Drosophila Yemanuclein is required for meiosis in the oocyte and paternal chromatin assembly in the zygote
Ahmed Algazeery

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Titre :
La Yemanucléine de Drosophile est nécessaire à la méiose ovocytaire et l’assemblage de la chromatine paternelle dans le zygote

JURY

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Ahmed Algazeery
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AE</td>
<td>Axial elements</td>
</tr>
<tr>
<td>Asf1</td>
<td>Anti-silencing factor 1</td>
</tr>
<tr>
<td>ATRX</td>
<td>α-thalassemia/mental retardation X-linked syndrome protein</td>
</tr>
<tr>
<td>Bbd</td>
<td>Bar body deficient</td>
</tr>
<tr>
<td>CAF-1</td>
<td>Chromatin assembly factor 1</td>
</tr>
<tr>
<td>CARs</td>
<td>Cohesin attachment regions</td>
</tr>
<tr>
<td>CE</td>
<td>Central elements</td>
</tr>
<tr>
<td>CHD</td>
<td>Chromodomain helicase DNA binding</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CO</td>
<td>Crossover</td>
</tr>
<tr>
<td>CR</td>
<td>Central region</td>
</tr>
<tr>
<td>DAXX</td>
<td>Death domain-associated protein</td>
</tr>
<tr>
<td>DCC</td>
<td>Dosage compensation complex</td>
</tr>
<tr>
<td>DDK</td>
<td>Dbf4-dependent Cdc7 kinase</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage repair</td>
</tr>
<tr>
<td>DLP</td>
<td>DAXX-like protein</td>
</tr>
<tr>
<td>DSBs</td>
<td>Double strand breaks</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double strand DNA</td>
</tr>
<tr>
<td>HATs</td>
<td>Histone acetyltransferases</td>
</tr>
<tr>
<td>HDACs</td>
<td>Histone deacetylases</td>
</tr>
<tr>
<td>HP1</td>
<td>Heterochromatin protein 1</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recommination</td>
</tr>
<tr>
<td>HRR</td>
<td>Homologous recombination repair</td>
</tr>
<tr>
<td>JNK</td>
<td>cJun N-terminal kinase</td>
</tr>
<tr>
<td>KMT</td>
<td>Lysine methyltransferases</td>
</tr>
<tr>
<td>LE</td>
<td>Lateral elements</td>
</tr>
<tr>
<td>MSCI</td>
<td>Meiotic sex chromosome inactivation</td>
</tr>
<tr>
<td>NAP1</td>
<td>Nucleosome assembly protein 1</td>
</tr>
<tr>
<td>NCO</td>
<td>Non crossover</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PEV</td>
<td>Position effect variegation</td>
</tr>
<tr>
<td>PTMs</td>
<td>Post translational modifications</td>
</tr>
<tr>
<td>RC</td>
<td>Replication coupled</td>
</tr>
<tr>
<td>RCAF</td>
<td>Replication coupled assembly factor</td>
</tr>
<tr>
<td>RI</td>
<td>Replication independent</td>
</tr>
<tr>
<td>RN</td>
<td>Recombination nodule</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>SC</td>
<td>Synaptonemal complex</td>
</tr>
<tr>
<td>SCR</td>
<td>Sperm chromatin remodeling</td>
</tr>
<tr>
<td>SDSA</td>
<td>Synthesis dependent strand annealing</td>
</tr>
<tr>
<td>SMC</td>
<td>Structural maintenance complex</td>
</tr>
<tr>
<td>SNBPs</td>
<td>Sperm nuclear basic proteins</td>
</tr>
<tr>
<td>SSA</td>
<td>Single-strand annealing</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single strand DNA</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-related modifier</td>
</tr>
<tr>
<td>Swi/Snf</td>
<td>Switching/sucrose non-fermenting</td>
</tr>
<tr>
<td>TF</td>
<td>Transversal elements</td>
</tr>
<tr>
<td>TP</td>
<td>Transition proteins</td>
</tr>
<tr>
<td>XNP</td>
<td>X-linked nuclear protein</td>
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Introduction
General introduction

All living organisms have life cycle—they are born, they grow, and eventually die. In order to prevent themselves from dying out, they reproduce. In most multicellular eukaryotes, reproduction requires two parents—father and mother. Sexual reproduction relies on two main processes, the formation of paternal and maternal reproductive cells (gametes) and fertilization that combines the father’s sperm and mother’s egg genetic materials through syngamy (Figure 1).

![Sexual reproduction cycle](image)

**Figure 1: Sexual reproduction cycle**
Sexual reproduction involves two main critical processes: meiosis that results in haploid reproductive cells (sperm and egg) and fertilization after which the haploid parental genomes gather in the zygote before they undergo syngamy.

Mitosis and meiosis are the two strategies used by eukaryotes to propagate the genome. Both mitosis and meiosis are preceded by a single round of DNA replication which generates one pair of sister chromatids from each original parent chromosome (Figure 2). Chromosomes are then maintained by sister chromatid cohesion throughout the prophase (Nasmyth, 2001). The two chromosomes are attached by their kinetochores to the spindle fibers apparatus that aligns the chromosomes on the equatorial plate and then separate them to opposite poles.

Despite similarities between the mitotic and meiotic cell divisions (Budd et al., 1989; Forsburg and Hodson, 2000), many characteristic features distinguish the two cell division forms. In the mitotic cell division, the duplicated genome set undergoes only one chromosome segregation round, resulting in diploid daughter cells. Therefore, by mitosis, two daughter
cells are produced from a single parent cell, each daughter cell being genetically identical to the original parent cell (Cnudde and Gerats, 2005; Zickler and Kleckner, 1998). In contrast to mitosis, the standard meiotic cell division process is unique in that it reduces the number of chromosomes by half, resulting in the formation of haploid gametes (Murakami and Keeney, 2008; Roeder, 1997). Studies revealed that meiosis is a highly conserved process in sexually reproducing organisms (Armstrong and Jones, 2003). The reduction of chromosome number is accomplished by following the single round of DNA replication phase by two consecutive rounds of meiotic chromosome segregation, meiosis I and meiosis II (Figure 2). Meiosis II, also called equational division, is most similar to the mitotic division, while genome set reduction is achieved by the first meiotic division (meiosis I) and so called reductional division (Figure 2). The meiotic pathway, at least in the male, ends with the production of four haploid cells different from a single parent cell, each daughter cell carries half the amount of parental genetic material (Cnudde and Gerats, 2005; Zickler and Kleckner, 1998).

The prophase of meiosis I was found to be extended compared to the prophase of the second meiotic division or to that of mitotic prophase. The extended duration of prophase I is not surprising given the complexity of events that must occur in order to segregate the homologous chromosomes. The homologous chromosomes must come into proximity, recognize each other as a homolog, intimately associate, and align with one another along their lengths and exchange their genetic information through a process called homologous recombination. At this stage they are called bivalent chromosomes. These major chromosomal events of meiosis, pairing, synopsis, and recombination are largely conserved across eukaryotes. These processes associated with many cytological changes further divide the meiotic prophase I into five substages: leptotene, zygotene, pachytene, diplotene and diakinesis (Pawlowski and Cande, 2005; Petronczki et al., 2003; Zickler and Kleckner, 1999).

One critical function of this exchange is that it provides a physical connection between the homologous chromosomes, the chiasma. Chiasmata allow chromosomes to align properly on the spindle and to separate accurately such that they will orient to opposite poles after prophase I is completed. Proper segregation of homologous chromosomes at anaphase I relies on chiasmata dissolution as a consequence of cohesion release from sister chromatid arms (Cai et al., 2003). Subsequent release of cohesion from sister chromatid centromeric regions is a prerequisite for the equational division of chromosomes at anaphase II (Chelysheva et al., 2005).
Figure 2: Schematic representation of a meiotic cell division as compared to mitosis

Before meiosis and mitosis the genome content of the dividing cell is duplicated by one round of DNA replication. In mitosis a single round of chromosome segregation occurs, whereas meiosis involves two chromosome segregation rounds. At the first meiotic division (reductional), homologous chromosomes are segregated, and at the second meiotic division (equational), sister chromatids are separated (Alberts et al., 2008).

Homologous recombination is initiated principally by the formation of meiotically induced Double Strand Breaks (DSBs). This process is a highly controlled process, in terms of both position and timing. Failing to initiate DSBs or initiating them at the wrong time or in the wrong place has severe consequences on the subsequent chromosome segregations and
disastrous consequences on the viability of the meiotic products (Lake et al., 2011). If the meiotic divisions were not properly executed, the organism fails to produce proper gametes and if fertilization occurs, it results in aneuploid embryos, an outcome that typically causes spontaneous abortion or birth defects (Mehta et al., 2012).

In eukaryotes, DNA replication and chromosome segregation are temporally separated. For successful cell division, these processes must be carefully coordinated. Several associations have to be established between parental chromosome pairs to keep them together from the time of their generation during S phase till the time they split during metaphase to anaphase transition. Therefore, the identification of the molecular mechanisms of sister chromatids cohesion is of central importance for understanding how the cell governs its meiotic cycle (Peters et al., 2008). Studies of these mechanisms revealed two main protein complexes responsible to ensure this association:
- The molecular ‘glue’ established during DNA-replication that keeps the sister chromatids together from S-phase to metaphase-anaphase transition is known as “cohesin”, a multiprotein complex which is believed to entrap the sister chromatids (Mehta et al., 2012).
- The synaptonemal complex (SC), a tri-partite proteinaceous structure that forms between homologous chromosomes during prophase I (Khetani and Bickel, 2007).

In spite of the similarity of the major meiotic events during both spermatogenesis and oogenesis, each process has its own complexity. Spermatogenesis is a continuous process that occurs throughout most of the life of the male (Cooper and Strich, 2011). In many animal species, the sperm DNA is packaged with specific non-histone proteins, protamines. At fertilization, these proteins have to be removed and replaced by maternally provided histones.

My thesis work is dedicated to unveil such mechanisms in an organism that provides powerful genetic tools, *Drosophila melanogaster*. We aimed in the present work to contribute to gain insight into the molecules and mechanisms that are involved in oocyte meiosis and paternal chromatin assembly in the zygote. The discovery and functional analysis of Yemanuclein (also called Yemanuclein-alpha) by Aït-Ahmed and collaborators have provided the basis for this work. Before the presentation of my experimental data, I will provide some bibliographical background on these critical events on which sexual reproduction relies, in the next two chapters.
Chapter 1: Molecular analysis of early meiotic prophase

Essentially, meiosis is a modified mitotic cell division. Despite the similarities between them in many mechanisms, the most notable difference is the two-steps chromosome segregation process that characterizes meiosis (Budd et al., 1989; Forsburg and Hodson, 2000). Chromosomes are first replicated during the period of pre-meiotic S phase that is similar to S phase in mitosis (Figure 3). However there is a key difference between S phase in mitosis and pre-meiotic S phase: pre-meiotic S phase takes much longer (25-30 minutes for mitotic S-phase vs. ~75 minutes for pre-meiotic S-phase) (Cha et al., 2000). This extra time may be needed for the proper establishment of the additional chromosome cohesion required for the subsequent critical meiotic processes. Indeed sister chromatids must have a specific cohesion that maintains them together until metaphase II (Petronczki et al., 2003).

![Figure 3: Chromosome segregation in mitosis and meiosis](image)

Prophase of meiosis I also takes much longer. During this lengthy phase, the homologous chromosomes condense into thread-like structures which first align and pair before becoming closely associated along their length by the tripartite proteinaceous structure known as the

*Chiasma connects the homolog*
synaptonemal complex (SC). This process is known as synapsis. The different stages of prophase I are distinguished based on the progression of synapsis. In leptotene the homologous chromosomes pair along their length before synapsis is initiated. The SC starts to form between paired homologous chromosomes at zygotene; this process is completed at pachytene and the SC starts to disassemble at diplotene. By the final stage of prophase I, diakinesis, the chromosomes begin to condense to achieve their shortest length at metaphase I. Meiotic recombination occurs during prophase I and the synaptonemal complex plays a key role in this critical event (Page and Hawley, 2004). Yemanuclein is a key player in these events (Meyer et al., 2010), this manuscript. Therefore in this chapter I will concentrate on the bibliographical background of these early meiotic events that are required for the formation of the bivalents, a basis for homologous chromosomes segregation at meiosis I.

I. Components of meiotic chromosome cohesion

DNA replication and chromosome segregation of eukaryotes are temporally separated. A remarkable feature of meiosis is a prolonged prophase arrest following formation of bivalent chromosomes especially in female meiosis in mammals. Indeed it lasts from around birth until ovulation, an interval that might be many months in the mouse and several decades in humans (Tachibana-Konwalski et al., 2010). Therefore, it is essential to keep the sister chromatids together from the time of their generation during S phase till the time they split at metaphase to anaphase transition. Without the cohesion between the two sister chromatids they could separate from each other prematurely. If the meiotic divisions are not properly executed, after fertilization aneuploid embryos may form, an outcome that typically causes spontaneous abortion or birth defects (Mehta et al., 2012). Identification of the molecular mechanisms that underlie sister chromatids cohesion is therefore of central importance (Peters et al., 2008). The identification of the molecules that are involved in this process is a first step in the unveiling of the mechanisms.

A. Structural maintenance of chromosomes (SMC) protein family

The first proteins that are essential for cohesion were identified by genetic screens for mutants that separate sister chromatids precociously before anaphase, during mitosis in the budding yeast *Saccharomyces cerevisiae* (Guacci et al., 1997; Michaelis et al., 1997) and during meiosis I in the fruit fly *Drosophila melanogaster* (Davis, 1971; Kerrebrock et al., 1992; Miyazaki and Orr-Weaver, 1992; Peters et al., 2008).
The cohesion between the two sister chromatids is achieved by specific protein complexes that have subunits called structural maintenance of chromosome (SMC) proteins (Haering et al., 2002; Hirano, 1998). SMC proteins are a family of large coiled-coil proteins, which are conserved in prokaryotes, eukaryotes and archea (Figure 4). In eukaryotes, six different SMC proteins have been discovered that form specific combinations, generating three heterodimers with different functions; Smc1/Smc3 heterodimers make up the structural basis for cohesins, Smc2 and Smc4 complexes forming condensins and a third complex with the two remaining SMC proteins, are Smc5 and Smc6 (Hirano, 2005b; Losada and Hirano, 2005; Nasmyth and Haering, 2005). SMC proteins are large complexes that regulate chromosome architecture and function. They maintain genome integrity by enabling post-replicative DNA repair, shaping chromosomes in preparation for cell division, and holding sister chromatids together to ensure that daughter cells receive a full complement of chromosomes (Seitan and Merkenschlager, 2012).

Figure 4: Architecture of the SMC complexes
A) The core of each SMC complex is formed by two SMC proteins; each contains a hinge domain which has ATPase head and an intramolecular antiparallel coiled coil that connects the two proteins.
B) Showing various SMC complexes composed of a specific SMC dimer and several non-SMC subunits: The SMC1/SMC3 cohesin, the condensin I and condensin II and the SMC5/6 complex (Wu and Yu, 2012).
The SMC proteins contain about 1,000 amino acids and share similar domain structures. The ATPase domain of each SMC protein is separated into N- and C-terminal halves by a long linker. The two nucleotide-binding Walker A and Walker B motifs reside in the two different ATPase halves. The SMC linker folds into an intra-molecular antiparallel coiled coil and allows the N-terminal ATPase half of an SMC protein to fold back to its C-terminal ATPase half and create a single globular ATPase head (Figure 4). The hinge domain at one end of the coiled coil mediates the heterodimerization of eukaryotic SMC proteins (Hirano, 2006; Losada and Hirano, 2005; Nasmyth and Haering, 2005). The two ATPase heads at the other end of the coiled coil can transiently interact with each other to bind and hydrolyze ATP. As revealed by electron microscopy, the SMC heterodimers can adopt different conformations, including V-shaped dimer and ring-like structures, possibly depending on the nucleotide-binding states of their ATPase heads (Anderson et al., 2002; Melby et al., 1998).

1. Condensin complex

Condensin is a pentameric complex, which comprises two members of the SMC family, SMC2 and SMC4, as well as three non-SMC regulatory subunits (Figure 4). In higher eukaryotes two condensin complexes were reported, condensin I and condensin II. Both condensins contain a heterodimer of SMC2 and SMC4 backbone but associate with different regulatory proteins. Condensin I comprises CAP-H/Barren, CAP-D2 and CAP-G whereas condensin II comprises CAP-H2, CAP-D3 and CAP-G2 (Hirano, 2005a; Ono et al., 2003; Yeong et al., 2003). Condensin I and II are both present in metazoans, however fungi have a single condensin complex, which is most similar to condensin I of metazoans. In Caenorhabditis elegans, a third condensin-like complex -the dosage compensation complex (DCC)- regulates the expression of X-linked genes (Jans et al., 2009).

2. SMC5/SMC6 complex

The SMC5/SMC6 complex (Figure 4) has a prominent role in the metabolism of meiotic DSBs and recombination but its exact function remains elusive (Farmer et al., 2011; Lehmann, 2005). Smc5- Smc6 complex is thought to promote the repair of DNA double-strand breaks (DSBs) by error-free sister-chromatid recombination (SCR), which makes it essential for genome stability and suppressing the inappropriate non-sister recombination events (De Piccoli et al., 2006). Recently, Smc5–Smc6 complex was found to localize to some specific chromosome regions during meiotic prophase I. The absence of Smc5–Smc6
complex in meiotic cells results in many catastrophic meiotic divisions as a consequence of unresolved linkages between homologous chromosomes (Farmer et al., 2011).

3. Cohesin complex

Cohesin is a large multiprotein complex (Figure 5) conserved from yeast to human (Table 1) (Haering and Nasmyth, 2003; Nasmyth, 2001). The spatiotemporal regulation of the association and dissociation of the cohesin complex to and from the sister chromatids respectively, is instrumental in faithful segregation of the sister chromatids during both mitosis and meiosis (Mehta et al., 2012). Cohesin complex localizes to the meiotic axis in many species (Cai et al., 2003; Eijpe et al., 2003; Klein et al., 1999; Page and Hawley, 2003; Severson et al., 2009). The major function of cohesin complex, as its name indicates, is to regulate sister chromatids cohesion both their proper cohesion and timely separation. In addition to its canonical function cohesin has been suggested by several lines of investigation in recent years to play additional roles in apoptosis, DNA-damage response, transcriptional regulation and hematopoiesis (Panigrahi et al., 2012).

Chromatin immunoprecipitation (ChIP) technique of the budding yeast genome revealed that cohesin binds to discrete cohesin attachment regions (CARs) on chromosome arms and to a larger domain surrounding centromeres (Blat and Kleckner, 1999; Megee et al., 1999; Peters et al., 2008; Tanaka et al., 1999). Importantly, cohesin was found to associate with chromosomes in budding yeast from late G1 phase until metaphase (Michaelis et al., 1997).

![Figure 5: The cohesin complex and its associated proteins](image)

Cohesin complex is composed of four subunits: a heterodimer of two very long SMC molecules (SMC1 and SMC3), associated with two smaller non-SMC subunits (kleisin family Mcd1/Scc1/Rad21 and stromalin family Scc3/SA1/SA2) proteins. Together these subunits form a large ring capable of topologically encircling DNA strands. Other proteins regulate cohesin’s binding to DNA and its residency there. The NIPBL/MAU2 dimer loads cohesin onto DNA, whereas WAPL/PDS5 release cohesin from chromosomes by opening the SMC3-RAD21 interface (Horsfield et al., 2012).
Structurally, cohesin complex is composed of four subunits (Figure 5): a heterodimer of two very long SMC protein molecules (SMC1 and SMC3), associated with two smaller non-SMC subunits (kleisin family Mcd1/Scc1/Rad21 and stromalin family Scc3/SA1/SA2) proteins (Nasmyth and Haering, 2005; Onn et al., 2008; Shintomi and Hirano, 2007). Kleisins are a superfamily of non SMC protein partners evolutionarily conserved from prokaryotes to humans (Schleiffer et al., 2003). The term kleisin is derived from the Greek word for closure (Xiong and Gerton, 2010). The SMC heterodimer and the non-SMC subunits form the complex which entraps the sister chromatids in its tri-partite ring structure (Figure 5) (Haering et al., 2002; Losada and Hirano, 2005).

### Table 1: Sister chromatid cohesion proteins

<table>
<thead>
<tr>
<th>Role in cohesion</th>
<th>Saccharomyces Cerevisiae</th>
<th>Schizosaccharomyces pombe</th>
<th>Drosophila melanogaster</th>
<th>Vertebrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cohesin complex subunits</strong></td>
<td>Scc1/Mcd1</td>
<td>Rad21</td>
<td>Rad21</td>
<td>Rad21</td>
</tr>
<tr>
<td></td>
<td>Scc3/Irr1</td>
<td>Psc3, Rec11</td>
<td>SA1</td>
<td>SA1, SA2, STAG3</td>
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<tr>
<td></td>
<td>Smc1</td>
<td>Psm1</td>
<td>Smc1</td>
<td>Smc1α, Smc1β</td>
</tr>
<tr>
<td></td>
<td>Smc3</td>
<td>Psm3</td>
<td>Smc3 (Cap)</td>
<td>Smc3</td>
</tr>
<tr>
<td></td>
<td>Rec8</td>
<td>Rec8</td>
<td>C(2)M*</td>
<td>Rec8, Rad21L**</td>
</tr>
<tr>
<td><strong>Loading</strong></td>
<td>Scc2</td>
<td>Mis4</td>
<td>Nipped-B</td>
<td>NIPBL</td>
</tr>
<tr>
<td></td>
<td>Scc4</td>
<td>Ssl3</td>
<td>Mau-2</td>
<td>Scc4/Mau-2</td>
</tr>
<tr>
<td><strong>Establishment</strong></td>
<td>Eco1/Ctf7</td>
<td>Esol</td>
<td>Deco</td>
<td>Esol1, Esol2</td>
</tr>
<tr>
<td><strong>Maintenance</strong></td>
<td>Pds5</td>
<td>Pds5</td>
<td>Pds5</td>
<td>Pds5A, Pds5B</td>
</tr>
<tr>
<td></td>
<td>Sgo1</td>
<td>Sgo1, Sgo2</td>
<td>Mei-S332</td>
<td>Sgo1, Sgo2</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>Rad61/Wpl1</td>
<td>Wpl1</td>
<td>Wapl</td>
<td>Wapl</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Sororin</td>
</tr>
<tr>
<td><strong>Dissolution</strong></td>
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<td>Cut1</td>
<td>Sse</td>
<td>Separase</td>
</tr>
<tr>
<td></td>
<td>Pds1</td>
<td>Cut2</td>
<td>Pim, Thr</td>
<td>Securin</td>
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<tr>
<td></td>
<td>Cdc5</td>
<td>Plo1</td>
<td>Polo</td>
<td>Plk1</td>
</tr>
</tbody>
</table>

(*) C(2)M is only distantly related to Rec8 and does not fulfill typical Rec8 function (Heidmann et al., 2004). (**) Rad21L is a newly identified kleisin whose properties are reminiscent of C(2)M (Ishiguro et al., 2011; Lee and Hirano, 2011; Llano et al., 2012). This table is taken from (Xiong and Gerton, 2010) with modifications, red indicates a meiosis-specific function.

Cohesin is a highly dynamic structure, the association and dissociation of cohesin complex to and from chromosomes alternate through every cell cycle (Figure 6). Cohesin is loaded onto unreplicated chromosomes in G1, followed by cohesion establishment during DNA replication in S phase and maintenance of cohesion in G2. In M phase sister, chromatids cohesion is resolved, and chromosomes segregate. It is noteworthy that in mammals two meiosis specific kleisins (Rec8 and Rad21L) have been identified that play essential roles in
meiosis I specific cohesion and homologous recombination. Interestingly in *Drosophila* there is no Rad21 paralogous sequence (see Table 1 for more details).

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**Figure 6: The cohesin cycle in mitosis and meiosis.**

A) At G1 phase of the cell cycle, the Scc2/Scc4 ensures the cohesin complex loading onto chromatin. Cohesion is established between sister chromatids by acetyltransferase Eco1. Upon entry into G2/M phase, cohesion is maintained by the proteins Pds5, sororin, and Wpl1. In mammalian cells, cohesin along chromosome arms is removed at the transition between prophase and metaphase via phosphorylation of Rad21 by Polo-like kinase 1. Centromeric cohesion is protected from removal by shugoshin and PP2A. At the onset of anaphase, a protease called separase cleaves the Mcd1/Scc1/Rad21 subunit, leading to the opening of the cohesin ring and the separation of sister chromatids (Xiong and Gerton, 2010).

B) Unique features of cohesin during meiosis: Cohesin (black bars) connects the two sister chromatids. Two pairs of sister chromatids, that is, two homologs, synapse thanks to the synaptonemal complex (SC; yellow) formation. Recombination occurs between non-sister chromatids. In anaphase I, cohesin complex in the chromosome arms is dissolved and the homologs segregate with cohesin only at the still intact centromeres. In anaphase II, centromeric cohesion is dissolved, and the sister chromatids segregate. Green arrowheads indicate orientation of kinetochores (Revenkova and Jessberger, 2005).

---

**i. Loading of cohesin at G1 phase**

The loading of the cohesin complex onto chromosomes is mediated by Scc2-Scc4 loading proteins, (Figure 6) (Ciosk et al., 2000; Takahashi et al., 2004). Scc2 was first discovered in
budding yeast (Ciosk et al., 2000) and it belongs to a family called chromosomal adherins. Scc2 homologs have been discovered in fission yeast (Mis4), Drosophila (Nipped-B), Xenopus and human (NIPBL) (Table 1) (Furuya et al., 1998; Gillespie and Hirano, 2004; Rollins et al., 1999; Tonkin et al., 2004). Scc4 protein was found in humans (Watrin et al., 2006) while its first homolog, Ssl3 was discovered in fission yeast. (Bernard et al., 2006). In C. elegans and Drosophila the Scc4 homologue is Mau-2 protein, which interacts with the Scc2 orthologs (Table 1) (Benard et al., 2004; Seitan et al., 2006). SCC2 and SCC4 and their orthologs are found to be specifically required for sister chromatids cohesion establishment and not its maintenance (Skibbens, 2005).

**ii. Establishment of cohesion at s-phase**

After having been loaded onto unreplicated DNA, the loaded cohesin then becomes cohesive during DNA replication and has been proposed to topologically embrace both sister chromatids inside its ring to establish sister-chromatid cohesion (Figure 6) (Cipak et al., 2008; Uhlmann, 2009). Cohesion can only be established once DNA has been replicated during S phase. Several lines of evidence point towards the direction that cohesion establishment is tightly coupled to DNA replication. Despite the mechanism by which cohesin is converted to the cohesive state during DNA replication is not completely understood, it is known that it requires the acetylation of Smc3 by the Eco1 family of acetyltransferases (Figure 6) (Skibbens et al., 1999). Eco1 contains a zinc finger in the N-terminal portion and an acetyltransferase domain in the C-terminal portion. Eco1 appears to be a global regulator of cohesion and it may be able to take advantage of the proximity of sisters during DNA replication to promote the cohesion (Xiong and Gerton, 2010). In humans, two paralogs of ECO1 were discovered (Table 1), ESCO1 and ESCO2. An acetyltransferase ortholog called Deco needed for centromeric cohesion has been identified in Drosophila (Hou et al., 2007; Williams et al., 2003).

**iii. Maintenance of cohesin ring**

After cohesion establishment in S phase, sister chromatids are held together by cohesin throughout G2. Independent of the cohesion establishment factors, several proteins (including Pds5, Sororin, and Wapl) (Table 1) were reported to maintain the sister chromatids cohesion following S phase (Figure 6) (Skibbens et al., 1999; Toth et al., 1999). In budding yeast, Pds5 is the essential gene required for cohesion maintenance during G2/M phase (Hartman et al., 2000; Panizza et al., 2000; Stead et al., 2003). In metazoans two PDS5 genes were identified,
Pds5A and Pds5B, Both Pds5A and B-deficient mice die at birth (Zhang et al., 2009; Zhang et al., 2007). Sororin is a vertebrate-specific component of the cohesin network (Rankin et al., 2005; Schmitz et al., 2007). Sororin was also suggested to be important for the DSBs repair in G2 phase (Schmitz et al., 2007). WPL1/RAD61/Wapl is the evolutionarily conserved gene involved in cohesion maintenance (Table 1). Wapl (wings apart like), first discovered in *Drosophila* (Verni et al., 2000) was found to promote the cohesin dissociation from chromosome arms in mammalian cells during prophase and increases its turnover on chromatin during interphase (Gandhi et al., 2006; Kueng et al., 2006).

**iv. Dissociation of cohesin at anaphase**

At the metaphase-anaphase transition (Figure 6), the proteolytic cleavage of the kleisin subunit by protease enzyme separase/Esp1 allows the cohesin ring to come apart and chromosomes to separate (Ciosk et al., 1998; Funabiki et al., 1996). In mammalian cells, cohesion between chromosome arms is removed after kleisin Rad21 phosphorylation by Polo kinase permits the cohesion to dissolve (Gimenez-Abian et al., 2004; Hauf et al., 2005). The presence of Polo kinase phosphorylation is of special importance during meiotic divisions, as it permits the non-destructive dissociation of cohesion between the sister chromatids at meiosis I leaving the centromeric cohesion maintained until meiosis II when it is targeted by separase (Figure 6) (Buonomo et al., 2000; Xiong and Gerton, 2010).

**B. Synaptonemal complex (SC)**

Synaptonemal complex (SC) is a meiosis-specific structure first identified by Fawcett and Moses in 1956 (Fawcett, 1956; Moses, 1956). It is found almost universally in sexually reproducing eukaryotic organisms (Anderson, 1925; Ashburner et al., 2005; Bridges, 1916). The ultrastructural analysis of the SC by transmission electron microscopy has revealed a tripartite proteinaceous structure (Figure 7). It comprises two chromosome axes (also called lateral elements – LE), surrounding a central element (CE). The axes of the two homologous chromosomes and the CE are connected along their entire length by fine fibrillar structures, the transverse filaments (TF), generating a zipper- or ladder-like structure. The TF and the CE together form the central region (CR) of the SC (Handel and Schimenti, 2010; Kouznetsova et al., 2011).
Figure 7: Model of synaptonemal complex structure.
A) Representative diagram showing a cross section of a segment of the SC with lateral elements (LE), transverse filaments (TF), central element (CE), and central region. Are also shown cohesins/condensins (blue ovals) and other LE proteins (green ovals) along the LEs (Page and Hawley, 2004).
B) Schematic view showing the orientation of different proteins that form SC in Drosophila, orientation disruptor (Ord), the axial element (AE)/LE component C(2)M and C(3)G (Lake and Hawley, 2012).
Synapsis initiates during early prophase I, where each pair of sister chromatids undergoes shortening along their longitudinal axes, resulting in the formation of ‘chromosome cores’ upon which the axial/lateral elements (AEs/LEs) of the SC assemble (Revenkova et al., 2004; Stack and Anderson, 2001). The cohesion between meiotic sister chromatids was reported by many evidences to play an essential role in assembly of the axial/lateral elements (AEs/LEs) of the SC (Cai et al., 2003; Klein et al., 1999; Pasierbek et al., 2001; Webber et al., 2004). During pachytene, SC central element proteins join each set of homologous AEs/LEs along their entire length resulting in synapsis of homologues (Page and Hawley, 2004). Synaptonemal complexes were found to stabilize all the counterpart associations from the period when the pairing interactions are lost until the crossovers are formed (Zickler and Kleckner, 1999). Despite the complexity and diversity of use of synaptonemal complexes, it appears a number of analogies between different species (Hunter, 2003). Proteins constituting synaptonemal complex were isolated by biochemical or genetic screens; they are described in the following paragraph.

II. Regulation of meiotic recombination

A. Initiation of meiotic recombination

1. Double strand breaks (DSBs) formation and repair

An important aspect that differentiates mitosis from meiosis is that levels of genetic recombination in meiosis are much higher (100-1000 fold) during meiosis than in mitosis. This difference is largely due to the programmed formation of DNA double stranded breaks (DSBs). Their formation is considered to be a tightly controlled process in terms of position, timing and number. The mechanism(s) that controls the DSB formation is still not completely understood (Lake and Hawley, 2012). However it is known that formation of DSBs at the wrong time or in the wrong place has severe consequences in the viability of meiotic products. A specific feature of meiosis is that DSBs are repaired by using the two homologs (Figure 8). This homologous repair of DSBs can result in two types of recombination outcomes. One type is crossing over (reciprocal exchange) while the other type results in gene conversion (Fogel and Hurst, 1967; Fogel and Mortimer, 1971; Holliday, 1974; Stadler, 1959). Several models have been proposed to explain both crossing over and gene conversion.
Figure 8: Major molecular events in meiotic prophase I.

Schematic representation shows the major chromosomal events of meiotic prophase I in *Saccharomyces cerevisiae* (Hochwagen and Amon, 2006) and *Drosophila melanogaster* (Joyce and McKim, 2011; Lake and Hawley, 2012). Prophase I involves three main processes; synapsis, double strand break (DSB) formation and DSB repair. These processes are largely conserved across eukaryotes and are associated with many cytological changes that further divide the meiotic prophase I into five substages: leptotene, zygotene, pachytene, diplotene and diakinesis. Meiotic recombination is achieved via a physical connection between the homologous chromosomes, the chiasma, which allows chromosomes to align properly on the spindle after prophase I is completed at diakinesis. Note the differences in the timing of DSBs formation between Yeast and Drosophila.
The first model of recombination (Holliday Model and Meselson-Radding Model) stipulated that recombination initiates via single-stranded DNA breaks (Holliday, 1964; Meselson and Radding, 1975). In 1981 it was observed that yeast transformation is stimulated up to 3000-fold by introducing a DSB into a plasmid (Orr-Weaver et al., 1981). Based on these experiments the double-strand break repair model was proposed to be the actual method for meiotic recombination. Today, the most accepted model for recombination is the double strand break (DSB) repair model (Szostak et al., 1983).

The DSB model requires breaks to be created in both strands of DNA in one chromatid (Figure 9). These breaks are then resected (exonuclease digested) in a 5’ to 3’ direction (Alani et al., 1990; Liu et al., 1995). This results in a single stranded DNA with a free 3’ end. This 3’ recombinogenic end invades a chromatid on the homologous chromosome, which results in displacement of one of the strands. The displaced strand can then act as a template for DNA polymerase to fill in the gap left after the strand invasion. Each strand is ligated to form double Holliday junctions that appear as cross bridges of single strands of DNA (Griffiths, 1993). Another model called SDSA (synthesis-dependent strand annealing) was proposed to account for the variability in gene conversion (Allers and Lichten, 2001).

2. Hotspots and coldspots

In *S. cerevisiae*, it was found that recombination is not evenly distributed along chromosomes. Therefore, the DSB events are also not uniformly distributed. The regions that experience high levels of recombination are known as hotspots and are associated with higher levels of DSB formation (Gerton et al., 2000; Goldway et al., 1993; Nicolas et al., 1989; Sun et al., 1989) while the regions with low levels of recombination (coldspots) show a much lower frequency of DSBs (Petes, 2001). Despite that all of the factors that determine the location of hotspots are not yet known, some features are common to many hotspots. For example, the incidence of hotspots was found to be correlated with promoters or in other areas of open chromatin, such as those that are transcriptionally active and more sensitive to nucleases (Baudat et al., 2000; Blat et al., 2002; Petes, 2001; Wu and Lichten, 1994). Few hotspots could be also detected within regions surrounding centromeres, telomeres and other transcriptionally silent areas (Blat et al., 2002; Gerton et al., 2000; Klein et al., 1999; Lambie and Roeder, 1988; Petes, 2001). Recently published evidence indicates that histone modifications such as the tri-methylation of lysine 4 on histone H3 creates the chromatin environment that forms a hotspot (Borde et al., 2009); this observation, may simply account
for the already known association between the hotspots and the transcriptionally active regions (Petes, 2001).

Figure 9: Double Holliday junction model for meiotic crossover or non-crossover formation. Double strand breaks are generated and their 5’ ends are resected to generate a 3’ overhang. A strand invasion event then generates a single-end invasion D loop intermediate. If the second end of the original DSB also engages with the homologue, a double Holliday junction is formed (shown on the left). The double Holliday junction can be resolved to form either a crossover or a non-crossover. Alternatively, the junction can be dissolved by double Holliday junction dissolution to form a non-crossover (Youds and Boulton, 2011).
3. Proteins required for DSB formation

Several key proteins have been identified to be required for the formation of DSBs. In *Saccharomyces cerevisiae*, ten proteins were found to be essential for DSB formation, these proteins include: Mre11, Rad50, and Xrs2, Ski8, Rec102, Rec104, Rec114, Mei4, Mer2, and Spo11 (Keeney, 2001). Mutations in any one of these ten recombination initiation genes confer similar phenotypes which involve a complete elimination of DSB formation. The four early recombination genes RAD50, XRS2, MRE11, and SKI8 (also known as REC103) function both in mitosis and meiosis (Keeney, 2001). Three of these genes form the MRE11-RAD50-XRS2/Nbs1 (MRX/N complex) (Andrews and Clarke, 2005; Chamankhah and Xiao, 1999; Usui et al., 2001).

*i. MRE11-RAD50-XRS2/Nbs1 (MRX/N complex)*

After DSB formation, the 5’ ends of the break are resected to produce 3’ single stranded overhangs. Resection is carried out by the MRX/N complex which comprises MRE11 (Puizina et al., 2004), RAD50 (Bleuyard et al., 2004) and XRS2/NBS1 (Waterworth et al., 2007).

**ii. Ski8 protein**

The fourth gene is Ski8, which was originally characterized for its role in degrading virus dsRNAs. Its loss of function mutation results in increased viral expression, and its name indicates “superkiller”. SKI8 is found to be involved in mitotic mRNA translation and stability (Brown et al., 2000; Searfoss and Wickner, 2000; Wickner, 1976). The role of Ski8 in mitotic RNA metabolism involves modulating 3’ to 5’ exonucleolytic degradation of damaged mRNAs that are not poly-adenylated (Frischmeyer et al., 2002; Searfoss and Wickner, 2000). In meiosis, however, it has been proposed to function as a scaffold protein with a role in assembling the DSB initiation complex Spo11-Rec102-Rec104 (Araki et al., 2001). Ski8 migrates from the cytosol to the nucleus during meiosis and it has been found to specifically localize to chromosomes during prophase I (Arora et al., 2004).

The products of the remaining six early recombination genes (Rec102, Rec104, Rec114, Rec107 [also known as Mer2], Mei4, and Spo11) function specifically in meiosis (Keeney, 2001; Malone et al., 1991; Malone and Esposito, 1981; Roeder, 1997; Weber and Byers, 1992).
iii. SPO11

Spo11 has been shown to be indispensable for meiotic recombination in various species including *Schizosaccharomyces pombe* (Lin and Smith, 1994), *Arabidopsis thaliana* (Grelon et al., 2001), *Drosophila melanogaster* (McKim and Hayashi-Hagihara, 1998), *Caenorhabditis elegans* (Dernburg et al., 1998), *Mus musculus* (Baudat et al., 2000) and in a variety of fungi including *Neurospora crassa* (Bowring et al., 2006), *Sordaria macrospora* (Storlazzi et al., 2003) and *Coprinus cinereus* (Celerin et al., 2000; Merino et al., 2000). SPO11 mutations in all these systems result in reduced recombination, sterility and/or reduced viability of gametes. Spo11 was shown to be homologous to topoisomerase VI, a subunit of archaeabacterial type II topoisomerase (Bergerat et al., 1997). It was shown to be responsible for the catalysis of DNA breaks (Keeney et al., 1997; Neale et al., 2005). The catalytic activity in *Saccharomyces* Spo11 is presumed to require tyrosine 135 (Keeney et al., 1997; Prieler et al., 2005). This tyrosine is substituted by a phenylalanine residue in some protists (Malik et al., 2007; Ramesh et al., 2005). Tyrosine and phenylalanine are both aromatic ring-containing amino acid residues; it has been proposed that this phenylalanine residue should perform the same biochemical activity as tyrosine (Diaz et al., 2002; Henderson and Keeney, 2004).

Although SPO11 encodes the key protein required for making the DSB, it is not sufficient to activate DSBs on its own, as proved by many studies at least 9 additional proteins are needed. These proteins include Mre11, Rad50, Xrs2, Mer2, Mei2, Rec102, Rec104 and Rec114 (Grelon et al., 2001; Keeney, 2001; Pecina et al., 2002).

iv. Rec104, Rec102, Rec114, Mei4, and Rec107 recombination proteins

Another family of recombination initiation proteins, despite they are absolutely required for DSB formation have specific functions that are largely unknown. This group includes Rec104, Rec102, Rec114, Mei4, and Rec107. Rec102 was found to mediate the Rec104 phosphorylation during meiosis, Rec104 protein having been shown to interact with the SC axial element protein Hop1 and to be required for mature SC formation (Hollingsworth and Johnson, 1993; Kee et al., 2004). Rec102 itself is phosphorylated but neither the kinase responsible nor the timing for this activation are presently known (Shcherbik et al., 2004). Rec102 and Rec104 also associate with the DNA on meiotic chromosomes and each requires the presence of the other for the complete Rec102/Rec104 complex loading. The localization of Rec102 and Rec104 to chromatin also requires Spo11 and Ski8 (Kee et al., 2004). Rec107 protein was found to show high abundance during meiotic prophase, in a Cdc7/Dbf4-
dependent manner, and phosphorylation on residues Ser11, Ser15, Ser19, Ser22, Ser29 (Sasanuma et al., 2008). Rec114 and Mei4 can associate with DSBs hotspots in a Spo11-independent manner suggesting that they may be the first to bind to the DNA. This binding is mediated by the Cdc7 phosphorylation of Rec107 protein (Sasanuma et al., 2008). Rec114 was suggested to have a role in attracting the late recombination factors (Bishop et al., 1999). Dmc1 is a homolog of the *E. coli* protein RecA that is involved in strand invasion during the later events of recombination (Bishop et al., 1992).

v. Role of DDK complex in meiotic progression

The catalytic component of the DDK (Dbf4-dependent Cdc7 kinase) complex is Cdc7 which phosphorylates and activates pre-replication components such as MCM helicase proteins during mitosis and meiosis (Sclafani, 2000). A possible link was suggested between DDK function and recombination initiation. Yeast mutants expressing only 15% of wild type levels of DDK display DSBs abolishment, despite they produced live, diploid, dyad spores (Matos et al., 2008). Similar phenotypes have been observed in *spo11 spo13* and *rec104 spo13* double mutant cells (Klapholz and Esposito, 1980a; Klapholz and Esposito, 1980b; Malone et al., 1991). This suggests DDK has another function after pre-meiotic replication to enable the formation of DSBs and to establish the monopolar spindle attachment required for the reductional division (Matos et al., 2008; Rabitsch et al., 2003; Toth et al., 2000). These experiments suggest that there is coordination between meiotic S-phase and recombination, but the exact mechanism, which coordinates these two critical events, is still not understood.

vi. Chromosome cohesion and homologous recombination

Many studies suggest the presence of a strong relationship between axis formation and recombination in many systems (Kleckner, 2006). Many recombination complexes are physically associated to the axis (Blat et al., 2002; Carpenter, 1975; Moens et al., 2007; Tesse et al., 2003). This physical association is thought to allow error-free coordination of chromosome recombination events. As prophase I progresses from early to mid pachytene, crossover complexes retain SC association whereas non-crossover complexes are released from the SC (Moens et al., 2007; Terasawa et al., 2007). Moreover, in *Arabidopsis* for example, the DSB formation takes place only when the axes are formed, suggesting a temporal link between these two processes (Sanchez-Moran et al., 2007).
vii. Synaptonemal complex proteins mediate meiotic recombination

During prophase I, homologous recombination manifests in form of developing recombination protein complexes (known as recombination nodules) that are closely associated with AEs of meiocytes (Carpenter, 1975; Moens et al., 2007). In mammals, about 250–300 early recombination nodules (ENs) are associated with the AEs before synapsis is initiated (Fraune et al., 2012). This observation gives a clear evidence for the close interdependency and the tight temporal correlation between the process of homologous recombination and the assembly of synaptonemal complex.

In budding yeast, mutations of the meiosis specific axis components Red1, Hop1 or the Rec8 cohesin subunit, result in an altered distribution of DSBs implying their essential role in the initiation and positioning of DSBs (Blat et al., 2002; Glynn et al., 2004). In yeast red1 mutants, AEs are not detectable and recombination is severely defective (Rockmill and Roeder, 1990; Xu et al., 1997). The recombination which does occur in these mutants is biased towards the sister chromatid, implying that Red1 mediates homologue bias in recombination (Xu et al., 1997). The Mek1 kinase localizes to the binding sites of Red1 and Hop1 and phosphorylates Red1 (Bailis and Roeder, 1998). It has recently been proposed that the role of Red1/Mek1 (and presumably Hop1) in establishing inter-homologues bias maybe to counteract the inter-sisters bias imposed by Rec8 and its role in sister chromatid cohesion (Kim et al., 2010).

B. DSBs repair

SPO11 induced DSBs trigger the meiotic equivalent DNA-damage response, which involves sensing of breaks, recruitment of repair proteins and processing of recombination intermediates (Figure 9). Because DSB repair is a fundamental process in both somatic and meiotic cells, homologous recombination repair (Hrr) proteins are the most conserved group of meiotic recombination proteins (Handel and Schimenti, 2010). DSBs repair is initiated by the removal of Spo11 from meiotic DSB ends. In yeast, the process is mediated by Sae2 and Mre11-Rad50-Xrs2-dependent endonucleolysis that releases Spo11 bound to a short nucleotide sequence (Neale and Keeney, 2006; Neale et al., 2005). Once Spo11 is removed, the 5’-ending strands are degraded to expose the 3’-ending single-stranded DNA (ssDNA). These tails, invading an intact homologous DNA duplex, are used as primers for DNA synthesis (La Volpe and Barchi, 2012). Strand invasion of homologous chromosome by the 3’ single strand is mediated by RAD51 and/or the meiosis-specific DMC1 (Masson et al., 1999; Masson and West, 2001); the ssDNA-binding (SSB) protein, replication protein A (RPA), is
an essential cofactor in this process (Kantake et al., 2003; Wang and Haber, 2004). RPA is a heterotrimeric protein involved in numerous DNA metabolic pathways including replication, repair, and recombination (Anciano Granadillo et al., 2010). It binds single-stranded DNA (ssDNA) during homologous recombination through interactions with a series of oligonucleotide/oligosaccharide binding-folds (OB-folds) that display a high affinity for ssDNA. This prevents DNA from winding back on itself allowing repair and prophase to occur properly (Bochkarev and Bochkareva, 2004; Joyce and McKim, 2009). Rad51 was reported to facilitate the formation of a physical filament connection between the invading DNA substrate and homologous duplex DNA template, leading to the generation of heteroduplex DNA (D-loop). After DNA is synthesized using the invading end as a primer, Rad51 dissociates from dsDNA to ensure DNA synthesis by exposing the 3’OH (Krejci et al., 2012).
III. Drosophila melanogaster as a model for meiotic studies

It is important to mention that in Drosophila, males and females use very different strategies to segregate their chromosomes, as meiotic recombination is specific for female meiosis. *Drosophila* female meiosis is an attractive model system to study the early events of meiosis because of powerful tools in genetics and cytology that can be used to identify and characterize the genes required for meiotic recombination (Mehrotra et al., 2008). Moreover, the ovary is arranged in accordance with developmental stage (Figure 10). The females reproductive system is represented by a pair of ovaries, each consisting of 14–16 reproductive units called ovarioles; each ovariole contains a chain of developing egg chambers that become more mature as they reach the posterior end of the ovary. At the anterior tip of each ovariole is the gerarium which is divided morphologically into four regions: region 1, region 2A, region 2B and region 3 (King, 1970). The germline stem cells reside in the anterior-most part of region 1, each stem cell divides asymmetrically to generate a cystoblast. The cystoblast undergoes four rounds of synchronized mitotic cell divisions with incomplete cytokinesis to produce a 16-cell interconnected cyst. All 16 cells of the cyst share the same cytoplasm through bridge structures called ring canals which ensure the exchange of intercellular material between the nutritive cells and the oocyte (Robinson et al., 1994).

The events of early meiotic prophase can be observed cytologically in context of oocyte development within the gerarium (Figure 11). Region 1 is concerned with the pre-meiotic DNA replication phase, after this, prophase and recombination are initiated (King, 1970; Mehrotra and McKim, 2006; Mehrotra et al., 2008). Prophase is initiated by pachytene. Early pachytene involves two processes: the full-length assembly of SC and DSB formation while DSBs repair occurs at mid-pachytene. Therefore, the three pachytene stages could be assigned to the three regions of the gerarium: 2A, 2B, and 3 (Mehrotra et al., 2008). In region 2A, up to four cells within the cyst initiate assembly of the SC. However, within the cyst, only two pro-oocytes have the potential to develop into a mature oocyte and enter into pachytene and assemble mature SC. Eventually, as pachytene progresses, one pro-oocyte of the two cells disassembles the SC and becomes a nurse cell. The proocyte that is selected to become the oocyte localizes at the posterior end of the developing egg chamber. Throughout regions 2A and 2B most of the oocytes are in pachytene, with the SC assembled between homologs along their entire lengths.
Figure 11: Oogenesis in Drosophila
The females reproductive system in Drosophila is represented by a pair of ovaries, each consisting of 14–16 reproductive units called ovarioles; each ovariole contains a chain of developing egg chambers that become more mature as they reach the posterior end of the ovary. At the anterior tip of each ovariole is the germarium where the events of early meiotic prophase can be observed. The remainder of the ovariole (stages 2–14) is termed the vitellarium.

Figure 10: Organization of the Drosophila germarium.
At the anterior tip of the germarium, a germline stem cell divides to produce a cystoblast (premeiotic region 1). The cystoblast undergoes four rounds of mitotic divisions to produce a 16-cell germine cyst. Within the cyst, up to four cells can initiate the formation of the synaptonemal complex (SC) (red) in zygotene, but only two pro-oocytes continue into meiosis and form a full-length SC in early pachytene (region 2A). At this stage programmed meiotic DSBs are induced and can be detected with an antibody to a phosphorylated form of a histone H2A variant, H2AV, at serine 137 (γ-H2AV) (blue circles denote DSBs detected with a γ-H2AV antibody). By early/mid-pachytene (region 2B), fewer DSBs (γ-H2AV foci) can be detected within the pro-oocytes. By mid-pachytene (region 3), one cell has been selected to become the oocyte (Lake and Hawley, 2012).
By region 3, oocyte determination has been completed since a single oocyte is usually evident and positioned toward the posterior of the cyst (Mehrotra et al., 2008).

A. Pairing of homologous chromosomes (synapsis) in Drosophila

In Drosophila, a pair of pro-oocytes enters in prophase at region 2A. Only one of them is selected by region 3 and defined by the presence of SC which is detected by antibodies against C(3)G transverse element (Figure 11) (Page and Hawley, 2001). C(3)G is a coiled-coil protein similar to proteins in budding yeast (ZIP1), C. elegans (SYP-1, SYP-2), and mammals (SYCP1) (Page and Hawley, 2004; Watts and Hoffmann, 2011). Zygotene pro-oocytes were identified by their patchy C(3)G staining, as opposed to the thread-like staining typical of pachytene (Figure 11). SC initiation was found to be linked strongly with cohesion proteins and has interesting parallels with synapsis initiation in budding yeast (Tanneti et al., 2011). The cohesion proteins components SMC1 and SMC3 were found to interact with C(2)M (Heidmann et al., 2004). C(2)M protein is a distantly related member of the kleisin family that includes Rec8 and Rad21 paralogues (Schleiffer et al., 2003). It was shown to be a lateral element component of the synaptonemal complex (Anderson et al., 2005). In wild-type, C(2)M was found to co-localize with C(3)G in most locations to the exception of the centromeres (Khetani and Bickel, 2007; Tanneti et al., 2011).

Ord is a meiosis-specific protein that localizes to chromosome arms and centromeres. It is required for chromosome cohesion and crossover formation (Anderson et al., 2005; Balicky et al., 2002; Khetani and Bickel, 2007; Webber et al., 2004). In ord mutant oocytes, the centromeres clustering is defective and the association of SC proteins with the centromeres is disrupted (Tanneti et al., 2011). Although Ord is found to be required for the cohesin accumulation onto centromeres, it is not essential for the initial loading of the cohesin subunits Smc1 and Smc3 to chromosome arms (Khetani and Bickel, 2007; Lake and Hawley, 2012).

B. Initiation of double strand breaks in Drosophila

The phosphorylated form of the H2Av variant (γ-H2Av) forms foci that have been used as markers for DSBs. By using an antibody that recognizes γ-H2AV at DSB sites, Mehrotra & McKim showed that γ-H2AV foci first appear in early pachytene after SC formation (Mehrotra and McKim, 2006). By counting the number of γ-H2AV foci, a gradual increase in the number of DSBs has been noticed as cysts mature in region 2A (Figure 11). This increase is followed by a decline in number as the cyst progresses from early to mid-pachytene (region
The γ-H2AV foci virtually disappear in the oocyte by the time the cyst reaches mid-pachytene (region 3). Because the gradual decrease in the number of γ-H2AV foci coincides with progression through pachytene, this decrease likely represents the process of DSB repair (Lake and Hawley, 2012). Whether the removal of γ-H2AV indicates that repair is complete is still unknown.

In *Drosophila*, in addition to the Spo11 ortholog Mei-W68 (McKim and Hayashi-Hagihara, 1998), Mei-P22 is reported to be required for DSB formation (Figure 12). Immunofluorescence analysis of an epitope-tagged Mei-P22 showed that during early pachytene after SC formation, Mei-P22 localizes to discrete foci prior to the time DSBs can be detected (Liu et al., 2002; Mehrotra and McKim, 2006). The percentage of Mei-P22 foci showed a partial overlap with the γ-H2AV marker (Mehrotra and McKim, 2006). Interestingly, in the absence of *mei-W68*, Mei-P22 is able to load onto the chromosomes, which indicates that Mei-P22 localization is independent of DSB formation (Liu et al., 2002). A third gene required for the induction of meiotically induced DSBs is *trem* (Lake et al., 2011). *Trade embargo* (*trem*), a 5-C2H2 zinc finger protein is expressed in early zygotene, localizes to chromatin in a thread-like fashion and is required for normal DSB levels. A point mutation in *trem* (*tremF9*) failed to localize Mei-P22 to discrete foci during pachytene. Because Mei-P22 can associate with chromatin in the absence of DSBs, these data suggest that Trem acts upstream of both Mei-P22 and Mei-W68 in the initiation of DSBs. The mechanism by which *trem* localizes Mei-P22 to discrete foci on the chromatin is still unknown (Lake and Hawley, 2012).

**C. Repair of double strand breaks in Drosophila**

In *Drosophila*, the DSBs repair genes process the recombination intermediates that can be resolved into either non-crossover (gene conversion) or crossover products (Haber, 2000). In order to make crossovers, two classes of genes named (crossover-specific genes) are required (Joyce and McKim, 2009).

The first class is precondition genes (Figure 12) that are responsible for determining which DSBs become crossovers and their distribution such as (*mei-218* and *rec*) (Bhagat et al., 2004; Joyce and McKim, 2009; Liu et al., 2000).
Figure 12: Major molecular events in meiotic recombination in Drosophila.

Homologous recombination HR involves three main processes: pairing of homologous chromosomes via synaptonemal complex, double strand break DSBs formation and DSBs repair. The SC is shown in green, the Drosophila genes names are shown along with human homologs in parentheses (Joyce and McKim, 2011).

The other class is called exchange genes, which produce crossovers (Figure 12). Four proteins have been identified in the exchange class: MEI-9, ERCC1, MUS312 and HDM (Joyce et al., 2009). Mutations in *mei-9*, *Ercc1*, *mus312*, and *hdm* result in a reduced and randomly distributed crossing over along chromosomes (Joyce et al., 2009). MEI-9 is the Drosophila homolog of human and yeast nucleotide excision repair (NER) (Sekelsky et al., 1995; Sijbers
et al., 1996). Mei-9 is required for 90% of all meiotic crossovers (Boyd et al., 1976). The HDM protein encodes a RPA-like protein (Joyce et al., 2009).

*Drosophila* has two checkpoint pathways that are activated to monitor delays with different meiotic events. The first is the ATR/MEI-41-dependent DSB repair checkpoint, which works when there is a defect in repairing DSBs (Joyce et al., 2011). When there are mutations in any of the DSB repair such as precondition genes or exchange genes, pachytene checkpoint delays the chromatin remodeling response to DSBs (Joyce and McKim, 2009).

The first part of my thesis work is dedicated to the characterization of Yemanuclein as a new actor in *Drosophila* meiotic recombination. We show that Yemanuclein is associated to the SC and that it interacts *in vivo* with cohesin complex components. Interestingly *yemanuclein* was not recovered in the numerous genetic screens that were undertaken to identify mutations that affect meiotic recombination.
Chapter 2: Chromatin reprogramming in sexual reproduction

The genetic information of a eukaryotic cell is encoded by long linear duplex polymers of deoxyribonucleic acid (DNA). DNA comprises huge information ranging from $10^6$ to $10^{11}$ base pairs (bp). This “database” is confined in about “2 meter length library” occupying only 5-20 µm in the cell nucleus (Widom, 1998). In order to fit this small space, the DNA filaments undergo tremendous compaction by wrapping around highly basic proteins called histones. The resulting DNA-histone complexes are nucleosomes, which are the building blocks of the macro-nucleoprotein structure called chromatin, which controls not only genetic inheritance, but also the activity of genes (Kornberg and Lorch, 1999).

I. Chromatin structure

A. Chromatin organization and genome dynamics

The nucleosomes constitute the first level of chromatin compaction and this contributes a relatively small fraction of the condensation needed to fit the typical genome into an interphase nucleus indicating that there are additional “higher order” levels of chromatin condensation (Figure 13). 'Higher-order' structures are poorly understood, but are known to rely on the interactions between nucleosomes and include additional assemblage of nucleosomes that assumes a reproducible conformation in 3D space (Woodcock and Ghosh, 2010).

Chromatin organization has a major effect on DNA readout, since the packaged DNA has to be made accessible for transcription, replication and DNA repair to occur properly. Moreover, some regions of the genome are differently accessible than others. Such differences in compaction state, at the same time, provide an opportunity for differential regulation of gene expression in programming different cell types that have to be remembered by a cell through multiple divisions.

DNA undergoes multiple levels of compaction that change over the course of the cell cycle (Figure 13). In interphase, chromosomes have a decondensed appearance, and occupy spatially distinct regions in the nucleus termed 'chromosome territories' (Cremer and Cremer, 2001). The use of DNA coloration dyes revealed that chromatin in interphase can be visualized at microscopic level into two relatively distinct forms, euchromatin and heterochromatin (Fransz et al., 2003). Euchromatin is characterized by a loose decondensed chromatin. The DNA is less densely packed during interphase and here the genes are in a so-
called permissive state for transcription. While, heterochromatin is initially referred as those chromatin regions that remain densely stained and are highly condensed. Heterochromatin can be either constitutive or facultative. Constitutive heterochromatin consists of non-coding and repetitive DNA sequences such as those present in the centromeric, pericentromeric and sub-telomeric regions. They persist in silenced state through cell divisions (Probst et al., 2009). Facultative heterochromatin on the other hand is not a permanent feature but is seen in some cells at certain developmental stages. It may result from transformation of euchromatin via specific epigenetic alterations at certain times, and contains loci activated at specific stages while repressed at others (Brown, 2002).

The concentration of DNA in different nuclear regions was found to be inversely correlated with the level of transcription, leading to the idea that gene expression is suppressed in heterochromatin. Recently, heterochromatin studies have led to a dramatic advance in
understanding epigenetic control of gene activity (Bernstein and Allis, 2005; Lund and van Lohuizen, 2004). Nevertheless, the precise molecular interactions and structural changes at chromatin higher-order folding remain barely known (Grigoryev et al., 2006). Differential gene expression could be explained by understanding the effect of heterochromatin regions. Studies in Drosophila revealed a phenomenon known as PEV for "position effect variegation" (Reuter et al., 1982; Reuter and Wolff, 1981). PEV is observed when a euchromatic gene is artificially relocated next to heterochromatin, which confers to the gene that comes in close proximity to heterochromatin, variegated expression. Silencing of certain genes may lead to phenotypic variegation in tissues. While the genetic information required for gene activation is present in all cells, the gene is active in a differential sporadic manner, being fully active in some cells, and completely inactive in others. This 'spreading' of repressive features along the chromosome results in a variegated expression pattern that can appear to be clonal or randomly speckled (Schotta et al., 2003). It is worth mentioning that transcriptionally active and silent regions may be positioned adjacent to each other; therefore, boundaries must exist to prevent two neighboring regions from influencing each other. These borders are called insulators and are DNA elements that prevent stimulation of transcription or silencing from one region to another (West et al., 2002).

Position-effect variegation is an excellent genetic tool to screen gene functions, many genes now known to be involved in chromatin dynamics have been successfully identified by using the phenomenon of PEV, such as suppressor of variegation 2-5, encoding the heterochromatin protein 1 (HP1) (James and Elgin, 1986). It was found that the physical tethering of HP1 to the heterochromatin regions ensures the chromatin condensation (Greil et al., 2003; Verschure et al., 2005), while paradoxically, via a potential interaction with RNA, HP1 has also been found to localize to some active genes (Cryderman et al., 2005; Piacentini et al., 2003). Therefore HP1 may be required for the expression of both its euchromatic and heterochromatic target genes. This broad distribution of HP1 makes it essential for various functions ranging from repressing transcription to long-range chromatin interactions.

**B. Nucleosome assembly**

1. Histones as the basic components

DNA and histones have very high and non-specific affinity to one another. Therefore, the proper assembly of nucleosomes requires that they have to be built in a stepwise fashion. This involves multiple intermediate stages and auxiliary factors (Figure 14). The DNA is first wrapped around two dimers of the histones H3 and H4 that act as a scaffold, it gives the
central 80 bp \((H3/H4)_2\) tetramer of the 147 bp of nucleosomal DNA that wraps into a unit known as tetramer or the tetrasome. The resulting tetrasome structure is then sandwiched between two dimers of histones H2A and H2B and wraps the additional 67 bp of DNA to give rise to the complete nucleosome core particle (Luger et al., 1997). Nucleosomes are spaced apart by a stretch of linker DNA that has an average length of ~50 bp. The linker region is bound by the non-core H1 histone, an interaction that contributes to overall compaction of the nucleosomal array. Histones interact with the phosphate backbone of DNA and therefore have no sequence specificity for their binding. The proper spacing of the nucleosomes therefore requires the activity of ATP-driven auxiliary factors known as ‘chromatin remodelers’ that move the DNA with respect to the histone octamer (Cairns, 2009).

**Figure 14: Schematic representation of stepwise assembly of the nucleosome core particle**
The core histones are color coded (yellow = H2A, red = H2B, blue = H3, green = H4) and cylinders represent helices (Dutnall, 2004).

Histones of the nucleosome unit present similar structures containing 2 distinct domains: the histone fold domain formed by three \(\alpha\)-helices connected by two loops, and the N-terminal tail domain consisting of 15-30 basic amino acids and protruding out from nucleosome surface (Khorasanizadeh, 2004). More than 120 direct atomic interactions between histones and DNA backbone, and nearly equal number of water-mediated interactions are distributed at the 14 super-helix locations at the interface of these sub-complexes, while multiple electrostatic and hydrophobic effects and hydrogen bonds are required for nucleosome formation (Kamakaka and Biggins, 2005; Luger, 2003).

As basic components of the nucleosome, histones have critical contribution in nucleosome structure. It is certain that modification on histone residues or alteration in primary sequence would affect nucleosome dynamics possibly via causing subtle structural variation or recruiting different chromatin-modifying factors, and hence influence the DNA accessibility and thereafter higher architecture.
2. Nucleosome assembly pathways

Histones assembly onto nucleosomes can be divided into two main pathways, a pathway coupled to DNA synthesis, which includes DNA replication and repair, called replication-coupled (RC) assembly and a pathway independent of DNA synthesis called replication-independent (RI) assembly.

i. Replication-coupled assembly

Ahead of the replication fork, nucleosomes must be temporarily disassembled or remodeled in order for the DNA replication machinery to gain access to the DNA. During DNA replication DNA unwinding is accompanied by transient disruption of parental nucleosomes. And following DNA replication during S-phase, the naked DNA is an available target for every DNA binding protein in the cell. Many studies reported that the assembly of replicated DNA into nucleosomes is coupled to the on-going DNA replication (McKnight and Miller, 1977; Stillman, 1986).

During S phase of the cell cycle, bulk DNA is replicated through a highly orchestrated multiple-component process that results in two copies of the genome. The newly synthesized core essential histones are strictly incorporated into DNA; these histones are so called canonical histones. Canonical histone proteins are encoded by replication-dependent genes and must rapidly reach high levels of expression during S phase. The majority of histone deposition takes place during S-phase and mainly involves the canonical histones. Thus, coupling nucleosome assembly to DNA replication within the cell is a vital process to establish a silent, closed chromatin conformation that helps to prevent aberrant gene expression (Loyola and Almouzni, 2004). It ensures proper inheritance of chromatin structure, propagation of epigenetic marks on histones to daughter cells and maintenance of genome integrity (Li et al., 2012).

ii. Replication-independent assembly

Independently to the DNA replication and under specific developmental conditions such as DNA repair, meiotic recombination or transcription initiation and termination, large families of histone variants are incorporated to the nucleosomes and alter their structure and dynamics. The histones that are already present in the chromatin are replaced by the histone variants through the replication-independent assembly pathway. Thus, the presence of such variants in chromatin adds a level of complexity to the regulation of chromatin structure (Talbert and Henikoff, 2010)
II. Regulation of chromatin structure

Chromatin is a highly dynamic structure that must keep the balance between being folded as much as needed and being accessible whenever necessary. The functional state of chromatin is ultimately regulated by the structural changes that make the underlying DNA molecule more or less accessible to the different biological processes such as DNA replication, transcription and DNA repair via its degree of compaction. DNA compaction depends on the status of its associated histones. The deposition of histones on DNA is the fundamental basis for epigenetic inheritance. It ensures that certain chromatin states are maintained inside a single cell and/or transmitted throughout cell divisions to daughter cells. This requires not only proteins that deposit histones on DNA but also proteins or complexes and biochemical processes mediated by many specific enzymes in order to maintain the chromatin modification status. The functional status of chromatin can be modulated through several interconnected regulatory mechanisms:

- incorporation of histone variants
- post-translational modifications of N-terminal tails that emerge from the nucleosome
- ATP-dependent chromatin remodeling
- histone chaperones

A. Histone Variants

Eukaryotes, beside canonical histones, have evolved various histone variants (Figure 15). In general, canonical histone genes have multiple copies that are highly similar in sequence. Moreover they are intron-less and they are expressed during S-phase. These histones are devoted to the DNA-synthesis process, or so called Replication Coupled (RC) chromatin assembly. They are highly conserved during evolution and are synthesized to provide the main supply of histones at DNA replication. In contrast histone variants or “replacement” histones have evolved from the corresponding canonical histones and differ from their canonical paralogues in primary sequence and the presence of introns in their genes (Zhu et al., 2012). Histone variants expression timing and deposition mechanisms are also different from canonical histones (Table 2). Histone variants are usually 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). Histone variants may therefore be incorporated into nucleosomes during the entire cell cycle and can impart unique properties to the nucleosomes they occupy (Kamakaka and Biggins, 2005). The incorporation of these histone variants is essential for nucleosomes
modulation. 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, DNA repair, condensation or kinetochore formation.

Histone variants may preferentially or specifically be expressed in certain tissues, for instance, the testis specific histone H3 variant (Witt et al., 1996). Some others are enriched at specific chromosomes or locations. As an example, CENP-A is an H3 variant that specifically localizes to centromeres (Palmer et al., 1991). Another such variant is macroH2A, which is enriched on the inactive human X-chromosome (Chadwick and Willard, 2002).

Histone variants were discovered on the basis of differences at the level of their primary sequence. Variants have been identified for all histone classes with the exception of histone H4 (Brown, 2001).

1. H2A variants

The H2A family (Table 2) contains a plethora of ‘universal variants’ found in almost all organisms. In mammals four H2A variants have been reported (H2A.Z, H2A.X, marcoH2A and H2A.Bbd) (Malik and Henikoff, 2003).

i. H2A.Z variant

H2A.Z is a highly conserved histone variant; it was found to have a single evolutionary origin different from all other H2A variants (Malik and Henikoff, 2003). H2A.Z differs from canonical H2A and other H2A variants mainly in its “docking” domain in the C-terminus and in the L1 loop where two H2A molecules contact each other (Arents et al., 1991; Luger et al., 1997; Suto et al., 2000). Genome-wide nucleosome occupancy studies in yeast (Mavrich et al., 2008a; Yuan et al., 2005) and flies (Mavrich et al., 2008b) showed a specific localization pattern of H2A.Z nucleosomes near the transcription start site. This has been proposed to be an epigenetic marker for directing or regulating the positioning of downstream nucleosomes. Therefore, ensuring the correct deposition of H2A.Z nucleosomes is crucial for the maintenance of epigenetic modifications at the transcription start site. (Gupta et al., 2008; Rando and Ahmad, 2007). H2AZ has also been shown to be essential in early embryonic development. Cell differentiation of murine inner cell mass (ICM) revealed that H2A.Z is first enriched at pericentromeric heterochromatin and subsequently enriched at other chromatin regions.
Figure 15: Schematic representation of histone variants

A) Structural domains of canonical histones and histone variants, the regions that differ in histone variants are shown in red (Allis et al., 2007b).

B) Genomic locations of some histone variants as identified by immunofluorescence and genome-wide ChIP studies (Banaszynski et al., 2010).

H2A.Z depletion in mice causes genome instability and disruption of HP1α localization at the pericentromeric regions, which suggests that HP1α function and pericentromeric heterochromatin identity are regulated by H2A.Z during early embryonic development (Rangasamy et al., 2003; Rangasamy et al., 2004). Interestingly, the only Drosophila H2A variant, known as H2Av, is paralogous to H2AZ (Talbert et al., 2012).
ii. H2A.X variant

H2A.X has a histone fold domain that is similar to the canonical H2A and differs from it only in having a unique C-terminal motif hydrophobic residue (Malik and Henikoff, 2003; Talbert and Henikoff, 2010). Despite that H2A.X has been implicated to play a role in meiosis, growth, tumor suppression and immune receptor rearrangements, its role in DNA double-strand breaks (DSB) repair makes it viewed as the “histone guardian of the genome” (Fernandez-Capetillo et al., 2004). The H2A.X serine residue at the γ-position of the C terminus can be phosphorylated (termed γ-H2A.X) as a result of DSB formation. Because DSB can potentially occur anywhere in the genome, H2A.X distributes randomly as well (Downs et al., 2000; Rogakou et al., 2000). During spermatogenesis (Figure 16), the pachytene stage is accompanied by γ-H2A.X enrichment at the sex chromosomes to initiate meiotic sex chromosome inactivation (MSCI) and X and Y chromosome condensation, which leads to the formation of a partially paired sex body. Interestingly, as a result of the knockout of H2A.X in mice, this entire process was impaired, resulting in sterility (Fernandez-Capetillo et al., 2003).

iii. macroH2A variant

MacroH2A is a vertebrate-specific H2A variant that differs from its corresponding canonical H2A in having a large C terminal domain (200 residues), termed “the macro domain”, that shares no sequence similarity with any other histone (Malik and Henikoff, 2003). In mammals, macroH2A is enriched on the inactive X chromosomes in females (Costanzi et al., 2000). Therefore it is thought to contribute to the maintenance of an inactive X chromosome. Some studies suggest that the C-terminal macro domain of macroH2A interferes with the binding of transcription factors but also with the N-terminal domain which impedes chromatin remodeling via SWI/SNF pathway (Angelov et al., 2003). In addition, macroH2A is enriched at developmental genes in human pluripotent cells in males and regulates the timing of HoxA activation. The distribution pattern of macroH2A at the Hox loci reveals its overlapping with PRC2 and therefore, marcoH2A is thought to cooperate with PRC2 and potentially acts as an epigenetic regulator of key developmental genes (Buschbeck et al., 2009). MacroH2A can also mediate gene silencing by inhibiting the catalytic activity and substrate binding capacity of PARP1, which is a nuclear enzyme that is involved in gene activation (Buschbeck et al., 2009; Ouararhni et al., 2006).
iv. H2A.Bbd variant

This histone variant was named because of its unique genomic distribution where it is deficient in inactive X chromosomes (bar body deficient) (Table 2 and figure 16). The unique subnuclear localization and key features of H2A.Bbd suggest that it is involved in gene activation. H2A.Bbd nucleosome has a truncated C-terminal docking domain (Chadwick and Willard, 2001). As a result it binds only 116 base pairs of DNA making the H2A.Bbd nucleosome less stable than the canonical nucleosome (Bao et al., 2004; Gautier et al., 2004). H2A.Bbd nucleosome not only lacks a small acidic region on the surface that is involved in transcriptional repression but also also K119, a residue that is often ubiquitinated on canonical H2A (Zhou et al., 2007).

v. H2AL variant

H2AL variant is a H2A.Bbd-like histone. It was recently identified in mouse spermatids. It has truncated C-terminal region and specifically marks pericentric regions (Ferguson et al., 2009; Govin et al., 2007). H2AL is also ectopically expressed in somatic cells and interact with the testis-specific H2B variant TH2B forming more labile nucleosomes than canonical H2A/H2B dimers (Govin et al., 2007; van Roijen et al., 1998; Zalensky et al., 2002).

vi. TH2A variant

TH2A is a testis-specific histone variant expressed and incorporated within spermatocytes chromatin during pachytene (Figure 16) and disappears in round spermatids but its exact function is still unknown (Meistrich et al., 1985; Rao et al., 1983).

2. H2B variants

In contrast to H3 and H2A histones, H2B isoforms are expressed in few tissues (Table 2), including sperm specific H2B variant (spH2B). In human, two variants of histone H2B specifically expressed in the testis have been described: TH2B and H2BFWT (Table 2) (Gineitis et al., 2000). Interestingly, TH2B was proposed to be a platform in specifying pericentric heterochromatin during late spermatogenesis (Table 2 and figure 16) (Govin et al., 2007). On the other hand, H2BFWT appears to be enriched at telomere interstitial blocks, and it has been proposed that it may serve as an epigenetic marker of telomeric identity in testis (Table 2 and figure 16) (Churikov et al., 2004).
3. H3 Histone variants

In human, eight histone H3 proteins were identified (Hamiche and Shuaib, 2012). They have been arranged on the basis of their incorporation onto chromatin, in two different groups: two replication-dependent canonical H3 histones (H3.1 and H3.2) and six replication-independent histone H3 variants (H3t, H3.3, CENP-A, H3.X, H3.Y and H3.5). It is noteworthy that H3.3, CENP-A, H3.X, and H3.Y are somatic histone variants, while H3t and H3.5 are testis specific variants (Hamiche and Shuaib, 2012). In yeast, a single H3 isoform has been detected equivalent to the mammalian H3.3 that can be deposited by both replication dependent and replication independent pathways (Hamiche and Shuaib, 2012). Thus, H3.3 gene is suggested to be the common ancestor which gave rise to all other non-centromeric major H3 variants (Postberg et al., 2010). *Drosophila* H3.2 variant would be the first derivative, followed by H3.1 and H3t that appeared on mammals (Malik and Henikoff, 2003; Postberg et al., 2010). H3.X and H3.Y were later detected in primates (Wiedemann et al., 2010). The amino acids differences between the different H3 histones may provide the specificity for their differential chromatin assembly and regulation.

*i. CENP-A*

CenH3 or CENP-A (Table 2) was identified as an H3 variant during co-purification with other core histones (Palmer et al., 1987; Sullivan et al., 1994). The other H3 variants, such as H3.1, H3.2 and H3.3, are at the same molecular weight and differ by only four to five amino acid residues (Palmer et al., 1987). In contrast, CENP-A has a variable N-terminus, with no sequence similarity to the N-terminal region of the other H3 variants (Sarma and Reinberg, 2005). Moreover, CENP-A shares only 50% identity in the histone fold domain with the other H3 histones (Malik and Henikoff, 2003). CENP-A is the first histone variant that was shown to identify specific chromatin regions, namely the centromeres (Henikoff et al., 2004). CENP-A is essential for kinetochore formation and chromosome segregation (Allshire and Karpen, 2008; Henikoff and Ahmad, 2005; Sarma and Reinberg, 2005).
<table>
<thead>
<tr>
<th>Histone</th>
<th>Localization</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2A</td>
<td>Genome-wide</td>
<td>canonical histone</td>
<td></td>
</tr>
<tr>
<td>H2A.Z</td>
<td>Regulatory elements, promoter, pericentric repeats</td>
<td>transcriptional activation, nucleosome instability</td>
<td>(Gupta et al., 2008; Jin et al., 2009)</td>
</tr>
<tr>
<td>H2A.X</td>
<td>XY Body, sites of double-strand DNA breaks</td>
<td>marker of DNA lesions</td>
<td>(Fernandez-Capetillo et al., 2004)</td>
</tr>
<tr>
<td>MacroH2A</td>
<td>Xi chromosome, promoters</td>
<td>X inactivation</td>
<td>(Costanzi et al., 2000; Pehrson and Fried, 1992)</td>
</tr>
<tr>
<td>H2A.Bbd</td>
<td>Excluded from Xi</td>
<td>destabilizing protein-DNA interaction</td>
<td>(Chadwick and Willard, 2001)</td>
</tr>
<tr>
<td>H2A.L</td>
<td>Pericentric repeats of spermatids</td>
<td>N.D.</td>
<td>(Govin et al., 2007)</td>
</tr>
<tr>
<td>TH2A</td>
<td>Testis</td>
<td>N.D.</td>
<td>(Meistrich et al., 1985; Rao et al., 1983)</td>
</tr>
<tr>
<td>H2B</td>
<td>Genome-wide</td>
<td>canonical histone</td>
<td></td>
</tr>
<tr>
<td>TH2B</td>
<td>Testis</td>
<td>pericentric heterochromatin.</td>
<td>(Govin et al., 2007)</td>
</tr>
<tr>
<td>H2BFWT</td>
<td>Testis</td>
<td>telomeric identity</td>
<td>(Churikov et al., 2004)</td>
</tr>
<tr>
<td>H3.1</td>
<td>Genome-wide</td>
<td>canonical histone (RC assembly)</td>
<td>(Tagami et al., 2004)</td>
</tr>
<tr>
<td>H3.2</td>
<td>Genome-wide</td>
<td>canonical histone (RC assembly)</td>
<td></td>
</tr>
<tr>
<td>H3.3</td>
<td>a) euchromatin, promoter, gene bodies</td>
<td>transcriptional activation (RI assembly)</td>
<td>(Ahmad and Henikoff, 2002; Jin et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>b) telomeres, pericentric repeats</td>
<td>N.D.</td>
<td>(Goldberg et al., 2010)</td>
</tr>
<tr>
<td>CENP-A</td>
<td>Centromere</td>
<td>kinetochore assembly</td>
<td>(Palmer et al., 1991; Sullivan et al., 1994)</td>
</tr>
<tr>
<td>H3T</td>
<td>Testis</td>
<td>N.D.</td>
<td>(Albig et al., 1996; Witt et al., 1996)</td>
</tr>
<tr>
<td>TH3 variant</td>
<td>Spermatogonia-specific</td>
<td>N.D.</td>
<td>(Meistrich et al., 1985; Trostle-Weige et al., 1984)</td>
</tr>
<tr>
<td>H3.X and H3.Y</td>
<td>Primates-specific</td>
<td>N.D.</td>
<td>(Wiedemann et al., 2010)</td>
</tr>
<tr>
<td>H3.5 variant</td>
<td>Hominid testis-specific</td>
<td>N.D.</td>
<td>(Schenk et al., 2011)</td>
</tr>
</tbody>
</table>

Table 2: Localizations and functions of canonical core histones and histone variants in mammals

Canonical histones in bold, N.D.–not determined, RC–replication-coupled, RI–replication-independent, Xi–inactive X chromosome.
ii. Histone H3t variant

The testis specific variant H3t (Table 2) has four amino acid substitutions (A24V, V71M, A98S, A111V) that distinguish it from its canonical H3.1 variant (Hamiche and Shuaib, 2012). The H3t variant has been identified as a human variant that is specifically expressed in the testis spermatocytes stage (Albig et al., 1996; Witt et al., 1996). H3t variant was reported recently to be present in HeLa cells (Govin et al., 2005), in the brain and in embryos (Govin et al., 2007); its exact function remains unknown.

iii. Histone TH3 variant

TH3 is specific to the rat and was strongly detected in spermatogonia and weakly in spermatocytes and round spermatids unlike other testis specific histones such as TH2A, H1T and TH2B (Meistrich et al., 1985; Trostle-Weige et al., 1984).


H3.X and H3.Y (Table 2), the recently identified histones in primates, display interesting changes in amino acids that are known to be modified in H3.1, H3.2, and H3.3 (Wiedemann et al., 2010).

v. Histone H3.5 variant

Another newly identified hominid-specific histone H3 variant, H3.5 is specifically expressed in testis (Table 2) and was shown to be associated with actively transcribed genes (Schenk et al., 2011).

vi. Histone variant H3.3

A specific chapter will be dedicated to H3.3 and its protein chaperones.

In conclusion we can say that the use of histone variants is an essential tool in the control of nucleosome and chromatin structure. The differential use of these variants has very important biological impacts. To illustrate this complex use of histone variants an interesting example is their use in the formation of the mammalian male germ cells where they are essential to regulate the complex events such as meiosis and histone replacements. These variants are with no doubt indispensable for male fertility.
The early meiotic events in spermatogenesis involve a number of epigenetic changes and differential incorporation of histone variants into paternal chromatin (Banaszynski et al., 2010).

B. Post-translational histone modifications
In addition to the diversity in nucleosome structure that comes from the usage of histone variants which have specialized roles in chromosome biology, chemical modifications of histones play essential roles. The histones of the nucleosomal core share structural histone fold domains but have unstructured N-terminal tails. Amino acid residues on these tails serve as substrates for the chemical modifications such as acetylation, methylation, ubiquitination, sumoylation, phosphorylation, ADP-ribosylation etc (Figure 17).

Prevalent post-translational histone modifications of the N- and C-termini of the core histones and their residue-specific epigenetic modifications include methylation (green M boxes) of lysine and arginine, acetylation (yellow A pentagon) of lysine, phosphorylation (red P sphere) of threonine or serine and ubiquitination (blue U triangle) of lysine residues (Graff and Mansuy, 2008).
These modifications are believed to act combinatorially as a marking system termed 'the histone code' (Strahl and Allis, 2000). They act through the recruitment of a class of factors known as 'chromatin readers' that alter chromatin in various ways. They occur mainly at the N-terminal tails. These short tails (less than 40 amino acids long) that emerge from the nucleosome are subject to multiple posttranslational modifications (PTMs) (Campos and Reinberg, 2009). PTMs can also be localized to the globular domain or the C-terminal (Figure 17). These changes are put in place and removed by many specific enzymes (Allis et al., 2007a). An overview of the most classical modifications is given here.

1. Histone acetylation

Histone acetylation was first discovered in the 1960’s (Allfrey et al., 1964). This led to the discovery of the link between the acetylation state of the histone and transcriptional activation. Histone acetylation within the histone tail neutralizes the tail’s positive charge, allowing the chromatin to relax which provides space for the transcriptional machinery to access the DNA. The acetylation reaction includes the transfer of an acetyl group from acetyl coenzyme A to amino group of a lysine residue on the histone (Roth et al., 2001). This reaction is catalyzed by a class of acetyltransferases; the first acetyltransferase to be discovered was HAT1 (Kleff et al., 1995). Although this enzyme was first found to localize to the cytoplasm, some studies confirmed its presence in the nucleus (Kelly et al., 2000). Two classes of enzymes reversibly regulate the acetylation state of the histone tail: histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Archer and Hodin, 1999). Histone deacetylases catalyze the removal of acetyl groups from the lysine on the tails of histones, which leads to chromatin condensation and transcriptional repression (Taunton et al., 1996).

2. Histone methylation

i. Overview

Methylation of histones can occur on lysine (K) or arginine (R) residues. The lysines can be modified by the addition of 1, 2 or 3 methyl group(s). This modification is implemented through the activity of histone lysine methyltransferases (KMTs) and removed by histone lysine demethylases (KDMs). The methylation does not affect the charge of histones and therefore does not have a direct effect on the structure of the nucleosome. The relationship between histone methylation and transcriptional state is complex and depends on the modified residue (Bannister and Kouzarides, 2011). Methyltransferases are grouped in three distinct
protein families: the PRMT1, protein arginine methyltransferase 1 family (catalyzing arginine methylation), the SET-domain family and the non-SET domain proteins DOT1/DOT1L (Ng et al., 2002). The SET domain was first identified in three Drosophila proteins: Suppressor of position effect variegation 3-9 (SU(VAR)3-9), Enhancer of zeste (E(Z) and Trithorax (Trx) (Jones and Gelbart, 1993; Stassen et al., 1995; Tschiersch et al., 1994). SUV39H lysine methyltransferase that specifically methylates lysine 9 on the N-terminal tail of H3 was the first to be discovered (Rea et al., 2000). By now several methyltransferases and their sites of modification have been identified (Kouzarides, 2007). In all cases the methyl donor is S-Adenosyl methionine (SAM) that is converted during the methylation reaction to S-Adenosyl homocysteine (SAH). There are six well characterized methylation sites on histones: H3 (K4, K9, K27, K36, K79) and H4K20. Methylation at H3K4, K36 and K79 is mostly coupled with active transcription (Lee and Shilatifard, 2007; Ruthenburg et al., 2007; Steger et al., 2008) whereas the H3K9, K27 and H4K20 trimethylation is linked to transcriptional repression (Ebert et al., 2006). Among these modifications H3K9 methylation is probably one of the best documented.

ii. H3K9 methylation and HP1 recruitment

Methylation of H3K9 is mostly associated with transcriptionally inactive chromatin (Peters et al., 2003; Schotta et al., 2002) but it is also found in transcribed regions of some active genes (Vakoc et al., 2005). Different methylation states (me1, me2 or me3) are found within different regions of the genome suggesting various functions of H3K9 methylation (Ebert et al., 2004). H3K9 di and trimethylation is involved in pericentromeric heterochromatin formation (Ebert et al., 2006). For this function, additional proteins are needed, first SUV39H methylating histone H3 at K9 creating a binding site for HP1 (heterochromatin protein 1) (Figure 18). HP1 binds via its chromodomain to di- and trimethylated H3K9 (Bannister et al., 2001; Lachner et al., 2001). After the initiation site has been introduced, heterochromatin can spread by the binding of SUV39H to HP1 that in return leads to more methylation of H3K9 (Maison and Almouzni, 2004). By H3K9 methylation tethering to HP1 (Figure 18), various effector proteins are recruited, which in turn regulate various chromosomal processes. Through the Swi6/HP1 pathway it is possible to carry out a dual function mediating both transcriptional silencing and activation of target loci (Cryderman et al., 2005; Huisinga et al., 2006; Lu et al., 2000; Vakoc et al., 2005; Yasuhara and Wakimoto, 2006). For example, HP1 recruitment by H3K9 methylation allows the RNAi machinery to spread and operate across large chromosomal domains. Once in place, it provides a mechanism to monitor, detect and
remove inappropriate, repeat-derived transcripts (Cam et al., 2005; Noma et al., 2004; Sugiyama et al., 2005). The defects in HP1 recruitment, especially those affecting the pericentromeric regions, result in mis-segregation of chromosomes (Allshire et al., 1995; Grewal et al., 1998; Kellum and Alberts, 1995; Peters et al., 2001). In *D. melanogaster*, centromeric heterochromatin can facilitate the achiasmate (non-exchange) segregation of chromosomes during meiosis (Karpen et al., 1996). Through Swi6, heterochromatin can directly recruit factors such as cohesin complex, which is essential for sister-chromatid cohesion (Bernard et al., 2001; Nonaka et al., 2002).

3. Histone ubiquitination and sumoylation

Protein ubiquitination is a key mechanism for the regulation of various cellular processes. Ubiquitin group is covalently attached to lysine residues. Ubiquitination involves three successive major classes of enzymes called E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase enzyme). The E1 enzyme mainly activates ubiquitin in a process dependent on ATP. The activated ubiquitin is then transferred to E2 enzyme via a thioester bridge. The E3 ubiquitin ligase complex then interacts with the E2 enzyme via the thioester bridge and transfers ubiquitin to the lysines of the protein (Deshaies and Joazeiro, 2009). Histones H2A and H2B are subjects to mono-ubiquitination mainly on the lysines 119 and 120 in mammals. The relationship between ubiquitination of histones and transcriptional state depends on the modified residue. In fact ubiquitination of H2A (H2AK119ub) on the body of genes is correlated with repression of transcription, whereas

![Figure 18: H3K9 methylation is essential for various biological processes and mediated by HP1 recruitment](image)

The Heterochromatin HP1 tethering by H3K9 methylation represents a platform for the recruitment of effectors across various extended domains which in turn regulate various chromosomal processes (Grewal and Jia, 2007).
ubiquitination of H2B (H2BK120ub) at promoters and coding regions is associated with transcriptionally active chromatin (Weake and Workman, 2008). Furthermore, ubiquitination of histones H2A and H2AX on lysine 63 is a key response to DNA damage (Srivastava et al., 2009). Sumoylation is a post-translational modification leading to a covalent bond of a small ubiquitin-related modifier (SUMO) on a target protein. The process of sumoylation is biochemically very similar to that of ubiquitination. Like ubiquitination, sumoylation is a reversible and dynamic process (Sarge and Park-Sarge, 2011).

4. Histone Phosphorylation

Histones can be phosphorylated on serine (S), threonine (T) and tyrosine (Y). The level of phosphorylation of histones is controlled by the activity of kinases and phosphatases enzymes. Phosphorylation of serine 10 of histone H3 (H3S10) is extensively studied and is found at high levels in active genes. Mitotic chromosomes, which are very condensed and transcriptionally inactive structures, are also rich in H3S10P (Johansen and Johansen, 2006). Phosphorylation of histone variant H2AX at serine 139, named γH2AX, is implemented very quickly after inducing double strand breaks in DNA (DSBs). It is involved in signaling the damage and is commonly used as a marker of DSB. Moreover, γH2AX was found to be required also for the accumulation of many subsequent DNA damage response (DDR) proteins at DSBs sites (Bonner et al., 2008). Thus γH2AX is believed to be the principal signaling protein involved in DDR and may play an important role in DNA repair (Yuan et al., 2010).

C. ATP-dependent Chromatin Remodelers

One question arising is how cells manage different chemical modifications and incorporate histone variants into the dense structure of chromatin. Furthermore, processes like transcription require access to DNA. By default, the access to DNA is virtually blocked by nucleosomes. In order to render DNA accessible, multiprotein machineries exist that utilize ATP hydrolysis to mobilize nucleosomes (Becker and Horz, 2002). Histones may then be moved to a new location or replaced by histone variants (Langst and Becker, 2004; Tomar et al., 2009; Workman, 2006).

ATP-dependent chromatin remodeling enzymes are large (>1 MDa) multi-component complexes (consisting of 4-17 subunits) that are highly conserved within eukaryotes (Tang et al., 2010). They are characterized by the presence of an ATPase subunit belonging to the superfamily II helicase-related proteins (Figure 19) (Singleton and Wigley, 2002). The first
complex to be described was the yeast SWI/SNF which was originally identified in two independent screens for mutants affecting either mating type switching or growth on sucrose (Sudarsanam and Winston, 2000; Workman and Kingston, 1998). Hence, the names Switching defective (SWI) and Sucrose non-fermenting (Snf) (Mohrmann and Verrijzer, 2005). The Snf2 helicase domain is conserved in these complexes and is capable of binding and hydrolyzing ATP (Eisen et al., 1995). Four main families have been described: Swi/Snf, Iswi, Ino80/SWR1, CHD (Hargreaves and Crabtree, 2011).

1. SWI/SNF family

Drosophila brahma (BRM), mammalian Brahma related gene 1 (BRG1), and yeast SNF2 are examples of proteins, which are categorized into the Swi/Snf family of proteins. In addition to the Snf2 helicase domain, all of these proteins possess a bromodomain, which has been reported to bind acetylated histone tails (Marfella and Imbalzano, 2007; Wang et al., 2007). Swi/Snf complexes are involved in various cellular processes such as DNA replication, repair and transcription (Wang et al., 2007).

2. ISWI family

Mammalian SNF2H and yeast Isw1 are examples of enzymes, which are categorized as Iswi (imitation switch) remodeling enzymes. In addition to the Snf2 helicase domain, proteins within this family possess a SANT (SWI3, ADA2, NCOR, TFIIIB) domain. This domain is reported to have the ability to bind histone tails (Marfella and Imbalzano, 2007; Wang et al., 2007). The chromatin accessibility complex (CHRAC) (Varga-Weisz et al., 1997), nucleosome remodeling factor (NURF) complex (Tsukiyama et al., 1995; Tsukiyama and Wu, 1995), and ATP-utilizing chromatin assembly and remodeling factor complex (ACF) (Ito et al., 1997) are examples of complexes which are classified as ISWI complexes. The Iswi protein within each complex acts as the ATPase subunit of the given complex (Elfring et al., 1994). While the Iswi family was initially identified in Drosophila, paralogues of the Drosophila Iswi proteins have been found in complexes in Yeast (McConnell et al., 2004; Tsukiyama et al., 1999; Vary et al., 2003), Xenopus (Guschin et al., 2000; MacCallum et al., 2002), and humans (Barak et al., 2003; Bochar et al., 2000; Hakimi et al., 2002; Langst and Becker, 2004; LeRoy et al., 1998; Poot et al., 2000; Strohner et al., 2001; Yasui et al., 2002).
Figure 19: ATP-dependent chromatin remodeling enzymes
A) Showing the domain structure of the four major classes of ATP-dependent chromatin remodeling complexes. Members of ATP-dependent remodeling enzyme share a conserved Snf2 helicase domain capable of binding and hydrolyzing ATP, image taken from (Jerzmanowski, 2007).

B) Schematic representation of possible action mechanisms of ATP-dependent chromatin remodeling enzymes that alter chromatin structure by utilizing the energy of ATP hydrolysis. These enzymes can alter chromatin structure by moving histones to a new location on the same piece of DNA (1), disrupting DNA histone contacts (2), moving histones to new DNA (3), or replacing histones with histone variants (4) (Mohrmann and Verrijzer, 2005).
3. CHD family

The CHD (Chromodomain helicase DNA binding) proteins are another example of ATP-dependent chromatin remodeling family. Like all ATP-dependent remodeling enzymes, members of this family possess a conserved Snf2 helicase domain. In the CHDs, this domain is C-terminal to tandem chromodomains believed to function in histone binding. The CHD family of proteins can be further divided into subfamilies based on the presence of additional domains. In addition to the double chromodomains and Snf2 helicase domain, CHD1 has a DNA-binding domain near its C-terminus (Marfella and Imbalzano, 2007). CHD1 has been reported to be required for H3.3 deposition on the paternal pronucleus (Konev et al., 2007). However these data are questioned by our recently published work. We believe that it is required for nucleosome organization on the newly assembled chromatin (this manuscript). CHD1 has been shown to play essential roles in transcription not only in yeast (Alen et al., 2002; Krogan et al., 2002; Simic et al., 2003) but also in Drosophila (McDaniel et al., 2008). Both human and yeast CHD1 are reported to bind histones methylated on H3 lysine 4, a hallmark of active transcription (Marfella and Imbalzano, 2007). CHD1 is reported to exhibit ATPase activity in yeast and Drosophila but Mouse CHD1 may also bear histone deacetylase activity (Marfella and Imbalzano, 2007).

4. INO80/SWR1 family

SRCAP (SNF2-related CREB-activator protein) and p400 are examples of proteins of this family categorized as Ino80 (inositol requiring 80) remodeling proteins that have a split ATPase domain (Bao and Shen, 2007; Wang et al., 2007). The Ino80 complex was first identified in yeast. This complex is reported to remodel chromatin, facilitate \textit{in vitro} transcription, and exhibit DNA helicase activity (Grewal and Jia, 2007). The DNA helicase activity of the complex has been attributed to the presence of RuvB proteins. The Ino80 complex is thought to be involved in both transcriptional regulation and DNA repair (Bao and Shen, 2007; Marfella and Imbalzano, 2007; Shen et al., 2000). Some recent studies revealed that INO80 complex is closely related to another ATP-dependent SWR1 complex which was the first identified H2A.Z chaperone (Kobor et al., 2004; Luk et al., 2010; Mizuguchi et al., 2004; Papamichos-Chronakis et al., 2011).
D. Histone chaperones

Histones deposition in chromatin is achieved by histone chaperones; the term “molecular chaperone” was first used by Ron Laskey to describe nuclear proteins in the extracts of frog oocytes that prevent incorrect interactions between histones and DNA (Laskey et al., 1978). Histone chaperones shield nonspecific interactions between the negatively charged DNA and the positively charged histones and promote specific interactions that lead to nucleosome assembly by forming an insoluble precipitate (Laskey and Earnshaw, 1980; Laskey et al., 1978). The function of histone chaperones is intimately coupled to the action of ATP-dependent chromatin remodeling machines to allow the ordered formation of the nucleosome structure in the chromatin assembly process (Eitoku et al., 2008; Haushalter and Kadonaga, 2003; Liu and Churchill, 2012; Park and Luger, 2008). Most histone chaperones are conserved in yeast, plants and animals (Hamiche and Shuaib, 2012; Polo and Almouzni, 2006; Zhu et al., 2012). Assembly of nucleosomes being a stepwise process that starts with H3-H4 deposition first onto DNA, followed by incorporation of two H2A-H2B dimers. Chaperones of the canonical histones are classified in two groups according to their affinity either toward H2A-H2B or H3-H4 (Gruss et al., 1993; Worcel et al., 1978).

An overview is given here for the well characterized chaperones of canonical histones. CENP-A chaperones are not considered here as they are highly specific for the centromeric region of the chromosome (Hamiche and Shuaib, 2012). A specific chapter is dedicated to H3.3 chaperones and the H3.3 variant as they have been part of my research work.

1. H2A-H2B histone chaperones

The main histone chaperones of H2A-H2B are nucleoplasmin and NAP1 (Nucleosome Assembly Protein 1). Nucleoplasmin is the typical Xenopus chaperone involved in histone storage. Drosophila NAP1 protein was found associated with histone H2A and H2B in a crude whole-embryo extract by co-immunoprecipitation assay (Ito et al., 1996). Only one NAP1 gene is present in yeast, however, higher eukaryotes, from Drosophila to human, have evolved NAP1 families comprising multiple members (Park and Luger, 2006). Other than binding with histones, NAP1 family proteins bind with other basic proteins. During spermatogenesis in Xenopus, histones, especially H2A-H2B, are largely replaced with various SP (Sperm-specific basic Protein). Incubation of NAP1 family members results in its interaction with different SP proteins and their release, leading to the final decondensation of sperm chromatin (Matsumoto et al., 1999).
2. H3-H4 histone chaperones

The analysis of the stepwise processes of nucleosome assembly and disassembly highlights the importance of the H3/H4 chaperones (Liu and Churchill, 2012). The chaperone Asf1 is the first chaperone identified to play a key role in supplying histones H3–H4 to the downstream chaperones, such as CAF-1 (Chromatin Assembly Factor 1) for nucleosome assembly (Figure 20) (Green et al., 2005; Hamiche and Shuaib, 2012; Tyler et al., 1999). The assembly of (H3–H4)_2 tetramers into nucleosomes is believed to be the rate limiting step of nucleosome assembly and is the key step in the inheritance of epigenetic information and maintenance of genome integrity (Li et al., 2012).

i. CAF-1 (Chromatin Assembly Factor-1)

CAF-1 was initially identified by biochemical fractionation of extracts derived from human HeLa cells (Smith and Stillman, 1989; Verreault et al., 1996). CAF-1 is the best-documented well-studied chaperone in divergent species, and is considered to be involved in the RC (Replication Coupled) chromatin assembly, DNA repair and heterochromatin silencing. It is an evolutionarily conserved factor, and in most species, it consists of three components: the large, mid and small subunits. In humans, the subunits correspond to p150, p60 and p48, in budding yeast to Cac1, Cac2 and Cac3, and in Arabidopsis to FAS1, FAS2 and MSI1 (Ridgway and Almouzni, 2000). The largest subunit of CAF-1, p150, interacts directly with the accessory factor in DNA polymerase complex, PCNA (Proliferating Cell Nuclear Antigen) (Figure 20). This provides insight into the molecular mechanism whereby CAF-1 is coupled to replication fork, and provides the first molecular link between nucleosome assembly and DNA replication (Shibahara and Stillman, 1999).

ii. ASF-1 (Anti-Silencing Factor 1)

The analysis of crude Drosophila embryo extract revealed that DNA replication-coupled chromatin assembly by CAF-1 requires a complex that comprises histones H3 and H4 and a factor homologous to yeast ASF1 (Tyler et al., 1999). Asf1 is the central H3/H4 chaperone that accompanies H3/H4 to the nucleus (Campos et al., 2010). It also acts as a histone ‘sink’ by buffering the majority of non-nucleosomal H3/H4 (Groth et al., 2005; Tagami et al., 2004). The eukaryotic ASF1 proteins are highly conserved through evolution in structure and function. Yeast and Drosophila encode one ASF1 protein, while human genome contains two Asf1 paralogues, Asf1a and Asf1b (Tamburini et al., 2005). In contrast to CAF-1 complex, ASF1 is unable on its own to promote RC nucleosome deposition (Loyola and Almouzni,
ASF1 is proposed to transfer nascent histones to CAF-1, the specialized histone deposition factor complex (Figure 20). Thus it represents the prototype of a histone donor chaperone that could effectively ensure a constant supply of histones at sites of nucleosome assembly (Loyola and Almouzni, 2004).

Figure 20: The de novo histones H3-H4 assembly through the Asf1-CAF-1 pathway (Jasencakova and Groth, 2011). See text for further details.
III. Histone variant H3.3, its chaperones and paternal chromatin assembly

A. Histone variant H3.3

Histone variant H3.3 differs from the canonical H3 by only four amino acid residues (Figure 21); three of these residues are clustered in the α2 helix of the histone fold domain and the other residue is in the N-terminal tail (Hake and Allis, 2006; Yuan and Zhu, 2012). In spite of the high sequence similarity between H3.3 and H3, these specific residues have been proposed to account for particular properties of histone H3.3 (Szenker et al., 2011). In the vertebrates and Drosophila, residues 87, 89 and 90 are S, V and M in H3, and A, I and G in H3.3 (Figure 21). The amino acid residues found at these positions in H3 and H3.3 vary between species but always distinguish H3 from H3.3. It is noteworthy that H3.3 histone variant is encoded by two intron-containing genes, h3.3A and h3.3B, the transcripts of which are regulated through polyadenylation and whose translation results in identical protein products (Wellman et al., 1987). H3.3 histones are enriched at transcriptionally active regions (Ahmad and Henikoff, 2002; Henikoff et al., 2009; Mito et al., 2005) as well as telomeres and pericentromeric regions (Drane et al., 2010; Goldberg et al., 2010; Wong et al., 2010; Wong et al., 2009). These studies indicate that histone variant H3.3 is incorporated in both transcriptionally active and inactive chromatin sites but it is still not known how these sites are functionally linked by its deposition (Szenker et al., 2011).

The dual nature of histone variant H3.3 makes it necessary for a variety of nuclear processes that specifically occur in germ cells and in early embryos development of metazoans (Banaszynski et al., 2010; Ooi and Henikoff, 2007; Orsi et al., 2009; Szenker et al., 2011). In Xenopus laevis embryos gastrulation depends on the critical involvement of H3.3 (Szenker et al., 2012). During mouse spermatogenesis, the majority of histone H3 is replaced by the variant H3.3 by the first meiotic prophase, where most of the mRNA required for the development of sperm is synthesized. It disappears just before complete inactivation of the genome, when the transition proteins and protamines replace almost all of histones (Akhmanova et al., 1995). Thus, the replacement of histone H3 by histone H3.3 could be important for the initiation and maintenance of an open chromatin during meiotic phase. Many studies revealed that H3.3 is notably required for the proper segregation of meiotic chromosomes in spermatocytes and for the global organization of early spermatid chromatin (Akhmanova et al., 1997; Sakai et al., 2009). The critical requirement of H3.3 after fertilization has also been extensively studied.
Figure 21: Schematic representation of histone H3 variants family
A) Showing the evolutionary conservation of histone variant H3.3 which is suggested to be the ancestor of all other H3 variants.
B) The alignment of amino acid sequences corresponding to human H3 variants; sequences are compared with the “ancestral” variant H3.3 (amino acid differences are highlighted) and the position numbers of amino acids that are different between H3.3 and H3.1/2 are indicated (Szenker et al., 2011).
In *Drosophila*, H3.3 deficient flies are viable but both male and female are sterile (Hodl and Basler, 2009; Sakai et al., 2009).

Interestingly the substitution of the four amino acids which distinguish *Drosophila* H3.3 from its canonical one (S31A, A87S, I89V and G90M), is not sufficient to rescue the fertility of H3.3 homozygous mutation; the fertility is rescued only after providing K4-methylated versions of H3.3 (Hodl and Basler, 2009). This suggests that not only the specific mere requirement of variant histone H3.3 but also K4 methylation of its residue are critical. In a more recent study, the same authors found that the null mutant flies for both H3.3 genes (His3.3A and His3.3B) rescued their fertility when they were provided with H3.2 expressed under the H3.3B promoter which suggests that the major difference between canonical and variant H3 histones resides in their mode of transcriptional regulation (Hodl and Basler, 2012). These results illustrate the complexity of the picture and the necessity of further investigations to fully understand the role of H3.3 and its epigenetic modifications. H3.3 variant deposition is mediated by a distinct group of histone chaperone complexes.

**B. Histone variant H3.3 chaperones**

The double incorporation of H3.3 in both transcriptionally silent and active chromatin is supported by the presence of two main histone chaperone complexes that mediate the H3.3 deposition. These two complexes are DAXX-ATRX and HIRA complexes.

1. **DAXX-ATRX complex**

The death domain-associated protein (DAXX) was shown to achieve H3.3 enrichment in heterochromatin (Wong et al., 2010). DAXX was first shown by Yang in 1997 to induce cell apoptosis through the cJun N-terminal kinase (JNK) pathway activation (Yang et al., 1997). It was initially identified as a cytoplasmic protein that interacts with FAS (fibroblast-associated surface antigen) (Salomoni and Khelifi, 2006), while some studies reported that DAXX resides primarily in the nucleus and participates in transcriptional regulation (Lanotte et al., 1991). In fact DAXX subcellular localization is submitted to regulation (Chen et al., 2009; Yeung et al., 2008).

DAXX protein cooperates with the SWI/SNF like ATP-dependent chromatin remodeling factor, α-thalassemia/mental retardation X-linked syndrome protein (ATRX) (Gibbons et al., 1995). ATRX protein contains an N-terminal ADD (ATRX-DNMT3-DNMT3L domain (Argentaro et al., 2007) and a C-terminal ATPase domain, which function in the regulation of its binding and enzymatic activities, respectively (Dhayalan et al., 2011). The ATRX ADD
recognizes histone-modification such as H3K9me3 (Figure 22) (Maze et al., 2013). ATRX chromatin remodeler with the H3.3 specific DAXX chaperone forms the DAXX-ATRX complex (Drane et al., 2010). It was found to regulate replication-independent histone H3.3 chromatin assembly at telomeres and pericentric heterochromatin (Figure 22) (Drane et al., 2010; Goldberg et al., 2010; Lewis et al., 2010; Newhart et al., 2012). In Drosophila, homologues have been identified for DAXX, DAXX-like protein (DLP) and ATRX, X-linked nuclear protein (XNP or dATRX). Unlike the mammalian ATRX, Drosophila XNP is found to mark the active transcribed genes with a major focus near heterochromatin of the X chromosome and interestingly colocalizes with H3.3 throughout the chromatin of somatic cells (Schneiderman et al., 2009).

![Figure 22: H3.3 deposition through DAXX-ATRX pathway](image)

**Figure 22: H3.3 deposition through DAXX-ATRX pathway**

The ATP-dependent chromatin remodeler ATRX recognizes the H3K4me0 and H3K9me3 histone marks and forms a complex with the H3.3 specific DAXX chaperone. The system deposits the replication-independent histone variant H3.3 at telomeric and pericentromeric heterochromatic regions of the genome (Maze et al., 2013).

2. HIRA

The isolation of H3.3 pre-deposition complexes identified a distinct factor, the histone regulator A, HIRA (Tagami et al., 2004). HIRA is a member of the HIR (Histone cell cycle Regulation Defective) proteins that were initially identified in yeast. In S. cerevisiae, the genes encoding Hir1p, Hir2p, Hir3p and Hpc2p were first identified as repressors of histone gene expression that are recruited to histone gene regulatory regions (Fillingham et al., 2009; Sherwood and Osley, 1991; Sherwood et al., 1993; Xu et al., 1992). In Schizosaccharomyces pombe, the HIRA orthologs Hip1 and Slm9 are involved in silencing some genes, long terminal repeat (LTR) retrotransposons, and transcription from cryptic promoters (Anderson...
et al., 2010; Anderson et al., 2009; Yamane et al., 2011). Indeed, Hip1/Asf1 contributes to heterochromatinization by promoting histone deacetylation and promotion of HP1 binding (Yamane et al., 2011). The HIRA complex is conserved through evolution both at the structural and functional level (Figure 23) (Amin et al., 2012).

**Figure 23: The evolutionary conservation of the HIR/HIRA complex subunits**

Schematic representation shows the conservation of HIR complex subunits from yeasts to human. In *S. cerevisiae* HIR complex is composed of Hir1, Hir2, Hir3 and Hpc2. The human HIRA complex is composed of the proteins HIRA, Ubinuclein1 and Cabin1. In *D. melanogaster*, HIRA partners are less well characterized. One of the partners has been identified by our laboratory in the course of my thesis work (see the Results section). Drawing after (Amin et al., 2012) with modifications.

In vertebrates, HIRA deposits H3.3 in a multicomponent complex manner that contains UBN1, CABIN1, and Anti-Silencing Factor 1a (ASF1a) (Goldberg et al., 2010; Tagami et al., 2004). Moreover, despite *S. cerevisiae* has only a single H3 that resembles H3.3, its deposition also involves a multicomponent complex that comprises Hir1p, Hir2p, Hir3p, Hpc2p and Asf1p (Green et al., 2005; Prochasson et al., 2005). Depending on species, there are 4 or 5 subunits in the core complex. Hir1p and Hir2p both display sequence similarities to metazoan HIRA; in fact Hir1p and Hir2p are encoded by genes that eventually merged as a single gene in metazoans (Green et al., 2005; Lorain et al., 1996; Prochasson et al., 2005). Hpc2p is orthologous to two related proteins in mammals, UBN1 and UBN2 (Balaji et al., 2009; Banumathy et al., 2009). The first member of the HPC2/UBN was identified in *Drosophila* (Ait-Ahmed et al., 1992). A proteomic analysis identified Yemanuclein in a HIRA complex as well as Asf1 (Moshkin et al., 2009). The Yemanuclein/HIRA complex has been confirmed by our work (this manuscript). However no evidence was provided that Asf1 is present in the complex that contains Yem as the reciprocal experiment was not done in the work reported by (Moshkin et al., 2009). Asf1p also has two counterparts in mammals, ASF1a and ASF1b (Sillje and Nigg, 2001) of which only ASF1a is included in the HIRA-containing complex (Tagami et al., 2004; Zhang et al., 2005). Whether Asf1 is in the HIRA core complex is still unclear. What is clearly established is that Human HIRA core complex is composed of at least three subunits, including HIRA, Ubinuclein 1 (UBN1) and Calcineurin-
binding protein 1 (CABIN1). CABIN1 is the yeast Hir3p similar sequence which, interestingly, is not found in the *Drosophila* genome (Amin et al., 2012). How the different subunits work and which one is the genuine histone H3.3 chaperone within the complex are real issues to be addressed in the near future.

C. Chromatin Remodeling of the Paternal Genome

At fertilization, sperm entry into the oocyte triggers the cell cycle of the dormant mature oocyte arrested at metaphase II to complete its second meiotic division. In *Drosophila*, ovulation provides the triggering signal for meiosis resumption of the metaphase I arrested oocyte. Once activated, the egg initiates crucial chromatin reprogramming events through a multi-step remodeling of the compacted and transcriptionally inactive sperm chromatin to form the male pronucleus. As soon as maternal and paternal haploid pronuclei are formed they fuse to yield the diploid zygote containing one functional copy of each parent genome opening the early window of embryonic development.

1. Compaction of paternal genome during spermatogenesis

The chromatin of mature mammalian spermatozoa differs markedly in composition and structure from somatic chromatin. It has an extremely specialized architecture; its genome is packaged into a volume that is typically 10% or less that of a somatic cell nucleus. To achieve this remarkable level of compaction, dramatic chromatin structural rearrangements occur during the late stages of spermatogenesis. As a result the genome is complexed with sperm-specific highly basic proteins called protamines (Braun, 2001). Protamines are the major sperm nuclear basic proteins (SNBPs) (Ausio, 1999; Balhorn, 2007). Protamines can be defined as relatively small proteins of up to 100 amino acids with a highly basic amino acid composition consisting predominantly of arginine residues (Ausio, 1999; Eirin-Lopez and Ausio, 2009). 90%–95% of sperm chromatin histones are replaced by protamines which not only facilitate the nuclear compaction necessary for motility but also protect the genome. Moreover it precludes the transcriptional activity (Carrell, 2012; Oliva, 2006). The structural transition from a highly dynamic canonical nucleohistone to a highly stable and compact nucleoprotamine is still poorly understood, but recent studies revealed that it is a multistep process assisted by chromatin remodeling complexes, highly germ line specific histone variants and post-translational modifications (PTMs) of the chromosomal proteins involved (Figure 24) (Churikov et al., 2004; Gaucher et al., 2010; Ishibashi et al., 2010).
Global histone hyperacetylation is essential for the structural transition from a highly dynamic canonical nucleohistone to a highly stable and compact nucleoprotamine (Gaucher et al., 2010). In both vertebrate and invertebrate organisms the process starts with an increase in the acetylation of sperm histones which relaxes chromatin structure. As a result histones replacement is enhanced with moderately basic transition proteins 1 and 2 (TP1 and TP2) and therefore, triggers the subsequent cascade of events which ends by protamine replacement (Rousseaux et al., 2011; Song et al., 2011). In addition to histone hyperacetylation, the histones of vertebrates were also found to be ubiquitinated prior to the protamine displacement. Histone ubiquitination has been recently shown to regulate nucleosome removal (Baarends et al., 1999; Lu et al., 2010). In addition proper deposition of protamines onto the DNA template is mediated by its phosphorylation (Lewis et al., 2003).

2. Reprogramming of paternal chromatin at fertilization

Before fertilization, the functional state of either the oocyte or the sperm genome is not yet realized due to two main raisons; first, as mentioned, the sperm genome is tightly condensed and is inaccessible for the different cellular processes. On the other hand the oocyte itself not completed its meiotic cycle. For these two raisons both genomes undergo a series of reprogramming events. At fertilization, the entry of the sperm into the oocyte cytoplasm leads to completion of maternal meiosis, resulting in formation of the polar body and the haploid maternal pronucleus. The situation is slightly different in Drosophila where female meiosis is arrested at metaphase I, its resumption depending on ovulation and not fertilization. At the same time as the maternal pronucleus forms, the paternal chromatin undergoes some drastic
reorganization. Notably, the major modification lies in the decondensation of the highly compacted protamine-containing sperm chromatin to produce an accessible haploid genome. The protamine/histone exchange must take place for DNA replication to take place (Piatti et al., 2011; Wu and Chu, 2008). This implies the stripping of SNBPs followed by de novo assembly of paternal nucleosomes by the deposition of maternally provided histones. This process is called sperm chromatin remodeling (SCR); it involves different asymmetric epigenetic changes, including differential DNA methylation, posttranslational modification of histone proteins and incorporation of histone variants (Banaszynski et al., 2010; Surani et al., 2007). One of the first steps achieved is the reduction of the protamine disulphide bonds to allow protamine removal and subsequent organization of the DNA in a nucleosomal structure (Kempisty et al., 2006; Oliva, 2006).

Once protamines are removed, a global reassembly of nucleosomes must occur in order to repackage the genome with maternally stored histones. Many recent studies in worms, flies and mice reported the replication-independent global histone incorporation of the histone H3.3 variant into paternal DNA during decondensation prior to the first replication cycle (Loppin et al., 2005; Ooi et al., 2006; Piatti et al., 2011; van der Heijden et al., 2005). Extensive studies in Drosophila showed that the de novo incorporation of histone H3.3 into paternal DNA during decondensation was mediated by HIRA (Loppin et al., 2005).

Figure 25: Biological events in the Drosophila fertilized egg
Schematic diagram of a Drosophila fertilized egg showing the events that take place from fertilization until the first mitotic division of the zygote. The oocyte that is arrested at metaphase I at the end of oogenesis resumes meiosis upon ovulation. Whether or not it is fertilized it completes its meiotic divisions, which yields to the formation of the female pronucleus. When the egg is fertilized, simultaneously to the completion of female meiosis, the paternal genome undergoes crucial chromatin reprogramming. HIRA complex mediates de novo assembly of nucleosomes with maternally provided histone H3.3 variant. This occurs after the protamines were removed by an unknown mechanism.
The point mutation changing the lysine residue at position 225 to an arginine residue in HIRA (named ‘sesame’ or ‘ssm’) leads to sterility in females as a result of defects of HIRA in the assembly of paternal chromatin at fertilization (Bonnefoy et al., 2007; Loppin et al., 2005). The role of HIRA is probably conserved in mammals, as mouse HIRA associates exclusively with the male pronucleus prior to pronuclear fusion (van der Heijden et al., 2005). In human cells, HIRA deposits H3.3 in a multicomponent complex manner that contains UBN1, CABIN1, and Anti-Silencing Factor 1a (ASF1a) (Goldberg et al., 2010; Tagami et al., 2004). Therefore other factors are expected to play a role in the deposition of H3.3 on paternal chromatin. As described above the Drosophila homologue for UBN1 is Yemanuclein (also called Yemanuclein-alpha or Yem-alpha). Yemanuclein was reported to be a DNA binding protein required for both oocyte meiosis and in the zygote (Ait-Ahmed et al., 1992; Meyer et al., 2010).

The second part of my thesis work is dedicated to the characterization of Yemanuclein as a HIRA partner in the assembly of paternal chromatin in the Drosophila zygote. We show in the present work that Yemanuclein is indeed a HIRA partner in paternal chromatin reprogramming (this manuscript). Surprisingly, ASF1 was reported not to be required in Drosophila, for the deposition of H3.3 during male pronucleus formation (Bonnefoy et al., 2007). This conclusion requires to be verified. Interestingly a physical interaction with the chromatin remodeling factor CHD1 has been implicated in aiding Drosophila HIRA in the deposition of this histone variant (Konev et al., 2007). Whereas CHD1 results in a phenotype similar to mutations in HIRA, in our experiments CHD1 is not found in the HIRA deposition complex (this manuscript).
Results and Discussion
Article 1:

Oocyte specific Yemanuclein is a new cohesin and synaptonemal complex associated protein
Oocyte specific Yemanuclein is a new cohesin and synaptonemal complex associated protein

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Running title: Yemanuclein in oocyte meiotic prophase

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cohesin; recombination; crossover interference
Summary

Meiosis is characterized by two chromosome segregation rounds (Meiosis I and II), which follow a single round of DNA replication, resulting in chromosome reduction. Chromosome number reduction occurs at meiosis I and relies on key structures, among which chiasmata that result from repair of double strand breaks (DSBs) between homologous chromatids. These events require synapsis between homologous chromosome axes which is achieved by the synaptonemal complex (SC), a key structure in recombination proficient meiosis. In Drosophila, recombination does not occur in male meiosis; accordingly SC specifically forms in the oocyte. In our earlier work we showed that Yemanuclein (Yem) is a maternally expressed protein; using a V478E mutation (yem\textsuperscript{1}) we were also able to unveil its critical role in sexual reproduction. In the present work we show that Yemanuclein is required at early meiosis I for the meiotic recombination process. Yem was found associated to the SC and the cohesin complex. Using the yem\textsuperscript{1} mutant allele we were able to show that different aspects of the crossover pathway were affected such as crossover distribution and interference. The strong genetic interaction of yem with the Spo11 homologue, mei-w68 and its impact on DSBs frequency and timing point to yem as a key player in meiotic recombination in Drosophila and given its conservation, presumably in other phyla as well.
Introduction

Meiosis is the specialized chromosome segregation process that drives germ cells to form haploid gametes. To achieve this result two successive segregation rounds that eventually halve the chromosome number follow a single round of DNA replication. Meiosis I is the key step in the chromosome segregation process that leads to haploidy; it relies on specific structures and molecules. The side-by-side organization of the sister kinetochores held by meiosis I specific cohesion in conjunction with a physical link between the homologous chromatids ensure the accurate segregation of the homologous centromeres at anaphase I of recombination proficient meiosis. Importantly the sister centromeres are held together until anaphase II due to meiosis specific cohesion ((Petronczki et al., 2003; Watanabe, 2005) and references therein). In most species the physical links that hold the homologous chromatids may be observed as cytological marks called chiasmata which result from the use of the homologous chromatid as template for double strand breaks (DSBs) repair. These meiotic events may be genetically measured as crossing-overs (CO), reviewed in (Phadnis et al., 2011). Resolution of recombination intermediates as COs relies on specific protein complexes. The first complex whose structure and function in meiotic recombination was recognized is the Synaptonemal Complex (SC), a zipper-like proteinaceous structure (Carpenter, 1975; Moses, 1969, 2006). The other complex is the cohesin complex that is important for many aspects of chromosome structure and function (Hirano, 2000; Wood et al., 2010). Importantly cohesin complex is essential in the organization of meiotic chromosomes, in particular it is intimately linked to the SC, the structure of which it contributes to form (Revenkova and Jessberger, 2006; Stack and Anderson, 2001). SC allows synopsis of the homologues and holds them together during the recombination process; therefore its integrity is required whenever the chromosome segregation process relies on
homologous recombination (Page and Hawley, 2004). In Drosophila, only oocyte chromosomes undergo recombination, accordingly SC is specific for female meiosis, males using a different strategy for chromosome segregation of its meiotic chromosomes (Thomas et al., 2005).

SC structure is highly conserved from yeast S. Cerevisiae to mammals. It is a tripartite structure composed of two lateral elements (LEs), a central element (CE) and transverse filaments (TFs) that cross the CE (Lake and Hawley, 2012; Page and Hawley, 2004; Zickler and Kleckner, 1999). Lateral elements originate from the axial elements (AEs), structures to which cohesins localize and organize the sister chromatids. Axial elements are considered as a basis for SC assembly (Lake and Hawley, 2012; Page and Hawley, 2004; Zickler and Kleckner, 1999). Interestingly in spite of the high conservation of the structure, the components are not necessarily conserved especially between Drosophila and other species. In Drosophila, the first components whose requirement for SC structure and crossover formation was reported were C(3)G (TF) and C(2)M (LE) (Manheim and McKim, 2003; Page and Hawley, 2001; Smith and King, 1968). Later, corona (cona), another component described as a CE has been reported, (Page et al., 2008). None of these proteins are conserved at the sequence level. Although C(2)M was shown to be distantly related to kleisins, the non SMC cohesin subunits, it does not share conspicuous sequence similarities with them (Heidmann et al., 2004). ORD is another SC component that is not related to any protein of other phyla. Interestingly in spite of its requirement for sister chromatids cohesion, gross SC morphology looks normal in ordnull germaria; however defects may be observed at an ultrastructural level. Anyhow SC disassembly occurs prematurely (by stage 1) in ordnull germaria (Webber et al., 2004). As a reference, wild type SC disassembly does not occur before stage 6 (Carpenter, 1975). However several groups have reported SC components retention far beyond this stage at specific sites and particularly at centromeric regions by
(Bisig et al., 2012; Qiao et al., 2012; Takeo and Hawley, 2012). SMC1 and SMC3, the canonical cohesins and Nipped-B, the Sec2 homologue, have all been shown to localize to the SC (Gause et al., 2008; Khetani and Bickel, 2007). How these complexes form, the possible existence of sub-complexes, how they contribute to the chromosome axes and the SC LEs is a complex issue. Moreover one has to keep in mind that new components keep being discovered, as reported in the present work.

In the present work we have investigated the role of Yemanuclein-alpha (whose name is simplified as Yemanuclein) during early meiotic prophase. We show that Yemanuclein (Yem) is an SC associated protein that interacts \textit{in vivo} with components of the cohesin complex. We also show that it is required for various aspects of meiotic recombination: crossover distribution, interference, DSBs timing and frequency. Moreover \textit{yem} and \textit{mei-w68}, \textit{spo11} homologue, display a strong genetic interaction.
Results

Yem-alpha is a new synaptonemal complex (SC) associated protein

Yem localization to the oocyte nucleus and its importance for female meiosis have already been recognized (Ait-Ahmed et al., 1992; Meyer et al., 2010). In the present work we aimed to investigate its implication in early meiotic prophase. We first addressed this question cytologically by immunostaining whole mount ovaries with antibodies against Yemanuclein. Yem detection was possible as early as region 2a of the germarium (Figures 1A). The various stages are schematically shown up to mid-prophase in the drawing of Figure 1B which is adapted from (King, 1970). Higher magnification of Yem stained egg chambers revealed ribbon-like structures typical of the synaptonemal complex (Figure 1A, d-e). Yem colocalization with well characterized SC components such as C(3)G and C(2)M was then assessed by co-staining with a monoclonal antibody against C(3)G and an antibody against a HA-tagged version of C(2)M (Manheim and McKim, 2003; Page and Hawley, 2001). As shown in Figure 1C Yemanuclein co-localized with C(3)G and C(2)M. Immunocytochemical detection of C(3)G and C(2)M was performed in the specific experimental conditions required for Yemanuclein, without fixation ((Ait-Ahmed et al., 1992; Capri et al., 1997; Meyer et al., 2010)). Therefore in an independent experiment C(2)M and C(3)G staining was performed in the classically used conditions. C(2)M was stained with a rabbit polyclonal antibody (Manheim and McKim, 2003) and the HA epitope tag antibody that recognizes the transgenic version (Figure 1D). C(3)G was revealed with the monoclonal antibody used above (Page and Hawley, 2001). As shown in Figure 1D, the typical staining of C(2)M and C(3)G was unchanged regardless of the fixation conditions. We can therefore unambiguously assign Yemanuclein localization to the SC.
Yemanuclein germarial localization depends on SC LEs and TFs

C(3)G transverse filament and C(2)M lateral element were shown to be indispensable for SC formation (Anderson et al., 2005). In their absence the threadlike structure typical of the SC is replaced by punctuate structures typical of SC components staining in mutant backgrounds (Manheim and McKim, 2003). As shown in Figure 2 (upper panel), Yemanuclein staining is strongly affected in germaria of females homozygous for c(2)MEP2115 or c(3)G68, two mutations that prevent SC formation (Manheim and McKim, 2003; Page and Hawley, 2001). A higher magnification of the c(2)MEP2115 germarium shows that the punctuate distribution of Yemanuclein is identical to that of C(3)G in the same background (Figure 2, lower panel). A similar observation was made for the localization of the Corona central element (Page et al., 2008). This result indicates that Yemanuclein localization to the chromosomes is highly dependent on SC integrity during the phase of recombination events. However after recombination is completed, Yemanuclein localization to the oocyte nucleus does not require SC any longer. Indeed from stage S3 on, Yemanuclein staining displayed no localization difference between mutant and wild type backgrounds (Figure 2). The similarity between Yemanuclein and C(3)G localization to the SC could also be established with the ord mutant background. ORD as well as C(2)M and SMC1 are considered to participate to AEs/LEs in Drosophila (Anderson et al., 2005; Khetani and Bickel, 2007; Manheim and McKim, 2003; Webber et al., 2004). In contrast to the c(2)M and c(3)G mutant backgrounds, the ord5/Df background does not totally abolish the formation of the SC thread like structures. However they are not as stable as they are in wild type backgrounds (Khetani and Bickel, 2007; Webber et al., 2004). As shown in Figure 2, in the ord5/Df background Yemanuclein staining became punctuate by stage 1 in a way similar to C(3)G. It is noteworthy that yem mutant backgrounds were with no effect on SC integrity (data not shown).
Crossover distribution and interference are defective in yem$^1$/+ oocytes

Yemanuclein DNA binding properties and its specific localization to the oocyte nucleus, the only recombination proficient germ cell in Drosophila, led us to hypothesize a role in meiotic recombination. Another striking feature is the frequent abnormal segregation of chromosome 4 in yem$^1$ oocytes (Meyer et al., 2010). This raised the possibility of defective recombination in this mutant as this feature is typical of meiotic recombination mutant backgrounds (Carpenter and Sandler, 1974). The finding that Yemanuclein localized to the SC further reinforced this hypothesis. A major pitfall to test this hypothesis was the lack of a straightforward assay on the progeny of females homozygous for the mutation, as the latter were essentially sterile (Meyer et al., 2010). However recombination being dose sensitive (Carpenter, 2003), we aimed to test this issue in the progeny of females heterozygous for yem1. Indeed a dominant effect on meiotic recombination was shown for mei-W68, c(3)G, mei-218, mei-9 (Carpenter, 2003; Carpenter and Sandler, 1974; Hinton, 1966; Mason, 1976; McKim and Hayashi-Hagihara, 1998; Page and Hawley, 2001). X chromosome was used for its sensitivity to recombination defects moreover it has convenient markers. Another important parameter to be considered in these experiments is the sensitivity of meiotic recombination to female age and environmental conditions such as temperature. To circumvent this problem, wild type recombination frequency was determined in our experimental conditions. As controls, we chose mutants whose dominant effect falls in different classes. The precondition mutations that affect the frequency and distribution of crossovers were tested in homozygous or heterozygous conditions. In heterozygous conditions, crossover locations were shown to be affected in mei-W68, mei-218, c(3)G mutant backgrounds (Supplemental Table 1). In contrast, heterozygous conditions for exchange mutations such as mei-9 known to affect specifically crossover resolution, result in a uniform decrease of recombination frequency along the chromosome (Carpenter and Sandler, 1974).
Yemanuclein association with SC led us to speculate an early function on crossover distribution. Therefore we first aimed to test whether yem\textsuperscript{1} like the precondition mutants affects crossover distribution along the chromosome. Recombination frequency was determined in wild type background for all the intervals tested on the X chromosome in the same experimental conditions as for the mutant backgrounds. Two genetic backgrounds were tested in this experiment: yem\textsuperscript{1}/+ and mei-W68\textsuperscript{1}/+. The ratio of recombination frequency between the progeny of heterozygous mothers and wild type recombination frequency is shown in Figure 3, panel A. In all the intervals tested, yem\textsuperscript{1}/+ and mei-W68\textsuperscript{1}/+ mothers displayed a similar effect on crossover frequency, that is an increase at telomeric and centromeric regions and a decrease in the central region. Therefore yem\textsuperscript{1} affects crossover distribution, and by definition yem falls in the precondition class of meiotic genes (Carpenter and Sandler, 1974).

We then aimed to analyse more specifically various meiotic mutants in a given interval. We chose to score recombination frequency in a small interval to reduce the probability of multiple events occurrence. Furthermore in these conditions one should be able to detect even subtle events if any and score a large number of flies to assess the statistical significance of the genetic data. The y-w interval located in the subtelomeric region of the X chromosome meets the criteria of interest as defined for these experiments. Moreover as shown in Figure 3A, the strongest effect of yem\textsuperscript{1} mutation was scored in this interval, a nearly 50% increase in recombination frequency; y-w interval was estimated to be 1.2 cM in our experiments whereas in the same experimental conditions recombination frequency was 1.7 cM for the progeny of yem\textsuperscript{1}/+ mothers. A similar increase was observed for progeny of mothers heterozygous for mei-W68\textsuperscript{1}, mei-218\textsuperscript{1}, c(3)G\textsuperscript{1}, mei-P22\textsuperscript{103} in the same interval. In contrast, a dramatic decrease was observed for the progeny of mei-9\textsuperscript{1}/+ mothers (Figure 3, panel B). Our results are in perfect agreement with those published earlier (Supplemental Table 1).
finding that mei-P22 falls in the precondition class is in good agreement with its role in double strand break (DSBs) formation, such as mei-W68 (Liu et al., 2002; McKim and Hayashi-Hagihara, 1998). These results are consistent with previously published results (supplemental Table 1) and therefore support the data on yem\(^{l}\) dominant precondition effect on meiotic recombination.

In order to gain understanding on how these so-called precondition mutations behave in combination with yem\(^{l}/+\), we performed an analysis of the recombination frequency in the \(yw\) interval for mei-218\(^{l}/+\); yem\(^{l}/+\) and mei-W68\(^{l}/+\); yem\(^{l}/+\) genotypes. The mei-9\(^{r}/+\); yem\(^{l}/+\) genotype was used as a control for the interaction of yem\(^{l}\) with an exchange mutation. The results are shown in panel C of Figure 3. In spite of being both classified as precondition mutations, the recombination frequency in the trans-heterozygotes differs greatly between mei-218\(^{l}/+\); yem\(^{l}/+\) and mei-W68\(^{l}/+\); yem\(^{l}/+\) genotypes. The combination of yem\(^{l}\) with mei-218 mutation results in a recombination frequency that is higher than that observed in the single mutant backgrounds. In contrast, a dramatic decrease was observed in the mei-W68\(^{l}/+\); yem\(^{l}/+\) background. The control mei-9\(^{r}/+\); yem\(^{l}/+\) genotype displayed a recombination frequency that was intermediate between the values observed in the single mutant backgrounds suggesting that the two mutations have independent actions. Therefore the mei-W68\(^{l}/+\); yem\(^{l}/+\) genotype reveals a strong genetic interaction of mei-w68 with yem unlike the other genes analysed here. This might reflect an incidence of yem\(^{l}\) mutation on the DSBs formation.

Another parameter we aimed to test is the impact of yem\(^{l}\) mutation on crossover interference. Crossover interference is a phenomenon that prevents two crossovers to be too close to each other. It is a way of controlling the DSBs outcome; first discovered in Drosophila it was then described in other organisms. Interference is inferred from the coefficient of coincidence (C), which is the ratio of observed to expected double recombinants, interference value being (1-
C). Therefore an increase in the coefficient of coincidence reflects defective crossover interference. C value was determined with the 3-point cross using cv-v-f markers (see X chromosome map, Figure 3A). It was determined as a function of maternal age in three different genetic backgrounds: wild type, yem\(^1\)/+ and mei-W68\(^{1}/+\). Two features were compelling from the analysis of the results: 1) the two mutant genotypes mimicked an age effect, 2) the two effects were additive as in 13-20 days old flies, yem\(^1\) mutation resulted in a more dramatic increase of coefficient of coincidence as if aging sensitized the flies. In conclusion yem\(^1\) affects performance of chromosomes for crossover interference.

**DSBs frequency and timing are dramatically modified in yem mutant background**

To gain further understanding on Yemanuclein role in the meiotic recombination pathway, we aimed to analyse DSBs in yem\(^1\) mutant background. To address this question we monitored the DSBs with anti \(\gamma\)-H2Av antibody. Similarly to H2AX which was shown to be phosphorylated in the presence of double strand breaks (DSBs) both in mitotic and meiotic mammalian cells (Mahadevaiah et al., 2001; Rogakou et al., 1999), Drosophila H2Av was shown to undergo the same modification \(\gamma\)-H2Av on serine 137 and was used as a marker for DSBs (Jang et al., 2003; Madigan et al., 2002). In Drosophila DSBs form in late region 2a after the SC is fully formed and are fully repaired by late pachytene in region 3 of the germarium that contains the so-called stage 1 oocyte (Jang et al., 2003; Mehrotra and McKim, 2006; Webber et al., 2004). This is schematically shown in Figure 4A and well illustrated in panel B that displays a wild type germarium stained for SC with an anti C(3)G monoclonal antibody (Page and Hawley, 2001)\} and DSBs with an anti \(\gamma\)-H2Av antibody (Mehrotra and McKim, 2006. The results obtained when yem\(^1\)/Df\(3\)450 ovaries were stained for DSBs are in striking contrast. Two features stand out: not only the number of \(\gamma\)-H2Av foci was reduced but most interestingly its timing was also strongly affected in the yem mutant.
background. The number of γ-H2Av foci was estimated as previously described (Jang, 2003 #80). As shown in Figure 4C, the peak of γ-H2Av was sharper and was restricted to cysts of region 2A. The question rose as to whether these breaks resulted from Mei-W68 activity. This issue was addressed by staining germaria that were mutant for both mei-w68 and yem. In the absence of Mei-W68 activity, the DSBs was not formed therefore their presence in the yem mutant background was dependent on Mei-W68 activity and not due to some other dysfunction such as unrepaired replication forks. In conclusion yem mutation impacted Mei-W68 recruitment and/or activity. Although the impact of SC mutations on DSBs frequency is controversial (Jang et al., 2003; Mehrotra and McKim, 2006; Webber et al., 2004), to our knowledge no meiotic mutation in Drosophila was ever reported to modify DSBs formation timing before the present report.

To gain insight into the basis of the genetic interaction observed between mei-w68′ and yem′, the DSBs were analysed in mei-W68′/+; yem′/+ germaria. As shown in Figure 4D, in this genetic background the γ-H2Av staining was decreased and localised in earlier germarial cysts than in wild type background. This observation might provide an explanation for the genetic interaction between mei-w68 and yem although the mechanism is difficult to suggest at this point of the work.

*Yemanuclein associates in vivo with components of the cohesin complex*

Yemanuclein association to the SC (this work) added to its DNA binding properties (Ait-Ahmed et al., 1992) led us to investigate its possible interaction with components of the cohesin complex. To address this question, we performed coimmunoprecipitation experiments using ovary protein extracts. We tested the *in vivo* association of Yemanuclein with SMC3, a canonical cohesion protein and C(2)M that was found as a complex with SMC3 (Heidmann et al., 2004). For this purpose we constructed a fly stock that bears both
gSmc3-ha and gc(2)M-myc transgenes described earlier (Heidmann et al., 2004). The immunoprecipitations were performed with antibodies against Yemanuclein endogenous protein and the epitope tags MYC (C(2)M) and HA (SMC3). The immunoprecipitates were probed with the corresponding antibodies but also with an antibody against the endogenous RAD21 kleisin (Gause et al., 2008). To assess the specificity of the immunoprecipitations and western blot experiments we used an antibody against the FLAG epitope that provides the negative control. As shown in Figure 5 (upper panel), in the control experiment using the FLAG antibody no signal was observed in the so-called immunoprecipitated fraction. Therefore we were confident that any detection in the extracts or the immunoprecipitated fractions could be considered as specific. The presence of YEM, C(2)M-MYC, SMC3-HA was assessed in the ovary protein extracts. The anti Yem polyclonal antibody significantly precipitated Yemanuclein but also SMC3-HA and C(2)M-MYC. The reciprocal experiments where on the one hand C(2)M-MYC pulled Yem and SMC3-HA and on the other hand Yem and C(2)-MYC were pulled by SMC3-HA allowed to propose that Yemanuclein, C(2)M and SMC3 proteins were in the same complex. SMC3-HA and C(2)M-MYC were already described in an earlier work to be within a same meiotic complex, as well as kleisin Rad21 (Heidmann et al., 2004). As shown in the lower panel of Figure 5, Rad21 was also found in SMC3-HA, C(2)M-MYC and Yem immunoprecipitates. Two different antibodies were used to immunoprecipitate Yemanuclein, both consistently pulled Rad21. It is noteworthy that immunoprecipitation with the anti YEM antibody gave the best results. We assume that a polyclonal antibody directed against the endogenous protein was more efficient than anti-tag antibodies. However all the above data are consistent and are supported by the controls. Therefore our data are in favour of an association of Yemanuclein to the meiotic cohesin complex.
Discussion

We report here that Yemanuclein is a new SC associated protein which was overlooked in classical genetic. Yemanuclein was first identified in differential screens for maternally expressed genes (Ait-Ahmed et al., 1992; Ait-Ahmed et al., 1988; Ait-Ahmed et al., 1987). The V478E mutant allele was recovered in a screen for female sterile mutations that fall in the genomic region which contains yem locus (Ait-Ahmed et al., 1992; Meyer et al., 2010). Yemanuclein properties raised the question of its role in early meiotic prophase especially in meiotic recombination.

_Yemanuclein is a new SC and cohesin associated protein_

Our results clearly show that Yemanuclein associates with the SC following a pattern that is very similar to that described for the two well characterized C(3)G and C(2)M proteins (Manheim and McKim, 2003; Page and Hawley, 2001). Moreover as previously described Yemanuclein follows a pattern that is completely similar to that of C(3)G in germaria that are mutant for c(3)G, c(2)M and ord (Manheim and McKim, 2003; Page and Hawley, 2001; Webber et al., 2004). Therefore its localization to the chromosomes at early meiotic stages is dependent on SC integrity. Interestingly Yemanuclein is an _in vivo_ partner of components of the cohesin complex. Cohesin is an essential component of the chromosome axes that eventually build the lateral elements (LEs) of the SC (Lee and Orr-Weaver, 2001; Revenkova and Jessberger, 2006). One would assume that Yem should be part of the axial elements and as such should be essential for SC formation or maintenance. Such is not the case. Even in mutants that are _yem^null_ (no staining at all with Yem antibodies), SC appears normal when probed with the C(3)G antibody (Data not shown). In contrast, C(2)M that was found in the same complex as Yemanuclein and whose involvement as an axial element was reported is required for SC formation (Anderson et al., 2005; Manheim and McKim, 2003). Another
difference between Yem and C(2)M is that C(2)M is not affected by c(3)G mutations for its localization to the chromosome axes (Manheim and McKim, 2003). This what one could expect from a typical axial element. The peculiarity of Yemanuclein is reminiscent of that of Rec8, a Rad21/Scc1 parologue in mammals which is absent in Drosophila. Rec8 was described as a meiosis specific kleisin that is essential for meiotic cohesion both in S. Pombe and S. Cerevisiae (Klein et al., 1999; Watanabe and Nurse, 1999). Moreover it was recognised in S. Cerevisiae as a component of the axial elements (Klein et al., 1999). In mammals the situation was controversial as to whether or not meiotic cohesin is a component of axial elements before a recent publication provided a convincing experiment where the two mammalian Rad21 paralogous sequences, Rec8 and Rad21L were simultaneously knocked out (Llano et al., 2012). In mice that are deficient for both meiosis specific kleisins, axial elements are strongly affected (Llano et al., 2012). However the situation is still controversial as the same Authors report no effect of this double knockout on sister chromatids cohesion which they justify as the absence of effect of this double deficiency on Rad21 localization to the sister chromatids (Herran et al., 2011; Llano et al., 2012), in contrast to other Authors claims (Ishiguro et al., 2011 ). In grasshoppers Rufás and collaborators have reported that a variety of complexes with cohesion components exist simultaneously and may fulfil different roles during meiosis (Calvente et al., 2013). We were not able to show any effect of yemI mutation on sister chromatids cohesion in spite of a detailed analysis of the mutant phenotypes (Meyer et al., 2010). In this work, we were not able either to show any effect on the SC assembly. Given how complex the situation is, more knowledge is required before being able to provide an explanation to our results. A recent review recapitulates our current knowledge on cohesin complexes during meiosis (McNicoll et al., 2013).
Crossover distribution and DSBs formation in yem\textsuperscript{1} mutant oocytes

Crossovers are essential for chromosome segregation in recombination proficient cells as they link the homologues, which results in their segregation at meiosis I. In absence of crossovers, non-disjunction may occur as the homologous chromosomes segregate randomly. Therefore the mechanisms that support crossover formation must be stringently regulated in particular to ensure that at least one crossing over forms between each homologous pair. Beyond the obligate crossing over, the spatial distribution of crossovers also is regulated. CO distribution is regulated at two levels: (1) at the level of DSBs formation, (2) at the level of their resolution into CO versus NCO. The relative number of NCO versus CO depends in particular on crossover interference, a phenomenon that prevents a new crossover to form too close to another crossover (Youds and Boulton, 2011). In spite of yem\textsuperscript{1} being a female sterile mutation, its effect on meiotic recombination could be tested thanks to the dominant effect of recombination defective mutations (Carpenter, 2003). We could show that yem\textsuperscript{1} affects crossover distribution along the X chromosome. It is noteworthy that all the meiotic mutations tested in heterozygous conditions have the same effect in the \(y-w\) interval to the exception of mei-9, the archetypal exchange mutation that affects late recombination stages, more precisely crossover resolution (Carpenter and Sandler, 1974). All these mutants affected in crossover distribution were classified as precondition mutants (Carpenter and Sandler, 1974). This classification does not tell much about what these precondition functions are. We can predict that the effect on crossover distribution is indirect. Indeed mutations that affect functions as diverse as SC formation (\(c(3)G\)), double strand break formation (\(mei-W68\)), DNA repair etc. affect crossover distribution (Bhagat et al., 2004). An interesting hypothesis was proposed to account for such a diverse class of mutations leading to similar defects in crossover distribution. In response to a decrease in the number of crossovers, a back up mechanism would allow crossover formation at a place where they are not usually authorized,
including the proximal regions (Bhagat et al., 2004). In C.elegans, a checkpoint has been reported that delays pachytene to allow crossovers to form even in chromosome locations where they normally do not occur (Carlton et al., 2006). Indeed even chromosome 4 may experience recombination in a context where crossover distribution is aberrant (Sandler and Szauter, 1978). As can be observed in our data in response to yem$^1$ mutation more crossovers form in the telomeric and centromeric regions of the chromosome and crossover interference is significantly affected in the mutant background. Interestingly yem$^1$ mutation affects not only the amount but also the timing of DSBs. How these effects translate into modifications of crossover interference and crossover distribution remains unclear and deserves further investigations.
Experimental Procedures

Fly stocks used in this work are given as a supplemental Table

Whole-Mount immunocytochemistry for Yemanuclein, SC and γ-His2Av detection

For Yemanuclein detection, immunostaining was performed with a polyclonal antibody developed against Yemanuclein-alpha, washing and incubation with fluorescent secondary antibodies were as described elsewhere (Ait-Ahmed et al., 1992; Capri et al., 1997; Meyer et al., 2010). Additional primary antibodies included mouse anti-C(3)G antibody used at 1:500 (Page and Hawley, 2001), rabbit anti-C(2)M antibody used at 1:400 (Manheim and McKim, 2003), and the monoclonal anti-HA tag at 1:200 (SIGMA). Rhodamine or FITC conjugated anti-rabbit and anti-mouse antibodies were from Jackson ImmunoResearch Labs Inc. The other secondary antibodies were coupled either to Alexa 488 and Alexa 555 (Molecular Probes) or Fluoprobes 546 (Interchim). Leica DMRA2, DMRXA and DM600B microscopes were used for conventional epifluorescence. Confocal microscopy was performed either with a Biorad device or a Zeiss LSM 510.

For γ-His2Av detection, females were dissected and fixed as described (Page and Hawley, 2001). Primary antibodies included mouse anti-C(3)G antibody used at 1:500 and rabbit anti-γ-His2Av antibody used at 1:500 (Mehrotra and McKim, 2006). The other secondary antibodies were coupled either to Alexa 555 (Molecular Probes) or Fluoprobes 546 (Interchim). Leica DM600B microscope was used for conventional epifluorescence. Confocal microscopy was performed either with a Biorad device or a Zeiss LSM 510.

Recombination frequency analysis

The flies were raised at 25 °C for all the crosses designed to measure crossing over (simple and double crossing over). All control experiments were carried out in exactly the same conditions as test experiments. Freshly hatched females were mated on the same day. After 5
days of egg laying the parents were transferred to fresh culture vials. Altogether three consecutive egg-laying periods were established until females were 20 days old. The progeny of each egg-laying period were analysed separately. This procedure allowed us to determine age-dependent effects on crossing over. X chromosome crossing over and interference analysis were assayed by crossing the different mutant stock (see supplemental table 2) with different combination of X chromosome markers to generate females heterozygotes for \textit{yem}^l, \textit{mei-W68}^l, \textit{mei-P22}^{103}, \textit{mei-218}^l, \textit{c(3)G}^l or \textit{mei-9}^l and heterozygous for \textit{yw cv y f car}.

For analysis of X chromosome crossing over (simple crossing over), only the first egg-laying period was used (0-5 days) to avoid the age-dependent effect. Statistical significance was assessed with Khi2 test. For all tests, \*\textit{P} < 0.05 \*\*\textit{P} < 0.01 and \*\*\*\textit{P} < 0.001.

For crossover interference analysis (double crossing over), the four different egg-laying period were used and compared (0-5, 5-9, 9-13 and 13-20 days). Coefficient of coincidence (C = 1 – crossover interference) was calculated as described elsewhere (Portin and Rantanen, 2000) with C = \(\frac{w}{n}\) \((w + x) (w + y)\). \(w\) is the number of flies with double crossovers, \(x\) and \(y\) are the numbers of flies with single crossovers for \(cv\) and \(v\), and \(v\) and \(f\) respectively, \(n\) is total fly number. Binomial \(t\)-test was used to calculate difference between coefficients of coincidence.

\[ t = |p1 - p2| \sqrt{\frac{(pq(n1+n2))/(n1n2)}{n1 + n2}} \]

where \(p1\) and \(p2\) represent coefficients of coincidence of the two populations, \(n1\) and \(n2\) represent the number of flies in the two populations.

\[ p = \frac{(p1n1 + p2n2)/(n1 + n2)}{q=1 - p} \]

For all tests, \*\textit{P} < 0.05 \*\*\textit{P} < 0.01 and \*\*\*\textit{P} < 0.001.

\textit{Immunoprecipitations and western blot analysis}

For coimmunoprecipitation experiments, we essentially used the protocol described in (Jager et al., 2001) with some modifications as indicated. 100 ovaries were dissected manually on

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ice in 250 μl ice cold lysis buffer. Lysis buffer was essentially as described earlier (Jager et al., 2001); it was supplemented with an EDTA-free protease inhibitor cocktail (Roche tablets) as recommended by the supplier. PMSF was also added to a 1mM final concentration. Before homogenization an equal volume of ice-cold lysis buffer was added. After centrifugation, the cleared homogenate was adjusted to 1 ml in lysis buffer. 2X30 μl were set aside to be used as input in the western blot experiments. The diluted cleared extracts were then submitted to the immunoprecipitation procedure with 50 μl G-Sepharose beads (SIGMA) pre-incubated with the primary antibodies. AS2 rabbit polyclonal (10 μl), M2 mouse anti-Flag (2 μl; Sigma), monoclonal anti-c-Myc (2 μl; Sigma), rabbit ChIP grade anti-HA (2 μl, Abcam). The anti-Flag antibody was used in a mock experiment to assess the specificity of the immunoprecipitation reactions.

The protein extracts and the retained fractions were then submitted to Western Blot analysis. SDS-Page electrophoresis was carried out on 8% acrylamide gels with a Bio-Rad device. Western blot was performed with standard procedures using either Pierce ECL Western Blotting Substrate (Thermo Scientific) or Millipore Immobilon Western Chemiluminescent substrate as recommended by the supplier. The following antibodies against the epitope tags were used: M2 anti-Flag (1:1000; Sigma), anti-c-Myc (1/400; Sigma), anti-HA (1:400, monoclonal Sigma). Rabbit polyclonals (kindly provided by Dale Dorsett) were used at a 1:100 dilution for YEM and 1:500 for RAD21) (Gause et al., 2008). Secondary antibodies were goat peroxidase-coupled anti-mouse and anti-rabbit antibodies (1:10000; Beckman).
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Figure Legends

Figure 1

Localization of Yemanuclein-alpha protein in wild-type (wt) germaria

Whole mount ovaries were stained as indicated in "Experimental procedures". The primary antibodies used were a rabbit polyclonal antiserum (Yemanuclein), a mouse monoclonal for C(3)G (Page and Hawley, 2001) and for C(2)M either a rabbit polyclonal antiserum or a mouse monoclonal Tag-HA for c(2)M-HA transgenic flies (Manheim and McKim, 2003). The secondary antibodies were anti-rabbit coupled to fluorescein, Alexa 488 or Alexa 555 and an anti-mouse coupled to fluorescein or Fluorochrome 546. For details see "Experimental procedures".

(A) Yemanuclein localization at early stages in a wild type ovariole (a-e). Pictures b and c display a higher magnification of the gerarium shown in a. (b) classical projection of confocal sections (R1 to R3, regions of the gerarium). In c and e, the stacks were treated with Volocity, the snapshots 3D reconstructions are shown. The pictures d and e show the typical ribbon-like localization of Yemanuclein in the 2 proocytes of a gerarium region 2a egg chamber and in a stage 3 egg chamber. (B) Schematic representation of an ovariole up to stage 6 (S6). FC: follicle cells. OO: oocyte. NC: nurse cells (King, 1970). (C) Projection of deconvolved z-series. Colocalization of Yem staining (green) with C(3)G and C(2)M staining (red). Lower panel displays higher magnifications of the geraria. (D) C(3)G and C(2)M staining in classical fixation conditions were provided to validate the suitability of our experimental conditions for SC components (see experimental procedures). For C(2)M, both the rabbit antibody against the endogenous protein (left) and the mouse antibody against HA tag of the transgenic C(2)M-HA were used (right). The projections of deconvolved z-series show the same C(3)G, C(2)M staining as above.
Figure 2

Yemanuclein localization in ovaries mutant for synaptonemal complex components

Each image represents a full projection from a deconvolved z-series with Yem staining in green and c(3)G staining in red.

(A, B) Localization of Yemanuclein-alpha protein in c(2)M and c(3)G mutant and wild type ovarioles (C). In c(2)M$^{EP2115}$ and c(3)G$^{68}$ mutant ovarioles (a,b) that affect formation of SC (Manheim and McKim, 2003), Yem canonical staining is absent from the nucleus at early stages (germarium) and reappears in the oocyte nucleus by stage 3 (arrowhead). (E) At higher magnification, in c(2)M$^{EP2115}$ mutant germaria, both C(3)G and Yem staining appears as fragmented and spotty signals (arrow). In contrast, in ord$^S$/Df mutant that affect maintenance of SC, normal C(3)G and Yem staining is observed at early germarial stages (F). However, this staining is severely disturbed at later stages, it looks fragmented in region 2B and spotty in region 3 (arrow). These localization defects are similar to those previously described for C(2)M and C(3)G in ord$S$/Df mutant germaria (Webber et al., 2004).
Figure 3

Recombination Frequency on chromosome X in mutant and wild type females

All the recombination tests were carried out in well controlled experimental conditions with females whose age was less than 5 days. Recombination frequency is in Centimorgans (CM).

A- Crossing over distribution along the X chromosome for yem1/+ (light grey), mei-W681/+ (dark grey) females. The ordinate represents the ratio of genetic distance observed in the mutant genotype over the genetic distance observed in the wild type (determined in our experimental conditions). The abscissa represents genetic intervals along the chromosome listed from distal to proximal; the wild type genetic distance (cM) is indicated for each interval. Statistical significance is assessed with the Khi2 test: *P < 0.05, **P < 0.01, and ***P < 0.001.

B, C- Recombination frequency in cM in the y-w interval analyzed in offspring of females with yem1/+ or control meiotic genotypes (B) and trans-heterozygous mutants (C). Wild type as determined in our experimental conditions is indicated in white, precondition genotypes and yem1/+ that display an increase in the recombination frequency are indicated in grey, the mei-9 pure exchange mutant that displays a decrease in recombination frequency, is in black. Statistical significance was determined with the Khi2 test *P < 0.05, **P < 0.01, and ***P < 0.001. (C) Note the high decrease of recombination rate in mei-W681/+; yem1/+ mutants in comparison to the other trans-heterozygous combinations of yem1 mutation.

D-Coefficient of coincidence as a function of maternal age in wild type (dark) and two mutant genetic backgrounds: yem1/+ (white), mei-W681/+ (grey) in the cv-v-f intervals. The calculations and statistical analyses are described in detail in ‘Material and Methods’ section.
Figure 4

Effect of *yem* mutation on γ-H2Av foci

A- Schematic view of a gerarium with the main events of the early meiotic prophase (Joyce and McKim, 2010; Lake and Hawley, 2012).

(B) Whole mount ovarioles were stained for C(3)G (red) and γ-H2Av (green). For each genotype, the γ-H2Av staining is shown separately on the right part. All Images represent a full projection of z-series. 3 different genotypes were analysed: wild type, *yem*/*Df*3450, *mei-W68*/mei-W68: *yem*/*Df*3450.

(C) Average number of γ-H2Av foci as a function of relative cyst age in wild-type and *yem*/*Df*3450 gerariums. The counting was performed as previously described (Mehrotra and McKim, 2006). As can be observed in panel B, *yem* background significantly affected the timing and overall γ-H2AV foci throughout the gerarium. Bars indicate standard error of the mean.

(D) γ-H2Av foci in mei-W68+/+; *yem*+/+ germaria. This experiment shows that in the trans-heterozygotes, the staining is different from the wild type staining.

Scale bar represents 20 microns.
Figure 5

*Yem associates in vivo with C(2)M, SMC3 and Rad21*

Ovary extracts were prepared from flies that express both SMC3-HA and C(2)M-MYC transgenes. The immunoprecipitations were performed with anti-YEM AS2 polyclonal antibody, anti HA tag and anti MYC tag antibodies. The mock immunoprecipitation was performed with an anti-FLAG monoclonal antibody. The protein extracts (E) and the immunoprecipitated (IP) fractions were submitted to Western blot analysis. YEM was revealed with the AS2 polyclonal antibody (1:100), SMC3 with anti HA (1:400), C(2)M with anti MYC (1:400) and FLAG was used at 1:1000. In the lower part of the Figure, RAD21 presence in the immunoprecipitated complexes was assessed with a rabbit polyclonal antibody (1:500, kindly provided by D. Dorsett). Two immunoprecipitates obtained with two different Yem antibodies consistently pulled RAD21.
Figure 1

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Figure 2
Figure 3
Figure 4

A

B

C

D

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Immunoprecipitations

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Western blot analysis

YEM
Myc
HA
FLAG

IP

E
HA
YEM
YEM
Myc

Rad21

Figure 5
## Supplemental Tables

### Supplemental Table 1 - Relevant data on meiotic defective mutants collected from the literature

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<td>y pm cv v f B (B+)</td>
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</tr>
<tr>
<td>RG13</td>
<td>y cv v/FM7a</td>
<td>This study</td>
</tr>
<tr>
<td>RG17</td>
<td>w cv/FM7a</td>
<td>This study</td>
</tr>
<tr>
<td>RG18</td>
<td>w cv b/FM7a</td>
<td>This study</td>
</tr>
<tr>
<td>RG21</td>
<td>f car/FM7a</td>
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<td>(Meyer et al., 2010)</td>
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### Supplemental Table 2 - Drosophila stocks used in this work

- nos-Gal4; P(UAS:gec(2)M[3sHA]) | KS. McKim
Supplemental Table 3 – Coefficient of coincidence determined in various genetic backgrounds

The differences between coefficients of coincidence were calculated using the binomial r-test. For all tests, *P < 0.05 **P < 0.01 and ***P < 0.001.

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<td>Mutations</td>
<td>Control</td>
<td>Mutations</td>
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<td>mei-W68&lt;sup&gt;1&lt;/sup&gt; /  &lt;sup&gt;1&lt;/sup</td>
<td>wt</td>
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<td>t = 5.6 ***</td>
<td>t = 5.48 ***</td>
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Supplemental references


Article 2:

Drosophila Yemanuclein and HIRA Cooperate for De Novo Assembly of H3.3-Containing Nucleosomes in the Male Pronucleus.
Drosophila Yemanuclein and HIRA Cooperate for De Novo Assembly of H3.3-Containing Nucleosomes in the Male Pronucleus

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Abstract

The differentiation of post-meiotic spermatids in animals is characterized by a unique reorganization of their nuclear architecture and chromatin composition. In many species, the formation of sperm nuclei involves the massive replacement of nucleosomes with protamines, followed by a phase of extreme nuclear compaction. At fertilization, the reconstitution of a nucleosome-based paternal chromatin after the removal of protamines requires the deposition of maternally provided histones before the first round of DNA replication. This process exclusively uses the histone H3 variant H3.3 and constitutes a unique case of genome-wide replication-independent (RI) de novo chromatin assembly. We have previously shown that the histone H3.3 chaperone HIRAP plays a central role for paternal chromatin assembly in Drosophila. Although several conserved HIRA-interacting proteins have been identified from yeast to human, their conservation in Drosophila, as well as their actual implication in this highly peculiar RI nucleosome assembly process, is an open question. Here, we show that Yemanuclein (YEM), the Drosophila member of the Hpc2/Ubinucin family, is essential for histone deposition in the male pronucleus, whereas loss of function alleles affect male pronucleus formation in a way remarkably similar to Hira mutants and abolish RI paternal chromatin assembly. In addition, we demonstrate that Hira and YEM proteins interact and are mutually dependent for their targeting to the decondensing male pronucleus. Finally, we show that the alternative ATRX/XNP-dependent H3.3 deposition pathway is not involved in paternal chromatin assembly, thus underlining the specific implication of the HIRA/YEM complex for this essential step of zygote formation.


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Introduction

Assembly of octameric nucleosomes in eukaryotic chromatin is a stepwise process where deposition of a histone H3-H4 heterotetramer precedes incorporation of two H2A-H2B dimers [1]. While the bulk of de novo chromatin assembly occurs during genome replication and mainly involves canonical histone H3, alternative, replication-independent (RI) chromatin assembly pathways use the conserved histone H3 variant H3.3 [2,3]. Canonical (or replicative) H3S (H3.1 and H3.2) in mammals, H3.2 in Drosophila are synthesized in early S phase and deposited at DNA replication forks by the trimeric CAF-1 (Chromatin Assembly Factor-1) complex [4]. In contrast, H3.3 is expressed throughout the cell cycle and is deposited at various genomic regions in a DNA-synthesis independent manner [3–6]. During the past decade, research on H3.3 has largely focused on the ability of this histone to be deposited at transcribed genes, opening the possibility that H3.3 could constitute an epigenetic mark of active chromatin [9–13]. Recent advances in the field have led to a more complex view of H3.3 biology. Although H3.3 is indeed enriched at transcribed gene bodies, it is now established that this histone is also deposited at various chromatin regions, such as regulatory elements, mammalian telomere repeats or satellite DNA blocks [3–7,14–18]. This surprising versatility of H3.3 could simply reflect its ability to be deposited in regions that are subjected to nucleosome depletion or rapid histone turnover [5,7,19].

In metazoa, H3.3 is also implicated in a variety of nuclear processes that specifically occur in germ cells and in early embryos [7,20–22]. In mouse spermatocytes, for instance, H3.3-containing nucleosomes are assembled on sex chromosomes during their inactivation and accumulate over the whole sex body [23].
Author Summary

Chromosome organization relies on a basic functional unit called the nucleosome, in which DNA is wrapped around a core of histone proteins. However, during male gamete formation, the majority of histones are 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 replaced by histones upon fertilization, in a replication-independent chromatin assembly process that requires the histone deposition factor HIRA. In this study, we identified the protein Yemanuclein (YEM) as a new partner of HIRA at fertilization. We show that, in eggs laid by yem mutant females, the male pronucleus fails to assemble its nucleosomes, resulting in the loss of paternal chromosomes at the first zygotic division. In addition, we found that YEM and HIRA are mutually dependent to perform chromatin assembly at fertilization, demonstrating that they tightly cooperate in vivo. Finally, we demonstrate that the replication-independent chromatin assembly factor ATRX/XNP is not involved in the assembly of paternal nucleosomes. In conclusion, our results shed new light into critical mechanisms controlling paternal chromosome formation at fertilization.

Moreover, an insertion mutation in the mouse H3.3.4 gene induces male subfertility, among other phenotypes [24]. Certain histone residues of H3.3 are also important for the establishment of heterochromatin during reprogramming in mouse zygotes [25]. Recently, knock-down experiments in Xenopus laevis demonstrated a specific and critical requirement of H3.3 during embryo gastrulation [26]. In Drosophila, H3.3 deficient animals are viable but are both male and female sterile [27,28]. H3.3 is notably required for the proper segregation of microchromosomes in spermatocytes [26] and for the global organization of early spermated chromatin [28,29].

A remarkable H3.3 deposition process also occurs during the decondensation of the male pronucleus at fertilization [21]. This unique, genome-wide assembly of H3.3 nucleosomes follows the rapid removal of sperm-specific nuclear basic proteins (SNBP) from the fertilizing sperm nucleus, after its delivery in the egg cytoplasm. In many animal species, during spermiogenesis, histones are progressively replaced with SNBP, such as the well-characterized protamines [30–32]. The nature and extent of this replacement is highly variable in metazoans [32]. In Drosophila, protamine-like proteins are encoded by two paralogous genes named Met35SAa and Met35SBb [33,34]. In this species, the vast majority of sperm DNA is packaged with protamines and with other non-histone SNBP [21,35], implying that de novo assembly of paternal nucleosomes at fertilization after SNBP removal must occur over the entire male genome.

We had previously shown that this unique RI assembly requires the conserved H3.3 histone chaperone HIRA [36,37]. Indeed, loss of function mutations in Hira are viable in Drosophila, but nucleosome 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 the development of gynogenetic haploid embryos [36,37]. In mice, HIRA is present in the decondensing male nucleus [38] and is most likely responsible for the strong paternal H3.3 enrichment observed in the zygote [38,39]. Recently, HIRA has been implicated in the formation of the male pronucleus in the crucian carp [40], confirming the widespread role of this histone chaperone in paternal nucleosome assembly at fertilization.

The Hiz/HIRA complex is composed of a small number of proteins that are conserved between yeast and human. In S. cerevisiae, the Hir chromosome assembly complex includes the HIRA-related proteins Hir1 and Hir2, Asf1 (Anti Silencing Factor 1), Hir3 and Hpc2 [41–43]. Hir3 is a poorly conserved protein related to His3 (S. pombe) and human CABIN1, but which does not seem to have an ortholog in Drosophila [43–45]. Hpc2 is functionally related to Hid4 in fission yeast and to the HIRA-associated proteins Ubicin1 and Ubicin2 (UB1/UB2) [8,14,46–48]. Interestingly, the strongest conservation between Hpc2 orthologs resides in a ~50 amino-acid domain called HIRD (Hpc2-Related Domain) or HUN (Hpc2-Ubicin1-5 domain) [44,48] and to a smaller domain called NHRD [49]. In Drosophila, Yemanuclein (YEM; also named Yemanuclein-9 [50,51]) is the only protein with a HIRD domain [44]. The yem gene has a strong ovarian expression and encodes a nuclear protein that accumulates in the germinal vesicle of ovarioles [51]. Recently, a mutant allele of yem (yem) has been characterized as a V478E replacement, which results in female sterility [52]. In this first report on YEM function, YEM was implicated in the segregation of chromosomes during the first female meiotic division but the sterility of mutant females suggested the existence of yet unknown roles for YEM [52]. In this paper, we have explored the implication of YEM in HIRA-dependent RI nucleosome assembly in the zygote. We show that the cooperation of YEM and HIRA in vivo is critical for the assembly of H3.3-containing nucleosomes in the male nucleus at fertilization.

Results

yem is a deletion allele of the yemanuclein gene

The original yem point mutation causes a single amino-acid replacement (V478E) in YEM protein (Figure 1A) [52]. This mutation induces female sterility but has no detectable effect on the level of yem transcripts in ovarioles nor on the accumulation of YEM protein in the oocyte nucleus (or germinal vesicle, GV) (Figure 1B, 1C). To obtain a more severe mutant allele of yem, we mobilized a P-element inserted near the transcriptional start site of the yem gene (Figure 1A). One of the imperfect excisions of this P-element generated a 3180 bp deletion (named yem) that spans the 5’ UTR and most of the coding region of yem. Accordingly, the yem allele induced female sterility in association with yem or with the large non-complementing deficiency Df(3R)350 (Table 1). In yem yem females, yem transcripts (corresponding to a region of the gene not covered by the yem deletion) were greatly reduced compared to yem yem females, and the YEM protein was not detected in the oocyte nucleus (Figure 1B, 1C). Finally, the female sterility of both yem mutant alleles was rescued by expressing a transgenic YEM protein tagged in its C-terminus with the Flag peptide (YEM-Flag) (Table 1). Taken together, these data suggest that yem is a null or at least a strong loss of function allele of yem.

YEM interacts with HIRA in vivo

The YEM protein has been previously detected in a HIRA complex purified from embryonic nuclear extracts [53], suggesting that it could represent the Drosophila ortholog of UB1/Hpc2. To more directly test the interaction of HIRA and YEM, we performed co-immunoprecipitation experiments using functional Flag-tagged and GFP-Flag-tagged transgenic versions of YEM and HIRA proteins, respectively. We confirmed that, in ovarian protein extracts, HIRA was able to co-immunoprecipitate with
YEM, and vice versa (Figure 2A). In the same experiments, however, the ATP-dependent chromatin remodeling factor CHD1 was not detected in the HIRA immune complex, in contrast to what was previously reported [54]. Although the reason for this apparent discrepancy with the study by Konve et al. is not clear, it reinforces the fact that, in our experimental conditions, HIRA and YEM show reproducible and specific interaction, confirming that these proteins are subunits of a common complex.

HIRA and YEM were previously shown to display a remarkable and specific accumulation in the nucleolus of the GV throughout oogenesis [36,51]. Similarly, immunodetection of the Flag-tagged versions of HIRA and YEM recapitulates their endogenous accumulation in the GV, where both proteins co-localize (Figure 2B). The oocyte nucleus is a large nucleus that essentially contains nucleolus, as the oocyte chromosomes remain confined within a small, compact structure called the karyosome [55]. Surprisingly, we observed that HIRA-Flag accumulation in the GV was completely abolished in yem<sup>-</sup>/Df(3R)3450 mutant oocytes. Conversely, we found that YEM-Flag was undetectable in the GV of about half of null Hira<sup>001</sup> mutant oocytes (Figure 2C). These effects could not be explained by reduced protein levels in mutant flies, as HIRA-Flag and YEM-Flag expression were apparently not affected in yem<sup>-</sup>/Df(3R)3450 and Hira<sup>001</sup> mutants, respectively (Figure 2D). These results indicate that YEM and HIRA are mutually required for their localization or for their stabilization in the oocyte and suggest that these proteins interact prior to their release in the egg cytoplasm, after GV breakdown. Taken together, these results confirm that YEM and HIRA belong to the same complex in vivo.

**Figure 1.** Mutations affecting the yem gene. (A) Schematic representation of the yem gene [51] and mutant alleles. yem<sup>1</sup> is a point mutation (V478E) [52] and yem<sup>1</sup> is a deletion that was generated by mobilizing the P-element insertion P[EPgy]2/Y23294 (red triangle). Coding sequence is in yellow and untranslated regions are in white. The YD1 [52], HRD/HUN [44,48] and NHRD [49] domains of YEM are indicated, as well as the position of primers used for RT-PCR analysis (green arrowheads). (B) RT-PCR analysis of yem expression in the indicated tissues and genotypes. RT-PCR amplification used the primer pair shown in (A). (C) Confocal images of wild-type or yem mutant egg chambers stained for DNA (blue) and with anti-YEM AS2 antibody (red). YEM protein accumulates in the germinal vesicle (arrowhead) in wild-type and yem<sup>-</sup>/Df(3R)3450 oocytes. In yem<sup>-</sup>/Df(3R)3450 mutant oocytes, only background staining is detected. Bar: 20 μm.

YEM is required for paternal chromatin assembly at fertilization

The female sterility associated with yem<sup>-</sup> or yem<sup>-</sup> mutations actually results from a maternal effect embryonic lethality phenotype. Indeed, eggs from yem<sup>-</sup>/Df(3R)3450 or yem<sup>-</sup>/Df(3R)3450 females (referred to as yem mutant eggs for simplicity) are normally fertilized and they initiate development, but the embryos systematically die before hatching (Table 1 and not shown). These features are reminiscent of the maternal effect embryonic lethality phenotype of Hira mutants, where embryos develop as non-viable syncytial haphloids after the loss of paternal chromosomes during the first zygotic division [36,37,56].

<table>
<thead>
<tr>
<th>Genotype of females</th>
<th>No. of eggs</th>
<th>No. of larvae</th>
<th>Hatch rate (%)</th>
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<td>758</td>
<td>85.3</td>
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<td>667</td>
<td>660</td>
<td>98.9</td>
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</table>

All yem mutant alleles are described in Figure 1. Df(3R)3450 is a large deficiency covering the yem locus. yem<sup>flg<sup>14</sup></sub> and yem<sup>-</sup> are two independent insertions of the same transgene.

DOI:10.1371/journal.pgen.1003285.t001
We thus examined male pronucleus formation in yem mutant eggs. In wild-type eggs, shortly after fertilization, while maternal chromosomes complete meiotic divisions, the decondensing male nucleus is strongly and specifically stained with an antibody recognizing acetylated histone H4, a mark of newly assembled chromatin [36,37]. Strikingly, we observed that in yem mutant eggs, acetylated H4 was practically not incorporated in the male pronucleus (Figure 3A). At pronuclear apposition, male pronuclei in yem mutant eggs always appeared round and condensed (Figure 3B), in a way identical to the male nucleus in Him mutants [36,37,57]. Paternal chromosomes subsequently failed to integrate the first zygotic division in yem eggs (Figure 3C), resulting in gynogenetic haploid development and embryonic lethality (Figure 3D). It should be mentioned however that exceptional gynogenetic development of adults can occur if the female pronucleus is diploid as the result of defective meiosis [52].

While the yem\_flag\textsuperscript{HIPv1} transgene efficiently rescued yem female sterility, another insertion of the same construct (yem\_flag\textsuperscript{HIPv1}) only restored fertility to very low levels, likely because of its weak expression (Table 1). Interestingly, in eggs laid by yem\textsuperscript{1}/ Df(3R)3450; yem\_flag\textsuperscript{HIPv1}\_females, the male pronucleus still appeared round and condensed but consistently incorporated...
significant levels of acetylated histone H4 (Figure 3B). This suggests that the level of maternal 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 [36,37]. To observe H3.3 deposition in the male pronucleus, we used a previously described, maternally expressed Flag-tagged transgenic version of H3.3 (H3.3-Flag) [37]. In contrast to control eggs, H3.3-Flag was not incorporated in paternal chromatin of ywm1 eggs, similarly to Hua mutants (Figure 4A). However, the female pronucleus in ywm eggs still incorporated low levels of H3.3-Flag during the first round of DNA replication, arguing that, like HIRA, YEM does not participate to the limited S phase deposition of H3.3 which occurs in replicating nuclei of early embryos [36] (Figure 4A). As a complementary approach, we analyzed the ywm mutant phenotype using a commercially available monoclonal anti-H3.3 antibody. In wild-type fertilized eggs, the antibody specifically stained the decondensing male pronucleus, but not the maternal chromosomes, thus confirming its specificity for H3.3 (Figure 4B). In agreement with the results obtained with H3.3-Flag, no staining was detected above background when Hua and ywm mutant eggs were stained with the anti-H3.3 antibody (Figure 4C). Altogether, these results demonstrate the critical requirement of YEM for the assembly of H3.3-containing nucleosomes on paternal DNA.

Although mutant ywm1/Df(3R)3450 and ywm1/Df(3R)3450 adults were viable, survival rates were reduced for ywm2/Df(3R)3450 individuals (Table S1) indicating that YEM also functions in somatic cells. Interestingly, the partial lethality of ywm2 mutant individuals was not aggravated when combined with the Hua101 null allele. Thus, HIRA and YEM do not have redundant functions but, instead, are obligate partners not only for male pronucleus chromatin assembly but presumably also for other somatic RI nucleosome assembly processes.

HIRA and YEM cooperate for their localization in 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 [37,39]. Strikingly, while robust HIRA-Flag staining is observed in the decondensing male nucleus in control eggs, HIRA-Flag was not detected in eggs from ywm1 and ywm2 females (n>20; Figure 5D). Thus, YEM is required for the recruitment of, or for the stabilization of HIRA in the male nucleus. As expected, paternal YEM-Flag was also detected in the decondensing male nucleus before pronuclear apposition (Figure 5B). However, in contrast to the homogeneous distribution of HIRA-Flag in the male nucleus, YEM-Flag appeared also enriched in a small number of discrete foci of unknown nature (Figure 5A). We verified that these foci localized neither to the centromeres nor to the telomeres of the male pronucleus (Figure 5C). Interestingly, the formation of these YEM-Flag foci appeared largely independent of HIRA, whereas the rest of YEM-Flag was not detected in a large majority of Hua mutant eggs (Figure 5B). Thus, with the exception of these discrete regions, our experiments demonstrate that HIRA and YEM are interdependent for their localization within the male pronucleus and for paternal chromatin assembly.

Drosophila ATRX/XNP is not involved in paternal nucleosome assembly

Several groups have recently established that in mammalian cells, RI H3.3 deposition is mediated by at least two distinct protein complexes. HIRA and its partners are involved in the enrichment of H3.3 at active genes and at upstream regulatory elements of both active and repressed genes [5]. In contrast, ATRX, a member of the SNF2 family of ATP-dependent chromatin remodeling factors and the histone chaperone DAXX (Death-Associated protein) are essentially responsible for the enrichment of H3.3 nucleosomes at heterochromatin loci [6,58–60]. In Drosophila, the ATRX homolog XNP (or dATRX) colocalizes with H3.3 throughout the chromatin of somatic cells [16]. To investigate the potential involvement of this chromatin remodeler in the assembly of paternal nucleosomes in the newly fertilized egg, we first determined its distribution in oocytes and eggs using a specific antibody recognizing both XNP isoforms [16]. Interestingly, XNP was found to accumulate in the oocyte nucleus, in a way remarkably similar to HIRA and YEM (Figure 6A). However, XNP was not observed in the decondensing male nucleus at fertilization (n>20) and the protein remained absent from early cleavage nuclei until their migration to the embryo periphery, at the synzygial blastoderm stage (Figure 6B and not shown). In addition, we observed that chromatin assembly in the male nucleus occurred normally in eggs from xnp1/xnp2 mutant females (n>20; Figure 6C). Finally, females homozygous for the semi-lethal allele xnp1, which abolishes the expression of the long XNP isoform [61], produced a limited amount of eggs that nevertheless hatched (not shown). We conclude that dATRX/XNP is most likely not involved in the assembly of paternal nucleosomes at fertilization.

Discussion

In human cells, the HIRA core complex is composed of at least three subunits, including HIRA, UBN1 and CABIN1 [43]. This complex is functionally involved in a large diversity of cellular and developmental processes that require dynamic histone turnover or de novo assembly of nucleosomes, independently of DNA synthesis. Although the HIRA complex mediates the deposition of the highly conserved H3.3 histone variant, its subunits display a comparatively weak overall conservation in animals. For instance, Drosophila does not seem to have any CABIN1 homolog and the highest conservation between UBN1 and YEM is mainly restricted to the small HHD domain. Despite this poor conservation, our work establishes Yemanuclein as a bona fide ortholog of Ubinuclein, by demonstrating its physical interaction with the HIRA histone chaperone and its critical requirement for H3.3 deposition during male pronucleus decondensation.

Paternal chromatin assembly is a major function of YEM

In contrast to the knock-out of the Hua gene in mouse, which is zygotic lethal in early embryos [62], null mutants of Drosophila Hua are viable but homozygous females are completely sterile [36]. This indicates that only the maternal contribution of Hua is essential, at least to form the male pronucleus. Our characterization of a null ywm2 allele allowed us to reach the same conclusion for YEM. Remarkably, the phenotype of the male pronucleus in eggs laid by ywm mutant females appeared indistinguishable to what we previously reported for Hua mutants. In both cases, RI deposition of H3.3-containing nucleosomes is practically abolished, typically preventing the full decondensation of the male nucleus and its integration into the zygotic nucleus. Thus, YEM and HIRA are equally required to assemble paternal nucleosomes at fertilization. This unique and major function of the HIRA complex is most likely conserved in animal groups where histones, and notably H3 and H4, are replaced with SNPs in sperm. This is for instance the case of mammals, where protamines package...
about 95% and 85% of mouse and human sperm DNA, respectively [30,32]. In fact, HIRA has been previously detected in the decondensing male nucleus at fertilization in mouse, which incorporates H3.3 before the first round of DNA replication [38,39]. We thus expect Ub-nucleolus 1/2 to be also involved in paternal chromatin assembly in mammals. In apparent contradiction with this prediction, a transgene expressing human UBN1 in the female germline could not rescue the sterility of yem mutant females (Figure S1 and not shown). However, this absence of complementation of YEM and UBN1 can be explained by the strong divergence of these orthologous proteins at the primary sequence level and it suggests that UBN1 can only function within its native, human HIRA complex. The apparent lack of a CABIN1 homolog in Drosophila also underlines the central role played by the HIRA-UBN1/YEM pair in the complex. Interestingly, while the implication of HIRA and UBN1 for RI deposition of H3.3 in rice was recently demonstrated in human cells, CABIN1 seemed to play only an auxiliary role in this context [63]. Possibly, CABIN1 could be important for human-specific functions of the HIRA complex, such as the formation of senescence-associated heterochromatin foci [45,64].

HIRA and YEM are interdependent to target the male nucleus

We had previously shown that HIRA specifically accumulates in the sperm nucleus shortly after its delivery in the egg cytoplasm [57]. Here, we have established that maternally expressed YEM similarly accumulates in the male nucleus at fertilization and until pronuclear apposition. Strikingly, we have also shown that HIRA and YEM are mutually dependent for their targeting to the male nucleus, strongly suggesting that these proteins physically interact during the assembly of paternal nucleosomes. However, nothing is known about the mechanism responsible for their rapid and specific localization in the fertilizing sperm nucleus, which is
delivered in the cytoplasm of the gigantic egg cell. We had previously established that the HIRA-dependent assembly of paternal nucleosomes occurs after the removal of sperm protamines [50]. This opens the simple possibility that the HIRA complex could recognize exposed sperm DNA immediately after the removal of SNRPs. Interestingly, pioneer work on YEM by Ait-Ahmed et al. had established that this maternal protein was able to bind DNA in vitro [51]. This property could be important to efficiently target the HIRA complex to sites of de novo nucleosome assembly in the decondensing male nucleus. This hypothesis has recently received indirect experimental support in human cultured cells [63]. In their study, Ray-Gallet et al. established that HIRA, UBN1 and CABIN1 were all individually able to bind DNA in vitro and they proposed that this remarkable property could allow the HIRA complex to target naked DNA for H3.3 deposition. Accordingly, this HIRA-dependent nucleosome gap-filling mechanism has been shown to participate in the maintenance of genome integrity [63], but could also be employed, at the genome-wide scale, for de novo assembly of paternal chromatin at fertilization.

Finally, the observation that YEM accumulates in discrete nuclear regions in both the male nucleus (this study) and the oocyte karyosome [52] opens the possibility that YEM could perform additional roles not related to nucleosome assembly.

Paternal chromatin assembly is specifically performed by the HIRA-YEM complex

Despite its expression in the female germline, we found that Drosophila ATRX/NXP is not targeted to the male nucleus and does not seem to play any role in male pronucleus formation. Among the 17 SNF2 type chromatin remodelers present in Drosophila [16], the Chromodomain-helicase-DNA-binding protein 1 (CHD1) is the only one that has been implicated in the remodelling of paternal chromatin at fertilization [21,54]. In contrast to Hira and yem, mutations in chd1 do not drastically affect H3.3 incorporation in paternal chromatin but still severely compromise the decondensation of the male nucleus, which appears aberrant in shape [21,54]. In contrast to the HIRA/CHD1 interaction reported by Kovec et al. [54], we could not
Figure 5. HIRA and YEM are interdependent for their recruitment to the male pronucleus. (A) YEM localizes to the decondensing male nucleus. Confocal images of eggs from yem-Flag & yem- females (upper panels) with the male nucleus (arrows) magnified in lower panels. YEM-Flag (green) is detected throughout the decondensing male nucleus but also accumulates in a small number of nuclear foci (arrowheads). Note that YEM-Flag is no longer detected at pronuclear apposition (right panels). Bar: 10 μm. (B) Hira mutations affect the general distribution of YEM-Flag in the male nucleus. Eggs from females of the indicated genotype were stained with anti-Flag antibodies and imaged as in (A). For each male nucleus, the presence of YEM-Flag (whole nucleus and/or foci) was evaluated and each category is represented as a percentage of the total number (n) of observed pronuclei. (C) YEM-Flag foci in the male nucleus do not colocalize with telomeres or centromeres. Upper panels: Nuclear foci of maternally expressed YEM-Flag (red arrow) do not localize to the cluster of telomeres marked with the paternal telomere marker GFP-K81 [69] (green arrow). Lower panels: YEM-Flag nuclear foci (red arrows) do not colocalize with paternal centromeres (green arrows) in the male nucleus. (D) Confocal sections of male pronuclei in eggs laid by 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 not detected. doi:10.1371/journal.pgen.1003285.g005

detect any interaction between these proteins in ovaries, using experimental conditions that permitted co-immunoprecipitation of HIRA and YEM. Our results thus suggest that the role of CHD1 in the male nucleus is distinct from the nucleosome assembly process mediated by the HIRA complex.

Although the implication of the HIRA histone chaperone in paternal chromatin assembly was firmly established a few years ago, it has remained unclear until now if this highly specialized RI assembly process also involved other subunits of the HIRA complex or other histone deposition pathways. In fact, we have previously reported that the histone chaperone ASFI [65], which is known to interact with both the CAF1 and HIRA complexes, was actually absent from the decondensing male nucleus [36]. Although the role, if any, of ASFI in paternal chromatin assembly awaits a proper functional characterization, we do not expect this histone chaperone to be directly involved in the assembly of
mucleosomes on paternal DNA. Accordingly, ASF1 has been previously shown to be dispensable for direct de novo RC or RI histone deposition in *Xenopus* egg extracts [66].

The complete failure of the male nucleus to assemble its chromatin in *Hin* or *jem* mutant eggs demonstrates that no other nucleosome assembly machinery can substitute for the HIRA-VEM complex in this peculiar context. However, the functional requirement of H3.3 itself in this process is not known. In *Drosophila*, H3.3 is not absolutely required for survival but it is essential for both male and female fertility [27,28]. Viability of *H3.3A; H3.3B* double null mutants could be explained by the fact that, in the absence of H3.3, canonical H3 can be assembled in a RI manner [28]. Although the mode of RI deposition of replicative H3 in these mutants is not known, it opens the possibility that HIRA could use canonical H3 in certain critical circumstances, such as a limiting availability of H3.3. This compensatory mechanism, however, is apparently not possible in *Drosophila* spermatocytes, where H3.3 is required for the correct segregation of chromosomes during meiotic divisions, underlining the importance of this variant for sexual reproduction [29]. Similarly, future work should aim at determining whether H3.3 is specifically required for the assembly of paternal nucleosomes at fertilization.
The Germinal Vesicle: A storage compartment for maternal nuclear proteins?

Both HIRA and YEM proteins, which are presumably expressed from germline nurse cells, display a remarkable accumulation in the oocyte nucleus during oogenesis [36,51]. Most of the volume of the large germinal vesicle is devoid of DNA as the maternal genome is tightly packaged within the karyosome. The presence of HIRA and YEM in the nucleoplasm of the GV is thus not related to nucleosome assembly. However, the fact that HIRA and YEM are mutually dependent for their accumulation in the GV suggests that they are stored in this compartment as a complex. In contrast to the null alleles, point mutations do not affect HIRA/YEM localization in the GV, suggesting that the mechanisms controlling their recruitment to the GV or to the male pronucleus are distinct. This could reflect the fact that the HIRA complex is active in the male pronucleus where these proteins are in a chromatin environment in contrast to their nucleoplasm distribution in the GV. Whether or not this transient accumulation of HIRA/YEM in the GV plays any role in the maturation of the complex before paternal chromatin assembly at fertilization remains to be tested. 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 [40,67]. It should be mentioned, however, that dATRX/XNP also accumulates in the GV despite its dispensability for paternal chromatin assembly. A recent study [68] reported the presence of several nuclear proteins in the GV with no known function in the oocyte, suggesting that this structure could serve as a storage compartment for a large number of nuclear proteins.

In conclusion, our characterization of Drosophila Yemanuclein demonstrates that this protein is a functional partner of HIRA in vivo. It also establishes that HIRA and YEM directly cooperate in the male nucleus for the genome-wide replacement of sperm protamines with H3.3-containing nucleosomes. The specific requirement of the HIRA complex in this unique developmental chromatin assembly process implies the existence of specific properties not shared with other H3.3-deposition pathways. In this regard, future work should explore the potentially conserved DNA binding property of the HIRA complex [51,63] and its potential role in targeting the fertilizing sperm nucleus in animals.

Materials and Methods

Flies

Flies were grown in standard conditions at 25°C. The w^{118} stock was used as a wild-type control in all experiments. The Hira^{wm} and Hira^{wm-} alleles and the Hira-flag transgenic constructs have been described earlier [36,37]. For the construction of the Hira-GFP-FLAG fusion gene, the gfp coding sequence was inserted between the Hira and Flag tag sequences of P{VP16-Hira-3xFlag} [37]. The yem^{wm} is a T>A substitution falling in the fifth exon of yem which results in a V478E mutation [51,52]. The GFP-RKI transgene is described in [69]. To mark paternal telomeres we used w^{118}/1; 3R{Ri-GFP-Rki}; 3R{Rki} males [69]. The w^{118}/1; 1.5E-GFP-CdH1.2 [70] stock has been kindly provided by Stefan Heidenmann. The Df(3R)S450 deficiency, the P{E(s)2}; EY23025 insertion and the ymp^{Rki} and ymp^{Rki-} mutant alleles [61] were obtained from the Bloomington Drosophila Stock Center.

Generation of the yem deletion allele

The yem^{wm} mutation was isolated after standard remobilization of the P[Epasc2]EY23025 element and selected for its non-complementation of the yem^{wm} chromosome. yem^{wm} is a 3180 bp deletion from position +2 in the 2'UTR (positions 24345 to 24345 in the genome), uncovering the first 5 exons and part of exon 6 of the yem gene. Note that we only refer in this study to the original gene model [51] identified as RA in Flybase (Flybase ID# FBgn0068913) and not to the recently published longer RB transcript (see Flybase.org).

RT–PCR

Total RNAs were extracted with the Trizol method (Invitrogen) from at least 30 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

yem-marker. The yem EcoRI fragment in blue script vector [51] was digested with Xba1 and Xbal. This fragment was replaced by a PCR amplification product with primers OA37 and OA38, bearing the Flag tag sequence in 3' of yem. Next, the resulted vector was digested with EcoRI and Xba1 and the yem-marker fragment was inserted into the Casper vector. Finally, SV40 polyadenylation signals were added to the previous construct as a Xba1-PstI fragment from the pCasper[AUG-tda] plasmid [71]. The resulting transgenesis construct is called HPI (for HoloProtein flanked with Flag). HPI1 (chromosome X), HPI2 (chromosome 2) and HPI3 (chromosome 3) are independent insertions of HPI. The yem-marker yem^{wm} chromosome was obtained by meiotic recombination. yem-marker yem^{wm} homozygous females (used in Figure 5) are fully fertile (not shown). pUASP-Ub1-4R. A human Ubel1 cDNA [47] was cloned into a BamHI site of the pUASP-attB vector [69]. Transgenic lines were established using the philo31 integrase method [72,73] in the M13Sp-RP-attP[Z/H-868] attP platform in polytene region 86F.

Primers

were dissected in PBS-Trition 0.1% and fixed at room temperature in 4% PFA in PBS for 25 minutes. Ovaries were then stained with propidium iodide and mounted as described above. Slides were observed under an LSM 510 META confocal microscope (Zeiss). Images were treated with LSM image browser, Image J or Photoshop CS2 (Adobe).

We used the following antibodies: AS2 anti-YEM antibody (1/169, [51,74]), M2 monoclonal anti-Flag antibody (1/500 in ovaries, 1/1000 in embryos; Sigma), anti-polyacetylated histone H4 (1/200; Millipore 06-389), monoclonal anti-H3.3 (H3F3B; 1/1000, Abnova), anti-XNP [16] (1/5000) and anti-UBN1 (1/200; [75]). Secondary antibodies were Alexa488 goat anti-mouse or goat anti-rabbit (1/1000, Invitrogen) and Cy3 donkey anti-rabbit (1/800, Millipore).

**Western blots**

50 µl of ovaries were homogenized in lysis buffer (15 mM Heps [pH 7.6], 10 mM KCl, 5 mM MgCl2, 0.3 mM EDTA, 0.3 mM EGTA, 350 mM Sucrose; 1 mM DTT) with protease inhibitors (Halt Protease Inhibitor Single Use Cocktail, Thermo Scientific; 1 mM PMSF). The protein extract was centrifuged, isolated from debris and stock in half volume of glycerol at ~80°C. 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), Peroxidase-coupled goat anti-mouse (1/10000; Beckman).

**Immunoprecipitations**

For co-immunoprecipitation experiments, we essentially used the protocol described in Jäger et al., 2001 [76] with some modifications as indicated. A hundred ovaries were dissected manually in 250 µl lysis buffer on ice. Lysis buffer was as described [76] to the exception of the protease inhibitors. In our conditions, Roche tablets of EDTA-free protease inhibitor cocktail were used as recommended by the supplier. PMSF was also added to a 1 mM final concentration. Before homogenization 250 µl ice-cold lysis buffer were added. The homogenates were cleared by centrifugation and the supernatant was adjusted to 1 ml in lysis buffer. The protein extracts were then submitted to the immunoprecipitation procedure after 2x30 µl were set aside to be used as input in western blot experiments. G-Sepharose beads (Sigma) were used as recommended by the supplier with the following antibodies at a 1/250 dilution: mouse monoclonal Flag M2 (Sigma) for HIRA and the AS2 rabbit polyclonal for YEM. Rabbit preimmune serum was used as negative control. Gel separation and western blots analysis were performed as indicated above. The rabbit GHD1 antibody (a gift from A. Loner) was used at a 1/250 dilution. Secondary antibodies were goat peroxidase-coupled anti-mouse and anti-rabbit antibodies (1/10000; Beckman). Revelation was performed with the Millipore Immobilon Western Chemiluminescent substrate as recommended by the supplier.

**Supporting Information**

Figure S1 Immunodetection of human UBN1 in egg chambers of Act5C-Gal4/+; pUASt-Ubn1-dSrf/+ females, UBN1 accumulates in the germinal vesicle (arrow).

![Figure S1](image)

**Table S1** All the indicated crosses were performed under standard conditions, at 25°C in several non-crowded vials. All the progeny from each cross were 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 normal viability of the y(w) insert allelle and of the y(w) allele. The y(w) allele is viable but shows lower survival rate than y(w). This sub-viability can be rescued with two y(w)/flag insertions, showing that it is indeed a specific effect of the y(w) mutation.

**Acknowledgments**

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**Author Contributions**

Conceived and designed the experiments: GAO OAA BL. Performed the experiments: GAO AA MC TL BS OB A. Performed the analysis: GAO OAA BL. Contributed reagents/materials/analysis tools: HG. Wrote the paper: GAO OAA BL.

**References**


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Supplementary Table 1. Viability of the yem mutant alleles

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<th>[Hu+] progeny (%)</th>
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<td>26* (42.6)</td>
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* Only w* progenies (that received the rescue transgene) were considered in these crosses.

* These are two independent repeats of the same experiment.

All the indicated crosses were performed under standard conditions, at 25°C in several non-crowded vials. All the progenies from each cross were considered, [Hu+] progenies (not carrying a balancer chromosome) were 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 normal 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.
**Supplementary Figure 1**

Immunodetection of human UBN1 in egg chambers of *Act5C-Gal4/+; pUASP-Ubn1-86F/+* females. UBN1 accumulates in the germinal vesicle (arrow).
Conclusion and Future Directions
Conclusion and future directions

Yemanuclein is a key maternal factor required specifically for sexual reproduction

The experimental part of my thesis work is dedicated to the functional analysis of Yemanuclein (formerly called Yemanuclein-alpha). Yemanuclein is encoded by a gene that was overlooked in screens for actors of Drosophila female meiosis. We reported its molecular characterization in an earlier work (Ait-Ahmed et al., 1992), and we showed that yemanuclein RNA is concentrated in the oocyte throughout meiosis I from the earliest germarial stages (Capri et al., 1997). Accordingly we show in the present work that Yem protein was detected in the oocyte nucleus throughout meiosis I. From its specificity for the oocyte nucleus and its affinity for DNA we assumed a role in female meiosis but lack of genetic tools eluded its function for two decades. The first mutation on yemanuclein gene was reported recently by our laboratory (Meyer et al., 2010). This point mutation that substitutes a Glutamic acid by a Valine in a relatively conserved domain (V478E) dramatically affects female fertility. More surprising is the production of exceptional progeny from eggs laid by females homozygous for the yem1 mutation as a result of parthenogenetic development (Meyer et al., 2010). Such a result supposes at least two defective steps in the sexual reproduction process of the mutant mothers. In other words the parthenogenetic adults developed from eggs in which the outcome of female meiosis was a diploid pronucleus. Moreover this diploid maternal pronucleus entered the first zygotic mitosis in absence of syngamy with the paternal counterpart. These considerations were the basis for the two directions of our research to gain insight into the functions affected in the mutant 1) during female meiosis, 2) during the process that results in the biological competence of the paternal pronucleus. Added to the previously published work, the present work contributes to unveil new functions for Yemanuclein that link the formation of the female pronucleus during meiosis and the reprogramming of the male pronucleus in the zygote. To our knowledge these observations have no precedent and highlight the exquisite coordination of events that lead to the formation of the zygotic nucleus.

Yemanuclein meiotic functions

Meiosis is a complex process that relies on two chromosome segregation steps. The specific step is meiosis I that results in the segregation of the homologous chromosomes. To achieve this end meiosis I relies on a specific organization of the chromosomes that is supported by highly specialized protein complexes: the synaptonemal complex and the cohesin complex.
Whereas the overall organization and the composition of the synaptonemal complex are specific for recombination proficient meiosis, the cohesin complex is a versatile structure whose overall composition is similar in mitotic, recombination proficient and non-proficient meiotic chromosomes. Interestingly, Yemanuclein interacts \textit{in vivo} with the two key complexes on which segregation of meiotic chromosome relies. The situation of Yemanuclein is unique in that it is dispensable for the formation of the Synaptonemal Complex. Moreover mutations on Yemanuclein including loss of function alleles do not seem to affect the SC morphology. Either Yemanuclein is loosely associated to the SC with no particular function for its structure or there might be defects that would be detectable at the ultrastructural level only. The functional implication of the interaction of Yemanuclein with the Cohesin Complex also remains to be elucidated. We showed in our previous work that the \textit{yem}\textsuperscript{1} mutation does not affect chromosome cohesion at meiosis I but obviously does affect monopolar orientation of the sister kinetochores. In \textit{S. Pombe}, the same cohesin subunit, the Rec8 kleisin that is paralogous to Rad21, fulfills both functions, cohesion and monopolar orientation whereas in \textit{S. Cerevisiae}, Rec8 is required only for cohesion (Petronczki et al., 2003). There is no Rec8 in Drosophila and in insects in general. How Drosophila ensures these meiosis specific functions is still elusive. The absence of effect of \textit{yem}\textsuperscript{1} mutation on cohesion may be obscured by possible functional redundancy. In contrast, Yemanuclein critical role at early meiosis is clearly demonstrated. Yem is required for the timely formation of the double strand breaks, a result supported by the evidence of a genetic interaction between \textit{yem} and \textit{mei-w68}, the Drosophila homologue of \textit{spo11}. The latter encodes the topoisomerase responsible for the formation of the double strand breaks. Therefore Yemanuclein function is essential for the initiation of the recombination process. Whether Yemanuclein role on DSBs formation depends on its association to the SC and the Cohesin Complex remains to be investigated.

\textbf{Yemanuclein zygotic function}

The following observations prompted us to investigate a possible role of Yemanuclein in the zygote:

- The presence of the Yemanuclein human orthologue, Ubinuclein, in the HIRA complex that supports H3.3 containing nucleosomes assembly (Tagami et al., 2004).
- The only known role for HIRA in Drosophila is precisely its requirement for chromatin assembly of the male pronucleus (Loppin et al., 2005).
- *yem*<sup>1</sup> homozygous mothers give rise to parthenogenetic progeny, a phenomenon that could be explained by defective reprogramming of the paternal pronucleus.

Our experimental data validate a role of Yemanuclein in the zygote. This role is very similar to that of HIRA in the assembly of H3.3-containing nucleosomes on the paternal pronucleus. Accordingly we were able to show an interaction between HIRA and Yemanuclein.

**Future Directions**

The originality of our work on Yemanuclein is its contribution to the unveiling of interesting features of sexual reproduction. To our knowledge, it is the first time that a gene whose specialization for sexual reproduction is so exquisite links two critical aspects: formation of a maternal pronucleus through meiosis and the paternal pronucleus through *de novo* chromatin assembly on the paternal genome. This ensures an interdependency of chromosome number reduction during female meiosis and diploidy restoration after the paternal pronucleus was reprogrammed and made competent for biological functions. Investigating the role of Yemanuclein in other organisms is one of the directions that might help to shed light on the mechanisms that underly its functions. Preliminary data on orthopterans allowed us to show a functional conservation of Yemanuclein during meiosis (collaboration with Julio Rufas, Madrid). More interestingly we aimed to investigate the conservation of Yemanuclein function in aphids, insects that are alternatively sexual or parthenogenetic depending on environmental conditions (collaboration with Denis Tagu, Rennes). The analysis of genes such as *yem* on these insects should provide critical information on the mechanisms that underlie the shift between parthenogenesis and sexual reproduction. Experimental analyses combined to the phylogeny (collaboration with Christophe Terzian, Lyon) will provide invaluable information on the structural and functional evolution of Yemanuclein family of proteins. The specificity acquired by Drosophila Yemanuclein for maternal functions is intriguing. A possible interpretation is that it might have been acquired from the adaptive use of the *Drosophila* protein for specific needs of sexual reproduction (Meyer et al., 2010). It is of special interest to investigate how widespread this specialization is, especially among insects (collaboration with Emmanuelle d’Alençon and Philippe Fournier).

The biochemical characterization of Yemanuclein family of proteins is at its beginning. The evidence that Yemanuclein and its orthologues in Yeast (HPC2) and Humans (UBN1) are part of the HIRA histone chaperone complex is very recent (Balaji et al., 2009; Banumathy et al.,
Yemanuclein was also shown to have DNA binding properties and to be associated to the kinetochores of chromosomes at metaphase I, a stage that is crucial for the reductional division (Ait-Ahmed et al., 1992; Meyer et al., 2010). On the one hand Yemanuclein is highly specialized for sexual reproduction; on the other hand it is involved in various biological complexes and functions. An important issue is the biochemical mechanisms that underlie the various functions. How are they linked? Can the various functions be assigned to specific domains of Yemanuclein? Our work paves the way to future investigations on Yemanuclein and evolution of sexual reproduction, a costly but well conserved strategy.
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**La Yemanucléine de Drosophile est nécessaire à la méiose ovocytaire et l’assemblage de la chromatine paternelle dans le zygote**

*Mots clés : Drosophile, ovocyte, méiose, complexe synaptonémal, cohésine, recombinaison, zygote, chromatine, H3.3, histone chaperone, HIRA*

La reproduction sexuée repose sur deux processus fondamentaux : la méiose qui permet la formation des gamètes dont le génome est haploïde et la syngamie qui permet, après fécondation, de restaurer la diploïdie par fusion des deux noyaux parentaux haploïdes. Alors que la méiose repose respectivement sur le génome maternel pour l’ovocyte et paternel pour le spermatozoïde, la restauration de la diploïdie dans le zygote repose exclusivement sur le génome maternel. Si un pronucleus maternel compétent pour la réplication est formé au terme de la méiose ovocytaire, le génome paternel quant à lui, n’acquiert cette compétence que sous l’influence de facteurs maternels. En effet, à la fin de la méiose, le génome paternel est « empaqueté » avec des protamines qui le rendent inactif pour toute fonction biologique, en particulier la réplication. L’éviction des protamines et leur remplacement par des histones maternelles sont des étapes indispensables à l’acquisition par le génome paternel de sa compétence à la réplication, préalable à la syngamie. Tous ces événements doivent être extrêmement coordonnés afin de permettre à un premier noyau zygotique comportant les deux lots de chromosomes parentaux de se former et d’entrer dans le premier cycle mitotique.

Notre laboratoire a identifié *yemanuclein-alpha*, aussi appelé *yemanuclein* (*yem*) dans un criblé moléculaire pour des gènes exprimés spécifiquement dans la lignée germinale féminelle, et son premier allèle muté *yem*¹. Cette mutation ponctuelle (V478E) a été identifiée dans un criblé génétique de « stérilité femelle ». Une descendance exceptionnelle observée chez les femelles *yem*¹, présente la propriété inattendue d’être parthénogénétique. Cette propriété révèle un double défaut chez le mutant : dans le processus de méiose ovocytaire qui conduit à la formation d’un pronucleus maternel haploïde mais aussi dans la formation d’un pronucleus paternel compétent pour la syngamie.

Mes travaux de thèse ont porté sur les deux aspects de la fonction de la yemanucléine. En conjuguant des méthodes de génétique, de biochimie, et de biologie cellulaire, nous avons pu mettre en évidence des fonctions essentielles de la yemanucléine dans les étapes initiales de la prophase méiotique de l’ovocyte de drosophile. Nous avons pu montrer que la yemanucléine joue un rôle clé dans la recombinaison méiotique et plus particulièrement dans la fréquence et la cinétique d’apparition des cassures double brin. Son association au complexe synaptonémal et au complexe cohésine, tous deux connus comme étant nécessaires à la ségrégation chromosomique, est un élément clé de cette fonction.

Outre cette fonction méiotique, la yemanucléine, facteur maternel, est aussi requise pour l’assemblage du génome de pronucleus paternel. Nous montrons dans ce manuscrit qu’elle joue ce rôle à travers son action dans un troisième complexe, en partenariat avec la protéine HIRA. Le complexe multiprotéique contenant la protéine HIRA est connu pour sa fonction de chaperon du variant de l’histone H3.3 et son rôle dans l’assemblage de la chromatine du pronucleus paternel. La yemanucléine est le premier membre de la famille HPC2/UBN1 caractérisé. Son rôle dans l’assemblage des nucléosomes découpé de la réplication est décrit pour la première fois dans ce manuscrit. C’est aussi la première fois qu’une protéine spécifique de la reproduction est décrite pour son implication à deux étapes clés de ce processus.
Drosophila Yemanuclein is required for meiosis in the oocyte and paternal chromatin assembly in the zygote

*Key words: Drosophila, oocyte, meiosis, synaptonemal complex, cohesin, recombination, zygote, chromatin, H3.3, histone chaperone, HIRA*

Sexual reproduction relies on two key events: formation of cells with a haploid genome through meiosis and restoration of diploidy through syngamy in the zygote. Meiosis completion is supported exclusively by the maternal genome for the oocyte and the paternal genome for the sperm cell. In contrast diploidy restoration in the zygote is entirely dependent on maternal factors. At the end of meiosis the maternal pronucleus is competent for replication, whereas the paternal genome is packed with protamines. These proteins need to be removed in the zygote and replaced by maternally provided histones before the paternal genome acquires competence for replication, a prerequisite for syngamy. All these events must be highly coordinated to allow the first zygotic nucleus to form with the two sets of parental chromosomes and enter the first mitotic cycle.

Our laboratory has identified *yemanuclein-alpha*, also called *yemanuclein (yem)* in a molecular screen for genes specifically expressed in the female germ line and its first mutant allele *yem*1, in a female sterile screen. The role played by *yem* not only in the meiotic process through which a haploid maternal pronucleus is formed but also in the zygotic process that makes a paternal pronucleus competent for syngamy, is underscored by the obtention of exceptional parthenogenetic progeny from *yem*1 mothers.

My thesis work is precisely dedicated to the analysis of both aspects of Yemanuclein function: in the oocyte and the zygote. Using genetic, biochemical and cell biology methods we were able to uncover essential functions of Yemanuclein in early meiotic prophase in the Drosophila oocyte. Using *yem*1 allele (V478E), we could show its requirement for meiotic recombination especially for the frequency and timing of the double strand breaks formation. Yemanuclein association with two protein complexes, the Synaptonemal Complex (SC) and the Cohesin complex known to be required for proper chromosome segregation, supports these findings. Beyond its meiotic function, Yemanuclein is also required in the zygote for assembly of paternal pronucleus chromatin. This is achieved through a third complex that acts as histone H3.3 chaperone. In the present manuscript we identify Yemanuclein as a partner of HIRA in its role in H3.3 nucleosome assembly and deposition on the paternal pronucleus. Interestingly Yemanuclein is the first member of the HPC2/UBN1 protein family ever characterized. The role of Yem/ HPC2/ UBN1 in replication independent chromatin remodeling remained elusive until very recently. Our work is original in that it is the first to report on a role of one member of this family in oocyte meiosis and paternal chromatin assembly in the zygote.

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