The roles of FANCD2 in the maintenance of common fragile site stability

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The roles of FANCD2 in the maintenance of common fragile site stability

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« Je sais que je ne sais rien. »

Socrate.

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ABBREVIATIONS

53BP1	p53-binding protein 1
ADH5	enzyme alcohol dehydrogenase 5
ALDH2	aldehyde dehydrogenase 2
AML	acute myeloid leukemia
AP-1	activator protein-1
APH	Aphidicolin
ATF4	activating transcription factor 4
ATR	ATM- and Rad-3-related protein kinase
ATRIP	ATR-interacting protein
BLM	Bloom syndrome helicase
BMF	Bone marrow failure
BOD1L	biorientation of chromosomes in cell division protein 1-like
BrdU	bromodeoxyuridine
CDK	cyclin-dependent kinase
CFS	common fragile sites
CHK1	Ser/Thr kinase checkpoint kinase-1
ChIP-seq	chromatin immunoprecipitation followed by sequencing
CHOP	C/EBP homology protein
CNVs	Copy number variants
CtIP	CtBP-interacting protein
CTRs	constant timing regions
DDC	DNA damage checkpoint
DEB	diepoxybutane
DNA	deoxyribonucleic acid
DRC	DNA replication checkpoint
eIF2α	eukaryotic initiation factor 2 alpha
ERFSs	early replication fragile sites
ETC	electron transport chain
FA	Fanconi Anemia
FANC pathway	Fanconi Anemia pathway

FL	fetal liver
FoSTeS	Fork Stalling and Template Switching
fs-UFB	fragile site-ultrafine bridge
G4	G-quadruplex
GINS	go ichi ni san
HPV	Human papillomavirus
HR	homologous recombination
HSC	hematopoietic stem cells
HSR	heat shock response
HU	Hydroxyurea
ICL	inter-strand crosslinks
IR	ionizing radiation
ISR	integrated stress response
JMs	joint molecules
MAMs	mitochondrial-associated membranes
MCM	minichromosome maintenance
MMBIR	Microhomology-Mediated Break-Induced Replication
MMC	mitomycin c
MMS	methyl-methane sulfonate
MRN	MRE11-RAD50-NBS1
mtDNA	mitochondrial DNA
MTS	matrix-targeting sequence
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NLS	nuclear localization sequence
ORC	origin recognition complex
OS	oxidative stress
OTCδ	mutant ornithine transcarbamylase
OXPHOS	oxidative phosphorylation
PERK	protein kinase R-like endoplasmic reticulum kinase
PICH	Plk1-interacting checkpoint helicase
PLK1	polo-like kinase-1
Pre-RC	pre-replicative complex

RFS	rare fragile sites
ROS	reactive oxygen species
RPA	replication protein A
RS	replicative stress
SF3B1	splicing factor 3B1
SMC1	structural maintenance of chromosomes protein 1
ssDNA	single strand DNA
TAD	topologically associated domain
TLS	translesion synthesis
TNFa	tumor necrosis factor alpha
TTRs	timing transition regions
UFB	ultrafine bridge
UPR	unfolded protein response
UPRER	endoplasmic reticulum unfolded protein response
UPRmt	mitochondria unfolded protein response
UV	ultraviolet radiation
XP	xeroderma pigmentosum

Résumé de thèse en Français :

Rôles de FANCD2 dans le maintien de la stabilité des sites fragiles communs.

Le manuscrit comporte une introduction, des résultats et enfin une discussion.
Tout d'abord l'introduction décrit tout d'abord le processus de réplication et les sources de stress réplicatif (SR) pouvant mener à l'instabilité des sites fragiles communs (SFC). Les différents facteurs impliqués dans l'instabilité des SFC sont présentés ainsi que les conséquences de cette instabilité. La deuxième partie de l'introduction porte sur le syndrome de l'Anémie de Fanconi (AF) causé par la perte fonctionnelle de la voie FANC présentée pour son rôle dans la réparation des ponts interbrins ainsi que pour ses fonctions non canoniques. Cette partie se conclut par le lien entre l'AF et les SFC et notamment l'instabilité accrue des SFC dans l'AF.

Les résultats sont présentés sous la forme d'un article et d'une annexe. Les résultats mettent en évidence une régulation par FANCD2 de la transcription des gènes présents au niveau des SFC évitant ainsi leur instabilité. Il est également montré que FANCD2 se relocalise spécifiquement au niveau du corps des gènes situés dans les SFC et que ce recrutement est dépendant de la transcription. La suppression de la transcription rend quasi nul le recrutement de FANCD2 et réduit significativement l'instabilité du SFC associé. Au cours de ce travail il a également été montré que la perte de FANCD2 provoque un stress mitochondrial qui induit l'expression des gènes des SFC. La diminution du stress mitochondrial atténue l'augmentation de l'expression des gènes des SFC ainsi que leur instabilité observée après déplétion de FANCD2. Le stress mitochondrial généré par la perte de FANCD2 active le facteur de transcription ATF4 qui est un composant de l'UPR. L'activation de l'UPR induit l'expression des gènes des SFC et la relocalisation de FANCD2 aux SFC. Notre étude montre que FANCD2 est essentiel afin de maintenir l'homéostasie de la cellule en couplant la réplication des SFC et le métabolisme énergétique.
En annexe sont présentés les résultats obtenus après immunoprécipitation de la chromatine par un anticorps dirigé contre l'acétylation de la lysine 16 de l'histone H4 (H4K16Ac) suivi d'un séquençage à haut débit. En résumé, ces résultats montrent que FANCD2 est nécessaire afin de maintenir cette marque au niveau des SFC après un stress réplicatif.

Le manuscrit se termine par une discussion générale qui aborde les nouveautés apportées par mon travail ainsi que les pistes qu'il ouvre et que nous souhaitons tester dans le futur.

INTRODUCTION

1 Les SFC, le talon d'Achille de la réplication de l'ADN.

Avant de se diviser, la cellule doit répliquer son ADN afin de transmettre aux deux cellules filles la même information génétique. La réplication de l'ADN se déroule durant la phase S et est initiée au niveau d'origines de réplication qui sont spatialement et temporellement régulées. Celles-ci ne sont pas définies par une séquence particulière chez les humains mais sont associées à un contexte chromatinien favorable (chromatine ouverte et active). Le complexe ORC se fixe en premier aux origines de réplication puis CDC6, CDT1 et MCM2-7 se fixent à ORC pour former le complexe de pré-réplication (pre-RC). Ce complexe se forme durant la phase G1 qui précède la phase S et est appelé « licensing ». Au cours du passage à la phase S, les pre-RC sont activés grâce à l'activité kinase de DDK et CDK qui permettent la liaison de plusieurs facteurs tels que CDC45 et GINS constituant la forme active des hélicases, intitulé le complexe CMG. Plusieurs autres protéines se lient aux origines formant une large machinerie appelée le réplisome qui permet la synthèse du brin d'ADN complémentaire lors de sa progression. Deux réplisomes démarrent à chaque origine et progressent dans une direction opposée créant ainsi une fourche de réplication bidirectionnelle.

Toutes les origines ne sont pas activées en même temps durant la phase S mais suivent un programme temporel de réplication. Les origines sont regroupées en unités de réplication qui sont-elles mêmes présentes dans des domaines ayant le même programme de réplication. Les régions répliquées tôt ,en phase S, sont associées à une transcription active et à l'euchromatine alors que les régions répliquées tardivement sont associées à une faible densité en gènes et à l'hétérochromatine. Le programme de réplication est très stable dans un type cellulaire mais peut être changé notamment au cours de la différenciation ou du développement. De plus, la cellule possède un excès de pre-RC et n'active qu'une partie d'entre eux au cours de la phase S. Cet excès en pre-RC permet à la cellule de s'adapter à son environnement et notamment en situation de SR qui se définie comme le ralentissement ou le blocage des fourches de réplications. Lorsqu'une fourche est ralentie ou bloquée, une origine à proximité, n'ayant pas

été jusqu'alors activée, peut l'être. Elle est appelée origine « dormante » et permet de répliquer la région et de maintenir la stabilité génomique par un mécanisme de compensation.

Le SR peut avoir plusieurs origines, exogènes ou endogènes. Les sources exogènes peuvent être les rayons ultraviolets, les radiations ionisantes ou encore des drogues chimiques. Beaucoup de ces drogues ont été développées afin de traiter les patients atteints de cancer par chimiothérapie pour bloquer la réplication des cellules cancéreuses. Ces composés peuvent créer des liaisons covalentes dans le même brin d'ADN comme le cisplatin ou entre les deux brins d'ADN comme la mitomycin C (MMC). D'autres composés ne touchent pas directement l'ADN mais interfèrent avec la machinerie de réplication comme l'aphidicoline (APH) qui se lie au site actif des ADN polymerases B et les inhibent ou encore l'hydroxyurée (HU) qui inhibe la ribonucleotide reductase et donc empêche la production de dNTP nécessaire à la réplication. Le SR peut être causé par des sources endogènes à la cellule. Les produits du métabolisme et les aldéhydes peuvent endommager l'ADN et bloquer la réplication. Certaines régions comme les séquences répétées ou les G-quadruplex forment des structures secondaires plus difficiles à passer pour les ADN polymérases. Certains facteurs, essentiels à la réplication, peuvent être limitants et donc empêcher le bon déroulement de la réplication tels que les nucléotides, les histones ou les chaperones d'histones. L'activation d'oncogènes provoque un SR détecté dès les premières étapes du développement tumorale.

Enfin, la transcription peut être un obstacle majeur pour la réplication. Ces deux processus sont généralement séparés temporellement et spatialement afin d'éviter leur rencontre mais celle-ci peut se produire notamment au niveau des SFC où la transcription des grands gènes, présents dans ces régions, nécessite plus d'un cycle cellulaire. Les rencontres face à face entre les deux machineries sont dommageables pour la cellule du fait de la formation de R-loops. Ceux-ci constituent des structures générées par l'hybridation de l'ARN néo-synthétisé avec l'ADN matrice laissant l'ADN non-matrice simple brin. Ces structures sont naturelles et ont des rôles physiologiques notamment dans la régulation de la transcription. Cependant, elles peuvent, en excès, engendrer une instabilité génétique. La cellule peut supprimer ces structures grâce à des enzymes spécialisées (RNase H) ou des hélicases.

Le SR est détecté et résolu par la cellule grâce à la protéine ATR, kinase essentielle à la réplication. ATR phosphoryle de nombreux substrats dont la kinase CHK1. L'activation de ATR/CHK1 permet de bloquer l'avancé du cycle cellulaire et ainsi de laisser le temps à la

cellule de terminer la réplication. Mais en présence d'un faible SR, ATR ne phosphoryle pas ses substrats nécessaires pour bloquer le cycle cellulaire. La cellule ne perçoit donc pas ce faible SR, ce qui peut être dommageable pour les régions plus sensibles au SR comme les SFC.

Les SFC sont définis comme étant des régions chromosomiques formant des discontinuités ou des cassures dans des chromosomes en métaphase après inhibition de la synthèse de l'ADN. Les SFC ont été découverts il y a plus de cinquante ans mais la compréhension des mécanismes causant leur instabilité a énormément progressé ces quinze dernières années. Aujourd'hui, on sait qu'un défaut de réplication est la cause de l'instabilité des SFC. Ce défaut de réplication est dû à différent paramètres épigénétiques expliquant pourquoi la fréquence de cassure des SFC est spécifique à chaque type cellulaire. Il a été montré que la région centrale des SFC est dépourvue d'origines dormantes qui ne peuvent donc pas être activées en cas de SR laissant la région non répliquée en mitose. La présence de grands gènes dans ces régions contribue également à l'instabilité des SFC. En effet, la transcription des grands gènes dans les SFC est nécessaire pour la cassure du site. Le modèle actuel propose que la transcription déplace les pre-RC des grands gènes, empêchant leur activation après SR.

La principale conséquence de l'instabilité des SFC est la formation de délétions et potentiellement la perte d'expression des gènes présents dans ces régions, décrits pour plusieurs d'entre eux, comme suppresseurs de tumeurs. Les dommages aux SFC est un évènement précoce dans le développement tumoral et la perte des gènes des SFC pourrait favoriser ce processus.

2 L'Anémie de Fanconi : un syndrome de prédisposition au cancer, modèle d'étude des SFC.

L'Anémie de Fanconi (AF) est une maladie génétique rare caractérisée par une aplasie médullaire, des malformations congénitales ainsi qu'une forte prédisposition au cancer (leucémies myéloïdes aigües et tumeurs solides de la tête et du cou). Les cellules des patients présentent une forte instabilité génétique qui semblerait être la cause de la prédisposition des patients au cancer. Ces cellules sont hypersensibles aux agents pontant l'ADN ce qui a mené à la découverte du rôle canonique de la voie FANC qui est la réparation des ponts inter-brins. La

maladie est en effet causée par la mutation biallélique d'un des gènes codant pour une protéine composant la voie FANC. Vingt et un gènes ont été identifiés jusqu'à présent mais d'autres pourraient être ajoutés puisque certains patients portent des mutations encore non identifiées. La voie FANC est une voie moléculaire pouvant être divisée en trois groupe : le premier groupe se nomme le FA core et permet l'ubiquitinylation du second groupe qui est l'hétérodimère FANCD2-FANCI. Une fois ubiquitinylé, FANCD2 et FANCI se relocalisent au niveau du dommage afin de coordonner sa réparation en interagissant avec les protéines du troisième groupe qui sont nécessaires à la recombinaison homologue et la synthèse translésionnelle.

Les chercheurs tentent aujourd'hui d'identifier quelle pourrait être la cause physiologique des ponts inter-brins contribuant au phénotype AF. Récemment, les aldéhydes, produits de manière physiologique ou après consommation d'alcool, ont été identifiés comme des cibles intéressantes contribuant à la maladie.

Les protéines FANC ont des fonctions indépendantes de la voie de réparation des ponts inter-brins. Plusieurs études se sont concentrées sur FANCD2, protéine clé de la voie FANC. Des études ont montré que FANCD2 est important dans la protection et le redémarrage des fourches bloquées, l'allumage des origines de réplication ainsi que dans la régulation de l'expression génique. Mais FANCD2, et d'autres protéines FANC, ont également des rôles cytoplasmiques notamment à la mitochondrie. En effet, il a été montré que les cellules AF ont une sensibilité accrue au stress oxydatif et des mitochondries produisant plus d'espèces réactives de l'oxygène (ERO). Récemment, il a été montré que la mitophagie est défaillante dans les cellules AF. Ces mitochondries défaillantes ne sont pas éliminées et recyclées, participant au stress oxydatif présent dans l'AF. Une autre conséquence de cette défaillance mitochondriale dans l'AF est le changement métabolique des cellules AF. En effet, il a été montré que les cellules AF utilisent préférentiellement un métabolisme glycolytique plutôt que la phosphorylation oxydative (OXPHOS) afin de produire de l'énergie. Ce changement de métabolisme est dû à un dysfonctionnement du complexe I de la chaine respiratoire au sein de la mitochondrie. Il est intéressant de noter que le métabolisme a un rôle primordial dans l'activation des cellules souches hématopoïétiques (CSH). Dans l'AF, l'activation des CSH provoque une augmentation de la production des ERO métaboliques ainsi qu'une accumulation des dommages à l'ADN causés par les ERO entrainant la mort de la majorité des HSC.

Dû à son rôle essentiel, la cellule possède des mécanismes de surveillance de l'activité mitochondriale. L'une d'entre elle est la voie de réponse aux protéines mal repliées (UPR). L'UPR a été d'abord décrit dans le réticulum endoplasmique et le cytoplasme mais une réponse similaire a été identifiée dans la mitochondrie. Les protéines mal repliées vont déclencher cette réponse qui va activer un programme transcriptionnel nucléaire qui a pour but de restaurer l'homéostasie de la cellule. Si le stress est trop important, la mort cellulaire par apoptose est alors induite. De manière intéressante, l'UPR a récemment été montré comme importante dans le maintien et la différenciation des CSH.

Les cassures chromosomiques observées dans les cellules de patients AF ne se produisent pas aléatoirement mais majoritairement aux SFC indiquant une sensibilité accrue de ces régions dans cette maladie. Il a été montré que FANCD2 se relocalise au niveau des SFC en mitose et est nécessaire au maintien de leur stabilité après un SR. Récemment, des études ont permis de mieux comprendre le rôle de FANCD2 aux SFC. En effet, il a été montré que FANCD2 facilite la réplication des SFC au niveau des régions riches en R-loops et que ces R-loops sont nécessaires à son recrutement. Au cours de ma thèse, nous avons étudié un nouvel aspect du rôle de FANCD2 dans le maintien de la stabilité des SFC via la régulation de la transcription des gènes présents dans ces régions. Nous nous sommes également intéressés aux modes de régulation de l'expression de ces gènes par FANCD2. Nous décrivons également pour la première fois à quels types de stress ceux-ci répondent.

RÉSULTATS

Puisqu'il a été montré que la transcription est impliquée dans l'instabilité des SFC, nous avons testé si FANCD2 pouvait réguler l'expression des gènes présents dans ces régions. Pour ce faire, nous avons utilisé la lignée cellulaire HCT116 où les SFC ont été entièrement caractérisés. Après déplétion de FANCD2 par ARN interférent, nous observons une augmentation de la transcription des gènes. Nous avons ensuite testé si cette augmentation de transcription est associée à une augmentation de l'instabilité des SFC correspondants. Le grand gène *FHIT* se localise au niveau du SFC FRA3B qui est le plus sensible dans cette lignée. Après déplétion de FANCD2, l'expression de *FHIT* est augmentée tout comme la fragilité de FRA3B. Nous avons également observé une augmentation de l'expression de *PARK2* qui est un grand gène se localisant au SFC FRA6E mais qui n'est pas fragile dans les HCT116. Nous avons donc testé

si l'augmentation de l'expression de *PARK2* après déplétion de FANCD2 influençait la stabilité de FRA6E. Après déplétion de FANCD2, l'instabilité de FRA6E est induite dans les HCT116.

Afin de déterminer si FANCD2 contrôle l'expression de ces gènes en se liant à leurs régions régulatrices, nous avons réalisé une immunoprécipitation de la chromatine grâce à un anticorps dirigé contre FANCD2 suivi d'un séquençage à haut débit (ChIP-seq). La ChIP a été réalisée en absence ou en présence d'un faible stress réplicatif (0,3uM APH), connu pour induire les cassures des SFC et la relocalisation de FANCD2 à la chromatine. Après analyse, nous avons observé que FANCD2 est préférentiellement lié aux gènes, quelle que soit la condition. Nous avons également identifié deux profils de liaison de FANCD2 aux gènes : l'un spécifique aux promoteurs et l'autre où FANCD2 s'étend sur tout le gène. Le second profil est fortement induit après traitement à l'APH et l'analyse de ces gènes montrent qu'ils sont associés, pour leur majorité, à des SFC connus. Grâce aux données disponibles sur ENCODE, nous avons trouvé une corrélation positive entre l'expression des gènes et le recrutement de FANCD2. De plus, en utilisant les marques chromatiniennes associées à la transcription, nous avons mis en évidence que FANCD2 ne se lie aux grands gènes que s'ils sont transcrits. Ces données suggèrent donc que la transcription est nécessaire au recrutement de FANCD2. Afin de confirmer cette analyse, nous avons utilisé une lignée HCT116 dans laquelle le promoteur du gène *FHIT* a été supprimé par CRISPR-Cas9 et donc n'exprimant plus ce gène. Nous avons observé qu'en absence de transcription, FANCD2 n'était quasiment plus présent sur le gène *FHIT*. De plus, dans des cellules transfectées avec un ARN interférent contrôle, l'absence de transcription réduit fortement l'instabilité de FRA3B confirmant un rôle majeur de la transcription dans l'instabilité des SFC. En l'absence de transcription, l'augmentation de l'instabilité après la suppression de FANCD2 n'est plus observée. Ces résultats indiquent que la transcription est nécessaire au recrutement de FANCD2 mais également pour son rôle aux SFC.

Puisque nous n'avons pas identifié FANCD2 au niveau des régions régulatrices des gènes des SFC, nous avons cherché un stress pouvant induire l'expression de ces gènes. Il a été montré que la perte de FANCD2 induit un SR, ainsi nous avons utilisé plusieurs agents induisant un SR et mesuré l'expression des gènes des SFC. Nous n'avons pas observé d'induction des gènes après SR sauf pour *WWOX* indiquant que le stress responsable de leur induction est différent. Plusieurs études ont mis en évidence le rôle important des protéines FANC dans la fonction mitochondriale. Nous avons également observé une dysfonction mitochondriale après la

suppression de FANCD2. En bloquant la chaîne respiratoire mitochondriale, nous diminuons l'expression des gènes des SFC indiquant que ces gènes répondent au métabolisme mitochondrial. Nous avons également mis en culture les cellules à une concentration en oxygène plus faible (3%), ce qui baisse le métabolisme cellulaire et l'activité mitochondriale. À une concentration en oxygène plus faible, l'augmentation de la transcription observée après suppression de FANCD2 à 20% d'oxygène est atténuée. Nous avons également observé une diminution des cassures chromosomiques lorsque les cellules poussent à une faible concentration d'oxygène. Ces résultats indiquent que les gènes des SFC répondent à un stress mitochondrial généré à la suite de la perte de FANCD2.

Nous nous sommes ensuite intéressé à l'UPR, qui est une voie activée après un stress mitochondrial, afin de restaurer l'homéostasie de la cellule. Nous avons trouvé que l'utilisation de drogues activant l'UPR (mitochondrial ou du réticulum endoplasmique) induit l'expression des gènes des SFC. De plus, la suppression de FANCD2 induit l'activation de la voie de l'UPR dépendante d'ATF4 qui est également activée après un stress mitochondrial. Ces données indiquent que l'UPR est activé après suppression de FANCD2, ce qui induit l'expression des gènes des SFC. Nous avons ensuite étudié le rôle de FANCD2 dans cette réponse. De manière intéressante, après l'activation de l'UPR, nous avons observé la formation de foyers FANCD2 dans les cellules et dont certains qui persistent jusqu'en mitose. Nous avons réalisé des ChIP suivis de qPCR et montré que FANCD2 était spécifiquement enrichi aux SFC après activation de l'UPR. Ces résultats montrent que les gènes des SFC répondent à l'activation de l'UPR et que FANCD2 est nécessaire afin de réguler cette réponse. De plus, FANCD2 se relocalise spécifiquement aux SFC après induction de l'UPR afin de prendre en charge les conflits entre transcription et réplication dans ces régions.

ANNEXE

En annexe sont présentés les résultats obtenus après analyse du ChIP-seq de l'histone H4 acétylée sur sa lysine 16 (H4K16Ac). Cette marque chromatinienne est associée à une transcription active, une chromatine ouverte et une réplication active. De plus, il a été montré que FANCD2 interagit avec l'acétyltransférase Tip60 qui acétyle H4K16. Nous avons donc testé si FANCD2 pouvait maintenir la stabilité des SFC via la régulation de la conformation de la chromatine dans ces régions sachant qu'il a été montré que la conformation de la chromatine est un facteur important dans la stabilité des SFC. Nous avons observé une diminution

d'H4K16Ac globale en l'absence de FANCD2 après un SR. Cette diminution est d'autant plus visible dans les régions où FANCD2 est recruté après APH, c'est-à-dire les SFC. Ces résultats indiquent que FANCD2 est nécessaire pour le maintien d'H4K16Ac dans les SFC. Ces résultats restent préliminaires et les mécanismes permettant d'expliquer cette diminution restent à clarifier. Cependant, la perte d'H4K16Ac dans les SFC met en évidence un changement de conformation de la chromatine qui est plus fermée après la perte de FANCD2 et qui pourrait en partie expliquer l'instabilité des SFC dans l'AF.

DISCUSSION

L'AF est une maladie génétique rare qui se caractérise par une forte instabilité génétique notamment aux SFC qui ont été décrits comme participant au développement tumoral. Mon travail avait pour but de mieux caractériser les rôles de FANCD2 dans le maintien de la stabilité des SFC. Nous avons mis en évidence une nouvelle fonction de FANCD2 aux SFC dans la régulation de l'expression des gènes présents dans les SFC. Nous avons également montré que la transcription de ces gènes est nécessaire au recrutement et à la fonction de FANCD2 aux SFC. Pour la première fois, nous avons mis en évidence une possible fonction des gènes des SFC dans la réponse au stress métabolique et à l'UPR. FANCD2 serait un checkpoint métabolique qui permet de coordonner réplication et métabolisme de la cellule. En son absence, un stress métabolique apparaît et la transcription des gènes des SFC est induite, créant des conflits entre réplication et transcription et une instabilité génétique.

L'UPR et son rôle dans la régulation du métabolisme a récemment été mis en évidence dans les CSH. L'activation de ces cellules nécessite un changement métabolique afin de fournir l'énergie suffisante pour proliférer. Cette activation induit également l'UPR qui doit être finement régulé afin de maintenir l'équilibre entre cellules souches et cellules en différenciation. Dans cette étude nous avons montré l'implication de FANCD2 dans l'UPR. Dans l'avenir, nous souhaitons tester, *in vivo,* le rôle de l'UPR dans l'AF et plus particulièrement dans les défauts hématopoïétiques observés dans cette pathologie.

INTRODUCTION

I. CFS, the Achille's heel of DNA replication.

1) Replicative stress: origin and consequences.

a) The replication initiation.

The cell has to completely and faithfully replicate its DNA before dividing to ensure the correct transmission of the genetic information. DNA replication is a fine regulated process in time and space, initiated from individual origins of replication that form bidirectional replication forks. The origin recognition complex (ORC), a complex of six different subunits that has been identified to bind to replication origins, is required for initiation of DNA replication in eukaryotic cells (Dutta and Bell, 1997). The ORC is evolutionary conserved but does not have a specific binding motif in humans (Vashee et al., 2003) even if its binding is not random but correlated with open and active chromatin (Miotto et al., 2016). AT-rich sequences, dinucleotide repeats, asymmetrical purine-pyrimidine sequences and matrix attachment region sequences in the chromatin context seem to facilitate the binding of ORC (Masai et al., 2010).

Additional proteins bind to ORC to form pre-replicative complexes (pre-RC) at replication origins. These proteins were identified through biochemical and genetic studies and are conserved from yeast to mammalian cells. The *Cdc6* gene is transcribed in late mitosis (Zwerschke et al., 1994) and its product is required for proper initiation of replication in yeast and human cells (Hereford and Hartwell, 1974; Saha et al., 1998). Similarly, Cdt1 has been found to be essential for the initiation of DNA replication in yeast (Hofmann and Beach, 1994). Requirement of Cdt1 in DNA replication has also been demonstrated in *Xenopus*, *Drosophilia* and human cells (Maiorano et al., 2000; Whittaker et al., 2000; Wohlschlegel et al., 2000). Cdt1 protein level peaks in G1 and disappears at the beginning of the S phase in human cells (Wohlschlegel et al., 2000). Minichromosome maintenance (MCM) genes were identified in a screen for mutations causing a high rate of minichromosome loss in yeast (Maine et al., 1984). Six MCM genes (2 to 7) appeared to have an important role in the initiation of replication (Tye, 1999). MCM2-7 proteins form a hexameric complex working as an helicase and assembling at

replication origins during early G1 phase and are important for replication initiation but also for elongation (Aparicio et al., 1997; Labib et al., 2000).

To sum-up, pre-RCs are composed by ORC, Cdc6, Cdt1 and MCM2-7 proteins. The formation of these complexes during the G1 phase prims the cell for replication and is called "licensing". ORC binds first to replication origins and this association persists throughout the cell cycle in yeast (Bell and Stillman, 1992). However, the assembly of ORC is different in human cells and is regulated by the dynamic binding of the ORC1 subunit to chromatin during G1 (Ladenburger et al., 2002). When the cells complete mitosis, Cdc6 and Cdt1 are loaded on to chromatin followed by the binding of the MCM complex (Nishitani and Lygerou, 2002).

Pre-RCs are activated at the G1/S phase transition under the control of DDK (Cdc7/Dbf4 kinase) and CDK (cyclin-dependent kinase) protein kinases (Zou and Stillman, 2000). DDK binds transiently to chromatin to phosphorylate the MCM complex, an essential step for the following binding of Cdc45 and GINS (go ichi ni san) complex to form the active replicative helicase (CMG complex) (Ilves et al., 2010; Masai et al., 2010; Moyer et al., 2006; Sheu and Stillman, 2006). Importantly, it has been shown that CDKs activity is also required for CMG complex formation in yeast (Tanaka et al., 2007). The CMG complex unwinds the parental duplex and allows the fixation of DNA polymerase α which performs limited synthesis to initiate replication at origins and Okazaki fragments on the lagging strand (Moyer et al., 2006). Then, polymerases δ and ε perform the bulk of chain elongation on the lagging and leading strands respectively (McElhinny, 2014). Additional proteins bind to this complex to form a large machinery referred to as the replisome, providing its stabilization and protection (Gambus et al., 2006).

To restrict replication to only once per cell cycle and avoid the use of origins that have already been used during the S phase, a process called re-replication, Cdc6 is eliminated from the nucleus (Saha et al., 1998) and Cdt1 is degraded and inhibited by a protein called geminin (Wohlschlegel et al., 2000) at the onset of S phase in human cells.

b) Origin activation: regulation in time and space.

All replication origins are not activated at the same time during the S phase but rather follow a replication timing program (Rhind and Gilbert, 2013). This program is dictated by the complex

organization of the DNA in the nucleus, transcriptional program and concentration of limiting factors (Fragkos et al., 2015). Replication origins are activated in clusters, called replication units or replicons, containing several origins (Cairns, 1966; Huberman and Riggs, 1968). These replicons are associated in replication domains with multiple origins firing with similar timing called constant timing regions (CTRs) and regions between CTRs called timing transition regions (TTRs) replicated by forks emerging from early CTRs toward late CTRs (Desprat et al., 2009; Farkash-Amar et al., 2008; Rhind and Gilbert, 2013) (**Fig. 1**). TTRs are believed to have low origin activity since insertion of an ectopic origin sequence or targeting histone modifications near origins in these regions failed to activate replication (Goren et al., 2008; Guan et al., 2009). However, origin activity has been detected within TTRs (Cadoret et al., 2008; Cayrou et al., 2011).

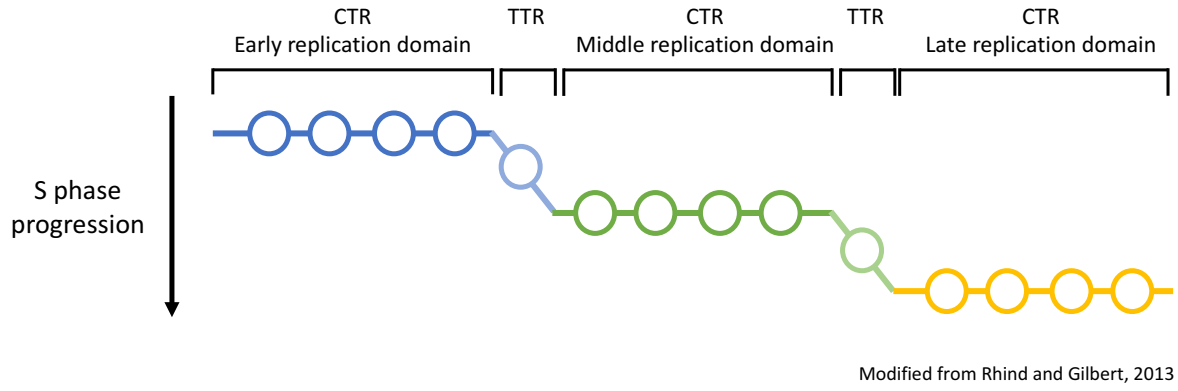


Figure 1: DNA replication timing.

Timing of a hypothetical region containing constant timing regions (CTRs) that replicate at different times during the S phase, and temporal transition regions (TTRs) between them.

Early replicating regions are rich in replication origins and associated with active transcription and active epigenetic marks of euchromatin whereas late replication is observed in regions with few replication origins, low gene density and heterochromatin (Cayrou et al., 2011; Goldman et al., 1984). Replication timing is compartmentalized in mammalian cells. Late replicating regions are located at the periphery of the nucleus, attached to the nuclear lamina, and early replicating regions taking place in the interior of the nucleus (Guelen et al., 2008). The notion that nuclear structure regulate DNA replication has been strengthened by the strong correlation between the three-dimensional organization of chromosomes and replication timing domains (Ryba et al., 2010). Chromatin domains interacting in the three-dimensional space were termed topologically associated domains (TADs) (Dixon et al., 2012). The model proposed is that

TADs are regulatory units of replication timing and TTRs represent the boundaries separating early and late TADs (Pope et al., 2014). These results support the conclusion that early and late replicating regions are spatially separated in the nucleus and replicated as structural and functional units.

Replication timing program is very stable in a given cell type but it can be flexible and changed in certain conditions such as during differentiation and development (Hansen et al., 2010). Importantly, the cell has an excess of pre-RCs and will activate only a subset of these pre-RCs during the initiation of replication (Taylor JH, 1977). When an origin is activated in a replication unit, all the other origins from the same replication unit are repressed by a process called negative origin interference which has been described in human cells (Lebofsky et al., 2006). The differential activation of pre-RCs confers to the cell a plasticity necessary to adapt to its environment. For example, when the replication fork progression is reduced or stalled, referred hereafter as replicative stress (RS), replication is still completed in a given time (which is about 6 to 8 hours for the S phase) to ensure genomic stability by activating dormant origins as a compensatory mechanism (**Fig. 2)** (Woodward et al., 2006). Dormant origins are defined as DNA replication origins that are licensed but do not fire during a normal cell cycle and that are activated if the neighboring replication fork is blocked (Ge et al., 2007).

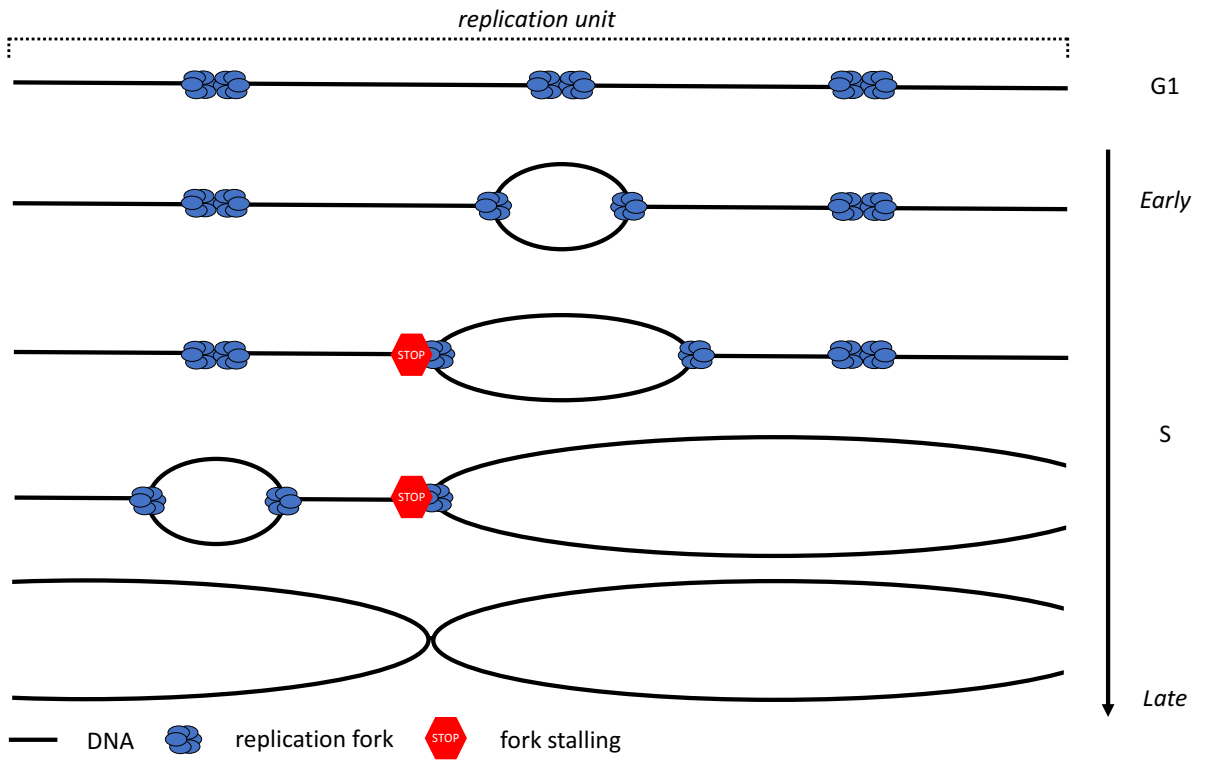


Figure 2: DNA replication completion after replicative stress.

Pre-RCs are licensed during the G1 phase. Some of them fire during the S phase. When a replication fork is blocked, a neighboring dormant origin can be activated to complete the replication.

A major challenge today is to understand what are the parameters influencing origin activation from all the licensed origins. Unlike in yeast, where replication starts at specific AT-rich DNA elements, analysis of *Drosophilia*, mouse, hamster and human cells revealed that replication origins tend to localize in GC-rich regions like CpG islands (Cayrou et al., 2011; Delgado et al., 1998). Interestingly, CpG islands are associated with transcription initiation in vertebrates connecting replication initiation with gene regulation (Deaton et al., 2011; MacAlpine et al., 2004). However, there is not a simple direct link between transcription and origin selection since not all active promoters are efficient sites of replication initiation (Cadoret et al., 2008; Prioleau et al., 2003). The contribution of transcription to replication origin activation, and the reverse, still not clear. Transcription is highly compartmentalized in mammalian nuclei and nascent transcription by RNA polymerase II occurs in foci known as transcription factories (Iborra et al., 1996; Jackson et al., 1993; Wansink et al., 1993). It has been shown in mouse that distal genes co-localize in the same transcription factory and dynamic movement into or out of these factories could regulate their expression (Osborne et al., 2004). We can hypothesize that transcription factors could bring close together replication origins by chromatin looping facilitating the access of replicative machinery at these sites (Chakalova et al., 2005). This

model is concordant with the finding that active genes tend to be early replicated whereas inactive genes replicate late (Goren and Cedar, 2003).

The finding that ectopic insertion of a known replication origin is still able to be recognized by the replicative machinery in human cells provide the evidence that the sequence have an important role in origin identification (Malott and Leffak, 1999; Paixao et al., 2004). G-quadruplex (G4) are a four-stranded DNA structure formed at G-rich DNA motifs. G4 have been detected at replication origins and are thought to have a major role in replication initiation (Besnard et al., 2012; Valton et al., 2014). How G4 favor replication initiation is still under investigation but it has been shown that G4 are correlated with nucleosome free regions that are accessible for the pre-RC assembly (Fenouil et al., 2012). It is possible that the folding of G4 could be used by the cell to regulate activation of the pre-RCs in different contexts (Cayrou et al., 2015).

c) Replicative stress.

The replisome can face different obstacles during its progression, with intracellular or extracellular origins, causing RS. Detection and response to RS is an important issue for the cell because the failure to deal with it can lead to different diseases and malignancy.

i) Sources of RS.

- <u>Exogenous sources:</u>

The most common exogenous sources of RS are ultraviolet radiations (UV), ionizing radiations (IR) and genotoxic compounds by inducing a broad spectrum of DNA lesions (reviewed in Vesela et al., 2017).

UV can cause physical barriers to replicative forks by inducing various DNA lesions such as cyclobutane pyrimidine dimers or pyrimidine 6-4 pyrimidone photoproducts (Cadet et al., 2005). UV-induced DNA lesions are almost exclusively removed by the nucleotide excision repair (NER) pathway. Patients with xeroderma pigmentosum (XP) are extremely sun sensitive

and have a very high risk of developing skin cancer due to genetic mutations in components of the NER cascade (Dupuy and Sarasin, 2015).

Different chemicals were developed to block cell replication with the aim of inhibit cancer growth. Crosslinking agents introduce covalently bonds between nucleotides located on the same strand (intrastrand crosslinks) like cisplatin, or opposite strands (interstrand crosslinks (ICLs)) like mitomycin C (MMC) and psoralens. These chemicals inhibit strand uncoiling or separation by the replicative fork thus blocking replication (Deans and West, 2013). ICLs are particularly toxic for the cell and mainly repaired by the Fanconi Anemia pathway (FANC pathway), which will be exposed in further details later in this manuscript. Alkylating agents, like methyl-methane sulfonate (MMS), attach an alkyl group to the DNA that represent a barrier for replicative forks (P. Groth et al., 2010).

Other compounds do not directly damage DNA but rather interfere with replication-related enzymes. Camptothecin inhibits the topoisomerase I which is required to relax DNA supercoiling generated by replication (Pommier, 2006). Camptothecin derivatives like topotecan and irinotecan are used as anti-cancer treatments. Aphidicolin (APH) is an inhibitor of B-family DNA polymerases binding to their active sites which leads to the uncoupling of helicases and polymerase activities, generating long stretches of single-stranded DNA (ssDNA) (Chang et al., 2006). Interestingly, APH is a specific inducer of common fragile sites (CFS) at low concentrations (Glover et al., 1984). Hydroxyurea (HU) inhibits the ribonucleotide reductase enzyme, thus blocking DNA replication by starving the DNA polymerases for deoxynucleoside triphosphates (Bianchi et al., 1986).

How patients acquire resistance to chemotherapeutic agents is a fundamental question in clinical research today.

- Byproducts of cellular metabolism

Folate metabolism, referred as one-carbon metabolism, is supporting different physiological processes such as nucleotide production, amino acid homeostasis, and epigenetic maintenance (Newman and Maddocks, 2017). This essential cycle also releases formaldehyde which is a potent DNA crosslinking agent (Burgos-Barragan et al., 2017). Thus, endogenous formaldehyde is a source of DNA damage and the cell has a defense mechanism composed of

two steps: first, formaldehyde can be removed by the enzyme alcohol dehydrogenase 5 (ADH5) and second, the repair of DNA damage that can be induced by this molecule is mediated by FANCD2 (Pontel et al., 2015). Other aldehydes are likely to be a source of DNA damage like acetaldehyde which is a by-product of several metabolic pathways but also massively produced after ethanol exposure (Garaycoechea et al., 2018). Interestingly, double-mutant mice deficient for the mitochondrial enzyme aldehyde dehydrogenase 2 (Aldh2) and Fancd2 are more sensitive to ethanol exposure, pointing out the critical role of FANCD2 as a protection against acetaldehyde-mediated DNA damage (Langevin et al., 2011).

A part of the one-carbon metabolism occurs in the mitochondria which is a potent producer of reactive oxygen species (ROS) due to the respiratory chain (Turrens, 2003). ROS are physiological and are important for cell communication but can, when in excess, damage mitochondrial and nuclear DNA (Croteau and Bohr, 1997). Interestingly, it has been shown that oxidative stress can reduce replication fork velocity, thus creating a RS (Wilhelm et al., 2016).

- Difficult to replicate regions

Repetitive DNA sequences can form secondary structures like slipped DNA, CTG slip-outs, triplex DNA and CAG slip-outs (Castel et al., 2010). Unusual non-B DNA structure formation is energetically favored on single stranded DNA, as for example during lagging strand replication, and can in turn interfere with the progression of the DNA polymerase (Mirkin and Mirkin, 2007). It is worth to note that expansion of repetitive DNA sequences in genes is implicated in many developmental and degenerative diseases by altering gene expression (either loss of or aberrant expression) (Gatchel and Zoghbi, 2005). For example, CGG repeat in the 5' UTR of the *FMR1* gene results in transcriptional silencing and loss of its protein product, FMRP, causing the fragile X syndrome FRAXA which is one of the most common form of inherited mental retardation (Bassell and Warren, 2008).

Counterintuitively to their role in replication initiation, G4 can inhibit replication fork progression (Cea et al., 2015; Paeschke et al., 2011). G4 formation occurs especially during DNA transitions like replication and transcription that use ssDNA as an intermediate, posing challenges to these processes (Maizels and Gray, 2013). In human cells, helicases such as BLM, WRN, FANCJ, CHL1, PIF1 and RTEL1 can recognize and unwind G4 (Ding et al., 2004; Fry and Loeb, 1999; London et al., 2008; Sanders, 2010; H. Sun et al., 1998; Y. Wu et al., 2012).

Deficiency in some of these helicases can cause genetic diseases, like Bloom, Werner or Fanconi Anemia syndromes, characterized by genomic instability notably at G4 sequences (Crabbe et al., 2004; London et al., 2008; Y. Wu et al., 2008). G4 importance in telomere maintenance has raised a strong interest for the design of G4-targeting drugs to inhibit cancer growth (Haider et al., 2011). However, G4 structures are not exclusively found at telomeres and could induce off-target effects.

Replication across G4 seems to require the intervention of specialized Y-family polymerases called translesion DNA synthesis (TLS) polymerases that contain a larger catalytic site allowing the passing through damaged templates but in a error-prone way (Goodman and Woodgate, 2013). It has been shown, *in vitro*, that the TLS polymerase polη can bind a G4 substrate and replicates across G4 with higher efficiency and fidelity than the B-family polymerase polε (Eddy et al., 2015).

G4 are also enriched in ribosomal DNA and promoter regions indicating that these structures have other important biological functions (Bochman et al., 2012; Tang et al., 2016).

- Limiting factors

Replication requires essential factors like nucleotides, replication machinery components, histones and histone chaperones which may become a source of RS if deregulated.

Regulation of the nucleotide pool is critical to ensure fork progression. Changes in this pool affect replication dynamics (Poli et al., 2012) and nucleotide depletion is thought to have a critical role in the early stages of cancer development (Bester et al., 2011).

Disruption of proteins composing the pre-RCs leads to genomic instability and genetic disorders. ORC2 deficiency destabilize replication dynamics in *Drosophila* (Loupart et al., 2000). *ORC1, 4, 6, Cdc6* and *Cdt1* mutations are found in the Meier-Gorlin syndrome which is characterized by short stature, microtia and hypoplasia/absence of the patella (Bicknell et al., 2011; Guernsey et al., 2011). Reducing the level of a single MCM leads to genomic instability in yeast (Liang et al., 1999). MCMs contribute to cancer progression since increased chromosome breaks after replicative stress and cancer predisposition is observed when Mcm4

function is compromised in mice and the MYCN transcription factor, amplified in neuroblastoma, upregulates MCM7 expression contributing to the proliferation of these cells (Shima et al., 2007; Shohet et al., 2002). A mutation in *MCM4* has been reported in individuals with immune deficiency, genome instability, and adrenal failure (Gineau et al., 2012; Hughes et al., 2012). Interestingly, excessive licensing in the same cell cycle (re-replication) is also promoting genetic instability and cancer (Hook et al., 2007). Excess Cdt1 in mice promotes cancer, and excess Cdt1 or Cdc6 in human cancer is correlated with excess genetic instability and a poorer prognosis (Arentson et al., 2002; Karakaidos et al., 2004).

Replication requires a large amount of histones because the parental nucleosome is disrupted ahead of the moving replication fork and the two new nucleosomes must assemble on the daughter strands with new and recycled histones to reproduce nucleosomal density (Groth et al., 2007). Formation of nucleosomes by parental and new histones is dependent on CAF-1 and the histone chaperone ASF1 (Groth et al., 2007), and disruption of these two proteins leads to severe genomic instability (Myung et al., 2003). Inhibition of histone biosynthesis impairs replication fork progression and generates DNA damage (Mejlvang et al., 2014).

Conflicts between replication and transcription:

Replication forks can pause during the normal process of replication at replication fork barriers (RFBs). DNA sequence at a RFB is not sufficient *per se* to stall replication (López-Estraño et al., 1999) and tightly bound proteins are thought to be responsible for replication fork stalling as for example the Tus protein which binds the terminator sequence in the *E. coli* genome (Hill and Marians, 1990) and the transcriptional machinery at ribosomal DNA in *E. coli* and human cells (Akamatsu and Kobayashi, 2015; French, 1992). Transcription has been identified as a factor that can impair replication fork progression (Prado and Aguilera, 2005), and to avoid conflicts, replication and transcription are generally temporarily and spatially separated to different domains (Wei et al., 1998). Strikingly, essential genes tend to be transcribed co-directionally to replication to preserve genome integrity (Sankar et al., 2016; Srivatsan et al., 2010) even if collisions can be a major source of gene evolution (Paul et al., 2013).

However, transcription can occur during S phase (Cho et al., 1998) and collisions can take place as illustrated by cells depleted for topoisomerase I showing breaks at the highly expressed *SRSF3* gene (Tuduri et al., 2009) or in regions called common fragile sites (CFS) with very

long genes that require more than one cell cycle to complete their transcription (Helmrich et al., 2011).

The replicative and transcriptional machineries can collide either head-on or co-directionally. Head-on collisions are particularly detrimental for DNA replication and genome stability (Sankar et al., 2016). Recently, the role of R-loops in head-on collisions has been highlighted (Hamperl et al., 2017; Lang et al., 2017). R-loops are generated by the hybridization of the nascent RNA with the template DNA, leaving the non-template DNA single-stranded. This process is natural and enriched at promoters and terminators but can be a threat for genome stability if persistent (Aguilera and García-Muse, 2012), notably after RS, by blocking replication fork progression at transcribed genes (Gomez-Gonzalez et al., 2009). When both machineries meet head-on, positive supercoil formation changes the topology of DNA between them and may promote R-loops formation (Gomez-Gonzalez et al., 2009). The resolution of these structures at head-on conflicts is essential for cell survival after a stress and avoid mutagenesis (Hamperl et al., 2017; Lang et al., 2017). RNA:DNA hybrid structures are removed in the cell by RNase H enzymes (Cerritelli and Crouch, 2009) or specialized helicases (Boué and Zakian, 2007). Interestingly, co-directional collisions were found to be less prone to form R-loops due to the clearance of such structures by active replication forks (Hamperl et al., 2017). Moreover, it has been shown in yeast that the cell can displace the RNA polymerase from the DNA and use the transcript as a replication primer (Pomerantz and O'Donnell, 2008).

The identification of unstable regions called early replication fragile sites (ERFSs) that colocalize with highly expressed genes strengthens the idea that transcription can be challenging for replication (Barlow et al., 2013).

Overexpression or constitutive activation of oncogenes:

Oncogenes are essential regulators of cell growth and can cause unregulated proliferation of the cell when overexpressed or constitutively activated. The activation of oncogenes is a major source of RS which is already detected in pre-cancerous lesions (Gorgoulis and Vassiliou, 2005). Different mechanisms underlying oncogene-induced RS have been identified: Myc overexpression for example alters DNA replication by increasing origin firing, origin density and asymmetrical fork progression, possibly by enhancing Cdc45 recruitment to replication origins (Srinivasan et al., 2013). Another interesting work focused on Cyclin E overexpression

and report that RS associated with this oncogene results from increased origin firing and increased interference between replication and transcription (Jones et al., 2012). A recent work further described the role of transcription in oncogene-induced RS by detecting aberrant firing of intragenic origins after oncogene activation leading to conflicts between replication and transcription (Macheret and Halazonetis, 2018).

In this first chapter, I have highlighted the importance for the cell to precisely coordinate replication and what are the different sources of RS. It is fundamental for the cell to detect and deal with RS to prevent the emergence of a pathological situation like for example tumor development.

ii) Detection and resolution of RS.

The different sources of RS described before result in replication fork stalling that will activate the S phase checkpoint, and more precisely the DNA replication checkpoint (DRC) which differs from the response to DNA damage (DDC) even if both of these pathways share many effectors (Técher et al., 2017). A major response to RS is the recruitment and the activation of the ATM- and Rad-3-related (ATR) protein kinase (Flynn and Zou, 2011). ATR, and its regulatory partner ATRIP (ATR-interacting protein), are recruited to replication protein A (RPA)-coated ssDNA generated by uncoupling of replicative MCM helicases from DNA polymerases (Cortez, et al., 2001; Lee Zou and Elledge, 2003). The length of ssDNA coated with RPA is critical for ATR activation. In *Xenopus* egg extracts, it has been shown that the uncoupling between replicative polymerases and helicases, using APH, cis-platinum or UV radiation, is necessary for ATR activation (Byun et al., 2005). ATR activation leads to the phosphorylation of many substrates like p53, BRCA1 and CHK1 to amplify the signal, delay cell cycle progression, start repair and recover stalled replication forks (Cimprich and Cortez, 2008; Siliciano et al., 1997; Tibbetts et al., 2000).

The Ser/Thr kinase checkpoint kinase-1 (CHK1) is the best studied substrate of ATR. CHK1 signals DNA damage to the rest of the nucleus after its activation by ATR at the replication fork thanks to its interaction with Claspin which brings ATR and CHK1 together at the replication fork (Kumagai and Dunphy, 2000). Phosphorylated RAD17 also interacts with Clapsin and is important for CHK1 activation (Wang et al., 2006). Once phosphorylated, CHK1 is released from chromatin and phosphorylates its substrates such as CDC25 phosphatases,

inhibiting their activity, preventing CDK activation and entry to mitosis (Furnari et al., 1997; Peng et al., 1997; Sanchez et al., 1997; Smits et al., 2006).

Different intensities of RS can affect replisome progression leading to an adapted response of the cell:

ATR and CHK1 are essential for S progression even in unperturbed conditions since their depletion leads to genomic instability (Casper et al., 2002; Syljuasen et al., 2005). As discussed before, RS has endogenous sources and ATR/CHK1 might be necessary to deal with these physiological stresses.

Upon mild RS, ATR activation does not lead to the phosphorylation of CHK1, p53 or RPA2 that are essential to block cell cycle progression (Koundrioukoff et al., 2013). Thus, the cell seems to be blind or to tolerate mild RS and cell cycle can progress. This situation may be detrimental for RS sensitive regions like CFS which are late replicating and can undergo through mitosis without completing their replication (Le Beau et al., 1998).

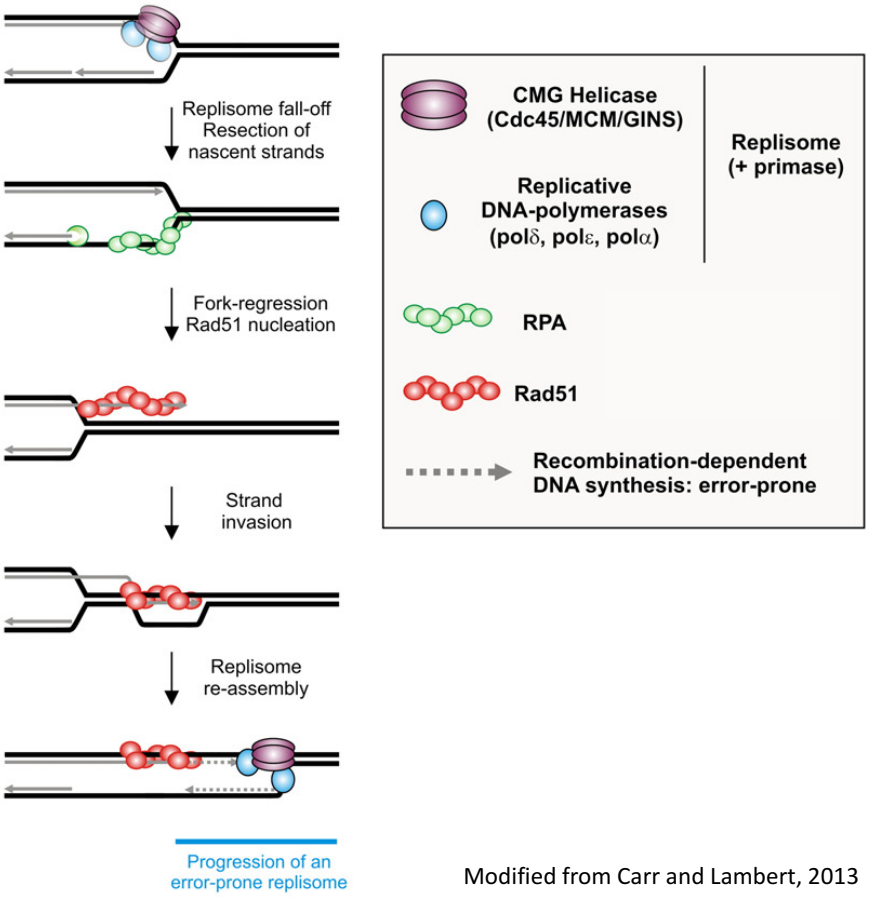
Upon stringent fork slowing, the checkpoint is completely activated, blocking cell cycle progression (Lossaint et al., 2013). Using yeast as a model, it has been shown that Mec1 and Rad53, the ATR and CHK1 homologs, are necessary to suppress late origin firing and maintain fork stability (Lopes et al., 2001; Santocanale and Diffley, 1998; Tercero and Diffley, 2001). Inhibition of origin firing upon RS by ATR prevents exhaustion of RPA and replication catastrophe (Toledo et al., 2014). It has been shown in yeast and *Xenopus* eggs extracts that ATR inhibits the binding of CDC45 to chromatin thus preventing origin firing (Aparicio et al., 1999; Costanzo et al., 2003). However, even if ATR signaling inhibits global origin firing under RS, it has been shown in *Xenopus* eggs extracts that it also promotes "local" origin firing close to stalled forks by the phosphorylation of MCM2 that allows the binding of the polo-like kinase-1 (PLK1) promoting CDC45 association to the chromatin (Trenz et al., 2008). This local activation of dormant origins by ATR might be used by the cell to complete replication of regions posing problems (Ge et al., 2007; Kawabata et al., 2011; Woodward et al., 2006).

Stalled forks can collapse if synthesis cannot be resumed and replisome is dissociated from the DNA. In this context, forks can be restarted by a mechanism called fork reversal or regression (reviewed in Neelsen and Lopes, 2015). This mechanism has been mostly described in yeast

and the precise role of the proteins involved in eukaryotes is still under investigation. However, homologous recombination (HR) is necessary for this process and has a major role at replication forks by acting at multiple levels: (i) repair of ssDNA gaps left behind moving forks, (ii) protection of nascent forks from resection, and (iii) rebuilding of a replisome at inactivated forks (Carr and Lambert, 2013).

The model of recombination-restarted replication (**Fig. 3**) implies a first step of resection of nascent strands which might help the regression of the fork. Different proteins have been shown to avoid excessive resection of the newly synthetized DNA like ATR, BRCA1, BRCA2, RAD51 and FANCD2 (Couch et al., 2013; Schlacher et al., 2011; Schlacher et al., 2012). After resection, RAD51 can nucleate on the exposed ssDNA and form a stable filament that promotes the invasion of the parental duplex DNA, to form a D-loop on which a replisome could be reassembled. Notably, BRCA2 can bind to RAD51 and promotes its filament assembly on ssDNA (Jensen et al., 2010).

Recombination-restarted replication is useful in regions that cannot activate dormant origins like fragile sites to complete their replication (Letessier et al., 2011; Ozeri-Galai et al., 2011). However, it has been shown that forks restarted by HR are error prone and could contribute to the generation of genomic instability (Lambert et al., 2005; Mizuno et al., 2013).



Modified from Carr and Lambert, 2013

Figure 3: Restart of fork collapse by HR.

Model for replication restart by HR: replisome dissociates from DNA and nascent strands are degraded. Fork regresses and RAD51 nucleates on the exposed DNA, promoting strand invasion for replisome rebuilding by HR.

Some regions in the genome, called CFS, are particularly sensitive to RS. Instability of these regions is thought to be implicated in the earliest steps of tumorigenesis (Bartkova et al., 2005; Gorgoulis and Vassiliou, 2005). Thus, characterizing the molecular basis of CFS instability is critical to better understand and fight cancer.

2) Fragile sites and the basis of CFS fragility.

Common Fragile Sites (CFS) are defined as chromosomal regions forming gaps or breaks on metaphase chromosomes after partial inhibition of DNA synthesis (Glover et al., 2005). The first fragile site was described in the 70s in the long arm of chromosome 16 and associated to the haptoglobin locus (Magnesis et al., 1970). It was later discovered that other fragile sites could be induced by specific cell culture conditions, for example when folic acid or thymidine was absent in the media, or when cells were treated with low doses of APH, linking

chromosome fragility to DNA replication stress (Glover et al., 1984; Jacky et al., 1983). This lead scientists to reconsider the importance of these breaks and define two categories of breaks: rare and common fragile sites (RFS and CFS, Glover et al., 1984). While RFS are observed in a small proportion of individuals and are inherited in a Mendelian manner, CFS are present in all individuals (Durkin and Glover, 2007). The instability of RFS is now well understood and relies on their unstable DNA sequence (CGG-repeat expansion or AT-rich minisatellite repeats) (Hewett et al., 1998). On the contrary, the basis of CFS instability has been matter of deep investigation and is still under debate today even if a significant progress in our understanding of the mechanisms regulating their instability has been made.

The different mechanisms presented here were discovered after long periods of investigation. Now, it becomes clear that CFS instability does not rely on only one of these mechanisms but on the combination of them.

a) A replicative problem.

An intrinsic replication difficulty has been the prime suspect in induction of CFS instability because it had been first discovered that CFS breakage was induced after perturbation of DNA replication (Glover et al., 2005).

The first hypothesis to explain the difficulty to replicate CFS was that CFS are composed of DNA sequences able to form secondary structures, and therefore hard to replicate. However, the implication of such sequences is still under debate (Le Tallec et al., 2014). Some studies claim that CFS are enriched in AT-rich sequences leading to a higher flexibility of DNA (Dillon et al., 2013; Fungtammasan et al., 2012; Lukusa and Fryns, 2008) while others failed to identify such sequences (Helmrich et al., 2006; Tsantoulis et al., 2008). Today, the model that CFS sequences *per se* can explain their fragility has been replaced by an epigenetic model accounting for the cell specific instability of these sites.

The well-studied CFS FRA3B, the most unstable fragile site when all cell types are considered, was found to be replicated late during the S phase using a combination of pulse-labeling cells with bromodeoxyuridine (BrdU) associated with Fluorescent In Situ Hybridation (FISH) (Le Beau et al., 1998). This study also showed that FRA3B replication was further delayed by adding APH resulting in a failure to complete DNA replication before mitosis. These findings

were validated for other CFS (Hellman et al., 2000; Palakodeti et al., 2004). However, CFS are not the only regions late-replicated in the genome. Something was missing to understand the replicative problem in CFS and a major understanding of CFS replication was achieved by the Pr. Debatisse's team, by mapping the initiation and termination events at FRA3B in lymphocytes and fibroblasts (Letessier et al., 2011). This work confirmed the late replication of FRA3B and explained the observation made many years ago by Murano *et al.* in 1989 that CFS breakage frequency was different between different cell types. Indeed, when analyzing the replication program in lymphocytes and fibroblasts, Letessier *et al.* observed that the central region of FRA3B, called the "FRA3B core", which is the most fragile part of the site, was free of initiation events in lymphocytes but not in fibroblasts even after APH treatment (**Fig. 4**). This paucity of initiation events correlates with the fragility in these two cell types (FRA3B being fragile in lymphoblasts but not or very weakly in fibroblasts), and exclude the idea that only the sequence features account for CFS instability. However, at least in some instances, the high AT rich content of specific CFSs can perturb, slow and stall replication forks (Ozeri-Galai et al., 2011).

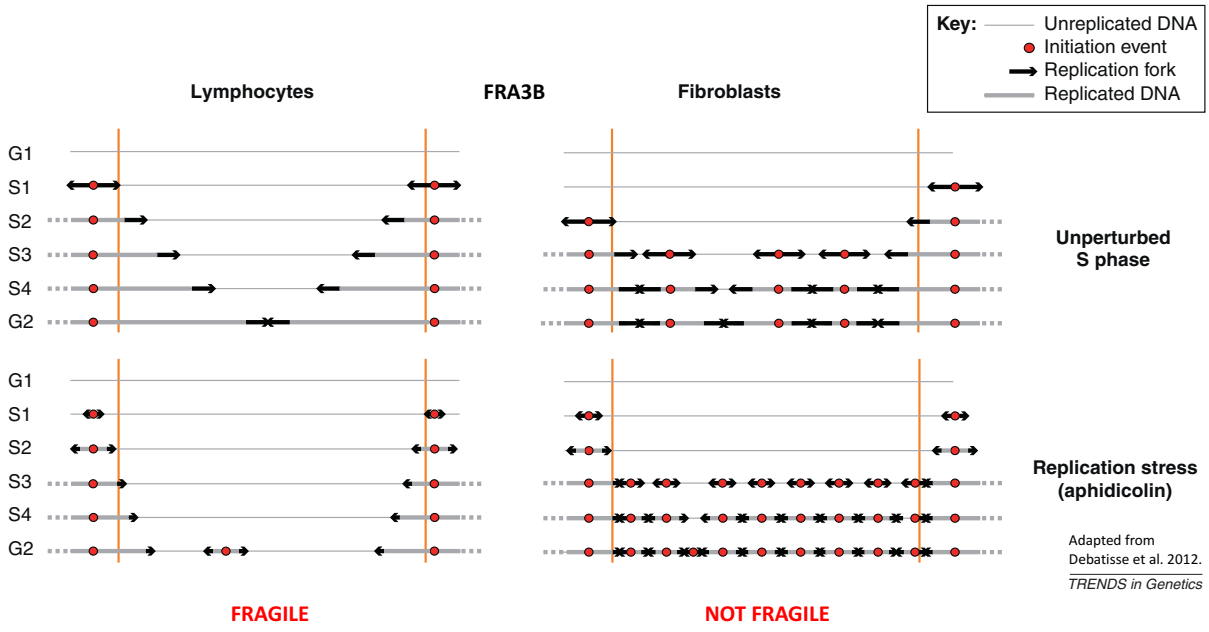


Figure 4: Cell specificity of CFS replication and fragility.

Replication timing of the CFS FRA3B in lymphocytes and fibroblasts. During an unperturbed S phase, replication is completed before mitosis in both cell types whereas, after a replicative stress, dormant origins fire to complete the replication of FRA3B in fibroblasts but not in lymphocytes.

b) Transcription.

The suggestion that CFS were associated with active gene transcription came with the observation that breaks induced by APH occurred in light G bands which tend to contain active genes (Hecht, 1988). It has also been observed that a large number of fragile sites can be induced by DNase I treatment which is known to target accessible chromatin sites associated with regions of active genes (Yunis and Hoffmann, 1989). Another evidence for the implication of transcription in CFS instability comes from the analysis of the fragile site Xp22.3, which maps in an active region and is breaking in both X chromosomes in females whereas Xq22.1, which is located in an inactivated region, is breaking only on the active X chromosome (Finley Austin, 1991).

The cloning and characterization of CFS lead to the identification of very large genes present in, or in close vicinity of, these regions which are conserved through evolution and frequently deleted in cancer (Smith et al., 2006) (**Table 1**).

Common Fragile Site	Gene associated	Size of the large gene (kb)
FRA3B	*FHIT*	1502
FRA3L	*LSAMP*	643
FRA4F	*CCSER1* *GRID2*	1475 1468
FRA6E	*PARK2*	1380
FRA7I	*CNTNAP2*	2305
FRA7K	*IMMP2L*	899
FRA16D	*WWOX*	1113
FRA20B	*MACROD2*	2058
FRAXC	*DMD*	2220

Table 1: Very large genes frequently deleted in tumors with their associated CFS.

List of the very large genes frequently deleted in cancer associated with CFS and their size.

Helmrich *et al*. showed that the transcription of these very large genes is a dilemma for the cell because, due to their size, their transcription requires more than one cell cycle. Thus, collision with the replicative machinery is inevitable. In agreement with this idea, they showed that CFS

breakage is present only if the gene is expressed in a given cell type. This study also revealed that CFS are preferential sites of R-loop formation which is exacerbated by APH treatment. These stable structures will perturb DNA polymerase progression even more in a situation of RS (Gomez-Gonzalez et al., 2009; Hamperl et al., 2017). Interestingly, the loss of DNA repair factors like BRCA1, BRCA2 or Fanconi Anemia proteins increases the formation of such structures (Bhatia et al., 2014; Hatchi et al., 2015; Schwab et al., 2015).

The contribution of collisions between transcriptional and replicative machineries is still under debate because CFS breakage is not always occurring inside the genes (LeTallec et al., 2013). However, even if the transcription of the very large genes is not sufficient to induce CFS breakage, it seems necessary. Interestingly, it has been shown in yeast that transcription can displace the components of pre-RC from DNA (Gros et al., 2015; Lõoke et al., 2010). The movement of the RNA polymerase in these very large genes which tend to be late replicated could explain the lack of dormant origins firing after a replicative stress (**Fig. 5**).

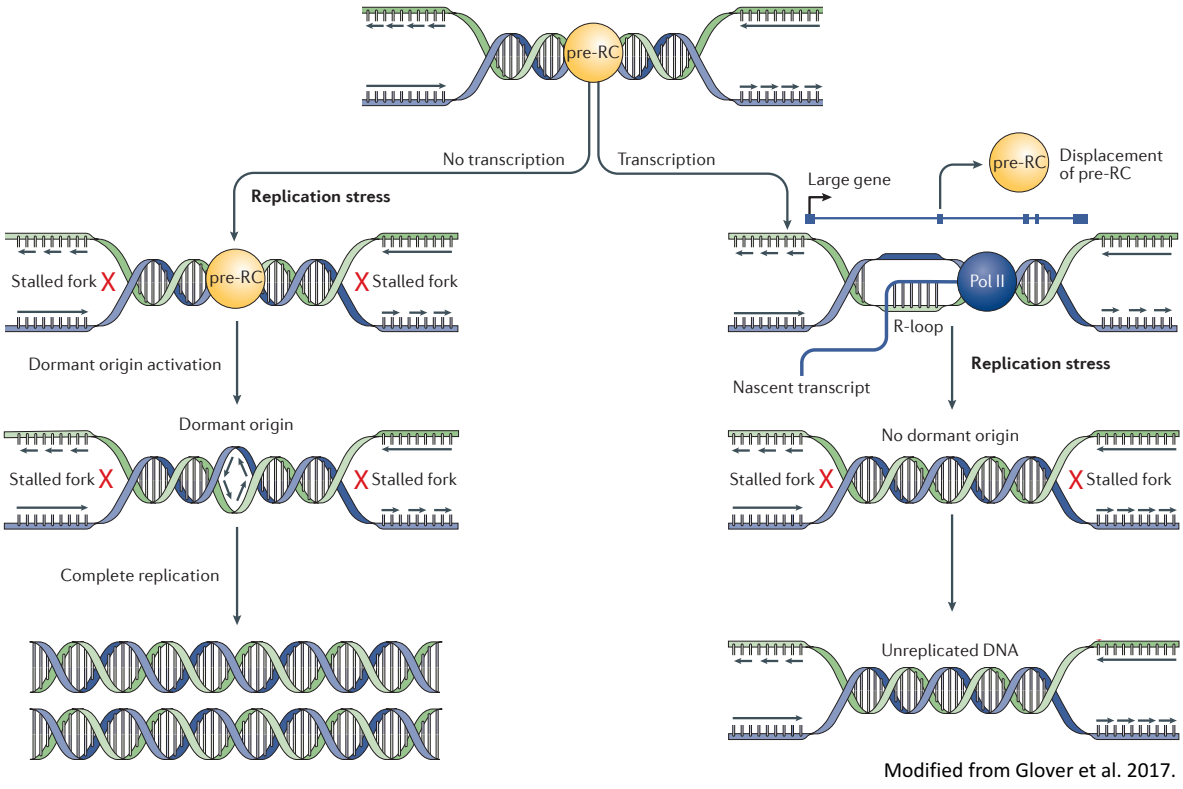


Figure 5: A model for CFS instability.

After replicative stress and without transcription, a dormant origin can be activated to complete CFS replication. In a situation of active transcription in the region, the transcriptional machinery will displace the pre-RC and impede the firing of a dormant origin and replication completion.

c) The chromatin environment.

Eukaryotic DNA is packaged into a nucleoprotein complex known as chromatin. Nucleosomes, which are 146 base pairs of DNA wrapped around a histone protein octamer, are the units of chromatin. Nucleosomes are highly dynamic because they can slide along DNA, be partially disassembled, subjected to post-translational modifications and their component histones can be replaced by their sequence variants (Lai and Pugh, 2017). Nucleosomes can interact with neighboring nucleosomes to form chromatin fibers. Fiber interactions contribute to the high degree of compaction observed in condensed chromosomes. This highly structured chromatin can limit the access of proteins to DNA and thus regulate replication, transcription and repair processes that are known to influence CFS stability (Ehrenhofer-Murray, 2004).

The chromatin structure surrounding the FRA3B breakpoints has been analyzed and nucleosomes in these regions shown not to be distributed randomly but in fixed and contiguous positions, termed "phased nucleosomes" (Mulvihill and Wang, 2004). Phased nucleosomes are usually found on either side of a nucleosome-free region which is associated with transcriptional regulatory regions like promoters, enhancers and terminators (C. Jiang and Pugh, 2009; Polach and Widom, 1995). Nucleosome phasing gradually diminishes when it moves away from the nucleosome-free region (Mavrich et al., 2008; Yuan et al., 2005). The *FHIT* gene is transcribed in the cell line used in Mulvihill and Wang study (lymphoblasts) and the two breakpoints studied are distant about 500 and 750 kb from the transcription starting site. Due to their location at a long distance from the transcription starting site, these breakpoint regions are expected to have randomly positioned nucleosomes which is not the case (Mulvihill and Wang, 2004). This particular nucleosome organization could restrict certain DNA sequences to be always wrapped or not within nucleosomes and could for example interfere with the binding of origin complex if the origins of replication are wrapped around the histones. The DNA sequence did not reveal strong nucleosome positioning elements, meaning that other factors are implicated in this positioning. Interestingly, APH treatment did not change chromatin conformation in these regions.

Histone acetylation is positively correlated with origin firing (Vogelauer et al., 2002) and is important for replication initiation site selection (Kemp et al., 2005). Histone acetylation has been examined for six CFS and was found to be less abundant in these regions compared to

surrounding regions (Y. Jiang et al., 2009). Moreover, increasing histone acetylation by the use of trichostatin, a histone deacetylase inhibitor, reduces CFS breakage. Thus, CFS are believed to be relatively compacted regions perturbing DNA replication progression.

Miotto et al. analyzed ORC binding sites and revealed that they are preferentially associated with an open and active chromatin defined as DNAse I hypersensitive regions and enriched for histone modifications such as H3K27ac, H3K9ac, H3K4me2, H3K4me1, and H3K4me3. The comparison between ORC2 binding and replication timing indicates that the density of ORC is higher in early replicating regions and decreases when regions are replicated lately. Strikingly, this study shows that CFS are nearly devoid of ORC2 binding sites, suggesting that these regions are in closed chromatin domains impeding ORC2 binding (Miotto et al., 2016). This analysis is consistent with the observation that CFS are devoid of replication origins able to fire and complete replication after a replicative stress (Letessier et al., 2011).

To strengthen the importance of chromatin conformation in CFS stability, a study showed that CFS breakage can be induced by calyculin A treatment (a protein phosphatase inhibitor), which provokes premature chromosome condensation, probably because of the inability of these unreplicated regions to condense properly (El Achkar et al., 2005).

d) Taking care of CFS: proteins implicated in CFS maintenance.

Several studies have discovered many proteins important to maintain CFS stability (summarized in **Table 2**). These proteins are known to be implicated in processes regulating CFS stability like DNA replication or response to RS.

One of the major proteins discovered to have such a role is ATR (Casper et al., 2002). Cells without ATR are much more sensitive to APH treatment and even non-treated cells show spontaneous CFS breaks, indicating that ATR is required for CFS stability during normal DNA replication. Consistently, inhibition of downstream effectors of ATR causes increased CFS instability, including CHK1, HUS1, Claspin and structural maintenance of chromosomes protein 1 (SMC1).

The two structure-specific endonucleases ERCC1 and MUS81-EME1 were shown to be specifically recruited to regions of late DNA synthesis during mitosis that correspond to CFS

to avoid segregation defects and subsequent G1 DNA damage accumulation (Naim et al., 2013). Moreover, MUS81 is necessary for DNA synthesis occurring at CFS in mitosis, a process termed MiDAS (mitotic DNA synthesis), which is dependent on the DNA polymerase POLD3 (Minocherhomji et al., 2015).

CFS are genomic regions difficult to replicate for the replicative DNA polymerase δ (Shah et al., 2009). To complete the replication of these regions, the cell can use specialized DNA polymerases. Three of these specialized polymerases are necessary for CFS stability: Polη (Bergoglio et al., 2013), Pol κ (Walsh et al., 2013) and REV3, the catalytic subunit of Pol ζ (Bhat et al., 2013).

HR is an essential process in the protection and restart of blocked replication forks and in the repair of double-strand breaks occurring during replication (Gelot et al., 2015). Accordingly, cells defective for HR show a reduction in the rate of replication fork progression (Daboussi et al., 2008). Interestingly, slowing of replication forks in these cells can be attributed to endogenous oxidative stress (Wilhelm et al., 2016). As CFS are regions prone to block replication forks and highly sensitive to RS, HR proteins are required to maintain CFS stability (Schwartz et al., 2005). Another recent finding that could explain the contribution of HR proteins to maintain CFS stability is the role of two HR proteins, BRCA1 and 2, in the prevention and/or resolution of R-loops (Bhatia et al., 2014; Hatchi et al., 2015).

Break-Induced Replication Repair (BIR) is a specific one-ended DNA DSB repair pathway dependent on POLD3, a subunit of DNA polymerase δ, used to repair collapsed DNA replication forks in cancer cells (Costantino et al., 2014). BIR seems to be implicated in CFS DNA replication repair since POLD3 has been identified to perform MiDAS at CFS (Minocherhomji et al., 2015). More recently, a study revealed that RAD52 has a role in human BIR (Sotiriou et al., 2016). In line with a function of BIR at CFS, RAD52 is detected at CFS on mitosis following RS and promotes MiDAS by recruiting MUS81 and POLD3 (Bhowmick et al., 2016).

FANCD2 is also necessary for CFS stability but its precise function will be discussed in a next section.

Protein	Function	Role in CFS expression*
DNA replication		
MUS81	Structure-specific endonuclease subunit	M phase replication
EME1	Structure-specific endonuclease subunit	M phase replication
RAD52	Homologous recombination protein	M phase replication
BLM	RecQ helicase	Suppresses inappropriate recombination
TOP1	Type I topoisomerase	Relieves torsional stress downstream of replication and transcriptional machineries
Cell cycle checkpoints		
ATR	Serine/threonine kinase	Cell cycle arrest in response to single-stranded, unreplicated DNA
ATM	Serine/threonine kinase	Cell cycle arrest in response to DNA DSBs
CHK1	Serine/threonine kinase	Cell cycle arrest in response to DNA damage or unreplicated DNA
Claspin	BRCA1 and CHK1 adapter protein	Facilitates the ATR-dependent phosphorylation of BRCA1 and CHK1; replication fork sensor
HUS1	Component of the 9-1-1 cell cycle checkpoint response complex	p53-dependent checkpoint activation and apoptosis
SNM1B	Metallo-β-lactamase superfamily protein	Promotes fork collapse; ATR activation
DNA repair		
RAD51	Recombinase	Homologous recombination
BRCA1	E3 ubiquitin–protein ligase	Genome surveillance
FANCD2	Fanconi anaemia-linked repair/replication protein	Facilitates replication
DNA-PK	Serine/threonine protein kinase	Nonhomologous end joining
Ligase IV	ATP-dependent DNA ligase	Nonhomologous end joining
XLF	XRCC4-like factor	Nonhomologous end joining
WRN	RecQ helicase	DSB repair
Translesion synthesis		
Pol η	Y family DNA polymerase	DNA synthesis at stalled forks in S phase
Pol κ	Y family DNA polymerase	Replication through repetitive elements
REV3	Polymerase ζ subunit	G2/M phase replication
Structural		
SMC1	Cohesin subunit	Prevents collapse of stalled replications forks

Modified from Glover et al. 2017.

Table 2: Proteins implicated in CFS stability.

List of the proteins that have been shown to be implicated in CFS stability maintenance.

3) Consequences of CFS fragility

a) In mitosis.

CFS are genomic regions which enter into mitosis with under-replicated DNA or unresolved DNA structures (Naim and Rosselli, 2009). Such conditions can lead to the formation of joint molecules (JMs) that can impede sister-chromatid disjunction generating segregation defects and subsequent genomic instability, notably anaphase bridges if not resolved (Wild and Matos, 2016). Anaphase bridges are defined as DNA connections linking segregating chromosomes during anaphase. There are two classes of anaphase bridges: DAPI-positive chromatin bridges and DAPI-negative ultrafine DNA bridges (UFBs). UFBs are detected by immunostaining of proteins coating these structures like the Bloom syndrome helicase (BLM) and the Plk1-interacting checkpoint helicase (PICH) which are important for their resolution UFBs (Chan et

al., 2007). PICH binding is increased by tension-induced DNA stretching which may explain the ability of PICH to recognize and stabilize DNA under tension in UFBs and facilitate their resolution (Biebricher et al., 2013).

UFBs have different origins and can be categorized in four groups: centromeric (c-UFBs), fragile sites (fs-UFBs), telomeric (t-UFBs), and ribosomal DNA (r-UFBs) (Liu et al., 2014). c-UFBs are the most abundant in unperturbed cells. They can be induced by topoisomerase II inhibition, suggesting that they reflect catenated sister chromatids (Wang et al., 2010). Instead, fs-UFBs are rare in unperturbed cells but are induced after APH treatment (Naim and Rosselli, 2009). fs-UFBs are characterized by the binding to their termini of the Fanconi anemia proteins FANCD2 and FANCI which are necessary to promote BLM assembly to these particular UFBs (Chan et al., 2009; Naim and Rosselli, 2009). However, the precise function of the interaction between these FANC proteins and BLM is still elusive. The structure-specific endonucleases ERCC1 and MUS81-EME1 colocalize with FANCD2 at CFS when the chromosomes start to condense to cut DNA and resolve JMs to prevent the formation of UFBs (Naim et al., 2013). Moreover, recent work revealed a role for the scaffold protein SLX4 in the recruitment of MUS81 and ERCC1 to CFS to promote faithful chromosome segregation (Guervilly et al., 2015).

Failed rescue of RS in mitosis will primarily lead to cell death or formation of p53-binding protein 1 (53BP1) nuclear bodies in the subsequent G1-phase of the cell cycle (Fragkos and Naim, 2017). 53BP1 accumulates at CFS after RS and sequester these regions in nuclear compartments in G1 (Lukas et al., 2011). These 53BP1 nuclear bodies could be chromatin domains assembled to repair DNA lesions in mitosis in the next cell cycle (Harrigan et al., 2011; Pedersen et al., 2015).

b) In cancer.

The link between CFS and cancer has been proposed since their discovery. Indeed, CFS were found to localize with cytogenetic breakpoints observed in tumors, suggesting that they could participate in tumorigenesis (De Braekeleer et al., 1985). Today we know that CFS instability is implicated in cancer development and constitute one of the earliest events of oncogenic transformation (Bartkova et al., 2005; Gorgoulis et al., 2005). Instability of CFS in the first steps of tumor growth is now explained because of their high sensitivity to RS occurring

following the activation of oncogene (Gorgoulis et al., 2005). It is important to note that the repertoire of CFS fragility will not be the same depending on the oncogene implicated in malignant transformation, thus adding another level of complexity in the contribution of CFS to cancer (Miron et al., 2015).

Copy number variants (CNVs) are genomic deletions and duplications spanning from tens of base pairs to more than 1 Mb. CNVs arise in cancer and contribute to cancer progression by conferring a selective growth advantage (Bignell et al., 2010). Importantly, it has been shown that RS induced *in vitro* by different agents like APH, HU or ionizing radiation can lead to deletions in CFS similar to those found in cancer (Arlt et al., 2009; Arlt et al., 2011; Durkin et al., 2008). By taking into account the cell replication timing and transcriptional program, it has become clear that CNV hotspots correspond to late replicated and transcribed large genes present in CFS (Wilson et al., 2015). However, how transcription of these large genes is susceptible to form CNV is still unclear. The model proposed is that CNV formation is caused by the repair of collapsed forks at active transcription sites by Fork Stalling and Template Switching (FoSTeS) and Microhomology-Mediated Break-Induced Replication (MMBIR) mechanisms, which leads to invasion of nascent DNA strands into ectopic locations (Bhowmick et al., 2016; Wilson et al., 2015).

Transcription of very large genes present in CFS seems to be a predictor for genomic instability (Wilson et al., 2015). In agreement with this notion, many of these genes are frequently rearranged in tumors and their expression is lost (Karras et al., 2017). However, the idea that CFS genes are tumor suppressors is still under debate. Indeed, many of the deletions observed in these genes occur in their large introns thus not affecting the protein function (Rajaram et al., 2013). Moreover, deletions occurring at CFS are mostly hemizygous deletions compared to deletions occurring at known recessive cancer genes which are mostly homozygous deletions (Bignell et al., 2010). The lack of inactivating point mutations characteristic of "classical tumor suppressors" within these genes supported the idea that CFS genes mutations are passengers in cancer development and deletions in these genes are just the reflect of RS. However, some homozygous deletions occur in CFS-genes and may contribute to oncogenesis. In line with this idea, different studies showed the role of many CFS genes like *FHIT, WWOX* and *PARK2* in genome maintenance and characterized their tumor-suppressor functions (Abu-Odeh et al., 2015; Saldivar et al., 2012). Based on these findings, a two-step model could unify both views:

in a first step, CFS genes are deleted and inactivated due to RS after oncogene activation, and then, in a second step, their loss will favor oncogenesis.

c) Viral integration.

Human papillomavirus (HPV) is the main cause of cervical carcinomas and its integration into the host genome represents a key factor to understand carcinogenesis (Pett and Coleman, 2007). Different studies reported that HPV integration occurred at different CFS in tumours and tumour cell lines (Gao et al., 2017; Thorland et al., 2000). The molecular mechanism of HPV integration is still under investigation but it is believed that HPV could use the repair machineries present in these regions (Hu et al., 2015).

4) A role of CFS?

CFS are conserved throughout evolution as they are conserved in different mammalian species (reviewed in Durkin and Glover, 2007) and probably in lower eukaryotes, including yeast which presents chromosome breakage after elimination of Mec1 (homolog of mammalian ATR) at specific regions of the genome called "replication slow zones" (Cha and Kleckner, 2002). Conservation of these unstable regions throughout evolution must have a relevance for the cell and different hypotheses exist trying to explain their conservation. Because CFS are the latest regions to be replicated, they could serve as a signal to indicate the end of replication and allow cell cycle to progress (Debatisse et al., 2006). Another hypothesis is that CFS conservation relies on a common function of the very large genes that they encompass. The nervous system could be a model to illustrate this hypothesis because many CFS genes are involved in neurodevelopment and deletions of these genes can lead to neurological disorders (Smith et al., 2006). Interestingly, recurrent DSB clusters in neural stem/progenitor cells were identified within genes that have the characteristics of CFS: long, transcribed and late replicating (Wei et al., 2016). The majority of these genes have common roles in neural cell adhesion and/or regulation of synapsis formation and function, and their selective breakage may be associated with cell plasticity. It is tempting to imagine a common function of CFS genes based on cell-type specificity. However, this common function is still to be determined and many CFS genes have poorly understood function such as the *LSAMP* gene (FRA3L) which is expressed in neuronal tissues but also surprisingly in fibroblasts.

II. Fanconi Anemia, a complex cancer predisposition syndrome, as a model to study CFS.

1) Repair of interstrand crosslinks by the FANC pathway: the canonical role.

a) First description of FA and phenotypes of FA patients.

In 1927 the three first Fanconi Anemia (FA) patients were described by the Swiss pediatrician Guido Fanconi who gave later his name to this disease (Lobitz and Velleuer, 2006). The three patients were brothers and died of a severe condition that resembled pernicious anemia which is a special kind of anemia characterized by enlarged red blood cells (erythrocyte macrocytosis), increased lysis of erythrocytes (hemolysis), and low levels of serum vitamin B12 (Fanconi, 1927). More than six decades later, the first FA gene was identified as *FANCC* using a functional complementation cloning approach (Strathdee et al., 1992). Following work used the same approach but also bioinformatical screening, proteomics, and whole-genome sequencing more recently, leading to the identification of 21 genes involved in FA so far (Gueiderikh et al., 2017) (**Table 3**).

FA gene alias (alternative name)	Estimated frequency in FA
FANCA	60%
FANCB	2% (X-linked)
FANCC	12%
FANCD1 (BRCA2)	2%
FANCD2	2%
FANCE	2%
FANCF	2%
FANCG	10%
FANCI	<2%
FANCJ (BRIP1)	<2%
FANCL	Rare
FANCM	Rare
FANCN (PALB2)	Rare
FANCO (RAD51C)	Rare
FANCP (SLX4)	Rare
FANCQ (ERCC4)	Rare
FANCR (RAD51)	Rare (dominant negative)
FANCS (BRCA1)	Rare
FANCT (UBE2T)	Rare
FANCU (XRCC2)	Rare
FANCV (REV7)	Rare
FANCW (RFWD3)	Rare

Table 3: Fanconi Anaemia genes.

List of the FA genes identified so far with their estimated frequency in FA patients. Source: HUGO Gene Nomenclature Committee.

Homozygous deletions in any one of these genes can cause FA but with a wide range of phenotypes which make more complex to identify the molecular defect at the basis of FA (Neveling et al., 2009). FA is an autosomal recessive disorder, with the exception of the X-linked *FANCB* gene and the dominant-negative mutation in the *FANCR* gene (also known as RAD51). FA incidence is estimated in 1/200 000 but, due to the founder effect, is higher in some populations like Ashkenazi Jews (1/30 000) and Afrikaners (1/22 000) (Dufour, 2017). *FANCA* represents the most commonly mutated gene in FA and mutations in *FANCC* and *FANCG* are the next most common. The majority of the other FA genes are rare and found in only one patient. It appears that the first individual described to be mutated for *FANCM* also carried biallelic *FANCA* mutations and that expression of FANCM failed to complement the defects in this patient, thus reconsidering *FANCM* as a *bona fide* FA gene (Singh et al., 2009). Recently, new patients carrying homozygous *FANCM* mutations have been identified but do not present the classical FA phenotype (no bone marrow failure) and rather show genomic

instability, early-onset of breast cancer in females and reduced fertility (spermatogenic failure and primary ovarian insufficiency) (Bogliolo et al., 2018; Catucci et al., 2018; Fouquet et al., 2017; Yin et al., 2018).

Classical clinical FA phenotypes are missing for some of the identified FA genes, like *FANCO*, *FANCR* and *FANCS*, which are called FA-like genes, opposed to *the bona* fide FA genes (Domchek et al., 2013; Meindl et al., 2010; Vaz et al., 2010; Wang et al., 2015). On the other hand, mutations in *FAAP16, FAAP20, FAAP24, FAAP100, USP1* and *UAF1,* phenocopy FA in cells and/or mouse models but mutations of these genes in patients have not been identified so far. Interestingly, new genes might be discovered because mutations in a subset of FA patients have not yet been associated with the known FA genes.

FA is a heterogeneous disorder with different signs and symptoms. The majority of FA patients present somatic abnormalities including absent or reduced fertility, skeletal malformations (thumb and radial ray), skin pigmentation defects, short stature, central nervous system abnormalities (small pituitary size), microcephaly and microphtalmia (Schneider et al., 2015). Nevertheless, many FA patients do not show or show only subtle abnormalities. Importantly, FA-D2 patients exhibit a more severe phenotype and residual protein is still present in cells from these patients, arguing for an essential role of FANCD2 in maintaining genome integrity (Kalb et al., 2007). In agreement, *Fancd2* knockout mice develop phenotypes not observed in mice mutated for the other FA genes like microphthalmia, perinatal lethality, more severe hypogonadism and tumor development (Houghtaling et al., 2003).

Bone marrow failure (BMF) is the major phenotype and cause of morbidity in FA, which represents the most common inherited BMF syndrome (Dokal and Vulliamy, 2010). All the hematopoietic cell lineages are affected in FA (pancytopenia) and patients develop BMF during the first decade of life and show increased predisposition to develop acute myeloid leukemia (700-fold risk compared to general population) (Butturini et al., 1994). BMF is currently treated by transplantation of hematopoietic stem cells from HLA-identical siblings or HLA-matched donors but patients cured for their anemia have a higher risk to develop solid tumors, especially vulvar, esophageal, and head and neck cancers (1 in 3 persons by the age of 48 years) (Rosenberg et al., 2003). Hematologic problems peak by the age of 20 years and are stable or decreasing thereafter while the risk of a solid tumor is low prior 20 years and then increases

progressively after that time (Rosenberg et al., 2003). Genomic instability in FA cells is thought to be the cause of cancer predisposition observed in FA patients.

b) ICL sensitivity.

FA cells show increased genomic instability and FA is usually diagnosed by detecting chromosome aberrations **(Fig. 6)** in peripheral blood cells after culture with an ICL-inducing agent such as mitomycin c (MMC) or diepoxybutane (DEB) (German et al., 1987). Due to their high sensitivity to ICL-inducing agents, management of cancer in FA patients must exclude treatment with these drugs.

Figure 6: Chromosome aberrations observed in FA patients after MMC treatment.
Examples of chromosomal aberrations observed in FA patients following MMC treatment.

The origin of ICL in FA has been matter of deep investigation and recent findings identified endogenous aldehydes as a potent source of instability in FA (Duxin and Walter, 2015) (**Fig. 7)**. Indeed, aldehydes produced by the cell (like acetaldehyde or formaldehyde) are highly reactive and can damage DNA (Voulgaridou et al., 2011). Interestingly, *Fancd2*$^{-/-}$ mice lacking enzymes detoxifying these aldehydes show profound BMF and mutated hematopoietic stem cells (HSC) (Garaycoechea et al., 2012; Pontel et al., 2015). To strengthen the idea that aldehydes play a role in FA, it has been shown that FA Japanese children carrying a variant allele in the aldehyde dehydrogenase 2 (*ALDH2*) gene display earlier-onset of BMF (Hira et al., 2013). A recent work showed that aldehyde-damaged HSCs are suppressed by p53 leading to BMF (Garaycoechea et al., 2018). Surprisingly, p53 suppression rescues HSC defects but does not result in further genomic instability.

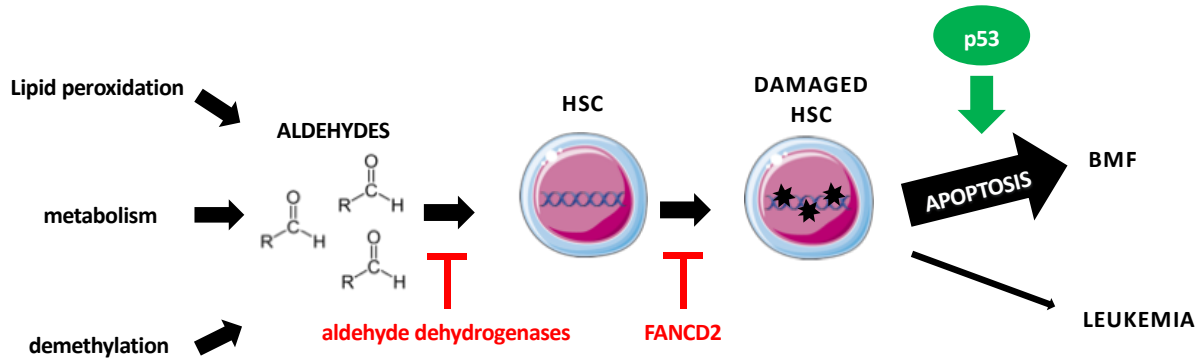


Figure 7: Model for HSC protection against endogenous aldehydes.

Aldehyde dehydrogenases activity protects DNA against the toxic effects of reactive aldehydes. If this first protection fails, FANCD2 neutralize the damage created by aldehydes. In the absence of these two protective pathways, the cell is eliminated by apoptosis through the activation of p53. However, some cells can escape and promote tumorigenesis.

c) The FANC pathway.

FA genes have been described to collaborate in a common DNA repair pathway necessary to repair ICL called the FANC pathway (**Fig. 8**). ICLs are very toxic for the cell as they block essential processes like DNA replication and transcription by inhibiting DNA strand separation. Resolution of ICLs implies the coordinated action of structure-specific endonucleases, translesion DNA polymerases and recombinases (Deans and West, 2011). The FANC pathway components can be subdivided in three groups: the first group is composed by 14 proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM, FANCT, FAAP100, MHF1, MHF2, FAAP20, and FAAP24), called the FA core complex, necessary to recognize the ICL and ubiquitylate the second group which is the heterodimer FANCD2-FANCI (referred here as FANCD2-I). Once ubiquitylated, this heterodimer will coordinate the repair of the ICL by the proteins of the third group composed of proteins involved in ICL incisions, translesion synthesis and HR repair (Ceccaldi et al., 2016).

All the FA proteins are conserved in vertebrates but through evolution, FANCM, SLX4 and BRCA2 are the most conserved and are present from yeast to human, indicating other important functions than ICL repair of these proteins (Garner and Smogorzewska, 2011). FANCL is conserved in vertebrates, flies, frogs, worms, plants and slime while the conservation of the other components of the FA core complex are lost or gained in different species (Zhang et al.,

2009). All the organisms that have FANCL also have FANCD2 and FANCI representing probably a minimal complex in order to ubiquitylate the FANCD2-I complex.

The repair of ICL by the FANC pathway occurs in different steps: (i) recognition of the ICL, (ii) recruitment of the FA core complex and ubiquitylation of FANCD2-I, (iii) incision of the ICL in one strand, (iv) filling of the gap created by the incision and translesion synthesis, and finally (v) the repaired strand can act as a template for reestablishing the replication fork *via* HR.

(i) ICL repair by the FA pathway occurs during the S phase (Kim et al., 2008). However, ICLs can also be repaired during the G1 phase of the cell cycle by nucleotide excision repair (NER) and the polymerase ζ (Sarkar et al., 2006). During replication, replisomes stall 20 nucleotides before an ICL probably due to the steric hindrance of CMG helicase which is ahead of the DNA polymerase. BRCA1 is essential to remove CMG from the stalled forks and enables the leading strand to reach within 1 nucleotide the ICL (a mechanism called "approach") (Long et al., 2014). The resulting structure is recognized by the DNA-binding FANCM-FAAP24 heterodimer which is necessary to recruit the FA core to the damaged site (Ciccia et al., 2007) through a direct interaction with FANCF (Deans and West, 2009). Deficiency in FANCM or FAAP24 impedes the loading of the FA core to the chromatin and abrogates FANCD2-I ubiquitylation even if the cytoplasmic FA core assembly is not affected (Kim et al., 2008). FANCM is associated with two histone-fold proteins, MHF1 and MHF2, which are necessary for FANCM stabilization and activity (Singh et al., 2010; Yan et al., 2010). After ICL induction, the FA core complex associates with the DNA helicase BLM which exists in a nuclear complex with topoisomerase IIIα, RMI1, and RMI2, forming a supercomplex termed BRAFT (Meetei et al., 2003). FANCM links the FA core complex and the Bloom's complex by its physical interaction with FANCF and RMI1 and Topoisomerase IIIα (Deans and West, 2009). Importantly, interaction of FANCM with the Bloom's complex is necessary to repair ICLs.

(ii) FANCM-FAAP24 interacts with the checkpoint protein HCLK2 independently of the FA core complex to ensure an efficient ATR/CHK1-mediated checkpoint signaling (Collis et al., 2008). It has been shown that ATR can phosphorylate different FANC proteins (Constantinou, 2012). FANCI phosphorylation by ATR is necessary for the mono-ubiquitylation and focus formation of FANCI and FANCD2 (Ishiai et al., 2008). Interestingly, a recent work refined the model of how FANCI phosphorylation can activate the FANCD2-I complex by identifying

FANCI-phosphorylated sites dependent of FANCD2 ubiquitylation and regulating FANCD2 de-ubiquitylation (Cheung et al., 2017). FANCD2 phosphorylation by ATR and CHK1 is necessary for its ubiquitylation and resistance to DNA crosslinking agents (Zhi et al., 2009). Phosphorylation of FANCA, FANCG, FANCE and FANCM by ATR have also been described and are essential for the activity of these proteins (Qiao et al., 2004; Singh et al., 2013; X. Wang et al., 2007). To sum up, ATR cooperates with FANCM and FAAP24 to recruit the FA core complex to the DNA damage and coordinate its repair.

Once recruited, the FA core complex mono-ubiquitylates the FANCD2-I heterodimer, which promotes its recruitment to the damage (Garcia-Higuera et al., 2001; Sims et al., 2007). Mono-ubiquitylation of both FANCI and FANCD2 are reciprocally dependent. The mono-ubiquitylation step is catalyzed by the ubiquitin ligase FANCL and the corresponding E2 ubiquitin-conjugating enzyme FANCT (UBE2T) (Alpi et al., 2008). The absence of any one of the FA core complex components impede the ubiquitylation of the FANCD2-I complex. Importantly, de-ubiquitylation by USP1 is also required for ICL repair and FANC pathway function (Oestergaard et al., 2007).

(iii) Ubiquitylated FANCD2 and FANCI control the cutting of DNA to release the ICL from one of the two parental strands, a process called “unhooking”, and convert the stalled fork into a DSB (Knipscheer et al., 2009). Two models of incision were proposed to explain the repair of a replication fork stalled by an ICL: one proposes that the first cleavage occurs in the leading strand while the other proposes that this first cleavage occurs in the lagging strand (Zhang and Walter, 2014). Because the average interorigin distance is large *in vivo*, it is believed that one replication fork is sufficient to repair ICL before the arrival of another fork. However, a recent study in *Xenopus* egg extracts proposed that ICL repair requires converging replication forks (Zhang et al., 2015).

The identification of the nuclease required for ICL unhooking has been matter of deep investigation. It has been shown that the FAN1 nuclease is recruited to stalled replication forks (MacKay et al., 2010). However, mutations in FAN1 do not cause FA as would be expected and a recent work showed that FAN1 recruitment by FANCD2 is dispensable for ICL repair (Lachaud et al., 2016). FAN1 is believed to prevent defective fork processing rather than defective ICL repair. The disruption of the docking protein SLX4 is causing FA in mice and biallelic mutations have been found in FA patients, thus *SLX4* has been assigned to FA genes

and named *FANCP* (Crossan et al., 2011; Kim et al., 2011). SLX4 is known to interact with the nucleases MUS81-EME1, XPF-ERCC1 and SLX1. Notably, specific mutations in the *ERCC4/XPF* gene have been identified in unclassified FA individuals and thus it has been renamed *FANCQ* (Bogliolo et al., 2013). Depending on the type of *ERCC4/XPF* mutation, NER or ICL repair will be affected, leading to one of the DNA repair disease xeroderma pigmentosum (XP), XFE progeroid syndrome or FA (Bogliolo et al., 2013). As MUS81-EME1 and XPF-ERCC1 were shown to be required to resolve ICLs, the model is that MUS81-EME1 is doing the first incision, creating a DSB, and XPF-ERCC1 is then doing the unhooking (Hanada et al., 2006; Niedernhofer et al., 2004). However, the mechanism by which endonucleases are recruited to the ICL remains uncertain and may be through the direct interaction of mono-ubiquitylated FANCD2 and UBZ4 motif of SLX4 or indirectly, through an intermediary (Klein Douwel et al., 2014; Yamamoto et al., 2011).

(iv) Once the ICL is unhooked from one strand, normal replicative DNA polymerases are not able to bypass the lesion but low-fidelity TLS polymerases that have larger binding pockets can. Lesion bypass often requires the sequential action of two different TLS polymerases: a first polymerase inserts a nucleotide across the damaged base ("insertion") and a second TLS polymerase extends the DNA from this nucleotide ("extension"). The TLS polymerase in charge of the insertion step is unknown, however the Y-family DNA polymerase REV1 is thought to have a major role in this step. REV1 is interacting with all other TLS polymerases and may serve as a platform during TLS (Lehmann et al., 2007). Consistently, cells defective for REV1 are more sensitive to ICLs (Sarkar et al., 2006). The extension is then carried out by Pol ζ, a B-family polymerase composed of a catalytic subunit, REV3, and a regulatory subunit, REV7 (also known as FANCV) (Räschle et al., 2008). Importantly, the FA core complex is necessary to recruit TLS polymerases to the ICL (Kim et al., 2012).

It is worth to mention here the role of the proliferating cell nuclear antigen (PCNA) in the recruitment of TLS polymerases. PCNA is ubiquitylated by RAD18 after replication fork stalling (Davies et al., 2008). Mono-ubiquitylated PCNA then coordinates the action of TLS polymerases (Lehmann et al., 2007). Interestingly, PCNA interacts with FANCD2 and promotes its ubiquitylation (Howlett et al., 2009).

(v) The intact strand generated by TLS is then used as a template for HR repair. The importance of HR in ICL repair is highlighted by the fact that different proteins known to be implicated in

HR repair are associated with a FA phenotype when mutated in patients: BRCA2 (FANCD1), BRIP1 (FANCJ), PALB2 (FANCN), RAD51C (FANCO), RAD51 (FANCR), BRCA1 (FANCS), and XRCC2 (FANCU).

HR is initiated by DNA 5'-end resection which occurs through the action of nucleases such as CtBP-interacting protein (CtIP), the MRE11-RAD50-NBS1 (MRN) complex, EXO1 or DNA2, and the BLM helicase (Nimonkar et al., 2011). FANCD2 has been shown to have a role in the regulation of resection through its interaction with CtIP (Murina et al., 2014; Unno et al., 2014). MRN activity is crucial for FANCD2 stability and function, showing a complex regulation between resection and FANCD2 (Roques et al., 2009). DNA 5'-end resection is a key step in the choice of DNA repair as it favors HR and suppresses non-homologous end joining (NHEJ) (Chapman et al., 2012). Cells defective for NHEJ are not sensitive to ICLs but it has been shown that NHEJ is stimulated in the absence of the FANC pathway and inhibition of NHEJ could rescue ICL sensitivity in FA cells (Pace et al., 2010). FANC pathway is believed to favor ICL repair by HR to avoid NHEJ which is more error-prone and toxic for the cell (Bunting et al., 2010).

Some HR proteins are present before the DSB formation at the ICL. BRCA1 is necessary to unload the CMG helicase and allows ICL repair (Long et al., 2014). Accordingly, FANCD2 foci formation is impaired in BRCA1-deficient cells after exposure to DNA crosslinking agents (Bunting et al., 2012). RAD51 is also recruited before DNA incisions possibly to prevent fork breakage/degradation and coordinate strand invasion when the DSB is formed (Long et al., 2011).

BRCA2 and PALB2 form a complex along with RAD51 to promote the formation of ssDNA nucleofilaments and strand invasion of homologous DNA template (Sy et al., 2009). The bi-orientation of chromosomes in cell division protein 1-like (BOD1L) protein stabilizes RAD51 nucleofilaments and its depletion results in ICL hypersensitivity (Higgs et al., 2015).

Then, repair synthesis, resolution of the HR intermediate and ligation resulting in an intact double-strand DNA complete the repair of ICL by the FA pathway.

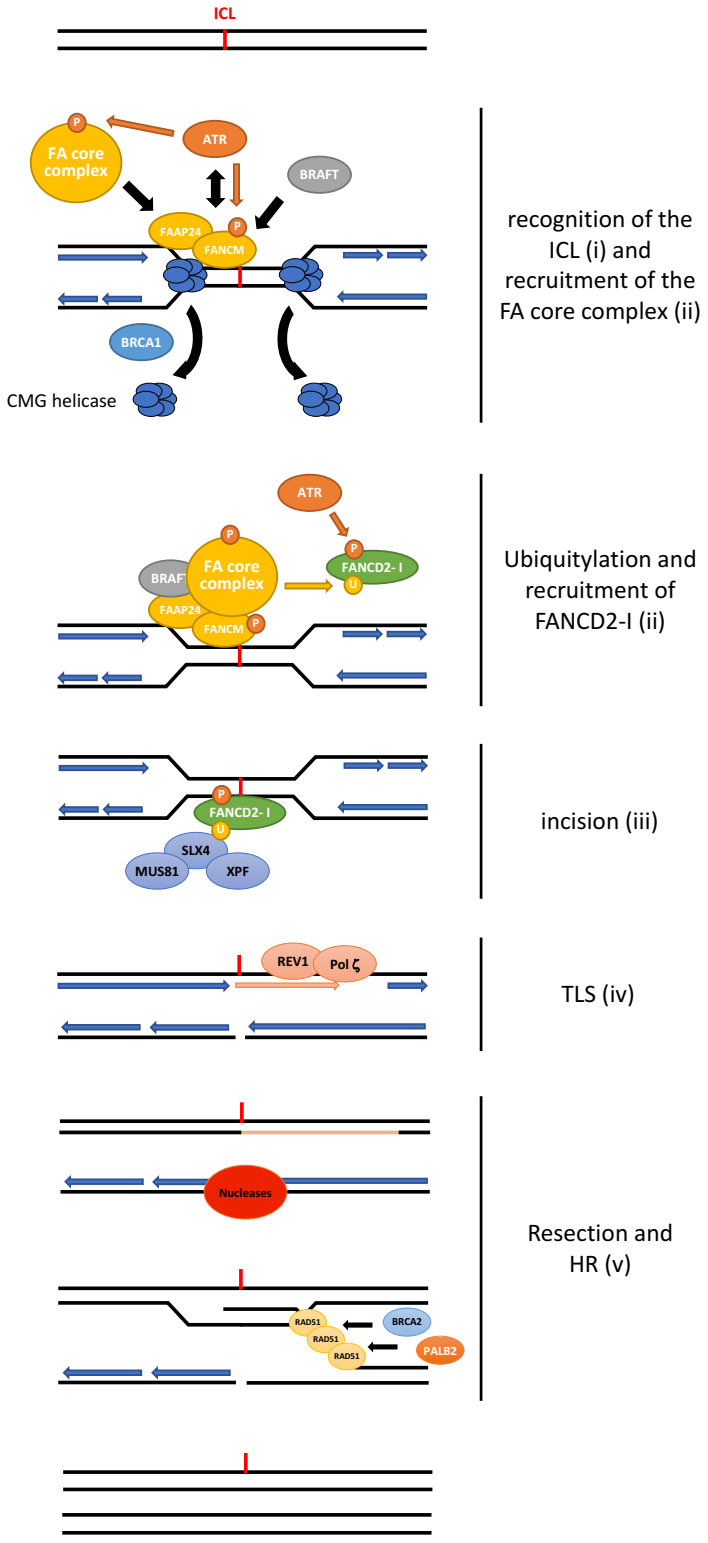


Figure 8: ICL repair by the FANC pathway.

During S phase, the FANC pathway resolves ICL through a multistage process starting with the unloading of the CMG helicase by BRCA1 and the recruitment of the FA core and BRAFT complex to the ICL by the heterodimer FANCM-FAAP24. The FA core ubiquitylates FANCD2 and FANCI, which orchestrates ICL incision by endonucleases. ATR phosphorylates several FANC proteins and is essential for their activities in this process. The gap left after the incision is filled by TLS polymerases and replication fork is restarted via HR.

2) Beyond the DNA repair function of FANC proteins.

In addition to their canonical role in ICL repair, FANC proteins have been recently described in other processes essential to preserve genomic integrity. Surprisingly, it appears that FANC proteins have important nuclear but also cytoplasmic roles and can even have independent functions from each other.

a) Nuclear functions.

i) Replication fork protection and origin firing.

As discussed before, HR is essential to allow blocked replication forks to restart and different FANC proteins are involved in this process. Interestingly, FANCD2 is ubiquitylated after APH or HU treatment which does not create DNA lesions like crosslinking agents but leads to replication stalling (Howlett et al., 2005). This finding attributes a possible global role of FANC proteins in replication fork recovery. In line with this idea, it has been shown that ubiquitylated FANCD2 is necessary to protect nascent DNA strands from MRE11 degradation after HU treatment (Schlacher et al., 2012). Moreover, ubiquitylated FANCD2 is necessary to recruit the FAN1 nuclease at stalled replication forks to resume replication and this function is not required for ICL repair (Lachaud et al., 2016).

In addition, FANCD2 seems to have independent functions from the classical FANC pathway regarding to DNA replication since it binds the MCM2-MCM7 replicative helicase independently of its ubiquitylation, but under the regulation of ATR, to monitor replisome movement after fork stalling (Lossaint et al., 2013). FANCI has also been found to be associated to replisomes after replication arrest but a recent work showed that FANCI could have a different role in replication compared to FANCD2 based on RS intensity (Chen et al., 2015). This study shows that, under low or mild RS, FANCI promotes origin firing whereas FANCD2 inhibits it probably through the binding to FANCI. However, during severe RS both proteins are necessary for replication fork restart at stalled forks and inhibition of dormant origin firing in the vicinity of stalled forks. The inhibition of origin firing by FANCD2 through FANCI inhibition is still to be confirmed as a study showed that FANCD2 performs this function along with BLM but independently of FANCI (Chaudhury et al., 2013). A role for non-ubiquitylated

FANCD2 in replication restart after RS is further supported by results showing its recruitment to chromatin after APH treatment in FANCC-defective cells to promote fork restart along with FANCJ and BRCA2 (Raghunandan et al., 2015).

To summarize, the FANC pathway as a whole, or its components, perform essential functions in protection against RS by regulating replication fork protection and restart as well as controlling origin firing in order to maintain genomic stability.

ii) FANC proteins and transcription regulation.

Different studies showed that FANCD2 can act as a transcription factor to activate or repress transcriptional activity. It has been shown that ubiquitylated FANCD2, in a complex with FANCP/SLX4, can bind to the promoter of the tumor suppressor *TAp63* gene and promote its transcription after DNA damage to induce cellular senescence (Park et al., 2013). Interestingly in this work, FANCD2 chromatin immunoprecipitation followed by sequencing (ChIP-seq) also revealed the binding of FANCD2 to *dNp63* and *BRCA2* promoters after DNA damage to induce their transcription and could participate in the tumor-suppressor activity of FANCD2 in squamous epithelium.

Cells from FA patients overproduce the tumor necrosis factor alpha (TNFα) cytokine and its inhibition by anti-TNFα antibodies can partially restore bone marrow defects *in vitro* (Dufour et al., 2003; Rosselli et al., 1994). TNFα triggers the activation of the transcription factor NF-κB which is constitutively activated in FA cells (Briot et al., 2008). To go further into the mechanisms leading to TNFα overproduction, a study showed that ubiquitylated FANCD2 can bind to the NF-κB consensus sequence in the promoter of *TNFα* to repress its activity (Matsushita et al., 2011). Moreover, this study showed that FANCD2 negatively regulates NF-κB transcriptional activity consistent with the repression of TNFα activation by FANCD2.

FANC proteins can also indirectly regulate transcription by modulating the recruitment of factors involved in transcription. A study showed that after oxidative stress induction, ubiquitylated FANCD2 is recruited to the promoters of antioxidant genes to increase the binding of the chromatin-remodeling protein BRG1 to open the chromatin for transcription (Du et al., 2012). Accordingly, expression of antioxidant genes is down-regulated in FA cells.

Interestingly, FANCD2 interacts with another chromatin-remodeling protein, the histone acetyltransferase Tip60 (Hejna et al., 2008), a protein known to be involved in transcription regulation (Ravens et al., 2015). Collaboration between FANCD2 and Tip60 in DNA repair has been shown to favor HR (Renaud et al., 2015). However, collaboration between FANCD2 and Tip60 in transcription has never been tested. Ubiquitylated FANCD2 is also necessary for nuclear localization and retention of the transcription factor FOXO3a to maintain HSC function (Li et al., 2015). Different FANC proteins were identified to directly interact with the transcriptional corepressor CtBP1 to regulate gene expression notably in the Wnt signaling pathway (Huard et al., 2013).

It now appears that FANC proteins orchestrate transcriptional or co-transcriptional events in order to prevent genomic instability. It has been shown that FANCI and FANCD2 stably associate with the splicing factor 3B1 (SF3B1) to regulate the dynamic distribution of splicing factors (Moriel-Carretero et al., 2017). Interestingly, splicing inhibition leads to the degradation of several DNA repair proteins including FANCD2 and FANCI, and FANC proteins interact with FoxF1, Hes1, Runx1 and Runx3 transcription factors that regulate stability and recruitment of FA complexes (Pradhan et al., 2015; Tremblay et al., 2008; Wang et al., 2014). Thus, transcription also seems to be important for the recruitment of FANC proteins. R-loops are three-stranded nucleic acid structures composed of a RNA-DNA hybrid and a displaced single-stranded DNA formed during transcription. They can be a threat to genome stability if persistent (Aguilera and García-Muse, 2012). Two studies revealed the role of the FANC pathway in resolving such structures. Schwab *et al.* showed that R-loop removal reduces FANCD2 foci formation and prevents transcription-induced replication stress observed in FA (Schwab et al., 2015). This study also demonstrates that R-loop resolution by the FANC pathway is carried out by the translocase activity of FANCM. Almost at the same time, Garcia-Rubio *et al.* reached the same conclusion showing an accumulation of R-loops in FA patient cell lines and that their removal suppressed genomic instability observed in FA (García-Rubio et al., 2015).

b) Mitochondrial dysfunction in FA.

Mitochondria are crucial organelles regulating essential processes like energy metabolism, amino acid production, apoptosis and even immunity (Spinelli and Haigis, 2018). Mitochondria are also a major source of ROS that are physiological products important for cell signaling but can threat DNA integrity if in excess (Sena and Chandel, 2012). Mitochondrial dysfunction is

known to be a common feature of genetic diseases associated with premature aging like Ataxia-Telangiectasia, Down Syndrome and FA (Pallardó et al., 2010). I will focus here on the different aspects of mitochondrial dysfunction implicated in FA.

i) Oxidative stress.

Dysregulated oxygen metabolism and its implication in FA disease has been proposed many years ago by a study showing that the frequency of chromosomal aberrations in FA was positively correlated to oxygen tension, indicating a possible dysfunction in oxygen-related functions (Joenje et al., 1981). The pro-oxidant state in FA has been shown by elevated activity of antioxidant enzymes in FA cells like phospholipid-hydroperoxide-glutathione-peroxidase (PHGPx) (Ruppitsch et al., 1997), Mn-containing superoxide dismutase (Mn-SOD), catalase and glutathione peroxidase (Gille et al., 1987) and also by elevated oxidized DNA and proteins due to ROS overproduction (Degan et al., 1995; Lyakhovich and Surralles, 2010). Moreover, excessive ROS production is accompanied by a defective detoxifying machinery and participates to the high sensitivity of FA cells to oxidative stress (OS), notably HSC (Y. Li et al., 2017; Mukhopadhyay et al., 2006).

The mechanisms by which FANC proteins are implicated in OS response is still unclear. However, a study showed that in response to OS, FANCD2 interacts and retains transcription factor FOXO3a to induce antioxidant gene expression (Li et al., 2010). Moreover, FANCD2 and FANCA can also form a complex with BRG1 within promoters of antioxidant genes to protect them from oxidative DNA damage and enhance antioxidant defenses (Du et al., 2012).

OS management in FA is a promising therapeutic issue as the use of antioxidants such as tempol or resveratrol decreases cancer incidence and hematopoietic defects in *Fancd2 (-/-)* mice (Zhang et al., 2008 and 2010). Moreover, OS is known to be implicated in the toxicity of ICL agents (Schaaf et al., 2002) and its reduction decreases ICL sensitivity of FA cells (Nagasawa and Little, 1983).

ii) Mitophagy.

Selective autophagy is an essential pathway that targets unwanted cytoplasmic contents (viruses, intracellular bacteria, damaged mitochondria and endoplasmic reticulum, lipid

droplets or peroxisomes) for engulfment by double-membraned vesicles (called autophagosomes) that are then destroyed by lysosomes. Recently, a study showed that FANCC is necessary for the clearance of viruses (virophagy) and damaged mitochondria (mitophagy) (Sumpter et al., 2016). This study demonstrates that FANCC interacts with the E3 ubiquitin ligase Parkin to promote Parkin-dependent mitophagy and this role is independent of its nuclear role in DNA damage. Interestingly, FANCC and FANCD2 were found in mitochondria and different FANC proteins were identified to be also required for mitophagy like FANCA, FANCF, FANCL, FANCD2, BRCA1 and BRCA2, suggesting that the FANC pathway participates in mitochondrial homeostasis (Sumpter et al., 2016). Another study revealed misbalance between mitochondrial fission and fusion in FA cells that could also account for defective mitophagy in these cells (Shyamsunder et al., 2016). Impaired mitophagy leads to increased OS which is often observed in neurodegenerative diseases due to the high metabolic activity of neurons (Lee et al., 2012).

It appears clear that FANC proteins have functions in the cytoplasm and in the nucleus. Even if these functions can be independent, they have a common objective which is the protection of the genome. For example, FANC proteins can protect the genome from mitochondrial ROS by removing damaged mitochondria. Both functions have to be considered to participate in the complex clinical phenotype of FA disease.

iii) Metabolism and stem cell maintenance.

Mitochondria are the principal site of aerobic ATP production through the mitochondrial oxidative phosphorylation (OXPHOS). OXPHOS takes place in the inner membrane of the mitochondria in eukaryotes and is composed by a series of protein complexes called electron transport chain (ETC). ETC is composed by four complexes necessary to transfer electrons from electron donors such as NADH or succinate to electron acceptors such as oxygen in redox reactions releasing energy which is used to produce ATP. Interestingly, decreased ATP and increased ROS production has been described in FA cells (Kumari et al., 2013) which is due to impaired complex I function of the ETC (Ravera et al., 2013). To avoid the use of damaged mitochondria and produce energy to sustain cellular functions, FA cells tend to use glycolysis (Cappelli et al., 2017). Interestingly, constraining FA cells to use OXPHOS with drugs leads to increased oxidative stress (Cappelli et al., 2017).

HSC metabolism has a pivotal role in dictating proliferation, differentiation or quiescence. HSCs are characterized by anaerobic metabolism and switch to aerobic metabolism during differentiation (Shyh-chang et al., 2013). Interestingly, it has been proposed that inducing HSCs to exit quiescence provoked increased metabolic ROS and DNA damage caused by ROS, leading to BMF in *Fanca -/-* mice (Walter et al., 2015). Moreover, p53 is activated upon metabolic stress and is thought to be necessary to mediate the glycolysis to OXPHOS switch in FA (Wei Du et al., 2016). As OXPHOS machinery is defective in FA cells and clearance of damaged mitochondria by mitophagy is impaired, mitochondrial dysfunction when HSC exit from dormancy can lead to DNA damage and HSC death (**Fig. 9**). HSC that will survive are more prone to have DNA mutations and could be a source of tumorigenesis.

Thus, metabolic control in FA HSCs could be a promising therapeutic issue. Recently, treatment of *Fancd2 -/-* mice with metformin has been shown to rescue HSC defects and to partially suppress cancer predisposition in *Fancd2 -/- Trp53 +/-* mice (Zhang et al., 2016). How metformin (which is primarily used to treat type 2 diabetes) is improving FA phenotype is still elusive but one possible explanation is that it may attenuate the metabolic response of HSCs when they enter the cell cycle. Another hypothesis is that metformin could react with aldehydes rendering them inert.

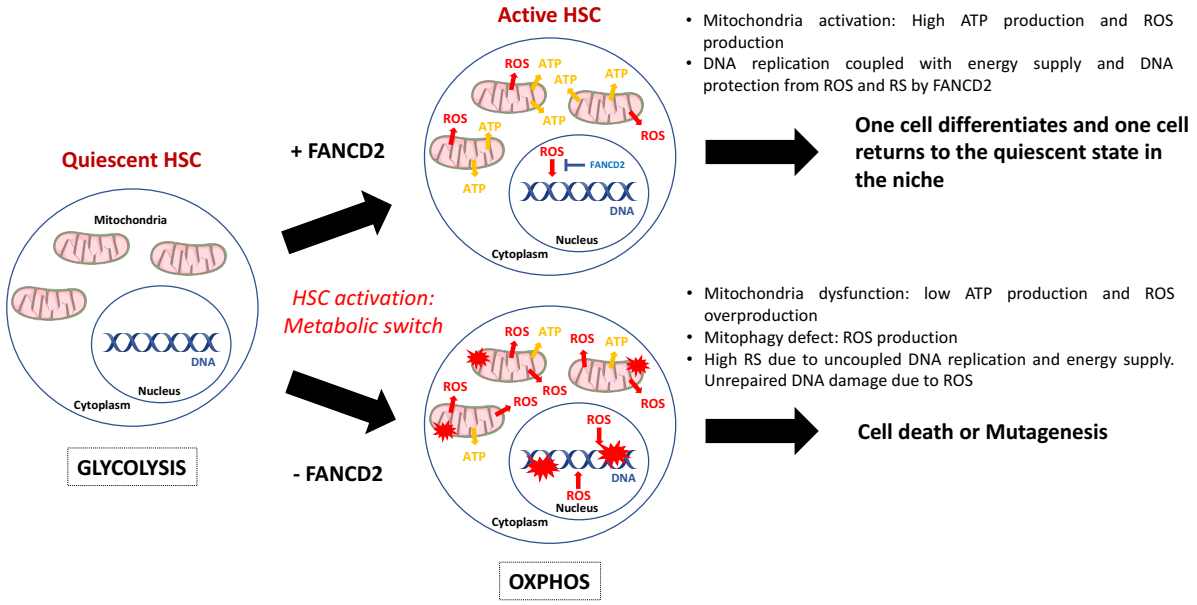


Figure 9: Model for HSC loss in FA after HSC activation.

Quiescent HSC relies in glycolytic metabolism. During differentiation, HSC switch to OXPHOS resulting in high ATP production and ROS release that can damage nuclear DNA. In the presence of FANCD2, DNA damage are resolved, and one cell differentiates while the other one returns to the quiescent state. In the absence of FANCD2, mitochondria are defective, generating ROS and not producing ATP. Moreover, damaged mitochondria are not cleared by mitophagy, generating more ROS. The cell has no energy to differentiate and DNA is damaged by ROS, leading to its death or mutagenesis.

iv) Mitochondria-nucleus crosstalk: the mitochondrial unfolded protein response.

Maintenance of protein homeostasis, known as proteostasis, is essential for cellular and organismal function and survival. Due to the importance of this process, the cell monitors proteostasis in the different subcellular compartments by complex pathways defined as unfolded protein responses (UPRs) (Jovaisaite et al., 2014). Perturbation of proteostasis activates the UPR that rapidly respond to recover homeostasis or induce cell death by activating a nuclear transcriptional program. UPR can be subdivided in three branches referring to the subcellular compartment that they monitor: endoplasmic reticulum (UPR^{ER}), cytosolic heat shock response (HSR), and mitochondria (UPR^{mt}). However, these three pathways are closely related and, for example, endoplasmic reticulum can communicate with mitochondria through contacts called mitochondrial-associated membranes (MAMs) (Kornmann et al., 2009). UPR^{ER} and HSR have been widely studied, enabling the characterization of the different signaling

proteins implicated in these protective responses **(Fig. 10)** (Vabulas et al., 2010; Walter and Ron, 2011).

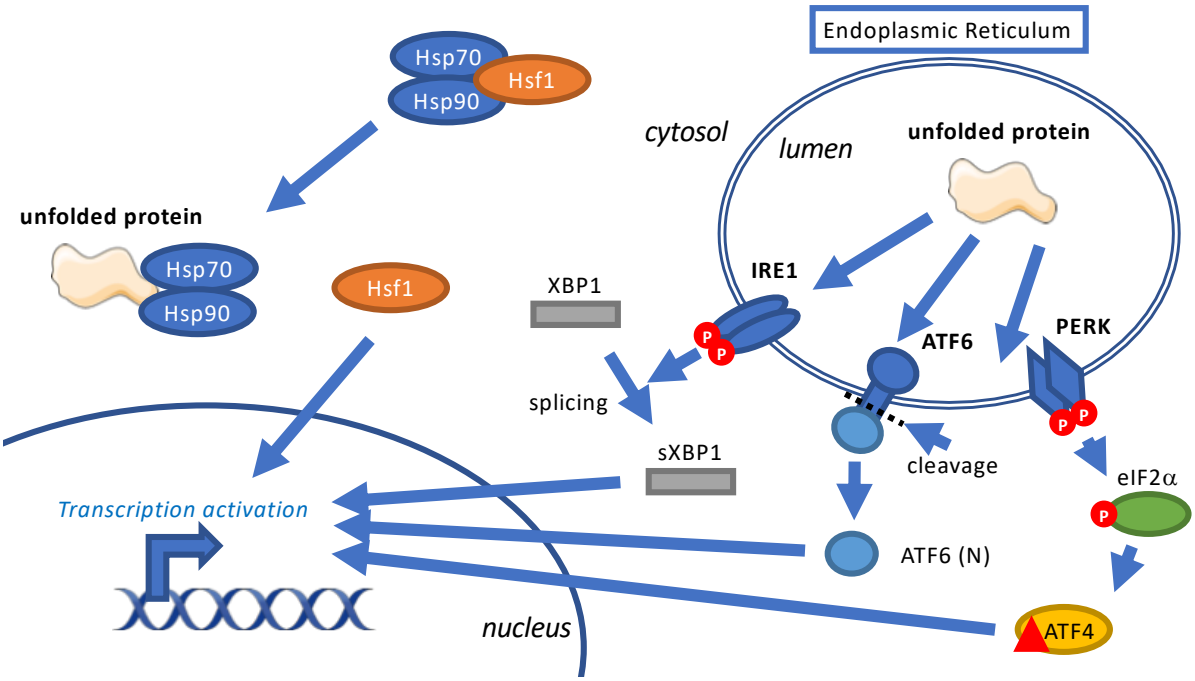


Figure 10: HSR and UPR^{ER}.

Heat Shock Response: The heat shock specific transcription factor Hsf1 is maintained in an inactive monomeric form in complexes with the chaperones Hsp70 and Hsp90. Protein unfolding results in the release of Hsf1 from chaperone inactivation. Monomeric Hsf1 trimerizes and is transported into the nucleus to activate heat shock gene transcription.

UPR^{ER}: ER stress activates the stress sensors ATF6, IRE1, and PERK, representing the three branches of the UPR^{ER}. ATF6 is a transcription factor synthesized as an ER-resident transmembrane protein. Upon accumulation of unfolded proteins, ATF6 is cleaved by proteases that liberates its N-terminal cytosolic fragment. ATF6(N) then moves into the nucleus to activate UPR target genes.

PERK is an ER-resident transmembrane protein kinase. Upon ER stress, PERK oligomerizes and phosphorylates itself and the translation initiation factor eIF2α, inactivating eIF2 and inhibiting mRNA translation, reducing the flux of protein entering the ER to alleviate ER stress. However, inhibition of eIF2α induce the translation of a few mRNAs including the transcription factor ATF4, targeting the transcription of CHOP which is a transcription factor controlling genes involved in apoptosis.

IRE1 is a bifunctional transmembrane kinase/endoribonuclease. Upon ER stress, its ribonuclease function is activated by IRE1 oligomerization in the ER membrane and cleaves the mRNA encoding a UPR-specific transcription factor called XBP1, giving rise to a spliced mRNA that is translated to the active forms of the transcription factor sXBP1.

The UPR^{mt} pathway:

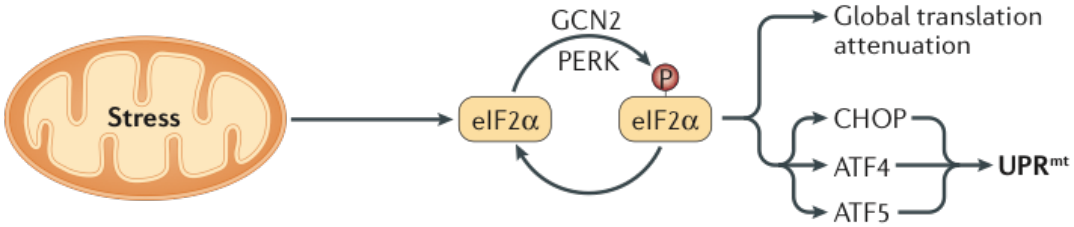
The vast majority of mitochondrial proteins are encoded in the nucleus and must be imported through the double membrane of the mitochondria (Herrmann and Neupert, 2013). Most mitochondrial proteins have an N-terminal matrix-targeting sequence (MTS) which is recognized by translocases to mediate their import and intramitochondrial sorting. Once in the mitochondria, MTS is cleaved and proteins undergo chaperone-assisted folding (Herrmann and Neupert, 2013).

The first evidence for the existence of a UPR^{mt} has been shown in mammalian cells with the description of a nuclear gene encoding mitochondria-localized chaperons, HSP60 and HSP10, controlled by a bi-directional promoter activated upon loss of the mtDNA and heat shock (Martinus et al., 1996; Ryan et al., 1997). However, most of the mechanistic studies have been done in the model organism *Caenorhabditis elegans* (Pellegrino et al., 2013). Works in this model organism identified the transcription factor ATFS-1 as the main UPR^{mt} regulator (Nargund et al., 2012). ATFS-1 contains both a nuclear localization sequence (NLS) and MTS. Under non-stressed condition, ATFS-1 is imported into the mitochondria for degradation by the LON protease while under mitochondrial stress it translocates to the nucleus to activate the transcription of protective genes in order to recover mitochondrial homeostasis (Nargund et al., 2012).

While several components of the UPR^{mt} in C. *elegans* are conserved, downstream signaling steps and transcription regulation are still elusive in mammals. The use of misfolding-prone deletion mutants of the mitochondrial protein ornithine transcarbamylase (OTCδ) helped to better characterize this pathway in mammals. Exogenously expressed OTCδ misfolds and accumulates in the matrix, activating the specific UPR^{mt} chaperone HSP60 controlled by the transcription factor CHOP (C/EBP homology protein) (Zhao et al., 2002). Because CHOP promoter has an activator protein-1 (AP-1) site, it has been suggested that Jun transcription factor is also implicated in the UPR^{mt} (Horibe and Hoogenraad, 2007). CHOP is known to be part of the integrated stress response (ISR) mediated by the eukaryotic initiation factor 2 alpha (eIF2α) kinase. eIF2α is phosphorylated by any of four different kinases GCN2, HRI, PERK and PKR responding to various cellular stresses which are amino acid deprivation, heme deficiency, ER protein misfolding or viral infection respectively (Pakos-Zebrucka et al., 2016).

eIF2α phosphorylation causes increase in the translation of activating transcription factor 4 (ATF4), activating several targets including CHOP (Ma et al., 2002). ATF4 and CHOP also induce the expression of the transcription factor ATF5 (Teske et al., 2013) which has been found to be involved in the mammalian UPR^{mt} (Fiorese et al., 2016) (**Fig. 11**).

Today, numerous studies have shown that UPR^{mt} can be activated by several ways resulting in mitochondrial dysfunction, including accumulation of unfolded proteins within the mitochondrial matrix but also conditions that perturb OXPHOS activity, promoting transcriptional activation to recover mitochondrial homeostasis (Shpilka and Haynes, 2018).



Modified from Shpilka and Haynes, 2018.

Figure 11: The UPR^{mt}.

Mitochondrial stress activates the UPR^{mt}. eIF2α is phosphorylated by GCN2 and PERK kinases, attenuating global translation but inducing preferential translation of ATF4 which activates *CHOP* and *ATF5* transcription to mediate UPR^{mt}.

However, other proteins involved are still to be found and recent findings suppose the existence of alternative pathways which are independent of CHOP activity (Papa and Germain, 2011). Recently, a study identified ATF4 activation upon mitochondrial stress by a non-canonical UPR^{mt} that is still unclear (Quirós et al., 2017).

<u>UPR^{mt} implications in physiology and disease:</u>

Under mitochondrial stress, UPR^{mt} promotes a metabolic shift to glycolysis by limiting OXPHOS gene transcription while increasing transcription of glycolysis components in yeast (Nargund et al., 2015). This function promotes efficient respiratory recovery during mitochondrial dysfunction and generation of ATP by glycolysis to promote survival. Moreover, loss of the TCA cycle enzyme FH1 induces ATF4 and the UPRmt in mammals and *C.elegans,*

reinforcing the notion of UPR^{mt} role in metabolic adaptation (Xusheng Wang et al., 2016). In human cells, it has been shown that mitochondrial dysfunction remodels the one carbon metabolism, activating ATF4 to promote serine biosynthesis and transsulfuration (Bao et al., 2016).

Physiologically, UPR^{mt} has been linked by many studies to longevity. It has been shown that mild mitochondrial perturbations that activate the UPR^{mt} during development result in increased longevity in worms, flies and mice (Copeland et al., 2009; Feng et al., 2001; Lee et al., 2003; Liu et al., 2005). However, UPR^{mt} has also been associated with diverse pathologies like myopathy, Alzheimer disease, depression and glioblastoma (Angelastro, 2017; Beck et al., 2016; Kambe and Miyata, 2015; Suomalainen et al., 2011). It seems that UPR^{mt} is strongly active during development but its activity decreases during ageing (Dillin et al., 2002). Decreased UPR^{mt} activity could explain the decline in mitochondrial quality and activity during ageing (Sun et al., 2016). However, counterbalancing decrease of UPR^{mt} activity could not be positive as recent studies showed that prolonged UPR^{mt} activation could facilitate the propagation of deleterious mitochondrial DNA mutations (Lin et al., 2016).

Another physiological function of the UPR^{mt}, and more generally of the UPR, is the maintenance of HSCs. HSCs resident in the adult bone marrow are predominantly maintained in a dormant state and have the lowest protein synthesis rate within the blood hierarchy of the bone marrow, protecting them from accumulating unfolded proteins (Signer et al., 2014). Interestingly, increased activity of the protein kinase R-like endoplasmic reticulum kinase (PERK) branch of the UPR has been observed in HSCs after ER stress compared to progenitors, leading to their apoptosis probably to avoid the propagation of damage and malignancy risk (Van Galen et al., 2014). This work highlights the precise regulation of the UPR in HSCs. Recently, a study showed that UPR^{mt} is activated upon HSC transition from quiescence to proliferation (Mohrin et al., 2018). Even if the underlying molecular mechanism of UPR implication in HSC maintenance is poorly understood, a recent study discovered a mitochondrial metabolic checkpoint regulated by the histone deacetylase SIRT7 (Mohrin et al., 2015). This study shows that SIRT7 is activated upon UPR^{mt} induction, repressing mitochondrial biogenesis and OXPHOS to coordinate the metabolism required for HSC maintenance. Interestingly, SIRT7 expression is reduced during ageing where HSC maintenance fails, but its reintroduction in aged HSCs could reduce UPR^{mt} and improve their regenerative capacity (Mohrin et al., 2015).

An interesting metabolic status of HSCs is existing in fetal liver (FL). Indeed, contrary to HSCs in the bone marrow, HSCs in the FL are actively expanding (Ema and Nakauchi, 2000). However, despite this elevated proliferation, FL-HSCs did not show ER stress. Studies showed that this phenomenon is due to the presence of bile acids in FL that protect FL-HSCs from ER stress by acting like a chemical chaperone (Sigurdsson et al., 2016). Thus, the use of chemical chaperones could be promising to prevent protein unfolding in HSC.

UPR has been linked to cancer (Ma and Hendershot, 2004). Several studies have reported UPR activation in various tumors (Fernandez et al., 2000; Shuda et al., 2003; Song et al., 2001). UPR activation is believed to protect tumor cells from apoptosis. Indeed, a study showed that blocking BiP function, a well-known UPR protein, inhibits tumor progression in mice (Jamora et al., 1996). Moreover, it has been shown that XBP1 is necessary for tumor growth under hypoxic conditions (Romero-Ramirez et al., 2004). These findings led researchers to target UPR in cancer and more particularly the UPR^{mt}, notably by inhibiting the HSP90 mitochondrial chaperone (Siegelin et al., 2011). Interestingly, ER stress has been shown to sensitize various solid-tumor cells to cisplatin (Chatterjee et al., 1997; Yamada et al., 1999). Cisplatin binds DNA but also proteins and is expected to affect protein folding. Concordantly, cisplatin is a potent activator of the UPR (Mandic et al., 2003).

Interestingly, several studies have linked UPR activation and DNA replication. Notably, it has been shown that PERK activation during ER stress leads to CHK1 phosphorylation (Malzer et al., 2010). Recently, it has been shown that PERK suppresses DNA synthesis by inhibiting both replication fork progression and replication origin firing via Clapsin and CHK1 activation during ER stress (Cabrera et al., 2017). CHK1 activation occurs in the absence of DNA damage and is independent of ATR (Cabrera et al., 2017). However, the precise mechanism of DNA replication inhibition by the UPR is still elusive.

To conclude, we have seen in this chapter that FANC proteins are important to maintain mitochondrial integrity and energy metabolism to avoid genomic instability that is thought to be the major cause of HSC loss and cancer predisposition in FA. In the next chapter, I will develop the different evidences showing increased CFS fragility in FA and how the FANC pathway may prevent chromosomal instability.

3) CFS instability in FA

As discussed above, DNA damage and chromosomal instability is having a major role in FA disease by promoting HSC exhaustion and cancer development. It has been shown in early studies that chromosomal breaks observed in FA were not random but colocalize with CFS (Fundia et al., 1994). This early report has been confirmed by molecular analysis and recent studies suggested that CFS breakage frequency as well as the distribution of chromosome aberrations could evolve with disease progression (Filipovi et al., 2016; Quentin et al., 2011; Schoder et al., 2010). These studies suggest a higher sensitivity of CFS in FA and a possible role of the FANC pathway in maintaining CFS stability. Indeed, it has been shown that CFS instability is increased in the absence of FANC proteins but the mechanisms by which the FANC pathway regulates CFS stability were unclear (Howlett et al., 2005). A few years later, it has been found that FANCD2 can persist in mitosis and form foci in metaphase chromosomes after RS induced by low doses of APH (Naim and Rosselli, 2009). These regions correspond to CFSs and it is supposed that FANCD2 is present there, along with the BLM helicase, to avoid chromatin bridges during mitosis and subsequent genome instability (Chan et al., 2009). With the aim of identifying the proteins that could collaborate with FANCD2 to prevent the formation of such structures, the Rosselli and Debatisse laboratories identified the two structure-specific endonucleases XPF-ERCC1 and MUS81-EME1 to colocalize with FANCD2 at CFS (Naim et al., 2013). It was shown that CFS breakage is an active process carried out by these endonucleases to promote faithful sister disjunction and preserve the integrity of the genome (Naim et al., 2013; Ying et al., 2013).

A recent study provided mechanistic insight into the role of FANCD2 in the maintenance of CFS. Indeed, after FANCD2 had been shown to control replisome function and protect replication forks during DNA replication (Lossaint et al., 2013; Schlacher et al., 2012), the Schildkraut group studied the role of FANCD2 in CFS replication (Madireddy et al., 2016). Interestingly, this study shows that FANCD2, independently of the FANC pathway, is necessary for efficient origin firing at the CFS FRA16D and to help the replication machinery to pass through the core of FRA16D by resolving R-loops which accumulate in the absence of FANCD2 and stall replication forks. Madireddy *et al.* findings are in line with previous observations of R-loop accumulation in FA (García-Rubio et al., 2015; Schwab et al., 2015). Moreover, R-loops were found to be critical for the recruitment of FANCD2 to CFS (Okamoto et al., 2018). This study shows that FANCD2 accumulates in the middle region of very large

genes at CFS after RS, and R-loop removal, or transcription inhibition, decreases FANCD2 binding to these genes. Moreover, FANCD2 is also recruited to transcribed large genes at CFS in avian cells indicating a conserved function of FANCD2 at CFS through evolution (Pentzold et al., 2018).

In vivo, CFS instability implication in FA has been established in the *Fanca* -/- mice model showing increased spontaneous CFS instability in megakaryocytes leading to DNA lesions and bridges, subsequent senescence and decreased platelet production (Pawlikowska et al., 2014).

To summarize, CFS instability is increased in FA and is suspected to contribute to genomic instability progression in this disease. Much efforts have been made recently to understand how the FANC pathway regulates CFS instability. Most of these studies focused their work on the replicative function of the FANC pathway and more particularly on its major actor, FANCD2. We hypothesized that FANCD2 could have an unexplored role in regulating the transcription of very large genes present in CFS to prevent transcription-replication collision and maintain their stability (**Fig. 12**). We also investigated what could be the function of the poorly characterized CFS genes, more particularly in the FA context. Because new functions of the FANC proteins outside the nucleus emerged and many CFS genes are associated with mitochondria, we tested whether metabolic stress could regulate CFS genes transcription.

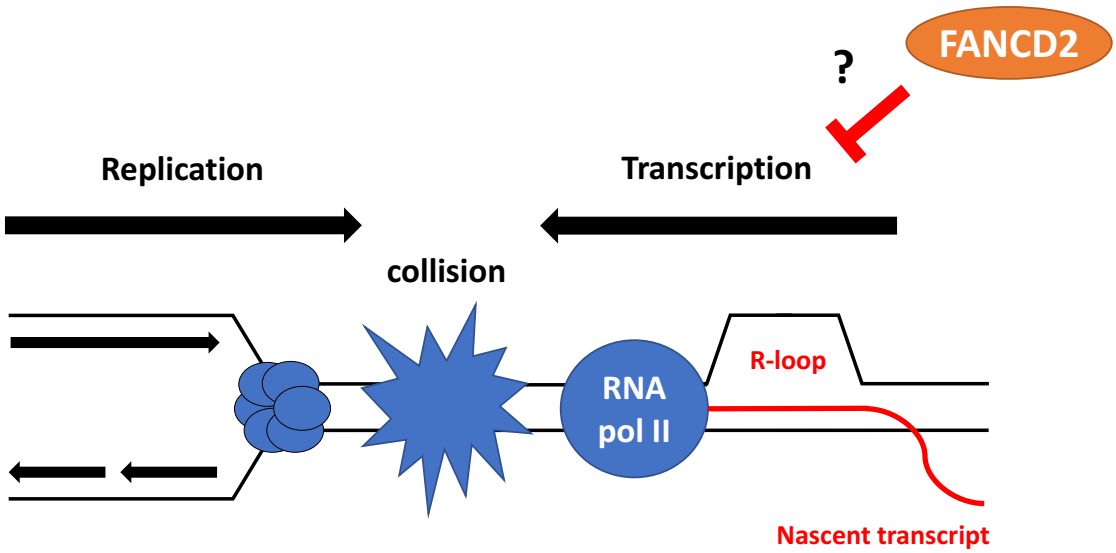


Figure 12: Working hypothesis: is FANCD2 regulating CFS gene transcription to avoid CFS instability?
The aim of my PhD is to characterise the role of FANCD2 in CFS gene transcription and its impact on CFS stability. We also investigated the function of CFS genes in the FA context.

ARTICLE

FANCD2 regulates common fragile site stability by tuning mitochondrial stress and the induction of CFS gene expression by the unfolded protein response.

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Keywords: replication stress, common fragile sites, Fanconi anemia, unfolded protein response

Running title: FANCD2 and the unfolded protein response in CFS maintenance

Summary

Common Fragile Sites (CFSs) are genomic regions that become unstable after replication stress and are frequently implicated in cancer-associated rearrangements. The majority of CFSs are found within very large conserved genes, the transcription of which contributes to CFS breakage. FANCD2, a key protein in the FA pathway (which is deficient in the Fanconi Anemia), maintains the stability of CFSs. In our study, we uncover a new role of FANCD2 in CFS stability maintenance. We show that FANCD2 depletion induces mitochondrial dysfunction, activating a specific unfolded protein response and CFS gene transcription, leading to CFS instability. We demonstrate that FANCD2 recruitment to CFSs depends on transcription and increases following UPR activation. In addition, in the absence of transcription, FANCD2 is not required to maintain CFS stability. Importantly, attenuation of mitochondrial metabolism reduces CFS transcription and breakage. We propose that FANCD2 promotes metabolic homeostasis and genome integrity by tuning the UPR, thus promoting CFS stability.

Introduction

Common fragile sites (CFSs) are genomic regions prone to form breaks and gaps on metaphase chromosomes after replication stress and drive genomic instability in the earliest steps of tumor development (Bignell et al., 2010; Durkin and Glover, 2007; Negrini et al., 2010). CFS instability is cell-type dependent and relies on the cell replication and transcription programs (Letessier et al., 2011; Wilson et al., 2015). Notably, the transcription of very large genes encompassing the CFSs contribute to fork-slowing/stalling, leading to their incomplete replication when cells enter mitosis (Helmrich et al., 2011; Wilson et al., 2015). Incomplete replication of CFSs leads to persistence of late replication or repair intermediates that are processed by structure-specific endonucleases and, if not timely and properly resolved, induces mitotic defects and genomic instability (Fragkos and Naim, 2017).

Among proteins involved in DNA replication/repair, a master regulator of CFS maintenance is FANCD2, a key protein in the FANC pathway; this pathway, composed by at least 21 proteins encoded by the *FANC* genes, is dysfunctional in Fanconi anemia (FA). FA is a rare chromosome instability disorder characterized by bone marrow failure, predisposition to acute myeloid leukemia and epithelial cancer, and hypersensitivity to DNA interstrand crosslinks (ICLs). Chromosomal aberrations in FA patients are not random but occur preferentially at CFSs (Schoder et al., 2010). FANCD2 has been shown to relocalize and form foci at CFSs during mitosis to prevent chromosomal abnormalities after replicative stress (Naim and Rosselli, 2009). In vivo, CFS instability following physiological replication stress is associated with impaired karyokinesis and megakaryocyte differentiation in *Fanca* -/- mice (Pawlikowska et al., 2014). Recent reports have highlighted the role of FANCD2 in coordinating replication and transcription, limiting R-loop formation and promoting CFS replication (Madireddy et al., 2016; Schwab et al., 2015). Therefore, failure to prevent or physiologically resolve R-loops and transcription-associated replication stress can be the cause of DNA damage and genomic instability that underlie the cancer predisposition of FA patients.

The FANC pathway is best known for its role in the repair of ICLs, but the physiological source of such lesions is unknown. Recently, aldehydes have been demonstrated to

have a role in inducing FA-associated phenotypes in mouse models (Garaycoechea et al., 2012), even when the kind of damage generated by these byproducts of cellular metabolism is unclear (Duxin and Walter, 2015). Interestingly, aldehydes induce R-loops in FA-depleted cells (Schwab et al., 2015). Another important effector of the FA clinical phenotype is the impaired redox metabolism associated with mitochondrial dysfunction, which is underscored by the fact that the tumor incidence and the hematopoietic defects in *Fanc*-deficient mice can be improved by antioxidant treatments (Q. S. Zhang et al., 2008, 2010). More recently, FANC proteins have been shown to regulate virophagy and mitophagy (Sumpter et al., 2016). Importantly, when hematopoietic stem cells (HSCs) are activated to enter the cell cycle, mitochondrial activity is increased to sustain their energy demand, and excessive ROS-mediated DNA damage participates in HSC attrition in FA (D. Walter et al., 2015). Thus, understanding how mitochondrial function is regulated and its impact on genome stability is important to better understand FA pathogenesis (Pagano et al., 2005).

Mitochondria are key organelles that regulate many aspects of cellular metabolism, including energy production and nucleotide and amino acid metabolism. They are bounded by a double membrane system with 4 distinct functional compartments, the outer and inner membranes, the intermembrane space and the matrix, and maintenance of the protein-folding environment in each compartment is fundamental for proper organelle function (Pellegrino et al., 2013). The components of the respiratory chain complexes required for oxidative phosphorylation (OXPHOS) activity are encoded by both mitochondrial and nuclear genomes, and coordinated expression from both genomes is crucial to allow the stoichiometric assembly and function of these complexes. Defective import, folding or assembly of these complexes are sensed by mitochondrial protein quality control systems that activate a feedback signaling pathway in order to recover mitochondrial homeostasis (Jovaisaite and Auwerx, 2015). Likewise, the cytosol and endoplasmic reticulum (ER) are exposed to nascent polypeptides and require dedicated protein-folding machinery. To adjust folding capacity and proteostasis, eukaryotic cells have evolved organelle-specific signaling pathways known as unfolded protein responses (UPRs).

In the present study, we uncover a new role of FANCD2 in CFS stability maintenance; specifically, it modulates a specific UPR pathway that induces the transcription of large

CFS genes. We show that FANCD2 attenuates the transcription of large CFS genes and their instability. FANCD2 binding to CFS is dependent on CFS gene transcription and increases in a dose-dependent manner. In addition, we show that FANCD2 is dispensable for maintaining CFS stability in the absence of transcription. Intriguingly, we demonstrate that CFS transcription depends on mitochondrial dysfunction and activation of the UPR. Attenuation of OXPHOS activity decreases CFS gene transcription and breakage in FA. Chemical activation of the mitochondrial and ER-specific UPR pathways induces CFS gene expression, which is dampened by FANCD2. UPR activation also induces FANCD2 relocalization at CFSs, showing its role in CFS regulation in response to the UPR. We propose that CFSs are part of a cellular response that senses mitochondrial and ER stress, and FANCD2, by tuning the UPR with CFS replication, promotes metabolic homeostasis and genome integrity.

Results

FANCD2 attenuates CFS gene expression and prevents CFS instability.

Since transcription is involved in CFS instability, we asked whether FANCD2 regulates the expression of the large genes encompassing these sites. To this end, we downregulated the expression of FANCD2 by siRNA in HCT116 cells, where CFSs have been previously characterized (Le Tallec et al., 2013). We first studied the *FHIT* gene, which encompasses FRA3B, the major CFS in this cell line. Interestingly, downregulation of FANCD2 increased the expression of FHIT, both at the mRNA and protein levels (Figure 1a). The increased *FHIT* expression was confirmed by using three independent siRNAs targeting FANCD2 (Figure 1b). FHIT expression was also increased after FANCD2 depletion in HeLa and RKO cells and after downregulation of the FANC core protein FANCA (Supp. Figure 1a and b). To test whether this was due to increased transcription, we used 5-ethynyl uridine (EU) to label newly synthesized RNA and measure nascent *FHIT* RNA transcripts. We found that the levels of nascent FHIT RNA were significantly higher in FANCD2-depleted cells than in the control (Figure 1c). Then, we wanted to evaluate whether increased *FHIT* transcription in FANCD2-depleted cells was associated with increased FRA3B instability. To do so, we used the fluorescence in situ hybridization (FISH) technique to specifically measure the frequency of breaks at FRA3B. Interestingly, consistent with the increased *FHIT*

transcription, downregulation of FANCD2 significantly increased FRA3B breakage (Figure 1d). To understand whether FANCD2 regulates the transcription of other mapped CFS genes, we analyzed the transcription of *WWOX* and *IMMP2L*, which encompass FRA16D and FRA7K, respectively. Downregulation of FANCD2 increased the transcription of both these large genes (Figure 1e). In contrast, expression of *PTPRG*, a large gene close to *FHIT* in the FRA3B region, was not changed by FANCD2 depletion (Figure 1e). *PARK2* is a large tumor suppressor gene encompassing the FRA6E CFS, which is reportedly not fragile in HCT116 cells (Le Tallec et al., 2013). Interestingly, we found that *PARK2* expression was also increased after FANCD2 depletion (Figure 1e). Therefore, we asked whether the increased transcription observed after the downregulation of FANCD2 could induce the instability of FRA6E. We found that siRNA-mediated downregulation of FANCD2 induced breakage of the otherwise stable FRA6E CFS (Figure 1f). Altogether, these results demonstrate that FANCD2 attenuates CFS gene transcription and the instability of the corresponding CFS.

FANCD2 binds to the gene body of large transcribed genes encompassing CFS and prevents CFS instability after replicative stress in a transcription-dependent manner.

To understand the role of FANCD2 in regulating CFS transcription, we analyzed FANCD2 genomic binding sites by chromatin immunoprecipitation sequencing (ChIP-seq) of the endogenous protein in samples that were untreated or had undergone replicative stress induced by low doses of aphidicolin (APH). In untreated conditions, we found that the majority of FANCD2-targeted sites were associated with genes and proximal upstream or downstream regions, whereas only a minority of FANCD2-targeted sites were intergenic (Figure 2a). After APH treatment, the number and density of peaks was increased, but the FANCD2 binding profile was similar, although the peaks were shifted toward gene bodies and the fraction of intergenic regions was higher (Figure 2a). When analyzing the sites bound by FANCD2 (active regions), we found that the average size of FANCD2-binding sites was higher than that of known transcription factors (Suppl. Fig. 2a). Indeed, we could distinguish a bimodal pattern of FANCD2 binding, one mode corresponding to narrow peaks or FANCD2-“specific sites” and another to broader FANCD2 peak coverage or “domains” (Figure 2b). The

first was enriched in promoter regions, while the latter was enriched in gene bodies (Suppl. Fig. 2b). We then analyzed the active regions where FANCD2 was most enriched after APH treatment (the top induced genes) and found that they were enriched in large genes, most of which corresponded to previously characterized CFSs (Table 1). Interestingly, we found that after APH treatment, FANCD2 was recruited to all the CFS genes described in HCT116; it was also recruited to some CFS genes such as *FHIT* and *WWOX* in untreated conditions, suggesting a role for FANCD2 in the maintenance of these sites, even without exogenous stress (Figure 2c). By comparing the top induced genes in our ChIP-seq data with those in previous studies in U2OS and DT40 cells (Okamoto et al., 2018; Pentzold et al., 2018), we found that the overlapping genes in the three datasets corresponded with large genes, most of which map to CFSs, suggesting that FANCD2 has a specificity toward large CFS genes (Suppl. Fig. 2c). The specific binding of FANCD2 to large genes was further supported by the median size of the genes bound by FANCD2 after APH treatment, which is much higher (251,656 bp) than the median size calculated for all the genes in the genome (23,590 bp) (Suppl. Fig. 2d).

Next, we asked whether FANCD2 binding was correlated with transcription. Interestingly, we found that FANCD2 enrichment at CFSs increased with gene expression level (Figure 2d). We then interrogated the available ENCODE data for the HCT116 cell line to analyze the *FHIT* gene, which is transcribed in these cells, and the large CFS gene *DMD*, which is not transcribed in this cell line (Suppl. Figure 2e). Contrary to *FHIT*, FANCD2 was not recruited to *DMD*, suggesting that transcription is required for FANCD2 binding to CFSs. To confirm this result in an unbiased manner and avoid nonspecific effects of transcription inhibitors, we analyzed HCT116 cells in which the *FHIT* promoter was deleted using the CRISPR/Cas9 system and *FHIT* expression was suppressed (*FHIT*-KO, Suppl. Fig. 2f). We performed FANCD2 ChIP followed by qPCR to analyze FANCD2 binding to the *FHIT* gene in *FHIT*-KO cells. Interestingly, FANCD2 binding to *FHIT* was almost abolished in the absence of transcription, whereas binding to other CFS genes was not affected (Figure 2e). We then asked whether FRA3B fragility was also suppressed. We analyzed metaphase spreads for the frequency of FRA3B breakage by FISH after APH treatment. As shown in Figure 2f, we found that FRA3B breaks were strongly reduced in *FHIT*-KO cells. Most importantly, in the absence of *FHIT* transcription, FANCD2 depletion did not

increase FRA3B breakage relative to control cell values, demonstrating that FANCD2 is required to prevent CFS breakage only when the gene is transcribed. Together, our data highlight that FANCD2 is targeted to CFS genes and prevents their fragility in a transcription-dependent manner.

CFS genes respond to mitochondrial dysfunction and activation of the unfolded protein response.

How is CFS gene transcription regulated by FANCD2? To understand whether an endogenous stress elicited by FANCD2 depletion may be the cause of the increased expression of CFS genes, we first treated the cells with different concentrations of drugs that induce replication fork slowing or stalling (APH, hydroxyurea (HU) or mitomycin C (MMC)) and measured CFS gene transcription. We found that replication stress induced by these drugs increased the transcription of only *WWOX* and not that of other CFS genes, suggesting that another type of stress could trigger their expression (Suppl. Fig. 3a).

Several studies have shown that FANC proteins function in the mitochondria and that they regulate mitophagy by interacting with Parkin, the protein encoded by *PARK2* (Sumpter et al., 2016). Therefore, we analyzed the effects of FANCD2 depletion on mitochondrial function. Downregulation of FANCD2 led to increased oxygen consumption, a decrease in ATP synthesis and ATP/AMP ratios, and a defect in electron transport between complexes I and III of the respiratory chain (Figure 3a). Moreover, we observed that the decline in ATP/AMP ratio was associated with increased lactate dehydrogenase (LDH) activity at time points after FANCD2 depletion, suggesting a shift to glycolytic metabolism to compensate for OXPHOS dysfunction (Suppl. Fig. 3b). Then, we asked whether the expression of CFS genes was dependent on mitochondrial OXPHOS activity. To do so, we used sodium azide (NaN_3), a mitochondrial respiration chain inhibitor. Treatment with NaN_3 decreased the expression of all tested CFS genes in both control and FANCD2-downregulated cells (Figure 3b). Finally, we wanted to ascertain whether physiological attenuation of the OXPHOS metabolism attenuates CFS gene expression. To this end, we cultured cells at low oxygen tension (3%) and measured CFS transcription. Compared to the corresponding levels in cells cultured in 20% O_2, the transcription levels of CFS genes

other than *PARK2* were significantly reduced in FANCD2-depleted cells at 3% O_2, (Figure 3c) while *PARK2* may be induced to promote the shift to glycolytic or fatty acid metabolism (Dorn, 2016; Esteban-Martínez et al., 2017). Interestingly, WWOX transcription was also reduced under these conditions, suggesting a decreased level of replication stress (Figure 3c). We then checked whether chromosome breakage was decreased at 3% O_2 after depletion of FANCD2. Remarkably, the frequency of total breaks on metaphase chromosomes was significantly rescued in FANCD2-depleted cells at 3% O_2 compared with cells at 20% O_2 (Figure 3d). Together, these data indicate that CFS genes respond to mitochondrial dysfunction induced after FANCD2 depletion and that attenuation of oxidative metabolism rescues CFS stability by attenuating CFS gene transcription and replication stress.

Organelle dysfunction is known to activate a conserved signaling cascade called the UPR, which communicates with the nucleus and activates a transcriptional program aimed at restoring organelle homeostasis. The main effector of the mitochondrial stress response in mammalian cells is the transcription factor ATF4 (Quirós et al., 2017). Therefore, we wanted to examine whether ATF4 was induced following FANCD2 depletion. Interestingly, FANCD2 downregulation induced *ATF4* transcription and its nuclear relocalization (Figure 4a), as well as the activation of its targets *CHAC1*, *PCK2*, and *PSAT1* (Figure 4b), indicating the activation of mitochondrial stress signaling. Consistently, siRNA-mediated downregulation of ATF4 decreased their expression and abrogated their induction after FANCD2 depletion (Figure 4b). However, ATF4 was not responsible for the increased CFS gene transcription observed after FANCD2 depletion (Suppl. Fig. 4a), suggesting that CFS genes may be regulated by FANCD2 in a distinct, parallel branch of the UPR. Mitochondria can communicate with other organelles such as the ER in regulating stress signaling. To obtain further insights, we pharmacologically activated the UPR using either carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a mitochondrial uncoupler that activates the mitochondrial UPR (UPR^{mt}), or thapsigargin (TG), a sarco/ER Ca^{2+}-ATPase (SERCA) inhibitor and a known inducer of ER stress (UPR^{ER}). Interestingly, both treatments induced the transcription of CFS genes, which was further increased after FANCD2 depletion, indicating that CFS genes respond to UPR activation and that FANCD2 attenuates their expression (Figure 4c). CCCP and TG treatments also induced the UPR genes *BiP* and *CHOP*, demonstrating their efficacy. However,

FANCD2 depletion did not induce the expression of these two genes. Rather, FANCD2 was necessary for *CHOP* induction after CCCP treatment and, to a lesser extent, after TG treatment (Figure 4d), showing its involvement in the UPR. ATF4 is a downstream target of eIF2alpha which is phosphorylated by the protein kinase R-like ER kinase (PERK) upon UPR activation, but we were not able to detect significant induction of eIF2alpha phosphorylation after FANCD2 depletion in basal conditions (Suppl. Fig. 4b). Accordingly, treatment with PERK inhibitor I (PERKi) failed to attenuate *ATF4* induction and CFS gene transcription after FANCD2 depletion, but inhibited *CHOP* and *BIP* expression after chemical UPR activation (Suppl. Fig. 4c). These results indicate that CFSs are activated by a distinct UPR pathway and that FANCD2 is necessary to modulate this response. Interestingly, it has already been reported that ATF4 can be activated by independent unidentified mechanisms (Münch and Harper, 2016; Quirós et al., 2017). To further support the role of FANCD2 in modulating the UPR, we looked at FANCD2 behavior after UPR induction by TG or CCCP treatment (Figure 4e). Interestingly, UPR activation induced FANCD2 relocalization in nuclear foci, some of which persisted in mitosis, similar to what was observed after APH treatment and suggesting that they correspond to CFSs (Suppl. Fig. 4d). To confirm FANCD2 recruitment to CFS genes after UPR activation, we performed FANCD2 ChIP-qPCR after TG treatment. Strikingly, we detected a significant enrichment of FANCD2 at the *FHIT* gene after TG treatment compared to untreated samples (Figure 4 f). Thus, CFS gene transcription is induced following UPR activation, which in turn promotes FANCD2 recruitment to these genes, relieving the associated stress. Altogether, these data highlight a new role of FANCD2 in mitonuclear communication, thereby attuning the UPR-mediated CFS gene transcription and preventing the associated CFS instability (Suppl. Fig. 4e).

Figure 1

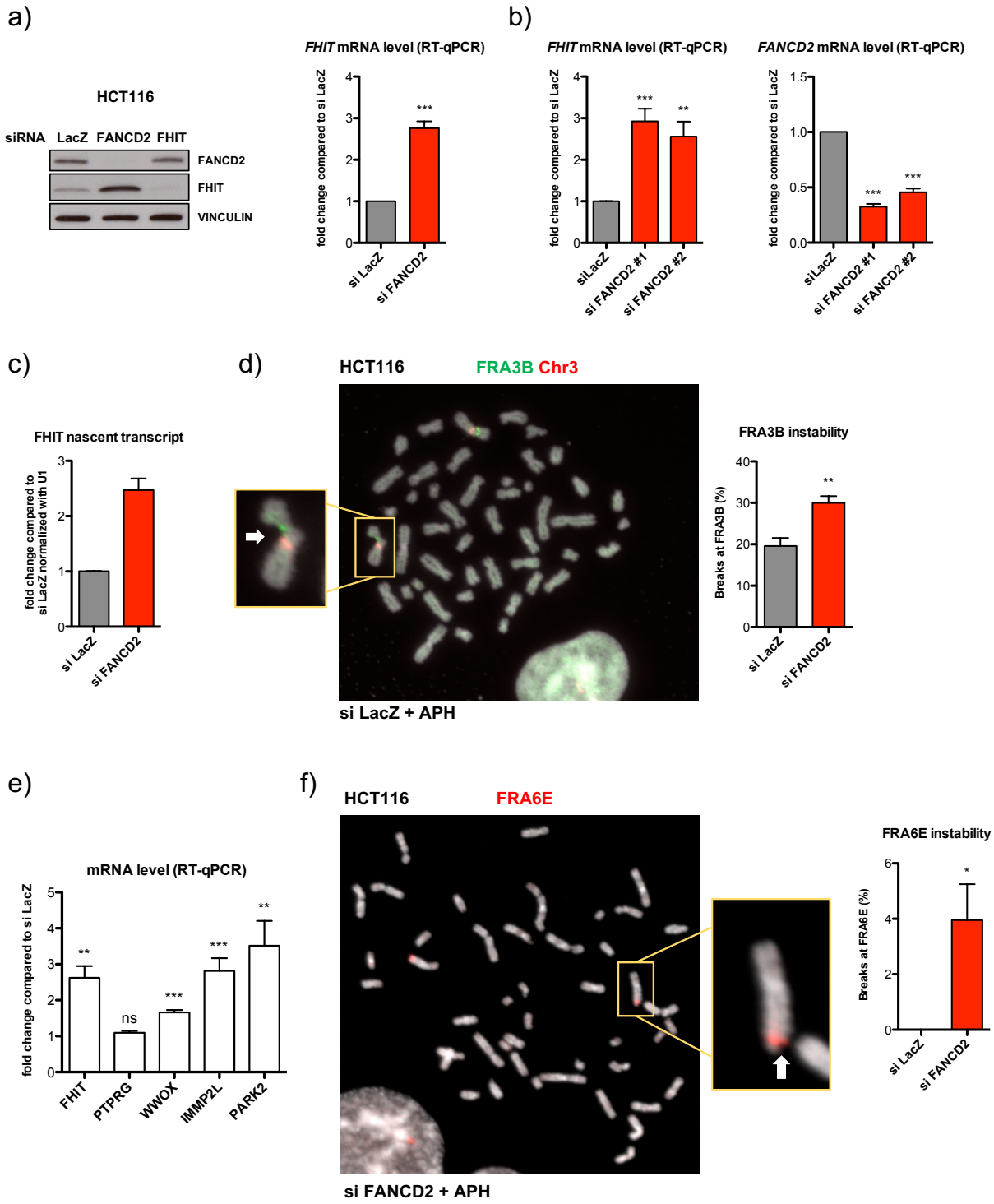
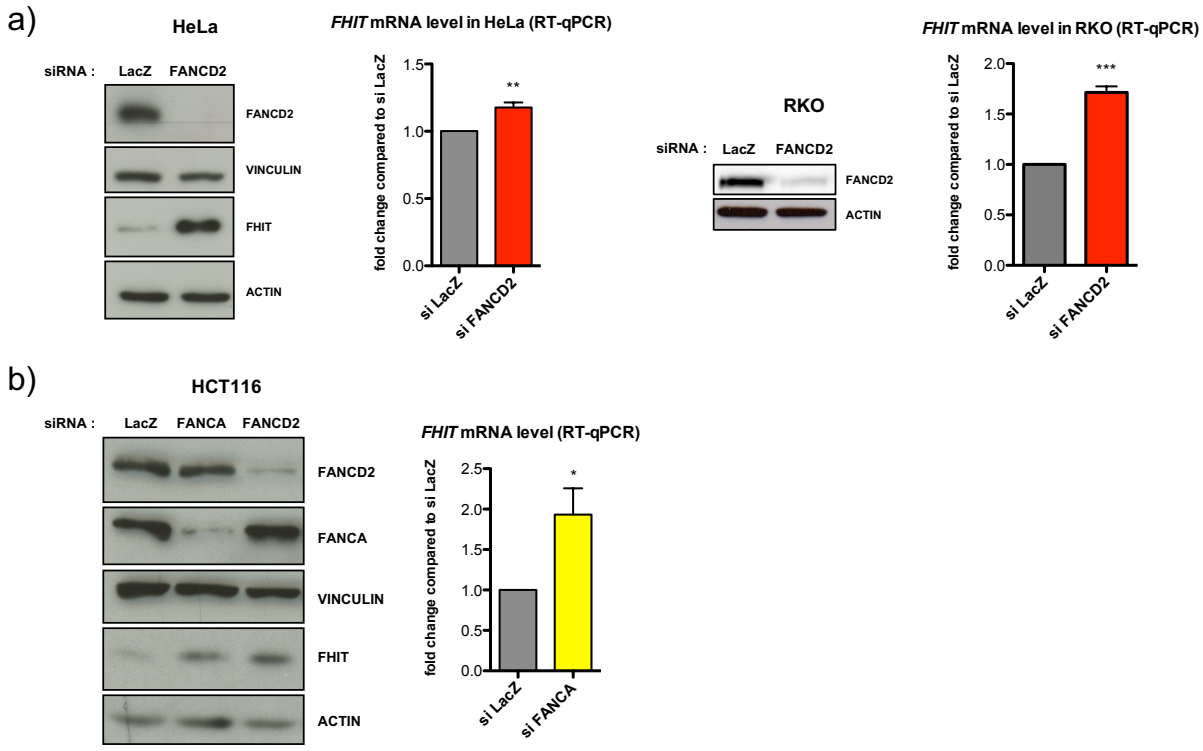

a)
HCT116
siRNA LacZ FANCD2 FHIT
FANCD2
FHIT
VINCULIN
FHIT mRNA level (RT-qPCR)
fold change compared to si LacZ
si LacZ
si FANCD2
b)
FHIT mRNA level (RT-qPCR)
FANCD2 mRNA level (RT-qPCR)
siLacZ
si FANCD2 #1
si FANCD2 #2
c)
FHIT nascent transcript
fold change compared to si LacZ normalized with U1
d)
HCT116
FRA3B Chr3
FRA3B instability
Breaks at FRA3B (%)
si LacZ + APH
e)
mRNA level (RT-qPCR)
FHIT
PTPRG
WWOX
IMMP2L
PARK2
ns
f)
HCT116
FRA6E
FRA6E instability
Breaks at FRA6E (%)
si FANCD2 + APH

Figure 1: FANCD2 attenuates CFS gene expression and prevents CFS instability.

a) Western blot detection of FANCD2 and FHIT protein levels in whole-cell lysates from control and FANCD2 and FHIT siRNA-transfected HCT116 cells. Vinculin served as a loading control. FHIT siRNA transfection was performed to verify the specificity of the FHIT antibody for the band it recognized. The *FHIT* mRNA level was measured by RT-qPCR after control or FANCD2 siRNA transfection (n=3).
b) RT-qPCR analysis of FHIT expression using three independent FANCD2 siRNAs. FANCD2 depletion was estimated by RT-qPCR (n=4).
c) Quantification of nascent EU-labeled *FHIT* transcripts after control or FANCD2 siRNA transfection by RT-qPCR normalized to *U1* (*RNU1-1*) RNA gene expression. (n=2)
d) Representative example of FISH analysis of metaphase spreads from cells treated with 0.3 μM APH, showing a break at FRA3B detected with the FHIT/FRA3B probe. The frequency of FRA3B induction is presented as the percentage of chromosome 3 homologs with breaks at FRA3B (n=4).
e) mRNA levels of large genes measured by RT-qPCR after siFANCD2 treatment compared to levels after siLacZ treatment (n=3).
f) An example of an FRA6E break in a metaphase spread from siFANCD2-transfected cells after treatment with 0.3 μM APH. Frequency of FRA6E induction presented as the percentage of chromosome 6 homologs with breaks at FRA6E (n=3).

Supplemental Figure 1



Supplemental Figure 1: FANCD2 attenuates CFS gene expression and prevents CFS instability.

a) Western blot of whole-cell lysate of control and FANCD2 siRNA-transfected HeLa and RKO cells. mRNA levels of *FHIT* measured by RT-qPCR after treatment with control or FANCD2 siRNA. (n=3 for HeLa cells and n=4 for RKO cells). Note that RKO cells expressed very low levels of FHIT and that we did not detect FHIT protein by Western blot in this cell line.

b) FHIT expression increases after FANCA depletion in HCT116 cells, observed at the protein level by Western blot and the mRNA level by RT-qPCR (n=5).

Figure 2

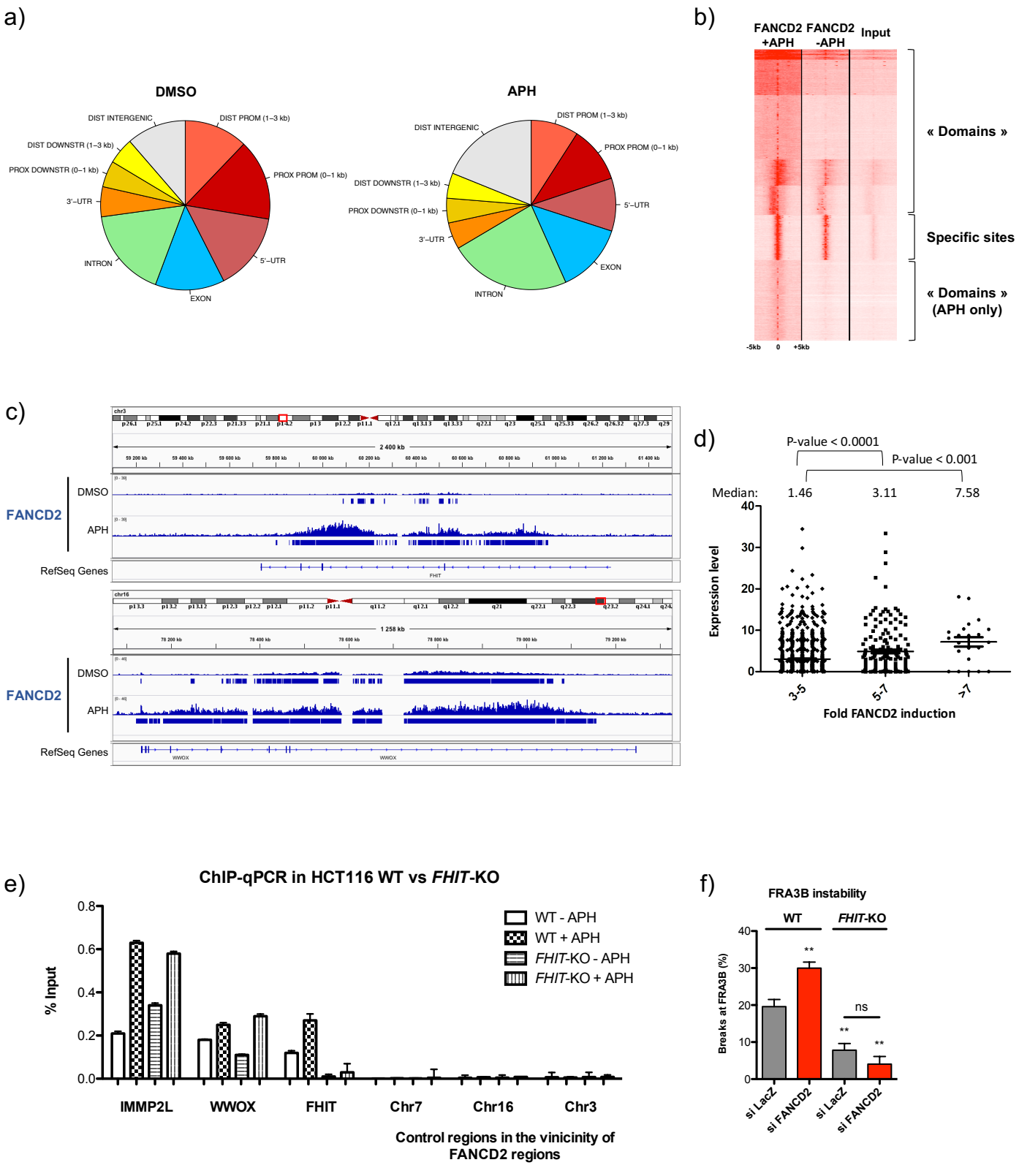
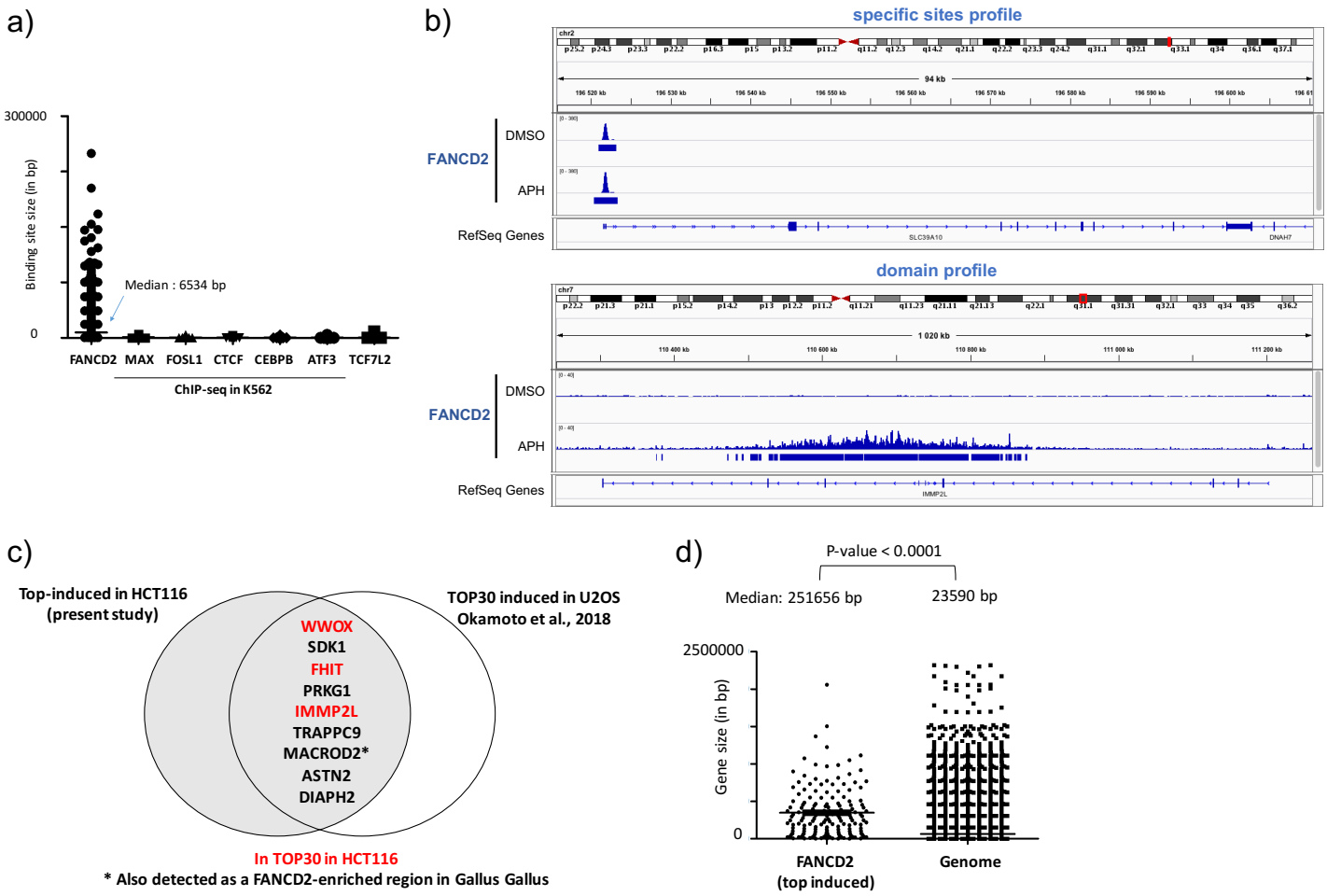

a)
DMSO
APH
DIST INTERGENIC
DIST PROM (1–3 kb)
DIST DOWNSTR (1–3 kb)
PROX DOWNSTR (0–1 kb)
PROX PROM (0–1 kb)
3'–UTR
5'–UTR
INTRON
EXON
b)
FANCD2 +APH
FANCD2 -APH
Input
« Domains »
Specific sites
« Domains » (APH only)
-5kb
0
+5kb
c)
FANCD2
DMSO
APH
RefSeq Genes
d)
P-value < 0.0001
P-value < 0.001
Median:
1.46
3.11
7.58
Expression level
Fold FANCD2 induction
e)
ChIP-qPCR in HCT116 WT vs FHIT-KO
% Input
WT - APH
WT + APH
FHIT-KO - APH
FHIT-KO + APH
IMMP2L
WWOX
FHIT
Chr7
Chr16
Chr3
Control regions in the vinicinity of FANCD2 regions
f)
FRA3B instability
WT
FHIT-KO
Breaks at FRA3B (%)
ns
si LacZ
si FANCD2

Figure 2: FANCD2 binds to the gene body of large transcribed genes encompassing CFSs and prevents CFS instability after replicative stress in a transcription-dependent manner.

(a) FANCD2 location peaks relative to genomic annotations are represented in a pie chart.
(b) Heatmap of FANCD2 peaks across transcription starting site (TSS) (+/- 5kb) clustered in domains and specific sites.
(c) IGV visualization of FANCD2 enrichment along *FHIT* and *WWOX* genes in the presence or absence of APH.
(d) FANCD2 binding fold induction according to gene expression level. Gene expression data were obtained from ENCODE.
(e) FANCD2 ChIP followed by qPCR in wild-type (WT) and *FHIT*-KO cells treated or not treated with 0.3 µM APH. The results are expressed as the percentage of Input (n=3).
(f) FRA3B instability in WT and *FHIT*-KO cells transfected with control or FANCD2 siRNA and treated with 0.3 µM APH (n=3).

Supplemental Figure 2



Supplemental Figure 2: FANCD2 binds to the gene body of large transcribed genes encompassing CFSs and prevents CFS instability after replicative stress in a transcription-dependent manner.

(a) Median size of FANCD2 binding sites compared with the size of known transcription factors. ChIP data for the indicated transcription factors were obtained from public ChIP-seq data available in the University of California, Santa Cruz (UCSC) database for the K562 cell line.

(b) IGV visualization of representative site-specific and domain binding profiles of FANCD2.

(c) Comparative analysis of top FANCD2-induced genes identified in the present study (HCT116) and those of two recent works realized in U2OS and DT40 cells. The genes common to TOP-30 in HCT116 and U2OS datasets are highlighted in red, and the genes that scored in TOP-30 in U2OS are highlighted in black. **MACROD2* is found in all three different studies.

(d) Comparison of the median gene size between FANCD2 top-induced genes and all genes present in the genome.

(e) IGV visualization of FANCD2 enrichment (present study), RNA polII profile and active transcription marks available in ENCODE for HCT116 cells at the two large genes *FHIT* and *DMD*.

(f) FHIT mRNA level detected by RT-qPCR in WT and *FHIT*-KO cells after control or FANCD2 siRNA transfection.

Figure 3

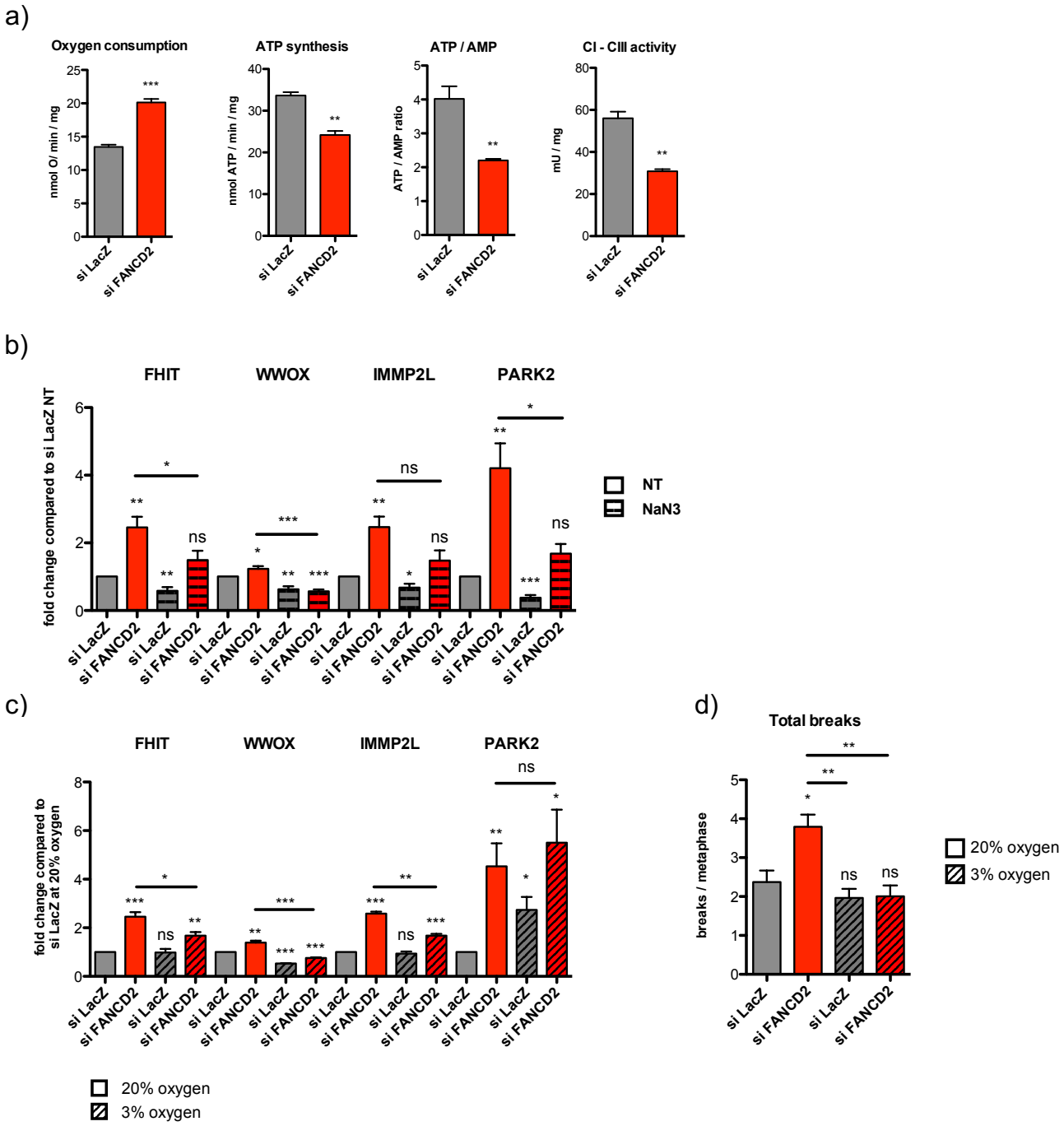

a)
Oxygen consumption
nmol O/ min / mg
ATP synthesis
nmol ATP / min / mg
ATP / AMP
ATP / AMP ratio
CI - CIII activity
mU / mg
si LacZ
si FANCD2
b)
FHIT
WWOX
IMMP2L
PARK2
fold change compared to si LacZ NT
NT
NaN3
c)
fold change compared to si LacZ at 20% oxygen
20% oxygen
3% oxygen
d)
Total breaks
breaks / metaphase

Figure 3: CFS genes respond to mitochondrial dysfunction induced after FANCD2 depletion.

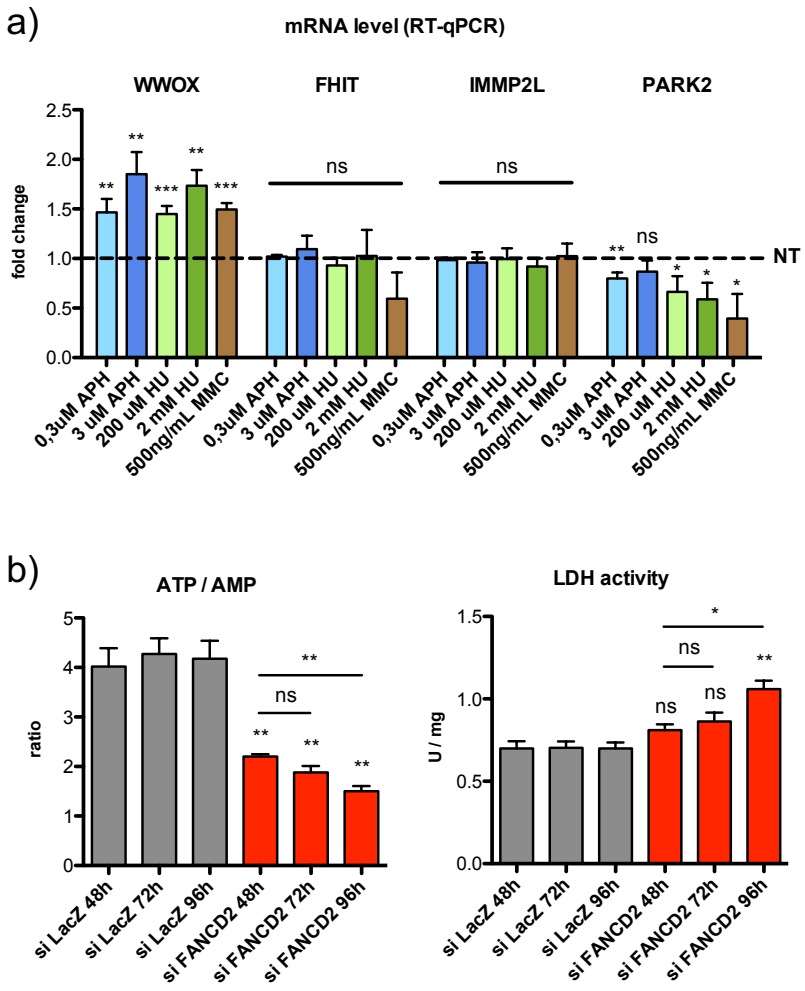
(a) Parameters for mitochondrial activity analyzed after control or FANCD2 siRNA transfection (n=3).

(b) Gene expression levels measured by RT-qPCR after control or FANCD2 siRNA transfection treated or not treated (NT) with 20 mM NaN3 for 10 hr (n=3).

(c) Gene expression levels measured by RT-qPCR after control or FANCD2 siRNA transfection in cells maintained at 20% or 3% oxygen concentration (n=3).

(d) Chromosome fragility in cells transfected with control or FANCD2 siRNA, treated with 0.3 μM APH and maintained at 20% or 3% oxygen. Total breaks are scored as the mean number of breaks per metaphase. At least 50 metaphases were analyzed per condition and experiment (n=4).

Supplemental Figure 3



Supplemental Figure 3: CFS genes respond to mitochondrial dysfunction induced after FANCD2 depletion.

(a) Expression levels of FANCD2 and CFS genes measured by RT-qPCR in cells treated for 16 hr with the indicated drugs relative to untreated cells (NT) (n=5).

(b) ATP/AMP ratio and LDH activity measured 48, 72 and 96 hr after transfection with control or FANCD2 siRNA (n=3).

Figure 4

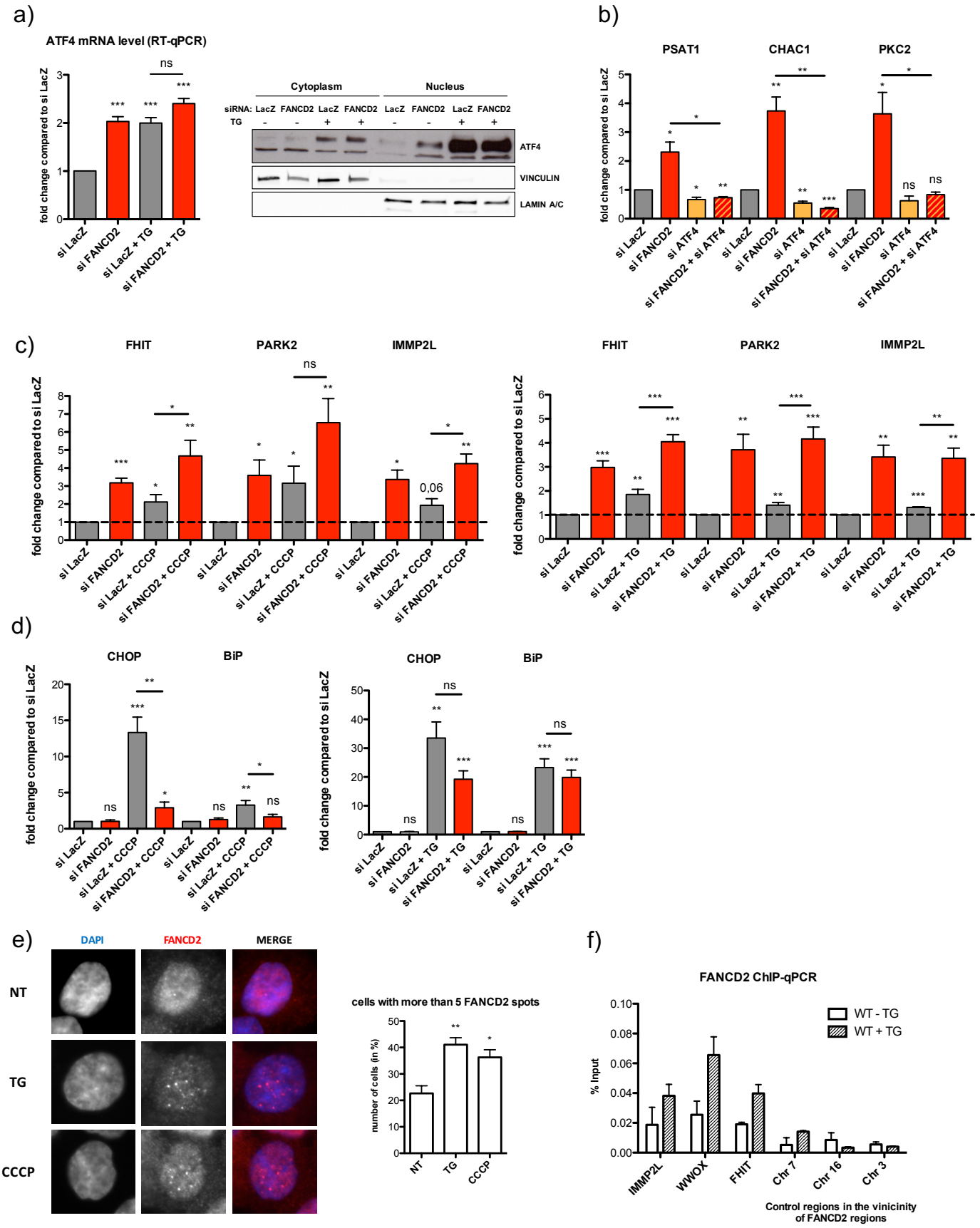

a)
ATF4 mRNA level (RT-qPCR)
fold change compared to si LacZ
si LacZ
si FANCD2
si LacZ + TG
si FANCD2 + TG
Cytoplasm
Nucleus
siRNA: LacZ FANCD2 LacZ FANCD2 LacZ FANCD2 LacZ FANCD2
TG - - + + - - + +
ATF4
VINCULIN
LAMIN A/C
b)
PSAT1
CHAC1
PKC2
si ATF4
si FANCD2 + si ATF4
c)
FHIT
PARK2
IMMP2L
si LacZ + CCCP
si FANCD2 + CCCP
0,06
si LacZ + TG
si FANCD2 + TG
d)
CHOP
BiP
e)
DAPI
FANCD2
MERGE
NT
TG
CCCP
cells with more than 5 FANCD2 spots
number of cells (in %)
f)
FANCD2 ChIP-qPCR
% Input
WT - TG
WT + TG
IMMP2L
WWOX
FHIT
Chr 7
Chr 16
Chr 3
Control regions in the vinicinity of FANCD2 regions

Figure 4: FANCD2 attenuates CFS gene expression activation by modulating a specific pathway of the unfolded protein response.

(a) ATF4 expression measured by RT-qPCR in siLacZ- and siFANCD2-transfected cells, treated or not treated with 1 mM TG (n=5). Western Blot detection of ATF4 in cytoplasmic and nuclear fractions of siLacZ- or siFANCD2-transfected cells treated or not treated with 1 mM TG. Vinculin and lamin A/C were used as loading controls for cytoplasmic and nuclear fractions, respectively.

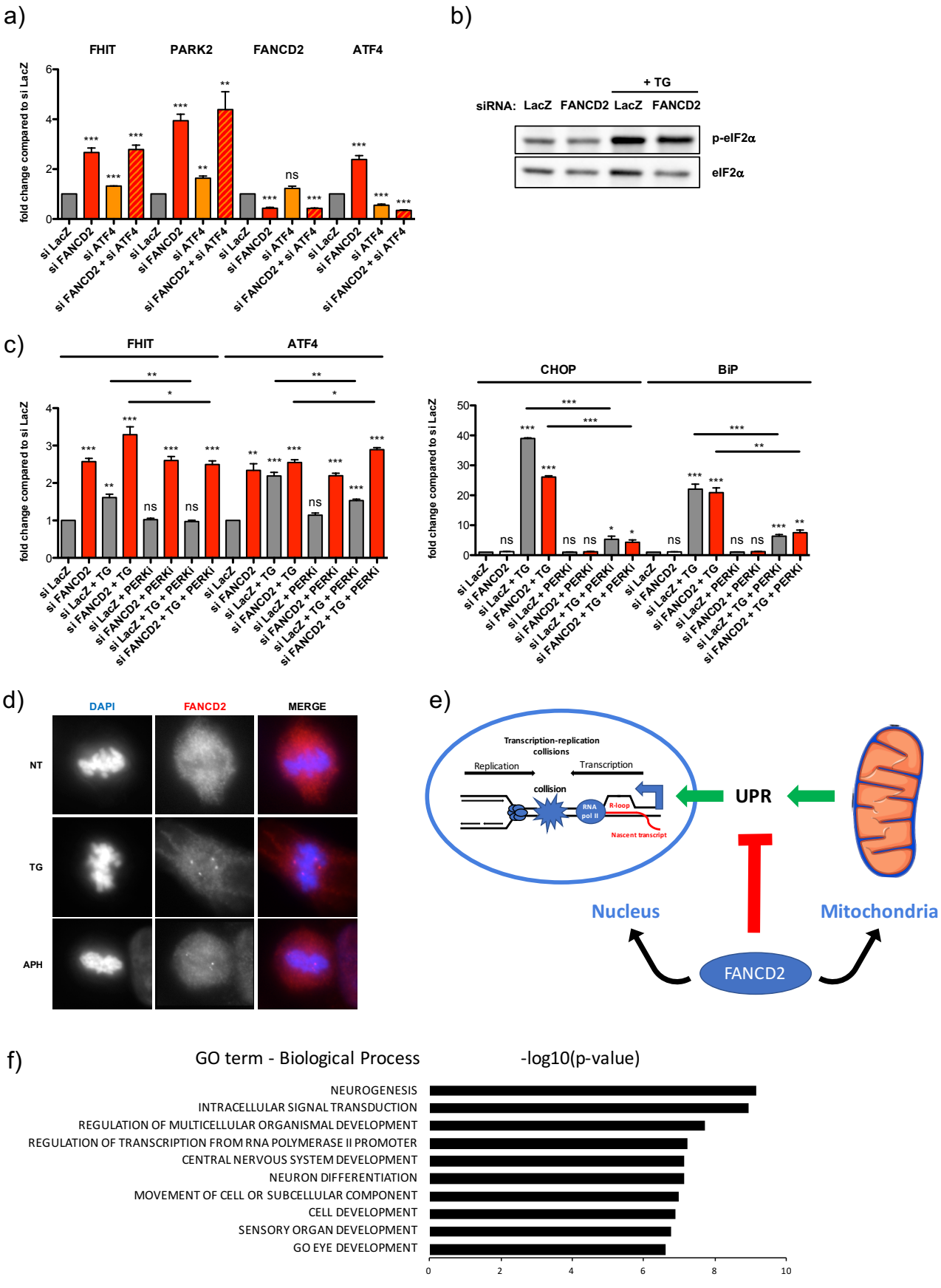
(b) Expression levels of ATF4 target genes measured by RT-qPCR after control and FANCD2 siRNA transfection (n=3).

(c, d) Expression levels measured by RT-qPCR of CFS genes (c) and UPR target genes (d) in siLacZ- and siFANCD2-transfected cells treated or not treated with 10uM CCCP or 1 mM TG (n=3).

(e) Immunofluorescence staining of FANCD2 in untreated cells (NT) or cells after treatment with TG or CCCP. FANCD2 foci were counted in at least 50 nuclei in each of four different experiments, and cells with more than 5 spots were quantified (n=4).

(f) FANCD2 ChIP followed by qPCR in cells treated or not treated with 1 mM TG. The results are expressed as the percentage of the input (n=3).

Supplemental Figure 4



Supplemental Figure 4: FANCD2 attenuates CFS gene expression activation by modulating a specific pathway of the unfolded protein response.

(a) Analysis of CFS gene expression after control, FANCD2, ATF4, or FANCD2+ATF4 siRNA transfection (n=3).
(b) Western blot of whole extracts of cells transfected with control or FANCD2 siRNA and treated or not treated with 1 mM TG.
(c) mRNA levels of FHIT and ATF4 measured by RT-qPCR in siLacZ- and siFANCD2-transfected cells left untreated or treated with 1 mM TG and/or 0.5 μM PERK inhibitor (PERKi) (n=3).
(d) Immunofluorescence staining of FANCD2 in metaphase cells treated or not treated (NT) with TG or APH.
(e) A model for FANCD2 roles in genome stability maintenance. FANCD2 shuttles between the nucleus and mitochondria, ensuring coordination between nuclear and mitochondrial activity. At the mitochondria, FANCD2 ensures mitochondrial activity to supply energy demand for cell proliferation. Mitochondrial stress and UPR activation induces FANCD2 relocalization and retention at CFSs, which promotes their replication. In its absence, mitochondrial dysfunction activates the UPR, which induces CFS gene transcription, increasing transcription-replication collisions and CFS instability.
(f) Gene Ontology analysis of FANCD2 top-induced genes represented as negative log 10 of p-value.

Table 1

Table1: FANCD2 top-induced genes

Rank	Gene ID	Genomic location/fragile site		Gene size (bases)	Description/Function
1	IMMP2L	7q31.1	FRA7K	899,887	Inner Mitochondrial Membrane Peptidase
2	DOCK1	10q26.2	FRA10F	547,109	Dedicator Of Cytokinesis 1, guanine nucleotide exchange factor, cell motility
3	EXOC4	7q33	FRA7H	813,523	Exocyst Complex Component 4, exocyst component, vesicle transport
4	FHIT	3p14.2	FRA3B	1,503,873	Fragile Histidine Triad, nucleotide metabolism, mitochondrial Ca++ uptake
5	SPATA17	1q41		240,373	Spermatogenesis Associated 17, calmodulin binding
6	PTPRG	3p14.2	(FRA3B)	736,045	Protein Tyrosine Phosphatase, Receptor Type G
7	SMYD3	1q44	FRA1I	758,003	KMT3E, Histone-Lysine N-Methyltransferase
8	DPYD	1p21.3	FRA1E	843,317	Dihydropyrimidine Dehydrogenase
9	PLCB1	18q12.2	FRA18A**	891,110	Phospholipase C Beta 1, intracellular transduction
10	DCDC1	11p13	FRA11E	539,442	Doublecortin, Golgi-derived vesicle transport
11	GRID1	10q23.2	putative CFS*	766,939	Glutamate Receptor, Ionotropic, Delta 1, synaptic plasticity
12	FARS2	6p25.1	FRA6B	510,566	Phenylalanyl-TRNA Synthetase 2, Mitochondrial, mitochondrial translation
13	RABGAP1L	1q25.1	FRA1G	835,899	GTP-hydrolysis activating protein (GAP) for small GTPase RAB22A
14	PGCP	8q22.1	FRA8B	504,428	Carboxypeptidase Q, Aminopeptidase
15	SHANK2	11q13.4	FRA11H?**	784,883	SH3 And Multiple Ankyrin Repeat Domains 2, synaptic transmission
16	PARD3B	2q33.3	FRA2I	1,074,370	Par-3 Family Cell Polarity Regulator Beta, asymmetrical cell division and cell polarity
17	NBEA	13q13.3		730,736	Neurobeachin, Lysosomal-Trafficking Regulator 2
18	FHOD3	18q12.2	FRA18A**	482,364	Formin Homology 2 Domain Containing 3, Interaction with SQSTM1
19	SLCO3A1	15q26.1		318,741	Solute Carrier Organic Anion Transporter Family Member 3A1,
20	XYLT1	16p12.3 FS***	putative folate sensitive	369,113	Xylosyltransferase 1, glycosaminoglycan metabolism in the ER
21	GPATCH2	1q41		204,111	G-Patch Domain Containing 2, spermatogenesis,
22	MAD1L1	7p22.3		417,452	Mitotic Arrest Deficient 1 Like 1
23	PRKG1	10q11.23	FRA10G?**	1,307,200	Protein Kinase, CGMP-Dependent, Type I, mediator of the nitric oxide (NO)/cGMP signaling pathway
24	MGMT	10q26.3		300,859	O-6-Methylguanine-DNA Methyltransferase, DNA repair
25	FOXP1	3p13		629,297	Forkhead Box P1, transcription factor
26	WWOX	16q23.1	FRA16D	1,113,255	WW Domain Containing Oxidoreductase, Putative oxidoreductase
27	SUCLG2	3p14.1		294,155	Succinate-CoA Ligase GDP-Forming Beta Subunit, Mitochondrial, TCA cycle
28	KIAA1328	18q12.2	FRA18A**	418,211	Hinderin, Competes with SMC1 for binding to SMC3
29	HDAC9	7p21.1		915,475	Histone Deacetylase 9
30	TBC1D22A	22q13.31	FRA22A?**	440,863	TBC1 Domain Family Member 22A, putative GTPase-activating protein for Rab family protein(s)
31	TRAPPC9	8q24.3	FRA8D	730,499	Trafficking Protein Particle Complex 9, vesicular transport from endoplasmic reticulum to Golgi
32	DIAPH2	Xq21.33	FRAXL	920,335	Diaphanous Related Formin 2, endosome dynamics

* Wilson et al., 2015; Pentzold et al., 2018

**cytogenetically characterized by Debacker and Frank Kooy, 2007

***cytogenetically characterized folate sensitive FS at 16p12.3, Sutherland, 1988

Discussion

Recent work highlighted the role of FANCD2 in enabling efficient replication of CFS by preventing or resolving R-loop formation (Maddireddy et al., 2016). In the present study, we reveal a new role of FANCD2 in CFS stability maintenance, which it accomplishes by coordinating nuclear and mitochondrial function, thus attenuating the transcription of large genes encompassing CFSs. We proved that transcription is required for FANCD2 recruitment to CFS genes, and that in the absence of transcription, FANCD2 is no longer required to prevent CFS instability. According to previous reports suggesting that transcription and R-loop formation may be involved in FANCD2 retention at CFSs, downstream of FANC pathway activation, we showed that FANCD2 enrichment at CFSs is proportional to the level of CFS gene transcription. It would be interesting in the future to understand how FANCD2 is targeted to these sites. One possible scenario is that FANCD2 interacts with components of the transcription machinery. Indeed, recent work has shown that FANCD2 and FANCI interact with and regulate the dynamics of splicing factors that are involved in R-loop processing (Moriel-Carretero et al., 2017). We also showed that FANCD2 enrichment at CFSs is increased after replication stress. Since FANCD2 is recruited to stalled replication forks (Lossaint et al., 2013), the encounters between replication and transcription and R-loop formation may generate the substrate for FANCD2 binding. The precise DNA or chromatin structure promoting FANCD2 binding is still to be defined.

We also discovered a new role of FANCD2 in modulating the UPR. Depletion of FANCD2 induces a specific UPR pathway that involves the ATF4 arm, a key regulator of the mitochondrial retrograde response (Quirós et al., 2017). Indeed, ATF4 protein and its main target genes were activated. Interestingly, these genes are involved in rewiring the mitochondrial metabolism, suggesting that activation of this pathway following FANCD2 depletion may be beneficial, at least in part, to recover from stress. However, we find that the expression of CFS genes is not dependent on ATF4. Strikingly, a closer inspection of the top FANCD2-induced genes revealed a strong enrichment of CFS genes with reported functions in mitochondrial activity, ER dynamics and secretory pathways (Table 1). In agreement, we showed that both

mitochondrial and ER stress induced their expression, which is attenuated by FANCD2. Therefore, it is likely that transient activation of these genes is also required to recover mitochondrial or ER homeostasis. We propose that transcription of these genes may interfere with their replication in a dose- and replicative stress-dependent manner. It is important to note that mitochondrial dysfunction can lead to replicative stress by a shortage or an unbalanced synthesis of nucleotides. We have found that *WWOX* gene expression is induced by replicative stress. In this scenario, mild mitochondrial dysfunction and UPR activation would be beneficial and constitute a feedback mechanism ensuring cellular homeostasis. However, prolonged or excessive UPR activation would lead to excessive transcription of CFS genes with concomitant replication stress and would generate a conflict between these two processes. Accordingly, FANCD2 binding to CFS genes is proportional to their level of transcription and increases with replication stress, suggesting that these two factors together promote FANCD2 binding and function to prevent CFS instability. We have demonstrated that in the presence of replication stress but the absence of transcription, CFS breakage is low and not dependent on FANCD2. Likewise, transcription is necessary but not sufficient to generate breakage, since CFS breaks are mostly induced by APH (even if they could break at very low frequency in unperturbed conditions).

We propose that CFS loci behave as both cis- and trans-acting components of the UPR that become unstable above a threshold of UPR activation and replication stress. This trigger would activate FANCD2 recruitment, constituting a sort of metabolic checkpoint. In the absence of FANCD2, chronic UPR activation could generate a biphasic feedforward loop in which mitochondrial and replication stress would allow for stress compensation in the short term but interfere with CFS replication later, inducing their breakage.

CFS fragility is cell-type dependent and relies on replication and transcription programs, both of which are developmentally regulated and determine cell fate and identity. Remarkably, Gene Ontology analysis of the top FANCD2-induced genes identified in our ChIP-seq revealed a strong enrichment in biological processes related to development, notably to that of the central nervous system (Suppl. Fig. 4f). Previous work has shown that recurrent double strand break (DSB) clusters in neural

stem/progenitor cells occur in large transcribed genes involved in neural function and synapsis and suggested that genomic alterations affecting these genes might generate neuronal diversity (P. C. Wei et al., 2016). Recent studies have highlighted the fact that dynamic UPR activation controls the balance between direct and indirect neurogenesis and regulates brain development (Laguesse et al., 2015). Therefore, the UPR, by regulating CFS genes, may induce metabolic reprogramming and plasticity during cell differentiation. Intriguingly, the UPR has also been shown to be a major pathway that regulates hematopoietic stem cell function and integrity (Mohrin et al., 2015; Sigurdsson and Miharada, 2018; Van Galen et al., 2014). It will be crucial in the future to better characterize how the FA pathway regulates the UPR during hematopoiesis and whether it modulates the expression and stability of specific CFSs in hematopoietic cells.

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Material and methods

Cell culture

The HCT116 cell line was maintained in McCoy's 5A medium (ATCC), and RKO and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) at 37°C in a humidified atmosphere under 5% CO_2. Media were supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin. All cell lines were purchased from the ATCC. HCT116 KO-FHIT cells generated by CRISPR/Cas9 genome editing were kindly gifted by Pr. M Debatisse.

For experiments requiring low oxygen conditions, cells were maintained in an incubator provided with an O_2 control system (HERAcell 150i, Thermo Scientific).

siRNA transfection

siRNA duplex oligonucleotides were purchased from Ambion to target FHIT (#AM16708) and from Eurogentec to target the other genes. siRNA sequences are provided in Supplementary Table 2. For all siRNA experiments, cells were transfected with siRNAs at a final concentration of 20 nM using Interferin (Polyplus) according to manufacturer's instructions. Following siRNA transfection, knockdown of gene expression was assessed by Western blot or qRT-PCR analysis. Unless otherwise indicated, cells were collected for total cell lysate preparation, subcellular fractionation, biochemical assays, and qRT-PCR analysis 48h after transfection.

Western blotting and subcellular fractionation

For total lysates, cells were disrupted in lysis buffer (50mM Tris-HCl, 20 mM NaCl, 1 mM $MgCl_2$, SDS 0.1%) containing a protease and phosphatase inhibitor cocktail (Roche) supplemented with 0.1% endonuclease (benzonase, Millipore) for 10 min at room temperature with rotation. For fractionation analysis, cells were lysed using the NE-PER kit (ThermoFisher) and following the manufacturer's instructions. Laemmli

buffer containing beta-mercaptoethanol was added, and samples were boiled for 5 min at 95°C. Samples were separated on SDS-PAGE denaturing gels (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with phosphate-buffered saline (PBS)-milk (5%) or PBS-bovine serum albumin (BSA) (3%) for 1 hr, and signals were visualized with WesternBright ECL (Advansta) on a digital imaging system (GeneGnome, Syngene) or using Amersham Hyperfilm ECL films (GE) on a table-top processor (Curix 60, AGFA). The antibodies used are listed in Supplementary Table 2.

Quantitative RT-PCR

Total cellular RNA was extracted with the ReliaPrep RNA Cell Miniprep System (Promega), and 1 µg of RNA was used to synthesize cDNA with the High-Capacity RNA-to-cDNA kit (ThermoFisher). PCR primers were purchased from Eurogentec and used in PCRs with SYBR Green Master Mix (ThermoFisher) on a QuantStudio 7 Flex (Applied Biosystems). Relative gene expression was calculated using the ΔΔCq calculation method and normalized to *GAPDH* expression. Values are represented as a fold change compared to the control transfection values (siLacZ). Primer sequences are available in Supplementary Table 2.

Cell treatments and chemicals

Replicative stress was induced by treatment with APH (Sigma A0781), HU (Sigma H8627) or MMC (Sigma M4287) for 16 hr at the indicated doses.

The UPR was induced by treatment with 1 mM TG (Interchim 42759J) or 10 µM CCCP (Sigma C2759) for 8 hr. PERK inhibitor I (Merck 516535) was added at 0.5 µM at the same time as TG treatment.

For OXPHOS inhibition, sodium azide (NaN_3, Sigma S8032) was used at 20 mM for 10 hr.

Immunofluorescence

Cells grown on glass cover slips were fixed in 4% formaldehyde for 15 min before permeabilization with 0.5% Triton for 10 min at room temperature. After blocking with 3% BSA in PBS containing 0.05% Tween 20, cells were stained overnight with the primary antibody against FANCD2 and then with a secondary antibody, anti-rabbit Alexa Fluor 594 (Invitrogen), for 1 hr at room temperature. Slides were mounted in DAKO mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (SouthernBiotech) and examined at a magnification of 63x using an epifluorescence microscope (Zeiss Axio Observer Z1) equipped with an ORCA-ER camera (Hamamatsu). The microscope and camera parameters were set for each series of experiments to avoid signal saturation. Image processing and analysis were performed using ImageJ software.

Nascent transcript analysis

Nascent transcripts were captured and analyzed using the Click-iT Nascent RNA Capture Kit from ThermoFisher following the manufacturer's instructions. Briefly, cells were seeded in a 6-well plate and transfected the next day. Next, 48 hr after transfection, cells were incubated with 0.5 mM 5-ethynyl uridine (EU) for 1 hr and harvested for RNA extraction. Then, 5 µg of RNA was biotinylated with 0.5 mM biotin azide and precipitated. Finally, 1 µg of biotinylated RNA was bound to 50 µL of streptavidin magnetic beads and used for cDNA synthesis and qPCR.

Metaphase spread preparation and FISH analysis

Forty-eight hours after transfection, cells were incubated or not incubated with 0.3 µM APH for 16 hr. Cells were then exposed to 100 ng/mL colcemid (Roche) for 3 hr, treated with hypotonic solution (0.075 M KCl) for 15 min and fixed with 3:1 ethanol/acetic acid overnight at -20°C. Cells were dropped onto slides and dried during the day. For FRA3B analysis, two FISH probes were used: the ZytoLight SPEC FHIT/CEN 3 dual color probe (ZytoLight) and labeled bacterial artificial chromosomes (BACs). Briefly, bacterial strains containing the BACs (RP11-170K19, RP11-495E23) were grown overnight at 37°C with 12.5 µg of chloramphenicol and extracted using the BACMAX

DNA purification kit (Epicentre). Then, DNA was sonicated to obtain fragments shorter than 400 bp, which were then labeled in green or red using the labeling kit PlatinumBright (Kreatech) following the manufacturer instructions. A PARK2 FISH probe (Empire Genomics) was used for FRA6E analysis. Briefly, slides were sequentially incubated in 70%, 90% and 100% ethanol for 2 min and dried. Then, 10 µL of each probe was added to the slide, and a cover slip was inverted on it with rubber cement on its edges to avoid dehydration. Slides were placed on an automatic hybridizer (Hybridizer, Dako), and slides were heated at 72°C for 2 min and then at 37°C for at least 16 hr. Coverslips were removed in wash buffer (0.5 SSC, 0.1% SDS) at 37°C, and slides were incubated in wash buffer for 5 min at 65°C to remove nonspecific signals. Slides were washed with PBS, and DAPI was added with mounting medium for microscope analysis.

Chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq)

ChIP-seq experiments were performed using Active Motif ChIP sequencing services. First, 1 x 10^7 HCT116 cells that had been treated or not treated with 0.3 µM APH were fixed in 11% formaldehyde for 15 min. After cell lysis, 30 µg of chromatin was used for immunoprecipitation using the FANCD2 antibody (Novus). Immunoprecipitated and input DNA were sequenced by Illumina sequencing, generating 75-nt sequence reads. More than 30 x 10^6 reads per condition were obtained, and a spike-in adjusted normalization method was applied. Peaks were called using the SICER algorithm and aligned to human genome build hg19. The Integrative Genomics Viewer (IGV) was used to visualize peaks from the genome.

ChIP followed by quantitative PCR

After preclearing with magnetic beads for 1 hr, the chromatin from an equivalent of 1 × 10^7 HCT116 cells was used for immunoprecipitation with FANCD2 antibody (Novus) or immunoglobulin G, as a control. After an overnight incubation at 4°C, the beads were washed and eluted in buffer E (25 mM Tris-HCl [pH 7.5], 5 mM EDTA, 0.5% SDS), and crosslinking was reversed at 65°C with proteinase K for 6 hr. The DNA was

then purified using the QIAquick PCR purification kit (QIAGEN) and eluted in 100 µl of distilled water. PCR primer pairs are listed in Supplementary Table 2.

Oxygen consumption measurements

Oxygen consumption was measured at 25°C in a closed chamber using an amperometric electrode (Unisense Microrespiration, Unisense A/S, Denmark). Cells were permeabilized with 0.03 mg/ml digitonin for 1 min, centrifuged for 9 min at 1000 rpm and resuspended in the appropriate buffer [Grasselli et al., 2010, J. endocrin.]. The same solution was used in the oxymetric measurements. For each experiment, 500,000 cells were used. Finally, 10 mM pyruvate plus 5 mM malate were added to stimulate the pathway by Complexes I, III and IV.

Electron transfer from Complex I to Complex III

The electron transfer from Complex I to Complex III was studied spectrophotometrically by following the reduction in cytochrome *c* at 550 nm. The molar extinction coefficient used for reduced cytochrome *c* was 1 mM^{-1} cm^{-1}. For each assay, 50 µg of total proteins was used. The assay medium contained 100 mM Tris-HCl pH 7.4 and 0.03% cytochrome *c*. The reaction was started with the addition of 0.7 mM NADH. If the electron transport between Complex I and Complex III is conserved, the electrons pass from NADH to Complex I, then to Complex III via coenzyme Q, and finally to cytochrome *c*.

ATP and AMP quantification

ATP and AMP were measured according to the enzyme coupling method of Bergmeyer et al. (Bergmeyer HU, Grassl M, Walter HE (1983) Methods of Enzymatic Analysis, Verlag-Chemie, Weinheim, p. 249). For ATP assays, medium contained 20 µg of sample, 50 mM Tris- HCl pH 8.0, 1 mM NADP, 10 mM $MgCl_2$, and 5 mM glucose in a final volume of 1 ml. Samples were analyzed spectrophotometrically before and after the addition of 4 µg of purified hexokinase/glucose-6-phosphate dehydrogenase (Boehringer). The rise in absorbance at 340 nm due to NADPH formation was proportional to the ATP concentration. For AMP assays, the medium contained 20 µg

of sample, 50 mM Tris-HCl pH 8.0, 1 mM NADH, 10 mM $MgCl_2$, 10 mM phosphoenolpyruvate (PEP), and 2 mM ATP in a final volume of 1 ml. Samples were analyzed spectrophotometrically before and after the addition of 4 µg of purified pyruvate kinase/LDH (Boehringer). The rise in absorbance at 340 nm due to NADH oxidation was proportional to the AMP concentration. For all biochemical experiments, protein concentrations were determined using the Bradford method.

Glycolytic enzyme assay

Enzymatic LDH (EC 1.1.1.27) activity was measured to quantified the glycolytic activity, and activity was expressed as IU/mg of total protein (micromoles/min/mg of protein). The reaction mixtures used for the determination contained 100 mM Tris-HCl pH 9, 5 mM pyruvate, 40 µM rotenone and 0.2 mM NADH.

Fo-F1 ATP synthase activity assay

Evaluation of the Fo-F1 ATP synthase activity was performed as previously described. Briefly, 200,000 cells were incubated for 10 min (min) in an appropriate medium, and ATP synthesis was induced by the addition of 0.1 mM ADP. The reaction was monitored every 30 seconds for 2 min in a luminometer (GloMax® 20/20n Luminometer, Promega Italia, Milan, Italy) for the luciferin/luciferase chemiluminescent method, with ATP standard solutions at concentrations between 10^{-8} and 10^{-5} M (luciferin/luciferase ATP bioluminescence assay kit CLSII, Roche, Basel, Switzerland). Data were expressed as nmol ATP produced/min/10^6 cells. The oxidative phosphorylation efficiency (P/O ratio) was calculated as the ratio between the concentration of the produced ATP and the amount of consumed oxygen in the presence of respiring substrate and ADP (Hinkle, 2005 BBA).

Statistical analysis

All quantitative data are represented as the mean ± SD of at least three independent experiments. Statistical significance was tested using a two-tailed Student's t test.

Statistical tests were performed using Prism (GraphPad software). P scores are indicated as *p<0.05, **p<0.01, and ***p<0.001 and ns for not significant (p>0.05).

Supplementary Table 2: siRNA sequences, antibody and primer lists.

siRNA	sequence
LacZ	CGU-CGA-CGG-AAU-ACU-UCG-A
FANCD2	GGA-GAU-UGA-UGG-UCU-ACU-A
FANCD2 #2	GAU-AAG-UUG-UCG-UCU-AUU-A
FANCD2 #3	GAA-CAA-AGG-AAG-CCG-GAA-U
FANCA (mix of 4 sequences)	GUU-AGA-GUU-UGC-UCA-GUA-U
	GAG-CCG-UGC-AGA-UCU-GUC-C
	CGC-UUU-GGC-UGC-UGG-AGU-A
	CGA-CAU-GCA-UGC-UGU-GGG-A

Protein	Company	Reference
ACTIN	Santa Cruz	1616
ATF4	Proteintech	10835-1-AP
eIF2a	Santa Cruz	133132
FANCA	Abcam	AB97578
FANCD2 (ChIP)	Novus Biologicals	NB100-182
FANCD2 (IF)	Abcam	AB108928
FANCD2 (WB)	Santa Cruz	20022
FHIT	Thermo Fisher	71-9000
LAMIN A/C	Santa Cruz	7292
P-eIF2a (Ser51)	Cell Signaling	3597S
VINCULIN	Abcam	AB18058

mRNA expression

ATF4	GTT-CTC-CAG-CGA-CAA-GGC-TA	ATC-CTG-CTT-GCT-GTT-GTT-GG
ATR	ACC-TCA-GCA-GTA-ATA-GTG-ATG-GA	GGC-CAC-TGT-ATT-CAA-GGG-AAA-T
BiP	TGT-TCA-ACC-AAT-TAT-CAG-CAA-ACT-C	TTC-TGC-TGT-ATC-CTC-TTC-ACC-AGT
CHAC1	GTG-GTG-ACG-CTC-CTT-GAA-GA	TTC-AGG-GCC-TTG-CTT-ACC-TG
CHOP	AGA-ACC-AGG-AAA-CGG-AAA-CAG-A	TCT-CCT-TCA-TGC-GCT-GCT-TT
FANCD2	CCA-TGG-TCA-CAG-CAC-CAA-TA	TCA-GCA-CAC-TGG-CAT-TTA-GC
FHIT	CGT-TCA-CGT-CCA-TGT-TCT-TC	CTC-CAA-GAG-GCA-GGA-AAG-TC

GAPDH	CCT-CAA-CGA-CCA-CTT-TGT-CA	TTC-CTC-TTG-TGC-TCT-TGC-TG
IMMP2L	TGA-AGG-AGA-TAT-TGT-CAG-AAC-CAT-AGG	GAT-CAC-CTT-CAA-CCC-AGA-TGT-GA
PARK2	ACC-TCA-GCA-GCT-CAG-TCC-TC	TGC-TGC-ACT-GTA-CCC-TGA-GT
PCK2	CAT-CCG-AAA-GCT-CCC-CAA-GT	GCT-CTC-TAC-TCG-TGC-CAC-AT
PSAT1	GTC-CAG-TGG-AGC-CCC-AAA-A	TGC-CTC-CCA-CAG-ACC-TAT-GC
U1	CCC-AGG-GCG-AGG-CTT-ATC-CA	CGA-ACG-CAG-TCC-CCC-ACT-AC
WWOX	AGT-GGC-AGG-AGA-TTT-GCC-AT	GTG-ACC-ACA-ACC-ACT-TTG-GC

ChIP-primers

IMMP2L	TCC-TCC-TGG-CTG-CTT-TCA-TG	GAG-CTG-TGA-GAA-GTG-TGC-CA
WWOX	CCA-CCT-TTT-GCC-GCA-GTA-AC	AGG-CCC-TGA-TGA-CAT-CTC-CT
FHIT	AGG-CAA-GTT-GAC-AAC-ACC-CA	CGA-AGC-CAG-GGT-TTG-CAA-TC
Chr7	ACC-TGA-CAG-AAC-CCA-GAT-GC	TGT-GGG-AGT-GAG-GGA-AAA-AG
Chr16	AAC-CAC-CCT-CCA-CAA-GAC-TG	CCG-TCT-CAA-TAG-GAG-GGA-CA
Chr3	CGC-ACT-TGC-TAT-TCC-CTC-AT	AAG-CTG-GCC-TGT-GAG-TAG-GA

Author contributions

PF carried out experiments, interpreted the data, and wrote the manuscript. BM performed ChIP-seq analysis and ChIP-qPCR experiments. SR and EC performed biochemical and metabolism analyses. VN conceived and supervised the project, performed initial experiments, interpreted data and wrote the manuscript, with input from other authors.

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ANNEX

Unpublished results:

FANCD2 is necessary to maintain H4K16 acetylation at CFS after replicative stress.

Chromatin conformation is impacting multiple biological processes including DNA transcription, replication, and repair which are factors implicated in CFS instability. Notably, it has been shown that CFS regions are characterized by histone hypoacetylation and increasing acetylation of these regions could reduce CFS instability (Jiang et al., 2009). We asked whether FANCD2 could maintain chromatin conformation to prevent CFS instability. Interestingly, FANCD2 interacts with the histone acetyltransferase Tip60 which acetylates the histone H4 on its lysine 16 (H4K16ac) to prevent the binding of 53BP1 during DNA repair (Renaud et al., 2015). To ascertain if FANCD2 loss could impact chromatin conformation, we performed H4K16ac ChIP-seq in presence or absence of FANCD2 with or without replicative stress (APH) in HCT116.

Whole genome analyses revealed that H4K16ac was enriched in gene regions in all the conditions tested (Figure A). However, depletion of FANCD2 increased H4K16ac at transcription starting sites (TSS) (Figure B). Moreover, in control siRNA conditions, APH treatment increased H4K16ac along genebodies whereas in the absence of FANCD2, APH treatment lead to a decrease in H4K16ac (Figure B). These results suggest that FANCD2 is necessary for the deposition of the H4K16ac mark through genebodies and its maintenance during replicative stress. The appearance of a "wave" of H4K16ac at TSS after FANCD2 depletion could represent chromatin opening necessary for transcriptional activation or a blockage of H4K16ac spreading in genebodies, leading to its accumulation at TSS.

To analyze H4K16ac at CFSs, we compared data obtained from FANCD2 and H4K16ac ChIP-seq experiments. We confirmed that CFSs regions are hypoacetylated compared with surrounding regions as shown for the FRA3B region (Figure C). Interestingly, we observed that FANCD2 is mostly enriched in the body of the genes present in hypoacetylated regions after replicative stress (Figure C). Then, we quantified H4K16ac abundance according to FANCD2 recruitment after replicative stress. Interestingly, we observed that H4K16ac levels were not

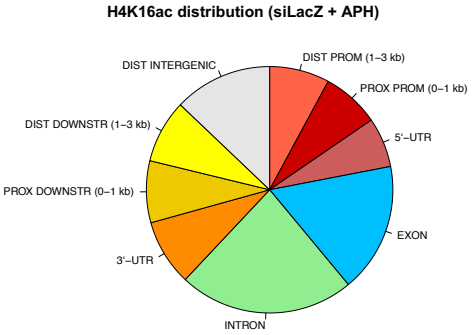
affected in genes where FANCD2 is not recruited but, on the contrary, they were diminished in the absence of FANCD2 in genes where FANCD2 is recruited after replicative stress (Figure D). These results indicate that FANCD2 is recruited to genes present in hypoacetylated regions and maintains H4K16ac mark after replicative stress.

Further work is necessary to determine the cause of H4K16ac loss after FANCD2 depletion. This loss could be due to a direct role of FANCD2 in the deposition of this mark through its interaction with an acetyltransferase (Tip60 for example) or by an indirect effect due to less active replicative forks passing through CFS genes and consequently, impaired deposition of this mark (Ruan et al., 2015). A low level of H4K16 acetylation is generally associated with low level gene transcription which is in line with the low expression of genes encompassing the CFS. However, our previous data reveal increased transcription of CFS genes after FANCD2 depletion which seems at odds with the decrease in H4K16ac. This conflictual situation, an increased transcription over a less accessible chromatin region, could be damageable for the cell and CFS stability. Even if much effort has to be done to understand the meaning of H4K16ac loss at CFS, FANCD2 seems to be a critical factor in maintaining epigenetic stability through these regions.

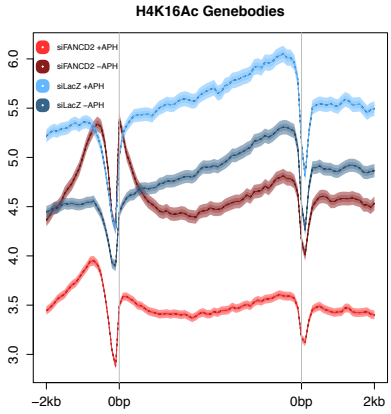
Finally, it is interesting to note that CFS are present in hypoacetylated regions that border more acetylated chromatin regions. These transition zones between different chromatin domains could be linked to replication timing and transition between early and late replicated regions. As our results indicate that FANCD2 is necessary to maintain chromatin conformation at CFS after replicative stress, we are analyzing, in collaboration with Cadoret's laboratory, if replication timing is changed through these regions in the absence of FANCD2.

Annex Figures

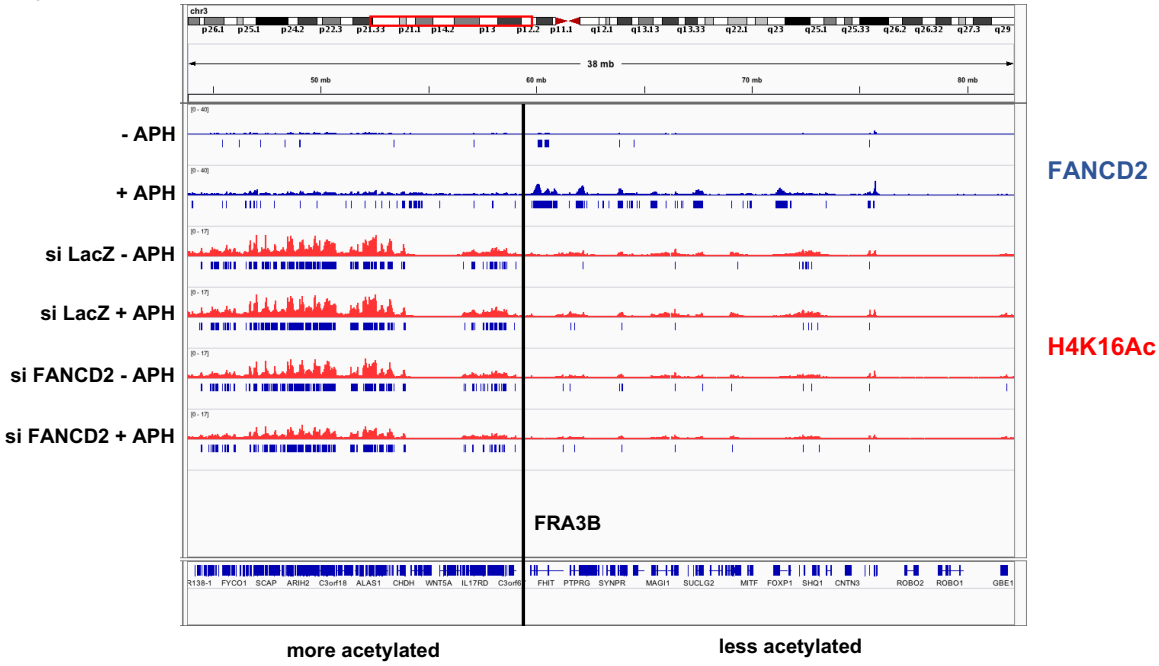
A)



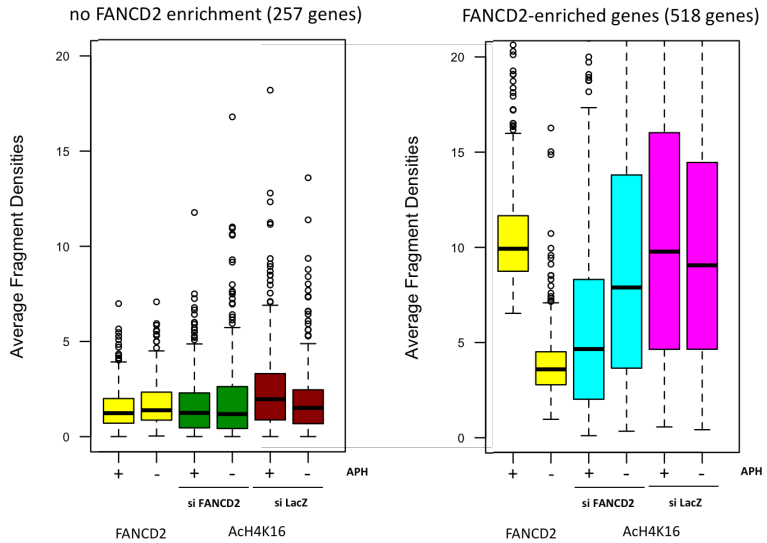
B)



C)



D)



Annex figures: FANCD2 is necessary to maintain H4K16 acetylation at CFS after replicative stress.

a) H4K16ac location peaks relative to genomic annotations are represented in a piechart.

b) H4K16ac peak distribution across gene bodies presented as average plots.

c) IGV visualization of FANCD2 and H4K16ac

d) FANCD2 and H4K16ac average fragment densities in genes. On the left chart is presented genes with no FANCD2 induction after APH treatment, and on the right chart is presented genes with FANCD2 induction after APH treatment.

DISCUSSION AND PERSPECTIVES

FANCD2 recruitment and role at large transcribed genes.

CFS stability maintenance has raised a strong interest from scientists as their instability is suspected to contribute to cancer development (Bignell et al., 2010). FANCD2 has been identified to participate in the maintenance of CFS throughout S-phase and mitosis (Howlett et al., 2005; Naim and Rosselli, 2009), even if its role in these regions was poorly understood. Recently, different studies have revealed the critical role of FANCD2 in facilitating CFS replication and thus preventing instability (Madireddy et al., 2016; Okamoto et al., 2018). During my PhD, we discovered a new role of FANCD2 in CFS stability maintenance by regulating CFS gene expression. We show that FANCD2 is recruited to the body of transcribed large genes after replicative stress. In the absence of transcription, CFS breakage is strongly reduced and FANCD2 recruitment is almost abolished. In addition, without transcription, FANCD2 is not required to maintain CFS stability. Based on these results, our model is that FANCD2 is recruited in a manner dependent on transcription to attenuate it and avoid replication-transcription collision leading to CFS instability. However, we still do not know what is the precise mechanism of FANCD2 recruitment to CFS genes. Different hypotheses can be suggested:

- R-loops which are by-products of transcription, could trigger FANCD2 recruitment to these genes and retain it to facilitate replication in these regions as proposed in a recent study (Okamoto et al., 2018).
- FANCD2 could also be recruited by the splicing machinery to regulate its distribution and avoid congestion through these regions (Moriel-Carretero et al., 2017).
- Another possibility to explain FANCD2 presence in these regions could be to deal with the topological stress created by conflicts between replication and transcription. FANCD2 could be required to alleviate DNA supercoiling created in the space where both machineries merge to avoid topological stress and breakage. The histone chaperone activity of FANCD2 (Sato et al., 2012) could regulate DNA transitions and access of specific enzymes like helicases, topoisomerases or DNA repair factors.
- FANCD2 role could also be related to origin firing. FANCD2 could be recruited to set up the firing of a dormant origin as a backup to complete replication (Madireddy et al., 2016).

- Finally, FANCD2 binding could be associated to damaged forks occurring at CFS and its role in fork protection and DNA repair.

These different situations are not mutually exclusive and may reflect the dynamic processes underlying replication fork stalling and resolution.

Recent studies reported ChIP-seq data using tagged FANCD2 protein (Okamoto et al., 2018; Pentzold et al., 2018). Working with endogenous FANCD2, we confirmed these findings identifying the top induced-FANCD2 regions after replicative stress as very large transcribed genes encompassing CFSs. We identified some large genes in common with these previous reports and differences that are likely due to the cell-type specificity of CFS (HCT116 *vs* U2OS *vs* DT40). Given the conservation of FANCD2 binding to CFSs in these three different studies, we propose that FANCD2 ChIP-seq could be used to identify CFSs in a given cell type.

In addition to CFS binding, we identified another binding profile of FANCD2 which is more specific to promoters. We analyzed the biological processes and phenotypes associated to the genes bound by FANCD2 in their promoter using the GREAT tool developed at Stanford University. Interestingly, we found a high similitude between human and mouse phenotypes identified from FANCD2-bound promoters and the FA phenotype, like bone development defects, cancer of cells originating from HSCs and genital abnormalities (**Fig. 13**). This analysis suggests that these genes have a major contribution in FA disease. To determine if FANCD2 regulates the transcription of these genes, we are currently performing an RNA-seq analysis that will be integrated to our ChIP-seq data.

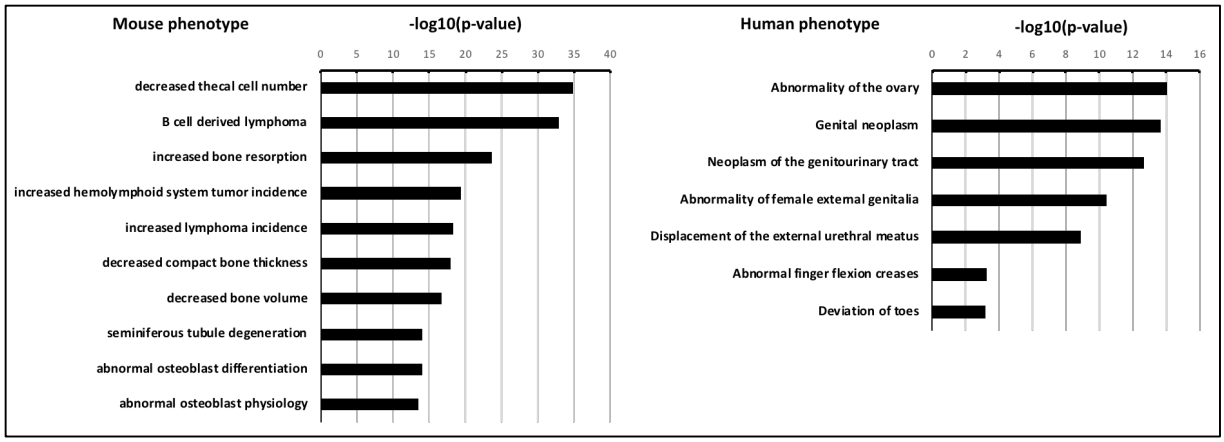


Figure 13: Genes bound by FANCD2 at their promoters may have a role in FA disease.

Gene Ontology analysis of FANCD2-bound promoters. Mouse and human phenotypes, on the left and right respectively, show high similarities with FA phenotypes.

FANCD2 regulation of CFS gene transcription.

We observed that FANCD2 relocalizes to large transcribed genes and that, in the absence of FANCD2, their transcription is increased. Thus, FANCD2 represses the transcription of these genes but it is not clear if this repression is direct or a response to a stress due to FANCD2 loss. The finding that CFS genes respond to mitochondrial dysfunction and more generally to the unfolded protein response (UPR) lead us to think that CFS gene transcription is increased by an indirect way after FANCD2 depletion and that these genes respond to different signals like metabolic, replicative or mitochondrial stresses. To strengthen this idea, we found that *PTPRG*, a gene close to *FHIT,* is also bound by FANCD2 after replicative stress but its transcription is not increased after FANCD2 depletion (even slightly decreased). Our idea is that FANCD2 role at CFS genes is associated with its DNA replication role preventing replication-transcription instability. CFS gene transcription increase after FANCD2 depletion is highly problematic for the cell due to the loss of FANCD2 replicative function at CFS and increased replication-transcription collisions. To better understand transcriptional regulation of CFS genes by FANCD2, we will integrate the RNA-seq results with our ChIP-seq data.

CFS instability is cell-type dependent and relies on replication and transcription programs (Helmrich et al., 2011; Letessier et al., 2011). Our results indicate that CFS genes respond to different signals linked to cell metabolism. In our studies, we used epithelial cells which use preferentially the OXPHOS metabolism. In these cells, *FHIT* and *PARK2*, genes linked to mitochondrial function, are expressed. On the contrary, in fibroblasts, these genes are not, or

almost not, expressed and cell metabolism is more dependent on glycolysis. We hypothesized that cell-type specificity of CFS gene expression, and consequent instability, could be dependent on cell metabolism. However, CFS gene function in this process is still poorly understood. It will be interesting to study the link between the metabolic switch, a situation well documented in HSCs, and CFS expression. During HSC activation, one cell remains quiescent and the other one differentiates. Metabolism is critical in this process as the quiescent cell maintains glycolytic metabolism whereas the proliferating cell increases OXPHOS and mitochondrial activity to produce large amounts of ATP. We can imagine that CFS breakage could be a determinant of CFS gene expression in daughter cells and therefore regulate cell metabolism and cell fate. After several activation cycles, CFS can break and cell loses its ability to activate CFS gene, impairing differentiation and leading to cell death.

FANCD2 and the UPR.

Through the years, the DNA repair FANC pathway has emerged to have a large range of functions like DNA replication, transcription, inflammation, immunity, metabolism and mitophagy. During my PhD, we have been searching for signals that could induce CFS gene transcription and identified the UPR as a good candidate. We had clues to test the UPR as an inducer of CFS genes. Most of the genes encompassing CFS are linked to mitochondria (*IMMP2L*, *PARK2*, *FHIT*) but also with secretory pathways (*EXOC4*) and neuronal development (*LSAMP*) which are closely related to the UPR function (Moore and Hollien 2012; Godin et al., 2016). Moreover, mitochondrial dysfunction has been widely described in FA and let us suppose that this signal could be triggering CFS gene expression.

We show that FANCD2 depletion leads to mitochondrial dysfunction and that attenuation of this stress could reduce the CFS gene expression increase. It has been shown that FANCD2 is imported into the mitochondria thanks to its interaction with the nucleoid complex protein Atad3 in mice (Zhang et al., 2017). However, the precise function of FANCD2 in the mitochondria is still poorly understood. Interestingly, mitochondrial DNA (mtDNA) has been found to harbor R-loops having a major role in its replication (Xu and Clayton, 1996). As proposed in the nucleus, FANCD2 could recognize R-loops in the mitochondria to ensure mtDNA replication.

It has been shown in yeast, that UPR^{mt} promotes a metabolic shift to glycolysis by limiting OXPHOS gene transcription while increasing transcription of glycolysis components (Nargund et al., 2015). Our data highlight the same metabolic switch in cells depleted for FANCD2 over time and let us suppose that the UPR^{mt} is activated upon FANCD2 depletion to allow cell survival and mitochondrial homeostasis. We show that the ATF4 pathway is activated in the absence of FANCD2, but CHOP induction is defective after UPR induction, meaning that FANCD2 could be necessary for optimal UPR activation. We also show that CFS gene transcription is increased after UPR induction. However, prolonged stress activation that cannot alleviate the stress because of the absence of FANCD2 causes excessive CFS gene transcription and CFS instability. CFS gene response seems to be broader than mitochondrial stress since endoplasmic reticulum stress could also induce their transcription. We are still working on the implication of FANCD2 in the UPR but we think that FANCD2 is able to maintain genomic stability, in addition to its DNA repair and replicative roles, by regulating UPR activation and therefore avoiding deregulated CFS gene transcription and subsequent CFS instability. Interestingly, we observed a FANCD2 foci formation after UPR induction that correspond to CFS genes. Upon UPR activation, CFS gene transcription is increased and FANCD2 could be recruited to mediate replication through these genes. In line with this idea, it has been shown that UPR induction can inhibit DNA replication (Cabrera et al., 2017).

We believe that a dysfunctional UPR response may be implicated in FA disease. Recently, different studies showed the critical role of UPR in the maintenance of HSCs (Van Galen et al., 2014; Mohrin et al., 2015; Sigurdsson and Miharada, 2018). Moreover, we obtained preliminary data by RNA-seq indicating a possible UPR deregulation in *Fanca -/-* mice compared to WT, strengthening the idea of UPR involvement in FA.

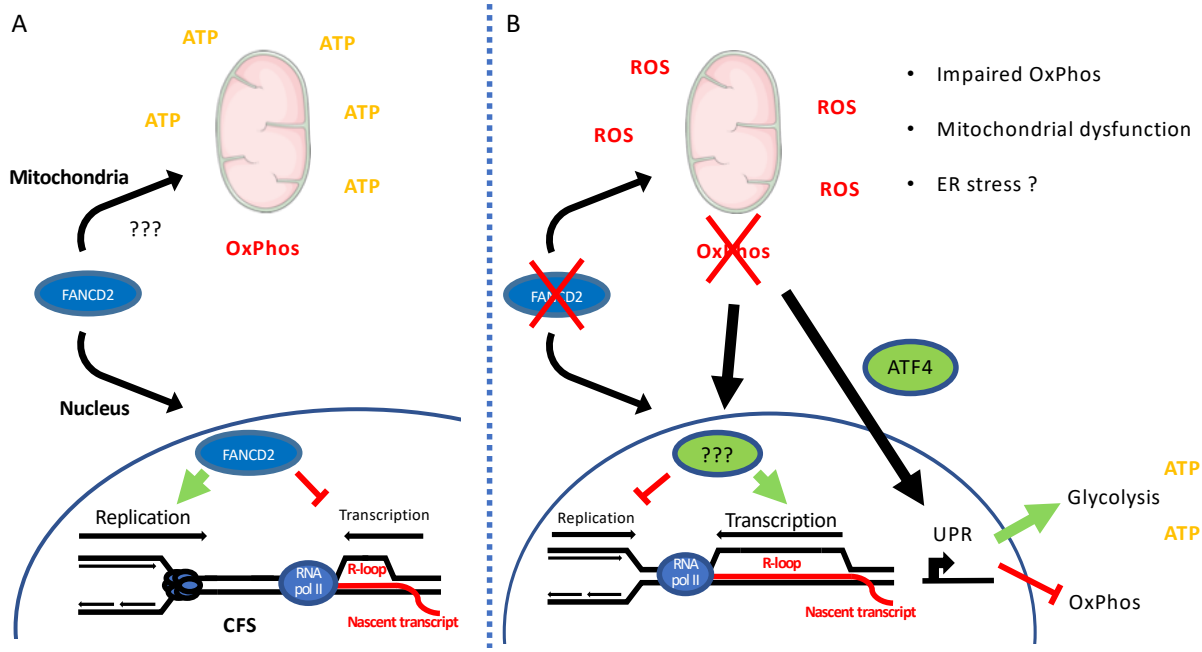


Figure 14: Model for the central role of FANCD2 in maintaining metabolic homeostasis and genome stability.

A. FANCD2 shuttles between the mitochondria and the nucleus and is necessary to sustain ATP production by OXPHOS in the mitochondria. However, its precise function is still elusive, and it is not known how it is targeted to the mitochondria. In the nucleus, FANCD2 promotes DNA replication at sites of replication-transcription encounters, by protecting stalled replication forks from degradation and promoting their restart. Notably, at CFS, FANCD2 helps the replication machinery to pass through the core of CFS by resolving R-loops. In our study, we show that FANCD2 is recruited to CFS only when CFS genes are transcribed and that in the absence of transcription is no longer required for CFS stability maintenance.

B. Mitochondrial function is altered in FA cells: OXPHOS is dysfunctional with a consequent overproduction of ROS, and mitophagy is impaired. After depletion of FANCD2, we observed an impaired OXPHOS activity and increased glycolysis over time confirming mitochondrial dysfunction. Mitochondrial stress activates a signalling pathway, called the UPR^{mt}, in order to decrease mitochondrial stress and recover mitochondrial homeostasis. Interestingly, CFS gene transcription is increased after FANCD2 depletion and is induced by mitochondrial or ER stress. Conversely, mitochondrial or ER stress induction leads to FANCD2 relocalization into nuclear foci and at CFS. Attenuating mitochondrial stress reduces CFS gene expression and CFS instability. Moreover, the ATF4 transcription factor, a key UPR^{mt} effector, is activated after FANCD2 depletion. However, CFS gene induction after FANCD2 depletion is likely regulated by other UPR arms that are still to be identified. Moreover, it will be interesting to test if CFS genes have a role in the UPR.

To conclude, we propose that FANCD2 maintains metabolic homeostasis and genomic stability by preventing mitochondrial stress and attuning CFS gene transcription and replication. In its absence, UPR and CFS genes are induced to restore mitochondrial homeostasis. Deregulated UPR activation and expression of CFS genes could in

turn induce CFS breakage and promote genomic instability. By moderating the UPR, FANCD2 prevents CFS expression and instability, and maintains mitochondrial homeostasis.

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université PARIS-SACLAY | ÉCOLE DOCTORALE Structure et dynamique des systèmes vivants (SDSV)

Titre : Rôles de FANCD2 dans le maintien de la stabilité des sites fragiles communs.

Mots clés : Cancer, Stabilité génomique, Fanconi, Sites fragiles communs, Métabolisme.

Résumé : Les sites fragiles communs (SFCs) sont des régions génomiques particulièrement sensibles au stress réplicatif et sont impliquées dans l'initiation et la progression du cancer. L'Anémie de Fanconi (AF) est une maladie génétique rare qui se caractérise principalement par une aplasie médullaire, des malformations congénitales ainsi qu'une forte prédisposition au cancer chez les patients (leucémies myéloïdes et tumeurs solides de la tête et du cou). L'instabilité génomique a été identifiée comme étant une source majeure de prédisposition des patients AF au cancer et les SFCs sont particulièrement sensibles dans cette maladie. L'AF est causée par la mutation de gènes codant des protéines participant à une voie moléculaire appelée voie FANC qui a été décrite dans la réparation des ponts inter-brins. Malgré l'importance de la voie FANC dans le maintien de la stabilité des SFCs, les mécanismes sous-jacents restent à élucider. Au cours de ma thèse, nous avons identifié un nouveau rôle de FANCD2 dans le maintien des SFCs. En effet, nous montrons que FANCD2 atténue l'expression des gènes présents au sein des SFCs maintenant leur stabilité. De plus, nous montrons que la transcription de ces gènes est nécessaire au recrutement et au rôle de FANCD2 au sein de ces régions. Enfin, nous avons identifié le stress métabolique comme étant le signal induisant l'expression des gènes des SFCs et que FANCD2 module cette réponse. La réduction de ce stress pourrait être une piste thérapeutique intéressante afin de prévenir l'instabilité des SFCs dans l'AF.

Title : The roles of FANCD2 in the maintenance of common fragile site stability.

Keywords : Cancer, Genomic stability, Fanconi, Common fragile sites, Metabolism.

Abstract : Common fragile sites (CFSs) are genomic regions prone to form breaks and gaps on metaphase chromosomes after replicative stress and promote genomic instability in the earliest steps of tumor development. Proteins involved in replication/repair of CFSs are necessary to prevent their instability. Among them is FANCD2, a key protein of the FANC pathway necessary to resolve inter-strand crosslinks and defective in Fanconi Anemia (FA). FA is a rare genomic instability disorder characterized by bone marrow failure, congenital abnormalities and predisposition to acute myeloid leukemia and epithelial cancer. Genomic instability in FA is supposed to predispose patients to cancers. Importantly, CFSs are more unstable in FA and chromosome breaks observed in FA cells occur preferentially at CFSs. During my PhD, we identified a new role of FANCD2 in CFS stability maintenance. We show that FANCD2 attenuates transcription of the large genes present at CFSs, preventing their instability. Moreover, we demonstrate that transcription is necessary for FANCD2 recruitment and function at CFSs. Importantly, we identified the metabolic stress as a signal triggering CFS gene expression and FANCD2 is necessary to modulate this response. Reducing this stress is a promising therapeutic issue to prevent CFS and genomic instability in FA.