histones (Bonnefoy *et al.*, 2007; Raja and Renkawitz-Pohl, 2005; Rathke *et al.*, 2007). Moreover, *Drosophila* SCR is a RI process that specifically involves the H3.3 variant (Loppin *et al.*, 2005). In *ssm* eggs, SCR is defective: although protamines are normally removed, the sperm derived nucleus does not incorporate histones. As a consequence, the male pronucleus does not fully decondense and does not replicate its DNA (Bonnefoy *et al.*, 2007; Loppin *et al.*, 2001; Loppin *et al.*, 2005). In *Drosophila*, HIRA is thus critical for the RI chromatin assembly of the whole paternal genome and specifically assembles H3.3 containing nucleosomes (Loppin *et al.*, 2005). In addition, *Drosophila* HIRA has also been implicated in H3.3 deposition at a regulatory site near a variegating *white* transgene inserted near centromeric heterochromatin (Nakayama *et al.*, 2007). Histone exchange at this site is dependent on the binding of the GAGA factor-FACT complex. In *ssm* flies, the silencing of this *white* transgene is enhanced, indicating that HIRA is involved in counteracting the spreading of heterochromatin in this locus (Nakayama *et al.*, 2007). Surprisingly, homozygous flies with a null allele of *Hira* are viable and female sterility is the only associated phenotype (Bonnefoy *et al.*, 2007). This surprising result indicates that any function of HIRA not related to SCR is dispensable in *Drosophila*. In mouse, the zygotic lethality of *Hira* knocked-out embryos does not allow testing the requirement of maternal HIRA for SCR. However, considering that mouse HIRA actually localizes to the decondensing male pronucleus and that SCR involves the massive deposition of H3.3 in this species (Torres-Padilla *et al.*, 2006; van der Heijden *et al.*, 2005), this critical function of HIRA is most probably conserved. In fact, HIRA is expected at least to play this role in a majority of species whose sperm contains non-nucleosomal chromatin. Some species, like the frog *Rana catesbeiana*, for instance, do not seem to contain protamines or protamine-like SNBPs but instead retain core histones in the sperm chromatin (Frehlick *et al.*, 2006). It is thus possible that HIRA is not required for SCR in these species. Similarly, *Xenopus* sperm chromatin retains H3 and H4 whereas H2A and H2B are replaced with protamin-like proteins. Since nucleoplasmin, a histone chaperone for H2A and H2B is necessary and sufficient for *Xenopus* SCR *in vitro* (Philpott and Leno, 1992; Philpott *et al.*, 1991), it suggests that this process does not actually require a H3/H4 RI assembly factor such as HIRA (Fig. 4).

The specific *Hira* mutant phenotype observed in *Drosophila* could result from a function of HIRA related to some peculiar features of SCR, rather than from a general RI nucleosome assembly defect. At least, we know that the removal of protamines itself does not seem to depend on HIRA because these SNBPs are normally removed in *Hira* mutant eggs (Bonnefoy *et al.*, 2007). The recent discovery that another nucleosome assembly factor, CHD1, was important for male pronucleus formation in *Drosophila* shed a new light on this process (Konev *et al.*, 2007). CHD1 (Chromo-ATPase/Helicase-DNA-binding protein 1) is an ATP-dependent nucleosome remodeling factor of the SNF2-like family of proteins, which is characterized by the presence of two chromodomains (Brown *et al.*, 2007; Hall and Georgel, 2007; Marfella and Imbalzano, 2007; Woodage *et al.*, 1997). *In vitro*, CHD1 facilitates the transfer of histones from the NAP-1 histone chaperone to DNA and allows the assembly of regularly spaced nucleosomes (Lusser *et al.*, 2005). *Drosophila* adults with no functional CHD1 survive but are sterile. In females, the sterility results from a 100% maternal effect embryonic lethality. As in embryos produced by *Hira* mutant females, the male nucleus in *chd1* mutant eggs is unable to participate in the formation of the zygote (Konev *et al.*, 2007). In contrast to *Hira* mutant eggs, where the male nucleus is always spherical and devoid of histones, the male nucleus in *chd1* mutant eggs adopts various shapes and histones are detected (Konev *et al.*, 2007) (G.A.O and B.L unpublished observations). Notably, H3.3 is detected in the paternal chromatin of *chd1* mutant eggs (Fig. 5), indicating that at least some HIRA-dependent histone deposition occurs in the absence of this motor protein. Thus, CHD1 could synergize with HIRA for the very rapid and massive RI nucleosome assembly activity required for SCR or could participate in the regular spacing of nucleosomes on paternal DNA.

Although the exact function of CHD1 at fertilization remains to be determined, it is remarkable that SCR, a process that occurs once in the life cycle and in a single nucleus, represents a critical task for at least two different nucleosome assembly factors. Understanding how these proteins are orchestrated *in vivo* for RI assembly over a whole genome is a fascinating question for future research.

Multiple assembly pathways involved in H3.3 deposition?

Although the functional characterization of H3.3 in metazoans awaits formal genetic analysis, it is now clear that this histone variant is involved in a variety of chromatin remodeling mechanisms. Whether these mechanisms rely on different nucleosome assembly pathways largely remains to be investigated. The fact that H3.3 is deposited independently of DNA synthesis is a major property that distinguishes it from H3, although at least one exception has been reported in the *Xenopus* oocyte where H3 seems to be RI deposited by a dynamic histone exchange process (Stewart *et al.*,

2006). Several lines of evidence indicate that H3.3 is deposited during S phase. In *Drosophila* cultured cells, overexpressed H3.3 is deposited at sites of DNA replication (Ahmad and Henikoff, 2002b). In early *Drosophila* embryos, during the rapid nuclear cleavages and before the onset of zygotic transcription, we have observed a relatively weak and uniform deposition of H3.3 in the chromatin of all nuclei that we interpret as S phase deposition (Bonnefoy *et al.*, 2007). Interestingly, this H3.3 deposition does not depend on the presence of the HIRA protein, opening the possibility that the CAF-1 complex could be responsible for the bulk of H3.3 nucleosome assembly during early *Drosophila* development. In this peculiar developmental context, where both H3 and H3.3 are stored in the egg and are thus available in large quantities, the RC assembly machinery seems to allow some deposition of the RI variant despite the fact that H3 is preferentially deposited (Bonnefoy *et al.*, 2007). More generally, the different models accounting for the propagation of epigenetic states through cell divisions also imply the deposition of H3.3 at DNA replication forks (Eitoku *et al.*, 2007; Hake and Allis, 2006; Henikoff *et al.*, 2004; Polo and Almouzni, 2006). However, the simple hypothesis that HIRA could participate in this task is challenged by our observations in fly embryos and thus deserves a real investigation.

Asf1 (Anti Silencing Factor 1) is a conserved histone chaperone involved in both RC and RI assembly pathways (reviewed in (De Koning *et al.*, 2007; Eitoku *et al.*, 2007; Mousson *et al.*, 2007). Several recent studies have showed that Asf1 interacts with a single H3-H4 dimer (Agez *et al.*, 2007; Antczak *et al.*, 2006; English *et al.*, 2005; Mousson *et al.*, 2005) suggesting that Asf1 could function in distributing H3-H4 or H3.3-H4 dimers to CAF-1 and HIRA, respectively. In addition, Asf1 plays a critical role for the unwinding of DNA replication forks by disrupting (H3-H4)2 tetramers (Natsume *et al.*, 2007) and by interacting with the putative replicative helicase MCM2-7 (Groth *et al.*, 2007). However, Asf1 is not directly involved in *de novo* RI or RC histone deposition in *Xenopus* egg extracts (Ray-Gallet *et al.*, 2007). Similarly, Asf1 is not detected in the decondensing male nucleus during *Drosophila* SCR (Bonnefoy *et al.*, 2007).

Interestingly, while TC assembly of H3.3 nucleosomes is well established, the histone chaperone responsible for this deposition remains elusive. In *Drosophila*, adults devoid of HIRA are viable suggesting that HIRA is not critical for TC assembly (Bonnefoy *et al.*, 2007). In addition, the absence of HIRA only causes a slightly delayed growth in chicken cells (Ahmad *et al.*, 2005). Spt6 and FACT are histone binding proteins that are involved in the reassembly of nucleosomes after the passage of the RNA polymerase II and thus represents interesting candidates for TC H3.3 deposition (Adkins and Tyler, 2006; Andrulis *et al.*, 2000; Belotserkovskaya *et al.*, 2004; Kaplan *et al.*, 2000). In addition to its role in SCR mentioned above, the CHD1 assembly factor has also been shown to affect H3.3 deposition in *Drosophila* blastoderm embryos suggesting its participation in TC assembly (Konev *et al.*, 2007).

The diversity of RI chromatin assembly processes should thus be reflected by the implication of various assembly factors, depending both on the model species as well as on the developmental or cellular processes considered. Understanding how these different factors

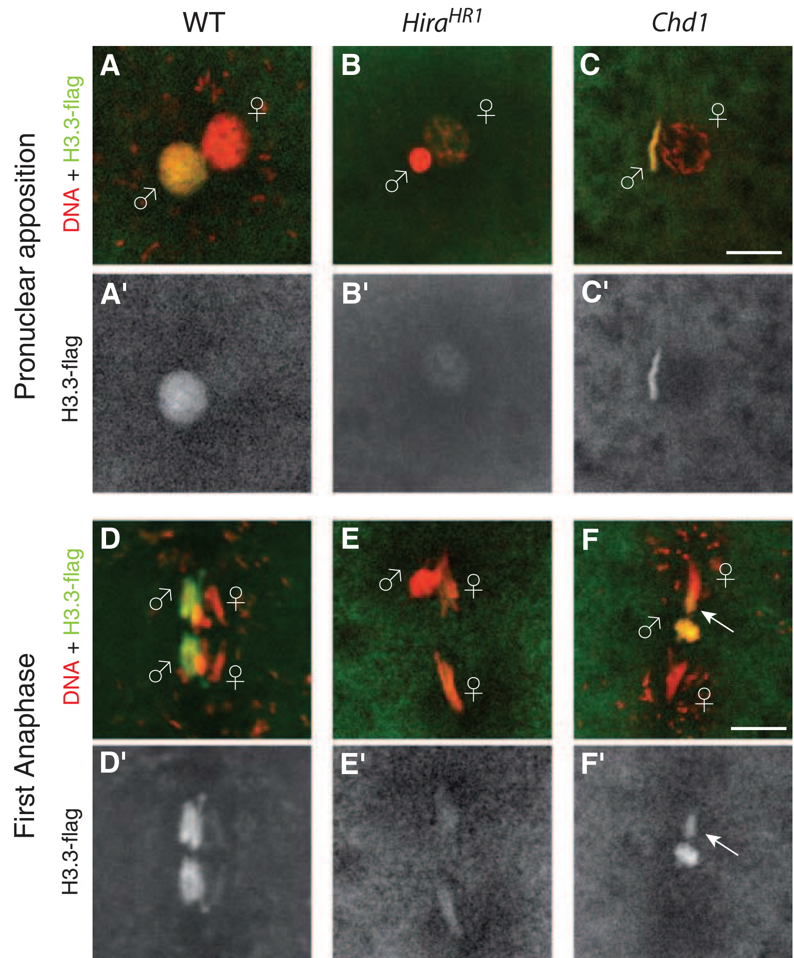

Fig. 5. HIRA and CHD1 are involved in *Drosophila* SCR. *Confocal images of eggs at the pronuclear apposition stage* **(A,B,C,A′,B′,C′)** *or embryos at the first zygotic anaphase* **(D,E,F,D′,E′,F′)** *stained for DNA (red) and with an anti-FLAG peptide antibody to detect maternally expressed H3.3-FLAG (green or gray).* **(A,A′)** *In eggs from wild type females, H3.3-FLAG is detected in the male pronucleus.* **(B,B′)** *In eggs laid by mutant* Hira^{HR1}/Hira^{HR1} *females, H3.3-FLAG is not detected in the abnormally condensed male nucleus.* **(C,C′)** *In eggs laid by* chd1[1]/Df(2L)Exel7014 *females (with no functional CHD1 protein), the male nucleus is aberrant in shape but stains for H3.3-FLAG (see also Konev* et al.*, 2007).* **(D,D′)** *During the first zygotic anaphase, paternal chromosomes still contain high levels of H3.3-FLAG.* **(E,E′)** *In* Hira^{HR1} *mutant eggs, the male nucleus is excluded from the first spindle that contains only maternal chromosomes.* **(F,F′)** *In* chd1 *mutants eggs, the male nucleus is occasionally incorporated in the first mitosis but paternal chromosomes (stained with H3.3-FLAG) segregation is defective (arrow). Wild-type males were used to fertilize females of indicated genotypes. All females used in these crosses contained a copy of a* H3.3-FLAG *transgene (Loppin* et al.*, 2005). DNA positive dots visible in (A,D,F) are* Wolbachia *endosymbiotic bacteria.*

cooperate and interact on the nucleosome assembly line will certainly need the forces of both biochemical and *in vivo* approaches.

Conclusion

Two levels of complexity challenge the dynamic nature of eukaryotic chromatin. The first level is common to most cells and includes the invariable remodeling events associated with the cell cycle, from DNA replication to cellular senescence. The diversity of remodeling processes that occur during development represents a second level of complexity, which is best illustrated by the dramatic reorganization of chromatin associated with the transmission of paternal DNA from one generation to another. The universal ability of eukaryotic cells to assemble nucleosomes independently of DNA replication drives this versatility. The H3.3 histone variant is at the heart of RI nucleosome assembly mechanisms. Being very close to its RC counterparts at the primary sequence level, H3.3 fulfills a neutral replacement role supported by its constitutive expression. In addition, the biochemical characterization of the H3.3 deposition pathway, the association of this variant with active PTMs, as well as its dynamic distribution over the genome have paved the road to establish a role in the epigenetic transmission of active chromatin states. Finally, developmental and genetic studies have unveiled unexpected roles for H3.3 or associated assembly factors in chromatin remodeling events essential for sexual reproduction. In this regard, the evolution of new functions for RI nucleosome assembly factors could be the key for the diversification of H3.3 roles. These different aspects of H3.3 biology must be considered to understand the evolutionary forces that shaped this histone and perpetuated it as one of the most conserved proteins in life.

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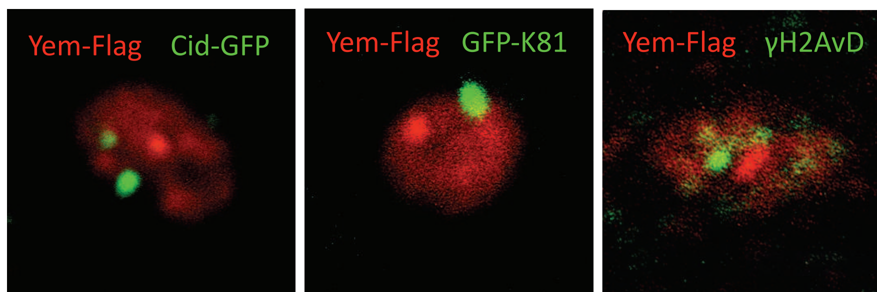


Figure 2. Les foyers d'accumulation de Yem ne correspondent pas aux centromères, aux télomères, ou aux sites de réparation de l'ADN.
Images confocales montrant des pronoyaux mâles dans des oeufs issus de femelles exprimant la protéine Yem-Flag. Le marquage révèle Yem-Flag (en rouge) et la protéine centromérique Cid-GFP, la protéine télomérique GFP-K81 ou le marqueur γ H2AvD (vert). Les foyers Yem-Flag ne coïncident avec la localisation d'aucune des protéines testées. Barre: 5µm.

Figure 1

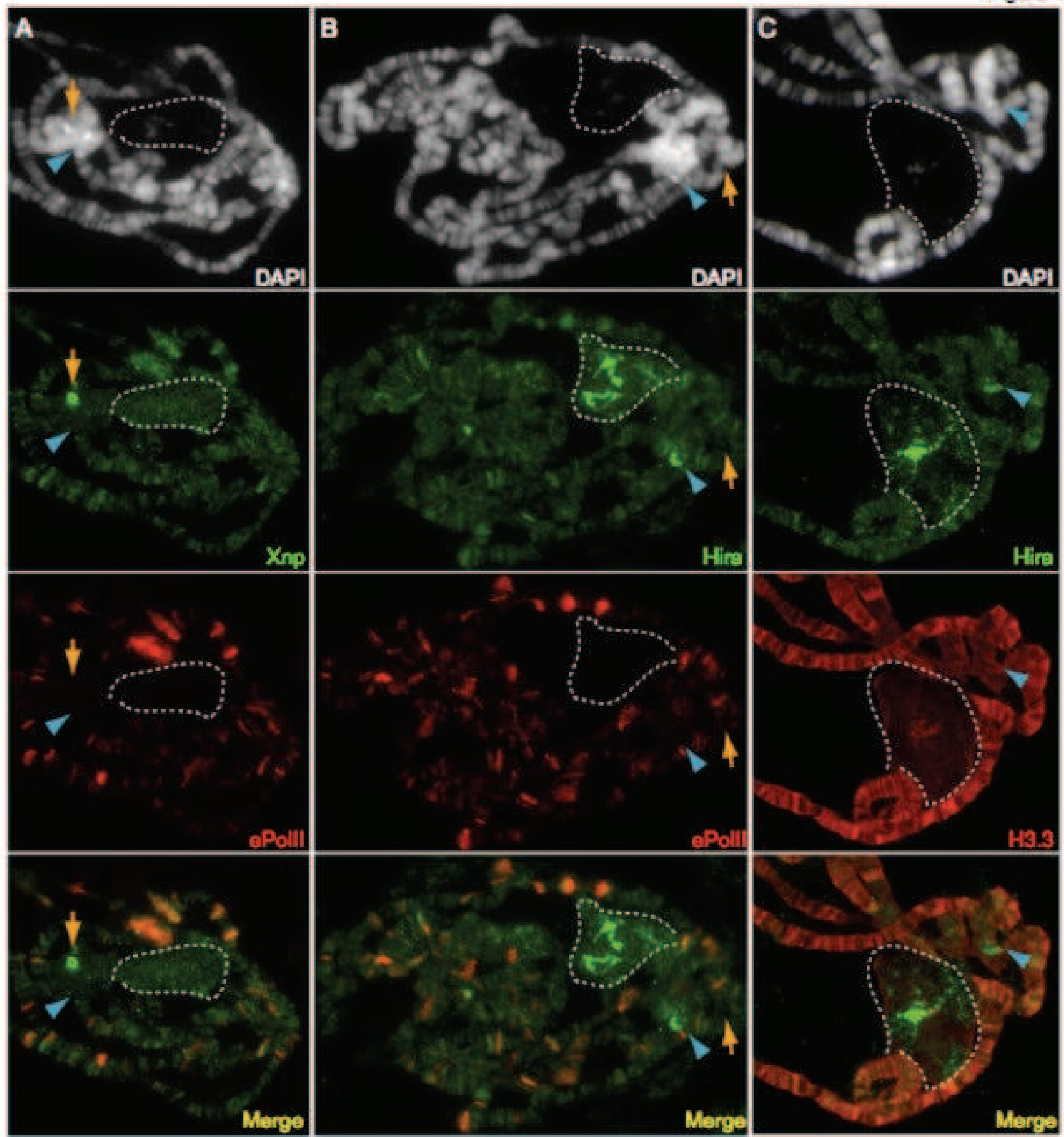


Figure 2

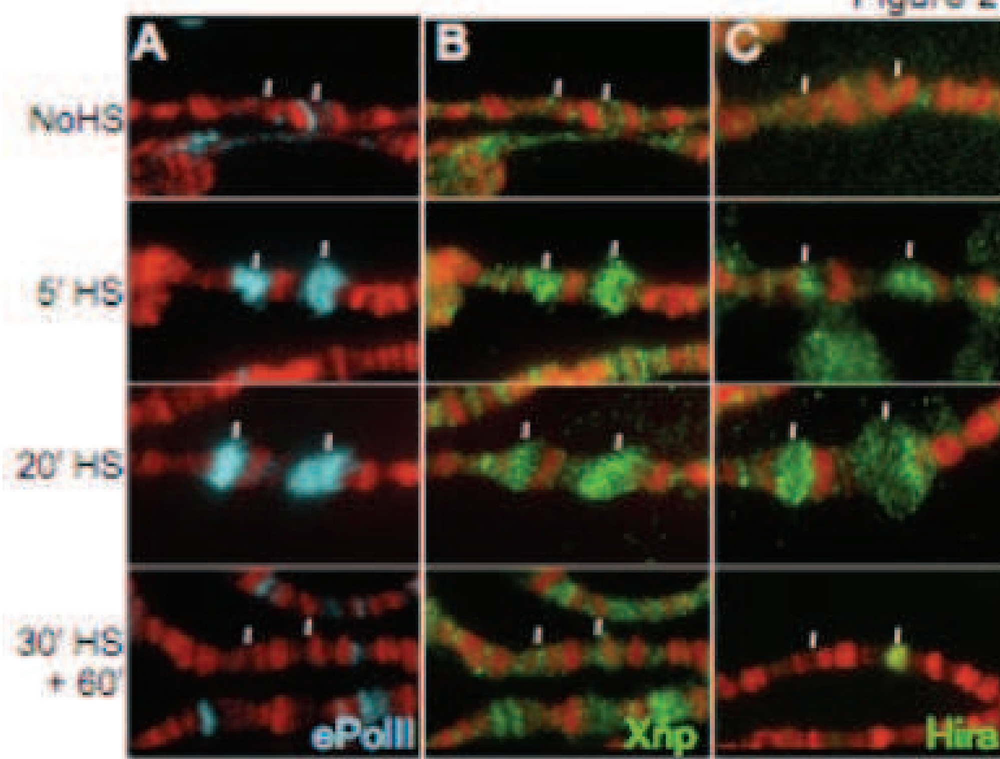
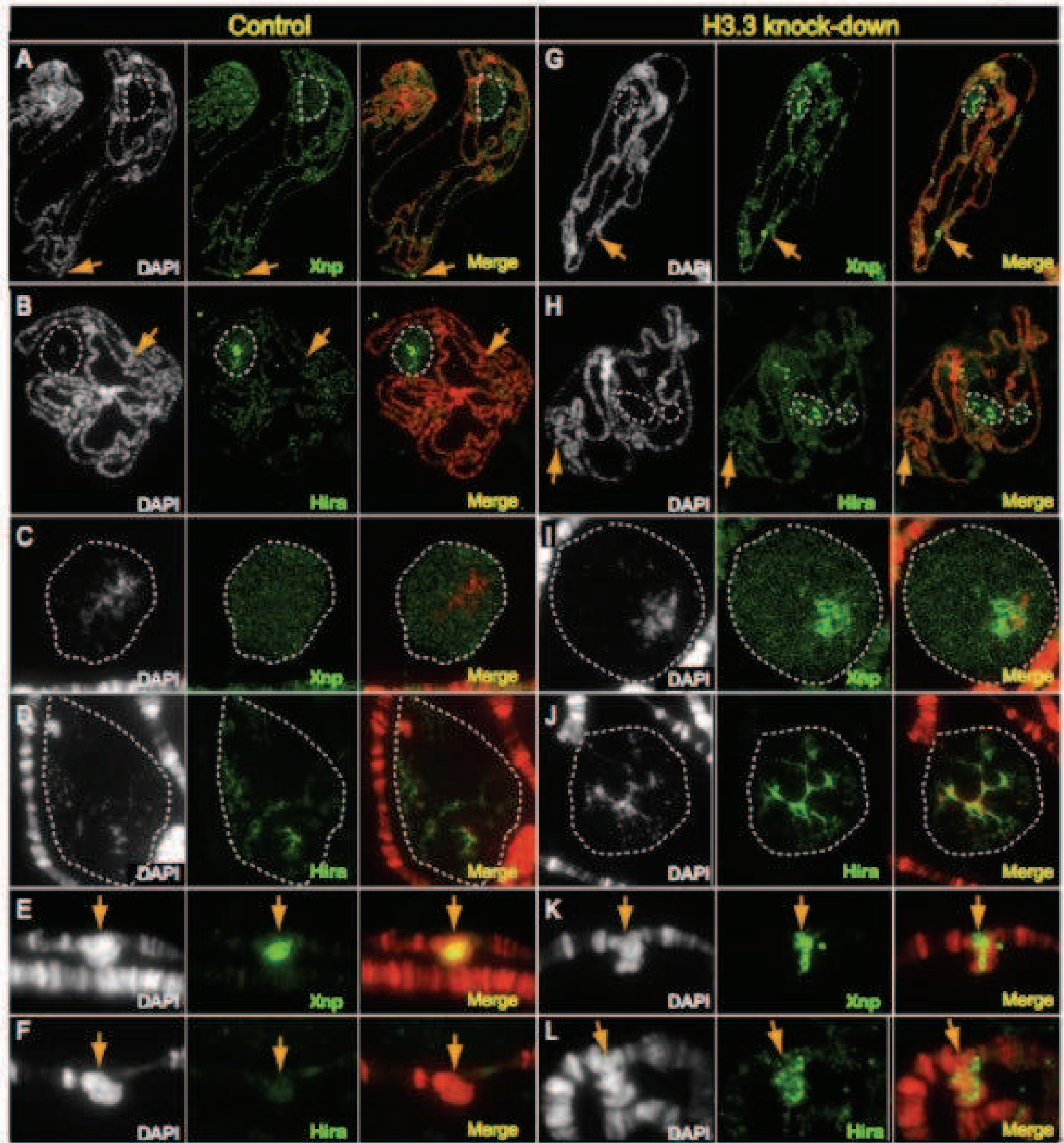


Figure 3



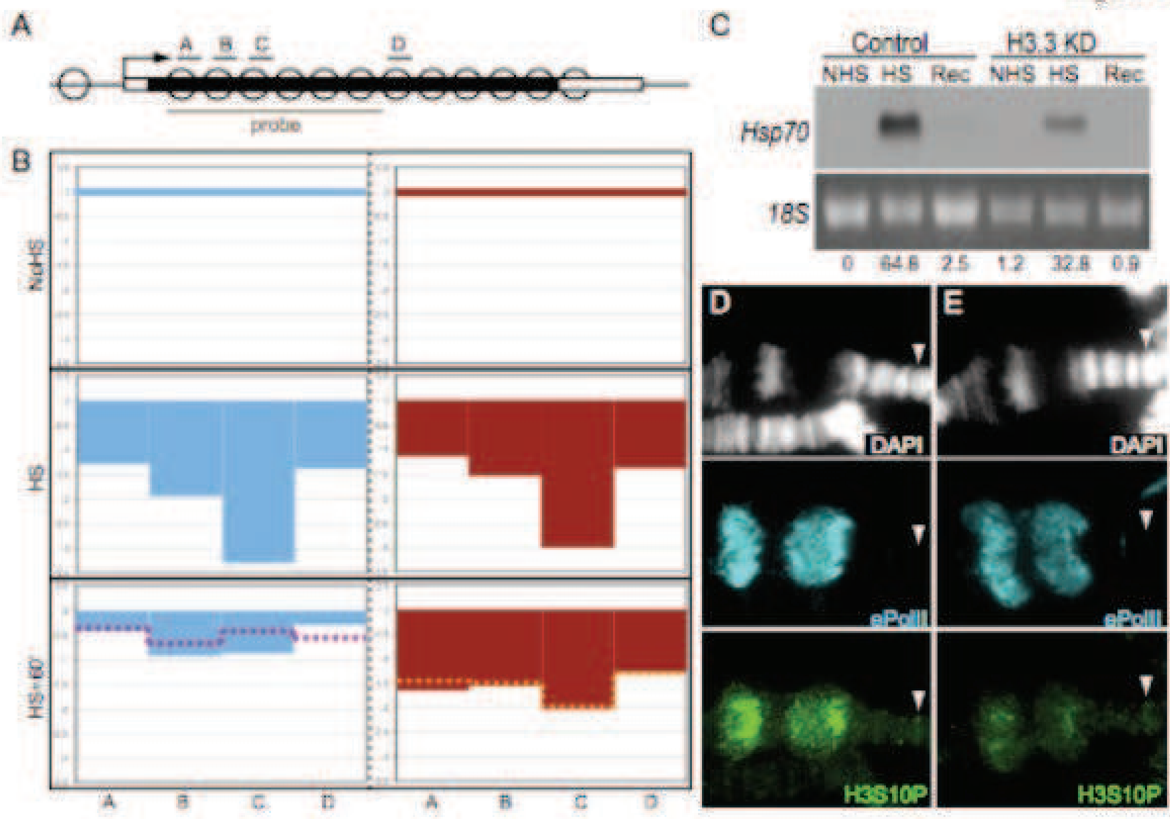


Figure 4
A
A B C D
probe
B
NoHS
HS
HS+60'
A B C D
A B C D
C
Control
H3.3 KD
NHS HS Rec NHS HS Rec
Hsp70
18S
0 64.6 2.5 1.2 32.8 0.9
D
E
DAPI
ePolII
H3S10P

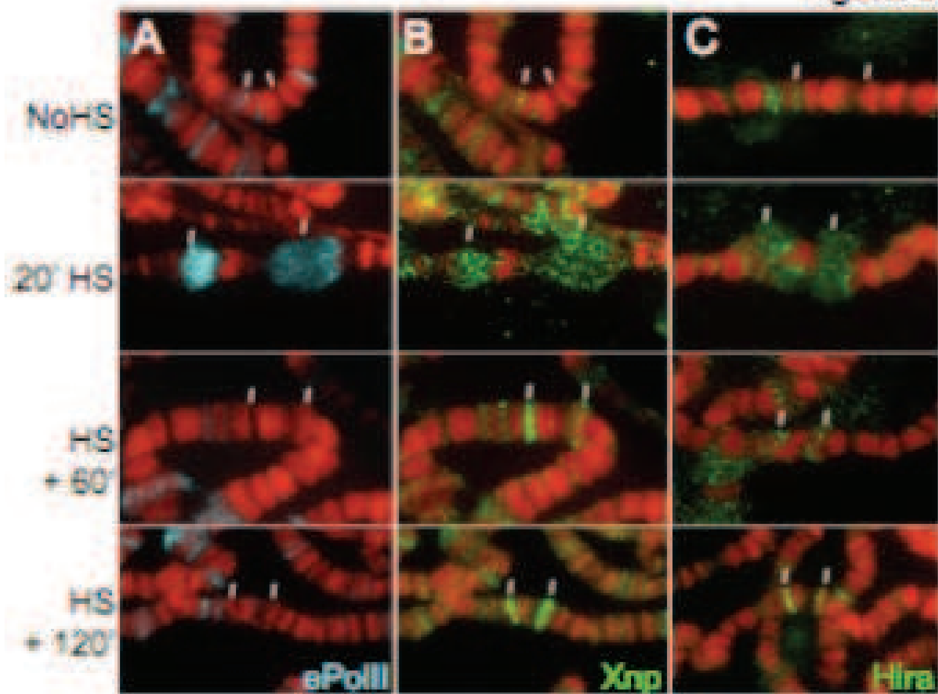
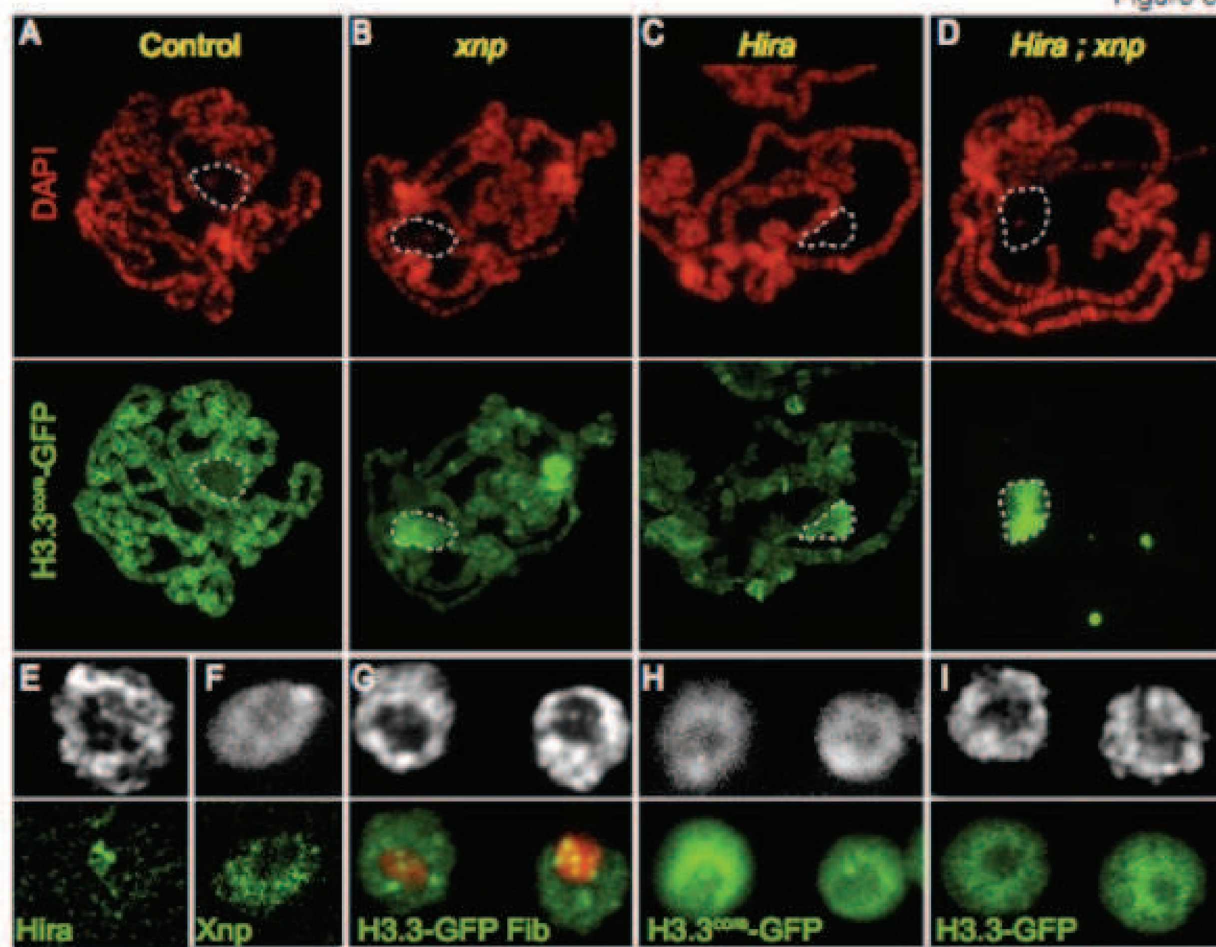
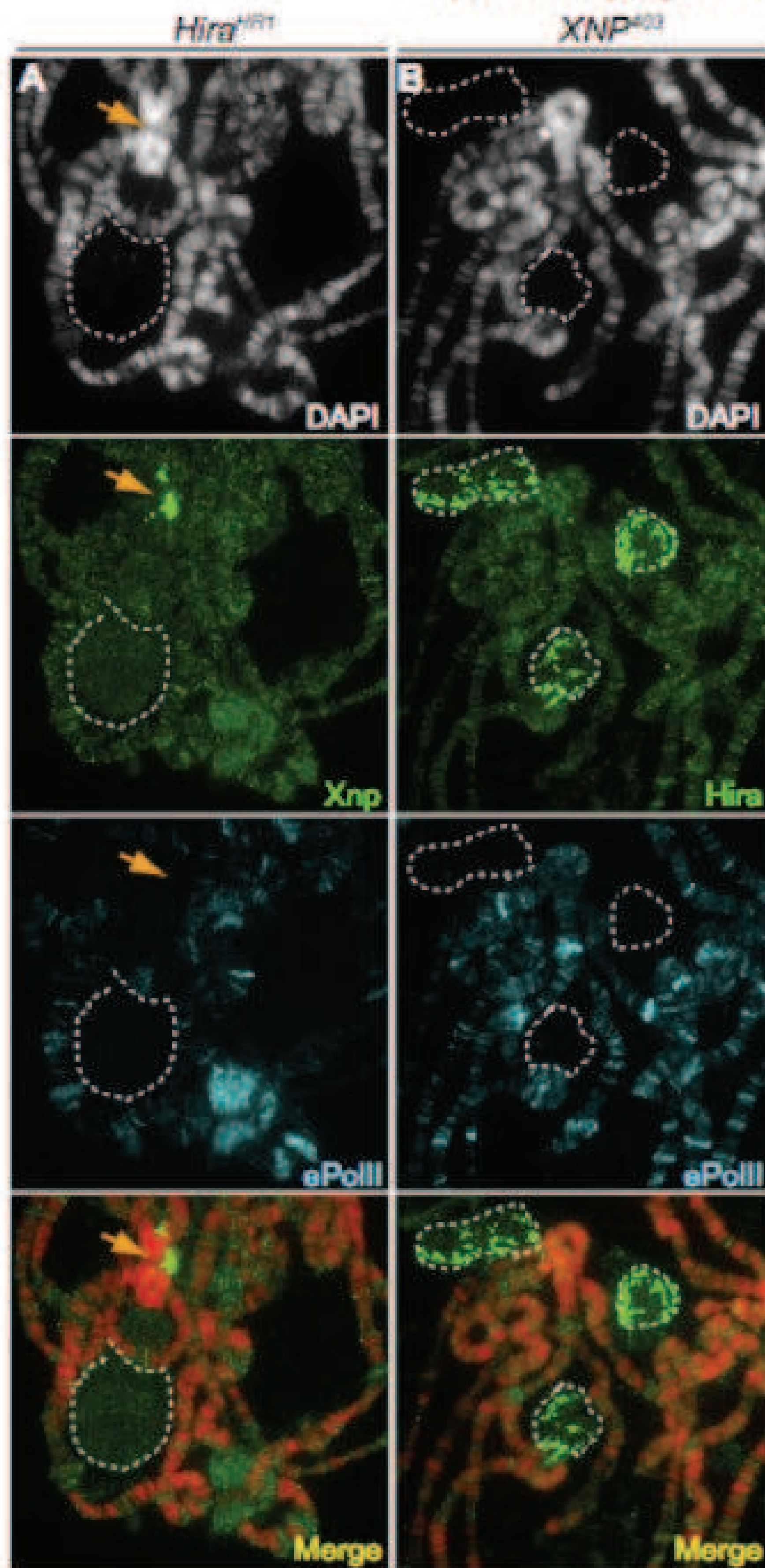


Figure 5
A
B
C
NoHS
20' HS
HS + 60'
HS + 120'
ePolII
Xnp
Hira

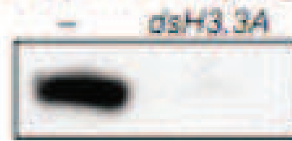
Figure 6



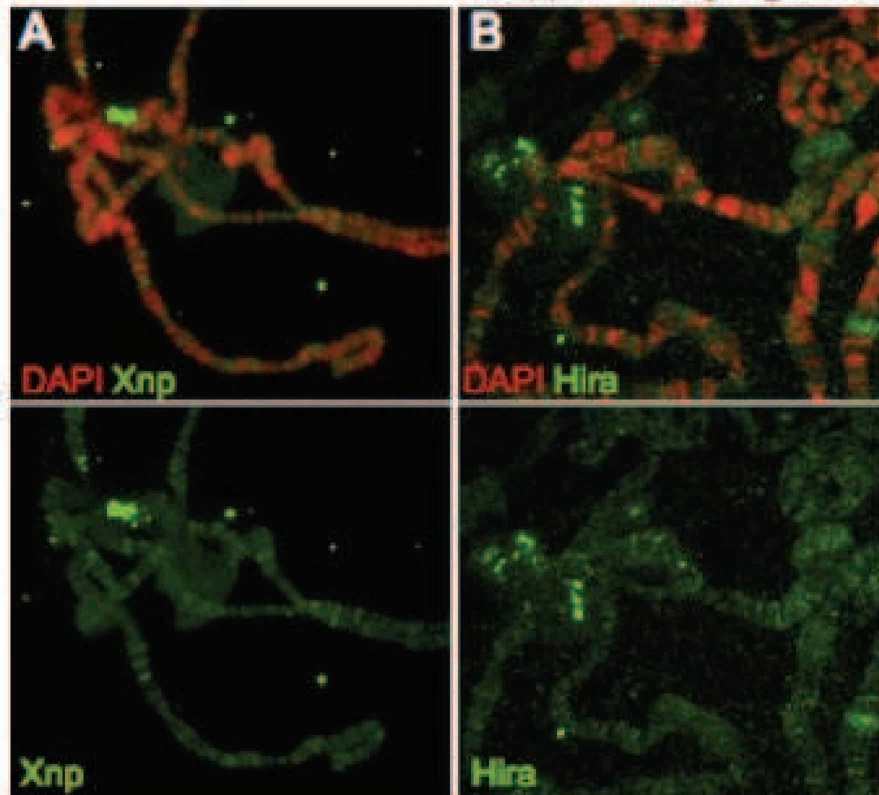
Supplementary Figure S1



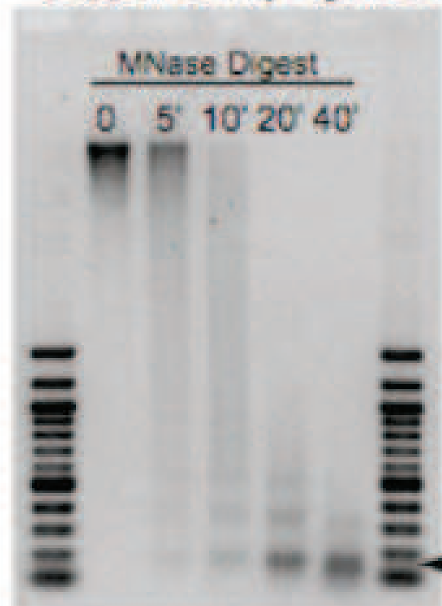
Supplementary Figure S2



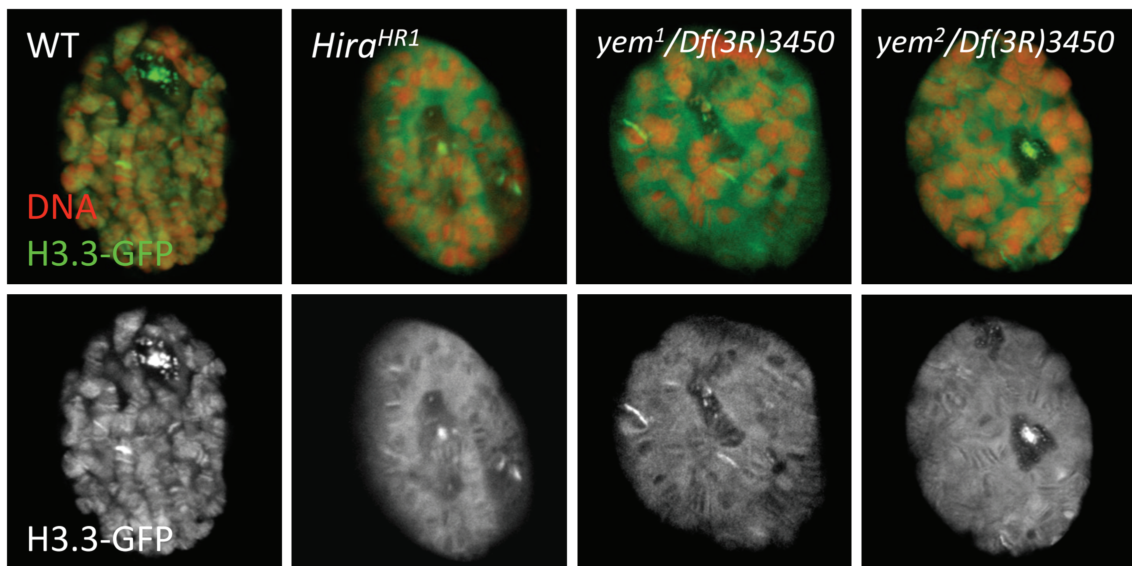
Supplementary Figure S3



Supplementary Figure S4



A.



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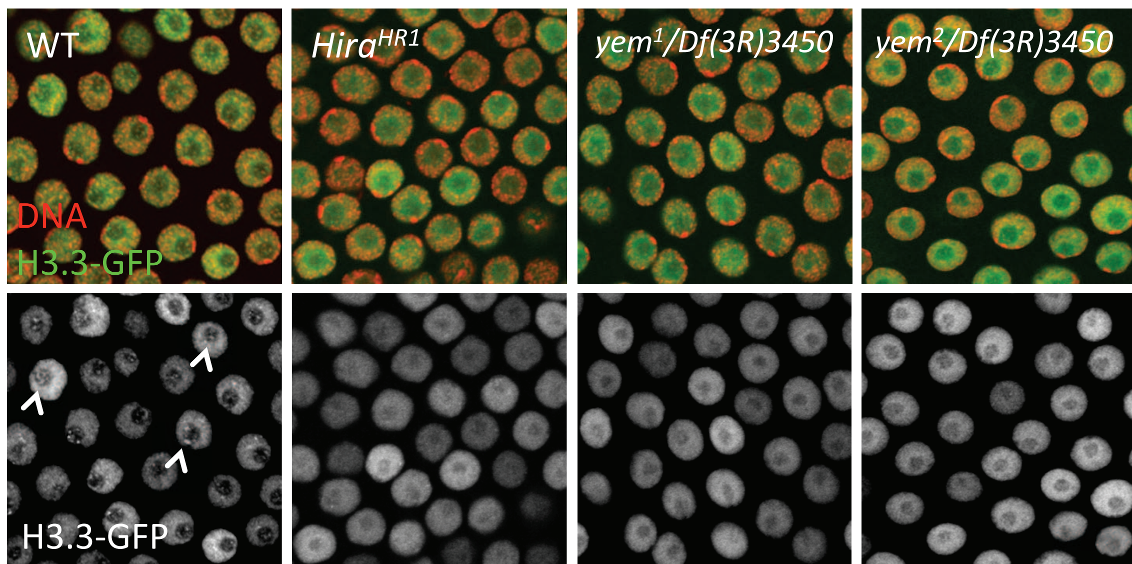

Figure 3. Yem est requis pour l'assemblage de H3.3 dans les cellules somatiques. L'expression de la protéine H3.3-GFP a été induite en contexte sauvage ou mutant pour Hira ou yem, dans des larves de stade 3 (A) ou des femelles adultes (B) par choc thermique. Un temps de chasse de 4 heures (A) ou 30 minutes (B) a été laissé avant dissection. Les images sont des photographies confocales de glandes salivaires (A) ou ovaires (B) marqués pour révèler l'ADN (rouge) et H3.3-GFP (vert). (A) L'incorporation de H3.3-GFP sur tous les chromosomes est fortement affectée en contexte mutant pour Hira comme pour yem. (B) De façon similaire, l'incorporation de H3.3-GFP dans le nucléole(flèches) est abolie chez ces mêmes mutants. Barres: 10µm.

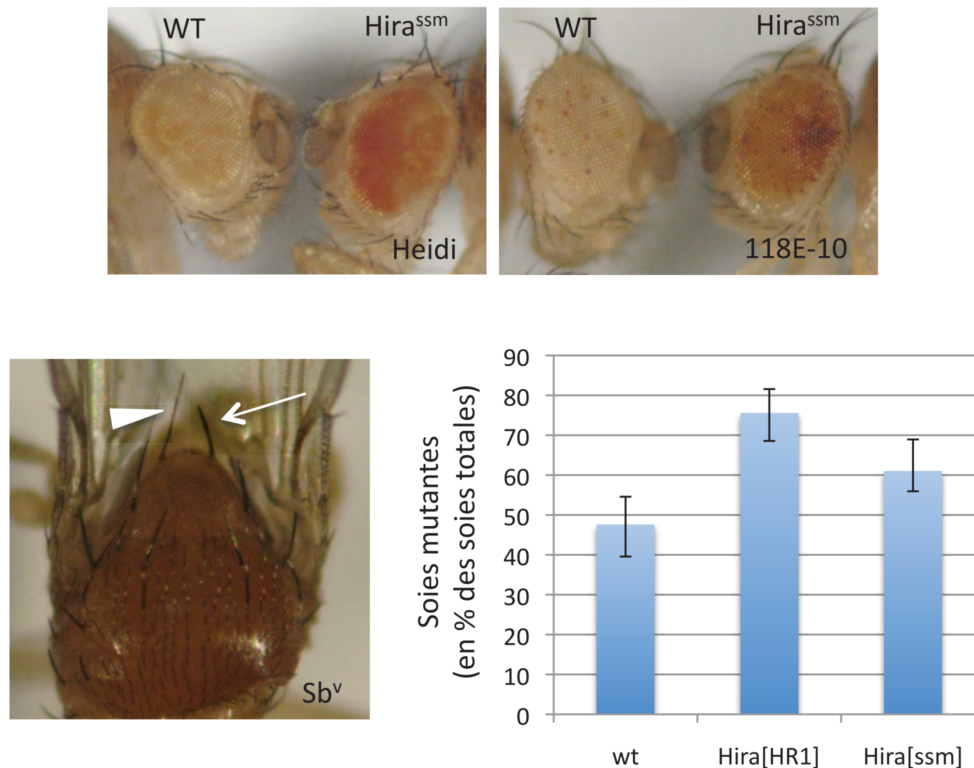

Figure 4. Les mutations de Hira sont des suppresseurs de variégation. L'aberration Sb^{v} et les insertions Heidi et 118E-10, qui présentent un profil d'expression soumis à la variégation ont été combinés avec les mutations Hira^{ssm} ou Hira^{HR1}. L'effet des mutants sur la variégation a été évalué chez des mâles hémizygotes. La variégation de l'allèle Sb a été mesurée en comparant les soies mutantes (flèches) et sauvages (tête de flèche): les résultats sont présentés dans le graphe.

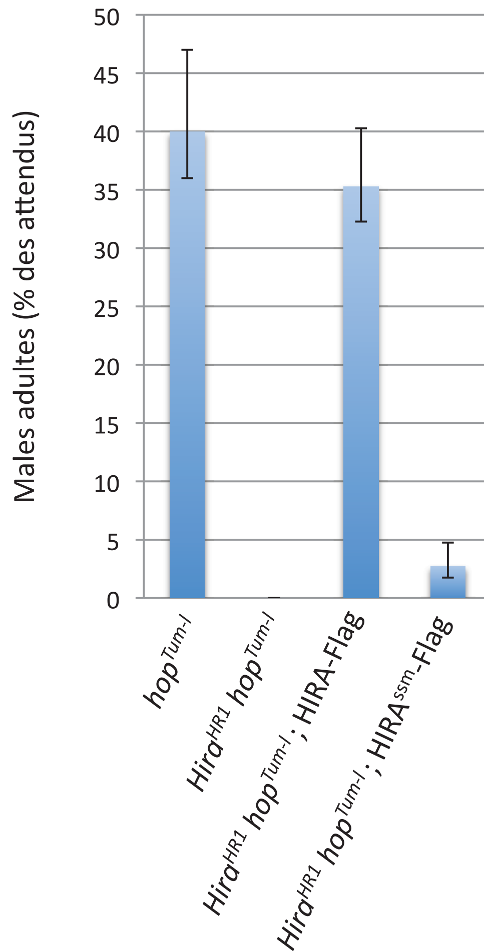

Figure 5. Intéraction létale entre les mutations affectant *Hira* et *hopscotch*$^{Tumoral\text{-}l}$ (*hop*$^{Tum\text{-}l}$).

Des femelles possédant à l'état hétérozygote les chromosomes *hop*$^{Tum\text{-}l}$ ou *Hira*^{HR1} *hop*$^{Tum\text{-}l}$ ont été croisées par des mâles sauvages (colonnes 1 et 2), ou portant des transgènes HIRA-Flag (colonne 3) ou *Hira*^{ssm}-Flag (colonne 4). Les mâles adultes survivants des génotypes indiqués ont été comptés. La double mutation *Hira*^{HR1} *hop*$^{Tum\text{-}l}$ est synthétique létale. Cet effet est sauvé par un transgène HIRA-Flag, mais ne l'est que faiblement par un transgène HIRA^{ssm}-Flag, démontrant qu'il dépend de HIRA.

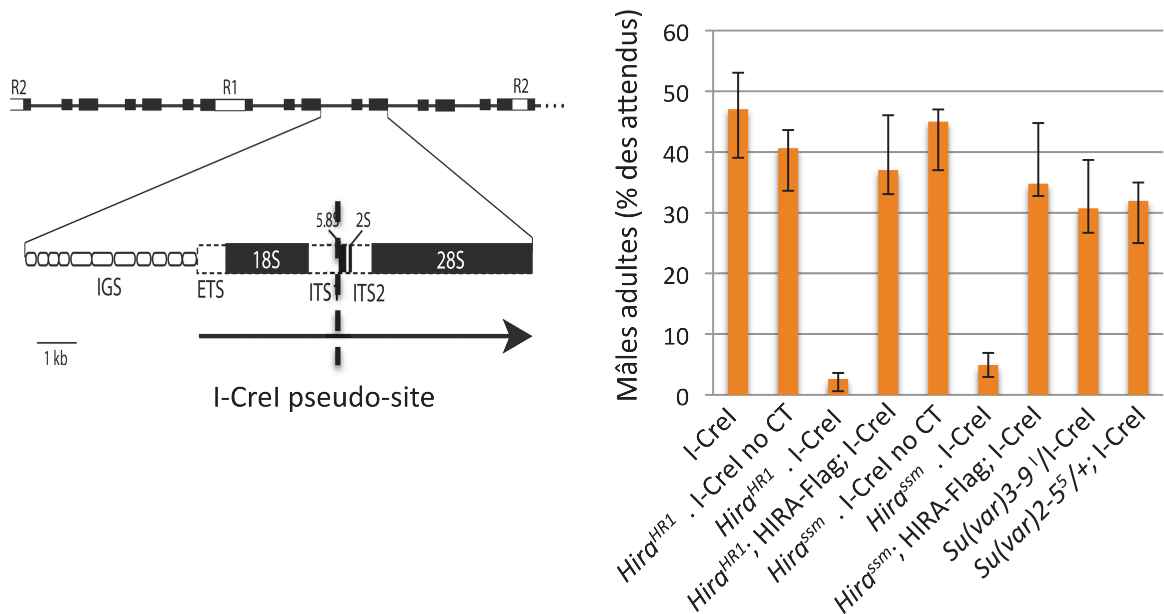

Figure 6. HIRA participe au maintien de l'intégrité du nucléole.
Test de survie après induction de la méganucléase I-CreI en contexte mutant pour *Hira*. I-CreI reconnaît un site de coupure dans chaque copie d'ADN ribosomique (schéma). L'expression de I-CreI a été induite par choc thermique (sauf pour les pistes no choc thermique, "no CT") dans des larves de stade 3 mutantes ou non pour $Hira^{HR1}$, $Hira^{ssm}$ (sauvées ou non par un transgène HIRA-Flag) ou hétérozygotes pour $Su(var)3\text{-}9^{1}$ ou $Su(var)205^{5}$. Les mâles adultes survivants ont été comptés.

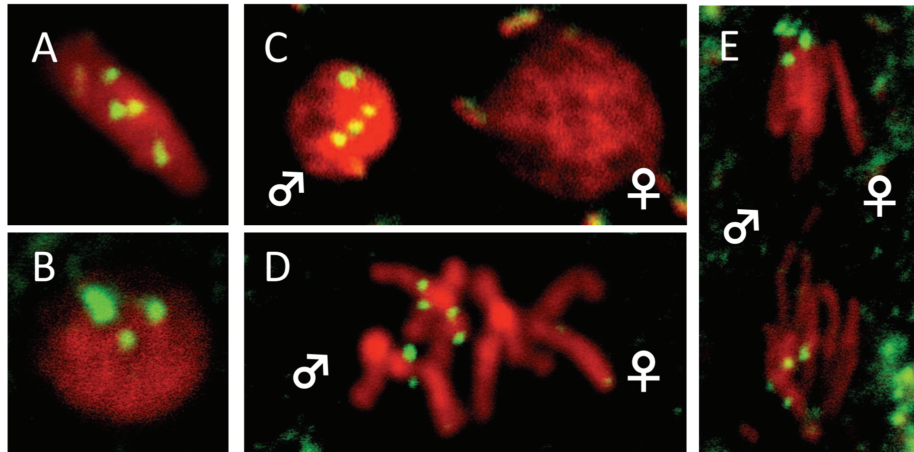

Figure 8. Transmission paternelle de Cid-GFP.
Images confocales d'oeufs issus de femelles sauvages croisées par des mâles exprimant Cid-GFP. L'ADN est en rouge, la GFP en vert. La protéine Cid-GFP est transmise paternellement et retrouvée dans le pronoyau mâle allongé (A) et en cours de décondensation (B) où les quatre centromères sont visibles. La protéine reste associée aux chromosomes patenrels pendant la première réplication zygotique et l'apposition des pronoyaux (C), la première métaphase (D) et anaphase (E) et est progressivement dilué au cours des premières divisions zygotiques (non montré). Barres: 10µm.

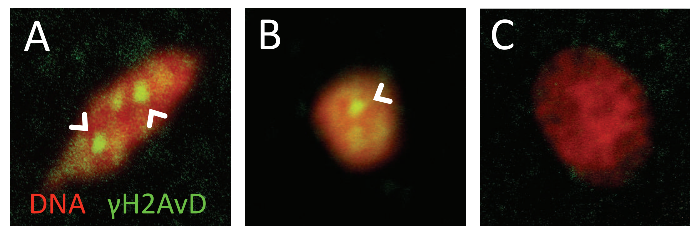

Figure 9. Accumulation de foyers H2AvD phosphorylés (γH2AvD) dans le pronoyau mâle.
Coupes confocales d'oeufs issus de femelles et mâles sauvages. Les oeufs ont été marqués pour révéler l'ADN (rouge) et l'histone γH2AvD (vert). L'histone γH2AvD est accumulée dans des foyers dans le pronoyau mâle allongé (A). Ce marquage est affaibli dans les pronoyaux arrondis plus matures (B) et disparaît dans les pronoyaux en cours de réplication (C), réflétant le caractère transitoire de ces foyers. Barre: 5µm.

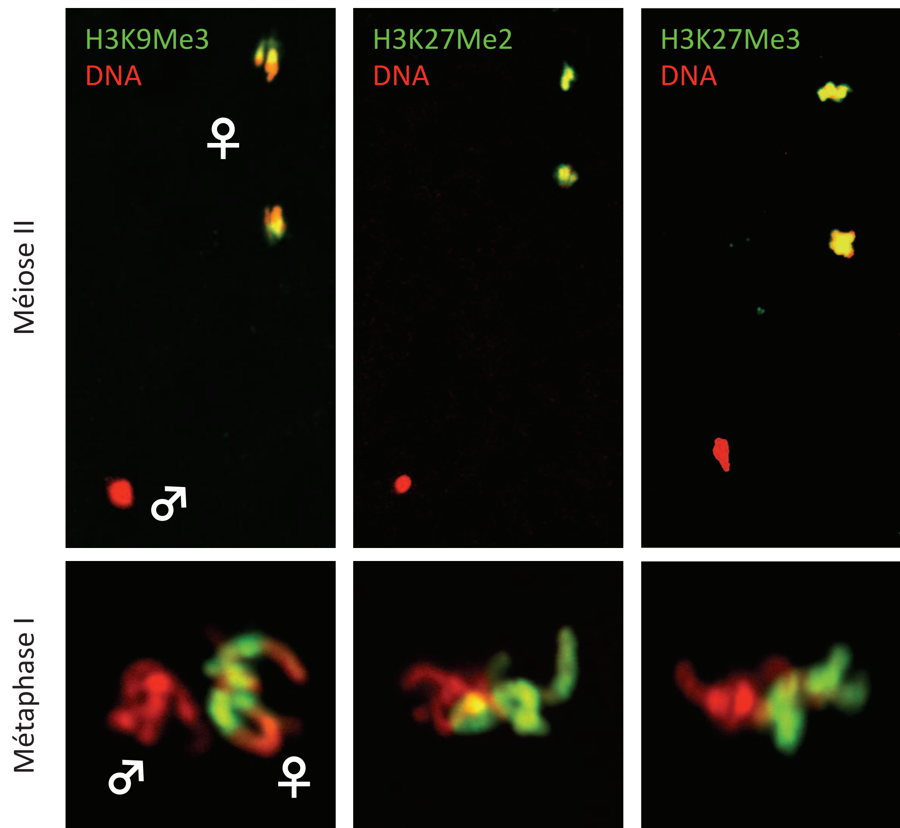

Figure 10. Distribution assymétrique de différentes méthylations de l'histone H3 entre les chromosomes paternels et maternels.
Images confocales d'oeufs pondus par des femelles sauvages et marqués pour révéler l'ADN en rouge et différentes méthylations de l'histone H3 (en vert). Les marques H3K9Me3, H3K27Me2 et H3K27Me3 marquent fortement les chromosomes maternels en méiose et pas pronoyaux mâles (Meiose II). Cette assymétrie persiste après la réplcation, pendant le premier cycle zygotique (Métaphase I) et 2 cycles plus tard au moins (non montré). Barres: 10µm.

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Drosophila I-R hybrid dysgenesis is associated with catastrophic meiosis and abnormal zygote formation

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Summary

The *Drosophila I-R* type of hybrid dysgenesis is a sterility syndrome (SF sterility) associated with the mobilization of the *I* retrotransposon in female germ cells. SF sterility results from a maternal-effect embryonic lethality whose origin has remained unclear since its discovery about 40 years ago. Here, we show that meiotic divisions in SF oocytes are catastrophic and systematically fail to produce a functional female pronucleus at fertilization. As a consequence, most embryos from SF females rapidly arrest their development with aneuploid or damaged nuclei, whereas others develop as non-viable, androgenetic haploid embryos. Finally, we show that, in contrast to mutants affecting the biogenesis of piRNAs, SF egg chambers do not accumulate persistent DNA double-strand breaks, suggesting that I-element activity might perturb the functional organization of meiotic chromosomes without triggering an early DNA damage response.

Key words: Hybrid dysgenesis, I element, Meiotic catastrophe, Haploid embryos, Meiotic DNA damage checkpoint

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Introduction

Transposable elements (TEs) are essential structural and regulatory components of genomes. Their ability to transpose provides a fundamental source of genetic variation but also represents a potential threat for genome integrity. Genomes have deployed a diversity of epigenetic defensive mechanisms against TEs and their concerted action results in the global, efficient and heritable repression of mobile elements throughout generations (Aravin et al., 2007; Siomi et al., 2008; Slotkin et al., 2007). In *Drosophila*, epigenetic control of TEs depends on histone modifications, chromatin structure, small RNA-based transcriptional silencing and DNA methylation (Aravin et al., 2007; Josse et al., 2007; Klenov et al., 2007; Dramard et al., 2007; Phalke et al., 2009; Klattenhoff et al., 2007; Klattenhoff et al., 2009; Li et al., 2009; Malone et al., 2009). Recent literature has abundantly described the mechanisms of Piwi-interacting small RNAs (piRNAs) biogenesis, as well as their essential role for the repression of TEs in germ cells (Brennecke et al., 2007; Vagin et al., 2006; Saito et al., 2006; Aravin et al., 2007; Siomi et al., 2008; Klattenhoff and Theurkauf, 2008). Accordingly, several families of TEs are derepressed in the germline of mutants affecting the piRNA pathway (Vagin et al., 2006; Chambeyron et al., 2008; Pane et al., 2007; Lim and Kai, 2007; Chen et al., 2007; Klattenhoff et al., 2007; Klattenhoff et al., 2009; Vagin et al., 2004). Remarkably, all these mutants are viable but induce female sterility associated with a complex phenotype including defects in germline stem cell maintenance, accumulation of germline DNA damage and aberrant egg axial patterning (Klattenhoff and Theurkauf, 2008). However, it is difficult to determine the actual contribution of TE activity to their complex sterility phenotype (Klattenhoff et al., 2007; Thomson and Lin, 2009).

In *Drosophila*, massive and deleterious TE germline mobilization is also observed in the progeny of certain intraspecific crosses. This phenomenon, known as hybrid dysgenesis, has long been recognized as a powerful experimental model for the study of TE regulation in a wild-type background (Bregliano et al., 1980). *Drosophila* hybrid dysgenesis systems are usually characterized by a severe gonadal atrophy in both sexes, resulting in sterility. These include the *D. melanogaster P-M* (P element) and *H-E* (hobo element) systems, as well as a hybrid dysgenesis in *D. virilis*, which involves several families of TEs (Kidwell and Novy, 1979; Blackman et al., 1987; Yannopoulos et al., 1987; Petrov et al., 1995; Blumenstiel and Hartl, 2005). The *I-R* type of hybrid dysgenesis is unique as it only occurs in females and does not result from a defective ovarian development. Instead, dysgenic females lay a normal amount of eggs but the resulting embryos fail to hatch (Picard and L'Héritier, 1971). The causative factor of this non-Mendelian female sterility is the *I* element, a 5.4 kb, non-LTR retrotransposon of the LINE (long interspersed nucleotidic element) superfamily of transposable elements (Bucheton et al., 1984). Most *D. melanogaster* strains are so-called *Inducer* (*I*) strains and contain about 10 transposition-competent but transcriptionally silenced *I* elements. Such functional *I* elements are absent from *Reactive* (*R*) strains that were established before the recent worldwide invasion of this retrotransposon in natural populations (Bucheton et al., 2002). Maternal transmission of piRNAs has been proposed to underlie the epigenetic repression of TEs revealed by *Drosophila* hybrid dysgenesis systems (Blumenstiel and Hartl, 2005; Brennecke et al., 2008; Chambeyron et al., 2008). In the case of the *I-R* system, maternal epigenetic protection is largely reduced in *R* strains, resulting in the expression of paternally transmitted *I* elements in the naive germline of dysgenic females (Brennecke et al., 2008; Chambeyron et al., 2008).

I-R hybrid dysgenesis occurs when *I* males are crossed with *R* females. The female progeny of this dysgenic cross, called SF (stérilité femelle) females, usually display a strong sterility phenotype associated with derepression of *I* elements. In addition, the *I-R* syndrome is characterized by a high mutation rate as well

as chromosomal non-disjunctions and rearrangements (Bucheton et al., 2002). In contrast to SF females, the genetically identical RSF females obtained from the reverse cross (*R* males with *I* females) show much lower expression of *I* elements and are fully fertile (Picard and L'Héritier, 1971; Bucheton et al., 2002). From the early work of Picard et al. (Picard et al., 1977) and Lavige (Lavige, 1986), it was established that embryos produced by SF females died through a strict maternal effect and frequently presented abnormal syncytial divisions. However, despite extensive research on this system, the nature of SF sterility has remained enigmatic since its discovery (Picard and L'Héritier, 1971).

In this paper, we have undertaken a detailed cytological study of *I-R* hybrid dysgenesis to determine the origin of SF sterility. Our study revealed that meiotic divisions are catastrophic in SF oocytes and eggs. This highly penetrant phenotype prevents the integration of the full set of maternal chromosomes in the zygote, resulting in non-viable embryos. We also show that, in contrast to mutants affecting the biogenesis of piRNAs, SF germ cells do not accumulate massive DNA damage during early oogenesis, suggesting that *I* activity perturbs the functional organization of meiotic chromosomes without activating the early germline DNA damage response.

Results

Meiotic catastrophe in eggs of SF females

We performed a cytological study of SF eggs and embryos to understand the nature of SF maternal-effect embryonic lethality. We used SF females that were not older than a week as SF sterility decreases progressively with age (see below). Consistent with early cytological studies (Lavige, 1986), we observed that a majority of syncytial SF embryos contained catastrophic mitotic figures with isolated or broken chromosomes and asynchronously dividing nuclei of various sizes (Fig. 1). In addition, in SF embryos, we observed that the polar body did not form the typical triploid rosette and contained many fragmented chromosomes (Fig. 1D–F). This last aspect of the phenotype suggested that meiosis was defective in SF eggs. We then turned to late oocytes to observe the first meiotic division. In *Drosophila*, the mature stage-14 oocyte is arrested in metaphase of meiosis I (King, 1970). To visualize the organization of meiotic chromosomes and the first meiotic spindle, we used control and SF females expressing the fluorescent centromeric protein EGFP-Cid (Schuh et al., 2007) or the microtubule-associated Jupiter-GFP (Buszczak et al., 2007), respectively. In fixed control stage-14 oocytes (n=30), meiotic chromosomes appeared as a slightly elongated mass of chromatin with non-exchange chromosomes occasionally separated towards the spindle poles (Fig. 2) (Theurkauf and Hawley, 1992). In about 80% of SF oocytes (22/28), the chromatin appeared fragmented and/or abnormally distributed into several small masses (Fig. 2). Some of these masses of chromatin were associated with an EGFP-Cid spot, whereas others were not, thus suggesting the presence of fragmented chromosomes. These isolated or fragmented chromosomes formed miniature spindle-like structures as revealed with the Jupiter-GFP marker (Fig. 2). In the rest of the SF oocytes (6/28), the first meiotic division was apparently normal, although the low resolution of meiosis I chromosomes did not allow the detection of possible more subtle defects.

We then analyzed very early SF eggs to observe the second meiotic division and pronuclear formation. Strikingly, meiosis II in SF eggs was almost systematically abnormal with bridges of chromatin connecting the separating chromatids in anaphase and telophase (Fig. 3C,D; Table 1). This defective separation of chromatids was followed by chromosome fragmentation and unequal segregation of meiotic products. Notably, the loss of genetic material in the female pronucleus was obvious at the pronuclear apposition stage. In control RSF eggs, apposed pronuclei appeared identical in size (Fig. 3E). In SF eggs, however, the female pronucleus was either small, fragmented in several smaller nuclei or, in some instances, did not form at all (Fig. 3F,G; data not shown). We thus concluded that, in eggs from SF females, defective meiotic divisions compromised the formation of a normal female pronucleus.

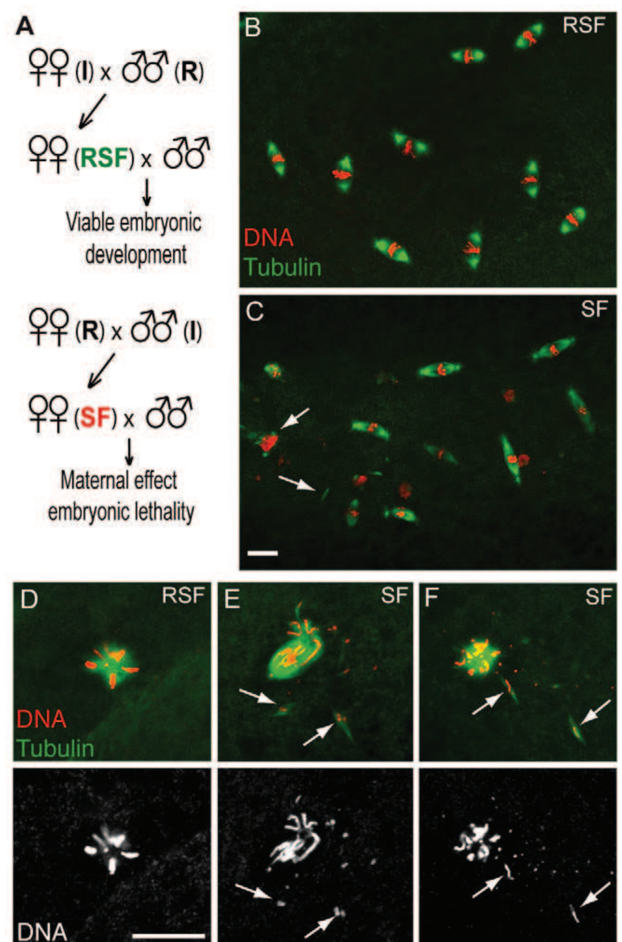

Fig. 1. The maternal-effect embryonic lethality associated with *I-R* hybrid dysgenesis. (**A**) Crossing scheme to obtain RSF (upper) and SF (lower) females. SF females lay eggs but the resulting embryos die before hatching. Genetically identical RSF females are fully fertile. (**B**,**C**) Confocal images of early syncytial embryos from RSF (B) or SF (C) females stained for Tubulin (green) and DNA (red). In contrast to the normal nuclear divisions observed in RSF embryos, SF embryos contain asynchronously dividing nuclei of various sizes and fragmented chromosomes (arrows). (**D–F**) In RSF embryos (D), fused polar bodies form a typical rosette of condensed chromosomes. In SF embryos (E,F), polar body organization is abnormal and many chromosomes are lost or fragmented (arrows). Scale bars: 15 μm.

Embryos from SF females develop with paternal chromosomes

In *Drosophila* fertilized eggs, pronuclei do not fuse but instead remain apposed during the first zygotic S phase and the paternal

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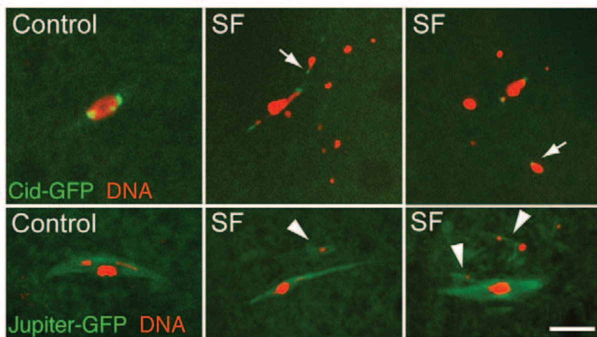

Fig. 2. Meiosis I is catastrophic in oocytes from SF females. Confocal images of meiosis I in stage-14 oocytes from females expressing the indicated marker. Control females are from the transgenic *EGFP-Cid* line and *Jupiter-GFP* line. SF females were obtained by crossing inducer *EGFP-Cid* or *Jupiter-GFP* males with *Charolles* females at 25°C. At day 5 of adult life, ovaries were dissected, fixed and stained for DNA (red). Scale bar: 10 µm. (**Top**) EGFP-tagged centromeric histone Cid (EGFP-Cid) marks centromeres in meiosis I chromosomes. In control oocytes, chromosomes show aligned centromeres in prometaphase. In SF oocytes, chromosomes appear fragmented or mislocalized. Centromeres are indicated with arrows. (**Bottom**) The control is an anastral first meiotic spindle in prometaphase marked with microtubule-associated Jupiter-GFP. In SF oocytes, mini-spindles organize around mislocalized or fragmented chromosomes (arrowheads).

and maternal sets of chromosomes enter mitosis as separate entities within a common mitotic spindle (Sonnenblick, 1950). In a majority of SF embryos at first mitosis, we observed that the spindle did not contain the full complement of chromosomes compared with RSF zygotes (Fig. 3H,I; Table 1). In other cases, some chromosomes were excluded from the spindle or lagged behind in anaphase of the first division (Fig. 3J,K; Table 1). To determine the identity of these absent or abnormal chromosomes, we stained SF eggs with an antibody directed against acetylated forms of histone H4 that preferentially marks paternal chromatin (Loppin et al., 2005a). We observed that, in SF eggs, from the pronuclear apposition until the end of the first zygotic division, the damaged or late chromosomes were systematically less-intensely stained than the unaffected chromosomes (Fig. 4A-F). In some cases, a single haploid set of strongly stained chromosomes was present at the first mitosis (Fig. 4E). We confirmed these observations by analyzing the progeny of transgenic SF females expressing the recombinant histone variant H3.3-Flag, a specific marker of paternal chromosomes at fertilization (Bonnefoy et al., 2007; Orsi et al., 2009). This experiment clearly confirmed the specific defective integration of maternal chromosomes in SF zygotes (supplementary material Fig. S1).

As previously reported by Picard et al. (Picard et al., 1977) and Lavige (Lavige, 1986), we observed that approximately 7% (n=1134) of SF embryos died at a late developmental stage as revealed by the fact that they turned brown after death and showed signs of organogenesis and cuticle deposition. By contrast, the rest of the unhatched eggs remained whitish, suggesting that they arrested development before cellularization (Fig. 4K). In the *Drosophila* mutant *maternal haploid* (*mh*), paternal chromosomes are unable to divide in anaphase of the first mitosis and form a chromatin bridge (Santamaria and Gans, 1980; Loppin et al., 2001). This frequently results in catastrophic early mitoses and most embryos die after a few rounds of nuclear divisions. However, a fraction of embryos escape this early arrest and

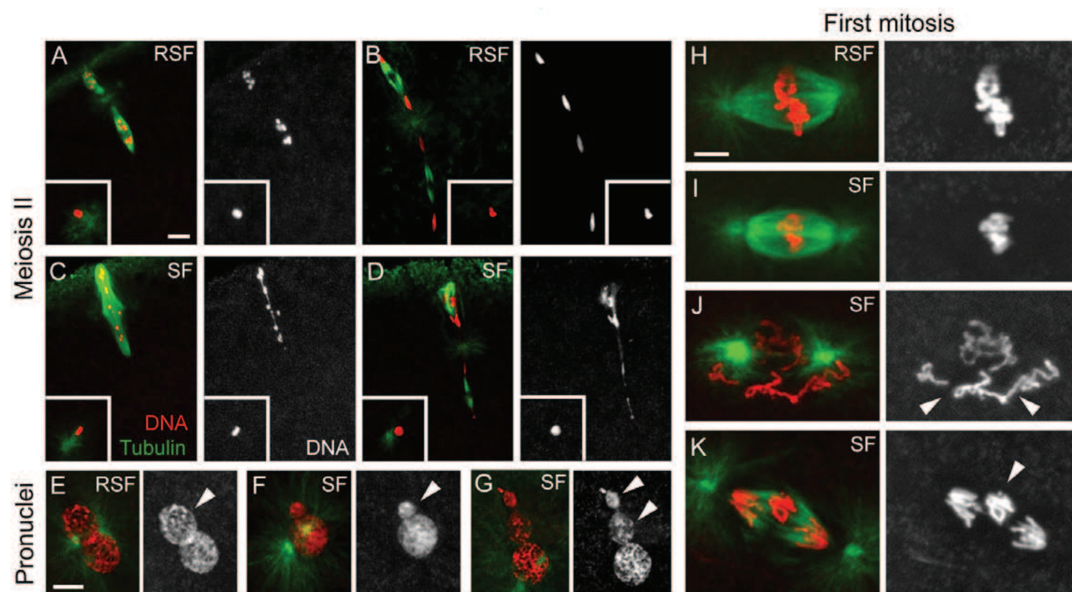

Fig. 3. Catastrophic meiosis and abnormal zygote formation in eggs from SF females. Confocal images of eggs and early embryos stained for Tubulin (green) and DNA (red). (**A–D**) Meiosis figures are shown with dorsal egg periphery at the top and the anterior end to the left. The corresponding male pronuclei are shown in insets. (A,B) RSF eggs in anaphase (A) or telophase (B) of the second meiotic division. (C,D) Meiosis II in SF eggs is catastrophic. Note the chromatin bridges in anaphase (C) and the unequal chromosome segregation in telophase (D). Loss of genetic material in the two innermost meiotic products is obvious in D. (**E**) Pronuclear apposition in an RSF egg. (**F**,**G**) In SF eggs, the female pronucleus looks abnormally small (F) or fragmented into several smaller nuclei (G). The female pronuclei are indicated with arrowheads. (**H–K**) First zygotic division. Metaphase of the first zygotic division in an RSF egg (H) containing the paternal and maternal chromosomes. First mitosis in an SF egg with either a reduced number of chromosomes (I), with chromosomes that appear excluded from the spindle (arrowheads in J) or with lagging chromosomes in anaphase (arrowheads in K). Scale bars: 10 µm.

Table 1. Phenotype quantification of SF eggs and embryos

	Meiosis II		First zygotic division		Cycle 2 –7 embryos		
	n	Abnormal (%)	*n*	Abnormal (%)	*n*	Aneuploid (%)	Haploid (%)
SF	40	97.5	56	96.4	90	82.2	11.1
RSF	41	2.4	25	0	50	0	0

SF or RSF females grown at 25°C were allowed to lay eggs between days 4 and 6 after emergence (day 1). Eggs at 0–1 hours were collected, fixed and stained for DNA. Phenotypes of SF eggs (meiosis II and zygote) and early embryos are described in the Results. *n*, the total number of eggs and/or embryos analyzed.

develop as non-viable, haploid gynogenetic embryos (Loppin et al., 2001). At the cytological level, early development of SF embryos appeared similar to *mh* embryos, with catastrophic syncytial divisions forming chromatin bridges (Fig. 1C; supplementary material Fig. S2). In addition, a minority of SF embryos developed beyond the blastoderm stage and contained normal mitotic figures but the nuclei were about half the size of control diploid nuclei (Fig. 4G-J). To demonstrate that these escaper embryos were actually haploid androgenetic embryos, we crossed SF females with males homozygous for the *K81* paternal effect mutation, which prevents the formation of functional paternal chromosomes in the progeny (Fuyama, 1984; Yasuda et al., 1995; Loppin et al., 2005b). As expected, these SF females failed to produce any brown embryos during their first week, confirming that late embryos from SF females developed with paternal chromosomes (Fig. 4L,M; supplementary material Table S1). In conclusion, our results demonstrated that most embryos from SF females die early with catastrophic mitoses, whereas a minority escape this early arrest as haploid androgenetic embryos.

Defective karyosome formation in SF oocytes

The meiotic phenotype observed in SF females prompted us to analyze the structure of the oocyte nucleus during SF oogenesis. In *Drosophila*, female meiosis initiates in region 2A of the germarium, at the anterior tip of each ovariole. After meiotic recombination, in later egg chambers, the oocyte nucleus enlarges while the condensed maternal chromosomes in prophase I of meiosis remain packaged within a subnuclear structure known as the karyosome (Spradling, 1993). In stage 6–9 control oocytes stained for DNA, the karyosome appeared as a round condensed structure within the unstained oocyte nucleus (Fig. 5A). By striking contrast, we observed that the karyosome was disorganized in a majority of SF oocytes (Fig. 5A; supplementary material Fig. S2). Typically, the SF karyosomes were fragmented and stretched along the inner side of the oocyte nuclear envelope. A remarkable and well-described feature of SF sterility is its modulation by age and temperature. Indeed, SF sterility is highest in young females but their fertility is progressively restored as they age (see supplementary material Table S1) (Picard and L'Héritier, 1971). In addition, SF sterility is strongest and lasts longer at relatively cooler temperatures and fertility can be transiently restored after a heat treatment (Bucheton, 1979). Interestingly, we observed that the penetrance and severity of the karyosome phenotype decreased with the age of SF females. In addition, most karyosomes were severely affected when SF females where placed at 18°C for 36 hours before dissection, whereas a heat treatment at 30°C dramatically suppressed the phenotype (Fig. 5B,C). Taken together, these observations suggest that defective karyosome formation in SF oocytes results in abnormal meiotic divisions.

SF germ cells do not accumulate unrepaired DNA double-strand breaks

In *Drosophila* female germ cells, the accumulation of unrepaired DNA double-strand breaks (DSBs) can trigger the activation of a well-characterized ATR-Chk2 (Mei-41-Lok) DNA damage response (Ghabrial and Schupbach, 1999; Abdu et al., 2002). In mutants that affect the repair of meiotic DNA DSBs, activation of the Chk2 checkpoint leads to a complex cellular response. This includes a specific disorganization of the karyosome and a strong egg ventralization phenotype that results from defective accumulation of the signaling protein Gurken in the oocyte (Ghabrial and Schupbach, 1999; Abdu et al., 2002). Interestingly, the Chk2 checkpoint is activated in the female germline of piRNA pathway mutants (Chen et al., 2007; Klattenhoff et al., 2007). In addition, these mutants are associated with egg patterning defects and defective karyosome formation (supplementary material Fig. S3) (Chen et al., 2007; Klattenhoff et al., 2007). Genetic analyses have demonstrated that, in these mutants, the checkpoint is not activated by meiotic DSBs, thus opening the possibility that these DNA damages could be induced by the activity of derepressed TEs (Chen et al., 2007; Klattenhoff et al., 2007; Klattenhoff et al., 2009).

As mutant stocks are generally not available in a reactive background, the activation of the checkpoint in SF germ cells could not be genetically tested. We thus examined the dorsal patterning of SF eggs to check for indications of DNA damage response. We observed that a fraction of SF eggs displayed a weak ventralization phenotype. In fact, fusion of egg dorsal appendages was only observed with very young SF females not older than 3 days (Table 2; Fig. 4K, arrow). Importantly, after a few days, SF females that were still fully sterile produced almost 100% of eggs with wild-type appendages. By clear contrast, *aub* or *armi* mutant females produced a majority of severely ventralized eggs throughout their life (Table 2). Interestingly, Van De Bor et al. (Van De Bor et al., 2005) have shown that *I* and *gurken* (*grk*) transcripts compete for the same RNA localization machinery in SF egg chambers, resulting in defective dorsoventral axis specification. This mechanism could indeed account for the ventralization of eggs produced by young SF females, where strong *I* transcription is expected to efficiently perturb *grk* mRNA localization. In conclusion, the egg patterning analysis did not support the hypothesis of early Chk2 checkpoint activation in SF germ cells. However, we wished to directly evaluate the impact of *I*-element activity on DNA integrity during early oogenesis. We thus stained SF and control ovaries with antibodies against the phosphorylated form of histone H2Av (γ-His2Av), which associates with DNA DSBs (Mehrotra and McKim, 2006). In wild-type or RSF ovaries, γ-His2Av foci were observed in oocytes of germarium regions 2A and 2B but were no longer detected in late-pachytene oocytes in their region 3 egg chambers (Fig. 6). In region 3 oocytes from *aub* mutant females, late-pachytene nuclei accumulated numerous γ-His2Av foci, as previously reported (Klattenhoff et al., 2007). By clear contrast,

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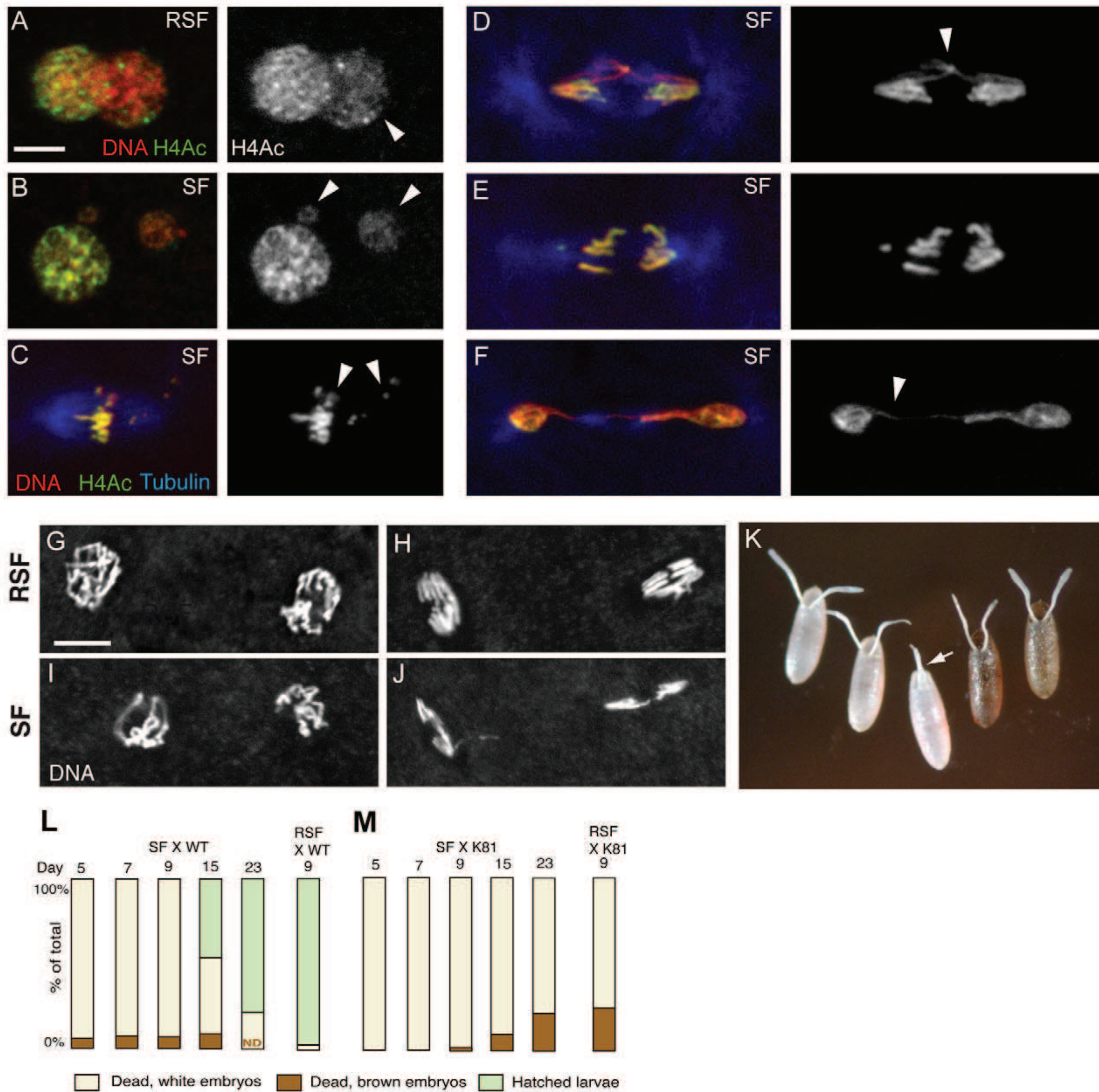

Fig. 4. Early loss of maternal chromosomes in SF embryos. (**A–J**) Confocal images of eggs and embryos stained with the indicated markers. (A,B) RSF (A) and SF (B) pronuclei stained with an anti-acetylated H4 antibody (green or white) that preferentially marks paternal chromatin (Loppin et al., 2005a). The female pronuclei are indicated with arrowheads. (C–F) First mitosis in SF eggs stained for acetylated H4 (green or white), DNA (red) and Tubulin (blue). Maternal chromosomes appear red and paternal chromosomes are yellow. Maternal chromosomes are abnormally positioned in the spindle or fragmented (arrowheads in C), lagging behind in anaphase or telophase (arrowheads in D and F) or absent (E). (G–J) Diploid nuclei from blastoderm RSF embryos in prophase (G) and anaphase (H). SF embryos that reach the blastoderm stage contain haploid nuclei (I, prophase; J, anaphase). Nuclei were stained with Propidium Iodide. (**K**) Unhatched eggs from SF females appear either whitish, indicative of early developmental arrest (the three eggs on the left), or brown, indicative of haploid development (the two eggs on the right). The arrow points to a weakly ventralized egg with the dorsal appendages fused at their base. (**L**) Diagrams showing the color phenotype of unhatched embryos produced from the same batch of SF females at the indicated days (day 1 is the day of emergence). Note that SF females progressively recover fertility as they age. ND, not determined. (**M**) When SF females are crossed with *K81* mutant males, brown embryos are not produced during the first week of life. Note that RSF females as well as aging SF females crossed with *K81* males produce an expected fraction of haploid gynogenetic embryos that turn brown after death. Scale bars: 10 μm. Numbers of examined embryos are in supplementary material Table S1.

such an accumulation of DNA DSBs was not observed in SF region 3 oocytes (n=10). In fact, half of region 3 SF oocytes were devoid of γ-His2Av foci, as in RSF controls. Interestingly, however, a few (1–3) γ-His2Av foci were observed in the other half of the late-pachytene SF oocytes but they never persisted beyond that stage. Thus, *I* activity either occasionally delays the repair of meiotic DSBs or, alternatively, generates a small number of non-persistent DSBs unrelated to meiotic recombination.

BicD aggregates are not observed in SF egg chambers

In wild-type inducer ovaries, endogenously expressed *I* transcripts are essentially sequestered in nurse cell nuclear foci in a piRNA-

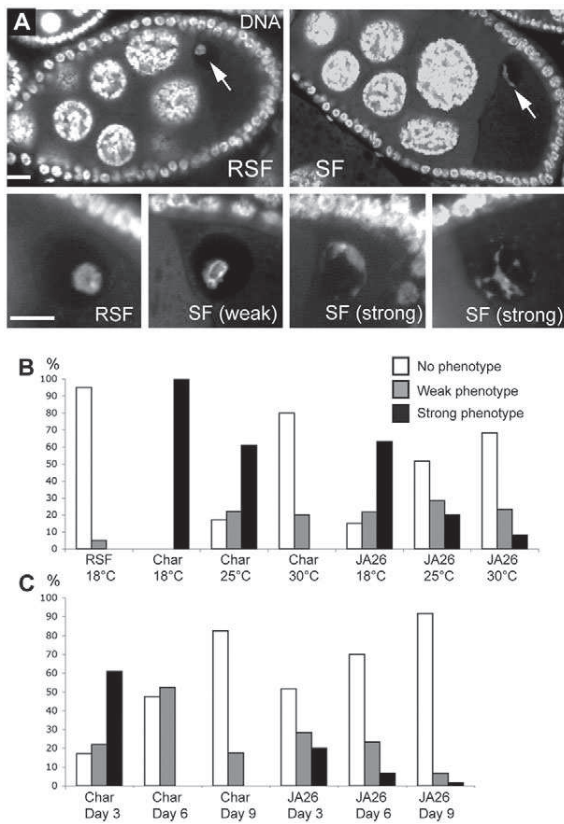

Fig. 5. Defective karyosome formation in SF oocytes. (**A**) Confocal images of stage 7–8 egg chambers dissected from RSF or SF females and stained for DNA (top). Egg chamber stages are from King (King, 1970). The oocyte is on the right, the karyosome is indicated with an arrow. (Bottom) In oocytes from RSF females, the karyosome appears spheric and condensed within the unstained oocyte nucleus. In SF oocytes, the karyosome is frequently abnormal, being slightly heterogeneous or elongated in aspect (weak phenotype) or displaying a severe distortion, fragmentation or attachment to the nuclear envelope (strong phenotype). (**B**) Effect of temperature on SF karyosome phenotype. Two-day-old RSF or SF females obtained at 25°C using the *Charolles* or *JA26* reactive stocks were placed at the indicated temperature for 36 hours before ovary dissection and DNA staining. For each condition, a minimum of 40 karyosomes from stage 6–9 oocytes were observed and classified according to the phenotypic classes described in A. Results are shown as a percentage of all observed karyosomes. (**C**) Effect of age on SF karyosome phenotype. SF females obtained at 25°C using the *Charolles* or *JA26* reactive stocks were dissected at the indicated age and ovaries were stained for DNA. Karyosome phenotype was analyzed as in B. Note that the same 25°C, 3-day-old SF female data is shown in B and C. Scale bars: 20 µm.

dependent manner (Chambeyron et al., 2008), whereas overexpressed GFP-labeled *I* transcripts have been shown to accumulate in cytoplasmic particles called pi-bodies that localize around nurse cell nuclei (Lim et al., 2009). In SF egg chambers, *I* transcripts are essentially transported in the oocyte (Seleme et al., 2005; Chambeyron et al., 2008). Recently, it has been shown that large ribonucleoprotein (RNP) aggregates of the dynein-motor machinery form in egg chambers of piRNA biogenesis mutants (Navarro et al., 2009). Interestingly, injected *I* transcripts accumulate in these aggregates, suggesting that they could serve as degradation sites for retrotransposon products, in the absence of

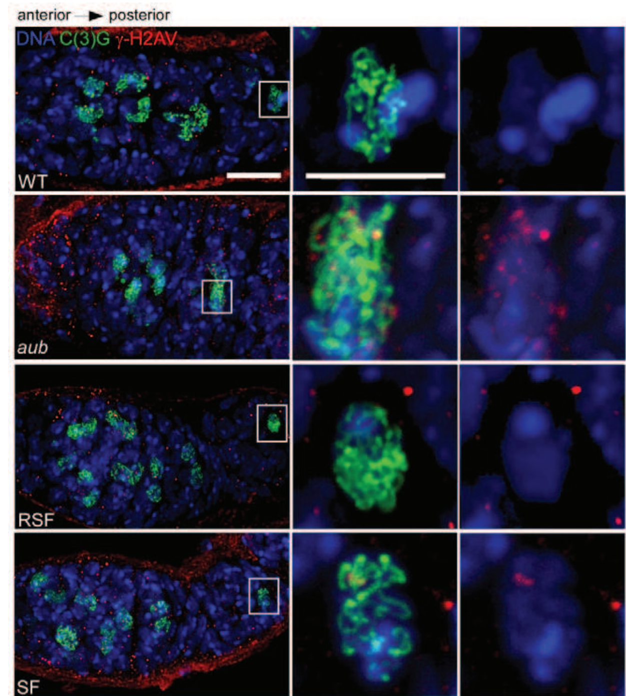

Fig. 6. γ-His2AvD distribution in the SF germline. Confocal images of wild-type (WT), aub^{QC42} or aub^{HN} (*aub*), RSF and SF germaria stained to visualize DNA (blue), C(3)G (green) and γ-His2AvD (red). Full views of germaria with their anterior tip on the left are shown in the left panels. Increased magnifications of late-pachytene oocytes (insets) are on the right. In WT and RSF germaria, γ-H2AvD foci are not detected in late-pachytene oocytes [identified by the C(3)G staining], indicating that meiotic DNA double-strand breaks are repaired at this stage. In generally disorganized *aub* mutant germaria, where oocyte determination is delayed, >10 γ-His2AvD foci accumulate in late-pachytene oocyte nuclei, shown here in an early region 3 oocyte. In SF germaria, 0–3 γ-His2AvD foci are observed in late-pachytene oocytes. A total of 10 late-pachytene oocytes were examined for each genotype. Scale bars: 5 µm.

piRNA biogenesis (Navarro et al., 2009). Furthermore, these authors have also shown that formation of these dynein aggregates was largely dependent upon the activation of the Chk2 checkpoint.

To investigate the possibility that these structures could form in SF egg chambers, we stained ovaries with anti-BicD or anti-Orb antibodies that were shown to accumulate in dynein aggregates (Navarro et al., 2009). We indeed observed aggregates in a large majority of *aub* or *armi* mutant egg chambers. By clear contrast, however, Orb or BicD aggregates were only rarely observed in SF and RSF egg chambers (Fig. 7A,B; data not shown). We conclude that *I*-element activity is not sufficient to trigger the formation of these aggregates in dysgenic ovaries. In the course of these experiments, we observed that the oocyte marker BicD was abnormally distributed in the germinal vesicle of a majority of *aub* and *armi* mutant stage 6–9 egg chambers (Fig. 7A,C). This phenotype was fully rescued in *aub mnk* double-mutant females, indicating that it was dependent on checkpoint activation (Fig. 7A,C). Importantly, we observed that, in SF and RSF oocytes, BicD was normally excluded from the germinal vesicle. Taken together, these results reinforce the conclusion that SF sterility is independent of Chk2 checkpoint activation and downstream cellular responses.

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Table 2. Egg patterning of SF eggs

Maternal genotype	Dorsal appendage phenotype (%) Wild-type	Fused	Absent	Hatch rate (%)	*n*
aub^{HN}/aub^{QC42}	23.1	52.4	24.5	0	481
$aub^{HN}\ mnk^{P6}/aub^{QC42}\ mnk^{P6}$	99.2	0.8	0	0	354
$armi^{1}/armi^{72.1}$	0.2	11.7	88.1	0	463
$mnk^{P6}/mnk^{P6};\ armi^{1}/armi^{72.1}$	76.6	16.1	7.3	0	137
RSF	100.0	0	0	96.2	498
SF (days 1–3)	69.3	30.4	0.3	0	743
SF (days 4–5)	99.2	0.8	0	0.2	651

Egg ventralization phenotypes are described in Staeva-Vieira et al. (Staeva-Vieira et al., 2003). SF females that emerged on day 1 were crossed with wild-type males and eggs from the same females were collected and analyzed after day 3 (days 1–3) and day 5 (days 4–5). The phenotype of eggs from other females remained unchanged over the same period of 5 days (data not shown).

Discussion

Extensive research on *Drosophila* hybrid dysgenesis systems has brought an essential contribution to the paradigm of TE epigenetic silencing. However, these models have comparatively received limited attention regarding the actual effect of TE activity in germ cells. In this context, the maternal-effect embryonic lethality associated with SF sterility appeared particularly difficult to link with *I* activity during oogenesis. In this study, we have shown that embryo lethality is a consequence of catastrophic meiosis in SF eggs. The loss or fragmentation of meiotic chromosomes leads to abnormal female pronucleus formation and prevents the subsequent development of viable diploid embryos. Instead, embryos from SF females initiate development with missing or damaged maternal chromosomes or with only the set of intact paternal chromosomes.

In contrast to the dramatic phenotype observed in eggs and embryos, SF oogenesis appeared relatively undisturbed by *I*-element activity. Our observation of meiosis prophase I progression in SF germaria has revealed the presence of a small number of non-persistent γ-His2Av foci in late-pachytene oocytes. These foci, supposedly associated with unrepaired DSBs, are thus the earliest phenotypic manifestation of *I* activity in SF germ cells that we were able to detect. Meiotic DSBs are normally repaired before the end of prophase and γ-His2Av foci are only exceptionally observed in wild-type region 3 oocytes (Mehrotra and McKim, 2006). The foci observed in SF oocytes could possibly result from a delay in the repair of DSBs induced by meiotic recombination, implying that *I* activity might disturb or slow down the normal repair process of meiotic DSBs. Alternatively, these DSBs could be directly generated by *I* retrotransposition. Indeed, in mammalian cells, retrotransposition of the *I*-related LINE 1 (L1) elements generates DNA DSBs associated with γ-His2AX foci (Bourc'his and Bestor, 2004; Belgnaoui et al., 2006; Gasior et al., 2006; Soper et al., 2008).

Whatever the origin of this DNA damage in early SF germ cells, they do not appear sufficient to trigger the activation of the Chk2-dependent checkpoint, at least as it is described for mutants affecting the repair of meiotic DSBs (Ghabrial and Schupbach, 1999; Abdu et al., 2002). For comparison, in certain hypomorphic alleles of meiotic DSB repair genes, the meiotic checkpoint is not activated despite the presence of about 7–10 persistent γ-His2Av foci (E.F.J. and K.S.M., unpublished data). The egg patterning analysis of SF eggs also supported the apparent absence of meiotic checkpoint activation in SF germ cells. Indeed, the weak ventralization phenotype observed with very young females disappeared after a few days despite the fact that dysgenic females remained fully sterile.

By contrast, SF egg chambers displayed a clear karyosome phenotype that was highly correlated with sterility. The morphology defect of SF karyosomes was reminiscent of the karyosomes in piRNA mutants. In these mutants, activation of the Chk2 checkpoint

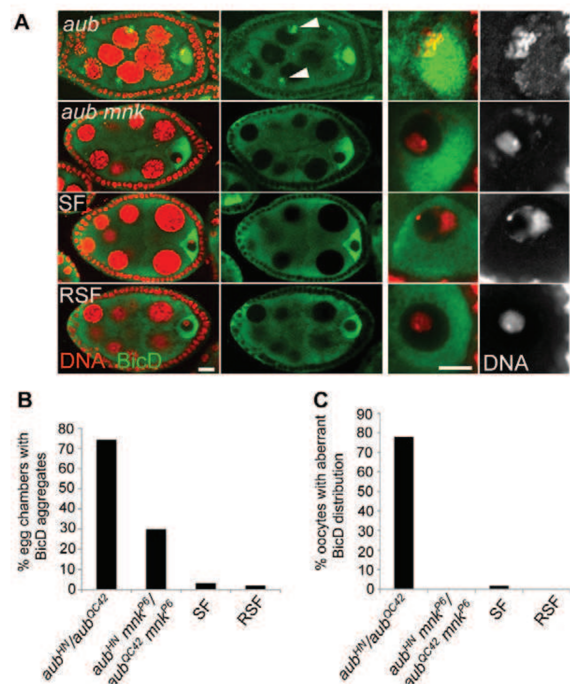

Fig. 7. BicD distribution is not affected in SF ovaries. (**A**) Confocal images of stage-9 egg chambers from aub^{HN}/aub^{QC42} (*aub*), $aub^{HN}\ mnk^{P6}/aub^{QC42}\ mnk^{P6}$ (*aub mnk*), SF and RSF females raised at 25°C. At days 3–5 of adult life, ovaries were dissected, fixed and stained for BicD (green) and DNA (red). BicD aggregates accumulate in egg chambers from control *aub* mutants (arrowheads) but not from double *aub mnk* mutants, SF or RSF females (left panels). Magnification of germinal vesicles of the same stage and genotype are shown in the right panels. Note that BicD is abnormally distributed within the germinal vesicle in *aub* mutant oocytes, whereas it is normally excluded from the oocyte nucleus in *aub mnk*, SF and RSF egg chambers. (**B**) Quantification of BicD aggregates. For each type of ovary, a minimum of 70 egg chambers at stages 6–9 were evaluated for presence or absence of BicD aggregates. (**C**) Quantification of aberrant BicD distribution. For each type of ovary, a minimum of 60 oocytes at stages 6–9 were analyzed. Note that karyosomes show a strong phenotype in *aub* mutants but appear normally shaped in *aub mnk* oocytes. Scale bars: 10 μm.

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is at least partially responsible for this phenotype, in a way similar to mutants affecting the repair of meiotic breaks (Ghabrial and Schupbach, 1999; Abdu et al., 2002; Staeva-Vieira et al., 2003). Indeed, we have observed that 97% (*n*=62) and 26% (*n*=94) of *aub mnk* and *mnk armi* karyosomes had a morphology rescued to wild-type, respectively (Fig. 7; data not shown).

Interestingly, DNA damage accumulation and karyosome defects in the absence of strong dorsoventral patterning defects have been described for mutants that affect both meiotic DNA damage repair and checkpoint signaling, such as *hus1* and *brca2* (Abdu et al., 2007; Klovstad et al., 2008). Similarly, germline derepression of TEs in the *tejas* mutant does not affect egg polarity (Patil and Kai, 2010). We thus cannot exclude that the karyosome defect in SF oocytes could reflect a partial or late DNA damage response, which would not trigger other known hallmarks of checkpoint activation, including egg ventralization. Indeed, in SF ovaries, *I* transcripts and ORF1 protein are first detected in germarium region 2A but they reach their highest levels in later-stage oocytes, where they presumably accumulate as RNPs (Seleme et al., 1999; Seleme et al., 2005). At these stages, however, any accumulation of DNA DSBs might go undetected with γ-His2Av antibodies. In this model, the DNA damage response could still cause the observed karyosome defect but would occur too late to significantly disturb Grk protein oocyte accumulation and dorsoventral axis specification.

In the alternative possibility, accumulation of *I* RNPs in the oocyte could directly affect karyosome formation without inducing any DNA damage response. However, and surprisingly, *I* products accumulate in the perinuclear cytoplasm of SF oocytes and do not appear to enter the nuclear compartment at cytologically detectable levels (Seleme et al., 1999; Seleme et al., 2005). Accordingly, GFP-tagged ORF1p remains cytoplasmic when transiently expressed in *Drosophila* cultured cells (Rashkova et al., 2002). Thus, only a minor fraction of *I* RNPs is expected to enter the oocyte nucleus in order to complete the retrotransposition process. This situation contrasts with the clear nuclear accumulation of L1 RNPs in $mael^{-/-}$ mutant mouse spermatocytes associated with DNA damage and chromosome asynapsis (Soper et al., 2008). In SF ovaries, we did not detect any gross defect in the distribution of the SC protein C(3)G in oocytes (supplementary material Fig. S4). However, the low resolution obtained with this kind of analysis (compared with mouse spermatocytes, for instance) cannot rule out the presence of undetected chromosome synapsis defects.

The modest effect of *I* activity on DNA integrity during early SF oogenesis contrasted with the situation observed in piRNA mutants where many TEs, including *I*, are derepressed. However, the origin of DNA damage in piRNA pathway mutants is not clear and the involvment of TEs in generating these breaks remains to be established (Chen et al., 2007; Klattenhoff et al., 2007; Klattenhoff and Theurkauf, 2008; Thomson and Lin, 2009). Genetic inactivation of the checkpoint does not restore the fertility of piRNA pathway mutant females (Table 2) (Klattenhoff et al., 2007). It thus indicates that additional, checkpoint-independent defects cause the female sterility in these mutants. Interestingly, embryos from *aub mnk* females display a specific and severe disorganization of cleavage nuclei that could explain the observed maternal-effect lethality (Blumenstiel et al., 2008). Furthermore, in *aub* and *spn-E* mutants, the *HeT-A* and *TART* retroelements involved in telomere maintenance are upregulated in the female germline and their retrotransposition to broken chromosome termini is increased, with potential consequences on chromosome stability (Savitsky et al., 2006). Finally, Piwi-family proteins are also involved in the biogenesis or processing of piRNAs directed against the 3′ UTR of a broad set of cellular transcripts, with possible regulatory functions (Robine et al., 2009). The overall phenotype of piRNA pathway mutants is thus expected to reflect this functional complexity, in contrast to *I-R* hybrid dysgenesis, where a single type of element is activated.

The meiotic defects we observed in SF oocytes and eggs are probably related to the chromosome rearrangements and non-disjunctions associated with *I-R* hybrid dysgenesis. Rearrangements are probably generated after illegitimate homologous recombination events between integrating *I* elements (Busseau et al., 1989; Prudhommeau and Proust, 1990; Proust et al., 1992). Considering the fact that these chromosomal aberrations were obtained in viable progeny from SF females, we suppose that more detrimental and frequent rearrangements are produced when SF females are still fully sterile. The accumulation of chromosomal rearrangements in oocyte nuclei could probably affect meiotic divisions by notably inducing non-disjunction and chromosome fragmentation events. In this model, the progressive *I* repression established in aging SF females would reduce the probability of these events occurring until oocyte chromosome architecture becomes compatible with normal meiosis.

Materials and Methods

Drosophila stocks

The w^{1118} standard inducer stock and the strong reactive wild-type stock *Charolles* were used to set up control or dysgenic crosses, unless otherwise specified. The *JA26 y w* reactive stock was provided by Alain Pelisson (Institute de Génétique Humaine, Montpellier, France). The *EGFP-Cid* stock (Schuh et al., 2007) and the *Jupiter-GFP* insertion (Buszczak et al., 2007) were obtained from Stefan Heidmann (University of Bayreuth, Bayreuth, Germany) and from the Carnegie Protein Trap Stock Collection (http://flytrap.med.yale.edu/), respectively. The mnk^{P6} stock was a gift from Tin Tin Su (Brodsky et al., 2004). The following alleles were obtained from the Bloomington *Drosophila* Stock Center at Indiana University (http://flystocks.bio.indiana.edu/): $armi^{1}$ and $armi^{72.1}$ (Cook et al., 2004), aub^{HN} and aub^{QC42} (Schupbach and Wieschaus, 1991), $krimp^{f06583}$ (Lim and Kai, 2007) and $mael^{r20}$ (Clegg et al., 1997). The following heterozygous or hemizygous females were used in this study: $armi^{1}$ $armi^{72.1}$ (*armi*), aub^{HN} aub^{QC42} (*aub*), $krimp^{f06583}$ *Df(2R)Exel6063* (*krimp*) and $mael^{r20}$ *Df(3L)ED230* (*mael*). The $K81^{2}$ paternal-effect embryonic lethal mutant is a small, viable deficiency that completely removes the *ms(3)K81* gene (Yasuda et al., 1995). *aub mnk* or *mnk armi* double-mutant females were obtained by standard crossing techniques and meiotic recombination.

Crosses and egg phenotype analysis

Control and dysgenic crosses were set up at the appropriate temperature using equal numbers of freshly emerged virgin males and females that were kept together throughout the experiment. Eggs were collected on agar plates, counted and, if necessary, the dorsal appendage phenotype was examined by direct observation under a stereomicroscope. Embryos were then allowed to develop for 3 days at 25°C before hatching rate and brown/white phenotype determination.

Egg collection, ovary dissection and immunofluorescence

Females that were no older than 1 week were allowed to lay eggs on agar plates in the presence of males at 25°C. Eggs were dechorionated in bleach and fixed as described (Loppin et al., 2001). Ovaries were dissected in TBST (TBS-0.15%, Triton X-100), fixed in a 1:1 mixture of heptane: 4% paraformaldehyde in PBS, rinsed in TBST and were immediately incubated with the primary antibodies as previously described (Bonnefoy et al., 2007). Antibodies and dilutions used were: anti-α-Tubulin (Sigma, T9026, 1/500); anti-H4Ac (Chemicon International, AB3062, 1/200); anti-Flag (Sigma, F3165, 1/1000); anti-H3K14Ac (Millipore, 06-911, 1/500); anti-C(3)G (kindly provided by R. S. Hawley, 1/500) (Page and Hawley, 2001); anti-γ-His2AvD (1/500) (Mehrotra and McKim, 2006); and anti-BicD (Developmental Studies Hybridoma Bank, 1B11-s, 1/200). DNA was stained with Propidium Iodide or Hoechst. Confocal images were acquired using either a LSM510 microscope (Carl Zeiss) or a Leica SP2 (for Fig. 5) and were processed with Adobe Photoshop software.

Karyosome defect assay

To analyze the effect of temperature on karyosome phenotype, 1-day-old SF females were kept at 25°C for 2 days and were then placed at 18°C, 25°C or 30°C for 36 hours before ovary dissection. To analyze the effect of age, SF females that were obtained at 25°C were aged for 3, 6 or 9 days before dissection. Stage 6–9 oocytes

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stained with Propidium Iodide and H3K14Ac were observed under a confocal microscope and karyosomes were classified into three phenotypical categories as described in Fig. 4. For each condition, a minimum of 40 karyosomes was observed.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/20/3515/DC1

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