Role of chromatin organization in double strand break repair at centromeres

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« Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less. »

Marie Curie (1867 - 1934)

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List of abbreviations

53BP1	Tumor suppressor p53-binding protein 1
6-4PPs	Pyrimidine 6-4 pyrimidone photoproducts
ACF1	ATP-dependent chromatin-remodeling factor 1
ADP	Adenosine diphosphate
AID	Activation-induced deaminase
ALC1	Chromodomain-helicase-DNA-binding *protein* 1-like
ALT	Alternative Lengthening of Telomeres
Alt-EJ	Alternative End Joining
APC/C	Anaphase promoting complex/cyclosome
APLF	Apratxin and PNK-like factor
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
ATRIP	ATR-interacting protein
ATRX	Alpha thalassemia/mental retardation syndrome X-linked chromatin remodeler
BACH1	BTB Domain And CNC Homolog 1
BAF	BRG1- or BRM-associated factors
BAK	Bcl-2 homologous antagonist killer
BAP1	BRCA1 associated *protein*-1
BARD1	BRCA1-associated RING domain protein 1
BAX	Bcl-2–associated X protein
BER	Base Excision Repair
BioID	Proximity-dependent Biotin Identification
BIR	Break-induced Replication
BirA	E. coli biotin ligase
BLM	Bloom syndrome protein
BRCA1-2	Breast Cancer 1-2
BRCC36	Lys-63-specific deubiquitinase B
BRE	BRCA1-A complex subunit BRE
BRG1	Transcription activator BRG1
BTR	BLM–TOP3A–RMI
CBP	CREB-binding protein
CCAN	Constitutive Centromere Associated Network
Cdc25C	M-phase inducer phosphatase 3
CDK	Cell cycle-dependent kinase
CDK1	Cell cycle-dependent kinase 1
CDK9	Cell cycle-dependent kinase 9
CENP	Centromere protein
CENP-A	Centromere protein A
CHD	Chromodomain-helicase-DNA binding
ChIP	Chromatin Immunoprecipitation
CHK1	Checkpoint kinase 1
CPDs	Cyclobutane pyrimidine dimers
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSB	Cockayne syndrome protein B
CSR	Class-switch recombination
CtBP	C-terminal-binding protein
CtIP	CtBP-interacting protein
CUL3	E3 ubiquitin ligase cullin-3
CYREN	Cell cycle regulator of non-homologous end joining
DAXX	Death domain-associated protein
dCas9	Dead Cas9
DDR	DNA damage response
dHJ	double Holliday Junction
DMC1	Meiotic recombination protein 1
DNA	Deoxyribonucleic acid
DNA2	DNA Replication Helicase/Nuclease 2
DNAPKcs	DNA-dependent protein kinase catalytic subunit
Dox	Doxycycline
DRIP	DNA-RNA immunoprecipitation
dRNAseH	Dead RNAseH
DSBR	Double Strand Break Repair
DSBs	Double strand breaks
dsDNA	Double stranded DNA
DSS1	Deleted in spilt hand/spilt foot 1
DUB	Deubiquitylating enzyme
EME1	Essential Meiotic Structure-Specific Endonuclease 1
ERCC1	Excision Repair 1, Endonuclease Non-Catalytic Subunit
ETAA1	Ewing's tumor-associated antigen 1
EXD2	Exonuclease 3'-5' domain-containing protein 2
EXO1	Exonuclease 1
EXO10	Exonuclease 10
EYA1	EYA transcriptional coactivator and phosphatase 1
EZH2	Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit
FACT	Facilitates chromatin transcription
FANCD2	Fanconi anemia group D2 protein
FANCI	Fanconi anemia group I protein
FANCJ	Fanconi anemia group J protein
FBH1	F-box DNA helicase 1
FBXL10	[Histone H3]-dimethyl-L-lysine(36) demethylase
FHA	Forkhead-associated
FISH	Fluorescence *in situ* hybridization

GCN5	Histone acetyltransferase GCN5
GEN1	GEN1 Holliday Junction 5' Flap Endonuclease
HAS	Helicase SANT
HAT	Histone Acetyl Transferase
HDAC1-2	Histone Deacetylase 1-2
HELB	DNA helicase B
HJ	Holliday junction
HJURP	Holliday junction recognition protein
HNRNPD	Heterogeneous nuclear ribonucleoprotein D
HP1α	Heterochromatin protein 1
HR	Homologous Recombination
HR-TIDE	Homologous Recombination based-TIDE
Hus1	Checkpoint protein HUS1
Ig	Immunoglobulin
INO80	Inositol requiring 80
IR	Ionizing Radiation
IRIF	Irradiation induced foci
ISWI	Imitation switch
KAP1	KRAB-associated protein-1
KDM2A	Lysine-specific demethylase 2A
KDM4A	Lysine-specific demethylase 4A
KDM4B	Lysine-specific demethylase 4B
KDM4D	Lysine-specific demethylase 4D
KDM5A	Lysine-specific demethylase 5A
KDM5B	Lysine-specific demethylase 5B
KEAP1	Kelch-like ECH-associated protein 1
KMN	KNL-1/Mis12 complex/Ndc80
KNL1	kinetochore scaffold 1
KNL2	kinetochore scaffold 2
KRAB	Krüppel associated box
L3MBTL1	Lethal(3)malignant brain tumor-like protein 1
LEDGF	Lens epithelium-derived growth factor
LIG3	DNA ligase 3
LIG4	DNA ligase 4
lncRNA	Long non coding RNA
LTGC	Long tract gene conversions
MBTD1	MBT domain-containing protein 1
MCC	Mitotic checkpoint complex
MDC1	Mediator of DNA damage checkpoint protein 1
MERIT40	BRISC and BRCA1-A complex member 1
MiDAS	Mitotic DNA synthesis
MLL	Mixed-lineage leukemia
MMEJ	Microhomology-mediated end-joining
MMR	Mismatch Repair
MMSET	Multiple myeloma SET domain
MRE11	Double-strand break repair protein MRE11
MRN	MRE11-RAD50-NBS1
MTA1	Metastasis-associated protein 1
gRNA	Guide RNA
HAC	Human artificial chromosome
MUS81	Crossover junction endonuclease MUS81
NBS1	Nibrin
NCS	Neocarzinostatin
NCS	Neocarzinostatin
NEDD8	Neural precursor cell expressed developmentally down-regulated protein 8
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NSD2	Nuclear receptor binding SET domain protein 2
OPT	Oct-1, PTF, transcription
OTUB1	Ubiquitin thioesterase OTUB1
OTUD5	OTU domain-containing protein 5
PALB2	Partner and localizer of BRCA2
PARI	PCNA-interacting partner
PARP	Poly ADP-ribose polymerase
PAXX	Paralogue of XRCC4 and XLF
PCNA	Proliferating cell nuclear antigen
PHF11	PHD finger protein 11
PIKK	Phosphatidylinositol 3-kinase
PML	Promyelocytic leukaemia
PNKP	Polynucleotide phosphatase/kinase
POLD1	DNA polymerase δ subunit 1
POLD3	DNA polymerase δ subunit 3
PP2A	Protein phosphatase 2A
PP4C	Protein Phosphatase 4 Catalytic Subunit
PP6	Protein phosphatase 6
PRC1	Polycomb repressive complex 1
PRDM2	PR domain zinc finger protein 2
PRMT7	Protein arginine N-methyltransferase 7
PTIP	PAX transcription activation domain interacting protein
PTM	Post translationnal modification
PUMA	p53 upregulated modulator of apoptosis
RADs	DNA repair proteins
RAG1-2	Recombination activating gene 1-2
RAP80	BRCA1-A complex subunit RAP80
RBX1	RING-box protein 1
RECQL5	ATP-dependent DNA helicase Q5
REV7	Revertibility protein 7
RIF1	Rap1-interacting factor 1
RING	Really Interesting New Gene
RMI	RecQ-mediated genome instability protein 1
RNA	Ribonucleic acid
RNFs	Ring finger proteins
ROS	Reactive oxygen species
RPA	Replication protein A
RSC	Chromatin structure remodeling

RSF1	Remodeling and spacing factor 1
RUVBL1	RuvB-like 1
SAC	Splindle Assembly Checkpoint
Sae2	DNA endonuclease SAE2
SDSA	Synthesis-dependent strand annealing
SDSA	Synthesis dependent strand annealing
SET7	Histone-lysine N-methyltransferase SETD7
SETD2	Histone-lysine N-methyltransferase SETD8
SETD8	Histone-lysine N-methyltransferase SETD8
SETDB1	Histone-lysine N-methyltransferase SETDB1
SHLD1-3	Shieldin complex subunit 1-3
SHM	Somatic hyper-mutation
SIRT1	Sirtuin 1
SIRT6	Sirtuin 6
SLX1	Structure-specific endonuclease subunit SLX1
SLX4	Structure-specific endonuclease subunit SLX4
SMARCAD1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1
SNF2	Transcription regulatory protein SNF2
SPO11	Meiotic recombination protein SPO11
SSA	Single strand annealing
ssDNA	single-stranded DNA
SUMO	Small Ubiquitin-like Modifier
SUV39H1	Histone-lysine N-methyltransferase SUV39H1
SUV39H2	Histone-lysine N-methyltransferase SUV39H2
SWI/SNF	Switch/sucrose-non-fermenting
SWR1	Swi2/Snf2-related 1
TC-HR	Transcription-coupled homologous recombination process
TCR	T-cell Receptor
TERRA	Telomeric repeat–containing RNA
TIDE	Tracking of Indels by Decomposition
TIRR	Tudor-interacting repair regulator protein
TNFAIP3	Tumor necrosis factor alpha-induced protein 3
TSS	Transcription start site
U2OS	Human Bone Osteosarcoma Epithelial Cells
UCHL3	Ubiquitin carboxyl-terminal hydrolase
UDR	Ubiquitylation-dependent recruitment
UHRF1	Ubiquitin-like containing PHD and RING finger domains 1
USP	Ubiquitin peptidase
UV	Ultra-violet
VCP	Valosin-containing protein
WHSC1	Nuclear receptor binding SET domain protein 2
WIP1	Protein phosphatase 1D
WRN	Werner syndrome ATP-dependent helicase
WSTF	Williams Syndrome Transcription Factor
XLF	Non-homologous end-joining factor 1
XPF	DNA repair endonuclease XPF
XPG	DNA repair endonuclease XPG
XRCC1-6	X-ray repair cross-complementing protein 1-6
ZMYND8	Protein kinase C-binding protein 1

Thesis summary in French

L’ADN dans les cellules est soumis à différents types de lésions pouvant affecter son intégrité. Les cassures double brin (CDBs) sont parmi les lésions les plus cytotoxiques puisque les deux brins d'ADN sont affectés. Par conséquent, leur réparation infidèle peut conduire à des réarrangements génomiques tels que des translocations chromosomiques, pouvant être à l'origine de nombreuses maladies dont le cancer.

Les cellules répondent aux CDBs en initiant une cascade de signalisation qui conduit à l'activation de points de contrôle du cycle cellulaire (Lukas et al., 2004). Les cellules entreprennent alors la réparation des CDBs principalement par deux voies : le Non-Homologous End Joining (NHEJ) qui relie simplement les extrémités libres de l'ADN, et la Recombinaison Homologue (HR) qui se sert de la chromatide sœur comme modèle pour réparer les CDBs de manière fidèle. La réparation par HR est composée de deux étapes principales : la résection des extrémités cassées (qui implique des facteurs tels que RPA) et l'invasion du brin homologue (impliquant des facteurs tels que RAD51 et ses partenaires BRCA1-PALB2-BRCA2). Alors que NHEJ a lieu tout au long du cycle cellulaire, HR est supprimée durant la phase G1 du cycle cellulaire (Orthwein et al., 2015), pour éviter une recombinaison non homologue, et ce par ubiquitination constitutive de PALB2, ce qui inhibe la formation du complexe BRCA1-PALB2-BRCA2, supprimant ainsi l'étape d'invasion du brin homologue.

Lorsqu'elles se produisent aux centromères, les CDBs peuvent entraîner une mauvaise ségrégation chromosomique des cellules filles pendant la division cellulaire. Cela peut conduire à un nombre anormal de chromosomes, appelé aneuploïdie, ainsi qu'à des réarrangements génomiques, caractéristiques de nombreuses maladies telles que les troubles du développement, les anomalies congénitales, l'infertilité, le vieillissement prématuré et le cancer (Barra and Fachinetti, 2018 ; Beh and Kalitsis, 2015). En effet, l'aneuploïdie peut provoquer une carcinogenèse en altérant l'équilibre entre oncogènes et suppresseurs de tumeurs. Environ 90% des tumeurs solides sont aneuploïdes (Compton et al., 2011). De plus, il a été montré que les centromères sont des points chauds de réarrangements chromosomiques dans les cellules de mammifères (Simi, et al., 1998) et leur fragilité intrinsèque pourrait contribuer aux cycles de rupture-fusion des centromères observés dans de nombreuses tumeurs solides (Martínez and van Wely, 2011). Par conséquent, la préservation de l'intégrité du centromère est une tâche difficile mais importante pour la cellule. Néanmoins, les mécanismes par lesquels les centromères conservent leur intégrité lorsqu'ils sont endommagés sont encore très peu connus.

Dans ce contexte, nous avons développé un système unique pour induire des CDBs spécifiquement aux séquences centromériques en utilisant la technique CRISPR/Cas9 (Tsouroula et al., 2016). Dans les cellules murines, les centromères contiennent des régions répétées, appelées satellites mineurs, sont caractérisés par H3K36me2 et H3K4me2 qui représentent généralement une chromatine active (Chan and Wong, 2012) et contiennent CENP-A, un variant d'histone H3 déposé exclusivement aux centromères. Notre laboratoire a montré que les CDBs dans la chromatine centromérique activent de manière surprenante à la fois l'étape de résection des extrémités de l'ADN et le recrutement de RAD51 tout au long du cycle cellulaire (Tsouroula et al., 2016). Étant donné que les régions centromère-kinétochore sont soumises à des tensions induites par le fuseau mitotique au cours de la mitose, il est concevable qu'il existe un besoin de réparation fidèle par HR dans ces régions à n'importe quel stade du cycle cellulaire, même si HR est généralement supprimée.

L'organisation de la chromatine est récemment apparue comme un acteur majeur dans le choix des voies de réparation de l'ADN. Dans ce contexte, mon projet de doctorat visait à étudier comment la structure unique de la chromatine centromérique permet la réparation des CDBs par HR en G1.

Afin d'étudier l'implication de H3K4me2 dans l'activation de HR en G1, nous avons soit inhibé l'histone méthytransférase SETD1A, soit dirigé l'histone déméthylase LSD1 aux centromères en la fusionnant à une Cas9 catalytiquement inactive (dCas9) et nous avons induit des CDBs. La réduction de H3K4me2 par les deux stratégies a considérablement diminué le recrutement des facteurs de résection tel que RPA et des facteurs d'invasion du brin homologue tels que RAD51 et BRCA1.

H3K4me2 est présent sur les sites de transcription (Wang et al., 2014) et l'ADN centromérique est transcrit, produisant de l'ARN non codant (Hédouin et al., 2017 ; Quénet et al., 2014). De plus, les cassures d'ADN au niveau des régions transcrites du génome favorisent la formation de R-loop (hybride d'ADN et ARN), ce qui à son tour facilite la réparation par HR en recrutant des facteurs tels que RPA et BRCA1 (Nguyen et al., 2017 ; Hill et al., 2014). Enfin, les centromères ont tendance à former des R-loops, ce qui est important pour leur intégrité (Castellano-Pozo et al., 2013 ; Kabeche et al., 2018). Nous avons donc émis l'hypothèse que la présence de H3K4me2 est à l'origine d'une transcription centromérique, qui peut donner lieu à la formation de R-loop, permettant ainsi de recruter des facteurs de HR. Nous avons d'abord vérifié que H3K4me2 a un rôle dans la transcription centromérique, en montrant que les niveaux d'ARN centromérique diminuent quand SETD1A est inhibé ou que LSD1 est dirigé aux centromères. Puis pour étudier le potentiel rôle de H3K4me2 et de la transcription dans la formation de R-loops au niveau des CDBs centromériques, nous avons quantifié le recrutement d'une RNAseH catalytiquement inactif (dRNAseH), qui va détecter les R-loops, aux

centromères après avoir inhibé SETD1A ou dirigé LSD1 aux centromères. Dans les deux cas, la formation de R-loops a été considérablement réduite, confirmant que la formation de R-loops centromériques a pour origine une transcription centromérique dépendante de H3K4me2. Enfin, pour déterminer si les R-loops ont un rôle dans la réparation par HR des CDBs centromériques en G1, nous avons quantifié le recrutement de BRCA1, RPA et RAD51 dans des cellules surexprimant la RNAseH WT, qui hydrolyse l'ARN des R-loops. Comme prévu, l'inhibition des R-loops aux centromères a conduit à une diminution du recrutement des facteurs BRCA1 et RPA aux cassures centromériques et par conséquent à la diminution du recrutement de RAD51.

Ces résultats montrent que H3K4me2 a un rôle dans la transcription centromérique et la formation de R-loops, qui conduisent au recrutement de facteurs permettant l'initiation de la réparation par HR aux CDBs centromériques en G1.

H3K4me2 est une caractéristique générale de la chromatine active mais n'est pourtant pas suffisant pour permettre la réparation par HR en G1 dans les lésions survenant à l'extérieur des centromères. Cette observation nous a incités à concentrer notre attention sur une caractéristique centromérique tout à fait unique, le variant d'histone H3 : CENP-A. Pour déterminer l'implication de CENP-A dans la réparation par HR dans les cellules en G1, nous avons quantifié le recrutement des facteurs HR aux CDBs centromériques après inhibition de CENP-A, HJURP ou MIS18 (cofacteur de HJURP). Nous avons constaté que le recrutement de RAD51 et de BRCA1 aux CDBs centromériques en G1 était significativement réduit. Cependant, le recrutement de RPA n'a pas été affecté, ce qui suggère que CENP-A a un rôle dans HR en aval de l'étape de résection des extrémités. Afin de déterminer si CENP-A est suffisant pour recruter RAD51 aux CDBs, nous avons dirigé CENP-A aux péricentromères en le fusionnant à dCas9 pour voir si cette région devient permissive au recrutement de RAD51 en G1. La présence de CENP-A aux CDBs péricentriques a considérablement augmenté le recrutement de RAD51. De même, diriger HJURP au locus LacO/IsceI, conduisant à l'incorporation de CENP-A dans cette région (Barnhart et al., 2011), a été suffisant pour augmenter le recrutement de RAD51 en G1 aux CDBs induites par IsceI (Soutoglou et al., 2007) suggérant un rôle direct de CENP-A dans le recrutement de RAD51 aux CDBs en G1.

Orthwein et al., 2015 a précédemment démontré qu'en phases S/G2, USP11 dé-ubiquitine PALB2 et favorise son interaction avec BRCA1 et BRCA2 sur les sites de cassures pour recruter RAD51 et permettre la réparation par HR. En G1 cependant, USP11 est dégradé après irradiation conduisant à une ubiquitination constitutive de PALB2 et à l'inhibition de la formation du complexe BRCA1-PALB2-

BRCA2, supprimant ainsi l'étape d'invasion du brin homologue. Puisque nous avons montré que RAD51 est recruté aux CDBs centromériques en G1, nous avons cherché à déterminer si la fraction restante de USP11 en G1 joue un rôle dans le recrutement de RAD51, peut être en interagissant avec des protéines centromériques. En effet, par des expériences d'immunoprécipitation, nous avons révélé une interaction constitutive entre CENP-A et HJURP avec USP11 dans les cellules en G1. De plus, nous avons observé une diminution de l'incorporation de CENP-A aux centromères en l'absence de USP11, suggérant le rôle de USP11 dans la formation des centromères. En outre, et conformément au rôle joué par USP11 dans le recrutement de RAD51 aux CDBs centromériques en G1, l'inhibition d'USP11 a réduit le recrutement de RAD51 aux CDBs centromériques mais pas aux CDBs péricentriques. Enfin, nous avons dirigé USP11 aux péricentromères en le fusionnant à dCas9 afin de déterminer si cette région devenait permissive au recrutement de RAD51 en G1. La présence d'USP11 aux CDBs péricentriques a considérablement augmenté le recrutement de RAD51, indiquant qu'USP11 est suffisant pour augmenter le recrutement de RAD51 aux CDBs.

Des études antérieures ont révélé que HJURP est ubiquitiné (Beltrao et al., 2012 ; Stes et al., 2014). Par conséquent, pour explorer davantage le mécanisme d'interaction de USP11 avec HJURP et CENP-A, nous avons cherché à déterminer si l'activité de dé-ubiquitination d'USP11 y jouait un rôle. Nous avons surexprimé Flag-USP11 et évalué l'ubiquitination de HJURP. Nos résultats ont montré que la surexpression d'USP11 diminue l'ubiquitination de HJURP. Ce résultat a été corroboré par une augmentation de l'ubiquitination de HJURP après inhibition d'USP11. Ceci nous a conduit à l'hypothèse que HJURP doit être dé-ubiquitine par USP11 pour interagir avec CENP-A. Pour tester cela, nous avons inhibé USP11 et déterminé comment cela affectait l'interaction entre CENP-A et HJURP. Nous avons constaté qu'en l'absence d'USP11, GFP-HJURP co-immunoprécipite moins abondamment CENP-A. Ce résultat a été en outre étayé par la mise en évidence que l'incorporation ectopique de GFP-CENP-A à la chromatine dans la région LacO quand HJURP-LacI y est présent était diminuée après inhibition d'USP11.

Ainsi, USP11 a un rôle constitutif au niveau des centromères en G1 en dé-ubiquitinant HJURP, pour faciliter son interaction avec CENP-A et, vraisemblablement, son incorporation aux centromères. Ces résultats suggèrent que USP11 pourrait être important pour la formation et l'intégrité des centromères. USP11 est recruté aux centromères en G1 par le biais de son interaction avec CENP-A et HJURP, ce qui permet exceptionnellement le recrutement de RAD51 spécifiquement aux CDBs centromériques en G1.

Pour déterminer si l'activation de la réparation par HR aux CDBs centromériques en G1 est délétère dans les répétitions centromériques ou si elle confère au contraire des avantages pour l'intégrité des centromères, nous avons bloqué la réparation par HR en inhibant RAD51, puis évalué l'instabilité génomique au niveau des centromères en étalant des chromosomes en métaphase, à partir de cellules dans lesquelles des CDBs centromériques ont été induits en G1. Bien qu'aucune différence dans la fréquence de translocations chromosomiques n'ait été trouvée après l'induction des CDB centromériques en présence de RAD51, l'inhibition de RAD51 a augmenté le taux de translocations chromosomiques et autres anomalies, prouvant ainsi que l'utilisation de la réparation par HR aux CDBs centromériques en G1 inhibe la formation d'anomalies délétères.

Les centromères sont essentiels pour assurer une ségrégation chromosomique correcte au cours de la division cellulaire. Une réparation infidèle des lésions centromériques peut altérer l'organisation du centromère, entraînant une instabilité chromosomique et des aneuploïdies, à l'origine de diverses maladies, dont le cancer. Bien que les centromères soient connus pour être des régions fragiles du génome, la manière dont ils conservent leur intégrité lorsqu'ils sont endommagés est peu étudiée. Ce projet a montré que lorsque des CDBs se produisent aux centromères, H3K4me2 permet une transcription centromérique et une augmentation de la formation de R-loops, conduisant à la résection des extrémités cassées. La présence de CENP-A et HJURP facilite la seconde étape de HR, le recrutement de RAD51, par l'interaction spécifique de HJURP avec USP11, qui peut alors dé-ubiquitiner PALB2 dans cette région et permettre le recrutement de RAD51 et donc l'achèvement de la réparation par HR en G1. Enfin, l'utilisation de la réparation par HR aux CDBs centromériques en G1 inhibe la formation d'anomalies délétères. Ainsi, nos résultats mettent en évidence un rôle important de l'organisation de la chromatine centromérique dans la réparation de l'ADN, permettant une succession d'événements menant à la réparation par HR en G1, et indiquent que les connaissances actuelles de l'influence de la chromatine sur le choix des voies de réparation de l'ADN sont loin d'être complètes.

INTRODUCTION

1. DNA lesions: Origin, physiological roles, and pathological consequences

1.1 Sources of DNA lesions

The primary objective of living organism is to deliver its genetic information intact and unchanged to the next generation. Preserving the integrity of DNA is therefore crucial for the development, proper cell function and life span of an organism. This must be achieved despite constant assaults by endogenous and environmental agents on the DNA, leading to various types of lesions. Indeed, each cell in the human body receives 10^3 to 10^6 DNA lesions per day (Lindahl, 1993; Lindahl and Barnes, 2000).

1.1.1 Endogenous sources of DNA lesions

Endogenous DNA damage can occur via cellular metabolic processes, such as by oxidative respiration producing reactive oxygen species (ROS), DNA hydrolysis, oxidation, alkylation, and methylation (Figure 1.A). These processes generate base modifications and consequently base-pairs mismatches and mutations upon replication (De Bont and van Larebeke, 2004; Kasai and Nishimura, 1984). Another endogenous source of damage is replication stress, defined as the slowing or stalling of replication fork progression (Zeman and Cimprich, 2014). It can originate from unrepaired DNA lesions, misincorporation of ribonucleotides, secondary DNA structures that are challenging for replication machinery, collision between transcription and replication or nucleotide depletions. If this replication stress persists, fork fails to restart and collapses leading to the formation of DNA double strand breaks (DSBs). Telomeres, as the ends of chromosomes, can appear similar to a broken part of DNA double-helix and consequently, can be recognized by the DNA repair machinery. A cap structure formed by a specific set of proteins protect telomeres from being recognized as breaks. However, in most mammalian cells the replication of telomeres cannot be completed, and they are shortened at each cell division (this process is further discussed in part 1.4.1. and in the review “How to maintain the integrity of the repetitive genome” (Mitrentsi, Yilmaz and Soutoglou, 2020). This shortening causes telomere capping defects and their subsequent recognition by DNA repair pathways, leading to chromosomal rearrangements (Celli and de Lange, 2005; Celli et al., 2006). Finally, DNA lesions can be advantageous and programmed by the cells in order to induce genetic variability

in meiosis and in the establishment of the immune system repertoire by V(D)J recombination, class-switching and somatic hypermutation (Alt et al., 2013; Baudat et al., 2013).

1.1.2 Exogenous sources of DNA lesions

Several environmental factors can also cause DNA lesions (Figure 1.A). For instance, the UV component of sunlight is one of the major external DNA damaging agents leading to different DNA lesions such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4PPs). These lesions can alter the structure of DNA and thus inhibit DNA replication or transcription (Lindahl, 1993). Ionizing radiation (IR) produced by radioactive decay of naturally radioactive compounds or by radiotherapy causes several types of lesions, including DSBs (Goodhead, 1999). Radiomimetic drugs used in cancer chemotherapy, such as bleomycin, or used for research purposes, such as neocarzinostatin (NCS) or phleomycin, have similar effects. Finally, genotoxic compounds in cigarette smoke induce different types of damage to the DNA and are a leading cause of several of the most common cancers in Western countries.

1.2 Different repair mechanisms for each type of DNA lesions

There are multiple types of DNA lesions: mismatches, single- or double-strand breaks, base modifications, etc. (Figure 1.B). Because each type of DNA damage can alter gene expression and cause genome instability by inducing mutations and chromosomal rearrangements, their accumulation can have important consequences for the life expectancy of an organism. Therefore, it becomes evident that cells must have acquired systems that repair DNA anomalies and thereby restore genome integrity (Friedberg, 2008; Lindahl and Barnes, 2000). In this context, a complex set of cellular surveillance and repair mechanisms has evolved to reverse or limit potentially deleterious DNA damage through the concerted action of specific proteins (Figure 1.B). Among these pathways are mismatch repair (MMR) for erroneous insertion, deletion and mis-incorporation of bases, nucleotide excision repair (NER) for bulky adducts such as 6-4PPs or CPDs, base excision repair (BER) for abasic sites, and DSB repair pathways (DSBR) for repairing DNA DSBs, that will be discussed in more details in the third part of the introduction (Rastogi et al., 2010). Cellular responses to DNA damage are complex and interrelated (Ciccia and Elledge, 2010). They influence the efficiency and manner by which damaged DNA is recognized and repaired, cell cycle progression, the coordination of DNA replication and cell division relative to the repair, and the decision point determining survival

or programmed death of cells carrying lesions. In fact, alterations in the genes required for recognizing, processing, and responding to DNA damage may result in an enhanced rate of accumulation of additional mutations, recombinational events, chromosomal abnormalities, and gene amplification (Loeb et al., 2003).

1.3 Physiological roles of DNA lesions

DNA lesions are a double-edged sword. Indeed, while genomic instability can have catastrophic consequences, mutations caused by DNA lesions can be beneficial for evolution as a source of genetic diversity. Moreover, as previously mentioned, these lesions can also be programmed by cells in order to induce genetic variability in meiosis and to produce an effective immune system (Figure 1.C).

1.3.1 In meiosis

Meiosis ensures the proper segregation of chromosomes during sexual reproduction in eukaryotes in order to generate haploid gametes. Prior to the first meiotic division, hundreds of DSBs are formed and their repair by homologous recombination (HR, described in details in part 3.1.) promotes homologous chromosomes pairing in order to exchange genetic information (Richardson et al., 2004) and increase genetic diversity (Baudat et al., 2013). The induction of DSBs is triggered by the SPO11 enzyme which recognizes and cuts specific genomic regions called “recombination hotspots” (Keeney et al., 1997). A key parameter in the targeting of SPO11 to genomics sites seems to be chromatin structure and organization (Lichten and de Massy, 2011; Smagulova et al., 2011). SPO11-induced DSBs promote HR through the meiosis-specific RAD51-like protein DMC1 (Richardson et al., 2004), leading to non-crossover and crossover products at the origin of the genetic diversity.

1.3.2 In immune repertoire establishment

The immune repertoire establishment involves three programmed genome alteration machanisms: V(D)J recombination, class-switch recombination (CSR) and somatic hyper-mutation (SHM) (Bassing and Alt, 2004; Schlissel et al., 2006), which occur to generate immunoglobulin (Ig) and T-cell receptor (TCR) diversity, thus allowing effective recognition of diverse pathogens and antigens. Ig and TCR proteins comprise variable regions that specify antigen binding, and constant regions that endow specific properties to the TCR or the various Ig classes to activate the immune response (Alt et al., 2013).

V(D)J recombination: The antigen-binding variable region of Igs and TCRs is composed of V(variable), D (diversity) and J (Joining) gene segments that are assembled in various combinations, resulting in different amino-acid sequences of the antigen binding regions of Igs and TCRs. Each segment is flanked by recombination-signal sequences that are recognized by the RAG1/RAG2 lymphocyte-specific endonucleases, which generates a blunt DSB at the signal sequence and a covalently-closed DNA hairpin at the coding end (Schatz and Baltimore, 2004). These segments are then fused through the non-homologous end joining (NHEJ) repair pathway (NHEJ is described in details in part 3.2.) (Bassing and Alt, 2004; Taccioli et al., 1993).

Class switch recombination and somatic hypermutation: Igs produced by B cells during V(D)J recombination are further processed by CSR and SHM (Alt et al., 2013). CSR recombines the constant region of Igs, modifying their function without affecting their affinity with antigens. This mechanism produces different antibody isotypes that can interact with different effector molecules and consequently increase the efficiency of the immune response. On the other hand, SHM introduces point mutations to the variable region leading to the production of higher-affinity antibodies (McKean et al., 1984). Both SHM and CSR are mediated by the activation-induced deaminase (AID) which catalyses the deamination of cytosine into uracil (Pavri and Nussenzweig, 2011) resulting in U:G mismatches that are processed by MMR and/or BER, thus creating an abasic site. In SHM, error-prone DNA polymerases are then recruited to fill in the gap and create mutations (Alt et al., 2013), while in CSR, they are further converted to nicks and eventually DSBs, which are later repaired by NHEJ (Alt et al., 2013; Bassing and Alt, 2004).

1.4 Consequences of DNA lesions in aging and pathology

When they are not programmed, DNA lesions can be deleterious and are associated with various pathological conditions. Depending the type of lesion and the site of occurrence, DNA lesions can induce senescence or cell death, which can lead to aging (Garinis et al., 2008); cancer, neurodegenerative disorders, inherited human diseases, immune deficiencies and infertility (Figure 1.C).

1.4.1 In aging

Aging is in part caused by the accumulation of DNA damage (Kirkwood, 2005; Schumacher et al., 2008). Indeed, various endogenously-arising DNA lesions accumulate with age in both the nuclear and mitochondrial genomes (Herbig et al., 2006; Schumacher et al., 2008; Sedelnikova

et al., 2004), paralleling a declining DNA repair capacity over time (Weissman et al., 2007; Yang et al., 2008). This accumulation induces cell senescence and apoptosis via DNA damage response pathways. Senescence corresponds to an irreversible growth arrest of a cell without complete shutdown of cellular or metabolic activities, whereas apoptosis, a type of programmed cell death. Both can occur when DNA repair mechanisms are unable to repair the damage. Further evidence comes from the fact that patients with defects in repair factors often display features of premature aging. Indeed, multiple repair factors play important roles at telomeres and consequently, defects in them result in telomere shortening and/or telomere dysfunction which can trigger senescence and apoptosis (d'Adda di Fagagna et al., 2004; Verdun and Karlseder, 2007). Moreover, telomeres shortening at each cell division can lead to the loss of the telomeric cap (Shelterin complex) and subsequently to their recognition as DSBs and the activation of the DNA damage response pathway, thus also leading to senescence or apoptosis (d'Adda di Fagagna et al., 2004; Longhese, 2008) (This process will be discussed longer in our review "How to maintain the integrity of the repetitive genome"). While cellular senescence and apoptosis are beneficial to an organism to reduce the accumulation of potentially harmful mutations, for older individuals they contribute to aging phenotypes by reducing tissue regeneration (cell renewal), loss of post-mitotic cells (e.g. neurons), and by senescent cells overexpressing proteins that affect local tissue microenvironments, disrupting tissue integrity (Campisi, 2003).

1.4.2 In cancer

Cancer is notably caused by DNA replication errors, transcriptional stress, unrepaired damage, mis-repaired damage, and acquired or inherited mutations. These phenomena can lead to DNA alterations that ultimately result in changes in expression of genes important for normal cellular functions and growth, including proto-oncogenes and tumour suppressor genes (Stratton et al., 2009). These modifications can originate from single point mutations, insertions, and deletions, and also from larger aberrations, such as alterations in chromosome number or chromosome translocations. Chromosome translocations arise from aberrantly re-joined DSBs from different genomic locations, which can result in the formation of fusion proteins with oncogenic potential when the fusion occurs between an oncogene and a transcriptionally strong promoter, turning the gene on in a contexed it is not normally expressed, or otherwise dysregulating gene expression of the gene (Roukos and Misteli, 2014). This has important consequences, since 20% of cancers are considered to be caused by chromosome translocations (Mitelman et al., 2007). Translocations are the primary driver of many haematological malignancies, and are

increasing being linked to solid cancers, such as prostate cancer (Mitelman et al., 2007). Moreover, for many cancers multiple genetic events occur in many different genes during the process of carcinogenesis, suggesting that an early and perhaps necessary event in cancer development is an underlying defect in mechanisms maintaining genomic stability (Gray and Druker, 2012; Hanahan and Weinberg, 2011; Nik-Zainal et al., 2016, 2012).

1.4.3 In neurodegenerative disorders

Neurons are generally associated with high ROS production because of a high mitochondrial respiration (Weissman et al., 2007). However, long-lived and post-mitotic neurons have a limited capacity for replacement in adulthood, which can potentially lead to accumulation of lesions. Indeed, accumulation of DNA lesions in neurons is associated with neurodegenerative disorders, including Alzheimer's, Huntington's and Parkinson's diseases and ataxias (Kulkarni and Wilson, 2008).

1.4.4 In inherited human diseases

Repetitive regions of the genome can be hotspots for DNA damage related pathologies. DNA-repeat instability is thought to arise from the repetitive nature of these sequences, which can form aberrant DNA-secondary-structure during DNA replication. These topological structures can lead to lesions. DNA-repair processes fixing these lesions can result in expansions or contractions of the DNA repeat sequences, usually a trinucleotide motif, which is involved in several neuromuscular and neurodegenerative diseases including Fragile X syndromes, Friedreich's ataxia, spinocerebellar ataxias, diabetes type 2, Creutzfeldt-Jakob disease, myotonic dystrophy and Huntington's disease (Kovtun and McMurray, 2008; Mirkin, 2007).

1.4.5 In immune deficiencies and infertility

As discussed before (part 1.3.2), genome rearrangements through programmed DNA lesions occur during immune-system development. Defect in this process can cause immune deficiency. For instance, lymphomas and leukaemias of B- and T-cell can result from impaired V(D)J recombination. Similarly, as meiotic recombination involves DSB generation, it is likely that defects in this process would cause human infertility (Alt et al., 2013; Matzuk and Lamb, 2008).

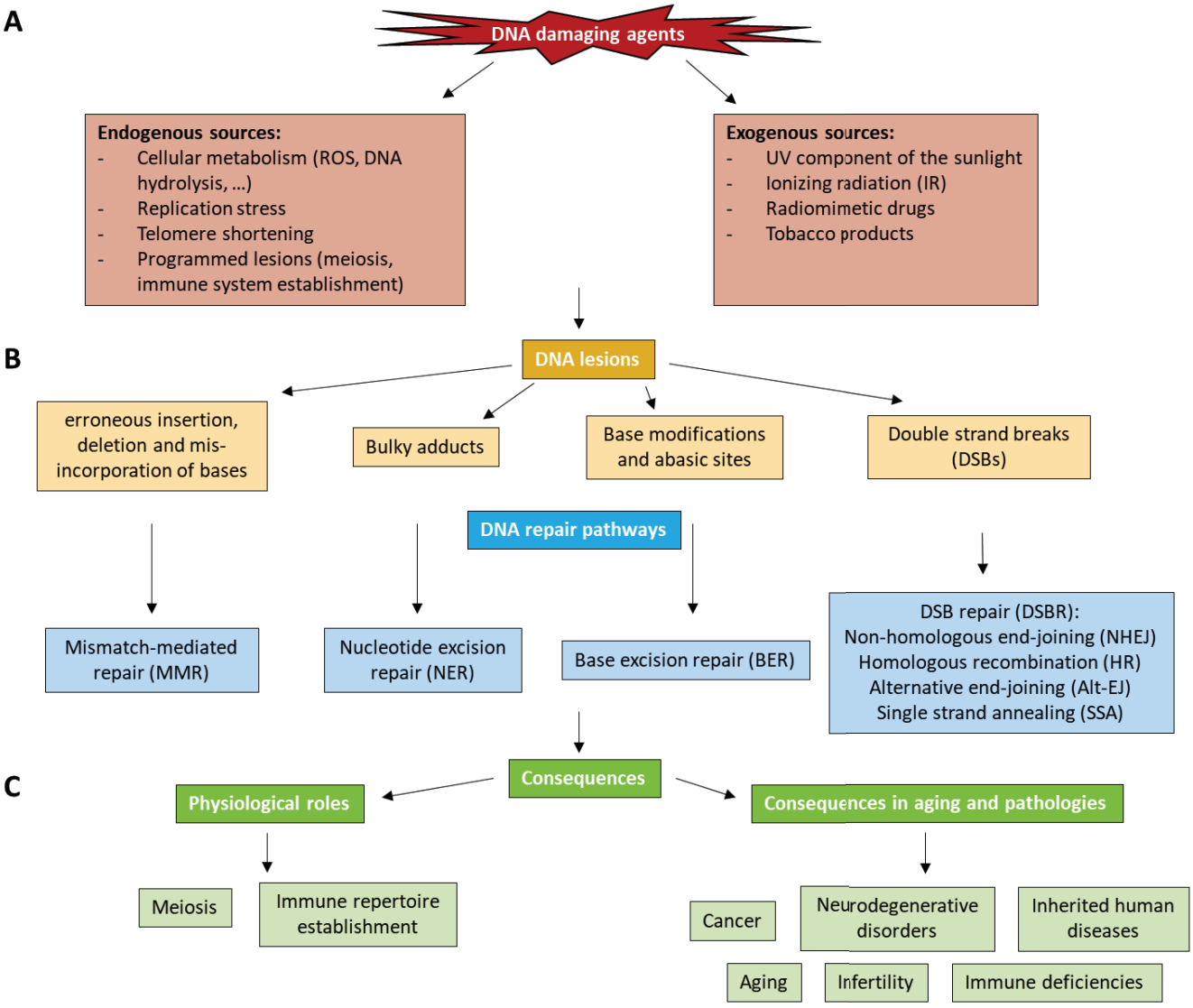


Figure 1: Different types of DNA lesions, originating from various damaging agents lead to distinct repair pathways activation and have diverse consequences.

(A) Sources of DNA damage can be exogenous or endogenous. **(B)** They lead to different types of DNA lesions that are repaired by specific repair pathways. (**C)** These DNA lesions can have physiological roles but also consequences in aging and pathologies.

2. Double strand breaks (DSBs) and the DNA damage response (DDR)

DSBs are among the most dangerous types of damage because they affect both strands of the DNA double helix. If left unrepaired, DSBs could lead to cell death or deregulated growth and cancer development, as they can cause large chromosomal alterations like loss of fragments or rearrangements such as translocations (Jackson and Bartek, 2009). Since multiple endogenous and exogenous factors described above can inflict DSBs (ROS, replication fork collapse, ionizing radiation, crosslinking agents that stall DNA polymerases, topoisomerase poisons, etc.) DSBs are frequently generated, with an estimated 10 to 50 DSBs per cell per day formed, depending on cell cycle and tissue (Vilenchik and Knudson, 2003). To overcome these damages, a complex set of cellular surveillance and repair mechanisms has evolved to reverse or limit potentially deleterious breaks, in a spatially and temporally controlled manner. This involves the DNA damage response (DDR) pathway, a signaling cascade that allows the recruitment of key factors of the repair process around broken ends and initiates cell cycle checkpoints to prevent cells from starting replication (the G1/S checkpoint), from replication progression (intra-S checkpoint) or from entering in mitosis (G2/M checkpoint), depending on the time when the break occurs (Bekker-Jensen and Mailand, 2010; Chapman et al., 2012a; Misteli and Soutoglou, 2009; Zhou and Elledge, 2000). The resulting cell cycle arrest gives the cells the necessary time to repair the damage before they divide, so as not to propagate the damage. Upon sensing of the breaks, the major transducers of the DDR -the kinases ATM, ATR and DNAPK- are recruited and activated. This activation allows the phosphorylation of numerous targets, leading to the propagation and the amplification of the signal and finally to cell cycle control, either giving time for DNA repair or triggering senescence or apoptosis.

2.1 DSB sensing

The DDR is primarily mediated by sensor proteins that recognize the DNA break: poly ADP-ribose polymerase (PARP), Ku70/Ku80 heterodimer, MRE11-RAD50-NBS1 (MRN) sensor complex and replication protein A (RPA) complex (Figure 2).

2.1.1 By PARP proteins

Upon their activation as a response to DNA damage, PARPs catalyse the PARylation (poly-ADP-ribose units attachment) of acceptor proteins, which promotes the recruitment of X-ray repair cross-complementing protein 1 (XRCC1) and DNA ligase 3 (LIG3) to break sites (El-

Khamisy et al., 2003; Mortusewicz et al., 2006) (Figure 2). PARP1 is involved in HR and alternative end joining (alt-EJ, described in detail in part 3.3.) repair pathways by recruiting the nuclease MRE11 and initiating end resection activity, an important step in HR which generates 3' single stranded DNA overhangs (See section 3.1.1 for more details) (Bryant et al., 2009; Paddock et al., 2011). On the other hand, PARP3 drives repair towards the NHEJ repair pathway by preventing excessive end-resection mediated by MRE11 (Beck et al., 2014) and by interacting with the histone chaperone APFL to accelerate the XRCC4-DNA ligase IV-mediated ligation (Grundy et al., 2013; Rulten et al., 2011). Recently, PARP1 was also shown to recruit the chromatin remodeler CHD2 at break sites to promote NHEJ repair (Luijsterburg et al., 2016).

2.1.2 By Ku70/Ku80 heterodimer

Ku70/Ku80 heterodimer rapidly localizes at DSBs where it loads and activate the catalytic subunit of DNAPK (DNAPKcs) to initiate repair by NHEJ (Drouet et al., 2005) (Figure 2). An early consequence of Ku70/Ku80 localisation at DSBs is to keep the DNA ends near each other before the repair and thus avoiding them from drifting apart.

2.1.3 By MRN complex

The MRN complex can bind directly to DNA through MRE11 DNA binding domains (D'Amours and Jackson, 2002), and trigger the activation and recruitment of ATM kinase (Figure 2). Additionally, MRE11 has a nuclease activity that initiates end-resection, a major step of HR (D'Amours and Jackson, 2002). Other members of the complex are also important for its correct functioning. RAD50's ATPase activity is necessary for both DNA binding but and for the stimulation of MRE11 nuclease activity (Bhaskara et al., 2007). NBS1 interacts directly with MRE11, stabilizes the MRE11/RAD50 complex and is responsible for its localization in the nucleus (Desai-Mehta et al., 2001). Furthermore, NBS1 interacts with MDC1 which triggers MRN recruitment at DSBs (Chapman and Jackson, 2008; Lukas et al., 2004; Spycher et al., 2008; Stucki et al., 2005) where it then promotes ATM activation (Falck et al., 2005) (discussed further in part 2.2.1).

2.1.4 By RPA complex

The replication protein A (RPA) complex is composed of three subunits (RPA1, 2 (also called 32), and 3). This complex has a very high affinity with single stranded DNA (ssDNA) and protects ssDNA from forming secondary structures (Maréchal and Zou, 2015). Its recruitment

at ssDNA causes both the localisation to ssDNA and the activation of ATR kinase and its co-activator ATR-interacting protein (ATRIP) (Cimprich and Cortez, 2008) (Figure 2). This activation contributes to Chk1-mediated cell cycle checkpoint and to the stabilisation of stalled replication forks. Additionally, RPA binds to resected DNA ends and participates in HR repair pathway.

2.2 Signal propagation

After the initial sensing of the DNA break, the signal is propagated by three members of the phosphatidylinositol 3-kinase (PI3K) family: ATM, ATR and DNAPK (Falck et al., 2005). These kinases trigger post-translational modifications (PTMs), notably the phosphorylation of downstream effectors in order to disseminate the damage signal and further recruit repair factors and to direct the regulation of cell cycle progression, apoptosis or senescence (Branzei and Foiani, 2008; Smith et al., 2010) (Figure 2).

2.2.1 ATM signaling

ATM is the major kinase mediating the phosphorylation of S139 on the histone variant H2AX (γ-H2AX), and the megabase spreading of γ-H2AX around the break (Falck et al., 2005; Tomimatsu et al., 2009). As previously mentioned, the recruitment of ATM at the sites of breaks is mediated by the MRN complex through its direct interaction with NBS1(Difilippantonio et al., 2005; Lee and Paull, 2004, 2005) (Figure 2). Moreover, the signal spreading factor MDC1 directly recognizes ATM-induced γ-H2AX and is able to recruit additional ATM molecules to the DSB sites, thus creating a positive feedback loop that mediates the signal spreading around the break (Savic et al., 2009; Stucki et al., 2005). Additional downstream DDR factors, such as 53BP1 and BRCA1, can also modulate the retention of ATM (Lee et al., 2010a).

ATM exists in the cells as an inactive homodimer. Upon damage, it is activated through auto-phosphorylation of S1981 that leads to its dissociation to active monomer (Bakkenist and Kastan, 2003). Further PTMs are necessary for ATM activation but are not necessary for its initial recruitment: three additional autophosphorylations (Bensimon et al., 2010; Kozlov et al., 2006, 2011) and acetylations by Tip60 on Lys3016 (Sun et al., 2005, 2007b). In addition to the phosphorylation of several target molecules which regulate DDR, ATM also phosphorylates checkpoint kinase 2 (Chk2), which activates Chk2 and allows the maintenance of G2 arrest following damage induction (Matsuoka et al., 2000; Saito et al., 2002). Indeed, Chk2 phosphorylates the phosphatase Cdc25C, which will be inactivated, and Cdc2, the kinase

subunit of Cdk1, will remain phosphorylated and therefore will not arrest the cell cycle (Ahn and Prives, 2002). ATM also phosphorylates p53 directly, or through Chk2 phosphorylation, leading to cell cycle arrest.

Mutations in ATM are responsible for the genomic instability disorder Ataxia-telangiectasia. ATM is considered as the major transducer of DDR. However, patients cells displaying mutations in ATM still show a partial DDR activation, suggesting a cooperation with other kinases such as ATR and DNAPK (Tomimatsu et al., 2009).

2.2.2 ATR signaling

ATR is recruited to ssDNA structures coated with RPA (Zou and Elledge, 2003) (Figure 2). ssDNA can arise from mis-coordination between DNA polymerase and helicase during DNA replication, and from DSB resection. RPA allows the recruitment of ATRIP that then interacts and promotes the recruitment of ATR at the sites of damage (Zou and Elledge, 2003). ATR also needs to be activated upon its recruitment at DSBs. This activation happens through the recognition of ssDNA-dsDNA junction by the Rad17-RCF2/4 complex, which in turn loads the Rad9-Rad1-Hus1 complex (9-1-1 complex) (Ellison and Stillman, 2003; Zou et al., 2003), that recruits TopBP1 to activate ATR and allow further signal spreading (Kumagai et al., 2006). In addition, ETAA1 was recently identified as an RPA-binding factor that stimulates ATR activity independently of TopBP1 in mammalian cells (Bass et al., 2016; Haahr et al., 2016; Lee et al., 2016). ATR can also be activated through ATM signaling, predominantly during S/G2 phases of the cell cycle (Jazayeri et al., 2006; Myers and Cortez, 2006). In addition to the phosphorylation of its interacting factors cited above, ATR activation leads to the phosphorylation of checkpoint kinase 1 (Chk1) and further cell cycle arrest through Cdc25 phosphorylation. This phosphorylation is facilitated by the adaptor protein Claspin that interacts upon damage with Chk1 in a TopBP1-dependent manner, thus mediating the interaction between Chk1 and ATR (Kumagai and Dunphy, 2003; Liu et al., 2006).

2.2.3 DNAPK signaling

DNAPK is a PI3K-like kinase composed of Ku70, Ku80 and DNAPK catalytic subunit proteins (DNAPKcs) and has been shown to play a major role in NHEJ. It can also phosphorylate H2AX and KAP1 following ionizing radiation and drive local chromatin decondensation near the DSB site, promoting DDR signaling (Caron et al., 2015; Falck et al., 2005; Lu et al., 2019a; Stiff et al., 2004). DNAPK is activated through DNA binding. DNAPK can also be phosphorylated by ATR, facilitating its activation (Yajima et al., 2006). It can act in concert with ATR and ATM

to phosphorylate RPA32 upon replication stress or ionizing radiation, which was shown to participate in the G2/M and intra-S checkpoints (Block et al., 2004; Liaw et al., 2011; Liu et al., 2012; Wang et al., 2001) (Figure 2).

2.3 Signal amplification and repair foci formation

The PI3K kinases activation leads to H2AX phosphorylation, generating γ-H2AX (Figure 2). This phosphorylation is considered a hallmark of the DDR and initiates the DDR signaling cascade. Indeed, MDC1 is recruited at the sites of break through direct recognition of γ-H2AX, and acts as a platform to recruit additional MRN-ATM complexes that can phosphorylate H2AX on adjacent nucleosomes, thus creating a positive feedback loop for DDR signal amplification and spreading around the break (Chapman and Jackson, 2008; Lukas et al., 2004; Spycher et al., 2008; Stucki et al., 2005). The spreading of DDR factors allows their visualization by microscopy as foci, termed irradiation induced foci (IRIF) (Kinner et al., 2008; Nagy and Soutoglou, 2009). MDC1 then triggers a second wave of protein accumulation at the sites of damage, by recruiting the ubiquitin ligase ring finger protein 8 (RNF8) to DSBs where it subsequently ubiquitinates H2A and H1 histones and promotes recruitment of downstream effectors such as 53BP1 and BRCA1 (Huen et al., 2007; Mailand et al., 2007; Thorslund et al., 2015). RNF8 recruitment to DSBs triggers the recruitment of an additional ubiquitin ligase, ring finger protein 168 (RNF168), which induces ubiquitination of H2A histones, thus enhancing 53BP1 and BRCA1 retention at DSBs (Doil et al., 2009; Mattiroli et al., 2012; Pinato et al., 2009). (The role of ubiquitination in DSB repair will be further discussed in part 3.6.).

H2AX phosphorylation occurs minutes after the induction of damage and is not only localized at the site of the break but extends along the length of the chromatin on either side of the break (Nakamura et al., 2010), until a gap in the H2AX nucleosome substrate appears (Bewersdorf et al., 2006). It has also been shown that γ-H2AX spreading was limited around a DSB by the factor Cohesin (Caron et al., 2012).

2.4 DDR outcomes

The major outcome of DDR is cell cycle arrest to give the cell time to repair the break before dividing (Figure 2). As mentioned earlier, ATM kinase phosphorylates Chk2 and p53, and ATR phosphorylates Chk1 (Bartek and Lukas, 2003), both triggering checkpoint activation that can lead to three outcomes: cell cycle arrest, senescence, or apoptosis.

2.4.1 Cell cycle arrest

As mentioned previously, Chk1 and Chk2 phosphorylate the phosphatase Cdc25C, which will be inactivated, thus blocking the dephosphorylation of CDK complexes, a step necessary for cell cycle progression (Bartek and Lukas, 2003). Moreover, arrest in G1 can be mediated by the p53 stabilization through its phosphorylation by ATM and Chk2. This can activate p21 transcription leading to CDK inhibition and subsequent G1/S transition blocking (Harper et al., 1993). Arrest is S phase can be mediated by progressive slowing down of replication fork progression and a decrease in the activation of replication origins (Grallert and Boye, 2008; Seiler et al., 2007). Arrest in G2 is due to the inhibition of the CDK1-Cyclin B complex, in response to the inhibition of the Cdc25 phosphatases (O'Connell et al., 2000) (Figure 2).

2.4.2 Senescence

Senescence corresponds to an irreversible growth arrest of a cell without complete shutdown of cellular or metabolic activities. It is mainly mediated by the persistent activation of DDR which triggers p53 phosphorylation, activation, and stabilization (Turenne et al., 2001), leading to the transcription of p21 (Figure 2).

2.4.3 Apoptosis

Programmed cell death is termed as apoptosis and occurs when DNA repair mechanisms are unable to repair the damage. Similar to senescence, apoptosis is mediated by the p53 protein phosphorylation, which activates the transcription of pro-apoptotic genes such as PUMA, BAX and BAK (Reinhardt and Schumacher, 2012) (Figure 2).

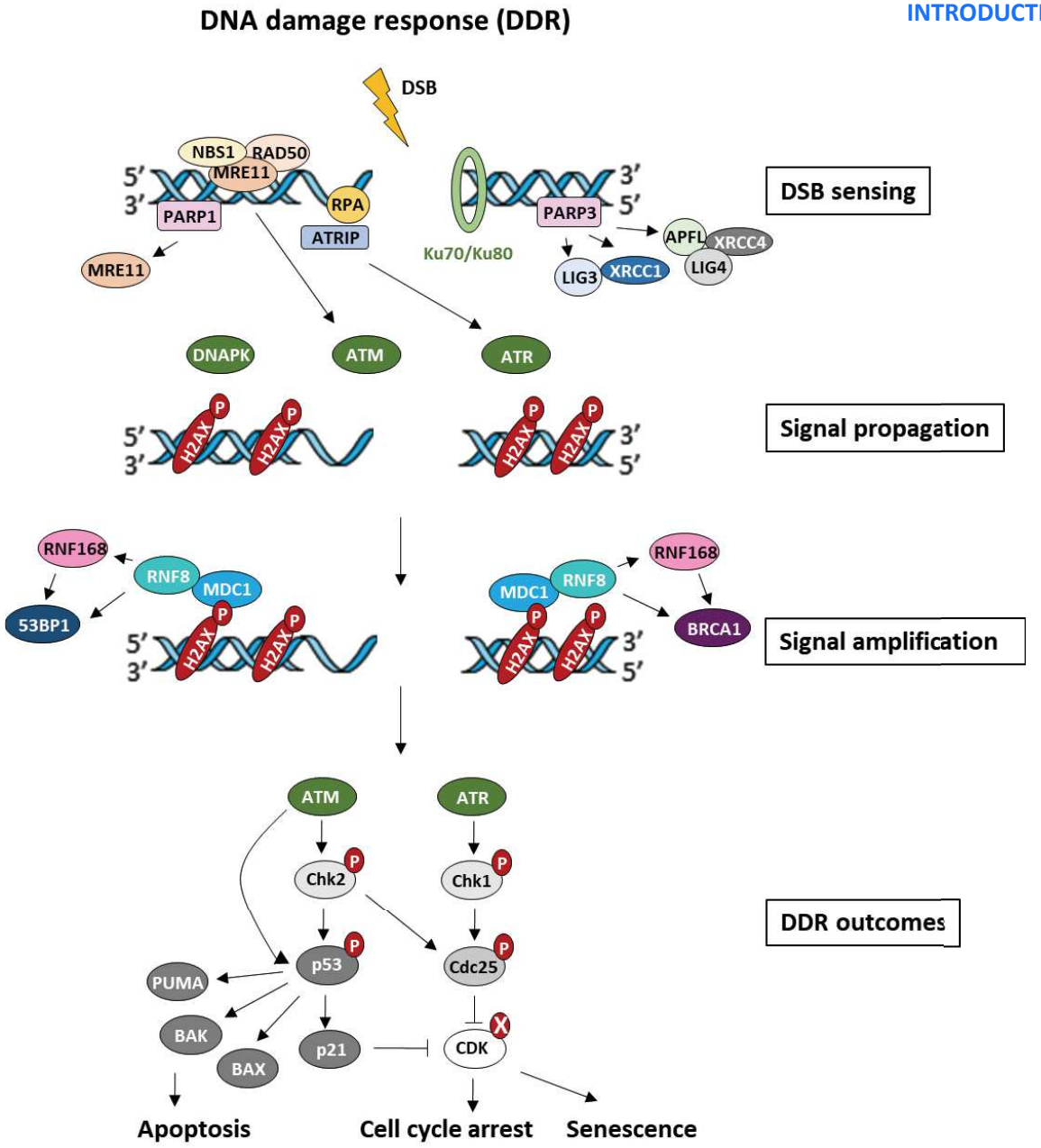


Figure 2: DNA damage response pathway (DDR)

(DSB sensing) DDR starts with the sensing of the break, a process that involves binding of either MRN complex, or Ku70/Ku80 heterodimer, or PARP factors, or RPA on a single stranded DNA. **(Signal propagation)** The PI3K kinases ATM, ATR and DNAPK are subsequently recruited through distinct mechanisms. ATM is mainly recruited through MRN complex and PARP1, ATR is mainly recruited by RPA through ATRIP, and DNAPK is mainly recruited by the Ku heterodimer. These kinases phosphorylate downstream effectors as well as the histone variant H2AX (γ-H2AX), leading to the signal propagation. **(Signal amplification)** γ-H2AX acts as a platform to recruit additional MRN-ATM complexes that can phosphorylate H2AX on adjacent nucleosomes, thus creating a positive feedback loop for DDR signal amplification. γ-H2AX is recognized by MDC1 that triggers a second wave of protein accumulation at the sites of damage, by recruiting RNF8 and RNF168 E3 ubiquitin ligases, leading to 53BP1 and BRCA1 recruitment. (**DDR outcomes)** Cell-cycle arrest is mediated by ATM and ATR kinases that phosphorylate Chk2 and Chk1, respectively. Chk2 phosphorylates p53 and Cdc25, leading to CDK inhibition to block the cell-cycle. Chk1 also blocks the cell cycle progression through phosphorylation of Cdc25. Persistent activation of DDR and CDK inhibition leads to senescence. On the other hand, p53-dependent transcription of pro-apoptotic factors PUMA, BAX and BAK can lead to apoptosis.

3. DSB repair pathways: mechanisms, role in translocations and regulation

DSBs are an important threat to the stability of the genome, potentially provoking chromosome rearrangements and disrupting gene structure and function. Given these potential dramatic consequences, DSBs should be repaired fast and faithfully. Several repair mechanisms have evolved to ensure that a certain engaged DSB repair pathway matches the cellular context in space and time, including cell cycle phase, the nature of the genomic sequence and the local chromatin environment (Chapman et al., 2012a). Mutations in DSB repair genes cause genomic instability in numerous diseases that are associated with cancer predisposition, developmental disorders and premature aging (Ciccia and Elledge, 2010). The two main DSB repair pathways are Homologous Recombination (HR) and classical Non-Homologous End-Joining (NHEJ). More highly mutagenic pathways are also involved in DSB repair: alternative End-Joining (alt-EJ), Single Strand Annealing (SSA) and Break-Induced Replication (BIR).

3.1 Homologous Recombination (HR)

HR uses an homologous sequence, mainly the homologous sister chromatid, as a template for repair, and is therefore known to be largely restricted to the S and G2 phases of the cell cycle (Takata et al., 1998). Because of this homology-based repair, HR is considered an error-free mechanism. The molecular mechanism of HR consists of resection that generates a 3'tail, which can then invade the double-stranded DNA homologous sequence, therefore forming a displacement loop (D-loop), leading to strand exchange (Figure 3).

3.1.1 End resection

DNA end resection is initiated by the MRN complex (MRX complex in yeast for XRS2) (D'Amours and Jackson, 2002; Symington and Gautier, 2011) (Figure 3). MRE11 endonuclease activity nicks the DNA strand of the break that has a free 5′ end, up to 300 nucleotides internal to the DNA end, and MRE11 3′–5′ exonuclease activity extends the nick towards the DNA end. MRE11 "short-range" endonuclease activity requires interaction with CtBP-interacting protein (CtIP) or Sae2 in yeast (Anand et al., 2016, 2019; Deshpande et al., 2016; Myler et al., 2017). This initial processing step is thought to displace Ku70/Ku80 from DNA ends. ATM also participates in end resection by stimulating the nucleolytic activity of CtIP and MRE11 (Geuting et al., 2013). CtIP cooperates with the Exonuclease 1 (EXO1), the

helicase Bloom syndrome protein (BLM) (and with the helicase Sgs1 in yeast), and the endonuclease DNA2 to create extensive single-stranded 3′ DNA overhangs, in a process called "long-range" resection (Daley et al., 2017; Eid et al., 2010; Liao et al., 2012; Makharashvili et al., 2014; Marrero and Symington, 2010; Nimonkar et al., 2011). Additional DNA end resection regulators have been described. DNA2 also interacts with WRN helicase to promote resection (Pinto et al., 2016). BRCA1-BARD1 ubiquitinates phosphorylated CtIP and enhances CtIP association to chromatin (Yu et al., 2006). BLM, MRN, RPA and PHF11 also stimulate the resection activity of EXO1 (Gong et al., 2017b; Nimonkar et al., 2011). The exonuclease EXD2 interacts with CtIP and MRE11 to promote resection (Broderick et al., 2016). On the other hand, the helicase HELB is recruited to resected ends through RPA and inhibits EXO1 and BLM-DNA2 activity (Tkáč et al., 2016).

3.1.2 Strand invasion

RPA wraps these resected ssDNAs, thereby protecting it from breakage and limiting interactions with ssDNA intermediates of other nuclear processes (Figure 3). RPA also forms a barrier to the loading of the RAD51 recombinase (RecA in yeast) and must be displaced by recombination mediators to allow HR repair (San Filippo et al., 2008). One of these recombination mediators is BRCA2 (with Rad52 in budding yeast, and Rad22 in *S. pombe* having a similar function) (Carreira and Kowalczykowski, 2011; Lisby et al., 2003; San Filippo et al., 2008; Sugawara et al., 2003). BRCA2, constitutively bound to the proteasomal component DSS1, interacts with ssDNA and can directly interact with RAD51 monomers. UCHL3 deubiquitinates RAD51 promoting its interaction with BRCA2 (Luo et al., 2016). It was recently reported that optimal loading of RAD51 on RPA-coated DNA ends depends on the DEK chromatin bound protein (Smith et al., 2017). BRCA2 also interacts with BRCA1–BARD1 via the PALB2 protein. The BRCA1-PALB2-BRCA2 complex along with DSS1 promotes the replacement of RPA and the loading of the recombinase RAD51 onto ssDNA (Xia et al., 2006; Zhao et al., 2015). PALB2 interacts with RNF168 in S/G2 facilitating HR (Luijsterburg et al., 2017). In addition, the recruitment of the BRCA1-PALB2-BRCA2 complex at the sites of damage is also dependent on CDK9 (Nepomuceno et al., 2017). Moreover, in S and G2 phases, the KEAP1-dependent ubiquitination of PALB2 is countered by USP11, thus allowing the formation of the BRCA1-PALB2-BRCA2 complex (Orthwein et al., 2015; Schoenfeld et al., 2004).

RAD51-bound ssDNA nucleoprotein filaments search for a homologous duplex DNA to invade and displace one strand, forming a D-loop and facilitating base-pairing with complementary homologous DNA sequences (Carreira et al., 2009; Jensen et al., 2010; Liu et al., 2010; Thorslund et al., 2010). BRCA1–BARD1 has recently been implicated in facilitating RAD51-mediated homologous pairing, indicating that BRCA1 promotes multiple HR steps (Zhao et al., 2017). RAD51 has five paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) that are recruited to the breaks with RAD51 in S/G2 phases and might work as cofactors of RAD51(Takata et al., 2001; Taylor et al., 2015; Thompson and Schild, 2001). In *S. cerevisiae*, Rad51 paralogues promote the stability of the Rad51 filament and restrain its disassembly by the antirecombinase Srs2 (Krejci et al., 2003; Liu et al., 2011). These relationships indicate that RAD51 filament stability is regulated to optimize the efficiency of HR. The RAD54 protein that is subsequently recruited promotes the stabilization of the D-loop and regulates the transition to DNA synthesis by dissociating RAD51 from DNA (Heyer et al., 2006).

3.1.3 Strand exchange resolution

The D-loop leads to three different HR sub-pathways: the classical HR pathway (DSBR) with formation of a double Holliday junction (dHJ), the synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR) (Figure 3). Depending on the polarity of dHJ resolution, this can result in crossing over between the recombining molecules, detected in somatic cells as a sister chromatid exchange (Sarbajna and West, 2014). This pathway is predominant in meiotic cells. dHJ can be dissolved through migration by the BTR (BLM–TOP3A–RMI) complex to separate the sister chromatids without a crossover. Alternatively, persistent dHJs can be resolved later in the cell cycle by HJ resolvases such as the SLX4/SLX1/Mus81/EME1 complex, and GEN1 (West et al., 2015). SDSA involves RAD51-mediated invasion by only one end of the DSB, whereas the second end is resected but remains passive (Pâques and Haber, 1999). How this asymmetry is established is not well understood. The non-invading second end of the break anneals with the displaced nascent strand and enables HR termination. Because it does not involve formation of a Holliday junction, SDSA is a non-crossover pathway, and is the predominant repair pathway in somatic cells. BIR occurs in the absence of a second end. In that case the entire chromosome is replicated (Heyer et al., 2010). This pathway can lead to loss-of-heterozygosity of all genetic information distal to the DSB.

3.1.4 Synthesis

Following D-loop formation, the free 3′ end of the invading strand engages a DNA polymerase to extend the nascent strand. DNA polymerase δ (Pol δ) plays a major role in this synthesis, but translesion DNA polymerases have also been implicated in competition with Pol δ (Kane et al., 2012; McVey et al., 2016).

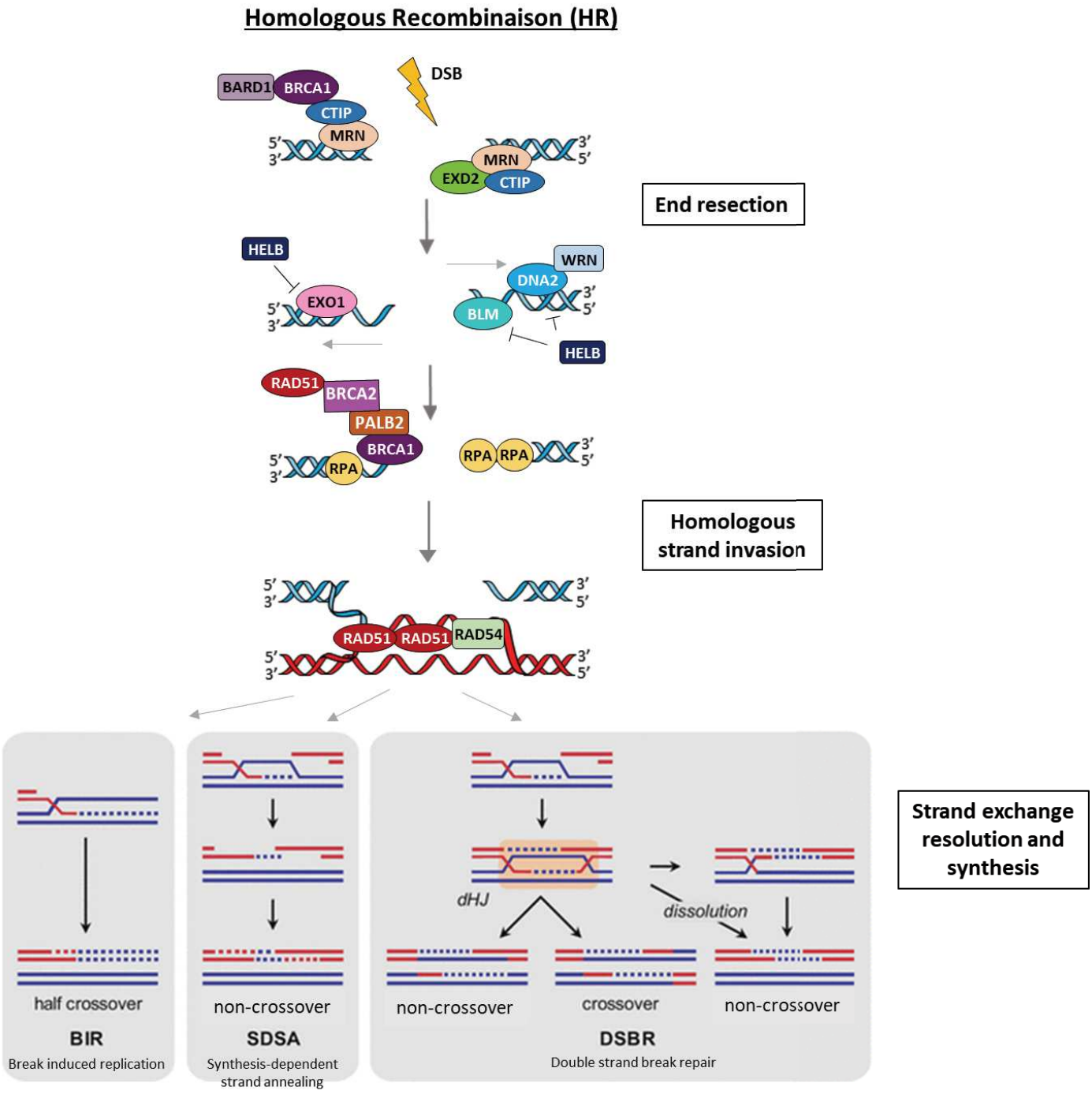


Figure 3: Homologous recombination (HR) pathway

(End resection) Break recognition is mediated by the MRN complex. CtIP is subsequently recruited to initiate short-range resection in parallel with MRE11. BRCA1-BARD1 enhance CtIP association to chromatin. EXD2 interacts with CtIP and MRE11 to promote resection. Then, the DNA ends are extensively processed by EXO1, BM and DNA2 nucleases. DNA2 interacts with WRN helicases to promote resection. HELB helicase limits EXO1 and BLM-DNA2 activity. **(Homologous strand invasion)** Resected ends are protected by RPA that is displaced by BRCA2. Strand invasion is then mediated by BRCA1-PALB2-BRCA2 complex that facilitates RAD51 loading. Rad54 is also recruited to stabilize the D-loop. **(Strand exchange resolution and synthesis)** Break induced replication **(**BIR) occurs in the absence of a second end. In that case the entire chromosome is replicated. Synthesis-dependent strand annealing (SDSA) involves invasion by only one end of the DSB, whereas the second end is resected but remains passive. The non-invading second end of the break anneals with the displaced nascent strand, leading to non-crossover. The classical double strand break repair (DSBR) involves a double Holliday junction (dHJ) formation. The dHJ can be either dissolved by branch migration leading to non-crossover products or resolved by endonucleolytic cleavage to produce non-crossover or crossover products. Strand exchange part adapted from (Sebesta and Krejci, 2016).

3.2 Non-homologous end joining (NHEJ)

Classical NHEJ does not require sequence homology, even though microhomologies can appear at the junctions and may help to align the broken ends (Roth and Wilson, 1986). DNA ends are protected from resection and can be precisely joined when they do not require any modification. They are imprecisely joined after processing in order to make ends ligatable, a process which may lead to the loss of a few base-pairs. NHEJ is therefore considered as a possible error-prone pathway (Davis and Chen, 2013). NHEJ is fast and active throughout the cell cycle (Rothkamm et al., 2003) (Figure 4).

3.2.1 Break recognition by Ku70/Ku80 heterodimer

NHEJ is initiated by the binding of the Ku70/Ku80 (also known as XRCC6/XRCC5) heterodimer to DSB ends (Figure 4). Ku has been shown to protect DNA from nucleases digestion (Foster et al., 2011) and to limit DSB mobility keeping the two broken ends together. Ku depletion leads to increased mobility and separation of the broken ends, which might increase translocation frequency (Roukos et al., 2013; Soutoglou et al., 2007).

3.2.2 DNAPKcs recruitment

Ku allows the recruitment of other NHEJ factors including DNAPKcs (Figure 4). Through its interaction with Ku, DNAPKcs kinase activity is stimulated and it phosphorylates Ku70/Ku80 (Chan et al., 1999), DNA ligase IV (LIG4, Wang et al., 2004), Artemis (Goodarzi et al., 2006; Ma et al., 2005), the associated scaffolding factors XRCC4 (Leber et al., 1998), XRCC4-like

factor (XLF, Yu et al., 2008), and the paralogue of XRCC4 and XLF (PAXX) (Ochi et al., 2015). DNAPKcs is also auto-phosphorylated and this leads to structural changes that are proposed to affect (promote or inhibit, according to the phosphorylated residues) the ability of DNA end-processing enzymes and ligases to access the DNA ends and promote NHEJ (Dobbs et al., 2010). DNAPKcs is also trans-phosphorylated by ATM, which is necessary to recruit the nuclease Artemis (Jiang et al., 2015).

3.2.3 End processing

Dependent on the free ends of broken DNA, simple ligation or end-processing can occur (Figure 4). Indeed, broken ends that are chemically modified with presence of blocking end groups (caused by irradiation for example), or secondary structure elements surrounding the break, such as hairpin produced during V(D)J recombination, necessitate additional processing (Deriano and Roth, 2013). Several enzymes have been implicated in end processing: the polynucleotide kinase phosphatase (PNKP), which can phosphorylate the 5' end and dephosphorylate the 3' end creating the correct chemical groups required for ligation (Chappell et al., 2002), some specialized DNA polymerases (λ and μ) that can fill in the gaps at the site of a DSB (Ramsden, 2011) or the nuclease Artemis that allows hairpin opening (Ma et al., 2002) and removal of ssDNA overhangs containing damaged nucleotides (Kurosawa and Adachi, 2010). Recently, Artemis has been shown to be also involved in completing resection in DSBs generated during G1 phase of the cell cycle (Biehs et al., 2017). WRN was also shown to interact with Ku heterodimer and XRCC4 to process the broken DNA ends with its 3’ to 5’ exonuclease activity (Cooper et al., 2000; Kusumoto et al., 2008; Perry et al., 2006).

3.2.4 End joining

The end joining, last step of NHEJ, is carried out by LIG4, which is stabilized by XRCC4 and stimulated by XLF (Andres et al., 2007; Riballo et al., 2009) (Figure 4). Ligase IV, XLF and XRCC4 can interact directly with the Ku complex. The paralogue of XRCC4 and XLF (PAXX) also interacts directly with Ku and promotes Ku-dependent ligation (Ochi et al., 2015). XRCC4 is essential for LIG4 stability and function, while XLF and PAXX have partially redundant scaffolding roles (Kumar et al., 2016; Zha et al., 2011).

Several accessory factors also regulate NHEJ. These include the MRN complex, which may assist in end bridging (Dinkelmann et al., 2009; Williams et al., 2008), apratxin and PNK-like factor (APLF), which interacts with Ku80 and with poly(ADP ribose)-modified proteins in the vicinity of the DSB (Grundy et al., 2013; Macrae et al., 2008; Rulten et al., 2011). Several

additional positive and negative regulators of Ku70/Ku80 have been identified (Arnoult et al., 2017; Liu et al., 2015c).

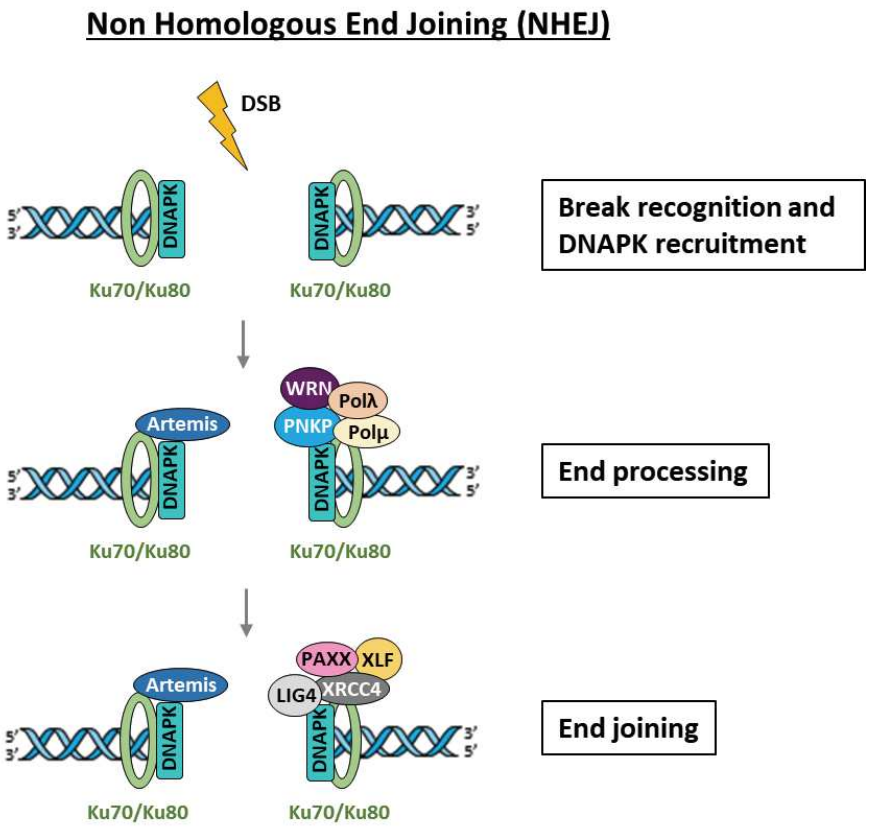


Figure 4: Non-homologous end –joining (NHEJ) pathway

DSB is recognized by Ku heterodimer that subsequently recruits DNAPKcs. DNA ends are then processed by different factors including Artemis, PNKP, WRN, Polλ and Polμ. Afterwards, XRCC4-LIG4-XLF/PAXX mediates the ligation of the two broken ends.

3.3 Alternative end-joining (Alt-EJ)

Alt-EJ, also called microhomology-mediated end-joining (MMEJ) is a NHEJ related rejoining mechanism that involves annealing at short sequence identities present near the DNA ends and that requires limited end resection (<100 bp) (Sfeir and Symington, 2015). It is a slower alternative pathway that is essentially activated in cells with impaired NHEJ (Mansour et al., 2013). Alt-EJ is activated during CSR and V(D)J recombination in NHEJ deficient cells and it increases the frequency of translocations when NHEJ is not functional (Yan et al., 2007; Zhu

et al., 2002). But alt-EJ was also reported to be activated when both HR and NHEJ are available (Truong et al., 2013), suggesting that this process might have a specific biological function during evolution, as well as being a mechanism used when classical NHEJ is not functional (Sfeir and Symington, 2015).

The recognition of DNA breaks requires PARP1 which competes with Ku for DNA binding (Audebert et al., 2004; Wang et al., 2006) (Figure 5). Alt-EJ involves short-range resection mediated by CtIP and MRE11 (Deriano et al., 2009; Ma et al., 2003; Rass et al., 2009; Truong et al., 2013; Xie et al., 2009). This resection can result in deletions making alt-EJ a highly mutagenic repair process (Mansour et al., 2010). RPA binding to resected ends prevents spontaneous annealing with micro-homologous sequences in yeast and mammalian cells (Ahrabi et al., 2016; Deng et al., 2014). Moreover, BRCA1 was shown not to affect alt-EJ in chicken B cells (Yun and Hiom, 2009), while BRCA1, BRCA2 and RAD51 work as a suppressor of this pathway in human cells (Ahrabi et al., 2016). Pol θ has been implicated in alt-EJ (Chan et al., 2010) and interacts with RAD51 to inhibit RAD51-mediated recombination, promoting alt-EJ at the expense of HR (Ceccaldi et al., 2015). The final ligation step is mediated by the XRCC1-Ligase III complex, although a role of Ligase I has also been described (Boboila et al., 2012; Dutta et al., 2017; Simsek et al., 2011). A recent study has reported that mouse B cells lacking RAD52 show increased CSR frequencies, in comparison to wild type cells, but fail to form CSR products with microhomology larger than 4 base pairs (Zan et al., 2017). Since the classical NHEJ is not associated with microhomology of >4 bp, this finding raises the interesting possibility that RAD52 contributes to alt-EJ during CSR and may compete with NHEJ in this context.

Alt-EJ is suggested to be at the origin of translocations in mouse cells. Indeed, depletion of CtIP and Ligase I and III decreased translocation frequency (Simsek et al., 2011; Zhang and Jasin, 2011), and several types of breast cancer and leukemias are characterized by microhomologies associated with alt-EJ (Stephens et al., 2009; Zhang and Rowley, 2006).

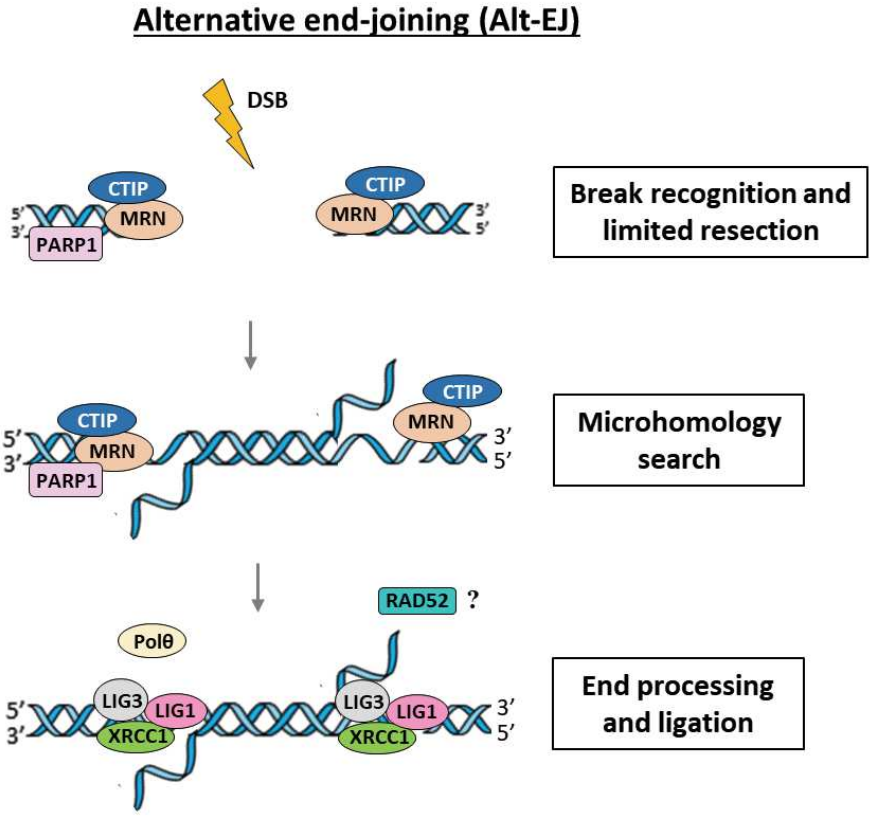


Figure 5: Alternative end-joining (Alt-EJ) pathway

Break recognition is mediated by PARP1 and the MRN complex. CtIP is recruited to initiate resection in parallel with MRE11. Resection is limited and leads to microhomology search. The ends are then ligated by XRCC1-LIG3 and LIG1. Polθ is involved in the filling of gaps before ligation.

3.4 Single Strand Annealing (SSA)

SSA is a mechanism that enables two homologous 3′-ssDNA ends, for example at tandem repeats, to be joined by annealing, at the cost of repeats deletion (Pâques and Haber, 1999). This mechanism does not need a donor sequence for the repair and hence does not involve strand invasion and RAD51 activity (Sung, 1997). SSA is therefore an error-prone pathway. SSA requires extensive DNA end resection by CtIP and RPA displacement to reveal complementary homologous sequences (Bhargava et al., 2016) (Figure 6). Annealing of the resected complementary ends is mediated by RAD52 (Rad52 in yeast) (Sugawara et al., 2003; Symington, 2002). After annealing, the non-complementary sequences are cleaved by XPF-ERCC1 (Rad1-Rad10 in yeast), and remaining gaps are filled and ligated to complete the repair of the DSB (Motycka et al., 2004; Shinohara et al., 1998).

The physiological role of SSA in cells is unclear, given that it is highly mutagenic. It might be used to repair breaks that have undergone extensive resection and they cannot be repaired by HR or alt-EJ, or maybe when the sister chromatid is not yet available for repair by HR.

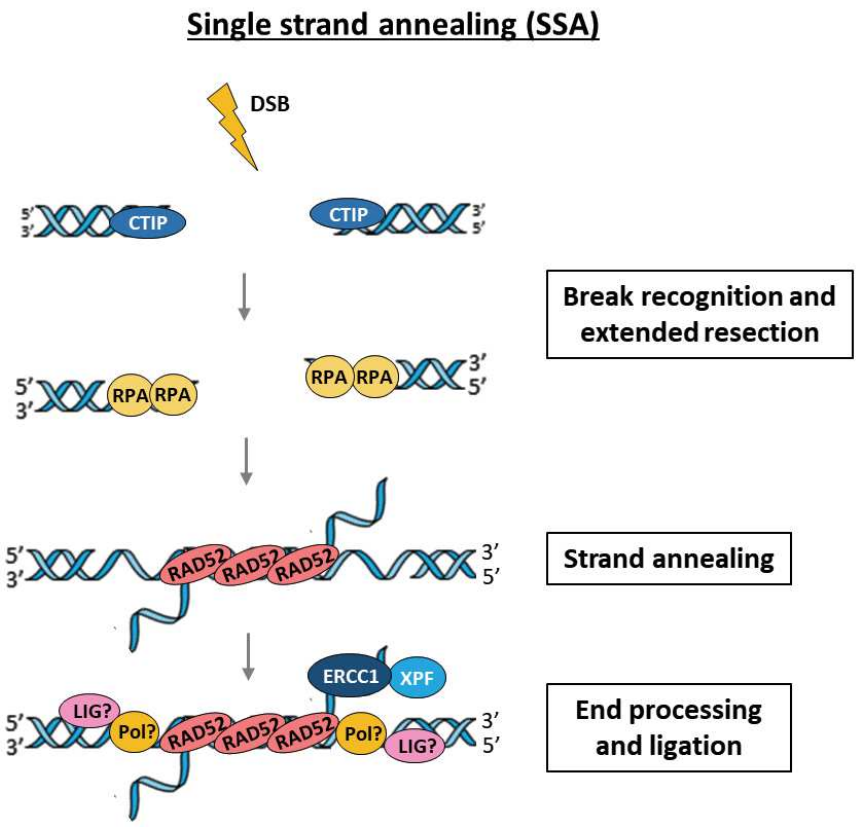


Figure 6: Single strand annealing (SSA) pathway

CtIP is involved in the first step of break recognition and resection. Resected ends are bound by RPA and the homologous ssDNA segments are annealed through RAD52. After annealing, the non-complementary sequences are cleaved by ERCC1-XPF complex and remaining gaps are filled and ligated.

3.5 Repair response to DSBs with one lost end: break-induced replication (BIR)

One-ended breaks can appear at the site of broken or collapsed replication forks, upon mis-segregation of fragmented chromosomes or because only one of the two ends of the DSB succeeds in strand invasion of a homologous sequence. In this case, there is no possibility of engaging error-free SDSA and there is no immediate partner for simple end-joining.

In this context, BIR process initiates the invasion of a single strand into a homologous DNA molecule followed by DNA synthesis that may continue until a disruptive event such as a

replication fork, or even to the end of the chromosome, frequently copying more than 100 kb from the donor chromosome (Mayle et al., 2015). This may lead to translocations and other chromosomal rearrangements. In *S. cerevisiae*, BIR is RAD51-dependent while in *S. pombe*, a Rad22-mediated form of BIR can occur at stalled forks (Nguyen et al., 2015). Emerging literature suggests that some BIR-like processes in mammalian cells are RAD51-independent. Indeed, RAD51-independent mitotic DNA synthesis (MiDAS), that occurs at common fragile sites (Bhowmick et al., 2016; Minocherhomji et al., 2015), is mediated by RAD52, DNA polymerase δ subunit 3 (POLD3) and the nuclease MUS81–EME1. In addition, RAD51-independent and Pol δ-mediated BIR can underly the alternative lengthening of telomeres (ALT) pathway (Dilley et al., 2016). Long tract gene conversions (LTGC) triggered at stalled mammalian replication forks can also be RAD51-independent (Willis et al., 2014).

3.6 Ubiquitination mediated signaling in DSB repair pathways

DSB response uses a multitude of PTMs to localize, modulate and ultimately clear DNA repair factors in a timely manner. Ubiquitination (Ub) and ubiquitin-like modifications (Ubl) such as SUMO and NEDD8 are well established as vital to the DSB response. Ubiquitin (Ub) is a highly conserved small protein, composed of 76 amino acids. Ubiquitination happens through a cascade of three steps: E1 (Ub-activating enzyme) activates Ub and transfers it to E2 (Ub-conjugating enzyme). Then E3 (Ub ligases) catalyze the ligation of the Ub with the substrate (Di Fiore et al., 2003; Pickart, 2001). Ubiquitin-mediated signaling is counterbalanced by the action of deubiquitinating enzymes (DUBs). Ubiquitin ligases and DUBs regulate turnover, activity and protein–protein interactions of DSB repair factors. A central function of Ub ligases/DUBs in DSB repair is to maintain the balance between NHEJ and HR pathways. Here is a non-exhaustive picture of Ub/DUB roles in DSB repair (Figure 7).

MRN: Pellino generates K63-Ub chains on NBS1 promoting ATM activation and HR repair (Ha et al., 2019). The DUB USP4 is recruited to DSBs through interaction with NBS1 where it regulates CtIP ubiquitination and recruitment at the break site (Liu et al., 2015a).

MDC1: Following recruitment to DSBs, MDC1 is SUMOylated by PIAS4 (Galanty et al., 2009, 2012; Luo et al., 2012; Yin et al., 2012), which promotes its recognition by the Ub ligase RNF4 (Sun et al., 2007a), which in turn promotes VCP/p97 dependent extraction of MDC1 from DSBs (Galanty et al., 2012; Garvin et al., 2019; Yin et al., 2012). The DUB USP7, on the other hand, interacts with and stabilizes the MDC1–MRN complex (Su et al., 2018).

Ku70/Ku80: Multiple Ub-E3 ligases ubiquitinate Ku70/Ku80 ultimately resulting in its eviction from the break by the VCP/p97 complex to terminate the repair (van den Boom et al., 2016; Kragelund et al., 2016). On the other hand, UCHL3 and OTUD5 de-ubiquitinate and stabilize Ku70/Ku80 at DSBs (de Vivo et al., 2019; Nishi et al., 2018).

RNF8: RNF8 is recruited to DSBs via its FHA domain that recognizes phosphorylated MDC1 (Kolas et al., 2007). It acts as a ubiquitin ligase and has a role both in HR and NHEJ. Indeed, it has been shown to ubiquitinate NBS1, which is required for effective HR repair (Lu et al., 2012). Its activity is also required to recruit BRCA1 (Hodge et al., 2016). Moreover, the removal of Ku protein is performed either by RNF8 or NEDD8 promoted ubiquitination of Ku (Brown et al., 2015; Postow et al., 2008). RNF8 also mono-ubiquitinates γ-H2AX to tether DNA repair factors at the break site (Yamamoto et al., 2017). The DUB USP3 and USP16 catalyze the disassembly of RNF8 and RNF168-generated ubiquitin chains (Joo et al., 2007; Nicassio et al., 2007).

RNF168: RNF168 is recruited at the break site in a RNF8 ubiquitination-dependent manner. RNF168 catalyzes the ubiquitination of histone H2A and H2AX (Mattiroli et al., 2012), which is important for the recruitment of 53BP1 (Fradet-Turcotte et al., 2013; Panier et al., 2012), the receptor-associated protein 80 (RAP80) and BRCA1 (Doil et al., 2009; Gatti et al., 2015; Stewart et al., 2009; Thorslund et al., 2015). RNF168 accumulation and Ub signaling at DSB is regulated by DUBs as A20 (Yang et al., 2018), USP14 (Sharma et al., 2018), UCHL3 (Zhang et al., 2017b), USP7 (Zhu et al., 2015) and USP34 (Sy et al., 2013).

53BP1: 53BP1 can directly recognize and bind RNF168-induced ubiquitinated H2A histones (Fradet-Turcotte et al., 2013). 53BP1 can bind H4K20me2 as well, which is also recognized by L3MBTL1 and JMJD2A (Acs et al., 2011; Mallette et al., 2012). To allow maximal 53BP1 recruitment, these factors need to be displaced from chromatin. L3MBTL1 is ubiquitinated by RNF8 and extracted by VCP/p97 from chromatin (Acs et al., 2011). This ubiquitination is antagonized by the DUB OTUB2 to limit excessive 53BP1 spreading (Kato et al., 2014; Nishi et al., 2014).

BRCA1: BRCA1 is recruited to ubiquitinated chromatin regions through its interaction with the BRCA1 A-complex (Huen et al., 2010). This complex contains RAP80, that is a reader of Ubc13 and RNF8-ubiquitinated histones after damage (Huen et al., 2010; Kolas et al., 2007; Wu et al., 2009) and contains Abraxas, BRCC36, BRE and MERIT40. BRCA1 recruitment at DSBs is also mediated by its major partner BARD1 through a number of different interacting

partners, including HP1γ (Wu et al., 2015). Ubiquitination of BARD1 disrupts interaction with HP1γ and therefore localization of BRCA1–BARD1 to DSBs. This inhibitory ubiquitination is removed by USP15 (Nishi et al., 2014; Peng et al., 2019). BRCA1 recruitment can be antagonized by USP1–UAF1. On the other hand, USP9X stabilizes BRCA1 (Lu et al., 2019b). BRCA1–BARD1 mediated mono-ubiquitination of H2A promotes displacement of 53BP1 and subsequent DNA end-resection. USP48 controls the extent of H2A ubiquitination to prevent hyper-resection (Uckelmann et al., 2018).

PALB2, BRCA2 and RAD51: The Cullin3 adapter KEAP1 prevents the formation of the complex BRCA1-PALB2-BRCA2 outside of S/G2 by ubiquitination of PALB2. This ubiquitination is countered by USP11 (Orthwein et al., 2015; Schoenfeld et al., 2004). USP11 turnover is regulated by KEAP1 in a cell cycle dependent manner, with USP11 protein levels at their lowest in G1. This prevents RAD51 loading from occurring outside of S/G2 phases. USP21 stabilizes the BRCA2–RAD51 complex by de-ubiquitinating BRCA2 (Liu et al., 2017). UCHL3 interacts with RAD51 and deubiquitinates residues that disrupt the RAD51–BRCA2 interaction (Luo et al., 2016). USP11 has also been shown to be recruited to DSBs by the NuRD complex in order to promote histone de-ubiquitination and deacetylation for proper chromatin remodeling and condensation post DSB induction (Ting et al., 2019).

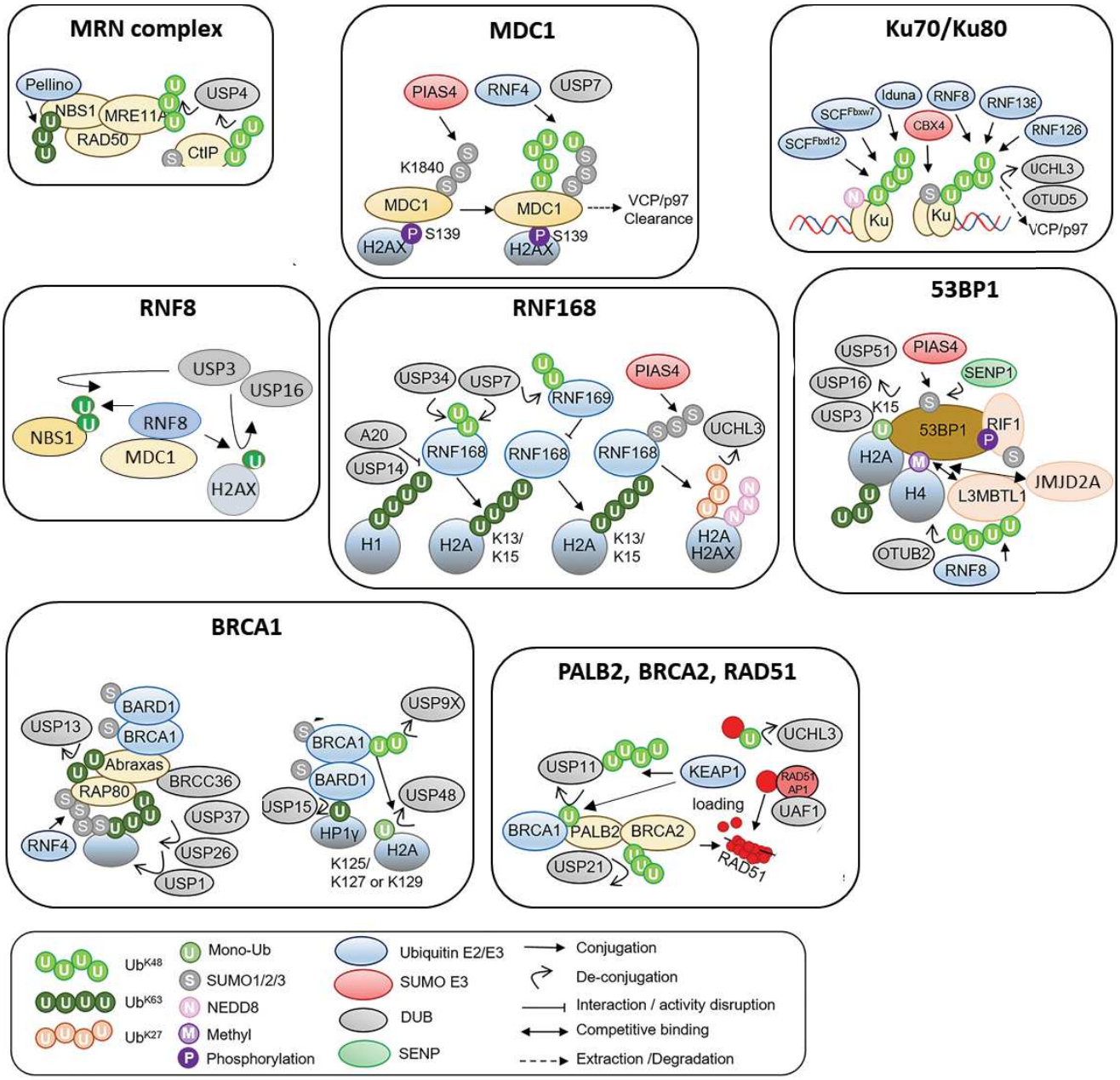


Figure 7: Ubiquitination mediated signaling in DSB repair pathways

Ubiquitin ligases and DUBs regulate turnover, activity and protein–protein interactions of DSB repair factors. Here is a non-exhaustive picture of Ub/DUB roles in the regulation of DSB repair factor. Detailed discussion in the main text. Adapted from (Garvin et al., 2019).

3.7 DSB repair pathways in the formation of translocations

Chromosomal translocations occur when broken DNA ends arising from DSBs on two heterologous chromosomes are improperly joined during the repair process. Translocations can lead to the activation of proto-oncogenes or the generation of novel fusion protein with oncogenic potential and are consequently associated with several tumor types. Indeed, they account for around 20% of cancer morbidity (Roukos and Misteli, 2014).

In mouse cells, initial studies on translocations formation focused on HR between repeats of different chromosomes. The induction of a DSB at a repeat on one chromosome did not lead to a translocation. The DSB was rather repaired by a simple gene conversion with the other chromosome without an exchange of material (Richardson et al., 1998). Subsequent studies also indicated that HR in mammalian cells is rarely associated with translocations (LaRocque et al., 2011; Richardson and Jasin, 2000a; Stark and Jasin, 2003). A DSB on one chromosome was sufficient to induce HR with another chromosome but it was not sufficient to drive translocation formation. However, the induction of two DSBs, one on each chromosome, led to the use of NHEJ and SSA and to the formation of reciprocal translocations (Elliott et al., 2005; Richardson and Jasin, 2000b; Weinstock et al., 2007). Indeed, SSA was proficient when the DSBs were induced close to identical sequences. But when DSB were induced in divergent sequences, SSA-mediated translocations dropped resulting in more NHEJ-mediated events. However, translocations were found to increase in the absence of LIG4 and Ku70 (Ferguson and Alt, 2001; Simsek and Jasin, 2010; Weinstock et al., 2007), suggesting that the NHEJ pathway has a protective role against translocations. Consistent with this, in the presence of microhomologies, translocations were found to occur with or without these canonical NHEJ components, and to be dependent on LIG3, CtIP and Pol θ, further pointing at a role for alt-EJ in the formation of translocations (Mateos-Gomez et al., 2015; Simsek and Jasin, 2010; Simsek et al., 2011; Zhang and Jasin, 2011).

While translocations induced in mouse cells arise by alt-EJ, translocations induced in human cells are characterized by little or no end processing at breakpoint junctions, suggesting the use of NHEJ (Brunet et al., 2009; Ghezraoui et al., 2014). Consistent with this, loss of LIG4 and XRCC4 reduced translocations. More recently, PARP3, involved in the recruitment of NHEJ factors, has also been shown to participate to translocation formation (Day et al., 2017). Moreover, loss of alt-EJ components LIG3 or CtIP was shown to not affect translocation frequency in human cells (Ghezraoui et al., 2014). On the other hand, PARP1 depletion has

been shown to reduce translocation in human cells, suggesting a role of alt-EJ in the formation of translocation (Audebert et al., 2004; Soni et al., 2014; Wray et al., 2013). Likewise, PARP1 overexpression has been found to increase translocations in some cell lines (Torres-Ruiz et al., 2017). In conclusion, c-NHEJ is directly implicated in translocation formation in human cells, although the contribution of alt-NHEJ cannot be excluded.

3.8 Regulation of DSB repair pathway choice

One question arising from the plurality of DSB repair processes concerns the way the most appropriate repair pathway is selected. NHEJ and HR, the most conservative pathways, are preferentially chosen, whereas SSA and alt-EJ, highly mutagenic pathways, are suggested to be used when the two main pathways are impaired or aborted. More precisely, selection of the adapted pathway is regulated by the cellular context, including the type and distribution of DNA lesions, local chromatin environment, the cell cycle phase, and the competition between specific pathway-related factors.

3.8.1 Role of the broken DNA end structure

The DNA-end structure is an important factor to determine the initial DSB repair pathway choice (Krenning et al., 2019). If the broken ends are blunt, Ku70/Ku80 heterodimer can bind and protect the DNA-end structure and facilitate NHEJ (Mimori and Hardin, 1986). However, if the broken ends contain ssDNA tails or single-stranded gaps close to DNA ends, it can block the binding of Ku70/Ku80 and directly lead to RPA loading and PARP activation, which can result in resection and the involvement of one of the resection-dependent repair pathways (HR, SSA or alt-EJ). Similarly, a DNA end that is chemically blocked may require processing. Finally, when a break is one-ended, the pathway used for its repair will necessarily be error-prone.

3.8.2 Role of end resection

DNA end resection plays a critical and essential role in determining the DSB repair pathway, because it is a key commitment step in HR, SSA and alt-EJ. The extent of resection needed for each pathway significantly differs; Alt-EJ requires end processing of a relatively small number of base pairs, whereas HR and SSA need extensive resection (Ceccaldi et al., 2016). There are many factors that can affect the commitment of resection in a cell-cycle dependent manner, which partly explains how HR is restricted to the S and G2 phases of the cell cycle.

3.8.2.1 Role of break recognition in regulating end resection

The first step of regulation starts from the break recognition either by Ku, which will favor NHEJ pathway, or by MRN that contains MRE11 which has a nuclease activity and can, together with CtIP, initiate end-resection. In yeast, depletion of Mre11 and Sae2/CtIP leads to an increased binding of Ku to DSBs (Zhang et al., 2007). Moreover, depletion of NHEJ factors leads to an increased Mre11 recruitment and subsequent end resection (Clerici et al., 2008; Zhang et al., 2007; Zierhut and Diffley, 2008). In line with this, Ku-deficient cells can start resection in G1 (Clerici et al., 2008; Zierhut and Diffley, 2008). Interestingly, Ku70 is also downregulated during meiosis where HR is the pathway used to establish genetic variability (Goedecke et al., 1999).

3.8.2.2 Role of BRCA1-53BP1 competition in regulating end resection

It has been recognized that an antagonistic relationship between 53BP1 and BRCA1 determines pathway choice (Bouwman et al., 2010; Bunting et al., 2010) (Figure 8). 53BP1 recognizes break induced RNF8- and RNF168 -dependent H2AK15-Ub with its UDR motif (Fradet-Turcotte et al., 2013), while its tandem Tudor domain binds H4K20me2 (Botuyan et al., 2006; Huyen et al., 2004; Sanders et al., 2004). 53BP1 is considered to promote NHEJ, and 53BP1 deficiency slightly reduces and delays NHEJ (Noon et al., 2010), but this effect is minor compared with the loss of core NHEJ factors (Xu et al.). Furthermore, 53BP1 is not required for NHEJ of most RAG-induced DNA breaks during V(D)J recombination (Ward et al., 2004), suggesting that 53BP1 is not a core NHEJ factor. 53BP1 forms foci around the break, thereby promoting the chromatin compaction, blocking the DNA nucleases access to the DNA ends (Bártová et al., 2019), and limiting the length of resection (Bunting et al., 2010; Chapman et al., 2012b; Xie et al., 2007; Zimmermann and de Lange, 2014). 53BP1 interacting factors RIF1 and PTIP also limit resection (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Feng et al., 2013; Gong et al., 2017b; Jowsey et al., 2004; Munoz et al., 2007; Silverman et al., 2004; Yan et al., 2011). Super-resolution imaging has demonstrated that 53BP1 focal enrichment is the most important in G1 phase cells and a progressive BRCA1-dependent exclusion of 53BP1 from damage sites occurs as cells transition through S phase (Chapman et al., 2012b). RPA foci are formed following 53BP1 repositioning (Kakarougkas et al., 2013). This suggests that 53BP1 limits the extent of resection and that the major role of BRCA1 is to overcome the barrier against resection established by 53BP1 (Shibata, 2017).

Indeed, BRCA1 promotes the dephosphorylation of 53BP1 through the PP4C phosphatase in G2, leading to RIF1 release and thus allowing resection (Isono et al., 2017). An additional regulatory mechanism that facilitates resection in S phase is the ubiquitination of RIF1 by the UHRF1 E3 ubiquitin ligase, which is specifically recruited to breaks through BRCA1 and induces its dissociation from 53BP1 (Zhang et al., 2016a). BRCA1/BARD1 recruitment depends on the recognition of H4K20me0 by BARD1 (Nakamura et al., 2019). The balance between BRCA1 and 53BP1 recruitment is affected by TIP60-mediated acetylation of residues close to H4K20, which can disrupt 53BP1 binding to H4K20me1 or H4K20me2 marks (Jacquet et al., 2016; Tang et al., 2013). Moreover, BRCA1–BARD1 ubiquitin ligase activity can ubiquitinate histone H2AK27, recruiting the chromatin remodeler SMARCAD1 and facilitating 53BP1 repositioning to promote resection (Costelloe et al., 2012; Densham et al., 2016). 53BP1 is also subject to direct regulation by TIRR, a protein that blocks the H4K20me-binding domain of 53BP1 (Botuyan et al., 2018; Dai et al., 2018; Drané et al., 2017; Zhang et al., 2017a).

Recent studies identified a 53BP1 effector complex, Shieldin, containing C20orf196 (SHLD1), FAM35A (SHLD2), CTC534A2.2 (SHLD3) and REV7. Shieldin complex is recruited at DSB sites in a 53BP1- and RIF1-dependent manner, and its SHLD2 subunit binds to ssDNA via domains analogous to those of RPA and antagonizes BRCA1 (Dev et al., 2018; Ghezraoui et al., 2018; Gupta et al., 2018; Mirman et al., 2018; Noordermeer et al., 2018). Interestingly, Shieldin has been shown to promote fill-in synthesis on ssDNA, potentially helping to blunt ssDNA tails (Mirman et al., 2018). Consistently, cells depleted of BRCA1 are characterized by impaired resection that leads to the inappropriate activation of NHEJ in S phase and to chromosomal rearrangements (Bunting et al., 2010). These rearrangements as well as the embryonic lethality of BRCA1-/- mice are rescued by depletion of 53BP1 (Bouwman et al., 2010; Bunting et al., 2010, 2012; Cao et al., 2009).

3.8.2.3 Role of cell cycle in regulating end resection

Cell cycle-dependent kinase (CDK) activity, which increases as cells enter S phase, provides activating signals to the resection machinery, through the phosphorylation of several substrates (Aylon et al., 2004; Caspari et al., 2002; Huertas et al., 2008; Ira et al., 2004; Tomimatsu et al., 2014) (Figure 8). CDK-mediated phosphorylation of CtIP is essential for efficient activation of the MRE11 nuclease and for its interaction with BRCA1 (Anand et al., 2016; Chen et al., 2008; Greenberg et al., 2006; Huertas et al., 2008). Moreover, CDK-dependent phosphorylation of NBS1 in S, G2 and M phases of the cell cycle seems to be necessary to promote HR (Falck et

al., 2012). In contrast to this study, it was recently reported that the same phosphorylation site of NBS1 is necessary for NHEJ activation at deprotected telomeres (Rai et al., 2017). CDK-dependent phosphorylation of EXO1 also promotes its activation and further resection (Tomimatsu et al., 2014).

In addition to CDK-mediated phosphorylation, several mechanisms are involved in cell cycle-dependent regulation of resection. In G1, DNA helicase B (HELB) is recruited to ssDNA by interacting with RPA and uses its 5′-3′ ssDNA translocase activity to inhibit EXO1 and BLM-DNA2, and therefore to inhibit resection, and is exported from the nucleus as cells approach S phase (Tkáč et al., 2016). Also, as mentioned before, in G1 PALB2 is ubiquitinated by the E3 ubiquitin ligase cullin-3 (CUL3)–RBX1 and the adaptor protein Kelch-like ECH-associated protein 1 (KEAP1), leading to its proteasome-mediated degradation, which prevents the assembly of the BRCA1–PALB2–BRCA2–RAD51 recombinase complex (Orthwein et al., 2015). In G2, on the other hand, Ubiquitin peptidase 11 (USP11) deubiquitinates PALB2, promoting the formation of stable BRCA1-PALB2-BRCA2 complexes, and thus the repair by HR, while in G1, USP11 is degraded upon IR-induced damages (Orthwein et al., 2015). Another study shows, however, that resection is essential for repair of complex DSBs in all phases of the cell-cycle (Averbeck et al., 2014).

As discussed above, 53BP1 together with Shieldin, limit end-resection and BRCA1 recruitment at DSB in G1, while BRCA1 recruitment leads to the exclusion of 53BP1 from DNA damage sites as cells transition through S phase. In S phase, the presence of 53BP1 at DSBs is down-regulated by the dilution of the H4K20me2 marks in newly replicated chromatin (Nakamura et al., 2019; Pellegrino et al., 2017; Saredi et al., 2016). In addition, BRCA1 changes the nature of 53BP1 foci in S phase, converting them from homogeneous domains into hollower spheres (Chapman et al., 2012b; Escribano-Díaz et al., 2013). In G2, 53BP1 foci appear again (Simonetta et al., 2018). NHEJ can be inhibited in S/G2 by CYREN if DSBs contain 5′ or 3′ overhangs (Arnoult et al., 2017). In contrast, CYREN/MRI stimulates NHEJ in G1 (Hung et al., 2018). Furthermore, cell-cycle dependent phosphorylation of BACH1, a cofactor of BRCA1, allows their binding only in S/G2 phase in order to promote resection (Dohrn et al., 2012). Cells enter in mitosis even in the presence of DNA damage (Rieder and Cole, 1998), but DSB signaling is attenuated in mitosis. Although MRN is recruited to DSBs and ATM is activated, the chromatin response is restricted to γ-H2AX modification and MDC1 recruitment, without activation of RNF8 and RNF168 or accumulation of BRCA1 or 53BP1 on chromatin (Giunta et al., 2010).

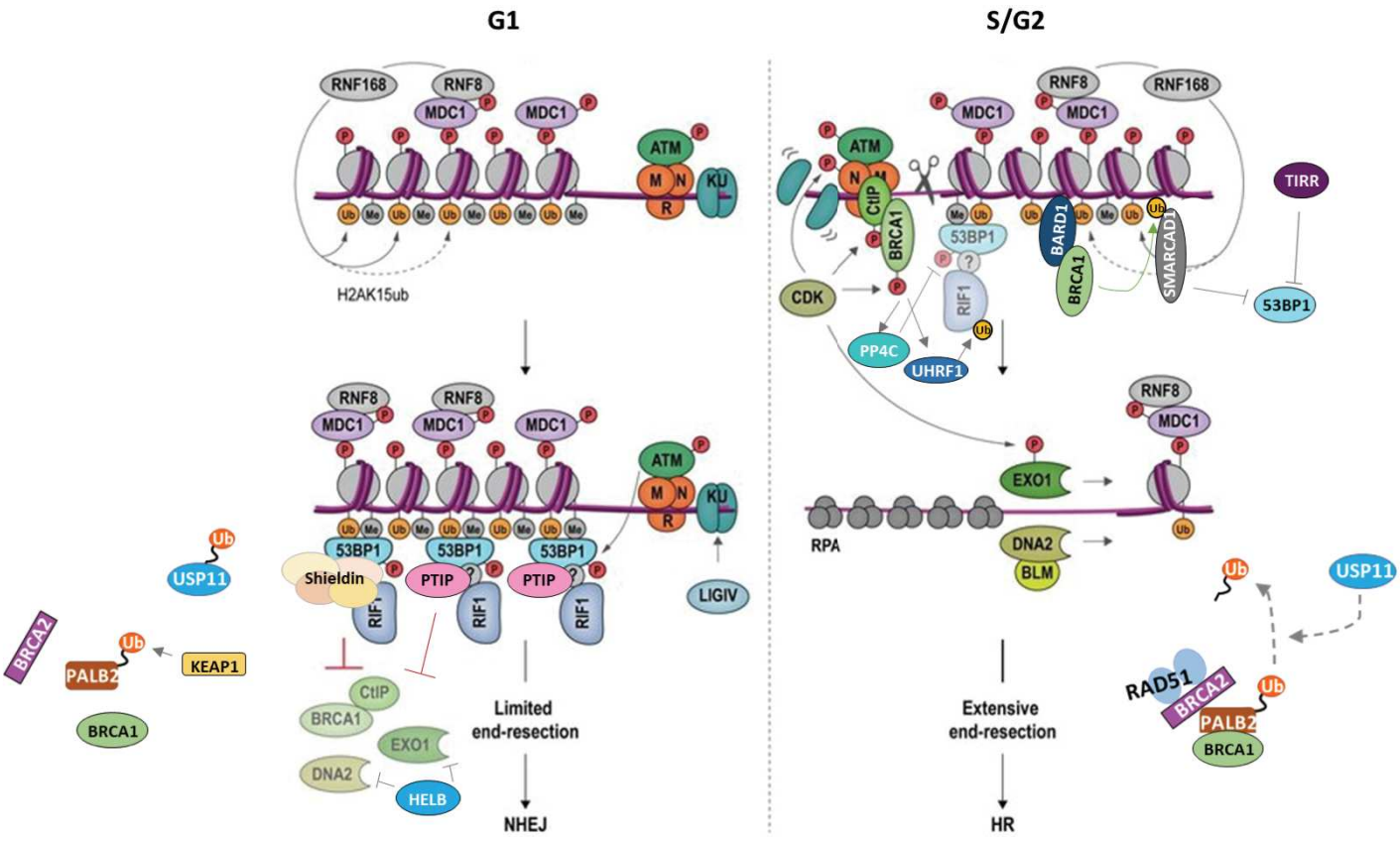


Figure 8: Role of BRCA1-53BP1 competition and cell cycle in regulating end resection

An antagonism between 53BP1 and BRCA1 regulates DSB repair pathway choice in mammalian cells in a cell cycle dependent manner. MRN binds DSBs and recruits ATM, which phosphorylates H2AX (γH2AX). γH2AX attracts MDC1, that recruits RNF8, which cooperates with RNF168 to catalyze protein ubiquitylation at DSBs. In G1, H2AK15ub, together with H4K20me2, mediates binding of 53BP1 at DSBs. In its ATM-phosphorylated form, 53BP1 interacts with RIF1 and PTIP that will limit resection and inhibits BRCA1-CtIP, EXO1 and DNA2. Shieldin complex, recruited in a 53BP1- and RIF1-dependent manner, antagonizes BRCA1. HELB inhibits EXO1 and BLM-DNA2. Moreover, KEAP1 ubiquitinates PALB2, which prevents the assembly of the BRCA1–PALB2–BRCA2–RAD51 recombinase complex. Limited end resection results in NHEJ repair. In S and G2 phases, CDK activity increases and H4K20me2 mark is diluted because of new histone deposition during DNA replication. CDK stimulates the endonucleolytic activity of the MRN complex, and the recruitment of BRCA1-CtIP to damaged chromatin. BRCA1 is also recruited through BARD1 recognition of H4K20me0. BRCA1 promotes the dephosphorylation of 53BP1 through the PP4C phosphatase, leading to RIF1 release. Besides, BRCA1 recruits UHRF1 that ubiquitinates RIF1 and induces its dissociation from 53BP1. Moreover, BRCA1–BARD1 ubiquitin ligase activity can ubiquitinate histone H2AK27, recruiting the chromatin remodeler SMARCAD1 and facilitating 53BP1 repositioning. 53BP1 is also directly regulated by TIRR. CDK-phosphorylated EXO1 and DNA2-BLM promote long-range resection, generating 3'-ssDNA overhangs. Finally, USP11 deubiquitinates PALB2, promoting the formation of stable BRCA1-PALB2-BRCA2 complexes. Extensive end resection results in HR repair. Adapted from (Fontana et al., 2018).

3.8.3 Role of RAD51 recruitment in the choice of homology-based repair pathway

Once resection has occurred, three pathways can be used: HR, SSA and alt-EJ. RPA binding suppresses alt-EJ (Deng et al., 2014), but it also needs to be removed by BRCA2 (and RAD52 in yeast) to allow RAD51 recruitment (Carreira and Kowalczykowski, 2011; Esashi et al., 2007; Moynahan et al., 2001; Sugawara et al., 2003; Symington, 2002). BRCA2 is the main mediator of RAD51 nucleofilament formation and strand exchange in mammalian cells (Ayoub et al., 2009; Carreira and Kowalczykowski, 2011; Esashi et al., 2007). Conversely, it has been shown that RAD51 nucleofilaments can be removed by the helicase PARI (and Srs2 in yeast) (Chiolo et al., 2005; Krejci et al., 2003; Moldovan et al., 2012). Srs2 depletion has been shown to reduce SSA and alt-EJ occurrence while increasing HR (Chiolo et al., 2005; Krejci et al., 2003). RAD51 filaments can also be disrupted by the helicases RECQL5, FANCJ and FBH1 (Islam et al., 2012; Simandlova et al., 2013; Sommers et al., 2009), favouring SSA and alt-EJ pathways. Additionally, Pol θ was shown to block RAD51 loading and thus favor alt-EJ (Mateos-Gomez et al., 2015). A competition between RAD51 and RAD52 recruitment also play a role in the choice of the repair pathway, RAD52 favoring SSA. Depletion of RAD51 has been shown to upregulate RAD52-mediated SSA activity (Bennardo et al., 2008), while depletion of RAD52 was shown to lead to a relocation of heterochromatic DSB at the periphery of heterochromatin core domain, where RAD51 is recruited (Tsouroula et al., 2016). Conversely, hyper-resected DSBs are increasingly unable to load RAD51, promoting RAD52 recruitment (Ochs et al., 2016).

3.8.4 Role of the chromatin state and nuclear position of the break

See part 4. and our review “How to maintain the genome in nuclear space” for a detailed discussion of this topic.

4. DSB repair in the context of chromatin and compartmentalized nucleus

DNA repair processes occur in the context of a complex chromatin environment, and this may have a broad influence on repair pathway choice. Indeed, chromatin is a highly dynamic and active participant in the repair process (Dabin et al., 2016). Changes in the chromatin originating from histone PTMs, histone variants composition and chromatin remodelers ensure accessibility to the damaged region and regulate the association and activity of repair factors, contributing to DSB repair pathway choice and coordination.

4.1 3D-organization of the chromatin

4.1.1 Different levels of compaction

Within the cell, DNA is folded into structures of increased complexity, compaction, and size (Hübner et al., 2013) (Figure 9). DNA is first wrapped around octamers of proteins called histones (H2A, H2B, H2 and H4), forming the nucleosomes (Fraser and Bickmore, 2007; Luger et al., 1997). The succession of nucleosomes and internucleosomal DNA, bound by the linker histone H1, forms a 10 nm fiber that appears as a beads-on-a-string structure on electron microscopy images and provides the first level of DNA compaction (Olins and Olins, 1974). This structure is further folded in a 30 nm fiber (Luger et al., 2012). This further compaction is due to the interaction of *cis* regulatory elements, leading to the formation of chromatin loops (Lieberman-Aiden et al., 2009; Rao et al., 2014). The highest level of compaction is reached in mitotic chromosomes.

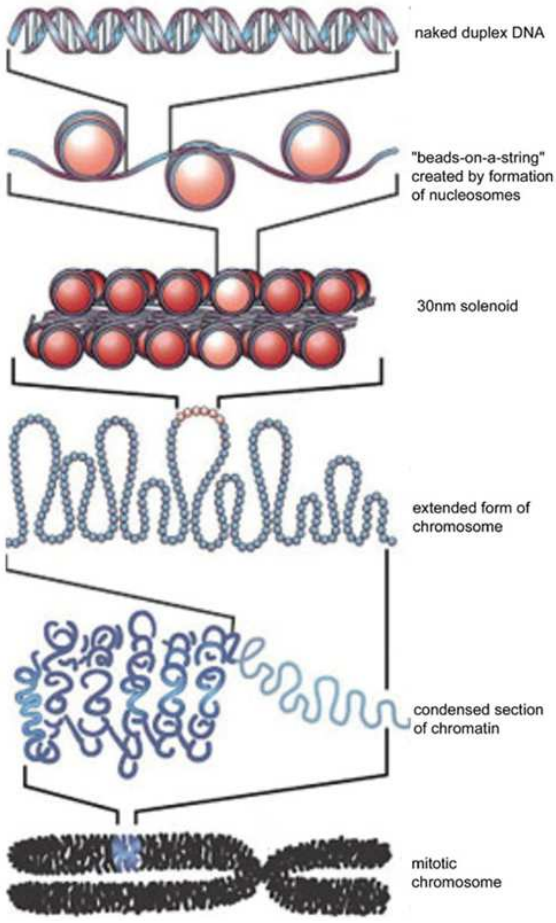


Figure 9: Different levels of chromatin compaction

The 10 nm fiber or 'beads on a string' represents the first level of eukaryotic DNA compaction, which is further compacted into the 30 nm fiber. The 30 nm fiber gets organized in loops that are further compacted. The highest level of compaction is reached in mitotic chromosomes. From: https://beyondthedish.wordpress.com/2012/05/25/stem-cell-differentiation-requires-proper-compaction-of-dna/

4.1.2 Chromatin remodelers

Alterations in chromatin structure are generated by distinct class of remodeling enzymes, specified as chromatin remodelers (Strahl and Allis, 2000). They can have a role in histone variant deposition and chromatin accessibility by sliding, evicting or depositing nucleosomes to facilitate DNA access (Clapier and Cairns, 2009). They are therefore involved in many processes such as transcription, replication, and DNA repair. These drivers have been categorized as readers, writers and erasers, and can either mediate histone PTMs (see below and in part 4.2. for their role in DSBs repair), or alter histone-DNA interaction through ATP hydrolysis (Clapier and Cairns, 2009). ATP dependent chromatin remodelers have been grouped into four families, on a structural basis: SWI/SNF (switch/sucrose-non-fermenting), ISWI (imitation switch), CHD (chromodomain-helicase-DNA binding) and INO80 (inositol

requiring 80). In addition to the SNF2-family ATPase domain, present in all remodeler families, SWI/SNF remodelers contain bromodomains, ISWI contain SANT-SLIDE modules, CHD contain tandem chromodomains and INO80 possess HAS (helicase SANT) domains. These domains define their target specificity. The role of this chromatin remodelers in DSB repair will be discussed in part 4.4.

4.1.3 Histone post-translational modifications (PTMs)

Among the different histone tails PTMs that exist, acetylation, phosphorylation and methylation are the most extensively studied (Lawrence et al., 2016). Acetylation of lysine residues is catalyzed by histone acetyltransferases (HATs) and aims to neutralize the positive charge of lysines, destabilizing the interaction between histones and DNA. This modification is therefore associated to an open and transcriptionally active chromatin state (Shahbazian and Grunstein, 2007). Consistently, deacetylation by histone deacetylases (HDACs) are often involved in transcriptional repression. Phosphorylation of serines, threonines and tyrosines by kinases add a negative charge that can destabilize the interaction between histones and DNA but also regulate recognition by histone binding partners (Rossetto et al., 2012). Phosphorylation is removed by phosphatases. Methylation of lysines and arginines by histone methyltransferases (HMTs) is essential in chromatin compaction and transcription regulation (Greer and Shi, 2012). Methylation is removed by histone demethylases. Different PTMs can be found on the same histone tail and work in concert to mediate specific processes. The role of histone PTMs in DSB repair will be discussed in part 4.2.

4.1.4 Histone variants composition

Histone variants can originate from an alternative splicing of the gene coding for the canonical histone, or from paralogue genes and present a different expression and distribution pattern (Buschbeck and Hake, 2017; Talbert and Henikoff, 2017). For example, CENP-A, an H3 histone variant, is uniquely localized at centromeres. Only H4 shows no known variant. Histones are normally incorporated in S phase during replication, while variants can be deposited throughout the cell cycle. The histone chaperones involved in histones and histone variants deposition can also be different. For instance, the chaperone HJURP is specific to the histone variant CENP-A. The different variants have different interacting partners, and they can carry specific PTMs. Therefore, they directly participate in the regulation of chromatin dynamics and functions. For example, the phosphorylation of the histone variant H2AX is key

for a proper DNA damage signaling. The role of histone variants in DSB repair will be discussed in part 4.3.

4.1.5 Euchromatin and heterochromatin

The concerted action of previously cited elements (chromatin remodelers, PTMs, histone variants) has created two structurally and functionally distinguishable territories: euchromatin and heterochromatin (Figure 10). The less condensed part of the chromatin corresponds to the euchromatin and is mainly associated with active transcription and distributed in the nuclear interior. Conversely, heterochromatin contains highly compacted regions of chromatin, is considered transcriptionally inactive and enriched at the nuclear periphery, around the nucleolus and at centromeres (Fraser and Bickmore, 2007). Euchromatin is generally enriched in acetylated histones H3 and H4, H3K4me and H3K36me (Grunstein, 1998; Litt et al., 2001; Noma K et al., 2001). Typically, H3K4me1 is enriched at active transcriptional enhancers, H3K4me3 marks the transcriptional start site (TSS) of active genes and H3K36me3 is highly enriched throughout the whole transcribed regions (Barski et al., 2007; Hon et al., 2009). On the other hand, heterochromatin is characterized by hypoacetylation of histones, and is divided into facultative and constitutive heterochromatin. Facultative heterochromatin is more plastic and can be subjected to transitions between open and compact states, for example with changing gene expression as cells transition through the differentiation process, and is marked by H3K27me3 (Trojer and Reinberg, 2007). Constitutive heterochromatin is always compact and is enriched in repetitive, gene-poor and late replicating DNA sequences such as in telomeres and centromeres (Fraser and Bickmore, 2007). It is characterized by H3K9me3 (Nakayama et al., 2001; Rea et al., 2000; Schotta et al., 2002; Tschiersch et al., 1994), and with the association of heterochromatin protein-1 (HP1) through H3K9me3 recognition (Bannister et al., 2001; Lachner and Jenuwein, 2002; Lachner et al., 2001). Thus, different chromatin structures are decorated by specific histone marks, different histone variants and are bounded by specific factors, which play a role in many functions, such as transcription regulation, replication and in DSBs repair.

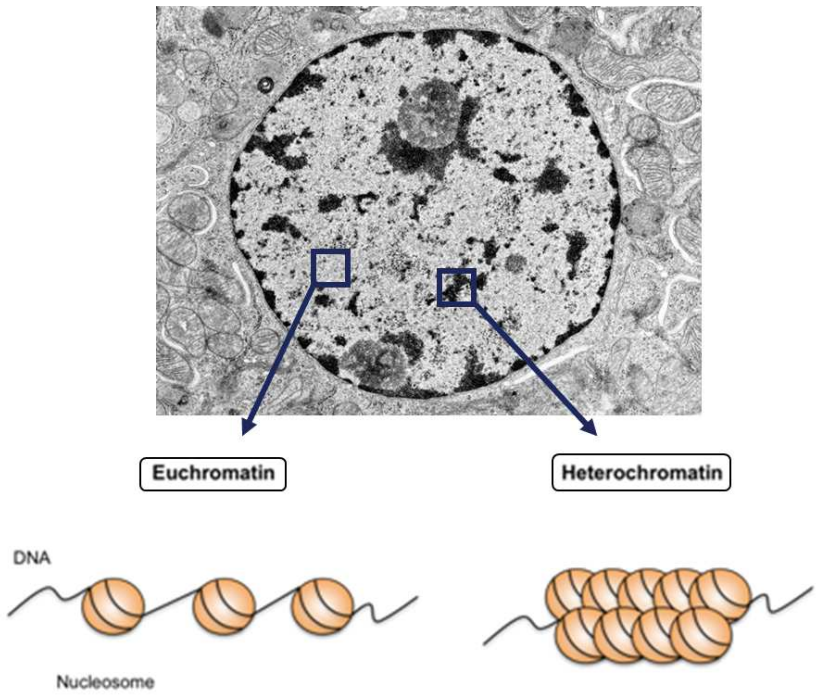


Figure 10: Euchromatin and Heterochromatin

The highly condensed nature of heterochromatin compared to euchromatin allows for their visual distinction using transmission electron microscopy (TEM) as shown in this TEM picture of a human nucleus (TEM picture adapted from Yale University: http://medcell.med.yale.edu/histology/cell_lab/euchromatin_and_heterochromatin.php Cartoons of chromatin adapted from MOJ Cell Science & Report: https://medcrave.org/index.php/MOJCSR/article/view/14976/28032

4.1.6 Nuclear compartments

The next level of chromatin organization consists of a compartmentalization of nucleus into distinct substructures (Figure 11). These compartments are not delimited by membranes but are rather defined by a specific subset of proteins associated with a specific function. The best-studied nuclear compartments are: the nuclear lamina, which maintains nuclear shape and anchors chromatin to the nuclear envelope (van Steensel and Belmont, 2017); the nuclear pore complexes, which regulate the transport of molecules between the nucleus and the cytoplasm (Beck and Hurt, 2017); the nucleolus, the site of rRNA synthesis and ribosomal assembly (Iarovaia et al., 2019); various nuclear bodies that play specific roles in nuclear processes, such as mRNA splicing and processing (Cajal bodies, Cleavage bodies, Nuclear speckles), protein degradation (Clastosomes), the heat shock response (Nuclear Stress bodies) and transcription (OPT domains, PML bodies) (Staněk and Fox, 2017); and, finally, the centromeric and

pericentric heterochromatin (Janssen et al., 2018). My work focuses on the study of DSBs repair in the centromeric sub-compartment of the nucleus. Pericentromeres correspond to genomic regions directly adjacent to centromeres and are characterized by repetitive sequences called major satellite repeats in mouse cells and satellite I, II and III in human cells. These regions contain H3K9me3, catalyzed by Suv3-9, H4K20me2/3, catalyzed by Suv4-20, the co-repressor KAP1, interacting with SETDB1 (histone methyltransferase), HDAC1 and HDAC2 (histone deacetylases) and CHD3/Mi-2a (CHD nucleosome remodeling factor) (Maison and Almouzni, 2004). Centromeres, also characterized by repetitive sequences, in this case termed minor satellite repeats in mouse cells and alpha satellites in human cells, present unique features to the rest of the genome, particularly the specific H3 histone variant CENP-A, high histone acetylation in combination with H3K4me2 and H3K36me2 histone modifications, that are more normally associated with active regions (McKinley and Cheeseman, 2016; Saksouk et al., 2015).

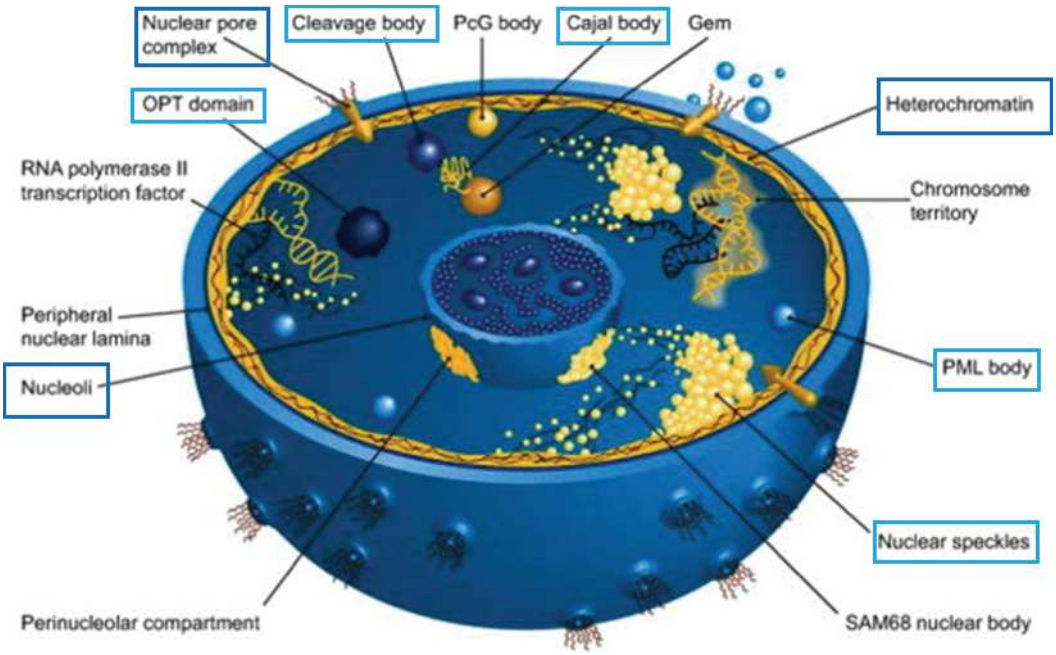


Figure 11: Nuclear compartments

The nucleus is organized into different substructures that are not delimited by membranes but are characterized by a specific set of proteins determining their unique biological function. These substructures are depicted in this picture. Adapted from (Frege and Uversky, 2015).

4.2 Histone PTMs in DSB repair

Histone modifications play roles in sensing, processing, and repairing damaged DNA. Histone PTMs can be dynamically regulated by DNA damage, as it is with the example of γ-H2AX. Thus, histone PTMs provide binding platforms for DDR factors to interact with chromatin at DNA damage sites. I will here summarize the involvement of histone PTMs, including phosphorylation, acetylation, methylation, and ubiquitination in DSB repair (Figure 12).

4.2.1 Phosphorylation

The first histone phosphorylation to be described in response to DSBs was H2AX phosphorylation on S139 to form γ-H2AX. As described previously, this phosphorylation can be induced by different DDR kinases including ATM, ATR and DNAPK. γ-H2AX covers sometimes more than 1 Mb of chromatin surrounding a DSB (Rogakou et al., 1998, 1999) and can be observed as a foci in response to DSB induction. As discussed previously, it promotes the recruitment of several DDR factors including MDC1, 53BP1, BRCA1 and the MRN complex. Mice deficient for H2AX, or incapable of S139 phosphorylation, are sensitive to DNA damage and display genome instability (Bassing et al., 2002; Celeste et al., 2002). To dismantle DDR complexes following repair, PP2A phosphatase dephosphorylates γ-H2AX. PP2A deficiency leads to an increase in γ-H2AX levels and defective repair. In addition, PP4, PP6 and WIP1 phosphatases also dephosphorylate γ-H2AX (Douglas et al., 2010; Macůrek et al., 2010; Moon et al., 2010; Nakada et al., 2008). Another site on H2AX, Y141, is phosphorylated by the kinase WSTF and is dephosphorylated by EYA1 (Cook et al., 2009; Xiao et al., 2009). This seems to act as a switch to apoptosis (Cook et al., 2009). H2AX phosphorylation on T101 has also been observed to occur in response to DNA damage and seems to play a role in DDR (Xie et al., 2010).

Apart from H2AX, some damage-associated phosphorylations have been identified on histone H2B (Fernandez-Capetillo et al., 2004; Lee et al., 2014): T129 in yeast and S14, but little is known about their involvement in DSB repair. During mitosis, Aurora-B kinase mediates the phosphorylation of H3S10, involved in chromatin compaction. Upon DNA damage, PARP1 inhibit the activity of Aurora-B leading to a reduction in H3S10p levels (Monaco et al., 2005; Sharma et al., 2015). Similarly, H3T11 is phosphorylated by CHK1 kinase but upon DNA damage, the level of this histone mark is reduced (Shimada et al., 2008), correlating with transcriptional repression of genes involved in the cell cycle including cyclin B1 and CDK1. Moreover, H4S1 and H4Y51 are phosphorylated upon DSB induction, and both of these marks

have been proposed to be involved in NHEJ (Cheung et al., 2005; Clouaire et al., 2018; Hossain et al., 2016).

4.2.2 Acetylation

H2AK15Ac induced by TIP60 upon DSB can compete with H2AK15Ub, which is necessary for 53BP1 binding, thus negatively affecting NHEJ (Jacquet et al., 2016). TIP60 can also acetylate H2AX on K5 upon damage (Ikura et al., 2007) and H2AX K5 hyperacetylation upon the depletion of the eraser SIRT1 results in defective DNA damage signaling (Yamagata and Kitabayashi, 2009). H2AX is also acetylated on K36 by p300 and CBP, which seems to be involved in resistance to irradiation (Jiang et al., 2010; Xie et al., 2010). H2B acetylation on K120 was also shown to be increased at DSB sites (Clouaire et al., 2018).

Histone H3 contains several acetylation sites that impact DSB repair, including H3K9, H3K14 and H3K18 and H3K23, catalyzed by GCN5 (Lee et al., 2010a). H3K14Ac and H3K18Ac recruit the SWI/SNF chromatin remodeling complex to damaged chromatin, which is required for the recruitment of Ku and therefore for efficient NHEJ (Ogiwara et al., 2011). Deacetylation of H4K16Ac and H3K56Ac by HDAC1 and HDAC2 promote NHEJ (Miller et al., 2010). H4K16Ac and H3K56Ac are also deacetylated by SIRT1 and SIRT6 respectively at DSB sites (McCord et al., 2009; O'Hagan et al., 2008; Toiber et al., 2013). A lack of SIRT6 causes defects in the recruitment of many DDR factors including BRCA1, 53BP1 and DNAPK (McCord et al., 2009; Toiber et al., 2013), suggesting that these chromatin marks and their regulators act early on in the DDR. HDAC1 also deacetylates linker histone H1 following DNA damage (Li et al., 2018a).

Several acetylation sites on histone H4 are also involved in the DDR. Indeed at DSB sites, H4 is hyperacetylated at K5, K8, K12, K16 either by TIP60 or CBP and p300 (Murr et al., 2006; Ogiwara et al., 2011). All three of these HATs were involved in promoting efficient NHEJ. TIP60 complex is also required for efficient HR as well. H4K16Ac, that is induced by the MOF acetyltransferase or TIP60, and was shown to increase at site of damage, plays an important role in modulating the recruitment of 53BP1 and BRCA1. H4K16Ac can be deacetylated by HDAC1 and HDAC2 (Miller et al., 2010). Loss of HDAC1 and HDAC2 rendered cells deficient for NHEJ.

4.2.3 Methylation

Methylations, in particular on lysine (K) residues, play important roles in transcription and in DNA repair (Black et al., 2012; Musselman et al., 2012). So far, only two methylations on arginine, H2AR3me2 and H4R3me2, have been linked to DDR. They are induced by PRMT7 and modulate the transcription of several DNA repair genes. SUV39H2 (KMT1B) di-methylates H2AXK134 and this may act in *cis* to promote γ-H2AX signaling by ATM (Karkhanis et al., 2012). Mouse cells deficient for SUV39H2 form less γ-H2AX and 53BP1 foci at break sites and are more sensitive to IR (Sone et al., 2014).

Demethylation of H3K4me3, an active chromatin mark, has been shown to occur at damaged sites and leads to transcriptional repression (Gong et al., 2017a; Li et al., 2014; Mosammaparast et al., 2013). This demethylation can happen through the activity of either KDM5B (JARID1B) or KDM5A (JARID1A or RBP2) (Gong et al., 2017a), and is required to recruit the ZMYND8-NuRD chromatin remodeling complex to DSB sites to repress transcription.

H3K9me3 is associated to heterochromatin. However, H3K9me2/3 can be induced at DSB site by SUV39H1 (KMT1A), SETDB1 (KMT1E) and PRDM2 (KMT8A or RIZ1) (Ayrapetov et al., 2014). In that case, it can recruit TIP60, which promotes acetylation and activation of ATM (Sun et al., 2009), and HP1, SUV39H1 and KAP1 (Ayrapetov et al., 2014). BARD1 can interact with HP1 to recruit BRCA1 and promote HR repair (Wu et al., 2015). Loss of SETDB1 or PRDM2, results in HR defects (Alagoz et al., 2015; Khurana et al., 2014). The H3K9 demethylases KDM4B (JMJD2B) and KDM4D (JMJD2D) also localize to damage sites in a PARP-dependent manner (Khoury-Haddad et al., 2014; Young et al., 2013). Although their role in DSB repair in unclear, loss of KDM4D results in defective ATM signaling, and in diminished HR and NHEJ.

As for H3K9me3, H3K27me3 is associated with repressive chromatin and transcriptional repression, and also accumulates at DSB sites (Chou et al., 2010; O'Hagan et al., 2008). Upon DNA damage, EZH2, an H3K27me2/3 writer, accumulates at promoters of actively transcribed genes in a PARP-dependent manner. H3K27M mutation, that blocks its methylation, inhibits NHEJ (Zhang et al., 2018). Similar to H3K9me3, formation of H3K27me3 at damage sites may inhibit transcription and promote DNA repair, but this role in DNA repair has not been well established.

The level of H3K36me3, unlike the other methylations, is unaltered by DNA damage, but the pre-existing mark is associated with DNA repair at active chromatin through the writers, erasers

and readers of methylated H3K36 (Aymard et al., 2014; Pfister et al., 2014). Notably, depletion of SETD2 (KMT3A), H3K36me3 writer, leads to a defect in damage signaling and in DNA end-resection, resulting in defective HR. LEDGF, which recognizes H3K36me3, interacts with CtIP to promote HR at DSB sites (Daugaard et al., 2012). Moreover, overexpression of KDM4A (JMJD2A), a H3K36me3 demethylase, reduces HR (Pfister et al., 2014). H3K36me2, on the other hand, can be induced at damage site through the activity of SETMAR methyltransferase and is required for the recruitment of NHEJ factors (Fnu et al., 2011). Upon damage induction, KDM2A and KDM4A, H3K36me2 erasers, are degraded, which might help maintaining H3K36 methylation at DNA breaks (Cao et al., 2016; Mallette et al., 2012). Therefore, the methylation status of the H3K36 histone residue can regulate the choice of DSB pathway since H3K36me3 is important for HR repair, while H3K36me2 seems to be involved in NHEJ.

K20 is the only reported residue on histone H4 to be methylated and involved in DNA repair (Jørgensen et al., 2013). H4K20me2, induced by either PR-SET7 (KMT5A or SETD8) or MMSET (KMT3G, WHSC1 or NSD2, is bound by the Polycomb protein L3MBTL1 and the histone demethylase KDM4A in the absence of damage. Upon damage, L3MBTL1 and KDM4A are evicted and H4K20me2 becomes available for the binding of 53BP1, which inhibit end-resection and favour NHEJ (Acs et al., 2011; Bunting et al., 2010; Mallette et al., 2012). As mentioned previously, H2AK15 becomes ubiquitinated and is also bound by 53BP1. On the other hand, H4K16ac provides a steric obstruction to 53BP1 for its binding to the adjacent methylation of H4K20me2, and recruits BRCA1, a factor promoting RAD51 recruitment to the DSBs, favoring HR.

4.2.4 Ubiquitination

H2A and H2B are the major histones targeted by ubiquitination in the DNA damage response. As previously mentioned, H2AK15 mono-ubiquitination by RNF8 and RNF168 helps the recruitment of 53BP1. H2A-K13/15Ub by RNF168 was also shown to protect reversed forks from MRE11-dependent degradation following replication stress (Schmid et al., 2018). H2A-K15Ub is also important for the recruitment of the BRCA1-A complex that will limit DNA end-resection and hyper-active HR (Coleman and Greenberg, 2011; Sobhian et al., 2007). A recent study has shown that phosphorylation of ubiquitin at Thr12 (pUbT12) of H2AK15ub inhibits 53BP1 recruitment at damage sites but is permissive for HR factors (Walser et al., 2020). BARD1 has an ubiquitin ligase function ubiquitinating H2AK127 and regulates

repositioning of 53BP1 to promote DNA end-resection (Densham et al., 2016; Kalb et al., 2014). Indeed, H2A K127/129Ub recruits the chromatin remodeler SMARCAD1 that is known to promote HR. H2AK127/129Ub can be removed by USP48 to limit DNA end-resection (Uckelmann et al., 2018). H2AK119 ubiquitination, induced by the polycomb repressive complex 1 (PRC1), plays an important role in the repression of transcription following DNA damage (Shanbhag et al., 2010). The complex FBXL10-RNF68-RNF2, that also promotes H2A K119 ubiquitination, is recruited to DNA damage sites and involved in transcriptional repression (Rona et al.). This transcription repression is reversible by the deubiquitination of H2AK119Ub by USP16 and BAP1 (Shanbhag et al., 2010; Yu et al., 2014). Many DUBs regulate H2A ubiquitination in DDR. The DUB OTUB1 regulates H2A-K15Ub levels upon damage, so that depletion of OTUB1 leads to persistent 53BP1 after DNA damage (Nakada et al., 2010). Another eraser, USP3, deubiquitinates H2A K13/15 in response to UV damage (Sharma et al., 2014). USP44 and USP51 decrease 53BP1 recruitment (Mosbech et al., 2013; Wang et al., 2016). USP26 and USP37 are involved in RNF8/RNF168 mediated HR pathway by preventing the excessive spreading of RAP80-BRCA1 and promoting the interaction of PALB2-BRCA1 (Typas et al., 2015). USP11 and DUB3 also deubiquitinate RNF8/RNF168 mediated H2AXUb (Delgado-Díaz et al., 2014; Yu et al., 2016). RNF20/RNF40 are recruited to damage sites in an ATM-dependent manner and monoubiquitinate H2BK120, which is likely required for both HR and NHEJ (Moyal et al., 2011; Nakamura et al., 2011).

Altogether, these PTMs are involved both in recruiting repair proteins and in modulating the nucleosome stability onto DNA. Recent literature has defined a ‘repair pathway histone code’ that identifies PTMs occurring during either HR or NHEJ (Figure 12). However, little is known about how the normal chromatin state is restored following repair and how defects in this process affect genome and epigenome integrity.

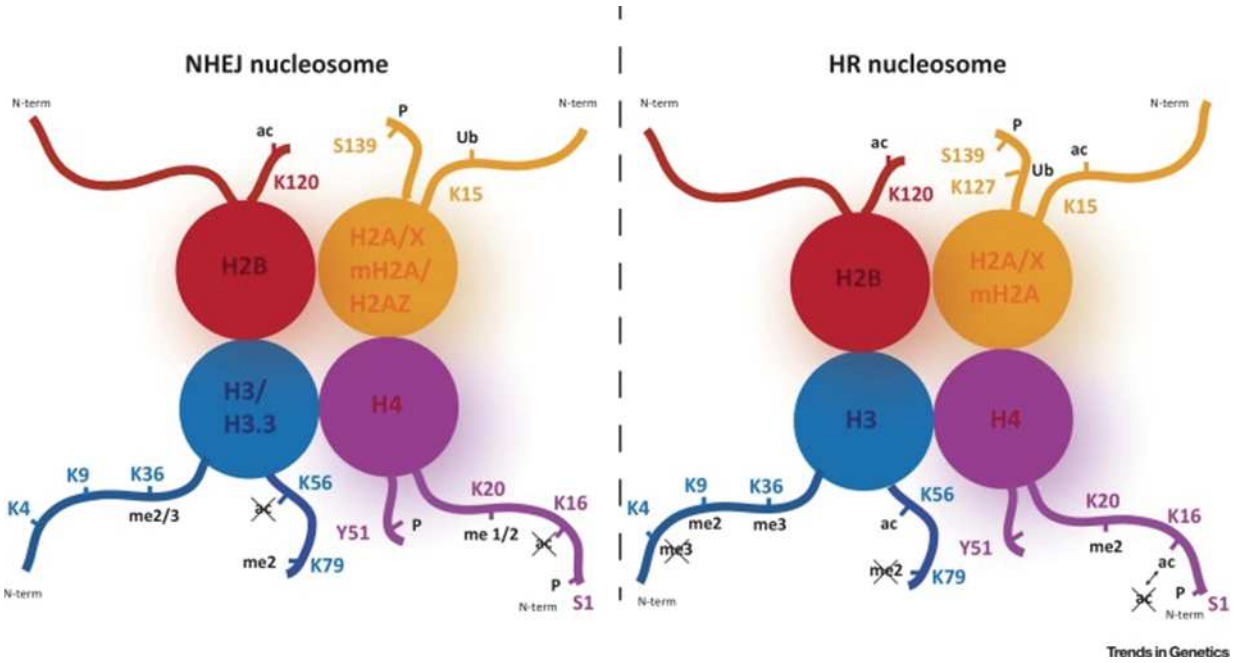


Figure 12: DSB Repair Histone Code

Nucleosomes exhibit a specific composition and post-translational modifications during repair by NHEJ and HR repair. The cross on top of a histone modification indicates the disappearance of this modification upon damage. N-term=N terminus; Ac=acetyl-; P=phospho-; Me=methyl-; Ub=ubiquitin. Detailed discussion in the main text. From (Clouaire and Legube, 2019).

4.3 Histone variants in DSB repair

Several histone variants are involved in DDR and DSB repair, and play a role in chromatin accessibility to repair factors and the restoration of the chromatin following repair (Polo, 2015; Soria et al., 2012) (Figure 13). The histone variant H2AX is the primary target of ATM, ATR, and DNAPK, and its phosphorylation is one of the earliest events following break induction (Rogakou et al., 1999). H2AX has been shown to be also deposited *de novo* at sites of DNA damage by the histone chaperone FACT in a repair-coupled manner (Piquet et al., 2018). The histone variant macroH2A1 is recruited transiently at DSBs where it was shown to participate in chromatin compaction through its interaction with the histone methyltransferase PRDM2 that induces H3K9me2 (Khurana et al., 2014; Timinszky et al., 2009). Moreover, it has been suggested to be required for BRCA1 recruitment on one hand (Khurana et al., 2014), but also linked with 53BP1 recruitment on the other hand (Xu et al., 2012a). H2AZ is transiently deposited at DSB sites by the chromatin remodeler p400 and is involved in the loading of Ku70/Ku80, thus stimulating NHEJ pathway (Xu et al., 2012b). H2AZ removal from the sites of break, by the histone chaperone ANP32e, is necessary for the induction of H4 acetylation to create a more open chromatin structure, and also seems to regulate resection to promote HR

(Gursoy-Yuzugullu et al., 2015; Xu et al., 2012b). When this removal is blocked, there is an increased CtIP-dependent end resection, accumulation of single-stranded DNA, and an increase in repair by alt-EJ (Gursoy-Yuzugullu et al., 2015).

Another histone variant, H3.3, was found deposited at sites of damage by the chromatin remodeler CHD2, where it contributes to efficiently recruit NHEJ core factors (Ku and XRCC4) (Luijsterburg et al., 2016). The H3 histone variants H3.1 and H3.3 were reported to be deposited *de novo* at UV-induced lesions sites (Adam et al., 2013; Dinant et al., 2013; Polo et al., 2006). Finally, CENP-A, the centromere specific H3 histone variant, was also suggested to be recruited at the sites of DSBs (Zeitlin et al., 2009) and its specific chaperone HJURP was first identified as being involved in homologous recombination process (Kato et al., 2007). However, more recent data did not confirm CENP-A recruitment at the sites of DNA lesions (Helfricht et al., 2013).

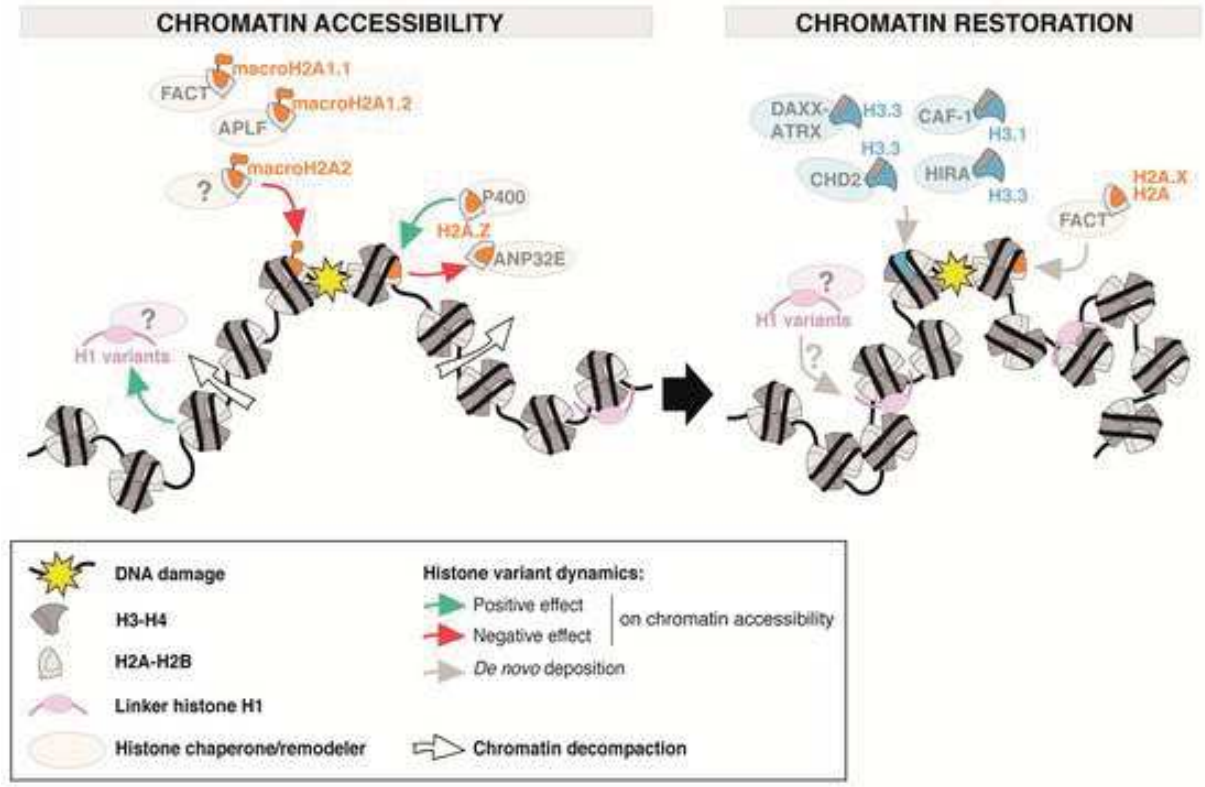


Figure 13: Histone variant in DSB repair

DNA damage induction leads to a transient decompaction to increase chromatin accessibility for repair. The deposition and eviction of specific histone variants in the damaged area positively (green arrows) or negatively (red arrows) contribute to chromatin accessibility. On the other hand, de novo deposition of histone variants (grey arrows) allows chromatin restoration. Histone chaperones and remodelers involved in these histone dynamics are indicated. It is important to note that histone variants involved in chromatin accessibility can persist and contribute to chromatin restoration, and some newly deposited histones can also increase chromatin accessibility. From (Ferrand et al., 2020).

4.4 Chromatin remodelers in DSB repair

In addition to histones PTMs and histone variants, chromatin remodelers also have mechanistic roles in DDR and DSBs repair. These chromatin remodelers are organized in four families: SWI/SNF family, INO80 family, CHD family and ISWI family. Here is a non-exhaustive list of their roles in DSB repair.

The SWI/SNF family remodelers are correlated with DDR activation and DSB repair mainly by inducing chromatin relaxation at the sites of breaks both in yeast and mammalian organisms (Papamichos-Chronakis et al., 2011). In human cells, BRG1 binds H2AX-containing nucleosomes via acetylated H3 and stimulates H2AX phosphorylation (Lee et al., 2010a). BRG1 recruits the GCN5 acetyltransferase and further increases H3 acetylation, thus creating a very accessible chromatin environment for DDR and DNA repair factors (Lee et al., 2010a). BRM1 is also recruited via CBP and p300-dependent H3 acetylation, and is required for the recruitment of Ku70/Ku80 (Ogiwara et al., 2011). Another remodeler that controls the accumulation of Ku70 is the BAF complex (Watanabe et al., 2014). In yeast, SWI/SNF and RSC complexes are recruited to DSBs mainly in S/G2 phases of the cell cycle and participate in DDR activation by remodeling nucleosomes at the break sites (Bennett and Peterson, 2015; Liang et al., 2007; Shim et al., 2007). Yeast SWI/SNF complex is recruited through the acetyltransferase activity of NuA4 complex and GCN5 acetyltransferase, and participates in HR repair (Bennett and Peterson, 2015). Absence of functional SWI/SNF has been shown to impair recruitment of MRX and delay the initiation of DNA end resection (Chai et al., 2005; Sinha et al., 2009; Wiest et al., 2017). RSC complex also participates to HR and is required after the strand invasion step (Chai et al., 2005). Another remodeler, Fun30, and its mammalian homologue SMARCAD1, are recruited at DSB sites and facilitate cell cycle-dependent DNA end resection by promoting extensive Exo1 and CtIP activity only in S-M phase and not in G1, restricting its ability to enhance resection only outside G1 (Bantele et al., 2017; Chen et al., 2012, 2016; Costelloe et al., 2012; Eapen et al., 2012).

INO80 and SWR1, the two complexes from the INO80 chromatin remodeler family in yeast, are both recruited to DSBs in a γ-H2AX dependent manner (Papamichos-Chronakis et al., 2011), while in mammals, INO80 recruitment is γ-H2AX independent (Kashiwaba et al., 2010). The INO80 complex was shown to participate in the maintenance of H2AX phosphorylation levels by antagonizing the SWR1 remodeler (Papamichos-Chronakis et al., 2011). In both yeast and mammals, INO80 is involved in resection (van Attikum et al., 2004; Gospodinov et al.,

2011) and stimulates the binding of RAD51 to resected DNA (Lademann et al., 2017). In yeast, INO80 mutants present a defective loading of RAD51 and RAD52. In mammals, INO80 depletion reduces RAD54B and XRCC3 transcription, affect thus also indirectly the repair process (Park et al., 2010; Tsukuda et al., 2005). Therefore, deficiency of the INO80 complex leads to hypersensitivity to DSB-inducing agents in yeast and HR defects in mammalian cells (Gospodinov et al., 2011; Shen et al., 2000, 2003). Moreover, INO80 is required for the eviction of H2AZ and γ-H2AX from the breaks, thus facilitating MRE11 binding, resection, and HR (van Attikum et al., 2004, 2007; Morrison et al., 2004; Tsukuda et al., 2005). On the other hand, SWR1 is involved in NHEJ by facilitating the recruitment of Ku proteins (van Attikum et al., 2007; Papamichos-Chronakis et al., 2011). Tip60 also belongs to this family of remodelers. p400, the catalytic subunit of this complex, is recruited to DSBs where it destabilizes the nucleosomes promoting RNF8 ubiquitination, BRCA1 and 53BP1 binding (Xu et al., 2010). P400 depletion did not affect resection process but impaired RAD51 recruitment (Courilleau et al., 2012) and increased of alt-NHEJ (Taty-Taty et al., 2016). Two other subunits of the Tip60 complex, RUVBL1 and MBTD1 were found to facilitate the release of 53BP1 thus promoting HR (Clarke et al., 2017; Jacquet et al., 2016).

In mammals, CHD1 is required for the recruitment of CtIP and HR repair (Kari et al., 2016). A CHD1-like protein named ALC1 localizes to DSBs and interacts with Ku70, XRCC1, and DNAPK (Ahel et al., 2009). CHD2 is recruited at the sites of damage through its interaction with PARP1, and is involved in the deposition of histone variant H3.3 at sites of DNA damage and in NHEJ (Luijsterburg et al., 2016). The NuRD complex catalytic subunit CHD4, and the non-catalytic subunit MTA1, stimulate the ubiquitination activity of RNF8 and subsequent recruitment of RNF168 and BRCA1 (Chou et al., 2010; Larsen et al., 2010; Polo et al., 2010; Smeenk et al., 2010). These remodelers are recruited to DSBs in a PARP-dependent manner. Two other subunits of this complex, the histone deacetylases HDAC1 and HDAC2, are also recruited at DSBs where they promote NHEJ (Miller et al., 2010). CHD3, the alternative catalytic subunit of NuRD complex, is released from heterochromatin after DSB induction (Goodarzi et al., 2011; Klement et al., 2014).

Finally, different subunits of the ISWI chromatin remodeling complexes are recruited to DSBs, changing chromatin structure and subsequently affecting DDR activation and repair (Aydin et al., 2014). The catalytic subunit of the different complexes from this family, SNF2H, promotes chromatin relaxation early in the repair process (Toiber et al., 2013). SNF2H is recruited to RNF20-ubiquitinated H2B in a PARP1 dependent manner and promotes the accumulation of

RNF168 (Nakamura et al., 2011; Smeenk et al., 2010). Its depletion impairs DNA end processing by CtIP and the recruitment of RAD51 and BRCA1 (Toiber et al., 2013; Vidi et al., 2014). SNF2H also promotes DDR signaling by enhancing the stability of H2AX (Atsumi et al., 2015). ACF1, a binding factor of SNF2H, is also recruited to DSBs where it is involved in both NHEJ and HR (Lan et al., 2010). Both factors are recruited to heterochromatic breaks where they are involved in chromatin relaxation after the disruption of KAP1-CHD3 interaction (Klement et al., 2014). Another interactor of SNF2H, RSF1 was shown to be recruited to DSBs where it promotes HR by recruiting RPA and RAD51 (Min et al., 2014). Upon breaks induction by IR, RSF1 recruits CENP-S and CENP-X centromeric proteins at the break sites, as well as the inter-strand crosslink repair proteins FANCD2 and FANCI (Pessina and Lowndes, 2014). CENP-S and CENP-X were suggested to promote the assembly of NHEJ factor XRCC4 (Helfricht et al., 2013), but their mechanism of action in the repair process is not clear.

4.5 Three-dimensional genome folding and looping in DSB repair

See review “How to maintain the genome in nuclear space”.

4.6 How to maintain the genome in nuclear space – Review

4.6.1 Interplay between transcription/R-loops and DNA repair

4.6.1.1 Interplay between transcription and DNA repair

See review “How to maintain the genome in nuclear space”.

4.6.1.2 Interplay between R-loops and DNA repair

An R-loop is a three-stranded nucleic acid structure arising from the invasion of an RNA strand in a double stranded DNA, forming a DNA:RNA hybrid and a displaced strand of DNA. R-loops occur frequently in genomes and play important roles in regulating gene expression (Castellano-Pozo et al., 2013; Ginno et al., 2012, 2013; Sanz et al., 2016). However, these structures have also been shown to contribute to DNA damage and genome instability, for example by interfering with the replication machinery (Gan et al., 2011). R-loops tend to form near transcriptionally active genes (Ginno et al., 2012), since they generally form during

transcription (Aguilera and García-Muse, 2012), but they can also form in trans, using an RNA transcribed at a distant genomic locus (Wahba et al., 2013).

Several studies have demonstrated a connection between R-loop formation and DSB repair (Figure 14). Indeed, recent studies have highlighted a role of RAD52 in RNA-templated DSB repair, that would also depend on a retro-transcriptase activity (Keskin et al., 2014; Mazina et al., 2017). Consistent with this, removal of the RNA part of R-loops by RNaseH overexpression impaired HR in budding yeast (Ohle et al., 2016) and impaired repair both by HR and NHEJ in human cells (Lu et al., 2018). Moreover, it was previously reported that DNA breaks at transcribed regions of the genome or *de novo* transcription from the break, promote R loop formation (Cohen et al., 2018; Ohle et al., 2016).

In addition to a role of R-loops in late steps of recombination, effects in DNA end resection have been observed. The impact of R-loop formation on DNA end resection has been controversial with some studies showing that they inhibit DNA end resection (Alfano et al., 2019; Costantino and Koshland, 2018; Ohle et al., 2016), and others showing that they promote end resection (Lu et al., 2018; Yasuhara et al., 2018). In *Schizosaccharomyces pombe*, transient R-loops formation was shown to be required for HR repair and their degradation regulates DNA end resection (Ohle et al., 2016). This study additionally suggested that R-loops are essential for maintaining repetitive DNA regions around DSBs. In budding yeast, R-loop structures block resection and prompt to asymmetric resection since only the side not involved in R-loop can be processed (Costantino and Koshland, 2018). In HeLa cells, the mRNA binding protein heterogeneous nuclear ribonucleoprotein D (HNRNPD) favors R-loop removal and regulate proper end-resection by favoring RPA phosphorylation (Alfano et al., 2019). In yeast, Senataxin ortholog Sen1 limits R-loops accumulation. In the absence of Sen1, R-loops drive Mre11-Dna2-dependent non-canonical DSB resection initiation (Rawal et al., 2020). On the other hand, in U2OS cells, Drosha drives the formation of R-loops around DSB sites and this facilitates end-resection (Lu et al., 2018). Also in human cells, RAD52 and XPG mediated R-loop processing, at transcriptionally active regions, promotes resection and therefore initiate repair by HR (Yasuhara et al., 2018). RAD52 recruits BRCA1 to antagonize the RIF1-53BP1 complex and the anti-resection activity of the Shieldin complex, suggesting that R-loops favor a BRCA1-mediated repair.

There is also evidence that R-loops may recruit factors that participate in DSB repair. The transcription-coupled homologous recombination process (TC-HR) is a BRCA1/2-independent alternative HR pathway, specific to actively transcribed regions, and based on the recruitment

of Cockayne syndrome protein B (CSB), that in turn recruits RAD52, which then recruits RAD51. CSB present a strong affinity for R-loops forming in these regions (Teng et al., 2018). R-loop have been demonstrated to facilitate HR by recruiting factors as RPA and BRCA1 (Hatchi et al., 2015; Hill et al., 2014; Nguyen et al., 2017). Finally, R-loop induction at DSBs has been suggested to stimulate strand invasion by either increasing RAD51 recruitment through recruitment of Senataxin (Cohen et al., 2018) or by promoting BRCA2 recruitment by its interaction with RNAaseH2 (D'Alessandro et al., 2018).

To reconciliate these contradictory findings, a possible explanation is that an intermediate level of R-loop formation is necessary for end resection, and both excessive R-loop formation or excessive R-loop resolution could lead to impairment of end resection. A second hypothesis is that pre-existent R-loops might block resection processivity and need to be removed by factors such as Senataxin, whereas DSBs-induced R-loop formation will not block resection since they are formed after resection creates ssDNA, and they can even recruit HR repair factors (Figure 14). Consistent with these ideas, a very recent study has shown that human EXO10 increases damage-induced long non coding RNA (dilncRNA) and R-loop levels at breaks sites, which reduces RPA binding to ssDNA and promote hyper-resection (Domingo-Prim et al., 2019). In that case, R-loops would promote resection and their removal would limit resection to allow proper HR repair.

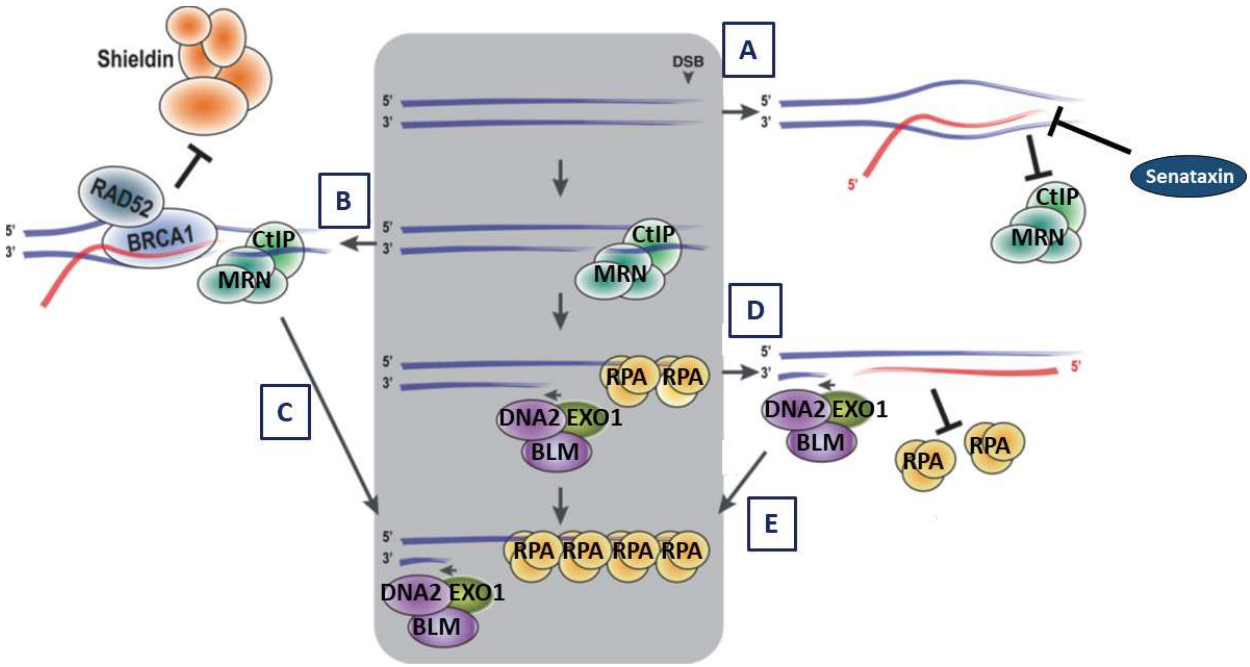


Figure 14: Putative effects of R-loop on DNA end resection

(A) When an R-loop is present at the site of the break it might block DNA end resection by blocking the first step of the process, the end-resection. R-loops can be removed by factors such as Senataxin. **(B)** If resection has already started, the presence of a R-loop can stimulate resection by attracting RAD52 and BRCA1. **(C)** These factors will antagonize the Shieldin complex, thus stimulating resection processivity. **(D)** Once resection is fully activated, de novo transcription would create ncRNAs that can pair with the ssDNA, forming R-loops. **(E)** R-loops will not affect resection and will facilitate repair. The presence of such structures might block RPA loading. Thus, elimination of the RNA will be required for activation of homologous recombination and limit DNA end resection. Adapted from (Jimeno et al., 2019).

4.6.2 DSB repair in nucleolus

See review “How to maintain the genome in nuclear space”.

4.6.3 DSB repair in heterochromatin

See review “How to maintain the genome in nuclear space”.

For more details:

Heterochromatin might need to decondense at damage induced sites to allow repair machinery recruitment (Figure 15). ATM phosphorylates KAP1 on serine 824, leading to the release of the remodeler CHD3, thus facilitating chromatin relaxation (Goodarzi et al., 2011; Noon et al., 2010; Ziv et al., 2006). Consistent with this, ATM inhibition leads to persistent breaks within heterochromatin, but has no effect on euchromatic DSBs (Goodarzi et al., 2008). ATM recruitment at DSB sites and activation was proposed to require 53BP1 (Kakarougkas et al., 2013; Noon et al., 2010). It was later proposed that the checkpoint kinase Chk2 phosphorylates KAP1, which will further disrupt KAP1 interaction with the phosphorylated HP1β and lead to chromatin decompaction (Bolderson et al., 2012; Kalousi et al., 2015). In addition, HP1 has been shown to be removed from chromatin upon DSB induction, unmasking the H3K9me3 histone modification and allowing the recruitment of the histone acetyl transferase (HAT) Tip60 that interacts with H3K9me3 (Sun et al., 2009) and that has been proposed to facilitate nucleosome removal and resection. However, recent data from our lab suggest that the chromatin expansion following DSBs is not a consequence of the eviction of heterochromatin proteins or repressive marks (Tsouroula et al., 2016). In contrast, we found that following DSBs induction at pericentromeres, H3K9me3, HP1s and KAP1 intensity are increased in G2 and RAD51 recruitment is significantly impaired upon simultaneous knockdown of HP1s or KAP1(Tsouroula et al., 2016). Moreover, these results correlate with previous studies showing a local increase of H3K9me3 in chromatin surrounding a single DSB (Ayrapetov et al., 2014), and recruitment of HP1s and KAP1 at lesions and their particular role in HR (Baldeyron et al., 2011; Geuting et al., 2013; Luijsterburg et al., 2009; Soria and Almouzni, 2013; Zarebski et al., 2009). Indeed, depletion of HP1α leads to impaired accumulation of 53BP1 and RAD51 at the sites of damage (Baldeyron et al., 2011). Other proteins related to heterochromatin (HDAC1, HDAC2, CHD4, MTA1, PRC1) are also recruited to DSBs and affect DSB repair (Chou et al., 2010; Ginjala et al., 2011; Ismail et al., 2010; Larsen et al., 2010; Miller et al., 2010; Polo et al., 2010; Smeenk et al., 2010). A hypothesis that can reconciliate these different findings is that HP1s and KAP1 are released from chromatin at early time points after damage induction to achieve chromatin relaxation, but they can be recruited again at later time points and have an active role in the repair process.

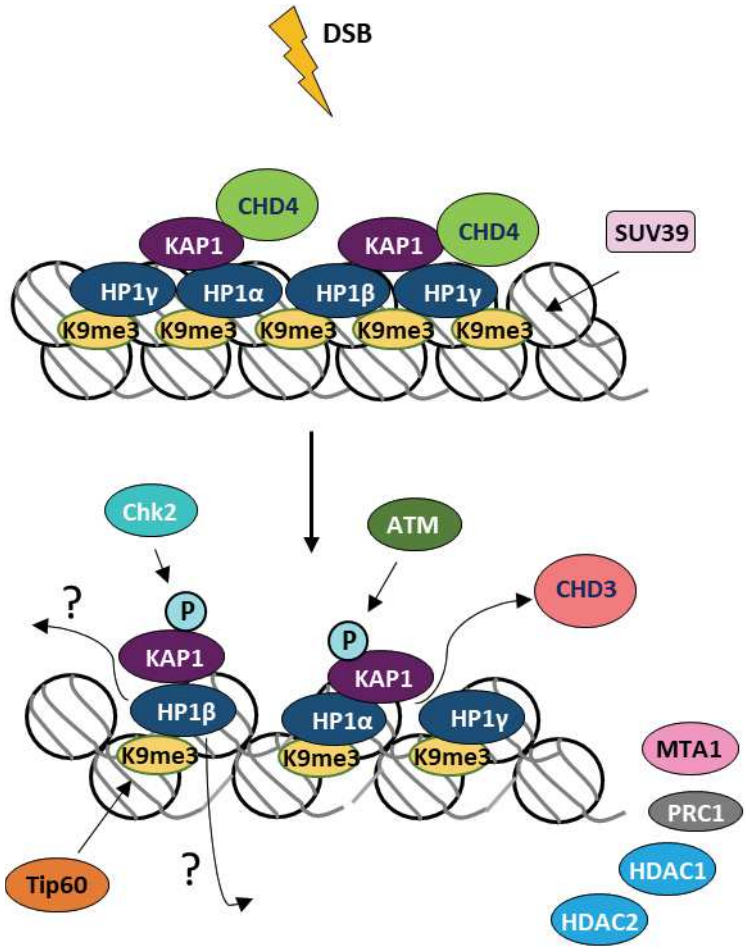


Figure 15: Chromatin expansion following DSBs in heterochromatin

KAP1- and HP1-rich heterochromatic domains prevent efficient DSB repair. Upon DSB induction, ATM phosphorylates KAP1, leading to the release of the remodeler CHD3, thus facilitating chromatin relaxation. Chk2 phosphorylates KAP1, which will further disrupt KAP1 interaction with the phosphorylated HP1β and lead to chromatin decompaction. HP1 has been shown to be removed from chromatin upon DSB induction, allowing the recruitment of Tip60 but recent data show that HP1s and KAP1 recruitment at breaks are increased instead. Other proteins related to heterochromatin (HDAC1, HDAC2, CHD4, MTA1, PRC1) are also recruited to DSBs and affect DSB repair. P represents phosphorylation events, K9me3 refers to H3K9me3 modification.

4.6.4 DSB mobility

See review “How to maintain the genome in nuclear space”.

4.7 How to maintain the integrity of the repetitive genome

4.7.1 Review in preparation "How to maintain the integrity of the repetitive genome"

4.7.2 Emphasis on centromere organization, function, and integrity

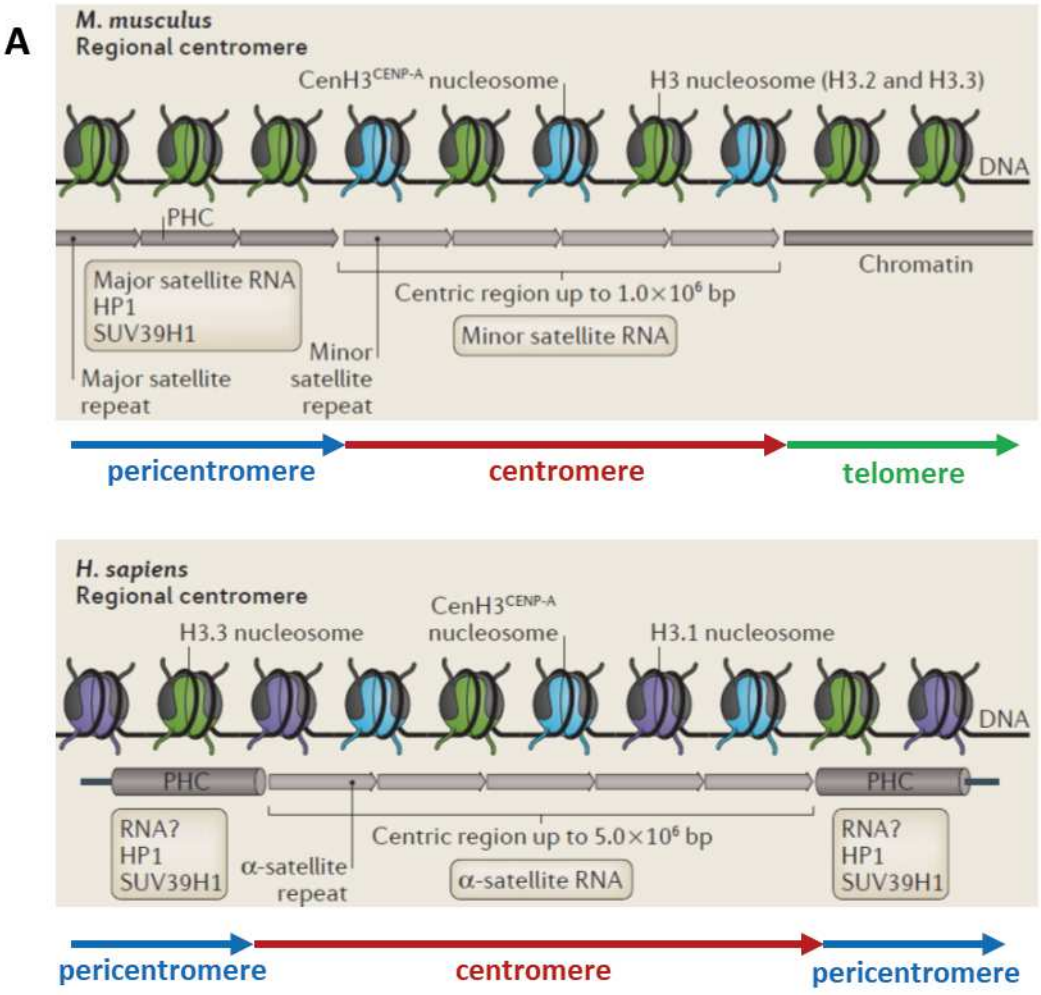
4.7.2.1 Centromeric organization

The centromeric region is essential for sister chromatids separation and proper chromosome segregation during cell division. In most Eukaryotes, centromeres contain mainly tandemly repeated DNA sequences (Figure 16.A). These structures known as satellite DNA can reach up to several megabases. In mouse cells, centromeres contain minor satellite repeats, up to one megabase long and contain 120 base pairs units. They are adjacent to a region called pericentromere, containing major satellite repeats that are 6 megabases long. Centromere is also adjacent to the telomere, since mouse chromosomes are acrocentric. Centromeres are organized as several entities surrounding pericentric heterochromatin clusters, formed by the coalescence of the pericentromeres from different chromosomes (Guenatri et al., 2004). In human cells, centromeres mainly contain alpha satellite repeats presenting 171 base pairs units and that can extend for megabases. Pericentric heterochromatin containing mainly satellites III are adjacent to centromeres. Although the alpha-satellite repeats promote the functional kinetochore assembly in the experimental condition of a human artificial chromosome (HAC) formation (Ohzeki et al., 2002), it has been shown that the DNA sequence is neither necessary nor sufficient for centromere identity. Indeed, a neocentromere can form within a non-centromeric locus of a chromosome on various DNA sequences (Fukagawa and Earnshaw, 2014; Marshall et al., 2008a, 2008b). This suggests that epigenetic marks play a key role in centromere specification. Centromeres are in fact defined by the deposition of the histone H3 variant centromeric protein A (CENP-A), which replaces canonical histone H3 in a part of centromeric nucleosomes (Buchwitz et al., 1999; Earnshaw and Rothfield, 1985, 1994) , and is considered the critical epigenetic mark for centromere specification.

In mammalian cells, CENP-A deposition at centromeres occurs in late telophase or early G1 (Jansen et al., 2007), and unlike canonical histones, its loading is uncoupled from DNA replication (Chen and Mellone, 2016; De Rop et al., 2012) (Figure 16.B). Blocks of CENP-A containing nucleosomes are interspersed with H3 containing nucleosomes (Blower et al., 2002). CENP-A deposition is carried out by its specific chaperone HJURP (Barnhart et al., 2011; Tachiwana et al., 2015) and is regulated by preloading complexes such as Mis18 complex

(Mis18α, Mis18β, and Mis18BP1/KNL2) in human cells (Dunleavy et al., 2009; Foltz et al., 2009; Fujita et al., 2007; Hayashi et al., 2004; Pan et al., 2019). In fact, targeting HJURP to a non-centromeric region induces CENP-A deposition and kinetochore assembly on the targeting region and forms a functional active centromere (Barnhart et al., 2011; Hori et al., 2013). CENP-A is essential for faithful chromosome segregation. Indeed, CENP-A depleted mice exhibit early embryonic lethality (Howman et al., 2000) and in most organisms, inactivation or depletion of CENP-A leads to chromosome mis-segregation (Blower and Karpen, 2001; Howman et al., 2000; Oegema et al., 2001; Régnier et al., 2005; Stoler et al., 1995; Takahashi et al., 2000). CENP-A presents different PTMs that contribute both to its deposition and function (Bui et al., 2017; Srivastava and Foltz, 2018; Srivastava et al., 2018). In particular, ubiquitination of CENP-A at lysine 124 was proposed to be required for CENP-A deposition at centromeres by HJURP (Niikura et al., 2015, 2016).

Centromeric chromatin contains other PTMs that are important for its function. H4 acetylation mediated by Mis18 complex primes or licenses the centromere prior to CENP-A deposition (Fujita et al., 2007), and H4 deacetylation in metaphase promotes condensation of centromeric chromatin (Wako et al., 2002). Centromeric chromatin also present H3 lysine 4 dimethylation (H3K4me2) (Sullivan and Karpen, 2004). H3K4me2 is required for targeting HJURP to the centromere and for kinetochore maintenance (Bergmann et al., 2011). Moreover, H3 lysine 36 dimethylation (H3K36me2) (Bergmann et al., 2011) was found in CENP-A nucleosomes. These two marks are usually associated with active transcription.


A
M. musculus
Regional centromere
CenH3CENP-A nucleosome
H3 nucleosome (H3.2 and H3.3)
DNA
PHC
Chromatin
Major satellite RNA
HP1
SUV39H1
Centric region up to 1.0×10⁶ bp
Minor satellite RNA
Major satellite repeat
Minor satellite repeat
pericentromere
centromere
telomere
H. sapiens
Regional centromere
H3.3 nucleosome
CenH3CENP-A nucleosome
H3.1 nucleosome
DNA
PHC
PHC
RNA?
HP1
SUV39H1
Centric region up to 5.0×10⁶ bp
α-satellite repeat
α-satellite RNA
RNA?
HP1
SUV39H1
pericentromere
centromere
pericentromere

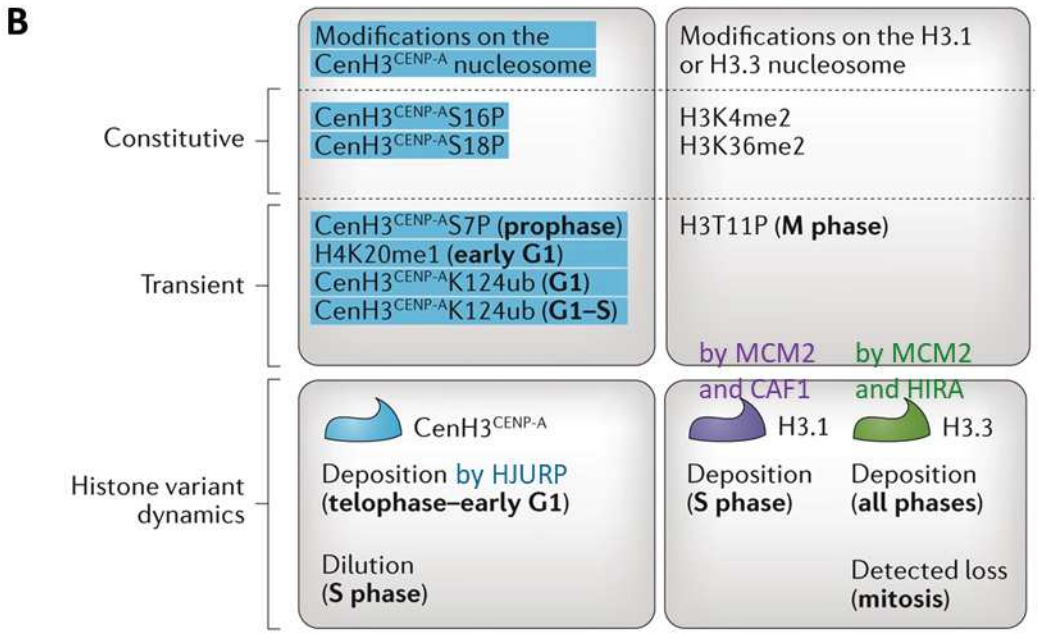

B
Modifications on the CenH3CENP-A nucleosome
Modifications on the H3.1 or H3.3 nucleosome
Constitutive
CenH3CENP-AS16P
CenH3CENP-AS18P
H3K4me2
H3K36me2
Transient
CenH3CENP-AS7P (prophase)
H4K20me1 (early G1)
CenH3CENP-AK124ub (G1)
CenH3CENP-AK124ub (G1–S)
H3T11P (M phase)
by MCM2 and CAF1
by MCM2 and HIRA
Histone variant dynamics
CenH3CENP-A
H3.1
H3.3
Deposition by HJURP (telophase–early G1)
Dilution (S phase)
Deposition (S phase)
Deposition (all phases)
Detected loss (mitosis)

Figure 16: Centromeric organization

(A) Mouse centromere contains minor satellite repeats and a unique variant of histone H3, CENP-A (blue nucleosomes, annoted $CenH3^{CENP-A}$). It Is adjacent to pericentromere, containing major satellite repeats, and telomere. Human centromere contains alpha satellite repeats and CENP-A. It is adjacent to pericentromeres containing mainly satellite III. **(B)** Centromeric histones present specific marks described here. CENP-A is deposited ate centromeres in late telophase-early G1, unlike canonical histones. Centromeric H3.1 is deposited by MCM2 and CAF1 in S phase, centromeric H3.3 is deposited by MCM2 and HIRA in all phases. Adapted from (Müller and Almouzni, 2017).

4.7.2.2 Role of centromeres in chromosome segregation during cell division

Centromeres recruit the kinetochore protein complex, which serves as a platform for the binding of the metaphase spindle microtubules during mitosis (Cheeseman and Desai, 2008; McKinley and Cheeseman, 2016; Nagpal and Fukagawa, 2016; Pesenti et al., 2016) (Figure 17). The kinetochore is composed of more than a hundred proteins (Tipton et al., 2012) divided in two subcomplexes: the constitutive centromere-associated network (CCAN) (Cheeseman and Desai, 2008; Perpelescu and Fukagawa, 2011; Takeuchi and Fukagawa, 2012), and the KMN network formed from the KNL1, the Mis12, and the Ndc80 complexes (Cheeseman and Desai, 2008; Cheeseman et al., 2006; Nagpal et al., 2015). CCAN proteins are constitutively localized at centromeres throughout the cell cycle and form a foundation for kinetochore assembly. The CCAN is composed of at least 16 proteins (CENP-C, -H, -I, -K, -L, -M, -N, -O, -P, -Q, -R, -S, -T, -U, -W, -X) (Nagpal and Fukagawa, 2016). The KMN complex is recruited to the CCAN during M-phase and binds directly to the spindle microtubules.

CENP-A also recruits spindle assembly checkpoint (SAC) proteins (Figure 17). The role of the SAC is to arrest the cell division process at the metaphase–anaphase transition, through a complex kinase-phosphatase signaling cascade, when a single chromosome pair is found to be unbound or mis-bound to the mitotic spindle, until accurate chromosome segregation can be guaranteed. The SAC kinase Aurora B phosphorylates the Ndc80 protein of each mis-bound sister pair to detach it from the spindle structure (Carmena et al., 2012). This phosphorylation will recruit Mps1 at the kinetochore (Zhu et al., 2013), which phosphorylates the protein Knl1 and recruit the mitotic checkpoint complex (MCC). This event ultimately results in the inhibition of the E3 ubiquitin ligase anaphase promoting complex/cyclosome (APC/C), hence prolonging the mitotic state (London and Biggins, 2014; Musacchio and Salmon, 2007). When the chromosomes are correctly attached, an enzymatic cleavage of the cohesion ring between

the two chromatids of one chromosome will separate the sister chromatids. Each kinetochore-bound chromatid then moves to a pole by depolymerization of the spindle microtubules and, in some eukaryotes, by additional motor protein activity.

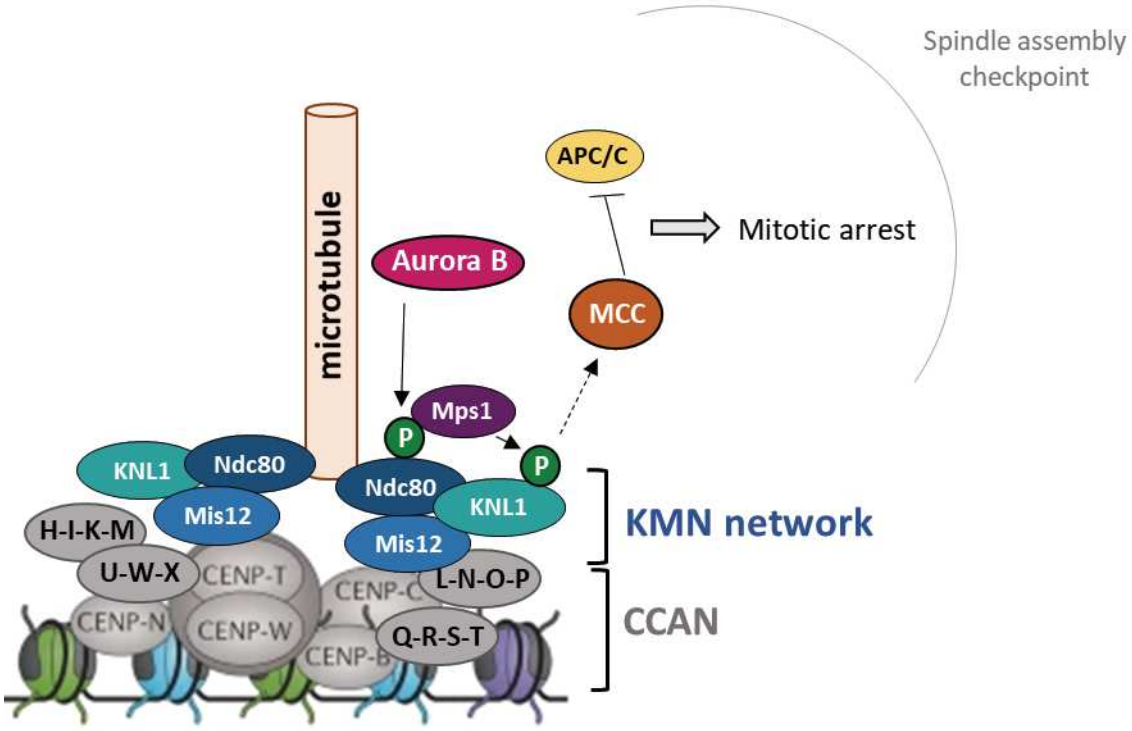


Figure 17: Kinetochore and spindle assembly checkpoint

The kinetochore complex is divided in two subcomplexes: the constitutive centromere-associated network (CCAN) containing 16 CENP factors, and the KMN network containing Ndc80, KNL1 and Mis12 factors. When a single chromosome pair is found to be unbound or mis-bound to the mitotic spindle, centromere recruits spindle assembly checkpoint (SAC) proteins to prolong mitotic state until correct attachment. Aurora B phosphorylates Ndc80, which recruits Mps1. Mps1 phosphorylates KNL1 and recruits the mitotic checkpoint complex (MCC) that will inhibit the E3 ubiquitin ligase anaphase promoting complex/cyclosome (APC/C) and block the cell cycle in mitosis.

4.7.2.3 Centromeric transcription

Centromeres, that were conceived not to have a transcriptional activity as being a constricted region, are in fact transcribed in non-coding RNAs (ncRNAs) (Bouzinba-Segard et al., 2006; Ideue et al., 2014; Quénet and Dalal, 2014). These ncRNAs have been proposed to have a role in the assembly of the kinetochore and in the targeting of CENP-A to centromeres by associating with soluble CENP-A and HJURP, and in the targeting of CENP-C (Du et al., 2010;

McNulty et al., 2017; Quénet and Dalal, 2014). Human centromeric RNAs have also been found to facilitate the preassembly of kinetochore proteins at the nucleolus, prior to their localization at the centromere (Wong et al., 2007). Moreover, it has been reported that upon genotoxic stress, there is a strong transcriptional activation of centromeric repeats in a manner that is dependent on p53, which is followed by disorganization of centromeric chromatin associated with the relocation of CENP-A (Hédouin et al., 2017). Centromeric ncRNAs have also been demonstrated to regulate the activity of Aurora B, but with opposite effects depending on the organism. In human cells, α-satellite RNAs suppress Aurora B's kinase activity (Ideue et al., 2014), while in murine cells, minor satellite RNAs are required for Aurora B's kinase activity (Ferri et al., 2009). Yet in both cases, impaired centromeric RNA leads to improper spindle attachments and chromosome missegregation (Bouzinba-Segard et al., 2006; Ideue et al., 2014).

The transcription process itself and the transcription factors involved seem to have a role in centromere function. For example, RNA pol II localizes to centromeres during mitosis in mammals (Chan and Wong, 2012; Liu et al., 2015b), and has been suggested to create an epigenetic environment favoring the deposition of CENP-A (Molina et al., 2016). FACT, a protein that associates with RNA pol II for centromeric transcription, binds to the histone fold domain of CENP-W and stimulates CENP-T/W complex deposition at the centromere (Prendergast et al., 2016).

Transcription has also a role in histone exchange, notably in the replication independent turnover of CENP-A (Schwartz and Ahmad, 2005). As mentioned above, centromeric transcription leads to a temporary removal of histones, providing a chance to re-incorporate specific variants with the help of chaperones that are associated with the RNA polymerase (Ahmad and Henikoff, 2002). H3.3, the placeholder of CENP-A, is loaded at centromeres in a transcription-coupled manner, after DNA replication and prior to new CENP-A loading (Dunleavy et al., 2011). It is not clear whether CENP-A loading is coupled to transcription as well, but ectopic CENP-A incorporation found in human cancer cells is associated with transcription-coupled H3.3 chaperones HIRA, DAXX and ATRX (Athwal et al., 2015; Nye et al., 2018).

As highlighted before, centromeres present H3K4me2 and H3K36me2 marks that are usually associated with active transcription and can potentially regulate centromeric transcription. Supporting this idea, mis-regulation of H3K4me2 mark was shown to reduce α-satellite RNAs levels and results in genomic instability (Huang et al., 2016).

Finally, it is important to note that centromeric transcription needs to be regulated and kept low. Overexpression of the centromeric transcripts leads to reduced levels of CENP-A, CENP-C and Aurora B at centromeres (Ling and Yuen, 2019) and reduced chromatin association of kinetochore proteins in yeast (Collins et al., 2005).

4.7.2.4 Centromeric integrity

See review "How to maintain the integrity of the repetitive genome".

4.7.2.5 Centromeric repair

See review "How to maintain the integrity of the repetitive genome".

4.7.2.6 Consequences of instability at centromeres

See review "How to maintain the integrity of the repetitive genome".

5. Brief summary

Maintaining the integrity of the genome is highly important for an organism's survival, with genomic instability linked life threating illnesses and infertility. However, cells need to maintain their genome while under assault from a great many endogenous and exogenous agents that can cause damage to the DNA. To get around this the cell has developed a complex system to detect and repair DNA damage. There are multiple repair pathways, which are under the control of many layers of regulation. While there are still many unknows, it has become increasingly clear that pathway choice is context specific, and is in part regulated by many factors, such as the type of damage, the status of the chromatin around the break site, DNA structure, the nuclear sub-compartment a break is in, and the phase of the cell cycle the break is detected in. Correct pathway choice is important as it has vital implications for the cell, since some pathways are error free whereas others are prone to errors. There are many questions remaining about the molecular mechanism of pathway choice and the regulation of DNA repair. During my PhD, I have been interested in elucidating some of these mechanisms at the crucial regions that are centromeres.

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Current Opinion in Cell Biology

How to maintain the genome in nuclear space

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Abstract

Genomic instability can be life-threatening. The fine balance between error-free and mutagenic DNA repair pathways is essential for maintaining genome integrity. Recent advances in DNA double-strand break induction and detection techniques have allowed the investigation of DNA damage and repair in the context of the highly complex nuclear structure. These studies have revealed that the 3D genome folding, nuclear compartmentalization and cytoskeletal components control the spatial distribution of DNA lesions within the nuclear space and dictates their mode of repair.

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Introduction

Genome integrity is continuously challenged by different damaging sources that affect DNA stability by leading to various types of DNA lesions. Unfaithful DNA repair leads to genomic rearrangements such as chromosomal translocations, aneuploidy, and indels that can be the origin of many diseases including developmental disorders, premature ageing and cancer [1]. Several pathways have evolved to detect DNA damage and limit its oncogenic potential by mediating their repair through the concerted action of specific proteins. double-strand breaks (DSBs) are repaired by nonhomologous end joining (NHEJ) which religates DNA free ends and homologous recombination (HR) that relies on extensive resection of the break, corresponding to a nucleolytic degradation of the 5′-terminated strand by exonucleases, to generate single-strand DNA that must be remodelled to load RAD51 to invade the sister chromatid and use it as a template to repair in an error-free manner [2]. Other alternative mechanisms exist, which soften the distinction between these two major pathways: single-strand annealing that requires resection but then uses a direct homologous sequence *in cis* for repair, and alternative NHEJ (Alt-NHEJ/microhomology-mediated end joining) that requires short-range resection to mediate end-joining by microhomology. These pathways are highly mutagenic and are believed to be activated when the primary pathways are perturbed [3]. More recently, it has been proposed that RNA-templated repair also occurs in yeast and possibly in higher eukaryotes [4]. The balance between these different pathways is tightly controlled, but the mechanism by which the balance is regulated in the nucleus is not well understood. The development of unique experimental systems to induce DSBs at specific genomic positions based on the use of restriction enzymes (as AsiSI), endonucleases (as I-SceI), zinc fingers, TALE nucleases, and the CRISPR/Cas9 [5] system, as well as new sequencing-based techniques to detect and map DSBs, such as BLESS, BLISS, DSB-Capture and END-seq [6–8], have allowed a better understanding of the DSB landscape across the genome and their preferred mode of repair. Facilitated by these new technologies, many recent studies have identified key factors regulating the DNA repair pathway choice. These studies have largely revealed that transcription status, chromatin organization, 3D nuclear position and 3D genome folding [9,10] are major players in DNA lesion signalling and regulation of repair pathway choice.

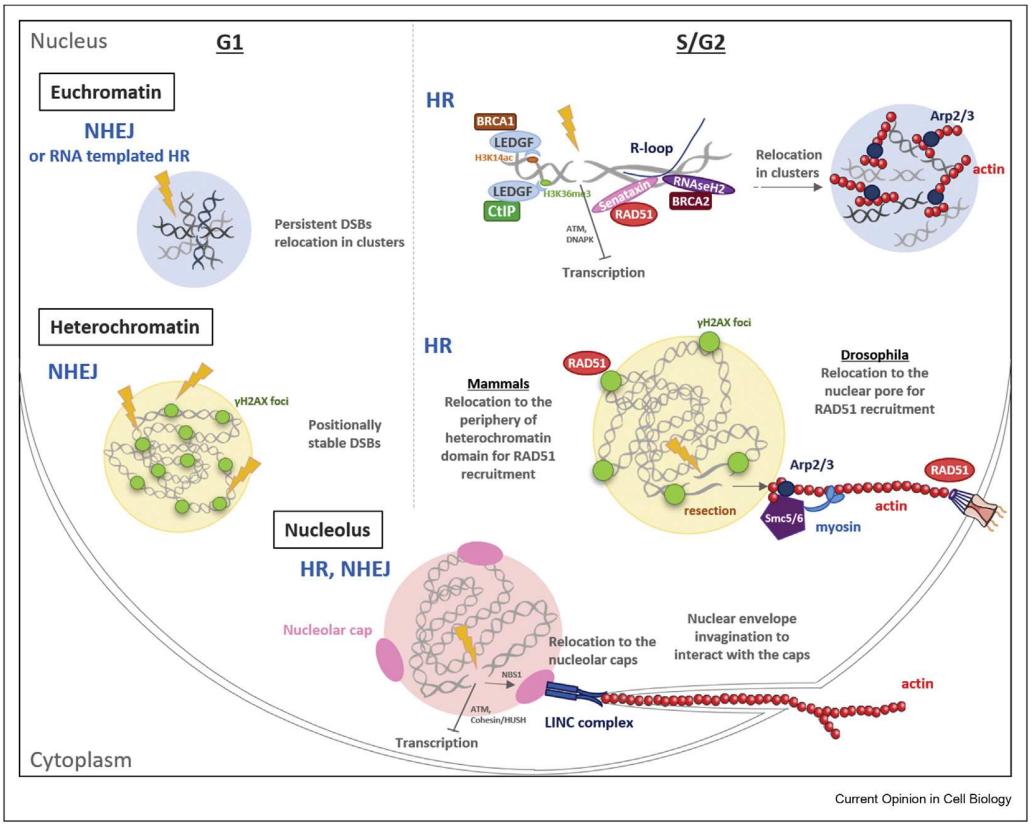
Interplay between transcription and DNA repair

Endogenous DSBs preferentially occur in the transcriptionally active regions of the genome, most probably as a consequence of abortive topoisomerase activity or replication fork stalling and collapse, after colliding with R loops (three-stranded structures composed of DNA-RNA hybrid) and secondary DNA structures such as G quadruplexes [11]. DSBs at active genes lead to transient transcriptional repression that depends on

ataxia telangiectasia mutated (ATM) signalling in response to clustered DSBs, or DNA-dependent protein kinase catalytic subunit (DNAPK) signalling in the case of unique nonclustered DSBs and promotes DNA repair by HR or NHEJ, depending on the cell cycle and chromatin remodelers recruited at the DSB site [12–21]. Interestingly, transcriptional repression has also been proposed to be essential in suppression of chromosomal translocations [22**].

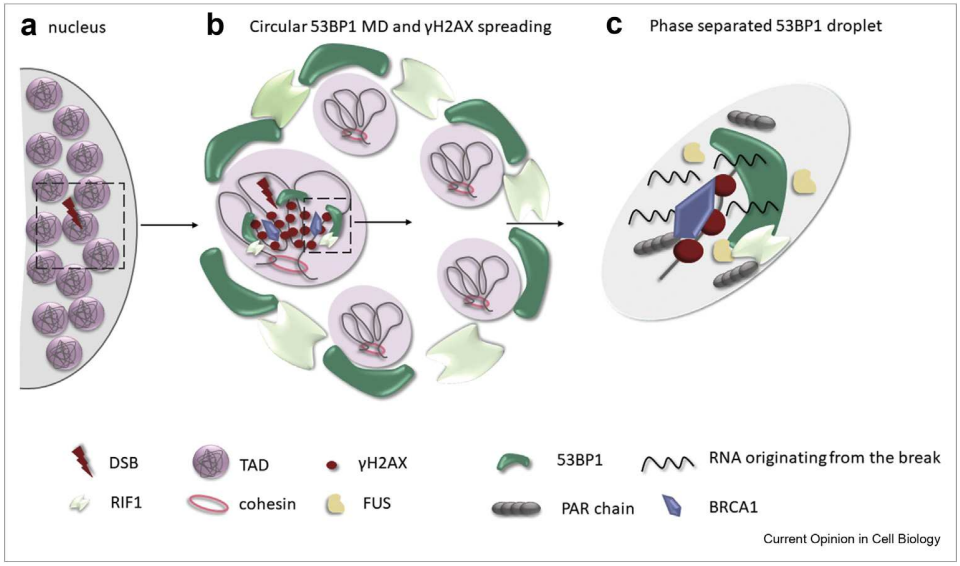
Although the stability of the active genomic regions is in constant jeopardy, DSBs at transcriptionally active loci are mainly repaired by the error-free HR in S/G2 phases of the cell cycle [23] (Figure 1). The mechanism underlying this preference involves the affinity of HR promoting factors such as BRCA1 and CtIP interacting protein LEDGF to active chromatin marks (H3K14ac and H3K36me3, respectively). More recently, it was shown that R loops-resolving proteins such as senataxin, that unwinds R-loops, and RNAseH2, that degrades the RNA engaged in R-loops, stimulate HR by either promoting the recruitment of RAD51(for senataxin) [24] or by directly interacting with BRCA2 (for RNAseH2) [25], revealing another way by which the error-free HR is promoted by transcription. But what happens to transcriptionally active regions in G1 phase of the cell cycle when HR is suppressed? One proposed mechanism was that DSBs in these genes persist and relocate in clusters possibly waiting to be repaired in post-replicative stages of the cell cycle [26] (Figure 1). Nevertheless, the repair process and kinetic of DSBs at active genes in G1 is still not well characterized. An arising concept is that RNA-templated repair, which does not necessitate homologous DNA template, may be used in G1[4]. Indeed, synthetic RNA oligonucleotides can act as templates for DSB repair in yeast and human cells [27]. Moreover, it was recently shown that DSBs can act as promoters leading to the transcription of noncoding RNAs [28**]. It is therefore interesting to speculate that even transcriptionally inactive genomic locations may transiently become active to access the benefit of HR. Extensive further work is needed to understand the role of RNA in DNA repair.

Figure 1



DNA repair pathway choice and spatial distribution of DSBs in euchromatin, heterochromatin and nucleolus in G1 and G2 phases of the cell cycle. Euchromatic DSBs occurring in G1 cluster to wait for postreplicative phases for repair. DSBs in mouse heterochromatin in G1 are positionally stable. Euchromatic DSBs in S/G2 are mainly repaired by HR and cluster in an actin-dependent manner. DSBs in heterochromatin in S/G2 relocate at the periphery of the HC domain or of the nucleus. In Drosophila, this process is actin- and myosin-dependent. DSBs at the nucleolar repeats result in the nuclear cap formation and are mainly repaired by HR. Cytoplasmic actin network ensures the invagination of the nuclear envelop to further interact with the nucleolar caps. DSB, double-strand breaks.

Figure 2



DSB microenvironment in the context of 3D genome folding. Chromosome architecture can regulate γH2AX spreading and DDR factors recruitment. (**a, b**) In the presence of a DSB, γH2AX is spreading inside a TAD till it reaches its boundaries. 53BP1 is organised in several nanodomains, that corresponds to TADs, it spreads into the surrounding chromatin and encircles the DNA lesion. RIF1 is then recruited at the border of the nanodomains and together with 53BP1 are vital for the built structure and for the protection of resected DNA ends inside this structure. (**c**) Each 53BP1 focus is at the same time a liquid droplet due to the highly disordered properties of 53BP1 and the RNA binding proteins such as FUS whose recruitment is regulated by Parylation. DSB, double-strand breaks; TADs, topologically associated domains.

DNA repair in heterochromatin

Heterochromatin contains highly compacted regions, is considered transcriptionally inactive and enriched at the nuclear periphery, around the nucleolus and at centromeres. Heterochromatin is divided into facultative heterochromatin, which is more plastic and can be subjected to transitions between open and compact state based on cell state; and constitutive heterochromatin, which is always compact and enriched in repetitive, gene-poor and late replicating DNA sequences such as telomeres and centromeres. These different chromatin structures are decorated by specific histone marks and bound by specific factors, which play a role in the choice of DSB repair. Constitutive heterochromatin is mainly composed of pericentromeric and centromeric repetitive sequences[29]. Because of the extreme compaction of the heterochromatin, it was hypothesized that repairing DSBs in this structure represents a challenge for access of repair factors. Therefore, heterochromatin may need to decondense to allow recruitment of the repair machinery. Although this was shown to be the case the mechanism by which it is achieved is not very clear. Some studies propose that Heterochromatin protein-1 (HP1) and other heterochromatin factors are released from heterochromatin in the presence of DNA damage [30–32]. Other studies suggest that the chromatin expansion following DSBs can occur without the eviction of heterochromatin proteins or repressive marks [33]. In agreement with the latter observation, several studies showed a local increase of H3K9me3 in chromatin surrounding a single DSB [34], and recruitment of HP1s and the Kruppel-associated box–associated protein-1 at lesions involved, for example, in the stimulation of DNA end resection [35].

The spatial arrangement of DNA lesions in heterochromatin and their pathway of repair has also been extensively investigated. In *Drosophila*, pericentromeric DSBs induced by irradiation were observed to initially relocate to the periphery of the heterochromatin domains [36] and later to the nuclear envelope (NE) [37] in a manner that depends on Suv39H1-mediated H3K9me3, on Slx5/Slx8 SUMO-targeted ubiquitin ligation (STUbLs) [38] and on the actin and myosin network [39**] (Figure 1). Since RAD51, the primary factor involved in homologous strand invasion, was shown to be recruited only after DSB relocation at the periphery of the domain and was mutually exclusive with HP1α [36,40], the hypothesis was that relocation is necessary to avoid recombination between repetitive sequences. More recently, our lab has demonstrated in mammalian cells that DSBs are positionally stable in G1 and recruit NHEJ factors, whereas in S/G2 phase, the use of HR involves the repositioning of the DSB at the periphery of the heterochromatin domain in a manner independent of chromatin relaxation [33] (Figure 1), in contrast to what was observed in *Drosophila* [36,37] but dependent on DSB end resection and active exclusion of RAD51 from the heterochromatin core [33]. Interestingly, we showed that in centromeric heterochromatin,

DSBs recruit both NHEJ and HR proteins throughout the cell cycle [33], suggesting that although both structures are condensed, their unique chromatin modifications and histone variant composition might influence the outcome of DNA repair. Indeed, centromeres present H3K36me2 and H3K4me2 marks associated with active chromatin, that might render these regions permissive to resection in G1. It is reasonable to consider that after an extensive broken end resection, the single strand may anneal directly to an adjacent repeat thus generating a contraction (loss of repeat units) or, if the annealing disengage, a synthesis-dependent strand annealing can generate an expansion (gain of repeat units), both leading to deleterious outcomes. But because centromeres from different chromosomes do not cluster, HR or any of the aforementioned resection-dependent mechanisms has minimal risk of leading to chromosomal translocations.

rDNA DSB repair

The nucleolus is a subnuclear compartment, well characterized mainly for its function on ribosome biogenesis. Nucleoli serve as the sites of ribosomal DNA (rDNA) transcription, and their structure in human cells involves clusters of tandem repeats (300 repeats in a haploid genome) that are located on the short p-arms of the five human acrocentric chromosomes (13, 14, 15, 21, 22). The unique chromatin structure in combination with the high transcriptional activity of the nucleoli has made it a favourite model for the study of the response to DSBs induced on the rDNA repeats [41–43]. Initial studies, have showed that damage by ionizing radiation was able to inhibit Pol-I transcription in an ATM-dependent manner, which further led to nucleolar reorganization [44] in an NBS1/treakle-dependent manner [45]. After transcription inhibition, the rDNA arrays segregate to the periphery of the nucleolus, forming the so-called nucleolar caps, which are structures that contain nucleoplasmic proteins and in the field represent the rDNA DSB relocation. Recent studies have used the I-PpoI meganuclease, or CRISPR/Cas9 system to induce specific DSBs at the rDNA repeats, and both of them resulted in the reorganization of the nucleolus and nucleolar cap formation [45–49]. Surprisingly, after the nucleolar caps formation NHEJ and HR can be activated both in G1 or S/G2 phases of the cell cycle [48] (Figure 1). A possible explanation is that undamaged rDNA repeats can be used as a template in cis, for the G1 cells, when the sister chromatid is not present in a similar to centromeres manner described previously. Another interesting feature of nucleolar DDR is its failure to activate a strong checkpoint response and to inhibit cell cycle progression [50**]. Moreover, most DDR factors except NBS1 are mainly detected at the nucleolar caps after relocation [50**]. Although it was mainly assumed that the DSB relocation at the nucleolus is concomitant with the transcriptional inhibition, a recent study reported that relocation and transcriptional inhibition can be uncoupled and that cohesin and human silencing complex control transcriptional repression in response to DNA damage [51**]. In this process, cohesin acts at an early step of the DSB response and human silencing hub, that is known to repress epigenetically retroviruses and LINES, is mediating H3K9me3 to ensure the complete transcriptional shutdown. Finally, there is evidence that as in DSBs [50**] induced in Drosophila heterochromatin, the actin network, has a role in ensuring the interaction of the rDNA DSBs with the nuclear periphery, and specially with the NE (Figure 1). This interaction is mediated by NE invaginations, in a linker of nucleoskeleton and cytoskeleton complex–dependent manner [39,51**]]. More specifically, this event happens downstream of transcriptional repression and is promoted by the SUN1 subunit of the linker of nucleoskeleton and cytoskeleton complex together with ARP3 and UNC-45, which are actin and myosin regulators [51**]. One has to point out that the NE invaginations are not DNA damage–induced but rather constitutive and therefore the NE might play a general role that is not only necessary in the presence of DNA damage.

Actin–myosin cytoskeleton involvement at the mobility of DSBs

A common feature of DSBs induced on repetitive regions, is their mobility to either the periphery of their domain or to the nuclear periphery. In all cases, it was suggested that the DSBs need to relocate to undergo HR. The concept that the nuclear environment and position can dictate the DNA repair pathway choice and DSBs migrate between nuclear compartments to use specific DNA repair pathways raises the question of whether this is achieved by an active or passive mechanism. DSB movement was reported to play roles in homology search, DSB isolation to prevent deleterious repair outcomes [33,37], DSB clustering in repair centres to reinforce the recruitment of repair factors or clustering owing to liquid droplet properties of repair foci [52**]. Another hypothesis is that targeting a DSB to the NE could transmit signals to the cytoplasm to coordinate cellular responses such as apoptosis [53]. In yeast, it is well accepted that persistent DSBs move to cluster together in a single RAD52 repair focus [54]and can relocalize to the NE [38,55–60]. Although in most of the studies DSB movement was attributed to passive diffusion, it was recently reported that they exert a nonlinear directional movement [61] and that microtubules' polymerization drives this process.

In higher eukaryotes, contradictory results have been published about DSB mobility for many years, most probably owing to the variability of the position that the DSB was induced and the nature of DSB induction. In

several studies, resection has been shown to be a determinant prerequisite for DSB mobility in yeast [62], Drosophila [36] and in mammalian cells [33], leading to the assumption that resected DNA ends are able to passively diffuse within the nuclear space. On the other hand, recent studies have highlighted the contribution of actin-related proteins and nuclear actin polymerization in DNA end resection, relocation and clustering of DSBs or stalled replication forks [63] through directed movement [39, 51, 64**]. More specifically in Drosophila, Arp2/3 or myosins are recruited to lesions in heterochromatin in an Smc5/6 complex-dependent manner and they subsequently move along the actin filaments to reach the nuclear pores [39**], whereas in euchromatin, short nuclear actin filaments assemble at DSB sites but to promote the clustering of DSB foci, without requiring myosin [39, 64**,]. Formation of transient nuclear actin filaments was shown in the context of various cellular response including DSB damage signalling [51**] [65]. In human cells, it has been shown that depending on the cell cycle phase, another factor other than Arp2/3, Formin 2, can promote actin filament assembly at DSB sites [26,66].

All together, these recent discoveries suggest that nuclear actin filaments can have distinct structures with specialized functions in response to DNA DSBs, depending on organism, cell cycle phase and chromatin organisation.

Three-dimensional genome folding and looping

A key feature of the mammalian cell nucleus is the nonrandom arrangement of the chromosomes in nuclear space. Chromosomes are confined in discrete territories and within them further levels of spatial organization are imposed to chromatin. Recent studies have coupled 3C to high-throughput sequencing to assess genome folding in a genome-wide scale, and one of the most striking discoveries was the organization in distinct folded modules namely topologically associated domains (TADs), whereby genomic interactions are strong within the domain and are sharply depleted across the boundary between two domains [67].

Is this spatial arrangement of the genome in 3D setting up the stage for DNA damage signalling and repair? Does it affect the DSB microenvironment? Recent studies have used super-resolution microscopy to resolve g-H2AX and 53BP1 foci upon ionizing radiation and have revealed that the foci are not homogenous structures they are rather divided in substructures that follow the TAD organization (Figure 2a, b). More specifically, Natale F. et al. [68] showed the existence of γH2AX chromatin nanodomains that are observed with a median length of around 75 kb of the genome, and those γH2AX nano-foci formation seemed to depend on CTCF, a factor highly bound at TAD boarders. Similarly, Ochs, et al. [69**] reported recently the existence of 53BP1 microdomains (53BP1-MDs). These 53BP1-MDs correspond to assemblies of 60–180 nm nanodomains of damaged chromatin that is labelled by a ring of 53BP1. In this process, 53BP1 is binding to compact chromatin sites, which colocalise with TADs, and this event is followed by RIF1 recruitment at the boundaries, to finally stabilize the 3D topology of the broken sites. In contrast, competitive resection promoting factors, such as BRCA1, are also recruited but not colocalizing with 53BP1 and RIF1 as they are mutually exclusive. Finally, the stabilized TAD-sized structures are arranged in an ordered, circular way (Figure 2b). A question arising from these data is why the existence of a structure like this is needed? One possible function for the 53BP1-MD may be to insulate the damaged from undamaged chromatin from adjacent lesions. This insulation would either reduce the risk of chromosomal translocations, or create a protective cave-like environment for the resected DNA by promoting local concentration of antiresection proteins such as Shieldin components. Shieldin is recruited at the nonchromatinized ss-DNA overhangs at the moment where decision for the pathway choice is made. It is possible that the binding of Shieldin to the 3' overhangs serves to protect the 5' overhang from nucleases, or competes with RPA for ssDNA binding, or leads to restructuring of the DNA that blocks the access of helicases, as a step after resection initiation. Alternatively, Shieldin counteracts resection through its interaction with CST/Pola/Primase leading to fill in synthesis at the DNA break.

Increasing evidence is arising on the regulation of γH2AX spreading by the initial chromatin conformation. Chip-seq data by Legube et al. [70,71] had already shown that γH2AX does not spread around a single DSB in a linear fashion but is characterized by mountains and valleys with the latter coinciding with CTCF binding possibly at TAD borders. Moreover, prior studies implicate involvement of cohesin and CTCF in the regulation of γH2AX spreading, suggesting that the pre-existing chromatin architecture surrounding a DSB promotes this process at nucleosomes that are brought into spatial proximity (reviewed in the studies by Legube et al. [70,71]).

Data from yeast models further support this hypothesis, as it was revealed that after a DSB is induced close to a centromeric cluster, γH2AX spreading occurs in trans, on different chromosomes [72]. The importance of the initial chromatin conformation on DSB repair, could also be relevant in other physiological processes of the cell, similar to the class switch recombination (CSR) for the generation of antibody isotypes and the V(D)J recombination of the immunoglobulin loci for antibody diversification, which both require programmed DSBs.

Immunoglobulins' (Igs) and T-cell receptors' repertoire is produced by B and T cells, respectively. To achieve different combinations of amino acid sequences on their antigen-binding regions, DSBs are induced next to the V(variable) D(diversity) J(joining) segments that are located at the Igs and T-cell receptors' N-terminal regions. The segments that occur are fused using the NHEJ pathway, to be further processed by CSR. In both cases, a DSB is generated and 3D folding has a role in the long-range interactions and the recombination event that follows. Recently, Vian et al. [73] showed a reduction in CSR after depletion of CTCF-binding sites at the igh SA locus of B-cells. Concerning V(D)J recombination, a study by Mora et al. [74] noted an enhancer-dependent framework in the igκ locus. In detail, this enhancer can regulate long-range chromatin interactions that connect sub TADS, which are finally shown to be important for the antibody repertoire production.

All these studies provide strong evidence that chromatin architecture and TADs affect DSB repair, opening up a new era in the field that needs to further be explored by 3C-based studies and super-resolution microscopy.

Conclusion and perspectives

It has become clear that the nuclear environment is an important determinant of DNA repair. Nevertheless, how this nuclear compartmentalization influences DNA repair pathway choice has to be further investigated. A concept that is arising is that the self-organizing properties of the nucleus might have a role. There is increasing evidence that membraneless nuclear organelles such as the nucleolus, chromocenters, Cajal and PML bodies have liquid-like properties, and they are suggested to be formed via liquid–liquid phase separation (LLPS) (reviewed in a study by Alberti et al. [75]), [76,77]. Interestingly, heterochromatic domains, enriched in H3K9me3 mark, such as the chromocenters, are proposed to phase separate, through HP1 oligomerization [76,77]. Considering the suggested roles of HP1 in DSB repair, phase separation of heterochromatin could be a mechanism that would control the accessibility of those domains to DNA repair factors, either by retaining or excluding them from the domain. Moreover, as it was recently shown that histone modifications contribute to phase separation to regulate chromatin compartmentalization [78], these modifications could also facilitate the response to the DNA damage. Last but not least, there is growing evidence that DNA repair foci are phase-separated after poly-ADP–ribose (PAR)–seeded liquid demixing [79]. More specifically, poly-ADP–ribose polymerase is forming PAR chains which together with FUS and other disordered RNA-binding proteins give the LLPS properties to these foci [79]. This event could be further supported by the fact that PAR marks increase at sites of DNA damage. Two recent studies indicate that 53BP1 foci phase separate and behave like liquid droplets, by fusion and fission events in a highly dynamic way [52**], which involves RNA molecules [28**](Figure 2c). It is possible that other DSB repair proteins are behaving in the same way, and this could also explain their original exclusion from other LLPS compartments, such as Rad51 from the heterochromatin domains. Obtaining more knowledge on this new topic will provide novel insights into fundamental questions on DSB repair. How important are the pre-existing biophysical properties of the nuclear compartments and the ones that are occurring after the DSB induction? An exciting question, which is gaining more and more attention and gives rise to a new the DNA repair field.

Conflict of interest statement

Nothing declared.

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Review 2: How to maintain the integrity of the repetitive genome

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INTRODUCTION

Because of their repetitive nature, tandemly repeated sequences such as centromeres, pericentromeres, ribosomal gene (rDNA) arrays and telomeres are highly prone to genomic instability ((Bzymek and Lovett, 2001; Mladenov et al., 2016). Those regions are extremely vulnerable to form DNA double-strand breaks (DSBs) and to improper DNA damage repair. Indeed breaks occur more frequently at repetitive regions than at other regions of the genome ((Knutsen et al., 2005), that is predicted to be partly due to the highly repetitive nature of the underlying DNA. DSBs can be fixed by two major mechanisms: nonhomologous end joining (NHEJ) and homologous recombination (HR) (Ciccia and Elledge, 2010). NHEJ ligates broken ends together, often resulting in small insertions and deletions at the break site and is therefore considered error prone. HR repair involves end resection of the DSB ends resulting in a single-stranded DNA sequence that can invade homologous sequence on the sister chromatid or homologous chromosome to repair in an error-free manner. However, the presence of up to millions of homologous repetitive sequences from different chromosomes renders the repair challenging. Indeed, recombination with a homologous repeat in cis or on nonhomologous chromosomes can result in abnormalities such as indels and chromosomal translocations. Moreover, the repetitive sequences are prone to form unusual secondary structures such as R-loops, harpin and G-quadruplexes, that are major sources of DNA damage and replication errors. These deleterious consequences are at the origin of many diseases. Here we will review the recent findings in how the genome is maintained in repetitive regions, with a focus on their integrity, repair, consequences of their instability and transcription and replication errors arising from these repetitive regions. Interestingly, a common feature of all these regions that has started being characterized recently, is that they are located in nuclear compartments which behave like membrane-less organelles through a liquid-liquid phase separation (LLPS). We will discuss the recent findings in this field and how the physical properties of these domains could also impact their integrity, as it has been reported that DNA repair foci themselves exhibit LLPS properties.

CENTROMERES

1. Integrity

In most eukaryotes, centromeres are composed of repetitive DNA sequences (Garavís et al., 2015a; McKinley and Cheeseman, 2016) and play an essential role in proper chromosome segregation during cell division as being the site of kinetochore assembly (Cheeseman, 2014). A recent study has reported sister chromatid exchange at human centromeric repeats at the

frequency of 5% in primary tissue culture cells, and higher levels in several cancer cell lines and during replicative senescence (Giunta and Funabiki, 2017). Centromere has been shown to be often the breakpoint of translocation (Wang et al., 2009), suggesting that DSBs type of damage at this site is not rare. The spindle-induced tensional forces during mitosis and mitotic spindle defects at the origin of lagging chromosomes and chromosomes bridges, are sources of fragility for centromeres (Guerrero et al., 2010) and can be at the origin of DSBs formation. Indeed, formation of lagging chromosomes has been linked to the accumulation of DNA damage markers, such as γH2AX, MDC1, 53BP1, and activation of the ATM/Chk2 response (Janssen et al., 2011). Repair of these DSBs through recombination-dependent pathways may disrupt centromere integrity in several ways, either by leading to sister chromatid exchange (Giunta and Funabiki, 2017), or by creating dicentric and acentric chromosomes when the homologous template is another chromosome, or, when the template is a repeat in cis, by resulting in DNA excision and loss of repeats. All these rearrangements can affect the formation of a functional kinetochore during mitosis, leading in turn to aneuploidies (Thompson et al., 2010). Moreover, centromere intrinsic instability is thought to be the cause of chromosomal rearrangements in mammalian cells (Simi et al., 1998) and of breakage-fusion cycles observed in some tumors (Martinez-A and van Wely, 2011). Besides, during DNA replication, the repetitive centromeric chromatin can forms complex secondary structures (Aze et al., 2016; Garavís et al., 2015a) and induce a stalled fork. This could make this region prone to replication errors and recombination events.

The role of centromeric repeats in centromere integrity is still not yet well understood. Although changes in the length of the repeats can affect centromere integrity (Jaco et al., 2008), centromere repeats are subjected to recombination events to suppress chromosomal rearrangement (Nakamura et al., 2008). This recombination can lead to loops formation that have an important role in the establishment of functional centromere (McFarlane and Humphrey, 2010), suggesting that recombination is essential for centromere maintenance. Moreover, even non satellite DNA can form functional neocentromeres implying that the repeats are neither sufficient nor required for centromere function (Warburton, 2004).

A key factor in centromere integrity is CENP-A, a H3 histone variant present exclusively at centromeres (Earnshaw and Rothfield, 1985). The overexpression of CENP-A is a major cause of centromere instability and a key link between centromere and cancers (Amato et al., 2009; Li et al., 2011; Tomonaga et al., 2003; Zhang et al., 2016b). Indeed, CENP-A overexpression correlates with a variety of tumors (Hu et al., 2010; Li et al., 2011; Qiu et al., 2013; Rajput et al., 2011; Sun et al., 2016; Tomonaga et al., 2003; Wu et al., 2012). One hypothesis suggests that

CENP-A overexpression leads to its erroneous incorporation at ectopic loci (Athwal et al., 2015; Lacoste et al., 2014; Nechemia-Arbely et al., 2017, 2019; Nye et al., 2018) in mammalian organisms, *Drosophila* and yeast (Choi et al., 2012; Gascoigne et al., 2011; Mendiburo et al., 2011), and can promote aneuploidy (Amato et al., 2009). It has been suggested that this overexpression and mis-localization prevent normal kinetochore assembly and thus alters proper chromosome segregation (Tomonaga et al., 2003). On the other side, CENP-A loss correlates with a drastic increase in centromere aberrations and leads to excision of centromeric repeats (Giunta and Funabiki, 2017).

2. Repair

Centromeric DSBs repair is still largely not understood. It has been proposed that DSB repair in centromeres is substantially improved compared to the repair in average genomic locations (Rief and Löbrich, 2002). This study suggests a faithful non-homologous end joining (NHEJ) dependent repair of centromeric DSBs, that would frequently rejoin correct break ends since centromeric DNA is not very dynamic (Rief and Löbrich, 2002). Yet our group has recently revealed that mouse centromeric DSBs can use both NHEJ and HR repair pathways throughout the cell cycle. Indeed, centromeric DSBs activate both DNA end resection and RAD51 recruitment, main HR repair steps, even in the absence of the sister chromatid in G1 (Tsouroula et al., 2016), with the hypothesis that a repeat in *cis* can be used as a template when the sister chromatid is absent. This mechanism would explain how centromeric recombination exist, is probably at the origin of the repetitive nature of the centromere and is necessary for the establishment of a functional centromere (McFarlane and Humphrey, 2010; Talbert and Henikoff, 2010). But it is important to mention that recombination at centromere is normally repressed during meiosis (Choo, 1998; Kuhl and Vader, 2019). Importantly, HR in *cis* does not necessarily result in the loss of repeats. Indeed, HR via synthesis dependent strand annealing (SDSA) or dissolution of **double Holliday junctions** by **branch migration** can mediate non-crossover repair (Karow et al., 2000; Ranjha et al., 2018).

Centromeres are characterized by a peculiar chromatin organization. Indeed, they present active chromatin marks, H3K36me2 and H3K4me2 (Chan and Wong, 2012) and are the unique structures containing CENP-A. It is possible that the presence of the mark H3K36me2 serves as a platform for H3K36me3, that promotes end-resection by recruiting CtIP through LEDGF (Aymard et al., 2014). Together with a preferential binding at centromeres of DNA2 (Li et al., 2018b) and centromeric recruitment of BLM during anaphase to allow proper segregation (Ke et al., 2011a), the centromeric environment might help creating a permissive region for resection.

Moreover, H3K4me2 role in centromeric transcription (Molina et al., 2016) contribute to centromere function and stability (McNulty et al., 2017; Quénet and Dalal, 2014) and CENP-A has been shown to increase tolerance to damage when overexpressed (Lacoste et al., 2014), and to re-localize to sites of DNA damage (Zeitlin et al., 2009), further highlighting that the repair depends on the environment of the break. In addition, a recent study has shown that centromeres can undergo recombination in primary human cells (Giunta and Funabiki, 2017) and that there may be active mechanisms to suppress centromeric recombination, involving core centromeric factors such as CENP-A and other CCAN proteins.

In yeast, centromere repeats promote break-induced replication (BIR) even in the absence of stress conditions (Nakamura et al., 2008; Tinline-Purvis et al., 2009). It was proposed that RAD51 and RAD52 control centromere fork stall and restart by directly controlling CENP-A deposition since it acts as a physical barrier for fork progression (Mitra et al., 2014). However, due to the highly repetitive nature of the centromere, recombination there may be particularly dangerous, as mentioned previously, since the HR pathway may use a repeat in cis or a repeat from a non-homologous chromosome. Indeed, this can lead to different forms of genomic instability such as aberrant centromere length and unequal or erroneous exchanges, that may directly impact genome integrity (Charlesworth et al., 1994).

3. Consequences of instability

Centromeric instability can be at the origin of different types of genomic rearrangements (Thompson et al., 2010), leading to different diseases including developmental disorders, premature aging and cancer(Barra and Fachinetti, 2018; Beh and Kalitsis, 2017). Centromeric instability can be at the origin of aging and cellular senescence, notably through the reduction or mislocalization of CENP-A (Hédouin et al., 2017; Lee et al., 2010b; Maehara et al., 2010), that might be mediated by changes in the transcription of the centromeric repeats (Giunta and Funabiki, 2017). Persistent centromeric DSBs have been also associated with senescence arrest (Hédouin et al., 2017). Centromeres are known to be often sites of aberrant rearrangement in cancers (Mitelman et al., 1997; Padilla-Nash et al., 2001). Among the variety of cancers displaying centromeric rearrangements, oral squamous cell carcinoma presents isochromosomes and unbalanced whole-arm translocations originating from centromeric breakpoints (Hermsen et al., 1996). Head and neck squamous cell carcinoma exhibits whole-arm translocations with again breakpoints close to the centromere (Martínez et al., 2012). Retinoblastoma is characterized by gain or loss of chromosome arms originating at centromeres (Chen et al., 2001). Various cancerous cell lines present centromeric recombination and other abnormalities in a manner

unrelated to chromosome missegregation (Giunta and Funabiki, 2017) as karyotypically stable cells also present centromeric rearrangements (Thompson and Compton, 2011) and misregulation during replication and in DNA damage repair (Takemura et al., 2006).

When centromeric rearrangement disrupt centromere function, a neocentromere assembles a functional kinetochore elsewhere on the remaining chromosome in order to restore the ability of the chromosome to segregate properly (Blom et al., 2010; Marshall et al., 2008a). Such neocentromere formation associated with chromosomal rearrangements is found in patients with developmental disorders (Burnside et al., 2011; Marshall et al., 2008a; Mascarenhas et al., 2008) and in many types of cancers including retinoblastoma (Morrissette et al., 2001), non-Hodgkin's lymphoma (Blom et al., 2010), acute myeloid leukemia (de Figueiredo et al., 2009), and lung cancer (Italiano et al., 2006). Interestingly, CENP-A is overexpressed in different types of cancer (Amato et al., 2009; Tomonaga et al., 2003; Wu et al., 2012). This CENP-A overexpression may enhance its ectopic incorporation and thereby facilitate neocentromere formation.

NUCLEOLUS

1. Integrity

The nucleolus is a subnuclear compartment hosting the transcription of the ribosomal DNA, that is located on the short p-arms of the five human acrocentric chromosomes (13, 14, 15, 21, 22) and is organized in clusters of tandem repeats forming the nucleolus organizer regions (NOR). Each unit on each chromosome contains a coding region with 18S, 5.8S, and 28S RNA genes. The rest of rDNA array display a non-coding intergenic spacer (IGS) region organized in repeats (McStay and Grummt, 2008). As a repetitive sequence, the rDNA is one of the most fragile regions of the genome and its instability can affect cellular functions such as senescence (Kobayashi, 2008; Tchurikov et al., 2015). NORs have been identified as hotspots for DSBs (Tchurikov et al., 2015). In human cells, the chromosomes carrying ribosomal DNA undergo translocations more frequently than other chromosomes (Denison et al., 2002; Therman et al., 1989). It has been suggested that the hotspots of DSBs in rDNA array coincide with the major CTCF binding sites and H3K4me3 marked regions. These regions are often located near to regions that possess specific epigenetic marks, including pericentromeres that also present hotspots of DSBs. This suggests that rDNA instability and breakage relate to different mechanisms of epigenetic regulation and 3D conformation (Tchurikov et al., 2015). rDNA cluster length presents a striking variability between and within human individuals (Stults et al., 2008), probably because of DSBs-induced homologous recombination with a repeat in cis. It has

been shown that in about 54% of solid tumors, rDNA clusters are altered before the start of the tumor expansion (Stults et al., 2009).

2. Repair

When induced by ionizing radiation (IR), rDNA DSBs result in ATM-dependent inhibition of Pol-I transcription, leading to nucleolar reorganization (Kruhlak et al., 2007) in an NBS1/treakle-dependent manner (Larsen et al., 2014). This reorganisation corresponds to the rDNA arrays relocation to the periphery of the nucleolus forming nucleolar caps that contain nucleoplasmic proteins. Similarly, rDNA DSBs induction by I-PpoI or CRISPR/Cas9 results in inhibition of RNA Pol-I transcription in a ATM-dependent manner, leading to reorganization of the nucleolus and nucleolar caps formation (Harding et al., 2015; van Sluis and McStay, 2015; Warmerdam et al., 2016). Relocation of rDNA breaks is suggested to be necessary for the accessibility of repair factors. Most DNA damage response (DDR) factors except NBS1 are mainly detected at the nucleolar caps after relocation (Korsholm et al., 2019). Both 53BP1, favoring NHEJ, and BRCA1, promoting HR, are recruited to the same nucleolar caps (van Sluis and McStay, 2015) with a preferential use of HR throughout the cell cycle (Harding et al., 2015), even in G1 phase of the cell cycle when the sister chromatid is absent. The most likely explanation is that undamaged rDNA repeats in *cis* can be used as a template in G1, similarly to the hypothesis suggested for centromeres.

Although rDNA DSB relocation in nucleolar caps was assumed to be dependent of the transcriptional inhibition, a recent study has reported that relocation and transcriptional inhibition can be uncoupled and that cohesin and human silencing complex control transcriptional repression in response to DNA damage (Marnef et al., 2019). In this process, cohesin acts at an early step of the DSB response and human silencing complex is mediating H3K9me3 to ensure the complete transcriptional shutdown.

In yeast, the repeats are essential for DNA damage repair and there loss leads to more sensitivity to DNA-damaging agents (Bartke et al., 2010). HR repair in the rDNA array has been suggested to result in loss of up to 90% of repeats (Warmerdam et al., 2016). But since nucleolar caps concentrate all the repeats of a single NOR in close proximity to each other, they may limit interchromosomal recombination and translocations.

In *Drosophila*, nuclear actin has been shown to form filaments to ensure the interaction of the rDNA DSBs with the nuclear envelope (Korsholm et al., 2019), mediated by NE invaginations (Caridi et al., 2018; Marnef et al., 2019). This event happens downstream of transcriptional

repression and is promoted by the SUN1 subunit of the linker of nucleoskeleton and cytoskeleton complex together with ARP3 and UNC-45, which are actin and myosin regulators (Marnef et al., 2019). This movement has been suggested to facilitate HR (Schrank et al., 2018).

3. Consequences of instability

Changes in rDNA repeat copy number is at the origin of instabilities seen in premature ageing, neurodegenerative disorders and cancer (Hallgren et al., 2014; Killen et al., 2009; Stults et al., 2009). Aged cells often show genome instability. Recent evidence suggests reduction of rDNA repeats as a feature of human aging (Ren et al., 2017; Zafiropoulos et al., 2005). For example, cells from patient having Bloom syndrome (mutated BLM) or Ataxia Telangiectasia (mutated ATM) present a high variability of the rDNA copy number due to mitotic hyper-recombination (Hallgren et al., 2014; Killen et al., 2009). Another possible model explaining how rDNA instability leads to cellular senescence is that DSBs induced recombination events can cause an accumulation of repair enzymes at the locus, which will activate the damage checkpoint control and block the cell cycle progression, inducing senescence (Kobayashi, 2008). Age-related neurodegenerative disorders, such as Hodgkin's disease (MacLeod et al., 2000) and Parkinson's disease (Rieker et al., 2011), show a deregulation in rDNA transcription and nucleolar function. Moreover, rDNA copy number variability has been associated with neurodegeneration. Indeed, an increased number was detected in patients with dementia with Lewy bodies (Hallgren et al., 2014) and elevated repeats of the 18S rDNA locus combined with increased silent chromatin marks in Alzheimer's disease patients (Pietrzak et al., 2011). Robertsonian translocations (ROBs) correspond to whole-arm translocation and centromeric fusion, as it happens for chromosome 21 in Down syndrome. ROBs in humans occur in the five chromosomes bearing NORs, suggesting that the hotspots of DSBs in rDNA arrays might provide the molecular basis for ROB (Tchurikov et al., 2015). Misregulation of ribosome biosynthesis is a recurrent feature in cancer and in rare genetic diseases as ribosomopathies (Narla and Ebert, 2010). Recent evidences suggest that many key proto-oncogenes and tumor suppressors play a direct role in the nucleolus and in RNA polymerase I transcription, and nucleolar misregulation is an important contributor to cancer (Derenzini et al., 2017; Diesch et al., 2014; Hannan et al., 2013; Orsolic et al., 2016). Moreover, enlarged and higher number of NORs correlate with an increased cell proliferation rate and tumor prognosis (Derenzini and Trerè, 1991; Gani, 1976).

PERICENTROMERES

1. Integrity

The pericentromeric heterochromatin domains are composed of different satellite subfamilies (HSATII, HSATIII, sn5, and β- and γ-satellite) in human cells (Miga, 2015), and of major satellite repeats in mouse cells. Chromosome rearrangements and breaks often involve pericentromeric regions, with a frequency of up to 40–60% in certain cancer cell lines such as colorectal carcinomas and adenocarcinomas derived cells (Knutsen et al., 2005, 2010). The distinction between pericentromeric and centromeric regions is often hindered because of the difficulties in aligning sequencing reads of repetitive DNA. Thus, instability assigned to centromeres can partly involve pericentromeric instability, and several cases of translocation involving centromeres in various tumors might involve pericentromeres.

Analyses of cancer tissues have shown that pericentromeric heterochromatin regions are more prone to translocations and copy number changes (Cramer et al., 2016; Hermsen et al., 1996; Jin et al., 2000). Rearrangements within the pericentromeric repeats seem to disrupt not only the local heterochromatin but also contribute to long-range changes to gene expression (Fournier et al., 2010). Changes in heterochromatin components can alter the nuclear compaction of pericentromeric repetitive DNA sequences, thereby increasing susceptibility to DNA damage. Changes in heterochromatic histone modifications can also directly affect DNA damage repair efficiency since many histone modifications have been implicated in promoting or inhibiting the recruitment of specific repair proteins (Price and D'Andrea, 2013), and can result in aberrant rates of transcription contributing to instability.

A key factor in pericentromere integrity is HP1. HP1 is essential for CENP-A assembly at centromeres in fission yeast (Folco et al., 2008) and for proper kinetochore-microtubule attachments and chromosome segregation in mitosis (Ekwall et al., 1995, 1996). In addition, yeast HP1 is required for sister chromatid cohesion (Nonaka et al., 2002; Yamagishi et al., 2008). In mammalian cells, HP1α helps mediate the assembly and maintenance of cohesin complexes (Kang and Lieberman, 2011; Perera and Taylor, 2010), and recruits and binds INCENP necessary for proper kinetochore-microtubule interactions in mitosis (Abe et al., 2016; Ainsztein et al., 1998; Kang and Lieberman, 2011; Perera and Taylor, 2010). Loss of H3K9 methyltransferases or HP1 homologs results in an increase in chromosome segregation errors in mice, Drosophila, and fission yeast (Ekwall et al., 1995, 1996; Peng and Karpen, 2009; Peters et al., 2002), strongly connected with cancer progression.

2. Repair

Pericentromeric repetitive sequences are tightly packaged, thus creating a very particular environment that needs a specialized repair process. Because of the extreme compaction of the heterochromatin, it was hypothesized that the access of repair factors is restricted. Thereby, heterochromatin needs to decondense at damage induced sites to allow repair machinery recruitment. HP1 has been shown to be removed from chromatin upon DSB induction, unmasking the H3K9me3 histone modification and allowing the recruitment of the histone acetyl transferase (HAT) Tip60 that interacts with H3K9me3 (Sun et al., 2009) and that has been proposed to facilitate nucleosome removal and resection. In addition, ATM phosphorylates KAP1 on serine 824, leading to the release of the remodeler CHD3, thus facilitating chromatin relaxation (Noon et al., 2010). It was later proposed that the checkpoint kinase Chk2 phosphorylates KAP1, which will further disrupt KAP1 interaction with the phosphorylated HP1b and lead to chromatin decompaction (Bolderson et al., 2012; Kalousi et al., 2015). However, our recent data suggest that the chromatin expansion following DSBs is not a consequence of the eviction of heterochromatin proteins or repressive marks (Tsouroula et al., 2016). In contrast, we found that following DSBs induction at pericentromeres, H3K9me3, HP1s and KAP1 intensity are increased in G2. Moreover, these results agree with previous studies showing a local increase of H3K9me3 in chromatin surrounding a single euchromatic DSB (Ayrapetov et al., 2014), and recruitment of HP1s and KAP1 at lesions involved, for example, in the stimulation of DNA end resection (Soria and Almouzni, 2013). In addition, HP1γ has been shown to help recruiting cohesin and BRCA1 to DSBs (Oka et al., 2011; Wu et al., 2015).

To prevent potentially dangerous recombination between repetitive sequences, heterochromatic regions have evolved specialized temporal and spatial responses to safely repair DSBs. Initially found in budding yeast and Drosophila (Chiolo et al., 2011; Torres-Rosell et al., 2007) and later demonstrated to also occur in mammals (Tsouroula et al., 2016), pericentromeric DSBs relocate to the periphery of heterochromatin core domain or nuclear periphery (Caridi et al., 2017). In *Drosophila*, pericentromeric DSBs induced by irradiation have been shown to relocate to the periphery of the heterochromatin domains (Chiolo et al., 2011) and later to the nuclear envelop (Ryu et al., 2015) in a process dependent on HP1 and its interactors, the SMC5/6 complex, the histone demethylase dKDM4A,(Caridi et al., 2018; Chiolo et al., 2011; Colmenares et al., 2017; Ryu et al., 2015), on Suv39H1-mediated H3K9me3, on Slx5/Slx8 SUMO-targeted ubiquitin ligation (STUbLs) (Horigome et al., 2016), on the actin and myosin network (Caridi et al., 2018), and on the chromatin relaxation(Caridi et al., 2018; Janssen et al., 2016). RAD51 was shown to be recruited only after DSB relocation at the periphery of the domain and to be mutually exclusive

with HP1α (Chiolo et al., 2011; Janssen et al., 2016). More recently, our lab has demonstrated in mouse cells that DSBs are positionally stable in G1 and recruit NHEJ factors, while in S/G2 phase, breaks relocate at the periphery of the heterochromatin domain to use HR, independently of chromatin relaxation (Tsouroula et al., 2016), but dependent on active exclusion of RAD51 from the heterochromatin core and on prior end resection (Tsouroula et al., 2016), indicating that early steps of the HR repair pathway are required to initiate DSB relocalization.

These findings suggest that physical constraints, imposed by heterochromatic organization of pericentromeric repeats, on the DNA damage response machinery are alleviated through either reorganization of local chromatin structure, or relocalization outside the core domain or at the nuclear periphery.

3. Consequences of instability

Instability in pericentromeric repeats has been implicated in many diseases and especially cancer progression. For example, head and neck squamous cell carcinoma presents pericentromeric DSBs (Martínez et al., 2012). Hematologic malignancies show a high frequency of the isochromosome i(17)q, coming from pericentromeric DSBs probably repaired by non-allelic homologous recombination (NAHR) (Barbouti et al., 2004).

Cancer progression and metastasis are associated with changes in the distribution of H3K9me2/me3 and HP1 at pericentromeric regions(De Koning et al., 2009; Dialynas et al., 2008; Slee et al., 2012; Vad-Nielsen et al., 2016) , and loss of these histone methylations results in an increased rate of tumorigenesis in mouse models (Braig et al., 2005; Peters et al., 2002). Immunodeficiency, Centromeric region instability, Facial anomalies syndrome (ICF) is characterized by mutations of DNMT3B, ZBTB24, CDCA7, or HELLS (Thijssen et al., 2015; Weemaes et al., 2013) and correlates with loss of DNA methylation, pericentromeric breaks, and rearrangements near the centromere with consequent whole-arm deletion. Impairment of DNMT3B interaction with CENP-C causes an overproduction of centromeric and pericentromeric transcripts (Gopalakrishnan et al., 2009).

Moreover, many cancers and genetic disorders are characterized by the transcriptional misregulation of the SatII and SatIII pericentromeric satellite sequences, and altered epigenetic state of pericentromeric chromatin (Eymery et al., 2009; Shumaker et al., 2006; Ting et al., 2011; Zhu et al., 2011).

TELOMERES

1. Integrity

Telomere integrity has an essential role in maintaining the genome stability, as their main function is to prevent the recognition of the natural ends of linear chromosomes as sites of DNA damage(Denchi and de Lange 2007). Human and mouse telomeres are composed of tandem repeat arrays of 5'-TTAGGG-3' sequences, which terminate in a single-stranded G-rich overhang, able to form secondary structures, called t-loops(Greider 1993, Doksani, Wu et al. 2013). Those repeats are bound by a six-subunit complex, Shelterin, which comprises the proteins TRF1, TRF2, POT1, TIN2, TPP1 and RAP1(de Lange 2005). Shelterin is repressing the DDR at telomeres by TPP1/POT1 mediated inhibition of ATM and ATR activation (Zimmermann, Kibe et al. 2014), and moreover, the TRF2 subunit protects them from NHEJ that could potentially lead to chromosome fusions, by ensuring the formation of a t-loop that will "hide" the telomere terminus(Doksani, Wu et al. 2013, Okamoto, Bartocci et al. 2013). This processing leads to the inability of DNA polymerases to duplicate the telomeres, which results to their shortening by approximately 50-100 bp on each cell division (Harley, Futcher et al. 1990). Cells have developed different telomere maintenance mechanisms, to avoid the limitation of their divisions' number that would arrest them in replicative senescence. To this end, stem cells and germ cells use the telomerase reverse transcriptase (TERT) enzyme that carries a telomerase RNA component (TERC) which is used as a template for *de novo* telomeric DNA synthesis (Greider and Blackburn 1985, Greider and Blackburn 1989, Mocellin, Pooley et al. 2013). Another telomere maintenance mechanism that is used mainly by cancer cells is the Alternative Lengthening of Telomeres (ALT) system (Bryan, Englezou et al. 1995, Bryan, Englezou et al. 1997, Dunham, Neumann et al. 2000, Pickett and Reddel 2015). ALT is used by 10% of cancers and is an HR- mediated maintenance pathway that is characterized by the formation of a PML body that contains DNA repair proteins at telomeres (ALT-associated PML bodies, APBs)(Cesare and Reddel 2010, Henson and Reddel 2010). ALT cells luck telomerase, and often ATRX protein, while in parallel show elevated numbers of TERRA RNA (Heaphy, de Wilde et al. 2011, Lovejoy, Li et al. 2012, Arora, Lee et al. 2014, Episkopou, Draskovic et al. 2014).

Failure of telomere maintenance and continued telomere shortening, lead to the presence of several dysfunctional telomeres able to cause genomic instability which is referred as telomere crisis (Artandi, Chang et al. 2000, Artandi and DePinho 2010, Maciejowski, Li et al. 2015). Consequently, many unprotected chromosome ends are formed and the chance that two dysfunctional telomeres find each other and fuse increases, and event that is characterized by p53 and RB pathways loss. We can observe then end-to-end fused dicentric chromosomes that lead

to genomic instability through mitotic mis-segragation(Hayashi, Cesare et al. 2012, Hayashi, Cesare et al. 2015) reviewed in (Maciejowski and de Lange 2017). Telomere fusions have been shown to occur by alt-NHEJ pathway both in mouse models and human cancer (Capper, Britt-Compton et al. 2007, Maser, Wong et al. 2007, Lin, Letsolo et al. 2010, Jones, Oh et al. 2014, Oh, Harvey et al. 2014). Except of the dicentric chromosome formation, many other genomic alterations occur due to cells under telomere crisis. For instance, breakage of dicentric chromosomes can be followed by a second fusion of the broken ends, referred as breakage-fusion-bridge (BFB) cycle. BFB cycles can give rise to translocations, when a broken DNA end invades to another chromosome and through break induced replication, copies a part of it(McClintock 1939, Riboni, Casati et al. 1997, Artandi, Chang et al. 2000, Gisselsson, Pettersson et al. 2000, Murnane 2006, Roger, Jones et al. 2013) .Moreover, daughter cells could inherit a broken dicentric chromosome, losing their heterozygosity. A very frequent telomere crisis event especially in cancers is the telomere-driven tetraploidy (reviewed in (Davoli and de Lange 2011)). In detail, persistant ATM and ATR signaling at damaged telomeres is able to prolong G2 phase, which results in an early entry into G1 after completely bypassing mitosis. This results to the reduplication of the genome in the next S phase, and tetraploidy(Davoli, Denchi et al. 2010, Davoli and de Lange 2012) . A potential reactivation of telomerase or the ALT pathway could help the cells overcome the telomere crisis and improve again the genome stability. Being able to cause several types of genomic alterations, preserving telomere integrity is crucial for the cells.

2. Repair

PML bodies are known to be associated with the Alternative Lengthening of Telomeres (ALT) pathway, which is a homologous recombination based pathway that is used by cells to maintain the integrity of their telomeres (Chung, Osterwald et al. 2012). During this process, many telomeres of different chromosomes are gathered on a PML body, forming APBs. Those bodies are suggested to maintain telomeres by changing their chromatin state to trigger ATM phosphorylation and therefore inducing a DNA damage response. Some years ago, it was reported that DSBs in ALT telomeres of U2OS cells can direct the movement and clustering of telomeres at the APBs. There, the breaks are being repaired by HR in a Rad51 and Hop2-MND1 dependent manner(Cho, Dilley et al. 2014). A more recent study has shown that in normal human fibroblasts, the depletion of PML was able to lead to chromosomal abnormalities and senescence, indicating the physiological role of these bodies in the stability of the genome(Marchesini, Matocci et al. 2016). Except from those effects it was recently reported that elongation of telomeres can happen during mitosis in the APB foci, and this is called mitotic DNA synthesis (MIDAS), a process that is mediated by BLM and Rad52(Ozer, Bhowmick et al. 2018, Min,

Wright et al. 2019). Concerning PML bodies, it was also shown that the depletion of the PML resulted in the decrease of HR (Yeung, Denissova et al. 2012). Moreover, it was reported that using IR in PML disrupted cells resulted in a delayed DSB response, revealed by the disappearance of gH2AX and 53BP1 foci(di Masi, Cilli et al. 2016). Collectively the above studies suggest that PML NBs are a nuclear compartment that favors HR and ensures the completion of the ALT-telomeres lengthening mechanism at the APBs.

3. Consequences of instability

The unique structure of telomeres and specifically the shortening of human telomeres, are highly linked to cancer development. Interestingly there are two opposite possible effects of telomere shortening, either suppression or progression of the tumor. The tumor suppressor pathway contains the silencing of the TERT component, which is able to inhibit telomerase activity, resulting to shortening of telomeres. As the telomeres lose their functionality, a process called replicative arrest and senescence is induced. In these cells Shelterin loading is insufficient and DDR is elevated at the shortened telomeres and they are led to senescence. Moreover, studies in mice demonstrated the limitation of tumor formation due to telomere shortening, when the p53 pathway is functional. Last but not least, it was shown that in leukemia and B cell lymphoma, the length of telomeres is longer, supporting the hypothesis of telomere shortening as a tumor suppressor mechanism. On the other hand, shortening of telomeres and failure of telomerase activation leads to telomere crisis, as described in [paragraph1]. Obviously, the high levels of chromosome rearrangements and genomic instability, are capable of leading to cancer initiation and progression.

TRANSCRIPTION AND REPLICATION ERRORS IN REPETITIVE REGIONS

Repetitive sequences are generally unstable and might form secondary structures that could induce replication fork stalling and high levels of recombination, that could in turn lead to DNA breakage (Branzei and Foiani, 2010; Pearson et al., 2005; Zhao et al., 2010).

Centromeric repeats are transcribed, producing non-coding RNA (Bouzinba-Segard et al., 2006; Hédouin et al., 2017; Quénet and Dalal, 2014). This transcription is suggested to contribute to centromere architecture and function (McNulty et al., 2017; Quénet and Dalal, 2014) and to RNA:DNA hybrids (R-loops) formation; and is therefore important for centromeric integrity (Castellano-Pozo et al., 2013; Kabeche et al., 2018). R-loops were previously reported to form at centromeres in mitosis, and to recruit RPA and ATR in order to stimulate Aurora B that will promote faithful chromosome segregation (Kabeche et al., 2018). But an increased expression of

centromeric repeats in mammalian cells can also compromise centromere structure and induce mitotic spindle defects and chromosome missegregation in mitosis(Bouzinba-Segard et al., 2006; Zhu et al., 2011). Loss of the H3K9 methyltransferases results in upregulated transcription of pericentromeric repeats that form R-loop and higher rates of insertions and deletions specifically at these repeats normally enriched for H3K9me2/me3 (Zeller et al., 2016). These R-loops can produce DNA damage by obstructing the progression of the replication machinery, leading to fork collapse and DSB formation (Aguilera and García-Muse, 2013). Satellite II pericentromeric repeats transcription has notably been associated to cancerous cells (Bersani et al., 2015).

Except from that, several DNA structures have been identified within the alpha-satellite of human centromeres, including single-stranded DNA, hairpins, triplexes, R-loops, and i-motifs(Aze et al., 2016; Garavís et al., 2015b, 2015a; Jonstrup et al., 2008; Kabeche et al., 2018; Ohno et al., 2002; Zhu et al., 1996). These complex secondary structures are formed during centromeric replication and transcription, and can be originated from replication-transcription conflicts, which represent a particularly vulnerable time to DNA damage and instability.

Inverted repeats (IRs) are mainly enriched in centromeres and rDNA arrays, while G-quadruplexes are particularly enriched in telomeres and centromeres (Čutová et al., 2020). Indeed, the guanine-rich sequence of both telomere and centromere can form a quadruplex based on G-quartets while the complementary cytosine-rich strand can fold into an intercalated tetramer called the i-motif.

Due to the repetitive nature of their DNA sequences, it is likely that repetitive regions have a highly complex DNA topology, which may lead to accumulation of DNA catenanes and formation of DNA loops between their repetitive sequences. During mitosis, centromeric DNA strands intertwine as a natural consequence of DNA replication, causing an accumulation of catenanes at centromeric regions. These structures play a role in preventing premature sister chromatid disjunction (Wang et al., 2010), but during anaphase they need to be resolved to preserve centromere stability. This catenated DNA also leads to the formation of a particular class of nucleosome-free DNA bridges defined centromeric ultra-fine bridges (cUFBs) (Chan et al., 2007).

Together with the ribosomal DNA (rDNA) and the chromosome fragile sites, telomeres are among the most challenging and difficult loci to replicate (Gadaleta and Noguchi 2017). Several features of their unique structure could potentially disturb the replication fork progression. For example, improper disassembly of the t-loop, or even the formation of other secondary structures, like G-quadruplexes could block the progression of the fork. Moreover, the replisome could be disturbed by the binding of the shelterin complex, but also from the formation of R-loops

associated with TERRA. All these make telomeres sensitive to replication stress (Lazzerini-Denchi and Sfeir 2016, Higa, Fujita et al. 2017). Several factors have been shown to be involved in the replication fork restart. In ALT cells, SMARCAL1, an ATP-dependent DNA-annealing helicase was shown to be enriched and able to inhibit ALT activity by initiating fork regression (Betous, Mason et al. 2012, Poole, Zhao et al. 2015, Cox, Marechal et al. 2016). Another factor that is implicated in alleviating replication stress in ALT telomeres is FANCM. Its ability to recruit other fanconi anemia-associated proteins leads to the initiation of replication fork reversal and thus restart of replication (Pan, Drosopoulos et al. 2017). Failure to restart a stalled replication form leads to the collapse of the fork, creating a DSB, which undergoes end resection process, mediated either by BLM-EXO1-DNA2 or the MRN complex. The resected ends have been shown to either be bound by PARP1 which lead to alt-NHEJ repair of the DSB, or to invade directly either another telomere or even itself and perform HR (Reviewed in (Sobinoff and Pickett 2017)).

PHASE SEPARATION

Growing evidence during the last years support the existence of liquid liquid phase separation (LLPS) in the nucleus. In general, liquid droplets have the ability to form a boundary at their surface which allows selective passage of molecules, and the existence of this interface has given rise to the possibility that droplets can form as compartments. Several studies indicated that nuclear compartments, including heterochromatin, nucleolus, stress granules, PML bodies etc. behave like membrane-less organelles in the nucleus (Banani, Rice et al. 2016, Shin and Brangwynne 2017). Interestingly, a few recent studies reported that DNA repair foci also exhibit LLPS properties.

1. Heterochromatin

One of the most studied nuclear compartments concerning its LLPS properties is heterochromatin. It was for many years believed that its highly compacted form is sufficient to give it the unique properties of forming distinct, membrane-less domains. Lately, though, more and more research groups support the idea that the formation of heterochromatin domains is mediated by LLPS. Moreover, many reports suggest a role of HP1α in this process. *In vitro* experiments in *Drosophila Melanogaster* showed that hp1a is initially diffused but progressively it can form spherical foci which are able to grow and undergo fusion events. This process requires hydrophobic interactions, and more specifically dimerization of Hp1a at the periphery of the heterochromatin domain (Strom, Emelyanov et al. 2017). Similarly, in mouse cells where

heterochromatin is in structure of chromocenters, FCS number and brightness analysis showed the existence of hp1a dimers (Hinde, Cardarelli et al. 2015) as well as reduced diffusion rates at the borders of the domain(Hinde, Cardarelli et al. 2015, Strom, Emelyanov et al. 2017). Interestingly, the oligomeric state of Hp1a is correlated with the compaction state of heterochromatin, meaning that higher HC compaction leads to the formation of dimers but also tetramers (Hinde, Cardarelli et al. 2015). Deeper analysis on the characteristics of hp1a domains, showed that phosphorylation of its N-terminal extension is important for the HC domain formation (Larson, Elnatan et al. 2017). Except from Drosophila and mouse heterochromatin, the S.Pombe swi6 protein that corresponds to hp1a promotes the HC formation in yeast by reshaping the nucleosome core (Sanulli, Trnka et al. 2019). Even though Hp1a leads to the formation of droplets but also optodroplets in drosophila, mouse and human cells, the stability of those droplets is under discussion in the field. It was recently reported that those optodroplets are not stable, and their characteristics resemble more to polymer globules than to liquid droplets. The characteristic of the polymer globules is that only the concentration of protein changes, but not their size(Erdel and Rippe 2018, Erdel, Rademacher et al. 2020). In both cases, phase separation seems to be the driving force in the formation of HC, with the role of hp1a being still under investigation and debate. Little is known about human heterochromatin that is organized in a different way and is not forming distinct foci. It would be very interesting to identify the properties of hp1a in this case. Does it still form higher oligomers? And do all its domains function similarly to mouse and drosophila variants? Last but not least, RNA from major satellites of mouse HC is shown to be involved in the phase separation of the domain(Huo, Ji et al. 2020). As human satellite repeats differ from major satellites and they are less transcribed, it is possible that this is additionally reducing their ability to form distinct phase separated heterochromatin foci. The fact that mouse and human heterochromatin spatial arrangement is so different, could be explored further in order to identify more factors involved in LLPS. For instance, it is still not known why mouse and *Drosophila* pericentromeric satellite repeats cluster and human do not, and understanding the mechanisms underlying this extremely unexpected feature, would possibly open up new ideas and aspects in the phase separation field.

2. Nucleolus

Nucleolus was the first nuclear compartment that was described as phase separated, in the previous century, in 1946, by Ehrenberg who showed its dependence on the temperature as well as its size and shape correlation with those of the nucleus (Ehrenberg 1946). In the last years this observation was confirmed by several reports. First, it was shown in Xenopus Oocytes that the tension and fluidity of the nucleolar surface could restructure it into spherical droplets upon

mechanical deformations, with F-actin having a role in this process (Brangwynne, Mitchison et al. 2011, Brangwynne 2013). Later, C. Elegance studies showed that the size of the nucleolus is depending on the size of the cell in a concentration of nucleolar components dependent manner (Weber and Brangwynne 2015). In addition, rRNA transcription stabilizes nucleoli by modulating thermodynamic parameters and thus phase separation (Berry, Weber et al. 2015). A more recent study showed that the nucleolus itself contains components that are in different liquid phases both in vivo and in vitro and suggested that phase separation gives rise to multiple layers of liquids, maybe in order to facilitate RNA processing in this compartment (Uppaluri, Weber et al. 2016). Since nucleolus was the first described LLPS compartment of the nucleus, with growing evidence supporting it, its properties were used to define the characteristics of LLPS in the cells. Subsequently, the maintenance of spherical shape, fusion and fission events but also existence of mobile molecules that undergo internal rearrangements were some of the criteria that were proposed and further opened the field (Hyman, Weber et al. 2014).

3. Telomeres (ALT-PML)

Telomeres of cancers that lack telomerease, are utilizing the ALT process, as explained previously. One of the characteristics of ALT, is the clustering of telomeres in PML bodies that are called ALT associated PML bodies (APBs). Similarly to nucleolus, APBs have been shown to contain LLPS separation properties. A study on PML bodies' phase separation showed that they are organized through interaction of small ubiquitin-like modification (SUMO) sites and SUMO-interacting motifs (SIMs) that exist in PML and its associated proteins(Banani, Rice et al. 2016). The stoichiometry of the SUMO and SIM scaffolds is important for the recruitment of the associated proteins for the biomolecular condensate formation (Ditlev, Case et al. 2018). PML bodies share with other condensates of the nucleus, like Cajal bodies, the general characteristic of undergoing liquid-liquid dimixing from the surrounding cytoplasm and nucleoplasm and thus assemble nuclear bodies (Banani, Lee et al. 2017, Wheeler and Hyman 2018). Interestingly, it was shown recently that one of the roles of the nucleoli LLPS is to compartmentalize protein quality of misfolded proteins and other factors in order to achieve their efficient clearance, controlling the genome integrity maintenance (Mediani, Guillen-Boixet et al. 2019). The existence of LLPS in PML bodies but also in other nuclear compartments is nowadays accepted, but its role in the cellular biology and the different processes of the cell, like DNA repair, mitosis etc. would be of great interest to further investigate.

4. DNA repair foci

Having discussed about the instability and repair of different repetitive elements, such as centromeres, pericentromeres, rDNA repeats and telomeres, but also their involvement in phase

separated domains, it is of high important to note that DNA repair foci themselves, probably acquire LLPS properties. Interestingly, two different groups have observed that 53BP1 foci undergo fusion and fission events, characteristic of phase separation, and are also capable of opto droplet formation. Moreover an important role of PAR chains and RNA in this process was indicated (Altmeyer, Neelsen et al. 2015, Kilic, Lezaja et al. 2019, Pessina, Giavazzi et al. 2019). Another factor that has been recently reported to undergo LLPS is Rad52 in yeast, where it was shown to form droplets that fused in a DNA damage-inducible intranuclear microtubule filament (DIM) dependent manner (Oshidari, Huang et al. 2020). This is just the beginning of the phase separation entering in DNA repair world, and several questions can arise. First of all, if is a global feature of DNA repair factors to form liquid droplets, and if not which are the mechanisms that lead to this choice for a part of them and not for the rest. What remains also unclear is whether the distinct foci of 53BP1 that fuse are coming from different DSBs that are clustered together, and if this is the case, why this is not deleterious for the cells. Finally, the LLPS properties of DNA repair factors could explain their exclusion from compartments like chromocenters or nucleolus after DSB induction (Chiolo, Minoda et al. 2011, van Sluis and McStay 2015, Tsouroula, Furst et al. 2016, van Sluis and McStay 2017, Caridi, D'Agostino et al. 2018), because of their different LLPS properties that do not allow them to remix. Future studies will allow to understand the relationship between LLPS and DNA repair, and unravel the mechanisms underlying the complexity of these processes.

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THESIS OBJECTIVES

DNA double strand breaks (DSBs) need to be repaired faithfully to avoid genomic rearrangements such as chromosomal translocations. Cells repair DSBs by two main pathways: Non-Homologous End Joining (NHEJ), which directly religates DNA free ends, and is considered as an error prone pathway since it does not require homologous chromatin strands; and Homologous Recombination (HR), which takes the sister chromatid as a template and uses the information that it encodes to repair the DSBs in an error-free manner. While NHEJ takes place throughout the cell cycle, HR is normally suppressed in G1, to avoid recombination with a non-homologous template (Ciccia and Elledge, 2010). The cell cycle related suppression of HR is partly controlled by the ubiquitination of PALB2. In S-G2, the de-ubiquitinase USP11 removes ubiquitin from PALB2, allowing PALB2 interaction with BRCA1-BRCA2, resulting in the binding of RAD51 to DNA lesions that performs homology search and strand invasion (Orthwein et al., 2015). In G1, however, USP11 was shown to be degraded in response to damage leading to increased PALB2 ubiquitination, and consequently inhibition of the BRCA1-PALB2-BRCA2 complex formation and suppression of the stand invasion step of HR (Orthwein et al., 2015) (Figure 18).

Centromeres are highly specialized genomic loci that play a crucial role in proper chromosome segregation during cell division. Being the site of kinetochore assembly, centromeres allow the interaction between microtubules of the mitotic spindle and sister chromatids (Cheeseman, 2014). Consequently, when occurring at centromeres, DSBs can cause the failure of proper chromosome segregation and lead to aneuploidies and genomic rearrangements (Thompson et al., 2010). Both of these events are hallmarks of many diseases such as developmental disorders, congenital abnormalities, infertility, premature aging and cancer (Barra and Fachinetti, 2018; Beh and Kalitsis, 2015). Indeed, aneuploidy can cause carcinogenesis by altering the balance of oncogenes and tumour suppressors. About 90% of solid tumours are aneuploid (Compton, 2011). Centromeres are hotspots for chromosomal breakage and rearrangements in mammalian cells (Simi et al., 1998) and their intrinsic fragility could contribute to centromere breakage-fusion cycles observed in many solid tumours (Martínez-A and van Wely, 2011). Therefore, preservation of centromere integrity is crucial for cell viability. Despite great interest in the field, little is known about how centromeres maintain their integrity when they are damaged.

We have recently revealed that DSBs in mouse centromeric heterochromatin intriguingly activate both DNA end resection and RAD51 recruitment throughout the cell cycle, even in G1, despite the absence of a sister chromatid (Tsouroula et al., 2016).

Chromatin organization has recently emerged as a major player in DNA repair pathway choice. Centromeres are composed of repetitive DNA elements called satellites, but their organization mostly relies on peculiar chromatin features rather than the underlying DNA sequence (McKinley and Cheeseman, 2016; Muller and Almouzni, 2017). Indeed, they are characterized by H3K36me2 and H3K4me2, marks of active chromatin (Chan and Wong, 2012), and nucleosomes that contain CENP-A (Earnshaw and Rothfield, 1985), a H3 variant deposited exclusively in G1 by its specific chaperone HJURP (Dunleavy et al., 2009; Foltz et al., 2009). H3K4me2 was demonstrated to play a role in CENP-A deposition and centromeric transcription. This transcription is suggested to contribute to centromere architecture, function and stability (McNulty et al., 2017; Quénet and Dalal, 2014), highlighting the important role of H3K4me2 in maintaining centromere integrity. CENP-A has been shown to be overexpressed in various tumours (Hu et al., 2010; Li et al., 2011; Qiu et al., 2013; Tomonaga et al., 2003; Wu et al., 2012), to re-localize to sites of DNA damage (Zeitlin et al., 2009) and to increase tolerance to damage when overexpressed (Lacoste et al., 2014). Besides, CENP-A loss correlates with a drastic increase in centromere aberrations and leads to excision of centromeric repeats (Giunta and Funabiki, 2017), pointing out a possible role of CENP-A in damage response process.

In this context, my PhD project aimed to investigate whether and how the unique organization of centromeric chromatin permits DSBs repair by HR pathway in G1. Using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology to induce DSBs specifically at centromeric sequences of mammalian cultured cells, my objective was to shed light on the exact molecular mechanisms underlying centromeric DSBs repair by HR in G1 both in human and mouse cells, and especially to elucidate the involvement of the centromeric chromatin organisation in this process.

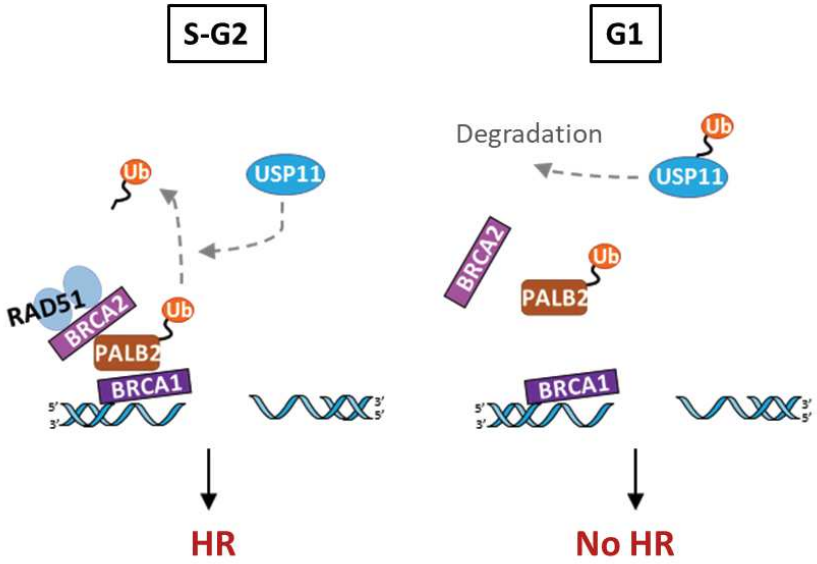

Figure 18: Schematic representation of cell cycle-regulated role of USP11 in HR repair

In S-G2, the deubiquitinase USP11 removes ubiquitin from PALB2, allowing PALB2 interaction with BRCA1-BRCA2, resulting in the binding of RAD51 to DNA lesions that performs homology search and strand invasion (Orthwein et al., 2015). In G1, USP11 is ubiquitinated and degraded in response to damage leading to increased PALB2 ubiquitination, and consequently inhibition of the BRCA1-PALB2-BRCA2 complex formation and suppression of the stand invasion step of HR (Orthwein et al., 2015).

RESULTS

In our study, we shed light on the molecular mechanisms that permits activation of HR repair at centromeric DSBs in G1. We show that H3K4me2, CENP-A and HJURP allow a succession of events leading to HR repair in G1. H3K4me2 promotes centromeric transcription and R-loop formation, which in turn facilitates end resection to occur at centromeric breaks. Further, we demonstrate that CENP-A and HJURP interact with USP11 in G1 allowing RAD51 recruitment to the breaks. Inhibition of RAD51 loading at centromeric DNA breaks in G1 leads to chromosomal translocations originating from the centromere and to loss of centromeric repeats suggesting that HR prevents the activation of mutagenic pathways and that the unique structure of centromeric chromatin is key in maintaining centromere integrity.

The results from this study are presented hereafter, in our recently submitted paper.

1. Submitted paper entitled “Licensing of homologous recombination in G1 preserves centromeric integrity”

Licensing of homologous recombination in G1 preserves centromeric integrity

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Centromeric integrity is key for proper chromosome segregation during cell division[1]. Centromeres have unique chromatin features that are essential for their maintenance[2,3]. Although they are intrinsically fragile and represent hotspots for chromosomal rearrangements[4,5], little is known about how their integrity in response to DNA damage is preserved. DNA repair by homologous recombination (HR) is suppressed in the G1 phase of the cell cycle, via degradation of the deubiquitinase USP11, to prevent formation of the RAD51/BRCA1/BRCA2 complex[6]. Here we demonstrate that DNA breaks occurring at centromeres in G1 do not trigger USP11 degradation and recruit the HR machinery, despite the absence of a sister chromatid. Mechanistically, we show that the unique centromere-specific histone H3 variant CENP-A, its chaperone HJURP in concert with H3K4me2 allow a succession of events leading to the licensing of HR in G1. H3K4me2 promotes DNA end-resection by allowing DNA damage-induced centromeric transcription and increased R-loop formation. CENP-A and HJURP interact with USP11 to render the centromeres uniquely accessible to RAD51 recruitment in G1. Finally, we show that inhibition of HR leads to centromeric instability and chromosomal translocations. Our results are consistent with a model in which licensing HR at centromeric breaks throughout the cell cycle prevents the activation of alternative mutagenic DNA repair pathways to preserve centromeric integrity.

Centromeres are highly repetitive genomic loci that play a key role in the proper segregation of chromosomes during cell division. Being the site of kinetochore assembly, centromeres allow the interaction between microtubules of the mitotic spindle and sister chromatids[7]. As such, their integrity is essential for cell viability. Centromeric instability can lead to aneuploidy and genomic rearrangements[8], both hallmarks of developmental disorders, congenital abnormalities, infertility, premature aging and cancer[1,9]. It is well established that centromeres are hotspots for chromosomal breakage and rearrangements in mammalian cells[4,10,11], and it has been proposed that their intrinsic fragility can contribute to centromere breakage-fusion cycles observed in many solid tumors[5]. Consistent with this, we have observed that mouse (NIH3T3) and human (U2OS) cells bare spontaneous centromeric DNA breaks, as determined by the co-localization of the DNA damage marker □-H2AX with the centromeric protein CENP-A or the anti-centromere antibody CREST (**Fig. S1A, S1B**). Despite great interest in the field, little is known about how centromeres maintain their integrity when they are damaged and which DNA repair pathways are involved.

To investigate how double-stranded DNA breaks (DSBs) arising at centromeres are repaired, we have previously developed a system to induce DSBs specifically at centromeres in mouse cells by co-expressing Cas9 with a guide RNA (gRNA) targeting the minor satellite repeats[12]. This system allows for the efficient generation of DSBs, which trigger the activation of the DNA damage response (**Fig. 1A and Fig. S2A**). Surprisingly, we demonstrated that in contrast to pericentric heterochromatin[12], Cas9-induced DSBs at centromeres recruit proteins of the homologous recombination (HR) machinery, such as RAD51[12], RPA and BRCA1 throughout the cell cycle (**Fig. 1A, S2B, S2D, S2F, S2H**), even in G1 where HR is believed to not be operational. This observation was conserved in human cells when DSBs were induced at alpha satellite repeats by Cas9 (**Fig. 1B, S2C, S2E, S2G**). To exclude the possibility that these observations are inherent to a system that induces multiple and clustered DSBs, we irradiated cells or cultured them in the presence of the DNA damaging agent neocarzinostatin (NCS). Remarkably, RAD51 is efficiently recruited at centromeric lesions after irradiation (IR) or NCS treatment, in both G1 and G2 (**Fig. 1C, 1D, S2I and S2J**), further supporting the notion that centromeric DSBs recruit the HR machinery throughout the cell cycle.

Even though centromeres are heterochromatinized, they are transcribed and contain active chromatin marks, such as H3K4me2[13–15]. To determine whether these features play a role in the

recruitment of the HR machinery in G1, we induced centromeric breaks in cells in which the histone methyltransferase SETD1A (a H3K4me2 writer) was depleted by siRNA (**Fig. S3A**) or in which we tethered the demethylase LSD1 (H3K4me2 eraser) to centromeres by fusing it to the catalytically inactive mutant of Cas9 (dCas9; **Fig. S3B**). Both strategies resulted in the reduction of H3K4me2 at centromeres (**Fig. S3C and S3D**) and in a substantial decrease in the recruitment of RAD51, RPA and BRCA1 in response to centromeric DSBs in G1 **(Fig. 2A and 2B**). This was dependent on the catalytic activity of SETD1A (**Fig. 2C and S3E**), further suggesting that the H3K4me2 mark *per se* facilitates the recruitment of the HR machinery in G1. The effect of H3K4me2 depletion on the recruitment of the HR machinery was specific to centromeric DSBs and had no effect on DSBs induced at pericentromeres (**Fig. S3F**), consistent with the fact that H3K4me2 is not present in these heterochromatinized repetitive regions.

H3K4me2 promotes transcription from centromeric repeats leading to the production of non-coding RNAs[13–17]. Consistent with this, we found that depletion of SETD1A or the tethering of LSD1 to centromeres led to a significant reduction in the production of centromeric non-coding RNAs (**Fig. S3G and S3H**). Interestingly, induction of DSBs at centromeres increased centromeric transcription by eight-fold (**Fig. 2D**), which was concomitant with a substantial increase in centromeric H3K4me2 (**Fig. 2E**).

DNA breaks at transcribed regions or *de novo* transcription from DSBs promote R-loop (DNA:RNA hybrid) formation, which in turn facilitates HR[18–20]. R-loops also form at centromeres and promote centromeric integrity[21,22]. To determine whether the increase in transcription in response to centromeric DSBs leads to the accumulation of R-loops, we expressed a catalytic mutant of RNAseH (dRNAseH) fused to GFP. We found that dRNAseH accumulated at centromeres in 12% of cells in the absence of DNA damage (**Fig. 2F and S3I**), suggesting that centromeres are prone to R-loop formation. Interestingly, DSB-induction at centromeres led to a four-fold increase in R-loop formation (**Fig. 2F and S3I**). This was confirmed by DRIP-qPCR using the S9.6 antibody, in conditions in which it specifically recognizes DNA-RNA hybrids (**Fig. 2G**). Notably, R-loop formation was substantially decreased upon SETD1A depletion or dCas9-LSD1 tethering, confirming that centromeric R-loop formation occurs through H3K4me2-dependent transcription (**Fig. S3J**) and does not merely reflect the binding of the gRNA/dCas9 complex to chromatin. To test the role of R-loop formation in HR initiation in response to centromeric DSBs in G1, we quantified the recruitment of BRCA1 and RPA in cells

overexpressing wild-type RNAseH. RNAseH directed inhibition of R-loop formation at centromeres led to a decrease in the recruitment of both HR factors (**Fig. 2H**), pointing to a role of R-loops in promoting DNA end-resection at centromeric DSBs. Together, these results show that DSB-induction at centromeres in G1 increases H3K4me2-dependent transcription and subsequent R-loop formation to facilitate DNA end-resection and HR initiation.

H3K4me2 is a general feature of active chromatin and decorates promoters and enhancers of transcribed genes[23]. Nevertheless, HR and particularly RAD51 binding is largely supressed in G1, even in transcribed regions[24], suggesting that H3K4me2 is not sufficient to allow complete activation of DNA repair through HR. This prompted us to focus our attention on unique features of centromeres, such as the presence of the histone H3 variant CENP-A, its associated chaperone HJURP and the cofactor MIS18, which promote incorporation of CENP-A to centromeres in early G1[25]. We thus quantified the recruitment of HR factors in response to centromeric DSBs in G1, in cells depleted of CENP-A, HJURP or MIS18 (**Fig. S4A**). Interestingly, recruitment of RAD51 was significantly reduced in all conditions (**Fig. 3A**). However, the recruitment of RPA was not affected (**Fig. S4B**), suggesting that the role of CENP-A and HJURP in HR occurs downstream of DNA end-resection and promotes the late stages of HR. To determine whether CENP-A and HJURP are sufficient to render non-centromeric DSBs permissive to RAD51 recruitment, we tethered CENP-A or HJURP through dCas9 to pericentromeres, where they are not normally present. Remarkably, tethering CENP-A or HJURP at pericentromeric DSBs doubled the recruitment of RAD51 in G1 (**Fig. 3B**). Similarly, tethering of HJURP to an ectopic LacO/IsceI locus by fusing it to LacI[26], leading to CENP-A incorporation and neocentromere formation at the LacO chromatin[27], was sufficient to increase RAD51 recruitment at I-SceI-induced breaks in G1 (**Fig. S4C**). This suggests that CENP-A and HJURP play a direct role in RAD51 recruitment at DSBs in G1, irrespective of the underlying DNA sequence. This mechanism operates strictly in G1 since CENP-A and HJURP depletion had no effect on RAD51 recruitment in G2 (**Fig. S4D**).

In S/G2, USP11 deubiquitinates PALB2 to promote its interaction with BRCA1 and BRCA2 at DSBs to recruit RAD51 and allow repair through HR[6]. In G1, however, □-irradiation-induced USP11 degradation prevents the formation of the BRCA1-PALB2-BRCA2 complex, suppressing HR[6]. In contrast to global DNA damage induced by □-Irradiation[6], we find that DSBs specifically localized at centromeres do not trigger substantial USP11 degradation (**Fig. S4E**), rendering USP11 as a

good candidate to promote RAD51 recruitment in G1 only in these regions. Indeed, USP11 depletion (**Fig. S4F**) led to a reduction in the recruitment of RAD51 in response to centromeric DSBs in G1 (**Fig. 3C**). Furthermore, tethering of USP11 at pericentric DSBs in G1 significantly increased RAD51 recruitment, indicating that USP11 is necessary and sufficient to enhance RAD51 recruitment at centromeric DSBs (**Fig. S4G**). To determine whether USP11 exerts its unique role at centromeric DSBs in G1 by interacting with CENP-A and HJURP, we performed co-immunoprecipitation experiments. Indeed, we find that USP11 interacts with both CENP-A (**Fig. 3D**) and HJURP (**Fig. 3E**). Consistent with this, tethering HJURP to a LacO array in NIH3T3 and U2OS cells resulted in increased USP11 recruitment (**Fig. 3F and S4H**). We then asked whether USP11 deubiquitinates HJURP and whether this regulates its interaction with CENP-A. We find that HJURP is constitutively ubiquitinated (**Fig. 3G**), in agreement with previous reports[28–30]. Furthermore, overexpression of USP11 resulted in a drastic reduction in the ubiquitination of HJURP (**Fig. 3G**) and depletion of USP11 had the opposite effect (**Fig. S4I**), suggesting that USP11 directly deubiquitinates HJURP. Remarkably, knockdown of USP11 reduced the interaction between HJURP and CENP-A (**Fig. 3H**) and impaired the incorporation of CENP-A at a neocentromere (LacO array) upon tethering of HJURP (**Fig. S4J**). These findings prompted us to determine whether USP11 controls *de novo* CENP-A deposition at centromeres. To this end, we quantified CENP-A incorporation in HeLa cells expressing CENP-A fused to the SNAP epitope tag[31] (**Fig. S4K**). As expected, HJURP depletion led to CENP-A misincorporation (**Fig. 3I and S4L**). Remarkably, USP11 depletion resulted in a striking defect in *de novo* CENP-A deposition (**Fig. 3I and S4L**), pointing to a fundamental role of USP11 in centromere maintenance. Altogether, these results suggest that USP11 has a constitutive role at centromeres in G1 by deubiquitinating HJURP to facilitate its binding with CENP-A and its incorporation to centromeres. The USP11/HJURP/CENP-A interaction might attract USP11 at centromeres, therefore licensing the recruitment of RAD51 to centromeric DSBs in G1.

Activation of HR at repetitive sequences is believed to be deleterious as it can trigger repeat contraction and/or expansion[32,33]. To determine whether the activation of HR at centromeric breaks is deleterious or confers a benefit for centromere integrity, we assessed genomic instability at centromeres upon induction of DSBs. Remarkably, we find that chemical inhibition of RAD51 results in a dramatic increase in the number of chromosomes with broken centromeres (**Fig. 4A**) and translocations (**Fig. 4B**). This suggests that licensing of HR throughout the cell cycle at centromeric DSBs prevents centromeric genomic instability and the onset of

chromosomal rearrangements. To decipher the underlying mechanism, we sought to determine which alternative DNA repair pathway is at play when RAD51 is inhibited or depleted. For this, we assessed the number of translocations occurring in conditions in which RAD51 is inhibited together with the inhibition of RAD52 that binds resected DNA ends to promote single strand annealing (SSA) or Break-Induced Replication (BIR). Interestingly, co-inhibition of RAD51 and RAD52 decreased the number of translocations observed when RAD51 is inhibited alone (**Fig. 4C**). Consistent with this, inhibition of RAD51 in the presence of centromeric DSBs results in a RAD52-dependent 10-fold increase in centromeric DNA content (**Fig. 4D**), suggesting that RAD52 is at the origin of these abnormalities. Furthermore, recruitment of RAD52 at centromeric DSBs in G1 was increased following RAD51 knockdown (**Fig. 4E**). These results suggest that activation of HR in response to DSBs occurring at centromeric repeats throughout the cell cycle prevents the action of additional mutagenic DNA repair pathways, which would otherwise lead to chromosomal abnormalities.

Our results point to an important role for the distinct chromatin organisation at centromeres in DNA repair, which allows the sequence of events leading to the licensing of HR in G1. We propose a model (**Fig. S5**) in which H3K4me2 permits an increase in centromeric transcription and R-loop formation in response to DSBs, which in turn promote DNA end-resection. CENP-A and HJURP facilitate DNA strand invasion by specifically interacting with USP11, allowing PALB2 deubiquitination and the subsequent recruitment of BRCA2-RAD51 to the resected DNA ends.

Contrary to the dogma that activation of HR in the absence of a sister chromatid is prone to trigger genomic rearrangements between repetitive sequences, our results demonstrate that the USP11/HJURP/CENP-A axis licenses the completion of HR in G1 to prevent the onset of centromeric instability (**Fig. S5**). It is possible that generation of R-loops at centromeres during mitosis[22] and their role in CENP-A deposition creates a unique environment at centromeres in G1, which is different from other repetitive sequences and necessitates the activation of HR.

The fact that activation of HR at centromeres does not promote chromosomal translocations suggests that centromeric repeats are copied *in cis* and not *in trans,* consistent with their spatially distant localization within the nucleus. In agreement with this, centromeric repeats in *S. pombe* undergo RAD51-dependent recombination even in the absence of stress to suppress chromosomal rearrangements[34]. Moreover, HR between centromeric repeats leads to closed

loops that can have an important role in the establishment of a functional centromere[35], suggesting that recombination might be essential for centromere maintenance. As CENP-A over-expression leads to a high resistance to DNA damage inducing drugs[36] and loss of CENP-A leads to centromeric instability[37], our results might aid the design of individualized cancer treatments, according to epigenetic markers such as CENP-A.

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Figure legends

Figure 1. Centromeric DSBs recruit RAD51 throughout the cell cycle. Immunofluorescence (IF) confocal analysis of **(A)** NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats and GFP-CENP-A and **(B)** U2OS cells expressing Cas9 + gRNA targeting alpha satellite repeats stained with DAPI and antibodies specific for γ-H2AX, CREST and RAD51 in G1 (EdU^- cells for NIH3T3 and double thymidine treated cells for U2OS cells) and G2 (RO-3306 in both cell lines) phases of the cell cycle (for more details about G1/G2 discrimination strategy see materials and methods). Percentage of cells with RAD51 colocalizing with GFP-CENP-A or CREST is shown as mean ± SD. Images are representative of 5-6 experiments with n=50 cells. IF confocal analysis of **(C)** NIH3T3 cells expressing GFP-CENP-A and **(D)** U2OS cells treated with IR (2Gy), stained with DAPI and antibodies specific for γ-H2AX, CREST and RAD51 in G1 and G2 as above. Percentage of cells with DSBs (γ-H2AX) and RAD51 colocalizing with GFP-CENP-A or CREST, corresponding to cells with at least on centromere colocalizing with γ-H2AX and RAD51, is shown as mean ± SD. Images are representative of 3 experiments with n=50 cells. For confocal images, scale bars represent 5 μm for G1 cells, 10 μm for G2 cells.

Figure 2. H3K4me2 supports DNA end-resection by promoting centromeric transcription and increased R-loop formation in response to DSBs. **(A)** Quantification of fold change of RPA, BRCA1 and RAD51 recruitment at centromeric DSBs in cells depleted of SETD1A (siSETD1A) relative to control (siSCR) in NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats. Data are the mean ± SD of 4-6 experiments with n=50 cells. **(B)** Quantification of fold change of RPA, BRCA1 and RAD51 recruitment at centromeric DSBs in NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats and co-expressing dCas9 fused to LSD1 (dCas9-LSD1) and relative to cells expressing dCas9 alone. Data are the mean ± SD of 3 experiments with n=50 cells. **(C)** Quantification of fold change of RPA recruitment at centromeric DSBs in cells depleted for SETD1A (siSETD1A) and reconstituted with WT SETD1A or with a truncated catalytically inactive mutant (SETD1A-ΔSET) relative to the control (siSCR). Data are the mean ± SD of 3 experiments with n=50 cells. **(D)** Quantification by RT-qPCR of fold change of centromeric RNA in the presence of DSBs in NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats, relative to cells expressing dCas9+ gRNA. Data are the mean ± SD of 2 experiments. **(E)** Enrichment of H3K4me2 at centromeres over the Input and relative to the no-antibody control, in the presence of DSBs in NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats, relative to cells expressing dCas9 + gRNA. Data are

the mean ± SD of 2 experiments. **(F)** Quantification of dRNAseH recruitment at centromeres in NIH3T3 cells expressing either dCas9 + gRNA targeting the minor satellite repeats or Cas9 + gRNA. Data are the mean ± SD of 3 experiments with n=50 cells. **(G)** Quantification of S9.6 enrichment at centromeres in NIH3T3 cells expressing dCas9 + gRNA targeting the minor satellite repeats or Cas9 + gRNA. Overexpression of RNAseH in each condition shows the specificity of the antibody. Data are the mean ± SD of 3 experiments with n=50 cells. **(H)** Immunofluorescence confocal analysis of NIH3T3 cells expressing either dRNAseH or RNAseH fused to GFP and Cas9 + gRNA targeting the minor satellite repeats, stained with DAPI and antibodies specific for 53BP1 and BRCA1. Quantification of fold change of BRCA1 and RPA recruitment at centromeric DSBs in cells expressing RNAseH relative to cells expressing dRNAseH is depicted on the right. Data are the mean ± SD of 3 experiments with n=50 cells. For all graphs represented as fold change, values represent mean ± SD of fold change calculated over the mean of control samples, and statistical significance was determined by t-test (*p<0,05, **p<0,01, ***p<0,001). For confocal images, scale bars represent 5 µm.

Figure 3. The USP11/HJURP/CENP-A axis licenses HR at centromeric DSBs in G1. **(A)** Quantification of fold change of RAD51 recruitment at centromeric DSBs in cells depleted of HJURP (siHJURP), CENP-A (siCENP-A), their combination (siHJURP+siCENP-A) and MIS18 (siMIS18), relative to control (siSCR) in NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats. Data are the mean ± SD of 4-5 experiments with n=50 cells. **(B)** Immunofluorescence (IF) confocal analysis of NIH3T3 cells in G1 expressing either dCas9 fused to GFP (GFP-dCas9) or dCas9 fused to GFP and CENP-A (GFP-dCas9-CENP-A) and Cas9 + gRNA targeting major satellites repeats, stained with DAPI and antibodies specific for γ-H2AX and RAD51. Quantification of the fold change of RAD51 recruitment at pericentromeric DSBs in cells expressing dCas9-CENP-A or dCas9-HJURP, relative to cells expressing dCas9 is depicted on the right. Data are the mean ± SD of 3 experiments with n=50 cells. **(C)** Quantification of the fold change of RAD51 recruitment at centromeric DSBs in cells depleted of USP11 (siUSP11), relative to control (siSCR) in NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats. Data are the mean ± SD of 4 experiments with n=50 cells. **(D)** Western blot analysis of USP11 and CENP-A after Immunoprecipitation (IP) of USP11 in NIH3T3 control cells (siSCR) or cells depleted from CENP-A (siCENP-A). The Input was 1% of the extract used for the IP. **(E)** Western blot analysis of USP11, GFP and GFP-HJURP after IP of GFP (GFP-IP) in NIH3T3 control cells (siSCR) or cells depleted from USP11 (siUSP11). The Input was 1 % of the extract used for the

IP. **(F)** IF confocal analysis of NIH3T3 lacO-Isce-I-tet cells expressing lacI fused to mCherry (mCherry-LacI) or fused to mCherry and HJURP (mCherry-lacI-HJURP) and co-expressing USP11 fused to GFP (GFP-USP11) and stained with DAPI. Quantification of percentage of cells with colocalization of GFP-USP11 with LacO locus in LacI or LacI-HJURP transfected cells is shown on the right. Data are the mean ± SD of 3 experiments with n=50 cells. **(G)** Western blot analysis of Ubiquitin (FK2 antibody) after IP of GFP (GFP-IP) under denaturing conditions in NIH3T3 cells expressing GFP or GFP-HJURP and co-expressing tagged USP11 (USP11-Flag) or the tag alone (Flag). The Input was 1% of the extract used for the IP. **(H)** Western blot analysis of USP11, CENP-A and GFP after IP of GFP (GFP-IP) or GFP-HJURP in NIH3T3 control cells (siSCR) or cells depleted from USP11 (siUSP11). The Input was 1 % of the extract used for the IP. **(I)** IF confocal analysis of *de novo* deposited CENP-A (CENP-A-SNAP; TMR Star in Red) and all CENP-A (old+new) (Green) in Hela expressing tagged CENP-A (CENP-A-SNAP) and depleted from HJURP (siHJURP), USP11 (siUSP11) or control cells (siSCR). Quantification of fold change of the percentage of cells with CENP-A-SNAP in cells depleted from HJURP (siHJURP) and USP11 (siUSP11) relative to control cells (siSCR) is shown on the right. Data are the mean ± SD of 4 experiments with n=200 cells. For all the graphs represented as fold change, values represent mean ± SD of fold change calculated over the mean of control samples. Statistical significance was determined by t-test (*p<0,05, **p<0,01, ***p<0,001, ****p<0,0001). For confocal images, scale bars represent 5 µm.

Figure 4: Licensing of HR at centromeric breaks throughout the cell cycle promotes centromeric integrity. **(A)** Quantification of the number of chromosomes with broken centromeres (lacking or with free centromeres) in cells expressing Cas9 + gRNA targeting the minor satellite repeats and treated with DMSO or RAD51 inhibitor (RAD51i). Data are the mean ± SD of 4 experiments, with n=50 metaphases. **(B)** Representative confocal image of metaphase spreads, after fluorescence *in situ* hybridization (FISH) with centromeric (PNA-Red) and telomeric (PNA-Green) probes, and stained with DAPI. The insert depicts a chromosomal translocation. Quantification of the number of translocations in cells expressing Cas9 + gRNA targeting the minor satellite repeats and treated with DMSO or RAD51 inhibitor (RAD51i) is shown on the right. Data are the mean ± SD of 5 experiments with n=50 metaphases. **(C)** Quantification of the number of translocations in cells expressing Cas9 + gRNA targeting the minor satellite repeats and treated with DMSO, RAD51 inhibitor (RAD51i), RAD52 inhibitor (RAD52i), or a combination of both. Data are the mean ± SD of 4 experiments with n=50

metaphases. **(D)** Quantification of fold enrichment of centromeric DNA after qPCR analysis in NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats and depleted of RAD51 (siRAD51) alone or together with RAD52 (siRAD51 + siRAD52) relative to the control (siSCR). Data are the mean ± SD of 3 experiments. **(E)** Immunofluorescence confocal analysis of NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats and RAD52 fused to GFP (RAD52-GFP) and stained with DAPI and antibodies specific for γ-H2AX and RAD51. Quantification of the percentage of cells with RAD51 and RAD52 recruitment at centromeric breaks in cells depleted of RAD51 (siRAD51) or control cells (siSCR) is shown on the right. Data are the mean ± SD of 4 experiments with n=50 cells. Data are the mean ± SD of 3 experiments with n=50 metaphases. For all graphs, statistical significance was determined by t-test (*p<0,05, **p<0,01, ***p<0,001). For confocal images, scale bars represent 5 µm.

Figure 1: Centromeric DSBs recruit RAD51 throughout the cell cycle

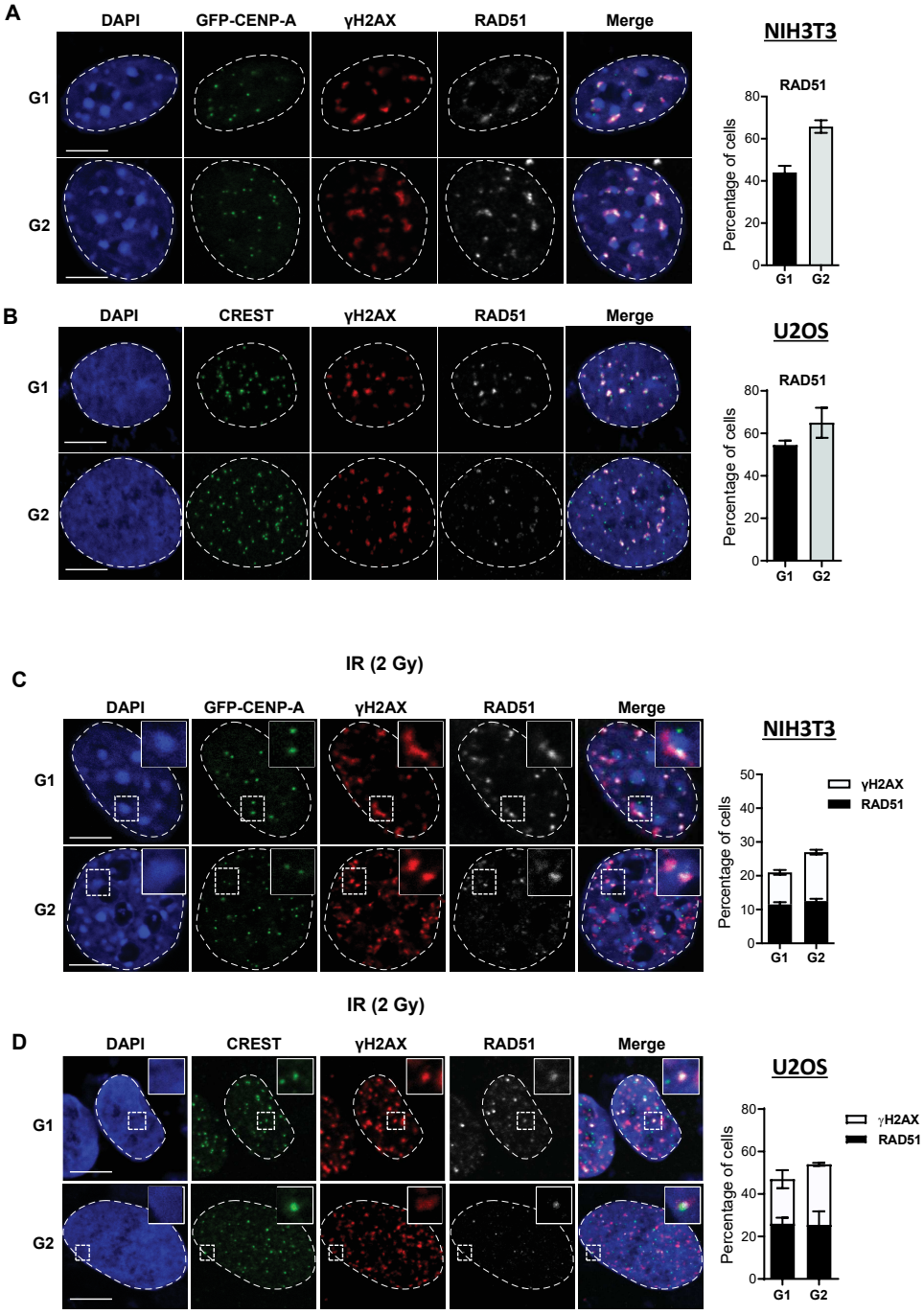


Figure 2: H3K4me2 supports DNA end-resection by promoting centromeric transcription and increased R-loop formation in response to DSBs

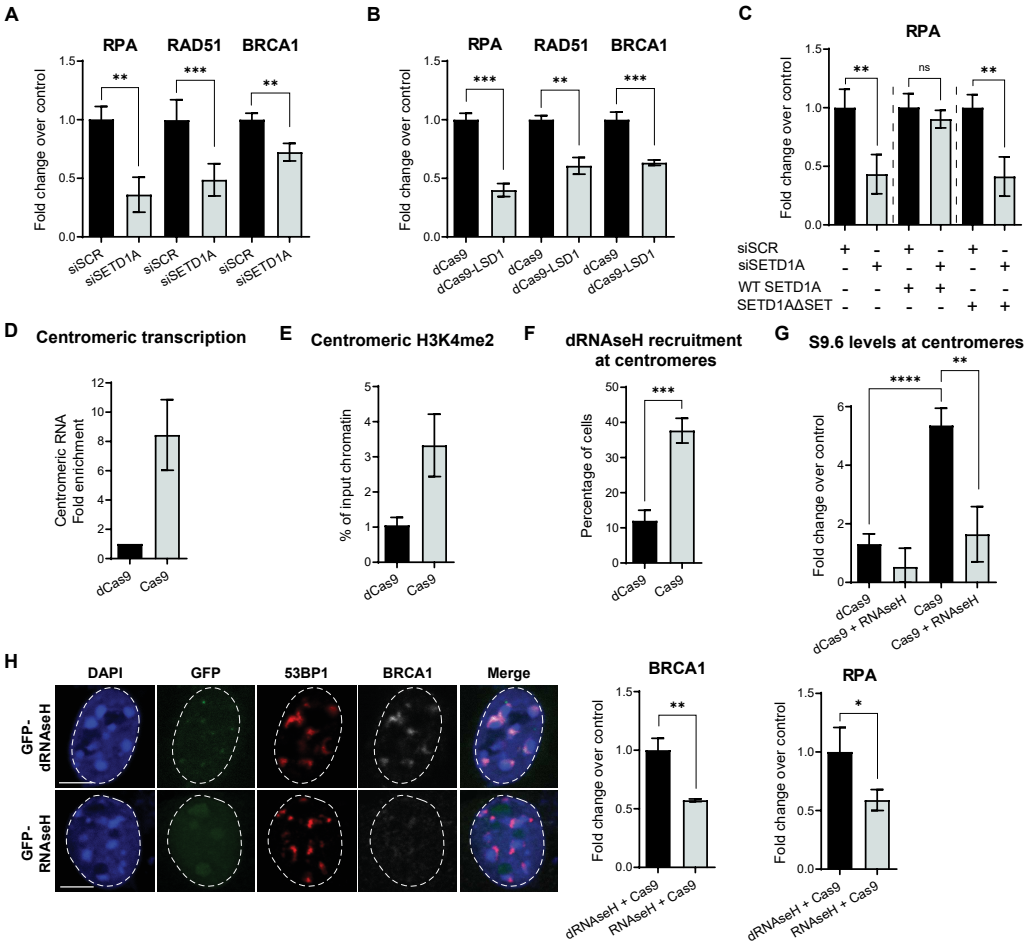


Figure 3: The USP11/HJURP/CENP-A axis licenses HR at centromeric DSBs in G1

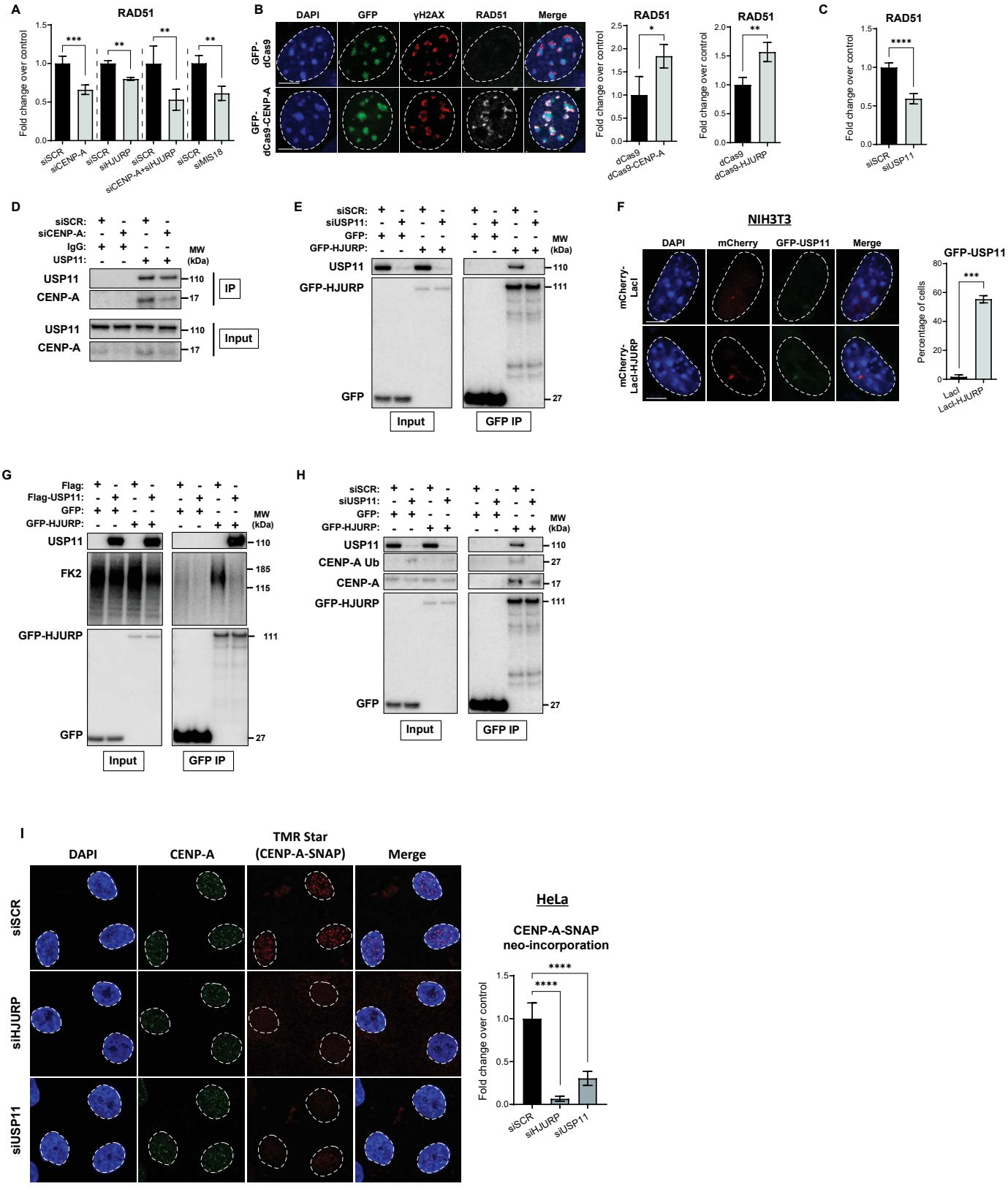
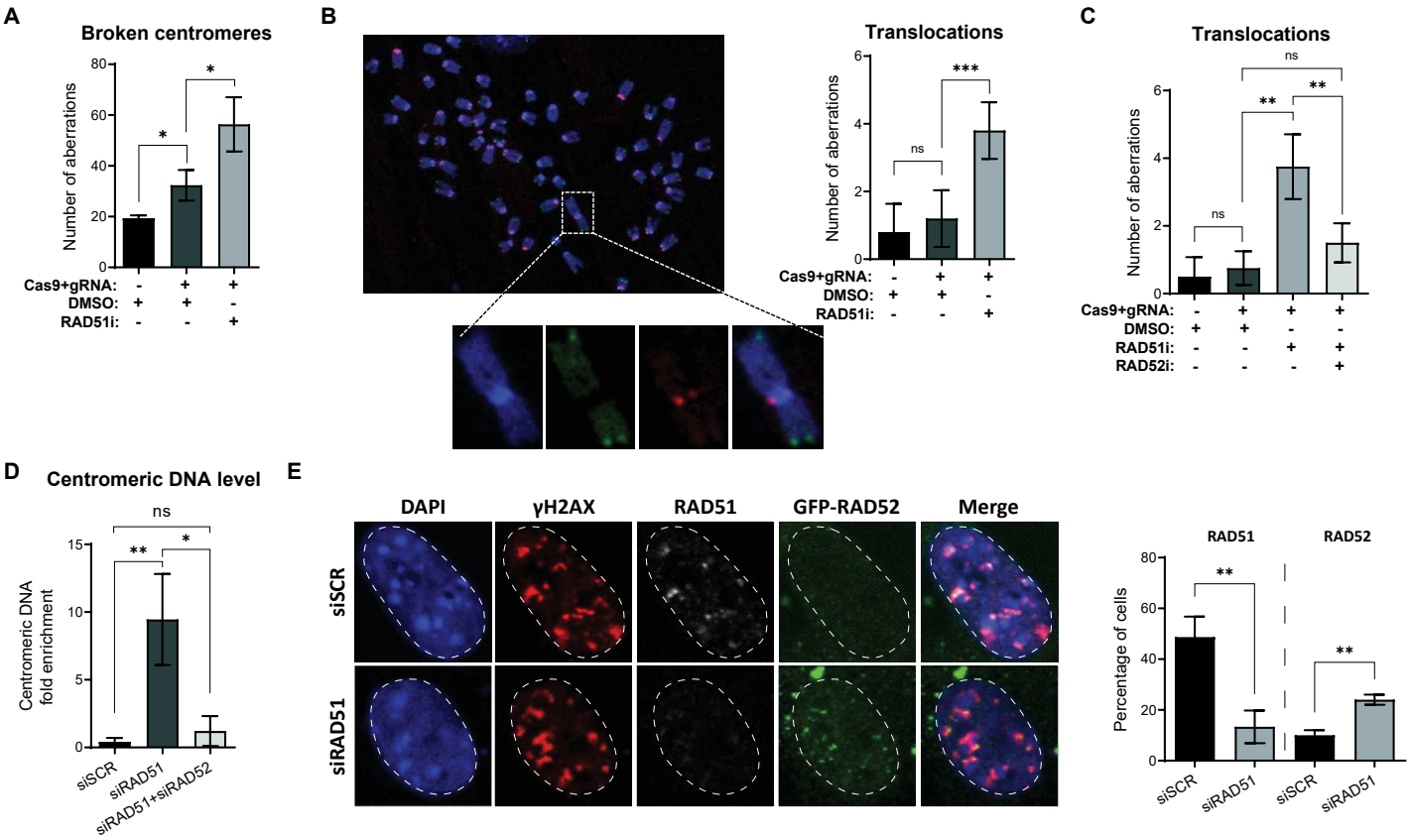


Figure 4: Licensing of HR at centromeric breaks throughout the cell cycle promotes centromeric integrity



SUPPLEMENTARY: Licensing of homologous recombination in G1 preserves centromeric integrity

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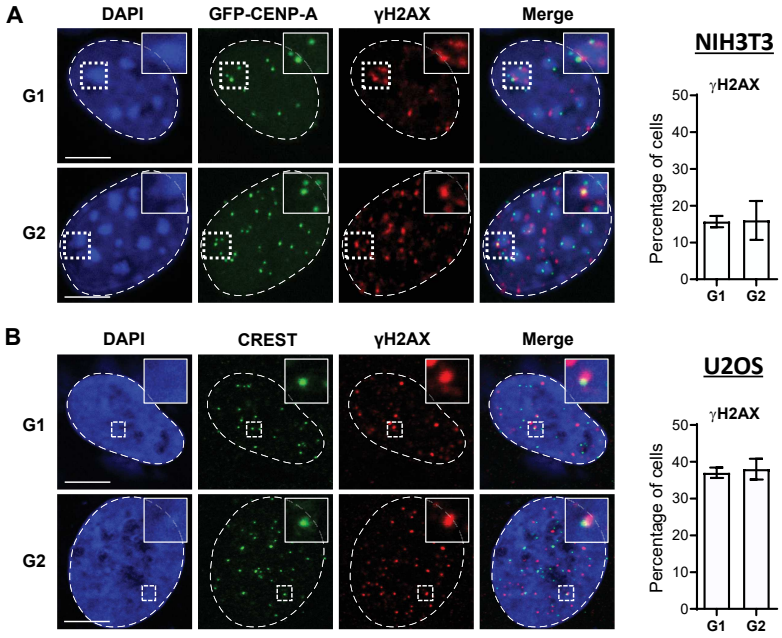
SUPPLEMENTARY Figure 1



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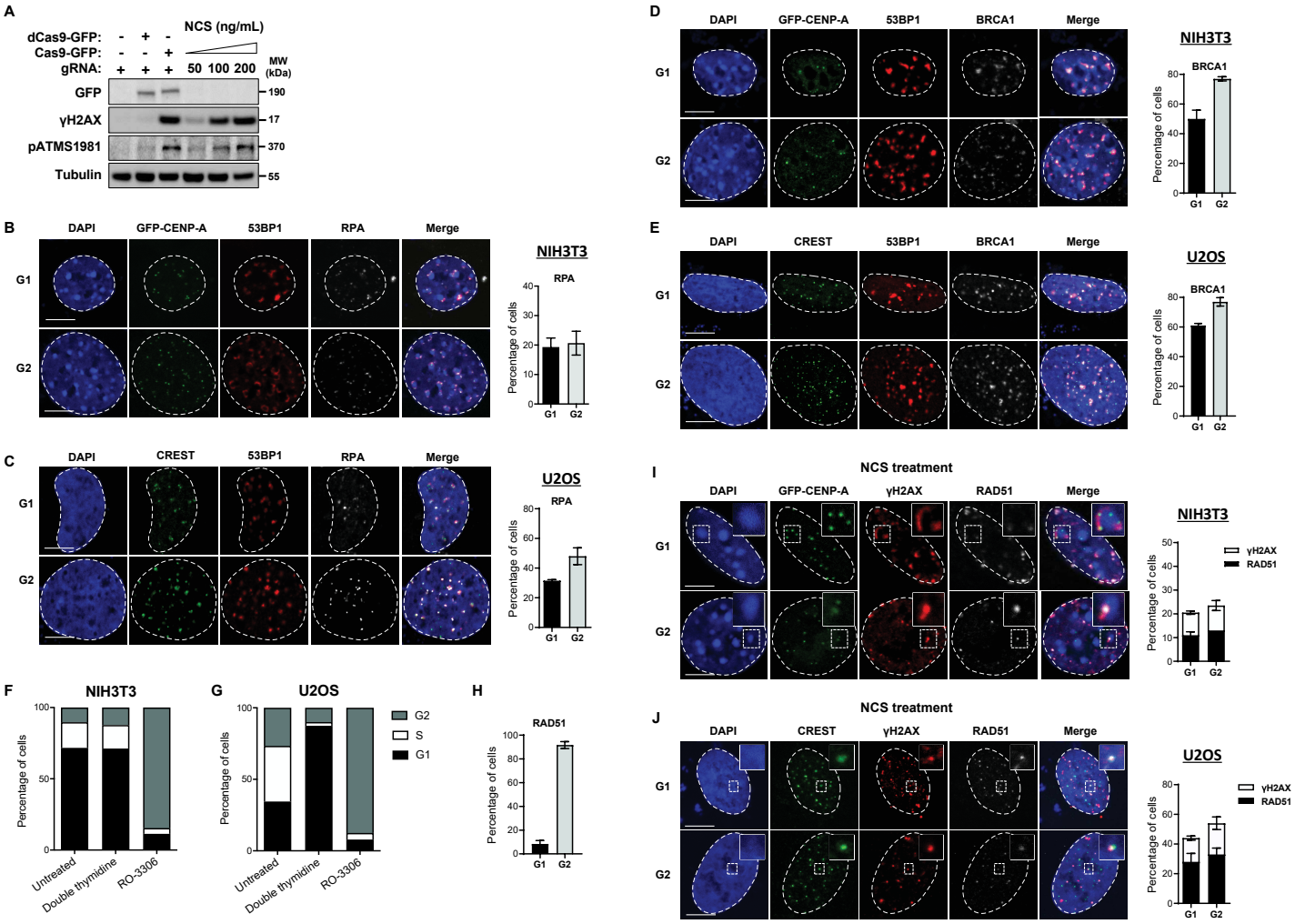

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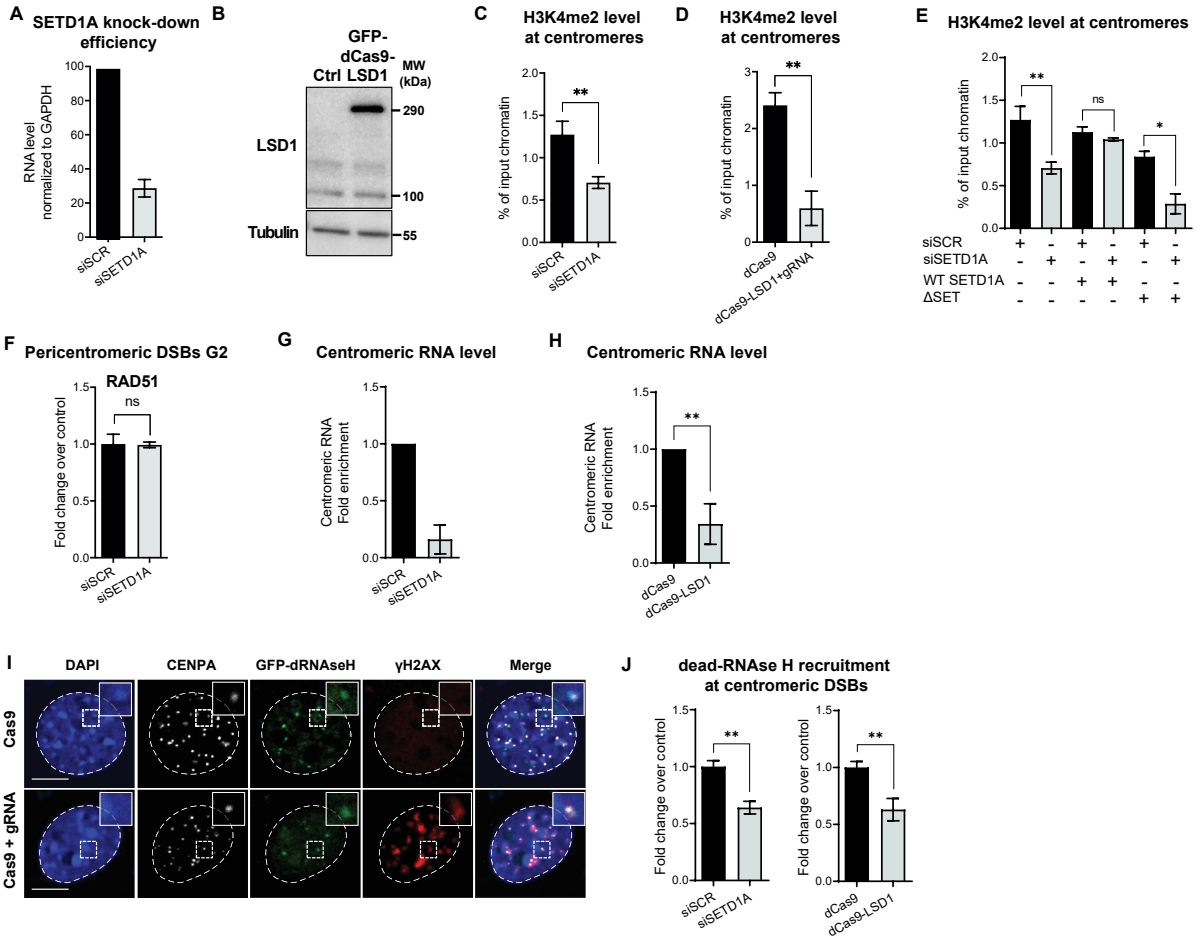

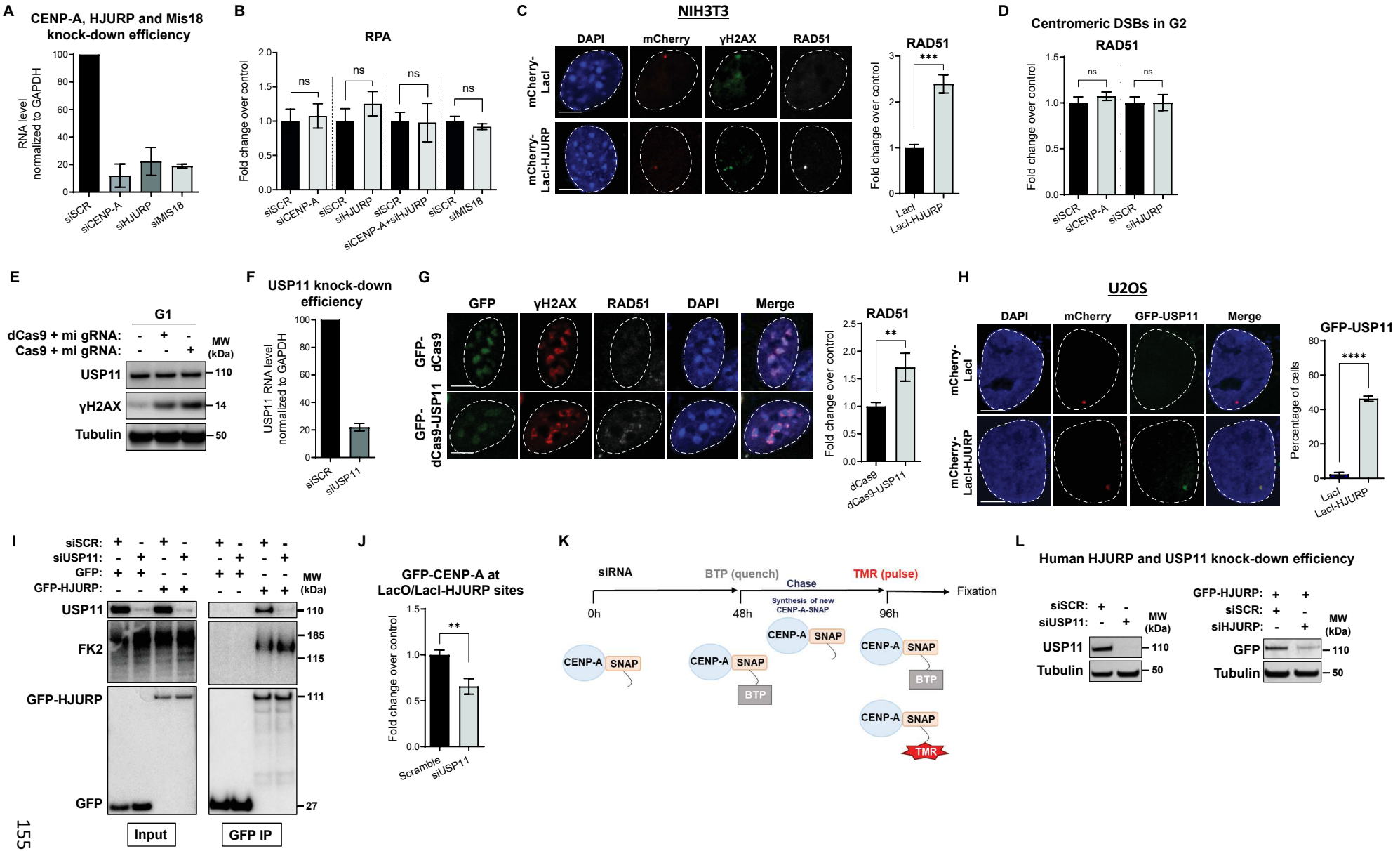
A
CENP-A, HJURP and Mis18 knock-down efficiency
RNA level normalized to GAPDH
siSCR
siCENP-A
siHJURP
siMIS18
B
RPA
Fold change over control
ns
siSCR
siCENP-A
siHJURP
siCENP-A+siHJURP
siMIS18
C
NIH3T3
DAPI
mCherry
γH2AX
RAD51
mCherry-LacI
mCherry-LacI-HJURP
RAD51
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LacI
LacI-HJURP
D
Centromeric DSBs in G2
RAD51
E
G1
dCas9 + mi gRNA:
Cas9 + mi gRNA:
MW (kDa)
USP11
γH2AX
Tubulin
110
14
50
F
USP11 knock-down efficiency
USP11 RNA level normalized to GAPDH
siSCR
siUSP11
G
GFP
γH2AX
RAD51
DAPI
Merge
GFP-dCas9
GFP-dCas9-USP11
RAD51
**
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GFP-USP11
Merge
mCherry-LacI
mCherry-LacI-HJURP
GFP-USP11
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Percentage of cells
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GFP:
GFP-HJURP:
MW (kDa)
USP11
FK2
GFP-HJURP
GFP
110
185
115
111
27
Input
GFP IP
J
GFP-CENP-A at LacO/LacI-HJURP sites
Fold change over control
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Scramble
siUSP11
K
siRNA
BTP (quench)
Chase
TMR (pulse)
Fixation
0h
48h
96h
Synthesis of new CENP-A-SNAP
CENP-A
SNAP
BTP
TMR
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Human HJURP and USP11 knock-down efficiency
siSCR:
siUSP11:
MW (kDa)
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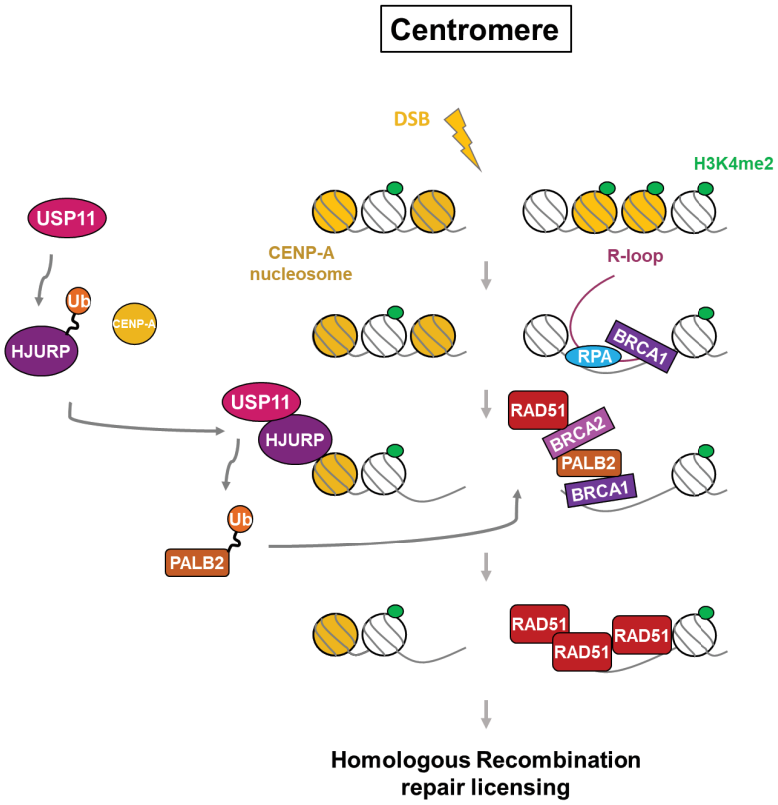
110
50

SUPPLEMENTARY Figure 5



Supplementary Figures legends

Supplementary Figure 1. **(A)** Immunofluorescence confocal analysis of **(A)** NIH3T3 cells expressing GFP-CENP-A and **(B)** U2OS cells stained with DAPI and antibodies specific for □-H2AX and CREST in G1 and G2 phases of the cell cycle. Percentage of cells with □-H2AX colocalizing with GFP-CENP-A or CREST, corresponding to cells with at least on centromere colocalizing with □-H2AX, is shown on the right as mean ± SD. Images are representative of 4 experiments with n=50 cells. For confocal images, scale bars represent 5 µm for G1 cells and 10 µm for G2 cells.

Supplementary Figure 2. **(A)** Western blot analysis using GFP, □-H2AX, phospho-ATM (pATMS1981) and tubulin in cells expressing dCas9-GFP, Cas9-GFP with a gRNA targeting minor satellite repeats or treated with the indicated concentrations of NCS. Immunofluorescence (IF) confocal analysis of **(B)** NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats and GFP-CENP-A and **(C)** U2OS cells expressing Cas9 + gRNA targeting alpha satellite repeats stained with DAPI and antibodies specific for 53BP1, CREST and RPA in G1 and G2 phases of the cell cycle. Percentage of cells with RPA colocalizing with GFP-CENP-A or CREST is shown on the right as mean ± SD. Images are representative of 4 experiments with n=50 cells. IF confocal analysis of **(D)** NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats and GFP-CENP-A and **(E)** U2OS cells expressing Cas9 + gRNA targeting alpha satellite repeats stained with DAPI and antibodies specific for 53BP1, CREST and BRCA1 in G1 and G2 phases of the cell cycle. Percentage of cells with BRCA1 colocalizing with GFP-CENP-A or CREST is shown on the right as mean ± SD. Images are representative of 3 experiments with n=50 cells. Cell cycle analysis by flow cytometry using propidium iodide and EdU in **(F)** NIH3T3 and **(G)** U2OS cells, either untreated or treated with double thymidine or RO-3306. **(H)** Percentage of cells with RAD51 recruitment at pericentromeric DSBs in NIH3T3 cells expressing Cas9 + gRNA targeting the major satellite repeats in G1 and G2. IF confocal analysis of **(I)** NIH3T3 cells expressing GFP-CENP-A and **(J)** U2OS cells treated with NCS (200 ng/ml), stained with DAPI and antibodies specific for □-H2AX, CREST and RAD51 in G1 and G2. Percentage of cells with DSBs (□-H2AX) and RAD51 colocalizing with GFP-CENP-A or CREST, corresponding to cells with at least on centromere colocalizing with □-H2AX and RAD51, is shown on the right as mean ± SD. Images are representative of 3 experiments with n=50 cells. For confocal images, scale bars represent 5 µm for G1 cells, 10 µm for G2 cells.

Supplementary Figure 3. **(A)** Quantification of SETD1A mRNA by RT-qPCR in cells depleted of SETD1A (siSETD1A), normalized to GAPDH and expressed as relative to control (siSCR). **(B)** Western blot analysis of LSD1 and tubulin in NIH3T3 cells expressing dCas9 fused to LSD1 and GFP (GFP-dCas9-LSD1) and control cells. **(C)** H3K4me2 enrichment at centromeres over the input and relative to the no-antibody control in cells depleted of SETD1A (siSETD1A) or in control cells (siSCR). Data are the mean ± SD of 3 experiments **(D)** H3K4me2 enrichment at centromeres over the input and relative to the no-antibody control in cells expressing dCas9 alone or fused to LSD1 (dCas9-LSD1) and a gRNA targeting the minor satellite repeats. Data are the mean ± SD of 3 experiments **(E)** H3K4me2 enrichment at centromeres over the input and relative to the no-antibody control in cells depleted for SETD1A (siSETD1A) and reconstituted with WT SETD1A or with a truncated catalytically inactive mutant (SETD1A-□SET) or in control cells (siSCR). Data are the mean ± SD of 3 experiments. **(F)** Quantification of fold change of RAD51 recruitment at pericentromeric DSBs in NIH3T3 cells expressing Cas9 + gRNA targeting the major satellite repeats and depleted of SETD1A (siSETD1A) and expressed as relative to control (siSCR). Data are the mean ± SD of 3 experiments. Quantification by RT-qPCR of fold change of centromeric RNA in NIH3T3 cells **(G)** depleted of SETD1A (siSETD1A) and expressed as relative to control (siSCR) and **(H)** expressing dCas9 alone or fused to LSD1 (dCas9-LSD1) and a gRNA targeting the minor satellite repeats. Data are the mean ± SD of 2-3 experiments. **(I)** Immunofluorescence confocal analysis of NIH3T3 cells expressing either dRNAseH fused to GFP (dRNAseH-GFP) and Cas9 alone or together with a gRNA targeting the minor satellite repeats and stained with DAPI and antibodies specific for □-H2AX and CENP-A. **(J)** Quantification of fold change of dRNAseH recruitment at centromeric DSBs in cells expressing Cas9 + gRNA targeting the minor satellite repeats and depleted of SETD1A (siSETD1A) relative to control (siSCR) or expressing dCas9 fused to LSD1 (dCas9-LSD1) and relative to dCas9 alone. Data are the mean ± SD of 3 experiments with n=50 cells. For all graphs represented as fold change, values represent mean ± SD of fold change calculated over the mean of control samples. Statistical significance was determined by t-test (*p<0,05, **p<0,01). For confocal images, scale bars represent 5 µm.

Supplementary Figure 4. **(A)** Quantification of CENP-A, HJURP and MIS18 mRNA levels by RT-qPCR in NIH3T3 cells depleted of CENP-A (siCENP-A), HJURP (siHJURP) and MIS18 (siMIS18), normalized to GAPDH and expressed as relative to control (siSCR). **(B)** Quantification of fold change of RPA recruitment at centromeric DSBs in cells depleted from HJURP (siHJURP), CENP-A (siCENP-A), their combination (siHJURP+siCENP-A) and MIS18 (siMIS18) relative to control

(siSCR) in NIH3T3 cells expressing Cas9 + gRNA targeting the minor satellite repeats. Data are the mean ± SD of 3 experiments. **(C)** Immunofluorescence (IF) confocal analysis of NIH3T3 lacO-Isce-I-tet cells expressing lacI fused to mCherry (mCherry-LacI) or fused to HJURP (mCherry-lacI-HJURP) and co-expressing I-SceI and stained with DAPI, and antibodies against□□-H2AX and RAD51. Quantification of fold change of RAD51 recruitment at lacO locus is shown on the right. Data are the mean ± SD of 3 experiments with n=50 cells). **(D)** Quantification of fold change of RAD51 recruitment in NIH3T3 cells in G2 and expressing Cas9 + gRNA targeting the minor satellite repeats and depleted from HJURP (siHJURP) and CENP-A (siCENP-A) relative to control (siSCR). Data are the mean ± SD of 3 experiments. **(E)** Western blot analysis of USP11, □-H2AX and tubulin in NIH3T3 cells in G1 and expressing dCas9 or Cas9 + gRNA targeting the minor satellite repeats. **(F)** Quantification of USP11 mRNA by RT-qPCR in cells depleted from USP11 (siUSP11), normalized to GAPDH and expressed as relative to control (siSCR). **(G)** IF confocal analysis of NIH3T3 cells in G1 expressing dCas9 fused to GFP (GFP-dCas9) or fused to USP11 and GFP (GFP-dCas9-USP11) and co-expressing Cas9 + gRNA targeting the major satellite repeats and stained with DAPI, and antibodies specific to□□-H2AX and RAD51. Quantification of fold change of RAD51 recruitment at pericentromeric DSBs in G1 is shown on the right. Data are the mean ± SD of 3 experiments. **(H)** IF confocal analysis of U2OS lacO-Isce-I-tet cells expressing lacI fused to mCherry (mCherry-LacI) or fused to HJURP (mCherry-lacI-HJURP) and co-expressing USP11 fused to GFP (GFP-USP11) and stained with DAPI. Quantification of percentage of cells with colocalization of GFP-USP11 with LacO locus is shown on the right. Data are the mean ± SD of 3 experiments with n=50 cells). **(I)** Western blot analysis of ubiquitin (FK2 antibody) after IP of GFP (GFP-IP) under denaturing conditions in NIH3T3 cells expressing GFP or GFP-HJURP and depleted of USP11 (siUSP11) or control cells (siSCR). The input was 1% of the extract used for the IP. **(J)** Quantification of fold change of CENP-A recruitment at LacO in cells expressing LacI-HJURP and depleted of USP11 (siUSP11) relative to control (siSCR) cells. Data are the mean ± SD of 3 experiments. **(K)** Schematic representation of the SNAP technology. **(L)** Western blot analysis of USP11, GFP and tubulin in HeLa cells expressing CENP-A-SNAP or CENP-A-SNAP + GFP-HJURP depleted of USP11 (siUSP11) or HJURP (siHJURP), compared to control (siSCR). For all graphs represented as fold change, values represent mean ± SD of fold change calculated over the mean of control samples. Statistical significance was determined by t-test (**p<0,01, ***p<0,001, ****p<0,0001). For confocal images, scale bars represent 5 µm.

Supplementary Figure 5. Schematic representation of the step-wise model for the licensing of HR in G1 at centromeres. For details see text.

Methods

Cell Culture and treatments.

NIH3T3 mouse fibroblasts were maintained in DMEM with high (4.5g/L) glucose, supplemented with 10% newborn calf serum and gentamycin (40 µg/ml). U2OS and HeLa human cells were maintained in DMEM with low (1 g/L) glucose, supplemented with 10% foetal calf serum and gentamycin (40 µg/ml). Neocarzinostatin (NCS; N9162-100 UG; Sigma) was added (50, 100 or 200 ng/ml) for 15 min, the medium was replaced, and 2 h later cells were harvested for western blot analysis or fixed for immunofluorescence analysis. DMSO, RAD51 inhibitor (553525; Calbiochem) or RAD52 inhibitor (E3768, Sigma Aldrich) were added (20 µM) 1 h before transfection and refreshed 6h before fixation or harvest. Irradiation (CellRad) was induced (2Gy) for 2 h before fixation.

Cell stage discrimination.

Cells were synchronized in the G2 phase of the cell cycle with the Cdk1 inhibitor IV (RO-3306; 217699; Calbiochem; 10 µM), which was added 8 h before transfection, for a total of 24 h. G1 experiments were performed in unsynchronized NIH3T3 cells, for which we have previously shown that close to 80% of cells are in G1 (Fig.S2F) and this percentage increases further after transfection with Cas9 (Tsouroula et al., 2016,). The IF quantifications were performed in NIH 3T3 cells negative for EdU staining (marker of S phase) and H3S10 staining (marker of G2), or in U2OS cells arrested in G1/S with double-thymidine (T1895; Sigma) block: 18 h thymidine treatment (2 mM), 9 h release, 16 h thymidine treatment (2 mM). Cell-cycle arrest was confirmed by flow cytometry. The double thymidine treatment did not later substantially the cell cycle in NIH3T3 cells (Fig.S2F).

Cell cycle analysis.

EdU incorporation and staining was performed according to the manufacturer's instructions (Click-iT EdU Flow Cytometry Assay Kit, Invitrogen). Cells were then treated with RNAse A (100 µg/ml) and stained with propidium iodide (40 µg/ml) for 30 min at 37°C. Data were collected on a FACSCalibur (Becton-Dickinson) and analyzed with FlowJo (TreeStar).

Transfection and siRNA Knockdown.

Transient transfections were performed with Lipofectamine 2000 (Invitrogen Life Technologies) or JetPei (Polyplus-transfection) for plasmids in mouse and human cells respectively, or

Lipofectamine RNAiMAX (Life Technologies) for siRNAs, following the manufacturer's instructions (see Supplemental Information Table S1 for siRNAs references, Table S2 for gRNA sequences and primers and Table S3 for plasmids). Knockdown efficiency was analyzed by western blot and/or RT-qPCR. All microscopy and western blot experiments were performed 72 h post-knockdown and 16 h post-transfection. For statistical analysis of all experiments, t tests were performed and errors bars represent standard deviation: *p < 0,05, **p < 0,01, ***p < 0,001, and ****p<0,0001

Real-Time qPCR.

RNA and cDNA were prepared using standard techniques. qPCR was performed in triplicate using SyberGreen (Qiagen) and a LightCycler 480 (Roche) as previously described (Pankotai et al., 2012). Transcript quantities were calculated relative to standard curves and normalized to GAPDH mRNA (see Supplemental Information Table S4 for primers).

Western Blot Analysis.

Proteins were fractionated by SDS-PAGE and transferred to Protran Nitrocellulose membranes (Sigma Aldrich) and blotted with antibodies listed in the Supplemental Information Table S5.

Immunofluorescence and Confocal Microscopy.

Cells were cultured on coverslips and pre-extracted in 0.1% Triton/1X PBS for 30 sec prior to fixation in 4% paraformaldehyde/1X PBS for 10 min, on ice. After a second fixation step in 4% paraformaldehyde/1X PBS for 10 min at room temperature, cells were permeabilized in 0.5% Triton/1X PBS for 10 min, blocked in 5% BSA/1X PBS-0.1% Tween for 1 h and incubated with primary antibody for 1 h (see Supplemental Information Table S5 for antibodies) and secondary antibody (Alexa Fluor, Life technologies) for 1 h. Cells were counterstained with DAPI (1 mg/ml) and mounted (Prolong Gold, Invitrogen) on slides. For EdU incorporation, the Click-iT EdU Alexa Fluor 488 or 594 Imaging Kit was used. Cells were observed on a confocal laser scanning microscope (TCS SP8; Leica) using a 63x objective.

Chromatin immunoprecipitation.

14 million cells were fixed by addition of Formaldehyde (Sigma; 1% final concentration) to the culture medium. Crosslinking was performed at room temperature for 10 min followed by quenching with 0.125 M glycine for 10 min at 4°C. Samples were washed twice with ice-cold PBS and collected by scraping. After centrifugation, an initial lysis was performed with buffer 1 (50 mM Tris pH8; 2 mM EDTA pH8; 0,1 % NP-40; 10 % glycerol; 1x Proteasome Inhibitor Cocktail

(PIC)) for 5 min at 4°C, followed by centrifugation and the second lysis step with buffer 2 (1 % SDS; 10 mM EDTA; 50 mM Tris-HCl pH8; 1x PIC). After sonication (E220 Focused-ultrasonicator, Covaris) to generate genomic DNA fragments with an average size of 300-700 bp, 1% input fraction was saved and chromatin (60 µg) was pre-cleared with BSA-pre-treated protein A/protein G Sepharose beads (Invitrogen) for 1 h at 4°C with overhead shaking. Next, immunoprecipitation (overnight at 4°C, with shaking) with 5 µg of antibody was performed. This was followed by pulldown with BSA-pre-treated protein A/protein G Sepharose beads (Invitrogen) and incubated for 4 h at 4°C, with rotation. Beads were washed twice with low salt buffer (0.1% SDS; 0,5% NP-40; 2 mM EDTA; 20 mM Tris pH8; 150 mM NaCl; 1x PIC), once with high salt buffer (0.1% SDS; 0,5% NP-40; 2 mM EDTA; 20 mM Tris pH8; 500 mM NaCl; 1x PIC), and once with LiCl/DOC wash buffer (10 mM Tris pH8; 0,5% sodium deoxycholate; 0,5% NP- 40, 250 mM LiCl; 1 mM EDTA; 1x PIC). Elution of the beads was then carried out twice (10 min each at room temperature, with rotation) in TE + 1% SDS + 0.1 M NaHCO3 and the eluted DNA was then subjected to NaCl (0.2 M) + RNaseA (50 µg/ml) treatment for 30 min at 37°C followed by Proteinase K treatment (0.07mg/ml) directly followed by reverse crosslinking overnight at 65°C, with shaking. DNA isolation was then carried out using the QIAquick PCR purification kit (QIAGEN), resuspended in elution buffer and qPCR was performed using SYBR Green mix (Roche) and analyzed on a medium- to high-throughput PCR platform (LightCycler 480 Instrument II, Roche).

Co-immunoprecipitation.

For USP11 immunoprecipitation, cells were transfected with non-targeting siRNA or siCENP-A for 72 h. Subsequently, cells were collected by scraping in ice cold 1x PBS. Cells were then washed once with ice-cold 1x PBS and lysed for 20 min at 4°C with lysis buffer (25 mM Tris pH 7.5; 1% Triton; 150 mM NaCl; 2 mM EDTA; 1x PIC and 10U/µL benzonase (Sigma Aldrich)). Lysates were then sonicated. After centrifugation at 12,000 RPM for 20 min at 4°C, 1% input fraction was saved, and the lysate was diluted three times with dilution buffer (25 mM Tris pH 7.5; 0.1% Triton; 150 mM NaCl; 2 mM EDTA; 1x PIC). This was followed by pre-clearing of the lysate with equilibrated protein A Sepharose beads (Invitrogen) for 1 h at 4°C, with rotation. After centrifugation, 2 µg of either IgG control or USP11 antibody was added for 2 h at 4°C, with rotation. Equilibrated protein A Sepharose beads (Invitrogen) were then added to lysates and incubated overnight at 4°C, with rotation. Beads were washed 3 times 10 min with lysis buffer (without benzonase) at 4°C, with rotation, and eluted in SDS sample buffer for 15 min at 75°C.

For GFP pull-down, cells were transfected with non-targeting siRNA or siUSP11 for 72 h, and/or with either GFP or GFP-HJURP plasmids for 24 h, combined or not with either Flag or Flag-USP11 plasmid transfection. Cells for GFP pull-down in denaturing conditions, for ubiquitination staining, were treated with MG132 (20µM, 1748, Bio-Techne) 8 h before collection. Subsequently, cells were collected by scraping in ice cold 1x PBS. Cells were then washed once with ice-cold 1x PBS and lysed either for 30 min at 4°C with lysis buffer (10 mM Tris pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.1% SDS; 1% Triton; 1% sodium deoxycholate; 2.5 mM MgCl2; 1x PIC and 1U/µL benzonase) or, for GFP pull-down in denaturing conditions, cells were resuspended in lysis buffer 1 (20 mM Tris pH 7.5; 50 mM NaCl; 0.5% NP-40; 1% sodium deoxycholate; 1 % SDS; 5 mM MgCl2; 1x PIC) and immediately in the lysis buffer 2 (20 mM Tris pH 7.5; 50 mM NaCl; 0.5% NP-40; 0.5% sodium deoxycholate; 0.5% SDS; 5 mM MgCl2; 1x PIC and 1U/µL benzonase) for 1 h at room temperature. After centrifugation at 12,000 RPM for 10 min at 4°C or room temperature, 1% input fraction was saved. This was followed by addition of the equilibrated GFP-Trap_A beads (Chromotek) and incubation either overnight at 4°C, with rotation, or 2 h at room temperature with rotation, respectively. Beads were washed 3 times with high salt wash buffer (10 mM Tris pH 7.5; 300 mM NaCl; 0.5 mM EDTA; 0.1% SDS;1x PIC) at 4°C, or 6 times with wash buffer (20 mM Tris pH 7.5; 150 mM NaCl; 0.5% NP-40; 0.5% sodium deoxycholate; 0.5% SDS; 1x PIC) at room temperature respectively. Lysates were eluted in SDS sample buffer for 10 min at 95°C.

Metaphase spreads and FISH.

After addition of Colcemid (0.02 µg/ml, 15210040; Fisher Scientific) for 6 h, medium and PBS 1x of the first wash were collected. Cells were trypsinized, harvested, and the pellet was resuspended in 0.06M KCl solution and incubated 30 min at 37°C. After centrifugation, the pellet was resuspended slowly in ethanol:glacial acetic acid (3:1) solution for fixation. This step was repeated 3 more times. Metaphase chromosomes were then spread on wet cold glass slides drop by drop, and air-dried. The spreads were fixed with 4% PFA for 4 min at 37°C, and treated with RNAse A solution (100 µg/ml in 2X SSC) 1h at 37°C. After Pepsin treatment (0.005%) for 4 min at 37°C, the spreads were fixed a second time, dehydrated through an ethanol series (70%, 85% and 100% ethanol for 2 min each) at room temperature and air-dried. Hybridization buffer (20 mM Na_2HPO_4, 20 mM Tris, 60% formamide, 0.1 µg/ml salmon sperm DNA in 2X SSC) containing 0.07 µM PNA telomeric probe Cy5 and 0.2 µM PNA centromeric probe Cy3 was applied to the slide. DNA was denatured at 85°C for 10 min and hybridization was carried out

at room temperature for 2 h. Slides were washed 2 times for 10 min in 2X SSC/0.1% Tween-20 at 55-60°C. DNA was counterstained with DAPI (1 mg/ml) and covered with mounting media (Prolong Gold, Invitrogen) and a coverslip. Images were acquired and analysed as described above. (See Supplemental Information Table S6 for FISH probes).

SNAP labelling of newly-synthetized CENP-A.

48 h after siRNA transfection, pre-existing CENP-A histones were quenched by incubating cells with 10 µM SNAP-cell Block (New England Biolabs) for 30 min at 37°C followed by a 30 min-wash in fresh medium at 37°C and a 48 h chase. Newly synthetized histones were then labeled by incubating cells with 2 µM SNAP-cell TMR star (New England Biolabs) for 15 min (pulse) followed by a 30 min-incubation in fresh medium at 37°C, 10 min of pre-extraction as described above, and 10 min of 4% PFA fixation. Images were acquired and analysed as described above.

DNA-RNA immunoprecipitation.

Cells were lysed and sonicated as described above (see Chromatin immunoprecipitation). Nucleic acids were then treated with RNAse A (200 µg/ml) for 30 min at 37°C, then with Proteinase K (20 mg/ml) 1 h at 65°C, and isolated using QIAquick PCR purification kit (QIAGEN). BSA-pretreated protein A Sepharose beads (Invitrogen) were incubated with 10 µg of S9.6 antibody in IP buffer (50 mM Hepes/KOH at pH 7.5; 0.14 M NaCl; 5 mM EDTA; 1% Triton X-100; 0.1% Na-Deoxycholate, ddH2O) at 4°C for 4 h, with rotation. 6 µg of digested nucleic acids were added to the mixture and gently rotated at 4°C overnight. Beads were recovered and washed successively with 1mL of lysis buffer (low salt, 50mMHepes/ KOH pH 7.5, 0.14 M NaCl, 5 mM EDTA pH 8, 1% Triton X-100, 0.1% Na-Deoxycholate), 1 mL of lysis buffer (high salt, 50 mM Hepes/KOH pH 7.5, 0.5 M NaCl, 5 mM EDTA pH 8, 1% Triton X- 100, 0.1% Na-Deoxycholate), 1 mL of wash buffer (10 mM Tris-HCl pH 8, 0.25 M LiCl, 0.5% NP-40, 0.5% Na-Deoxycholate, 1 mM EDTA pH 8), and 1 mL of TE (100 mM Tris-HCl pH 8, 10 mM EDTA pH 8) at 4°C, twice. Elution was performed in 100 µL of elution buffer (50mMTris-HCl pH 8, 10mMEDTA, 1% SDS) for 15 min at 65°C. After purification using QIAquick PCR purification kit, DNA was eluted in 50 µL of elution buffer (5 mM Tris-HCl pH 8.5), and analyzed by qPCR as described above.

Plasmid Construction.

The DNA sequences encoding Cas9 (Cong et al., 2013), dCas9 (Cong et al., 2013), LSD1, CENP-A, HJURP, USP11, EGFP, and mCherry, were amplified by PCR and cloned by megawhop cloning (Miyazaki, 2011). Individual gRNAs (Table S4) were cloned into a vector containing the U6 promoter followed by a gRNA scaffold. All plasmids (Table S5) were assembled by golden gate cloning (Engler et al., 2009). wtCas9 was amplified from pX330-U6-Chimeric_BB-CBh-hSpCas9 and dCas9 was generated by mutagenesis and amplified from pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A). pX330-U6-Chimeric_BB-CBh-hSpCas9 and pX335-U6- Chimeric_BB-CBh-hSpCas9n(D10A) were a gift from Feng Zhang (Addgene plasmids # 42230 and # 42335). See Table S3 for plasmid details and sequences.

Table S1

siRNA used*	
siRNA	**Reference**
scramble	D-001810-01
mouse SETD1A	L-051358-01
mouse CENP-A	L-044345-00
mouse HJURP	L-057537-00
mouse MIS18	L-065775-01
mouse USP11	L-064114-01
mouse RAD51	L-062730-00
mouse RAD52	L-043751-00
mouse PolQ	L-050773-01
human HJURP	L-015443-00
human USP11	L-006063-00

* All siRNAs (ON-TARGETplus SMARTpool siRNAs) were purchased from Dharmacon.

Table S2

gRNAs			
gRNA	**Sequence**	**Primers**	
Mouse minor satellite	AAAACACATTCGTTGGAAAC	Fwd	AAAGAAGACAAACCGAAAACACATTCGTTGGAAACGTTTAAGTCTTCTTT
		Rev	AAAGAAGACTTAAACGTTTCCAACGAATGTGTTTTCGGTTTGTCTTCTTT
Mouse Major satellite	GAAATGTCCACTGTAGGACG	Fwd	AAAGAAGACAAACCGAAATGTCCACTGTAGGACGGTTTAAGTCTTCTTT
		Rev	AAAGAAGACTTAAACTGATTTTCAGTTTTCTCGCCGGTTTGTCTTCTTT
Human alpha satellite	GAATCTGCAAGTGGATATT	Fwd	AAAGAAGACAAACCGGAATCTGCAAGTGGATATTGTTTAAGTCTTCTTT
		Rev	AAAGAAGACTTAAACAATATCCACTTGCAGATTCCGGTTTGTCTTCTTT

Table S3

Plasmid used *	
CMVp-Cas9-EGFP-SV40p-PuroR-pA	
CMVp-dCas9-EGFP-SV40-PuroR-pA	
U6p-gRNA(Ma-Sat#3)-CMVp-Cas9-mCherry-SV40p-HygroR-pA	
U6p-gRNA (Mi-Sat#2)-CMVp-Cas9-mCherry-SV40p-HygroR-pA	
U6p-gRNA(Ma-Sat#3)-CMVp-dCas9-mCherry-SV40p-HygroR-pA	
U6p-gRNA (Mi-Sat#2)-CMVp-dCas9-mCherry-SV40p-HygroR-pA	
U6p-gRNA (Mi-Sat#2)-CMVp-EGFP-dCas9-LSD1-SV40p-HygroR-pA	
U6p-gRNA(Ma-Sat#3)-CMVp-EGFP-dCas9-CENPA-SV40p-PuroR-pA	
U6p-gRNA(Ma-Sat#3)-CMVp-EGFP-dCas9-HJURP-SV40p-HygroR-pA	
U6p-gRNA(Ma-Sat#3)-CMVp-EGFP-dCas9-USP11-SV40p-PuroR-pA	
CMVp-EGFP-USP11-SV40-PuroR-pA	
CMVp-EGFP-SV40-PuroR-pA	
GFP-RNAseH	Gift from Dr. Andrés Aguilera
GFP-dRNAseH	Gift from Dr. Andrés Aguilera
WT SETD1A	Gift from Dr. David Skalnik and Dr. Grant Stewart
ΔSET	Gift from Dr. David Skalnik and Dr. Grant Stewart
GFP-CENPA	Gift from Dr. Bui Minh
GFP-HJURP	Gift from Dr. Bui Minh.
mcherry-LacI	Soutoglou and Misteli, Science, 2008
mCherry-Laci-HJURP	Gift from Dr. Daniel Richard Foltz
Flag-USP11	pQFlag-USP11 WT puroR (from Addgene)
RAD52-turboGFP	Gift from Dr. Madalena Tarsounas

* Plasmids and sequences available upon request.

Table S4

Primer used for ChIP		
Target	**Forward primer**	**Reverse primer**
B-actin	AAGAGCTATGAGCTGCC	ACTCCTGCTTGCTGATCC
Minor Satellites	CATGGAAAATGATAAAAACC	CATCTAATATGTTCTACAGTGTGG

Primer used for RT-qPCR		
Target	**Forward primer**	**Reverse primer**
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
minor satellites	GAACATATTAGATGAGTGAGTTAC	GTTCTACAAATCCCGTTTCCAAC
SETD1A	AAACCAGCTCAAGTTTCGGAAG	TTTTCCCGCATGTCCGCTAC
CENP-A	TTACATGCTGGTCGGGTCAC	GGCACCGTGTAGCCAGTATT
HJURP	GCGGCTGATAGCGAAGTACAA	CCTTCTGGAGCTTGCCCATTTA
MIS18	TTGCGCAGCGTCTCCTGTAA	ACTTTCAACGGCTTCAACGC
USP11	GTGTTGCACCAGACAAGATGA	AACCCTCAACCGGCTCAATC

Table S5

Antibodies used		
Antibody	**Company (reference)**	**Dilution ***
γ-H2AX (H2AX S139)	Abcam (ab22551)	1:1000 (IF & WB)
53BP1	Novus (NB100-304)	1:1000 (IF)
pATM (S1981)	RockLand (200-301-400)	1:1000 (WB)
RAD51	Calbiochem (PC130)	1:100 (IF)
EGFP	Abcam (6673-100)	1:3000 (WB)
RPA32	Novus Biologicals (600-565)	1:250 (IF)
α Tubulin	Sigma Aldrich (T5168)	1:5000 (WB)
mouse CENP-A	Cell Signaling Technology (#2048)	1:500 (IF & WB)
CREST	Antibodies online (15-234)	1:500 (IF)
BRCA1	Gift from Dr. Andre Nussenzweig	1:200 (IF)
LSD1	Abcam (ab17721)	1:1000 (WB)
H3K4me2	Active motif (39141)	5µg for ChIP
USP11	Abcam (ab109232)	1:1000 (WB)
Lamin A	Abcam (ab26300)	1:1000 (WB)
FK2	Millipore (04-263)	1:1000 (WB)

* IF= Immunofluorescence, WB= Western Blot

Table S6

Probes for FISH		
Probe	**Company (reference)**	**sequence**
PNA centromeric CENPB probe Cy3	PNA bio (F3002)	ATTCGTTGGAAACGGGA
TelG-Alexa488 PNA FISH probe	PANAGENE (F1008)	G-rich probe (repeats of TTAGGG)

2. Complementary results to Figure 2: H3K4me2 supports DNA-end-resection by promoting centromeric transcription and increased R-loop formation in response to DSBs

To investigate the involvement of H3K4me2 in HR activation in G2 as well and determine if this part of the mechanism is cell cycle specific, we depleted the histone methyltransferase SETD1A (a H3K4me2 writer) and we induced DSBs to centromeric DNA using WT Cas9 and the corresponding guide RNA in G2 cells. Interestingly, H3K4me2 depletion slightly reduced RAD51 recruitment at centromeric DSBs in G2 as well (Figure 19.A), suggesting that H3K4me2-dependent R-loop formation plays a role in centromeric resection throughout the cell cycle. To verify that the effect of SETD1A on HR repair factors recruitment is specific to H3K4me2 and not a secondary effect, we rescued SETD1A depletion with either WT SETD1A or ΔSET (methyltransferase activity inactive). Similarly to what we observed for RPA, our results showed that while WT SETD1A rescues BRCA1 loss at centromeric DSBs, ΔSET does not (Figure 19.B), suggesting that it is the H3K4me2 mark per se that facilitates HR initiation in G1.

To verify if the role of R-loops in the HR repair initiation at centromeric DSBs in G1 is further impacting the HR process, we quantified the recruitment of RAD51 in cells overexpressing WT RNAseH. As expected, inhibition of R-loops at centromeres led to a decrease in the recruitment of RAD51 at centromeric breaks (Figure 19.C).

These results further confirm the specificity of this process to centromeres and highlight further the role of H3K4me2-dependent R-loop formation in centromeric HR repair process.

3. Complementary results to Figure 3: The USP11/HJURP/CENP-A axis licenses HR at centromeric DSBs in G1

Consistent with the specific interaction of USP11 with HJURP and CENP-A, USP11 depletion did not affect RAD51 recruitment at pericentromeric DSBs (Figure 20.A).

HJURP interacts with CENP-A through its Scm3 region, that has two sites for potential ubiquitylation (Figure 20.B). To further decipher the role of USP11 in the modulation of the interaction between CENP-A and HJURP, we aimed to determine if these sites are deubiquitylated by USP11. We have created and expressed a GFP-tagged Scm3, and after verifying that this region alone also interacts with USP11 by immunoprecipitation (Figure 20.C), we checked GFP-Scm3 ubiquitylation upon USP11 overexpression (Figure 20.D). As before, Scm3 ubiquitylation decreased when USP11 was overexpressed, suggesting that USP11 allows

the interaction between CENP-A and HJURP by deubiquitylating the domain if HJURP that is interacting with CENP-A.

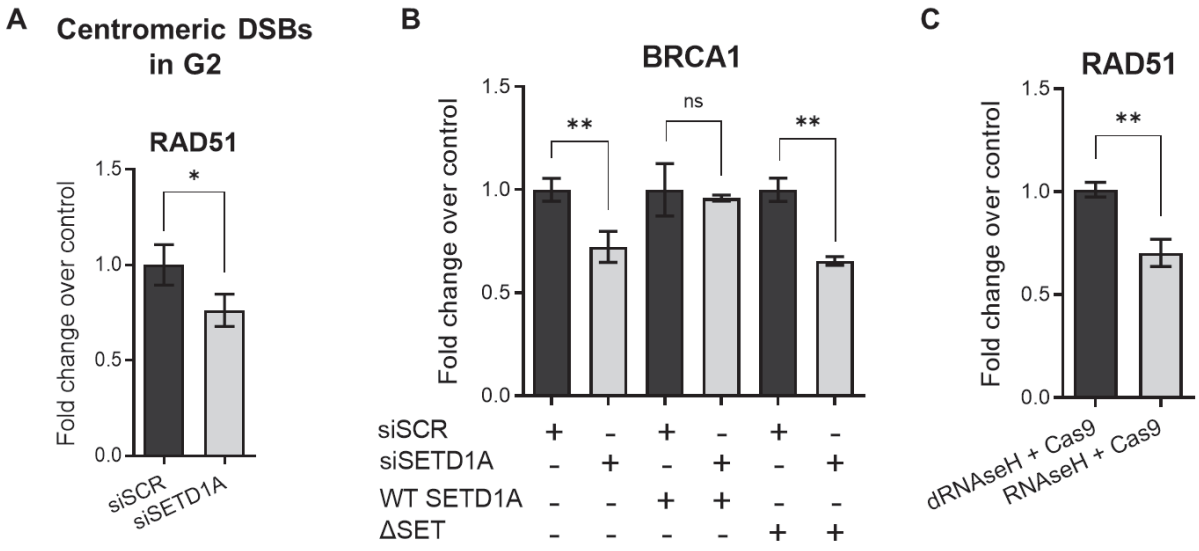


Figure 19: H3K4me2 supports DNA-end-resection by promoting centromeric transcription and increased R-loop formation in response to DSBs

(A) Quantification of fold change of RAD51 recruitment at centromeric DSBs in NIH3T3 cells synchronized in G2, depleted of SETD1A (siSETD1A) relative to control (siSCR) and expressing Cas9 + gRNA targeting the minor satellite repeats. Data are the mean ± SD of 3 experiments with n=50 cells. **(B)** Quantification of fold change of BRCA1 recruitment at centromeric DSBs in cells depleted for SETD1A (siSETD1A) and reconstituted with WT SETD1A or with a truncated catalytically inactive mutant (SETD1A-DSET) relative to the control (siSCR). Data are the mean ± SD of 3 experiments with n=50 cells. **(C)** Quantification of fold change of RAD51 recruitment at centromeric DSBs in cells expressing RNAseH relative to cells expressing dRNAseH. Data are the mean ± SD of 3 experiments with n=50 cells. For all graphs, values represent mean ± SD of fold change calculated over the mean of control samples, and statistical significance was determined by t-test (*p<0,05, **p<0,01).

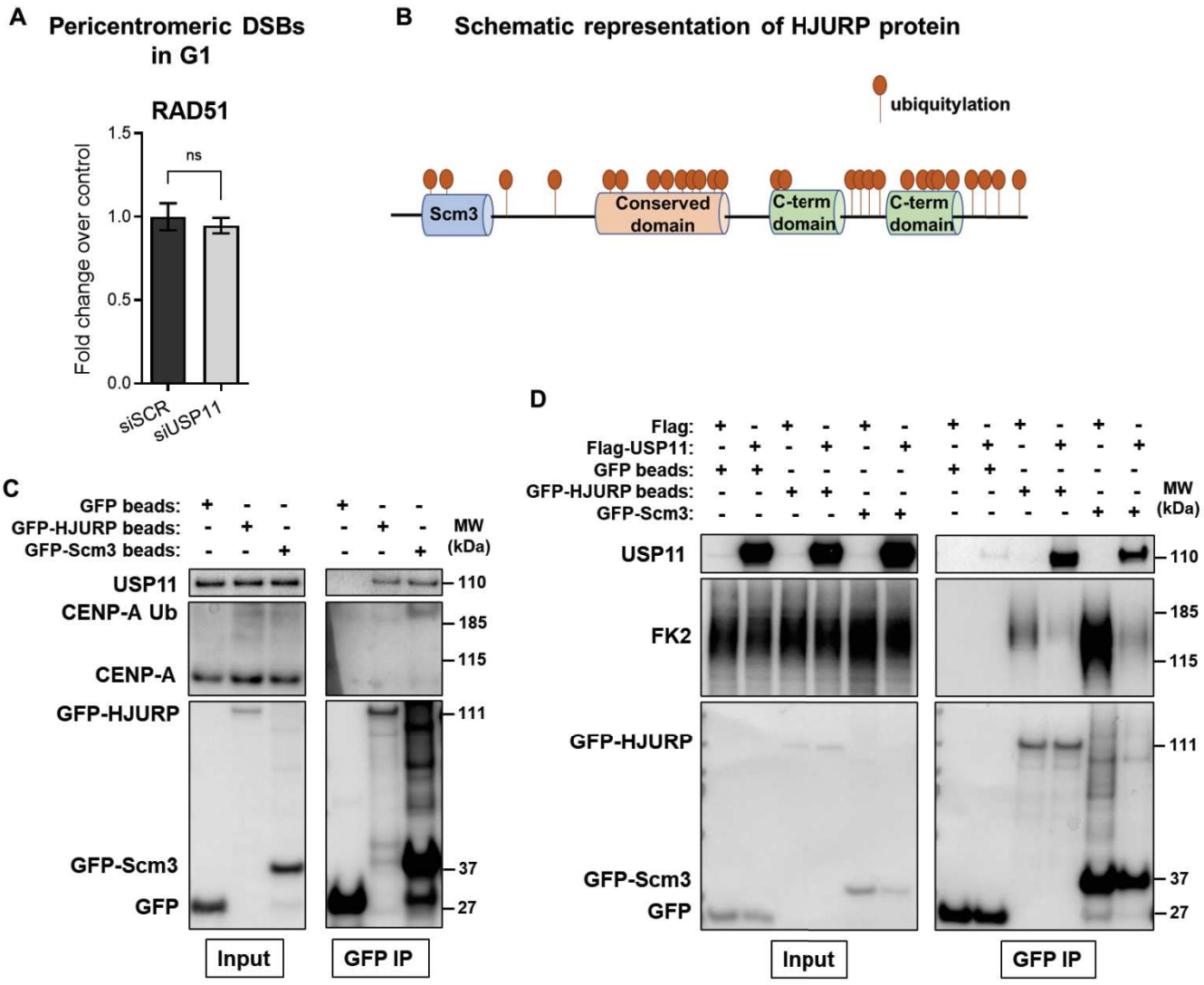


Figure 20: The USP11/HJURP/CENP-A axis licenses HR at centromeric DSBs in G1

(A) Quantification of the fold change of RAD51 recruitment at pericentromeric DSBs in cells depleted of USP11 (siUSP11), relative to control (siSCR) in NIH3T3 cells expressing Cas9 + gRNA targeting the major satellite repeats. Data are the mean ± SD of 3 experiments with n=50 cells. Data represent mean ± SD of fold change calculated over the mean of control of 4 experiments with n=200 cells. (B) Schematic representation of HJURP protein with its different domains and ubiquitination sites. (C) Western blot analysis of USP11, GFP, GFP-HJURP, GFP-Scm3 and CENP-A after IP of GFP (GFP-IP) in NIH3T3 cells. The Input was 1 % of the extract used for the IP. (D) Western blot analysis of ubiquitin (FK2 antibody) after IP of GFP (GFP-IP) under denaturing conditions in NIH3T3 cells expressing GFP or GFP-HJURP or GFP-Scm3 and co-expressing tagged USP11 (USP11-Flag) or the tag alone (Flag). The input was 1% of the extract used for the IP.

4. Complementary results to Figure 4: Licensing of HR at centromeric breaks throughout the cell cycle promotes centromeric integrity

Licensing of HR throughout the cell cycle at centromeric DSBs prevents centromeric genomic instability and the onset of chromosomal rearrangements. To decipher the underlying mechanism, we sought to determine which alternative DNA repair pathways are at play when RAD51 is inhibited or depleted. For this, we assessed, by centromeric and telomeric FISH on metaphase spreads, the number of translocations occurring after centromeric DSBs induction in conditions in which RAD51 is inhibited together with the depletion of proteins representative of different DNA repair pathways. Except from the role of RAD52 described in the paper, the number of translocations also depended on classical NHEJ, as inhibition of DNAPK reduced the number of translocations induced upon RAD51-inhibition (Figure 21).

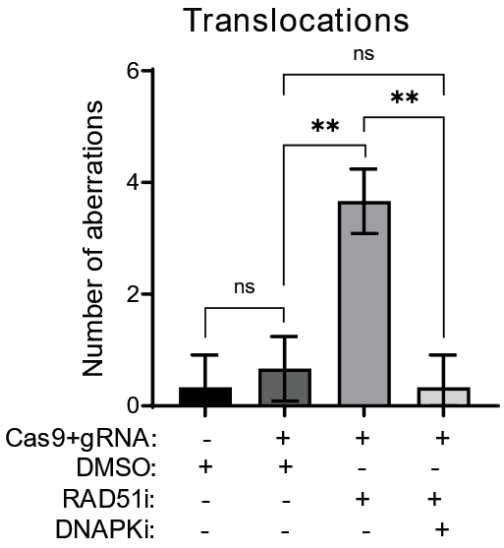


Figure 21: Licensing of HR at centromeric breaks throughout the cell cycle promotes centromeric integrity

Quantification of the number of translocations in cells expressing Cas9 + gRNA targeting the minor satellite repeats and treated with DMSO, RAD51 inhibitor (RAD51i), or a combination of RAD51i and DNAPK inhibitor (DNAPKi). Data are the mean ± SD of 4 experiments with n=50 metaphases.

5. Role of recombination in CENPA deposition

It has been proposed in yeast that Mre11, Rad51 and Rad52 maintain centromere function by regulating CENP-A^{CaCse4} levels at the programmed fork stall sites of early replicating centromeres, as CENP-A acts as a physical barrier for fork progression (Mitra et al., 2014). Another study has shown that yeast Rad51 localizes at centromeres and suppresses rearrangements of centromeric repeats that result in isochromosome formation (Nakamura et al., 2008). Moreover, homologous recombination between centromeric repeats leads to closed loops

that can have an important role in the establishment of a functional centromere (McFarlane and Humphrey, 2010), suggesting that recombination is an essential event in centromere maintenance. In light of these data, we have been wondering whether human MRE11, RAD51 and RAD52 play a role in promoting CENP-A deposition at centromeres. Using HeLa cells stably expressing CENP-A-SNAP, we quenched pre-existing CENP-A, depleted either MRE11 or RAD51 or RAD52 (Figure 22.A and 22.B) and checked *de novo* deposition of CENP-A by a TMR pulse. MRE11 depletion resulted in a striking defect in *de novo* CENP-A deposition, while RAD51 and RAD52 depletion led to a mild defect in CENP-A deposition (Figure 22.C). Our very preliminary findings suggest that these factors, present at centromeric regions for a maintenance purpose, might be directly available in case of break occurrence, further deepening our understanding of centromere-specific repair mechanism.

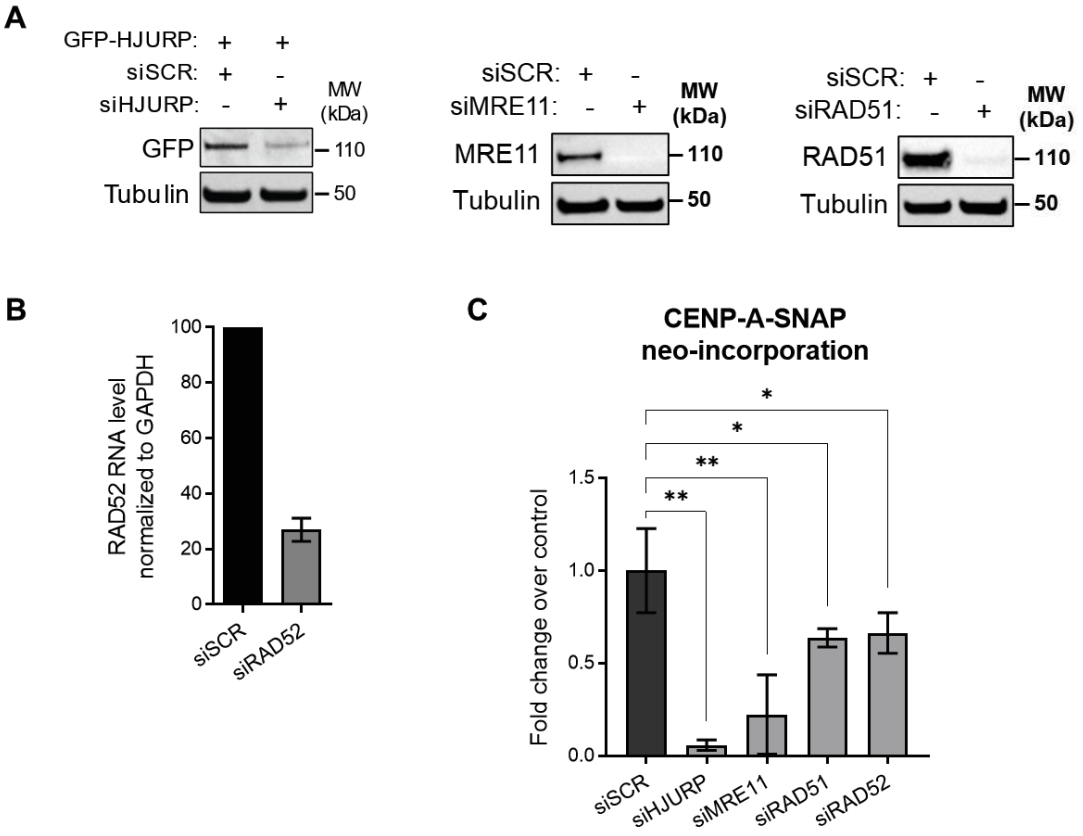


Figure 22: Role of recombination in CENPA deposition

(A) Western blot analysis of GFP, MRE11, RAD51 and tubulin in HeLa cells expressing CENP-A-SNAP or CENP-A-SNAP + GFP-HJURP depleted of HJURP (siHJURP) or MRE11 (siMRE11) or RAD51 (siRAD51) compared to control (siSCR). **(B)** Quantification of USP11 mRNA by RT-qPCR in cells depleted from USP11 (siUSP11), normalized to GAPDH and expressed as relative to control (siSCR). (C) Quantification of fold change of the percentage of cells with CENP-A-SNAP in cells depleted from HJURP (siHJURP) or MRE11 (siMRE11) or RAD51 (siRAD51) or RAD52 (siRAD52) relative to control cells (siSCR). Data are the mean ± SD of 4 experiments with n=200 cells. Values represent mean ± SD of fold change calculated over the mean of control samples. Statistical significance was determined by t-test (*p<0,05, **p<0,01).

DISCUSSION

Centromeres are essential to ensure proper chromosome segregation during cell division. However, centromeres are known to often be sites of aberrant rearrangement in cancers (Mitelman et al., 1997; Padilla-Nash et al., 2001), and to be often the breakpoint of translocation (Wang et al., 2009), suggesting that DSBs damage at this site is not rare. Unfaithful repair of centromeric lesions can alter centromere organization leading to chromosomal instability and aneuploidies, which can result in a range of diseases, including cancer. About 90% of solid tumours are aneuploid (Compton, 2011) with aneuploidy leading to carcinogenesis by altering the balance between oncogenes and tumour suppressors. Although it is known that centromeres are fragile parts of the genome, how they maintain their integrity when damaged had remained elusive. Here, we find that when DSBs occur at centromeres, H3K4me2 allows DNA damage induced centromeric transcription and increased R-loop formation, leading to end resection. We also find that the presence of CENP-A and HJURP facilitate RAD51 recruitment by specifically interacting with USP11, which deubiquitinates PALB2 and allows the recruitment of RAD51 to the resected DNA and hence a possible completion of HR repair in G1, when HR is normally suppressed. Finally, we find that recruitment of HR repair factors at centromeric breaks inhibits activation of mutagenic pathways and the formation of deleterious abnormalities such as chromosomal translocations. Our results point to an important role for the distinct chromatin organisation at centromeres for DNA repair, which allows the succession of events leading to HR repair in G1 and indicate that the current knowledge of the influence of chromatin on DNA repair pathway choice is far from complete. Moreover, our latest results on the role of USP11 and recombination on CENP-A deposition at centromeres open the way to a new understanding of centromere establishment.

1. H3K4me2 and R-loop role in DSB resection and repair

Centromeres contain H3K4me2, a histone modification associated with active chromatin (Wang et al., 2014). We demonstrate that reduction in H3K4me2 at centromeres by SETD1A depletion drastically decreases the end resection marker RPA binding at centromeric DSBs in G1 cells. This, however, is not due to a global defect in H3K4me2/3 throughout the genome and, therefore in the transcriptional state of the cell, because direct tethering of dCas9-LSD1 at centromeres leads to H3K4me2 reduction specifically at centromeres and had a similar effect on end resection.

Moreover, we demonstrate that RPA recruitment at centromeric breaks is rescued when SETD1A-depleted cells are complemented with the WT protein but not with ΔSET. This result argues that the role of SETD1A on centromeric DSBs repair is distinct from the non-catalytic function of SETD1A on the regulation of the DNA damage response, shown before, which does not depend on the SET domain but instead on the FLOS domain (Hoshii et al., 2018). Our observations are in line with the role of SETD1A promoting H3K4 methylation at replication forks, which enhances BOD1L and FANCD2 recruitment to stabilize RAD51 nucleofilaments and prevent replication fork degradation (Higgs et al., 2018).

We show here that H3K4me2 plays a positive role in DNA-end resection by promoting transcription and R-loop formation. Supporting this idea, mis-regulation of H3K4me2 has been shown to reduce α-satellite RNAs levels and result in genomic instability (Huang et al., 2016). Centromeric repeats are transcribed, producing non-coding RNA (Bouzinba-Segard et al., 2006; Hédouin et al., 2017; Quénet and Dalal, 2014). R-loops were previously reported to form at centromeres in mitosis, and to recruit RPA and ATR in order to stimulate Aurora B to enable faithful chromosome segregation (Kabeche et al., 2018). Moreover, R-loops are tightly linked to histone H3 S10 phosphorylation (H3S10P), a mark that significantly accumulates at centromeres (Castellano-Pozo et al., 2013).

It was previously reported that DNA breaks located at transcribed regions of the genome or *de novo* transcription from the break itself, promote R-loop formation (Cohen et al., 2018; Ohle et al., 2016), which in turn facilitates HR by recruiting factors such as RPA and BRCA1 (Hatchi et al., 2015; Hill et al., 2014; Nguyen et al., 2017). Consistent with these findings, we demonstrate that centromeric DSBs increase centromeric transcription and R-loop formation further promoting RPA and BRCA1 binding.

None of the previous studies have implicated an increase in H3K4me2 in break-induced R-loop formation. The exact mechanism that leads to such R-loops formation remains unclear and may be related to *de novo* RNA Pol II loading at DNA ends. In our study, we show that centromeric DSBs induce an increase of centromeric H3K4me2. Our findings are reminiscent of a very recent study in *Caenorhabditis elegans* suggesting a DNA-damage-induced H3K4me2, that is necessary to regulate the transcription of genes post-repair (Wang et al., 2020). But this transient increase of H3K4me2 was dependent of the completion of DNA repair. Our findings are not contradictory to the study of Mosammaparast et al. showing that LSD1 is recruited at DSBs sites, where it reduces H3K4me2 and promotes 53BP1 foci formation, as this mechanism is specific to S/G2 phase and does not happen in G1 (Mosammaparast et al., 2013), since LSD1 levels are very low in G1 and are increased during cell cycle progression (Lv et al., 2010).

Several studies have demonstrated a connection between R-loop formation and DSB repair. Consistent with this, R-loops destabilization by RNaseH overexpression impaired HR in budding yeast (Ohle et al., 2016) and impaired repair by both HR and NHEJ in human cells (Lu et al., 2018). In addition to a role for R-loops in late steps of recombination, effects in DNA end resection have been observed. The impact of R-loop formation on DNA end resection has been controversial with some studies showing that they inhibit DNA end resection (Alfano et al., 2019; Costantino and Koshland, 2018; Ohle et al., 2016), and others showing that they promote end resection (Lu et al., 2018; Yasuhara et al., 2018). In *Schizosaccharomyces pombe*, transient R-loops formation has been shown to be required for HR repair and their degradation regulates DNA end resection (Ohle et al., 2016). This study additionally suggest that R-loops are essential for maintaining repetitive DNA regions around DSBs. In budding yeast, R-loop structures block resection and prompt to asymmetric resection since only the side not involved in R-loop can be processed (Costantino and Koshland, 2018). In HeLa cells, mRNA binding protein HNRNPD favors R-loop removal and regulate proper end-resection by favoring RPA phosphorylation (Alfano et al., 2019). On the other hand in U2OS cells, Drosha drives the formation of R-loops around DSB sites and this facilitates end-resection (Lu et al., 2018). Also in human cells, RAD52 and XPG mediated R-loop processing, at transcriptionally active regions, promotes resection and therefore initiating repair by HR (Yasuhara et al., 2018). RAD52 recruits BRCA1 to antagonize the RIF1-53BP1 complex and the anti-resection activity of the Shieldin complex, suggesting that R-loops favor a BRCA1-mediated repair. Other recent studies have highlighted a role of RAD52 in RNA-templated DSB repair (Keskin et al., 2014; Mazina et al., 2017). In yeast, in the absence of Senataxin ortholog Sen1, R-loops drive Mre11-Dna2-dependent non-canonical DSB resection initiation (Rawal et al., 2020). To reconciliate these contradictory findings, a possible explanation is that pre-existing R-loops might block resection processivity and need to be removed, whereas DSBs-induced R-loop formation will not block resection since they are formed after resection creates ssDNA, and they can promote the resection by regulating the recruitment of specific repair factors. Our observations are in line with studies suggesting that the R-loop orientation, stability, and chromatin structure around the break determines the impact of R-loops on DNA end resection.

Our observations are also in agreement with studies reporting that R-loop induction at DSBs stimulates strand invasion by either increasing RAD51 loading through recruitment of Senataxin (Cohen et al., 2018) or by promoting BRCA2 recruitment by its interaction with RNAaseH2 (D'Alessandro et al., 2018). Additionally, a recent study shows that transcription-coupled homologous recombination process (TC-HR) is a BRCA1/2-independent alternative HR pathway, specific to actively transcribed regions, and based on the recruitment of Cockayne

syndrome protein B (CSB), that in turn recruits RAD52, which then recruits RAD51 (Teng et al., 2018). CSB present a strong affinity for R-loops forming in these regions (Teng et al., 2018). Furthermore, RPA recruitment is probably specific to DSBs-dependent R-loop formation, and therefore not opposite to the absence of RPA during centromeric DNA replication (Aze et al., 2016).

Short telomeres (Graf et al., 2017) or TRF2-depleted telomeres (Porro et al., 2014) express high levels of the telomeric non-coding RNA known as TERRA. TERRA forms R-loops at very short telomeres and R-loop persistence at short telomeres contributes to the activation of DDR and promotes recruitment of the RAD51 recombinase (Graf et al., 2017). TERRA also forms R-loops which stimulates HR in ALT cells (Arora et al., 2014). Finally, TERRA R-loops can form post-transcription in *trans* in a RAD51-dependent manner (Feretzaki et al., 2020). Since our work is very reminiscent of these findings, one hypothesis is that centromeric R-loop formation might also depend on RAD51.

Since H3K4me2 decorates active promoters throughout the genome, a question that arises is whether DSBs at these regions can also promote resection in G1. Indeed, HR is globally suppressed in G1 (Orthwein et al., 2015), and DNA end resection is limited in G1 by the CDK requirement for MRN activation (Anand et al., 2016; Chen et al., 2008; Falck et al., 2012; Greenberg et al., 2006; Huertas et al., 2008) and by 53BP1 (Chapman et al., 2012b). However, end resection does occur in G1, where it is essential for repair of complex DSBs (Averbeck et al., 2014; Zhou et al., 2014), but to a lesser extent than in S/G2. It is possible, therefore, that the limited resection at centromeres is sufficient to trigger RAD51 and homology search as the homologous sequence is very abundant. Furthermore, it is possible that the presence of H3K36me2 serves as a platform for H3K36me3 that recruits CtIP through LEDGF (Aymard et al., 2014; Daugaard et al., 2012). Thus, the combination of H3K4me2 and H3K36me2 on the same nucleosomes, a unique feature of centromeres, together with a preferential binding at centromeres of DNA2 (Li et al., 2018b), centromeric recruitment of BLM during anaphase to allow proper segregation (Ke et al., 2011b), and transcription-dependent BLM recruitment at DSBs where it fosters resection (Cohen et al., 2020) might help in creating a permissive environment for resection.

Another elegant hypothesis about the role of R-loops on HR repair is that these structures might increase strand displacement thus favouring homologous recombination.

2. CENP-A/HJURP/USP11 axis regulation

We show that the mechanism involving HJURP and CENP-A operates strictly in G1 since their depletion had no effect on RAD51 recruitment in G2. These results are in line with the fact that HJURP presence at centromere is transient, limiting CENP-A deposition in G1 (Dunleavy et al., 2009; Foltz et al., 2009). Thus, USP11 recruitment at centromeres via interaction with HJURP can explain why this specific HR process would only occur in G1. Another hypothesis is that since USP11 is not degraded in G2 and is recruited at DSBs site, and additional recruitment through HJURP and CENP-A will not show a significant increase in RAD51 loading.

Ubiquitination of CENP-A at lysine 124 has been proposed to be required for CENP-A deposition at centromeres by HJURP (Niikura et al., 2015, 2016). However, the exact molecular mechanism explaining the role of this ubiquitination remains unclear. Previous studies have revealed that HJURP is also ubiquitinated (Akimov et al., 2018; Beltrao et al., 2012; Stes et al., 2014), but the function of this ubiquitination is unknown. We show here that HJURP needs to be deubiquitinated to interact with CENP-A. We hypothesize that these mechanisms can involve a related regulation.

3. Template options for centromeric HR repair in G1

An important question raised by our study concerns the nature of the template used for homologous recombination in G1, given the absence of sister chromatid during this phase of the cell cycle. A possible scenario is that, since centromeric repeat sequences are identical between the different chromosomes, another centromere could serve as template. However, centromeres from different chromosomes are spatially isolated during interphase of mouse cells (Guenatri et al., 2004), hence HR or any resection-dependent mechanisms has minimal risk of leading to chromosomal translocations during interphase. A second possibility is that a repeat in *cis*, i.e. a repeat from the same centromere, can be used as template. But the problem arising from this notion is that after an extensive broken end resection, the single strand may anneal directly to an adjacent repeat thus generating a contraction (loss of repeat units) or, if the annealing disengage, a synthesis dependent strand annealing can generate an expansion leading to repeat gain (Khristich and Mirkin, 2020; Pâques et al., 1998; Read, 2004). It is therefore possible that RAD51 binding promotes error-free copying of centromeric repeats in *cis*, by inhibiting RAD52-dependent annealing of adjacent repeats. Our data are in line with this idea, since RAD51 inhibition leads to increase the recruitment of RAD52 at centromeric DSBs, and to increase RAD52-dependent centromeric expansion. Importantly, HR in *cis* does not necessarily result in

the loss of repeats. Indeed, HR via synthesis dependent strand annealing (SDSA) or dissolution of double Holliday junctions by branch migration can mediate non-crossover repair (Karow et al., 2000; Ranjha et al., 2018).

The role of centromeric repeats in centromere integrity has been discussed for many years without leading to a clear understanding. Indeed, although changes in the length of the repeats can affect centromere integrity (Jaco et al., 2008), centromere repeats undergo RAD51 dependent recombination even in the absence of stress conditions to suppress chromosomal rearrangement (Nakamura et al., 2008), and homologous recombination between centromeric repeats leads to closed loops that can have an important role in the establishment of a functional centromere (McFarlane and Humphrey, 2010), suggesting that recombination is an essential event in centromere maintenance. Moreover, the identification of functional neocentromeres lacking satellite DNA leads to the prediction that the repeats are neither sufficient nor required for centromere function (Warburton, 2004). Here we show that RAD51 inhibition leads to an increase in the formation of translocations originating from the centromeres, while RAD51 and RAD52 co-inhibition rescues this effect, suggesting that utilization of HR suppresses mutagenic SSA or BIR.

An arising concept is that RNA-templated repair, which does not necessitate homologous DNA template, may be used in G1 (Meers et al., 2016). In support, synthetic RNA oligonucleotides can act as templates for DSB repair in yeast and human cells (Keskin et al., 2014). Satellite repeats in centromeric regions are known to be transcribed (Bouzinba-Segard et al., 2006) and this transcription is suggested to contribute to centromere architecture and function (McNulty et al., 2017; Quénet and Dalal, 2014). We hypothesize that an RNA-templated repair at centromeres is consistent with a minimal risk of translocations and loss of repeats, although this requires further investigation.

4. HR repair at centromeres in G1, risky but necessary?

Due to the highly repetitive nature of the centromere, repair of DSBs through recombination-dependent pathways may disrupt centromere integrity in several ways. Either by leading to sister chromatid exchange (Giunta and Funabiki, 2017), or by creating translocations when the homologous template is another chromosome, or, when the template is a repeat in *cis*, by resulting in contraction or expansion of repeats (Khristich and Mirkin, 2020; Pâques et al., 1998; Read, 2004).

Here we show that activation of HR at centromeric breaks confers some benefits for centromere integrity, notably by suppressing mutagenic pathways and preventing translocations. Consistent with our data, work in yeast demonstrates that mitotic centromeres are subjected to RAD51-dependent HR to maintain genome stability (Zafar et al., 2017), and that an MRE11-dependent DSB repair pathway maintains repetitive regions and MRE11 lacking strains endure RAD52-dependent expansions (Sundararajan et al., 2010). Moreover, both HR and NHEJ accurately repair lesions at expanded CAG repeats and prevent repeat contractions, expansions, and chromosomal breakage (Sundararajan et al., 2010).

Why is HR repair activated at centromeric DSBs in G1 despite the risk of errors due to the repetitive nature of the centromeres? One hypothesis is that the pathway choice is set in mitosis, since R-loops form at centromeres in mitosis (Kabeche et al., 2018). It is also possible that those R-loops might need to be fixed fast, directly in G1, since single stranded DNA is more susceptible to damage. Moreover, centromere-kinetochore regions are subjected to spindle-induced tensional forces during mitosis and to the appearance of mitotic spindle defects at the origin of uncorrected merotelic attachments, rendering centromeres fragile sites (Guerrero et al., 2010). Therefore, cells might choose a faithful repair process by HR at centromeric lesions directly after mitosis, in G1, to ensure a proper repair at any cell cycle stage even if HR is generally suppressed.

We showed that in absence of RAD51, translocations can occur through NHEJ, SSA or BIR pathways. However, centromeres from different chromosomes are spatially isolated during interphase of mouse cells (Guenatri et al., 2004), hence the risk of chromosomal translocations during interphase is minimal. Although it is difficult to determine at which stage of the cell cycle the translocations that we observe occur, it is known that when a break persists because one of the main mechanisms NHEJ and HR is not working, there can be some movement of the break (Aymard et al., 2017; Evdokimova et al., 2018; Kilic et al., 2019; Lisby et al., 2003; Miné-Hattab and Rothstein, 2012; Ryu et al., 2015; Schrank et al., 2018) possibly leading to translocations. If a break stays unrepaired in mitosis, then translocations can occur there as well.

5. Role of recombination in CENP-A deposition

Our very preliminary results suggest that MRE11, RAD51 and RAD52 might have a role in CENP-A neo-incorporation. In agreement with our data, it has been proposed in yeast that Mre11, Rad51 and Rad52 maintain centromere function by regulating CENP-ACaCse4 levels at the programmed fork stall sites of early replicating centromeres, as CENP-A act as a physical barrier for fork progression (Mitra et al., 2014). Moreover, yeast centromere repeats promote break-

induced replication (BIR) even in the absence of stress conditions (Nakamura et al., 2008; Tinline-Purvis et al., 2009). Altogether, these data suggest that factors such as MRE11, RAD51, RAD52 and USP11 might be present at centromeres, and therefore, directly available for repair. A recently published work has realised a screen to identify human CENP-A assembly and maintenance factors, and MRE11 was identified as a candidate gene that affect the loading of new CENP-A at the centromere. However, this screen did not show a role for RAD51 and RAD52 in CENP-A neo-incorporation (Mitra et al., 2020).

H3K4me2 has been suggested to be required for targeting HJURP to the centromere and for kinetochore maintenance (Bergmann et al., 2011). Indeed, H3K4me2 depletion causes kinetochores to suffer a rapid loss of transcription of the α-satellite DNA and to no longer efficiently recruit HJURP, which affect CENP-A incorporation. Our results raise the hypothesis that H3K4me2-dependent R-loop formation might involve the recruitment of factors such as MRE11 that seems to be required for CENP-A deposition.

6. Functional interplay between centromere and nucleolus, another compartment with active HR in G1

Activation of HR in G1 has also been observed in the nucleolus, the nuclear sub-compartment containing ribosomal DNA (rDNA) (van Sluis and McStay, 2015). Indeed, endonuclease-induced DSBs specifically at nucleolar rDNA repeats relocate towards the nucleolar periphery in order to be accessible by both NHEJ and HR repair factors in G1 (van Sluis and McStay, 2015). It is therefore possible that a similar mechanism to the one we observed at centromeres takes place in nucleolus during G1. Interestingly, a common feature of centromeres and nucleoli, besides their repetitive nature, is transcription and R-loop formation (Castellano-Pozo et al., 2013; Velichko et al., 2019; Zhou et al., 2020). Moreover, accumulating evidence suggests a functional interplay between centromeres and nucleoli. Indeed, centromeres are often positioned at the periphery of nucleoli, probably through nucleolar proteins interaction with CENP-A (Foltz et al., 2006), and this association has been linked to genome stability (Padeken et al., 2013). Moreover, HJURP and CENP-A can localise at interphase nucleoli (Dunleavy et al., 2009; Hédouin et al., 2017; Kwenda et al., 2016), as do several other centromeric components, probably to be stored there and to provide a regulatory mechanism for their timely release into the nucleoplasm in mitosis and G1 (Kwenda et al., 2016; Wong et al., 2007). Thus, the presence of R-loop and CENP-A/HJURP in this compartment might allow a similar repair mechanism than the one we found at centromeres.

7. Limitations of the Cas9 induced breaks system in repetitive regions

A potential limitation of our study lies in the fact that we induce centromeric DSBs by an experimental system, taking advantage of the CRISPR/Cas9 technology, which can lead to persistent and high number of centromeric DBSs. Nevertheless, as IR and NCS treated cells also present centromeric breaks that recruit HR factors such as RPA and RAD51, it is most likely that we have discovered a functionally relevant repair mechanism.

8. CENP-A overexpression in ectopic incorporation sites and cancer

CENP-A overexpression leads to its erroneous incorporation at ectopic loci (Athwal et al., 2015; Lacoste et al., 2014; Nechemia-Arbely et al., 2017, 2019; Nye et al., 2018) in mammalian organisms, *Drosophila* and yeast (Choi et al., 2012; Gascoigne et al., 2011; Mendiburo et al., 2011). It has been suggested that overexpression and mis-localization of CENP-A to ectopic regions in cancer cells prevent normal kinetochore assembly and thus alters proper chromosome segregation (Amato et al., 2009; Tomonaga et al., 2003). Nonetheless, our findings involving HJURP/CENP-A in HR repair process provide new insights that could explain why CENP-A is found to be overexpressed in many cancer cells (Hu et al., 2010; Li et al., 2011; Qiu et al., 2013; Rajput et al., 2011; Sun et al., 2016; Tomonaga et al., 2003; Wu et al., 2012). Indeed, CENP-A overexpression in cancer cells can result from a defense mechanism of the cell by increasing HR dependent repair, or CENP-A overexpression can promote excessive recombination at ectopic incorporation sites leading to genomic instability at the origin of the cancer. Our finding can also give an insight into how exogenous overexpression of CENP-A leads to high resistance to drugs inducing DNA breaks (Lacoste et al., 2014). Indeed, overexpression of CENP-A might allow tolerance to damage by favoring repair. Besides, the role of CENP-A in centromeric HR can explain why CENP-A loss correlates with a drastic increase in centromere aberrations and leads to excision of centromeric repeats (Giunta and Funabiki, 2017).

This work paves the way for clinical assays to adapt and individualize cancer treatments according to epigenetic markers such as CENP-A. Indeed, when combined with the fact that ectopic overexpression of CENP-A leads to high resistance to Topoisomerase I inhibitors (Lacoste et al., 2014), our observations alert against the clinical use of PARP inhibitors, Topoisomerase I inhibitors or other drugs that induce lesions that are mainly repaired by HR. In fact, if CENP-A is overexpressed, tumors targeted by these drugs are more likely not to respond to the treatment (Lacoste et al., 2014). Therefore, if with CENP-A overexpression HR is high and NHEJ balance is reduced, then Topoisomerase II inhibitors that target NHEJ deficient cells

would be more beneficial. Further exploring these hypotheses could be a tremendous advance for research and key in finding new diagnostic and treatment options for cancer therapy.

PERSPECTIVES

1. DNA end-resection and the role of R-loops

My work has revealed that in G1 centromeric DSBs uniquely activate DNA end resection. Nevertheless, it remains unclear which nucleases are involved in centromeric resection and how they are recruited at centromeric DSBs in G1. Centromeres are enriched in H3K36me2, which can serve as a platform for H3K36me3, which in turn recruits CtIP (Aymard et al., 2014; Daugaard et al., 2012). Moreover, DNA2 has been shown to preferentially bind at centromeres (Li et al., 2018b); and centromeres recruit BLM during anaphase to allow proper segregation (Ke et al., 2011a). To investigate whether these or other known DNA end resection proteins play a role in centromeric DSB end resection in G1, we will deplete NIH3T3 cells of MRE11, CtIP, BLM, EXO1 and DNA2, both individually and in combination. To assay for their effect on end resection, we will assess the impact on RPA and RAD51 recruitment on centromeric DSBs in G1.

It is also known that transcription promotes the recruitment of BRCA1 in expense of 53BP1 recruitment (Aymard et al., 2014). To test this idea in centromeric DSBs in G1, we will locally increase transcription, through the tethering of dCas9-VP64 at centromeres, or decrease transcription, through the tethering of dCas9-KRAB at centromeres and determine how it affects 53BP1 and BRCA1 recruitment. To further assay the role of transcription, we will remove R-loops by dCas9-RNAseH tethering and determine how this affects 53BP1 recruitment.

Since H3K4me2 decorates active promoters throughout the genome, the question that arises is whether DSBs at these regions can also licence DNA end-resection in G1, or more specifically, whether all the break sites forming R-loops can activate resection in G1. To investigate this, we will utilize super-resolution imaging to assess if at DSBs induced by NCS or IR in G1, R-loops form mainly on H3K4me2 regions of the genome and whether these DSBs recruit more RPA and BRCA1 than other DSBs in the nucleus. To this end, we will compare the degree of colocalization of active chromatin (H3K4me2) with γ-H2AX and GFP-dRNAseH (used as a proxy for R-loop detection) to that of inactive chromatin (H3K9me2/3) with γ-H2AX and GFP-dRNAseH. Moreover, we will assess the RPA and BRCA1 intensity and foci formation in H3K4me2 regions with R-loops in G1 cells.

To verify the above point by another method, we will induce DSBs at selected H3K4me2-enriched promoters in NHH3T3 or U2OS cells and compare them with DSBs induced in regions that lack H3K4me2 (H3K9me2/3 or H3K27me3) using Cas9 and guide RNAs targeting these promoters. Using ChIP and DRIP in cells arrested in G1, we will compare the enrichment of R-loops and the recruitment of RPA and BRCA1 to these different sites.

Even if the H3K4me2-enriched promoter regions are predicted to be more prone to resection compared to silenced promoters, they should not be able to complete HR since RAD51 recruitment is inhibited in G1. To verify this, we will assess the recruitment of RAD51 in these DSBs by ChIP. To investigate how these resected DNA ends are repaired, we will assess the recruitment of factors that bind to ssDNA and are involved in SSA/ BIR (RAD52), alt-EJ (pol theta). Since our results show that tethering of CENP-A or HJURP is sufficient to increase RAD51 recruitment on resected DSBs in G1, we will express dCas9-CENP-A or dCas9-HJURP together with specific guide RNAs targeting these regions and, by ChIP, verify if they become more prone to RAD51 recruitment and whether this affects the recruitment of factors involved in alternative pathways.

As mentioned in the discussion, TERRA R-loops can form post-transcriptionally in a RAD51-dependent manner (Feretzaki et al., 2020). We hypothesized that centromeric R-loop formation might also depend on RAD51, which would represent another mechanism of RAD51 recruitment at centromeres and its availability for HR repair. To test this idea, we will deplete or inhibit RAD51 and check if this affects GFP-dRNAseH recruitment at centromeric DSBs or verify R-loop formation by DRIP. Since RAD52 is often recruited at resected DSBs in the absence of RAD51, we can also perform this experiment in conditions that both RAD51 and RAD52 are inhibited or depleted.

Another open question from our study relates to the kinetics of the increase of centromeric H3K4me2 levels in the presence of DSBs, and whether this increase depends on SETD1A or other H3K4me2 writers. A very recent study in *Caenorhabditis elegans* demonstrates a DNA-damage-induced increase of H3K4me2, which is necessary to regulate the transcription of genes post-repair (Wang et al., 2020). This transient increase of H3K4me2, however, was shown to depend on the completion of DNA repair. Since DSBs at centromeres lead to an increase in H3K4me2 and subsequent increase in R-loop formation, we assume that it occurs at the beginning of the repair process. To assess in detail the kinetics of H3K4me2 increase in response to centromeric DSBs, we will quantify H3K4me2 levels at centromere by ChIP at different time points following DSBs induction. Next, we will perform a series of experiments to determine

whether SETD1A or other known H3K4me2 writers (eg MLL/COMPASS) interact with repair factors or are recruited through the break-induced chromatin modifications.

As mentioned in the introduction and discussion, centromeres are also enriched in H3K36me2. Interestingly, H3K36me3 has been shown to promote HR (Aymard et al., 2014) and H3K36me2 can be converted to H3K36me3. To investigate the involvement of this histone mark in HR at centromeric DSBs in G1, we can induce H3K36me2 demethylation after tethering of the demethylase KDM2A at centromeres by fusing it to dCas9 and check if this will reduce HR factors recruitment.

2. Role of USP11 and ubiquitination in CENP-A/HJURP interaction

We show that the interaction of USP11 with HJURP and CENP-A allows RAD51 recruitment at centromeric DSBs in G1. It is unclear, however, whether this interaction occurs in the nucleoplasm or at centromeres. To investigate this, we will test whether USP11 is recruited at centromeres in early G1 when HJURP deposits CENP-A. To this end, we will co-express mCherry-USP11 and GFP-HJURP and visualize them, in cells arrested in mitosis and after a short release in early G1, when HJURP is known to associate transiently with centromeres. Alternatively, we can tether dCas9-HJURP at centromeres and verify if this is sufficient to recruit mCherry-USP11.

We also show that USP11 de-ubiquitinates HJURP and that USP11 is necessary for HJURP-CENP-A interaction. It would be therefore interesting to determine whether the USP11/HJURP interaction depends on USP11 catalytic activity and if de-ubiquitination of HJURP is necessary for HJURP/CENP-A interaction. To answer this, we can perform co-immunoprecipitation experiments in cells expressing GFP-HJURP together with either wt Flag-USP11 or Flag-USP11 dead mutant and quantify the degree of their interaction. Moreover, we can quantify the extent of HJURP/CENP-A interaction in cells depleted of USP11 and reconstituted with the wt or the dead mutant.

3. Nature of the template for centromeric HR repair

As suggested in the discussion, the template for HR repair can be either another centromere, or a repeat in *cis*, or centromeric RNA. We can envisage several experiments to address this point. If the template is another centromere, this would involve DSBs' movement, since mouse centromeres are spatially isolated during interphase. Centromeric DSBs foci movement can be

followed by live microscopy (as described in Schrank et al., 2018). If the template is a repeat in *cis*, this would probably lead to loss or gain of centromeric DNA repeat, which we can visualize by southern blot. A shift in the fragments size or intensity would suggest a change in the number of repeats.

Finally, to test whether centromeric HR requires DNA or RNA template as a donor for repair, we will induce centromeric DSBs in cells transfected with DNA or *in vitro* transcribed RNA template containing a unique sequence flanked by arms that share homology with the centromeric minor satellite repeats. Single cells clones will be then isolated and will all be tested for the incorporation of the unique sequence by PCR amplification or Southern blot. Transfecting such a DNA template would also allow us to show the end point of HR, the recombination *per se*.

In the discussion, we hypothesized that an RNA-templated repair at centromeres is consistent with a minimal risk of translocations and loss of repeats. If the experiments described above show the use of an RNA template, we can then determine by metaphase spread and centromeric FISH if more or less translocations occur following centromeric DSBs, compared to no template given.

4. Does HR repair happen spontaneously at centromeres?

In order to further validate the biological relevance of our work, we aim to determine if endogenous centromeric DSBs occur and how they are repaired. We can inhibit RAD51 alone, without DSB induction, and verify centromeric loss and translocations occurrence on metaphase spreads on which centromeric and telomeric FISH has been performed.

5. Which pathway takes place in the absence of HR?

Our results suggest that licencing of HR repair at centromeres throughout the cell cycle protects the centromeres from activation of mutagenic pathways. Indeed, we demonstrate that RAD51 depletion leads to enhanced recruitment of RAD52, which is involved in SSA or BIR pathways. Moreover, inhibition of RAD52 in conditions that RAD51 is inhibited, reduces the numbers of chromosomal translocations, suggesting that at least a fraction of the translocations is generated in a RAD52-dependent manner. To decipher whether NHEJ or Alt-EJ also promote genomic instability in the absence of RAD51, we have quantified chromosomal translocations in cells inhibited for both RAD51 and DNAPK or RAD51 and Pol theta. Interestingly, inhibition of DNAPK rescues the effect of RAD51 inhibition on translocation occurrence, showing a role for NHEJ in translocations formation. On the other hand, depletion of Pol theta had no effect on the

number of translocations (data not shown). Nevertheless, WB analysis revealed that the siRNA mediated depletion of Pol theta was partial, thus possibly explaining the lack of defects on chromosomal translocation formation (data not shown). Consequently, further experiments are required to clarify the role of Pol theta. To investigate whether RAD51 inhibition leads to an increase in binding of NHEJ factors at centromeric DSBs, we will quantify Ku80 colocalization with centromeres at centromeric breaks upon RAD51 depletion and compare this to non-treated cells.

Another question raised by our data is whether R-loops suppress NHEJ, which is considered the fastest DNA repair pathway, by promoting HR. To answer this, we will assess whether the tethering of RNAseH at centromeres by fusing RNAseH to dCas9 (dCas9-RNAseH), to decrease R-loops at centromeres, leads to an increase of NHEJ-dependent translocations. Additionally, the increase in translocations that we observe following RAD51 inhibition could be due to the fact that resection has already occurred, and that resected DNA ends will utilize alternative pathways, such as SSA or alt-EJ, for their repair. Thus, to examine whether inhibiting HR from the resection step leads to centromeric genomic rearrangements we would perform the above experiments in the presence of inhibitors of DNA end resection, such as Mirin.

As discussed previously, since centromeres are spatially isolated during interphase, translocations either occur in mitosis or involve the movement of the broken ends in interphase. This question can be answered by following centromeric DSBs foci movement upon HR inhibition, by live cell microscopy.

6. Role of USP11 and recombination in CENP-A deposition

To further analyse the possible role of MRE11, RAD51 and RAD52 in *de novo* CENP-A centromeric deposition, we aim to investigate their interaction with CENP-A by co-immunoprecipitation experiments. Since these potential interactions might be very transient, we could utilize the BioID technology, and couple CENP-A to BirA, biotinylate, purify with streptavidin beads and search for these factors by western blot.

To investigate if the role of MRE11 on CENP-A *de novo* deposition involves the MRE11 catalytic activity, we will reconstitute MRE11 depleted cells, with WT MRE11 or catalytically inactive mutants and will quantify the percentage of cells with *de novo* deposited CENP-A using the SNAP system. Moreover, we can tether endogenous RAD51 to centromeres by tethering the BRC3 domain of BRCA2 fused to dCas9 (Tsouroula et al., 2016) and assess whether this

increases CENP-A neo-incorporation. Furthermore, we can tether other factors such as wt and mutant isoforms of MRE11.

Interestingly, in a recently published study, an siRNA screen was performed to identify human CENP-A assembly and maintenance factors. Among the proteins identified, KEAP1, the E3 ubiquitin ligase that is counteracted by USP11, was found to be important for the maintenance of old CENP-A (Mitra et al., 2020; Orthwein et al., 2015). It is therefore possible that KEAP1 ubiquitinates CENP-A to facilitate its maintenance at the centromere. To verify whether USP11/KEAP1 opposite activities are functioning to keep a balance between the old and the *de novo* incorporated CENP-A we will first overexpress USP11 and determine whether this leads to the loss of old CENP-A and increased neo-incorporation using the SNAP system. Subsequently, we will co-deplete KEAP1 and USP11 and see if the effect of siUSP11 on CENP-A's new deposition is rescued.

7. Functional interplay between centromere and nucleolus

As mentioned in the discussion, the nucleolus is another compartment which can activate HR in G1 (van Sluis and McStay, 2015), has R-loops (Castellano-Pozo et al., 2013; Velichko et al., 2019; Zhou et al., 2020), and contains centromeric factors such as CENP-A and HJURP (Dunleavy et al., 2009; Hédouin et al., 2017; Kwenda et al., 2016). Therefore, it will be very informative to verify if a similar mechanism of HR repair occurs at the nucleolus as the one we identify at centromeres. This will be achieved by reproducing our key centromeric experiments in nucleoli. For example, we can investigate the impact of R-loop inhibition on RPA, BRCA1 and RAD51 recruitment, and of CENP-A and HJURP depletion on RAD51 recruitment in nucleoli.

8. CENP-A overexpression in ectopic incorporation sites and in cancer

CENP-A overexpression can lead to its ectopic incorporation in non-centromeric regions in mammalian organisms, *Drosophila* and yeast (Choi et al., 2012; Gascoigne et al., 2011; Mendiburo et al., 2011), and can promote aneuploidy (Amato et al., 2009). Intriguingly, mammalian CENP-A was shown to re-localize to sites of DNA damage (Zeitlin et al., 2009) and to increase tolerance to damage when overexpressed (Lacoste et al., 2014). CENP-A has also been reported to be overexpressed and mistargeted in various human cancers (Rajput et al., 2011; Tomonaga et al., 2003). Our results imply that CENP-A is involved in determining HR repair as the pathway of choice at centromeric DSBs.

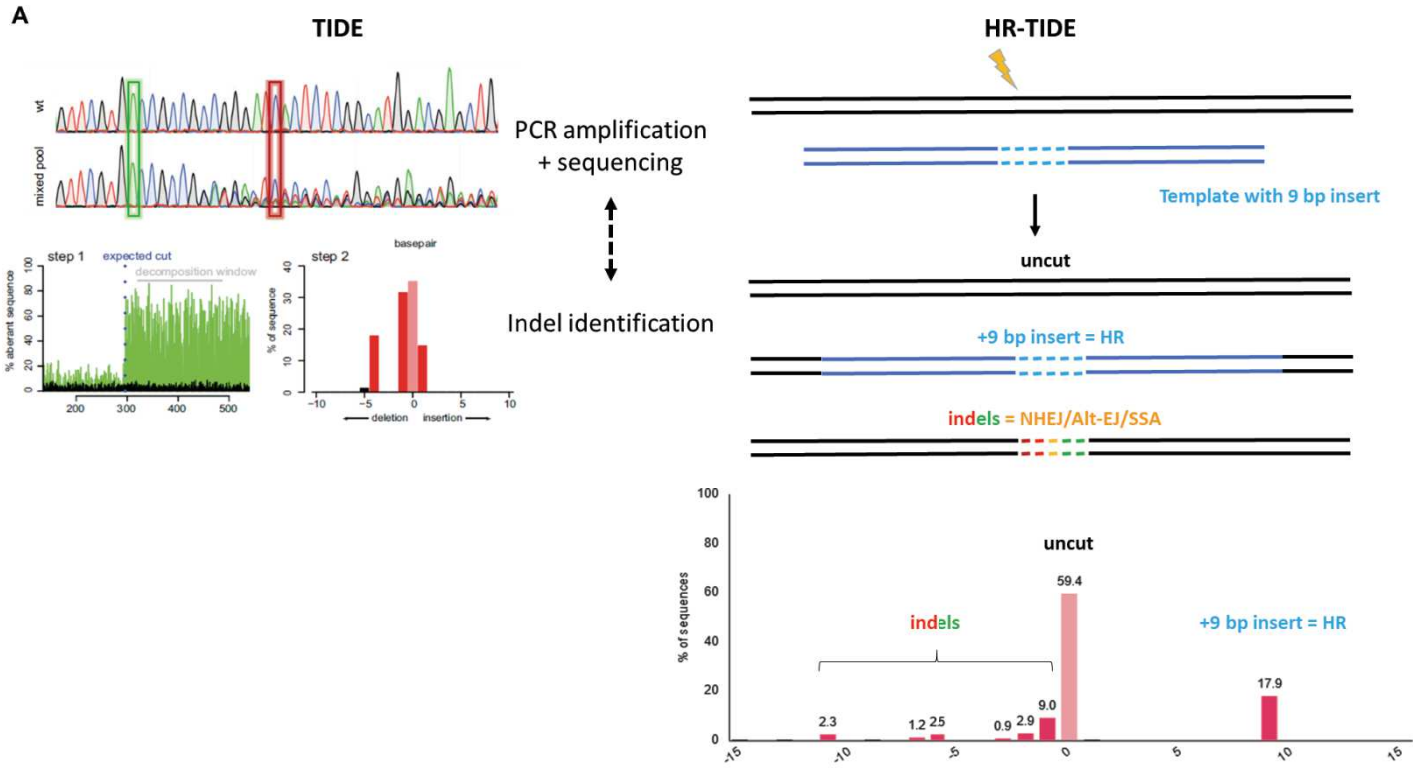
To assess whether CENP-A's role in HR is responsible for CENP-A's overexpression in cancer cells and for the increase in damage tolerance in cells overexpressing CENP-A, we sought to determine if known sites of CENP-A mis-incorporation upon overexpression are more prone to HR, using an adapted version of Tracking of Indels by Decomposition (TIDE) (Brinkman et al., 2014), Homologous Recombination based-TIDE (HR-TIDE). In our adaptation, the knock-in efficiency at endogenous loci mediated by error-free HR is measured (Figure 23.A). To this end, we selected several known loci in HeLa cells in which CENP-A is mis-incorporated after its overexpression (Lacoste. et al., 2014, Table 3) and we designed guide RNAs to induce DSBs in these locations (Figure 23.B). To assess HR efficiency, we took advantage of TIDE's sensitivity for measuring up to 9 bp insertions at the DNA after break induction. For this, we created homologous templates, which consist of a 1000 bp homology sequence (500 bp each side of the break) and a 9 bp unique DNA sequence, which is inserted by HR at each genomic location (Figure 23.A). The cells will be synchronized in different phases of the cell cycle and co-transfected with a specific guide RNA, Cas9 and the corresponding HR template. Subsequently, each locus will be amplified by PCR and subjected to TIDE. The frequency of the 9 bp insertion at each genomic location indicates the HR frequency, which will be compared to control cells with endogenous levels of CENP-A, where CENP-A is only bound at centromeres. We will perform these experiments in HeLa cells stably expressing mCherry-CENP-A expressed under the control of doxycycline (Dox) (Figure 23.C and 23.D). Therefore, we will be able to compare recombination occurrence in chosen loci in conditions of normal CENP-A expression (-Dox) or CENP-A overexpression (+Dox). We would like to first check by standard TIDE how CENP-A overexpression affects indels (insertion and deletion) frequency and nature. We will then verify by HR-TIDE if CENP-A overexpression and misincorporation at ectopic sites increases HR at those sites.

It was shown that CENP-A ectopic incorporation is dependent on ATRX, HIRA and DAXX (Athwal et al., 2015; Nye et al., 2018) but not HJURP. Therefore, the above experiments will decipher whether CENP-A presence alone on chromatin is sufficient to increase knock in efficiency by HR, possibly by interacting alone with USP11. Alternatively, one can hypothesize that even though HJURP does not deposit CENP-A in these sites it still interacts with it. This can be verified by tethering LacI-CENP-A to a LacO array and assessing whether HJURP is further recruited. Potential HR process at ectopic sites may also involve a direct link between CENP-A and RAD51 (Mitra et al., 2014).

Consistent with this idea, we find that cancer databases analyses obtained with CellMinerCDB show a strong correlation between the expression levels of CENP-A and RAD51 in cancers

(Figure 24), suggesting that these factors might be co-regulated or involved in a same process. We would like to extend these studies and determine, through utilizing various cancer databases, if abnormalities arise from sites of CENP-A ectopic incorporation in tumour cells overexpressing CENP-A. We can also verify if BRCA1 or HR repair factors-defective tumour cells have centromeric aberrations.

Finally, we would like to determine if CENP-A in HR repair leads to resistance to DNA break-inducing cancer treatments. To address this, we will correlate CENP-A expression with resistance/sensitivity to irradiation and chemotherapy drugs (Camptothecin, NCS, Olaparib, etc) using survival assays in our HeLa cell line expressing Dox-inducible mCherry-CENPA. Alternatively, we will examine whether tumours particularly resistant to chemotherapeutic drugs or PARP inhibitors exert high levels of CENP-A or HJURP compared to tumours more sensitive to these treatments, using cancer databases.


A
TIDE
wt
mixed pool
PCR amplification
+ sequencing
Indel identification
step 1
expected cut
decomposition window
% aberant sequence
step 2
basepair
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deletion
insertion
HR-TIDE
Template with 9 bp insert
uncut
+9 bp insert = HR
indels = NHEJ/Alt-EJ/SSA
% of sequences
indels
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17.9
9.0
2.3
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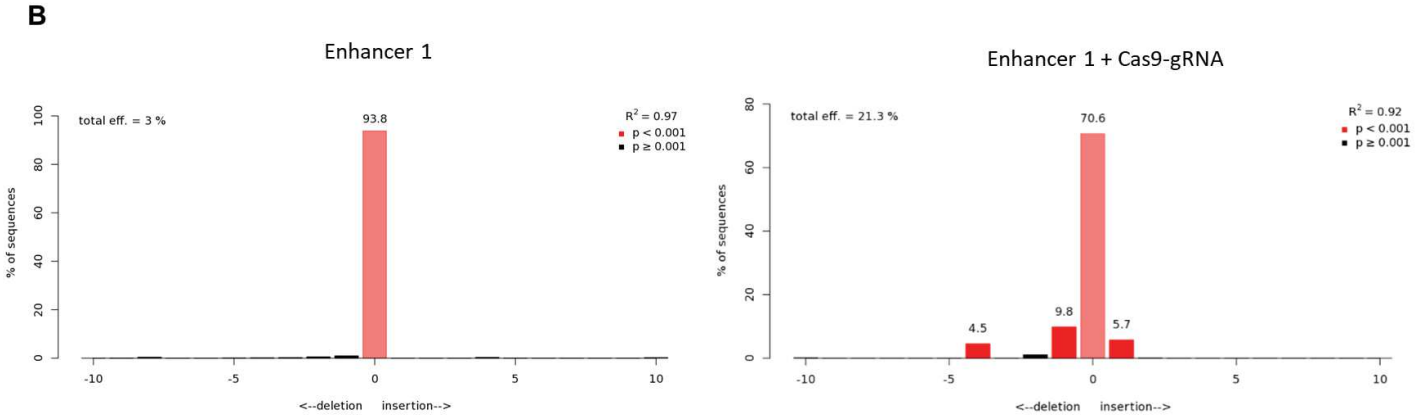
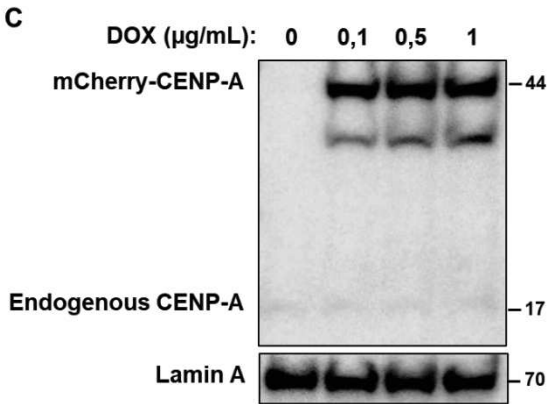
2.5
0.9
2.9


B
Enhancer 1
total eff. = 3 %
93.8
R² = 0.97
p < 0.001
p ≥ 0.001
% of sequences
<--deletion
insertion-->
Enhancer 1 + Cas9-gRNA
total eff. = 21.3 %
70.6
9.8
4.5
5.7
R² = 0.92


C
DOX (µg/mL): 0 0,1 0,5 1
mCherry-CENP-A
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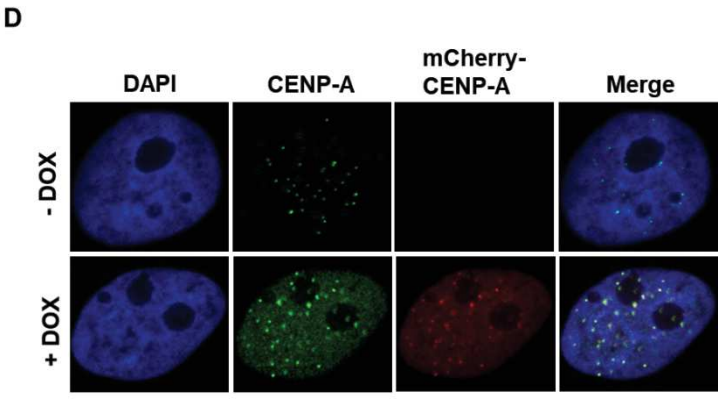
D
DAPI
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Merge
- DOX
+ DOX

Figure 23: HR-TIDE experiment to determine whether known sites of CENP-A mis-incorporation upon overexpression are more prone to HR

(A) Schematic description of TIDE (Tracking of Indels by Decomposition) and our HR-TIDE methods. After DSB induction, PCR amplification of the targeted region and sanger sequencing, the TIDE algorithm aligns and analyses the mixed pool of sequences arising from the breaks in DSBs-induced sample compared to the control sample. This analysis gives a graph showing the percentage of sequences that have a given indel. In HR-TIDE, we co-transfect a template containing 9 bp insert. If HR occurs, the 9 bp insertion will appear in the graph, while the rest of the indels correspond to repair through either NHEJ, or SSA or alt-EJ. **(B)** Example of the Enhancer 1 region where we have induced DSBs. Break induction leads to indels formation. **(C)** Western blot analysis of CENP-A and lamin A, as a control, in HeLa cells expressing mCherry-CENP-A under the control of doxycycline (DOX). **(D)** HeLa cells expressing mCherry-CENP-A under the control of DOX, treated or not with DOX, and stained with DAPI and antibodies specific for CENP-A.

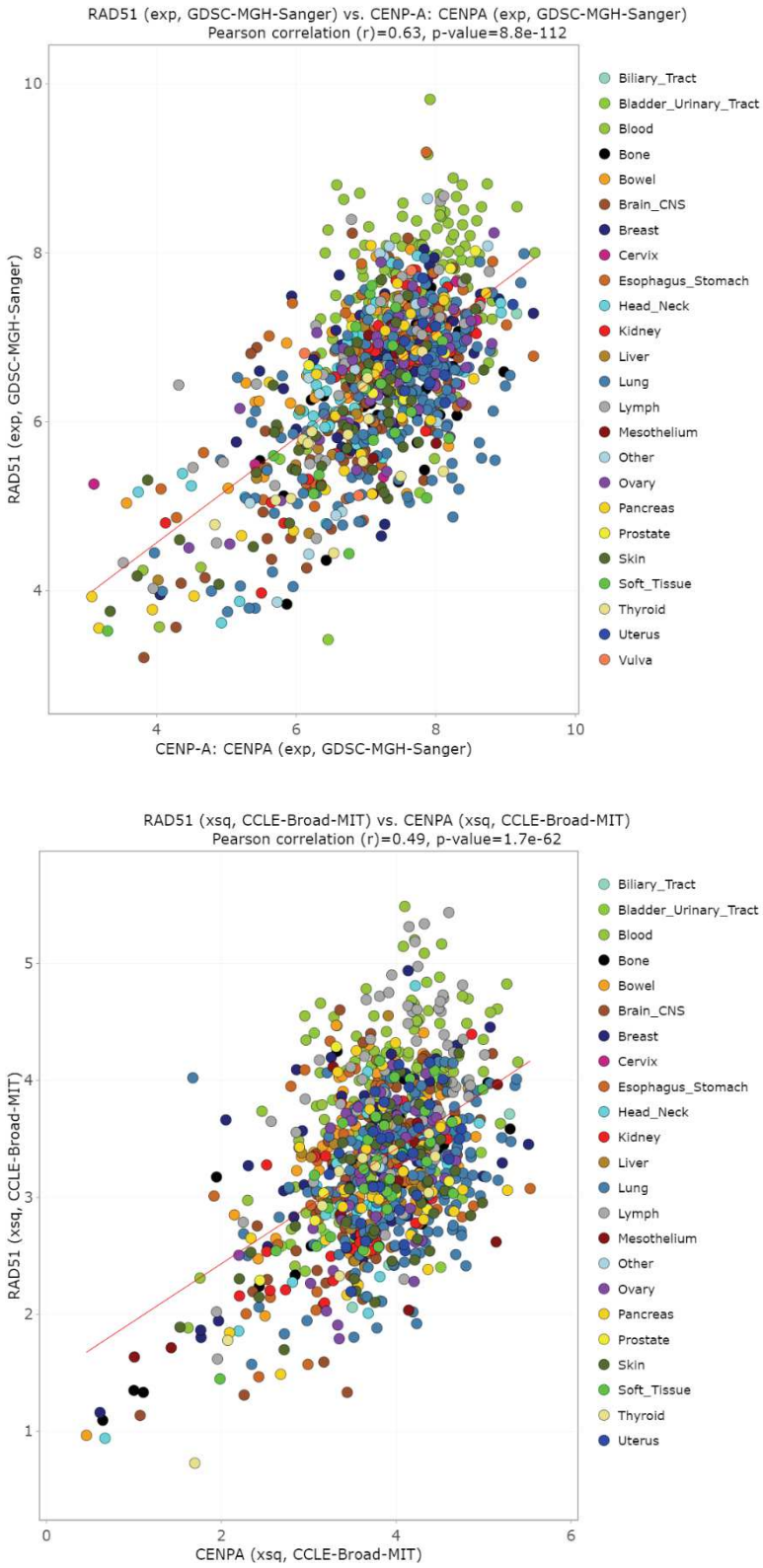

Figure 24: Cancer databases analyses obtained with CellMinerCDB shows a correlation between high CENP-A and RAD51 expression in cancer.

The upper panel is obtained by analyzing the GDSC-MGH-Sanger dataset, the lower panel is obtained by analyzing the CCLE-Broad-MIT dataset. Each point represents the relative expression levels of CENP-A and RAD51 in a specific cancer tissue.

SUPPLEMENTARY MATERIALS AND METHODS

Treatments.

As for DMSO, RAD51 inhibitor, and RAD52 inhibitor, DNAPK inhibitor (N-1537, Sigma Aldrich) was added (20 µM) 1 h before transfection and refreshed 6h before harvest. The concentration of doxycycline chosen for HR-TIDE experiment was 0.5 µg/mL and was added 24h before the transfection of gRNA-Cas9 and template plasmids and refreshed every 24h. Plasmids for TIDE and HR-TIDE experiments were transfected for 48h. siRNA used are in Table 1.

Plasmid Construction.

The GFP-Scm3 plasmid (Table 2) was generated by amplification of the Scm3 domain of GFP-HJURP plasmid and cloned into a pXPA vector by megawhop cloning (Miyazaki, 2011). The gRNA plasmids (sequences in Table 3) were constructed and amplified by PCR and cloned by megawhop cloning into a vector containing the U6 promoter followed by a gRNA scaffold. The template plasmids were amplified from genomic DNA by PCR in two part to insert the 9 bp thanks to the primers. The two parts were then assembled and amplified by PCR and cloned into a pXPA vector by megawhop. All plasmids were assembled by golden gate cloning (Engler et al., 2009).

TIDE and HR-TIDE.

Cells were collected and genomic DNA was extract with NucleoSpin DNA RapidLyse (Machery-Nagel). After amplification of targeted regions by PCR (primers in Table 3), amplicons were isolated with AMPure XP Beads (A63881, Beckman Coulter) and sent for sanger sequencing (primers in Table 3). In the case of HR-TIDE, amplification primers were chosen to be outside of the template sequence on the genome in order to not amplify the given template. TIDE analyses were then realised with the TIDE tool online.

Table 1

siRNA used*	
siRNA	**Reference**
Human RAD51	L-003530-00
Human RAD52	L-011760-00
human HJURP	L-015443-00
human MRE11	L-009271-00

* All siRNAs (ON-TARGETplus SMARTpool siRNAs) were purchased from Dharmacon.

Table 2

Plasmid used
CMVp-EGFP-Scm3-SV40p-HygroR-pA

Table 3

Name	Position (GRCh37)	gRNA	Primers for HR-TIDE PCR		Primers for HR-TIDE sequencing	
CTCF 2	chr15:41074866-41074930	AGTCCTTATTTAGCACACTT	Fwd	AAACATGAGCCAGGTGTGGT	Fwd	CAACCCCAGAATCCGTCCTC
			Rev	CTGTCAGTGTGTGTAGGTGCT	Rev	AGCTAGCACCTCCTTTCCCT
CTCF 4	chr17:57924318-57924381	AGTCATTCCCCACCTTCAGG	Fwd	GGGAAGGCAAGAGGATGATT	Fwd	GCTCTGGCTTATTCTGGGCA
			Rev	CTGCCTTAGTCTTGGCCTTC	Rev	GCACTACTGCCAAGAGGAGT
CTCF 5	chr7:131198408-131198464	AGTGGTGACATCATGCATGG	Fwd	CTCAGAGGCCAACATGCAGA	Fwd	TATTTGGGAGTTCCGCACCC
			Rev	GCTAGGATTACAGGTGCCCG	Rev	CACTTCCTGGGCACTTCACT
Enhancer 1	chr11:34296394-34296454	TGGCTGGCAACCTCTGCCTG	Fwd	TGACAGAAGCCAAGGTCACA	Fwd	GGCACACCTCAGCTGGATAA
			Rev	ATTCAGGGGAGGCATGTGAC	Rev	TGGGGTAGGAGGGGCATTTA
Enhancer 2	chr7:80171947-80172018	ATGTCACCCAGAGAAAGCGT	Fwd	GGGACATCACAGTAGCTTCACA	Fwd	GGACACCAAAGGATTCCAAAACC
			Rev	ACCATGTCAGGATGGGCATG	Rev	CCAAACCCCCAAATTTCCCAT
Enhancer 4	chr12:53289131-53289202	TAGATGCACCAGAACCTGGA	Fwd	AGATCGCGCTACTGCACTAC	Fwd	CTGCCTCACAGGATCGTTGG
			Rev	ACACAGTGCCTCCCTAGCTA	Rev	GCCCTCAGCAAGAATCAGGT
Enhancer 5	chr10:99331549-99331613	GTTCCCTAGCAGATTAACCT	Fwd	ATTCTCCTGCAGCTCTGTGG	Fwd	CCTGCCTCCTCCATCAGTTG
			Rev	CTCCAGCCCAGATGACAGTG	Rev	TCTCTGATCACAGCTCACGC
MYC internal	chr8:128751388-128751443	GGAGGAAACGCTAAAGCCCA	Fwd	GGAGATGGTGACCGAGCTG	Fwd	CCAGCAGCGACTCTGGTAAG
			Rev	GCAAAGCACATTCCCAAGCA	Rev	CAAAAGTCCAAGAGGGCGGG
MYC downstream	chr8:128755507-128755572	ACTCTCCATTCCAAATAATC	Fwd	ACTCTCCTTGCTTCCATCCT	Fwd	TTGGCACGTCATATAGGCGA
			Rev	ACAAACCACAGAAAACCCCCT	Rev	CTCCCCTTCACGTCCGATTC
Control	chr13:36074330-36074389	ATCCAGGGTTGCTTTCCACT	Fwd	TTCCCTTTTGTCCAGGCACT	Fwd	GCAGCTGACCTTTGCAGTTC
			Rev	GGACAAAGTGGAGCAAAATGC	Rev	TTTCCCCCTCTTCCCTGACA

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Role of chromatin organization in Double Strand Break repair at centromeres

Résumé

Les cassures double brins d'ADN (CDBs) sont parmi les lésions les plus dangereuses car elles peuvent causer des translocations chromosomiques. Quand elles ont lieu aux centromères, les CDBs peuvent perturber la ségrégation correcte des chromosomes au cours de la division cellulaire. Ceci est notamment à l'origine d'aneuploïdie et de réarrangements génomiques, caractéristiques principales the nombreuses maladies, dont le cancer. Dans ce contexte, la compréhension du processus de réparation des CDBs aux centromères présente d'importantes implications cliniques. Tirant avantage du système CRISPR/Cas9 afin d'induire des CDBs aux centromères de cellules de mammifères, nous avons montré que, contrairement à tout autre CDB survenant dans le génome, les CDBs centromériques peuvent être réparées par recombinaison homologue (RH) en phase G1 du cycle cellulaire, malgré l'absence de la chromatide sœur. Nous avons montré que H3K4me2 permet une transcription centromérique, augmentant ainsi la formation de R-loops et donnant lieu au recrutement de facteurs de résection. CENP-A, un variant d'histone H3 spécifique aux centromères, et sa chaperonne HJURP interagissent avec USP11, une dé-ubiquitinase permettant le recrutement de facteurs impliqués dans l'invasion du brin homologue. De plus, nous avons montré que la réparation par RH aux centromères en G1 permet d'inhiber la formation de translocations chromosomiques.

Mots clés : Centromères, CDB, CRISPR/Cas9, RH, chromatine, H3K4me2, CENP-A, HJURP

Résumé en anglais

DNA Double Strand Breaks (DSBs) are among the most deleterious lesions that DNA can endure, especially because they can lead to chromosomal translocations. When happening at centromeres, DSBs can interfere with the proper chromosome segregation during cell division, leading to aneuploidy and rearrangements, hallmarks of many diseases including cancer. Thus, understanding the process of DSBs repair in centromeres has important clinical implications. Using the CRISPR/Cas9 system to generate DSBs at centromeres of mammalian cells, we have demonstrated that in contrast to any other DSBs, centromeric DSBs can be repaired by Homologous Recombination (HR) in G1 phase of the cell cycle despite the absence of the sister chromatid. We showed that H3K4me2 allows damage-induced centromeric transcription and increased R-loop formation, leading to the recruitment of DNA-end resection factors. The histone variant CENP-A, specific to centromeric chromatin, and its chaperone HJURP interact with the deubiquitinase USP11 which allows the strand invasion step and thus the completion of HR repair. Moreover, we provided evidence that utilization of HR at centromeric breaks in G1 inhibits the formation of deleterious chromosomal translocations.

Keywords: Centromere, DSB, CRISPR/Cas9, HR, chromatin, H3K4me2, CENP-A, HJURP