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Ioanna MITRENTSI

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Double Strand Break repair in human heterochromatin

THÈSE dirigée par :

Dr. SOUTOGLOU Evi	DR, IGBMC, université de Strasbourg

RAPPORTEURS :

Dr. Jeggo Penny	Professor, Genome Damage and Stability Centre, University of Sussex
Dr. D'adda di Fagagna Fabrizzio	DR, IFOM, the FIRC Institute of Molecular Oncology Foundation
Dr. Larsen Dorthe Helena	DR, Danish Cancer Society Research Cente

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AUTRES MEMBRES DU JURY :

Dr. SEXTON Tom	CR, IGBMC, université de Strasbourg

"One should use common words to say uncommon things"

Arthur Schopenhauer

To my mother…

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Abbreviations

53BP1	p53-Binding Protein 1
AID	Activation-Induced cytidine
ALT	Alternative Lengthening of
Alt-EJ	Alternative End-Joining
ATM	Ataxia - Telangiectasia
ATR	Ataxia Telangiectasia and Rad3
ATRIP	ATR Interacting Protein
BACH1	BTB domain and CNC Homolog 1
BIR	Break-Induced Replication
BLM	Bloom helicase
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
BRCT	BRCA1 C
CAF-1	Chromatin Assembly
CDK	Cyclin-Dependent-Kinase
CENP-A	Centromere Protein A
Chk1	Checkpoint
Chk2	Checkpoint
cNHEJ	classical NHEJ
CRISPR	Clustered Regularly Interspaced Short Palindromic
CSR	Class Switch
DDR	DNA Damage Response
D-loop	Displacement-
DNAPK	DNA-dependent Protein
DNAPKcs	DNAPK catalytic subunit
DSB	Double Strand
ERCC1	Excision repair cross-complementing
EXD2	Exonuclease 3’-5’ domain-containing protein 2
EXO1	Exonuclease 1
FRAP	Fluorescent recovery after
FRET	
gRNA	guide RNA
HATs	Histone Acetyltransferases
HDACs	histone
HJ	Holliday-
HJURP	Holliday Junction Recognition
HMGA1	
HMGN1	High Mobility Group Nucleosome Binding
HP1	Heterochromatin Protein 1
HR	Homologous
Igs	Immunoglobulins
IR	Ionizing

KAP1	KRAB-associated protein-1
LINC	Linker of Nucleoskeleton and
MDC1	Mediator of DNA damage checkpoint protein 1
MRN	Mre11-RAD50-
MRX	Mre11-RAD50-
NBS1	Nijmegen Breakage
NHEJ	Non-Homologous End
NuRD	Nucleosome Remodeling
PALB2	Partner and localizer of
PAM	Protospacer Adjacent Motif
PARGs	poly-ADP-ribose glycohydrolases
PARPs	poly-ADP-ribosyl
PAXX	Paralog of XRCC4 and
PcG	Polycomb Group
PNKP	Polynucleotide Kinase/Phosphatase
PTIP	PAX Transactivation activation domain- Interacting
PTMs	Post-Translational Modifications
RECQ5	RecQ Like
RIF1	RAP1-interacting factor 1
RNF8	Ring Finger
ROS	Reactive Oxygen Species
RPA	Replication
SDSA	Synthesis-Dependent Strand
SETDB1	SET Domain Bifurcated 1
SHM	Somatic hypermutation
SMARCAD1	SWI/
SSA	Single Strand Annealing
ssDNA	single-stranded
SWI/SNF	Switching defective/sucrose non-fermenting
TADs	Topologically Associated Domains
TCR	T cell receptors
TopBP1	Topoisomerase II Binding Protein 1
TSS	Transcriptional Start Site
UCHL3	Ubiquitin carboxyl-terminal hydrolase isozyme
USP11	Ubiquitin specific
UV	Ultraviolet
WRN	Werner syndrome helicase/exonuclease
XLF	XRCC4-like
XRCC1	X-ray repair cross-complementing protein 1
XRCC3	X-ray repair cross-complementing protein 3
XRCC4	X-ray repair cross-complementing protein 4

1. Introduction

1.1 DNA lesions: causes and genome integrity

DNA, or deoxyribonucleic acid, is the molecule that contains the genetic information which are essential for the biological processes of all organisms. It is therefore necessary to preserve its integrity in order to achieve proper cell function and survival. A variety of types of damaging agents constantly assault the genome, causing different types of lesions at a rate of 10^3 to 10^6 per cell per day (Lindahl 1993). More specifically endogenous or exogenous damaging agents can cause double strand breaks (DSBs), single strand breaks (SSBs), as well as a range of base modifications or loss (Hoeijmakers 2001, Aguilera and García-Muse 2013) (Figure 1).

The cause of the damage is either endogenous, which occurs from normal metabolic processes of the cell, or from exogenous sources, where damage is caused by external DNA damaging agents. Examples of endogenous sources of damage occurring under physiological conditions include cellular metabolism and DNA replication. In detail, hydrolysis reactions in cell metabolism can lead to either the depurination of the DNA, where a base is lost, or its deamination where a base is converted to another one (Lindahl 1993). Concerning the by-products of cellular metabolism, reactive oxygen species (ROS) are produced during the oxidative respiration, and a build-up of these molecules leads to mutations and base-pair mismatches (Kasai and Nishimura 1984). Cells are also challenged during the replication process, as they face slowing or stalling of the replication fork progression (reviewed in (Zeman and Cimprich 2014)). This is known as replication stress, and when it persists the fork might fail to restart, often resulting in fork collapses, which if unresolved lead to the formation of DSBs. Replication stress can originate from multiple sources, including unrepaired DNA lesions, misincorporation of ribonucleotides, secondary DNA structures, nucleotide depletions or collision between replication and transcription. Consequently, fork collapse is considered the major source of endogenous DSBs. Telomeres pose another unique challenge to maintaining the integrity of the genome. Telomeres are the ends of chromosomes thus resemble a half of a DSB. However, “repair” of telomeres would lead to altering the genome and genomic instability. For example many unprotected chromosome ends are formed and the chance that two dysfunctional telomeres find each other and fuse increases. Therefore, the cells have developed mechanisms to protect the end of the chromosomal DNA sequence from being recognized as DSBs, by creating a telomere cap structure (de Lange 2002). Although, during telomere shortening, which occurs since telomeres are unable to be fully replicated each cell cycle,

capping defects are observed and the de-protected telomeres are processed as DSB, leading to genomic instability. I discuss in detail telomere integrity maintenance in the review (Yilmaz*, Mitrentsi* et al, *in preparation*, p40). Finally, not all breaks in the genome are harmful or unwanted. Programmed DNA lesions are induced by the cells for the induction of genetic variability in meiosis (important for species survival) and for the establishment of the immune responses repertoire (which is vital for protecting an organisms by producing an immune system response to the widest possible range of pathogens) by V(D)J recombination, class-switching and somatic hypermutation (Alt, Zhang et al. 2013, Baudat, Imai et al. 2013).

Exogenous sources of DNA damage are often environmental factors. A prominent example is sunlight. Among the different types of UV radiation, the UV-B component of the sunlight can lead to the formation of photoproducts, such as cyclobutane pyrimide dimers (CPDs), which cause structural alterations and affect the DNA replication and/or transcription process (Lindahl 1993). Another source of exogenous damage is Ionizing radiation (IR), which results from radioactive decay of naturally radioactive compounds or by medical exposures (such as radiotherapy). IR leads to different types of lesions, with DSBs being the most dangerous and cytotoxic (Lichter, Cremer et al. 1988). DNA lesions also originate from genotoxic chemicals, such as radiomimetic drugs, which are commonly used in DNA repair studies (for example neocarnizostatin (NCS) or phleomycin), and in cancer therapies (for example, DNA topoisomerase inhibitors such as etoposide or camptothecin). Finally, tobacco products represent the most common and well-known genotoxic chemicals, which produce DNA lesions and are very often are the cause of various cancers.

All of the DNA lesions being created by damaging agents can lead to genomic instability. To avoid this outcome, the cells have evolved DNA repair pathways that are specialized for the repair of each type of lesion. The single stranded DNA (ssDNA) damages are resolved by: the mismatch repair (MMR) pathway, which repairs insertions, deletions and mis-incorporation of bases; the base excision repair (BER) pathway, which removes small non-helix distorting base lesions; and the nucleotide excision repair (NER) pathway, which removes bulky adducts such as CPDs. For DSBs, which are the less frequent but among the most cytotoxic lesions, Homologous Recombination (HR) and non-homologous end joining (NHEJ) are the two major pathways used. These pathways are explained in detail, together with the alterative pathways, in section 1.5.

1.2 Physiological roles of DNA lesions

As mentioned before, programmed DNA lesions are desired and occur to ensure proper cell functioning of some important cell types. Their role is to create genetic variability during the following processes:

1.2.1 Meiosis

Meiosis ensures the proper segregation of chromosomes during sexual reproduction in eukaryotes. Hundreds of DSBs are formed during the first meiotic prophase, and they are necessary for a proper chromosome segregation and exchange of genetic material between homologous chromosomes, increasing genome diversity(Baudat, Imai et al. 2013). These DSBs are induced by the Spo11 enzyme at specific genomic regions that are called "recombination hotspots'' (Keeney, Giroux et al. 1997). HR is then activated and instead of the sister chromatid, the homologous chromosome is used as a template. This results in gene conversion with reciprocal exchanges (crossovers) or without reciprocal exchanges, promoting genetic diversity (reviewed in (Baudat and de Massy 2007, Hyppa and Smith 2010).

1.2.2 V(D)J recombination

The immune system has developed several strategies to ensure recognition of "non-self" molecules. The production of a highly diverse repertoire of immunoglobulins (Igs: B cell receptors and antibodies) and T cell receptors (TCR), is catalyzed by a mechanism that is termed V(D)J recombination (Alt, Zhang et al. 2013). Both the Igs and TCRs contain a variable region at their N-terminal that consists of the V (variable), D (diversity) and J (joining) gene segments. In order to produce different amino-acid sequences at the antigen binding regions of Igs and TCRs and to specifically recognize different pathogens, those segments are assembled in various combinations. The V(D)J recombination process is activated after specific DSB induction by the lymphocyte-specific endonucleases RAG1 and RAG2 next to the targeted segment (Schatz, Oettinger et al. 1989, Oettinger, Schatz et al. 1990). These segments are then fused through the NHEJ repair pathway(Taccioli, Rathbun et al. 1993) (NHEJ is described in detail in Section 1.5.2).

1.2.3 Class switch recombination and somatic hypermutation

After the production of immunoglobulins by B cells through the V(D)J recombination process, two more somatic alterations take place in these cells, to ensure the variability of B cell receptors and antibodies. The two mechanisms responsible for this are the class switch recombination (CSR) and the somatic hypermutation (SHM). Both mechanisms require the AID enzyme, a cytidine deaminase that catalyzes the deamination of cytosin to uracil in a transcription dependent manner (reviewed in (Pavri and Nussenzweig 2011). SHM affects the variable region by inducing specific point mutations that finally lead to the production of higher affinity antibodies (McKean, Huppi et al. 1984). Conversely, CSR is responsible for the recombination of the IGs constant regions, to allow the production of different antibody isotypes, thus increasing the immune response efficiency. Mechanistically, after AID action, the uracil residues are recognized and removed by the UNG enzyme (a uracil-DNA glucosylase), creating an abasic site. In SHM, error-prone DNA polymerases fill the gaps in the DNA, leading to the creation of mutations. On the other hand, during CSR, the abasic sites are converted into nicks, then eventually DSBs, which are ultimately joined by NHEJ (Alt, Zhang et al. 2013).

1. 3. Role of DNA lesions in ageing and pathology

DNA lesions are repaired by several different mechanisms, to preserve genome integrity (Figure 1). This is of great importance, beacuse if they are not properly repaired, DNA lesions can lead to accelerated ageing (e.g. progeria syndromes) or cancer. Different types of DNA lesions have different outcomes. For example, mutagenic lesions or lesions leading to insertions and deletions favor cancer development (Lengauer, Kinzler et al. 1998), whereas cytotoxic lesions tend to result in senescence or apoptosis, which is linked to ageing (reviewed in (Garinis, van der Horst et al. 2008)). A common driver of tumorigenesis is chromosomal translocations, which originated from DSBs that where unfaithfully rejoined. The outcome of this event is a fusion between two different chromosomes or chromosomal regions. This can create a chimeric fusion protein with oncogenic potential, a fusion of an oncogene to a strong promoter or the disruption of a tumor-suppressor gene (Roukos and Misteli 2014). The frequency of translocations and the partner choice is affected by several factors, including the timing of break repaired and the spatial organization of the genome. In mammals DSBs are positionally stable and require their translocation partner to be in close spatial proximity (Kruhlak, Celeste et al. 2006, Soutoglou, Dorn et al. 2007, Jakob, Splinter

et al. 2009, Roukos, Voss et al. 2013), whereas in yeast the breaks are more mobile and they can find partners throughout the nucleus (Mine-Hattab and Rothstein 2012). Interestingly, the frequency of translocation is also depending on the DNA repair pathway choice, as NHEJ has a protective role against translocations (Difilippantonio, Zhu et al. 2000, Gao, Ferguson et al. 2000), whereas alternative end-joining and the homologous-recombination based pathways like single strand annealing (SSA) are more prone to generate them(Elliott, Richardson et al. 2005).

In addition to ageing and cancer, DNA lesions are involved in neurodegenerative disorders(Kulkarni and Wilson 2008). Our nervous system has a limited capacity to proliferate in adulthood, leading to the accumulation of damage. Since the cells are blocked in G0 phase of the cell cycle, the NHEJ pathway, which is a more error prone mechanism, is activated to repair the damage (Rass, Ahel et al. 2007). Moreover, neurons exhibit high mitochondrial respiration rates, and consequently have high levels of ROS, which are responsible for the formation of lesions associated with Alzheimer's and Parkinson's diseases (Kulkarni and Wilson 2008). Last but not least, defects in the repair of the programmed DNA lesions (see section 1.2) can affect meiosis and immune system related processes, therefore leading to infertility or immune deficiencies (Alt, Zhang et al. 2013).

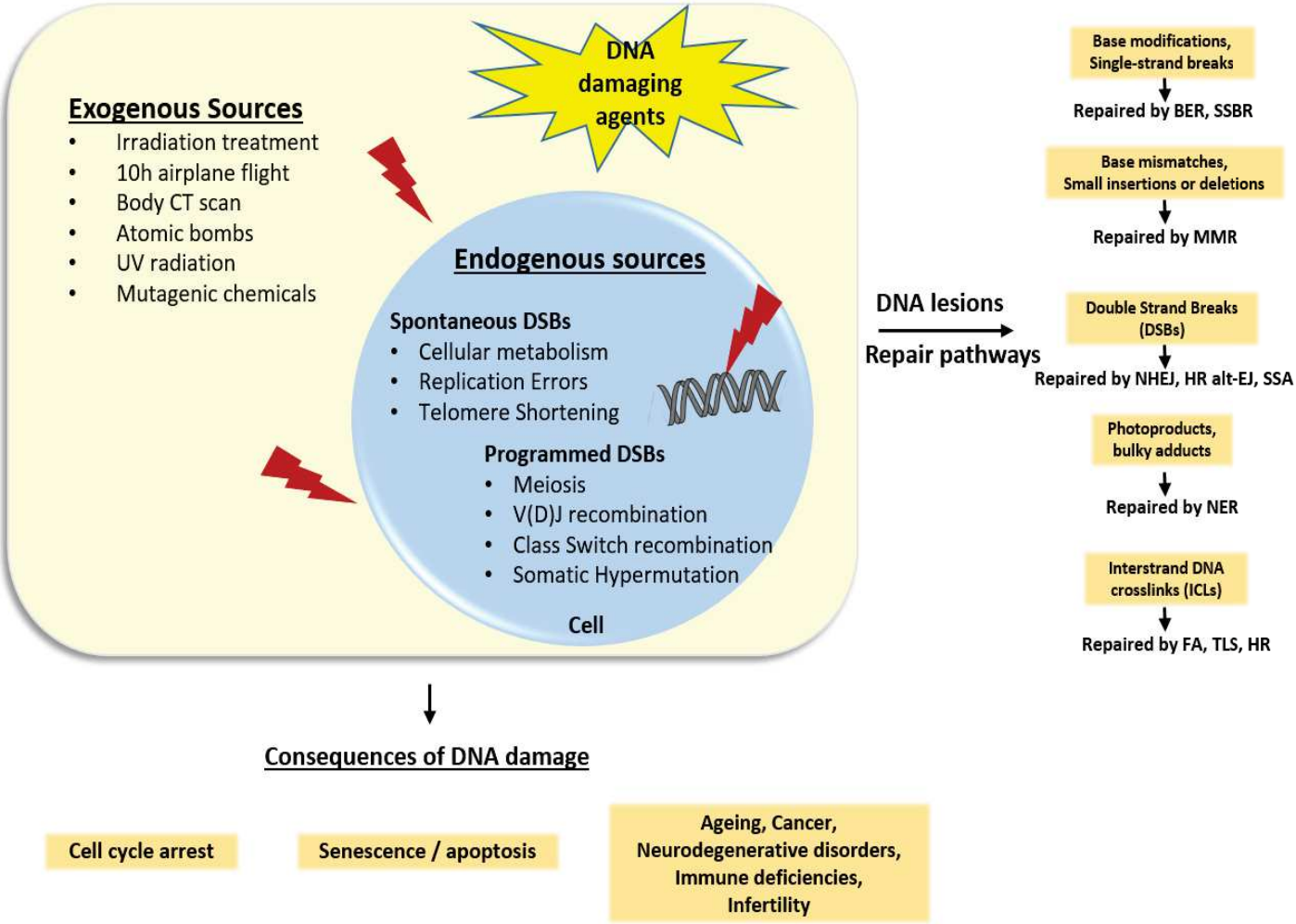

Figure 1: DNA lesions created by different damaging agents activate distinct repair pathways and can lead to different outcomes. DNA damaging agents can be endogenous or exogenous. They cause different types of DNA lesions, such as modified bases, single strand breaks, double strand breaks or base mismatches. Each type of lesion is repaired by a specific DNA repair pathway. Damage induction leads to cell cycle arrest in order to allow the cell to repair the break. In case the breaks are not properly repaired the cells can be led to apoptosis, senescence, or genomic rearrangements and thus cancer and other pathological conditions.

1.4 Double Strand breaks and the DNA Damage Response (DDR) pathway

DSBs are the most cytotoxic type of DNA lesion since they affect both strands of the double helix, triggering genomic instability, chromosomal translocations and therefore cancer and ageing(Jackson and Bartek 2009). Several DNA repair pathways have evolved to resolve DSBs, with NHEJ and HR being the major pathways utilized (described in detail in section 1.5). Before

their activation, the DSBs activate a common signaling cascade, the DNA Damage Response (DDR) pathway. The DDR is a network of processes that allows DSB recognition and for the activation of checkpoints to pause cell cycle progression and, thus, provide time for the cell to repair the breaks before its division to stop the damage being passed on to daughter cells (Bekker-Jensen and Mailand 2010, Chapman, Taylor et al. 2012). The major steps of the DDR signaling cascade are the DSB recognition, the signal transduction and amplification and finally the cell cycle arrest, and are discussed below. If repair is not possible, senescence and apoptosis are two other possible DDR outcomes (Figure 2).

1.4.1 DSB recognition

Several sensors have been implicated in the recognition of a DSB. First the Ku70-Ku80 heterodimer binds around the double stranded DNA ends rapidly and with high affinity. There it loads and activates DNA-dependent protein kinase's catalytic subunit (DNAPKcs) to initiate repair by NHEJ (Drouet, Delteil et al. 2005). In contrast, the MRN complex (MRX in yeast), composed of Mre11, Rad50 and NBS1/XRS2 proteins, can bind directly to the double stranded DNA ends through Mre11s binding domains (D'Amours and Jackson 2002). Mre11 is also implicated in recruiting and activating the ATM kinase and in initiating end-resection, which is a major step of HR (described in section 1.5.1). Of the other MRN complex player, Rad50 is a member of SMC family of ATPases and can also bind directly to DNA (D'Amours and Jackson 2002). Its ATPase activity is important both for the DNA binding and for stimulating the Mre11 nuclease activity (Alani, Padmore et al. 1990, Bhaskara, Dupré et al. 2007). The Mre11/Rad50 complex is stabilized through the direct interaction of NBS1 with Mre11 (D'Amours and Jackson 2002). Through this interaction NBS1 localizes the complex in the nucleus. Moreover, MRN is recruited to the DSBs after an interaction of NBS1 with MDC1 (Lukas, Melander et al. 2004, Stucki, Clapperton et al. 2005, Chapman and Jackson 2008, Spycher, Miller et al. 2008), which among other things promotes ATM kinase activation. DSBs can also be sensed through Parylation, a post translational modification that is used as a signal transducer in several pathways (Reviewed in (Hochegger, Dejsuphong et al. 2006). Briefly, it consists of a covalent addition of poly-ADP-ribose on protein substrates which is catalyzed by poly-ADP-ribosyl polymerases (PARPs) and removed by poly-ADP-ribose glycohydrolases (PARGs) (Beck, Robert et al. 2014). PARP inhibition is increases the cells' sensitivity to DNA damage (Boulton 1999, Audebert, Salles et al.

2004, Rulten, Fisher et al. 2011). Moreover, PARP1 and PARP3 have been shown to be rapidly recruited to DSBs (Haince, Kozlov et al. 2007, Boehler, Gauthier et al. 2011, Rulten, Fisher et al. 2011, Langelier, Planck et al. 2012). PARP1 participates in the early DDR signaling, by promoting the recruitment of MRN complex and ATM kinase to the sites of DSBs (Haince, Kozlov et al. 2007). PARP1 favors both HR and alternative end joining (alt-EJ) by recruiting Mre11, and thus facilitating end-resection, while at the same time minimizing the recruitment of NHEJ complexes at the sites of damage (Hochegger, Dejsuphong et al. 2006, Wang, Wu et al. 2006, Bryant, Petermann et al. 2009, Paddock, Bauman et al. 2011). In contrast, PARP3 promotes NHEJ repair in two ways. First, it interacts with APLF, a histone chaperon, to promote NHEJ, by accelerating the XRCC-4-DNA ligase IV- mediated ligation (Rulten, Fisher et al. 2011, Grundy, Rulten et al. 2013). Second, PARP3 interacts with the Ku heterodimer, leading to the protection of the DNA end from end resection (Beck, Robert et al. 2014). In conclusion, parylation through different PARPs can affect both the DDR and the DNA repair pathway choice.

1.4.2 Signal transduction

After recognition of a DSB, the different sensors allow the recruitment and activation of different kinases: ATM, ATR and DNAPK (Falck, Coates et al. 2005). All three are members of the phosphatidylinositol 3-kinase related kinase (PIKK) protein family and they are implicated in the phosphorylation of several DDR mediators. These phosphorylation events both allow the amplification of the DDR and the regulation of the cell cycle progression, apoptosis or senescence. Even though the three kinases have the ability to phosphorylate the histone variant H2AX on S139 (γ-H2AX) in mammalian cells, ATM is the major kinase responsible for spreading of γ-H2AX megabases around the break (Falck, Coates et al. 2005, Tomimatsu, Mukherjee et al. 2009). γ-H2AX is important in the DDR, aid signal amplification and recruits repair factors to the site of damage. The major actor in the recruitment of ATM at the break sites is the MRN complex and, more specifically, the direct interaction of ATM with NBS1 (Difilippantonio, Celeste et al. 2005, Lee 2005).The retention of ATM at the damage sites is also mediated through an MDC1 dependent positive feedback loop. In detail, the spreading factor MDC1 recognizes γH2AX through its C-terminal tandem BRCT repeats and recruits additional ATM molecules at the sites of DSBs (Stucki, Clapperton et al. 2005, Savic, Yin et al. 2009). It has also been proposed that downstream factors such as 53BP1 or BRCA1 can also modulate ATM retention (Lee, Park et al. 2010). ATM

activation upon DNA damage is controlled by several mechanisms. Normally, ATM exists in cells as an inactive homodimer, but upon damage, and after auto-phosphorylation on S1981, it is dissociated to an active monomer (Bakkenist and Kastan 2003). This active form of ATM is subjected to further post translational modifications, including its autophosporylation at different residues (Pellegrini, Celeste et al. 2006, So, Davis et al. 2009) and its acetylation by the TIP60 acetyltransferase, both important for its activation (Sun, Xu et al. 2007, Yan, Boboila et al. 2007, Sun, Jiang et al. 2009). Once ATM is activated, it can directly phosphorylate its target substrates, including Chk2 and p53, which maintain cell cycle arrest in G2 phase (Saito, Goodarzi et al. 2002). Interestingly, p53 is also directly phosphorylated by Chk2 itself in order to block the cell cycle and possibly the two kinases work synergistically to ensure its activation.

While ATM is strictly recruited to DSBs, ATR responds to damage related to DNA replication, e.g. stalled replication forks (Falck, Coates et al. 2005, Cimprich and Cortez 2008). It is recruited at ssDNA and junctions between ssDNA and double stranded DNA (dsDNA), through an interaction of its cofactor ATRIP and RPA (Zou 2003). RPA is a trimeric complex that binds to ssDNA to protect it from degradation and secondary structure formation. It then allows the recruitment of ATRIP, which promotes the recruitment of ATR at the break sites (Zou and Elledge 2003). ATR recruitment at the sites of damage is not sufficient for its activation, which requires its colocalization with a ring shaped complex Rad9-Rad1-Hus1 (9-1-1), a mechanism dependent on Rad17 (Ellison and Stillman 2003, Zou 2003). Even though in yeast it has been shown that the 9-1-1 complex can directly activate ATR (Majka, Niedziela-Majka et al. 2006), in mammalian cells some additional activators have been identified. One of them is TopBP1, which can stimulate ATR activity even in the absence of damage (Kumagai, Lee et al. 2006, Mordes, Glick et al. 2008, Navadgi-Patil and Burgers 2008). Moreover, ETAA1 is an RPA-binding factor that also stimulates ATR activity (Bass, Luzwick et al. 2016, Lee, Zhou et al. 2016). One of the major target substrates of ATR after its activation is the phosphorylation of Chk1. Upon DNA damage, the adaptor protein Claspin interacts with Chk1 in a TopBp1-dependent manner, leading to the interaction between Chk1 and ATR (Kumagai and Dunphy 2003, Liu, Bekker-Jensen et al. 2006). After phosphorylation, Chk1 dissociates from chromatin and phosphorylates its targets (Smits, Reaper et al. 2006), the Cdc25 phosphatases, to block cell cycle progression.

The third kinase involved in the signal transduction part of DDR, DNAPK, is a PI3K-like kinase composed of Ku70, Ku80 and DNAPKcs proteins. DNAPK has a major role in NHEJ, as it is

involved in the stabilization and tethering of the two broken ends before their ligation (more details in section 1.5.2). During DDR signaling though, it works cooperatively with ATM to phosphorylate H2AX (Falck, Coates et al. 2005, Caron, Choudjaye et al. 2015). Finally, DNAPK phosphorylates one of the subunits of RPA (RPA2) upon replication stress and IR, leading to an S or G2/M cell cycle arrest (Wang, Guan et al. 2001, Block, Yu et al. 2004, Liaw, Lee et al. 2011, Liu, Opiyo et al. 2012).

1.4.3 Signal amplification and spreading

As explained previously, ATM, ATR and DNAPK phosphorylate H2AX to produce γH2AX (Rogakou, Pilch et al. 1998) and this is the first step for the initiation of DDR signaling. Indeed, it allows the recruitment of MDC1, which mediates the recruitment of further MRN-ATM complexes resulting in the phosphorylation of more H2AX on adjacent nucleosomes. Through this positive feedback loop the signal is amplified by spreading γH2AX for more than 2 Megabases around the DSB site (Rogakou, Boon et al. 1999, Lukas, Melander et al. 2004, Stucki, Clapperton et al. 2005, Chapman and Jackson 2008, Spycher, Miller et al. 2008). MDC1 can then recruit additional factors, including RNF8 and RNF168, two E3 ubiquitn ligases that induce ubiquitination of H2A histones, thus enhancing 53BP1 and BRCA1 retention at DSBs (Huen, Grant et al. 2007, Mailand, Bekker-Jensen et al. 2007, Doil, Mailand et al. 2009, Thorslund, Ripplinger et al. 2015). In general, the spreading of DDR factors allows their visualization by microscopy (Nagy and Soutoglou 2009).

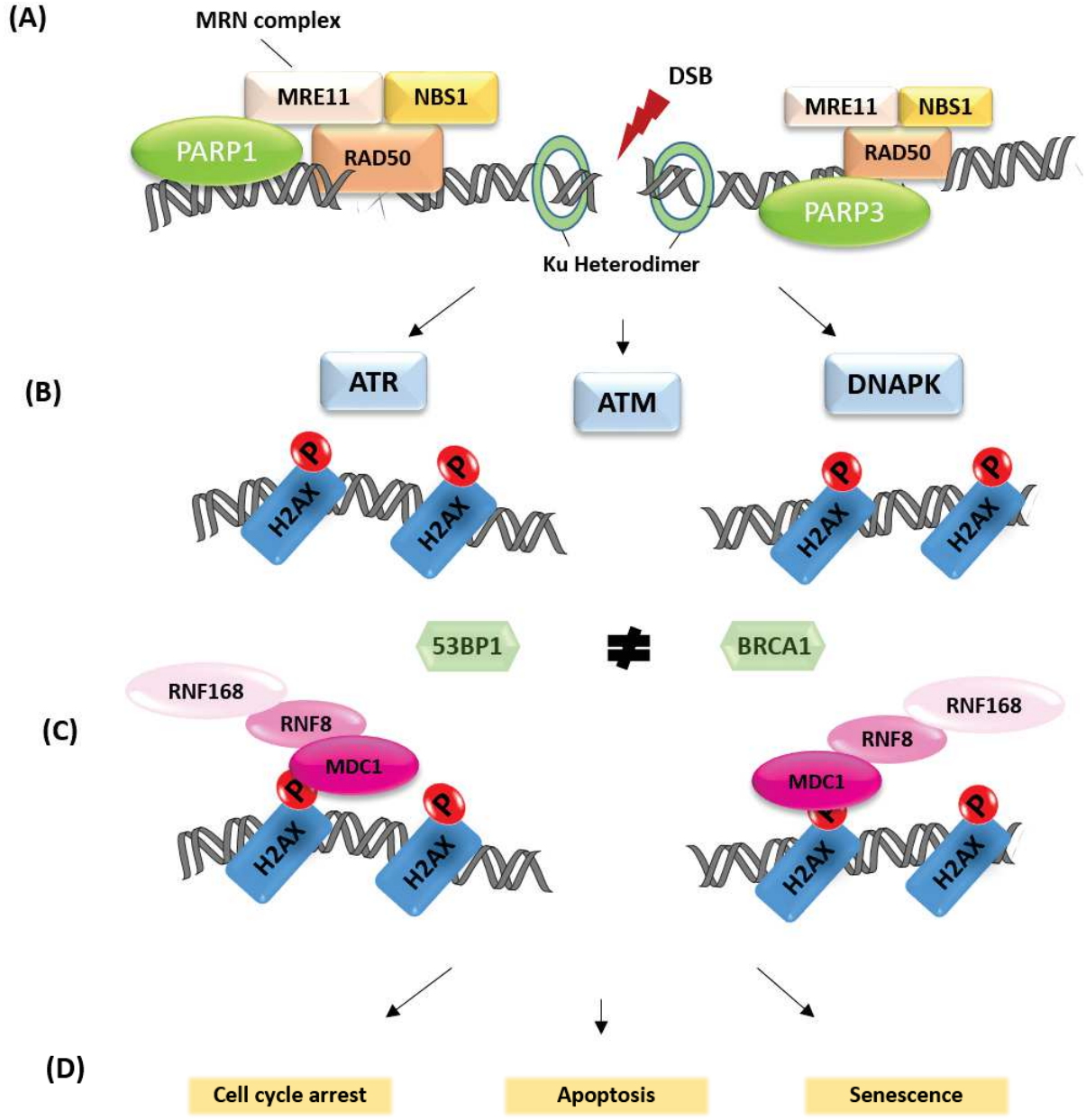

Figure 2: DDR pathway activation A. DDR starts with recognition of the break, with the binding of MRN complex or Ku heterodimer facilitated by PARP1 or PARP3, respectively (DSB recognition). **B.** The Plk3-like kinases ATM, ATR and DNAPK are subsequently recruited and phosphorylate downstream factors and the histone variant H2AX (γH2AX) (Signal transduction). C.Recognition of γH2AX by MDC1 that promotes RNF8 and RNF168 E3 ubiquitin ligases and thus 53BP1 and BRCA1 recruitment (Signal amplification). (D)DDR will ultimately lead to cell-cycle arrest, apoptosis or senescence (DDR outcomes)

1.4.4 DDR outcomes

DDR signaling through ATM and ATR leads to the phosphorylation and activation of Chk2 / p53 and Chk1 kinases respectively (Bartek and Lukas 2003), leading to a temporary cell cycle arrest, enabling the cell to repair the break before dividing. Chk1 phosphorylates Cdc25 phosphatases, an event that leads to their degradation which in turn blocks the dephosphorylation of CDK complexes, an important step for cell cycle progression (Bartek and Lukas 2003). Cdc25 targeting through Chk1 leads to cell cycle arrest either at the G1/S transition, S phase or G2/M transition (Bartek and Lukas 2003). Chk2 can also target Cdc25, leading to cell cycle arrest (Bartek and Lukas 2003), and can also phosphorylate and stabilize p53, with three possible outcomes: temporarily blocking the cell cycle progression in G1/S, induction of apoptosis when the DSB cannot be repaired, or induction of senescence where the damaged cells remain metabolically active but their proliferation is blocked to prevent propagation of the damage within the tissue (d'Adda di Fagagna 2008) (Figure 3).

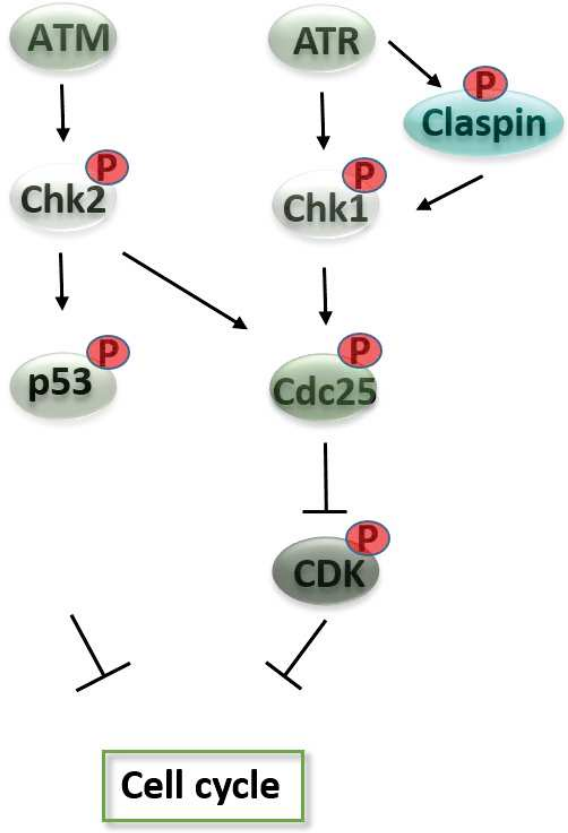

Figure 3. DDR leads to Cell-cycle arrest Cell cycle arrest is mediated by ATM and ATR kinases that phosphorylate Chk2 and Chk1, respectively. Chk2 phosphorylates p53 and Cdc25 to block the cell-cycle. Chk1 also blocks the cell cycle progression through phosphorylation of Cdc25.

1.5 Double Strand Break repair pathways

DSBs, could be extremely hazardous to the cells as they can lead to genomic instability and cancer. Their faithful and fast repair is really crucial to avoid these events. In order to achieve that, the cells have evolved different DNA repair mechanisms, which can take place depending on the chromatin structure of the break site, its location in the nucleus and the phase of the cell cycle at this specific moment that the DSB was induced (Chapman, Taylor et al. 2012). The two main pathways that are used to repair a DSB after the DDR activation are HR and NHEJ. Alternative DSB repair pathways have also been identified and they include the alt-EJ, SSA and Break-Induced Replication (BIR).

1.5.1 Homologous Recombination (HR)

HR is the main pathway that is utilized to repair breaks induced physiologically during meiosis or replication errors, and is a conserved pathway from yeast to mammals (Jasin, de Villiers et al. 1985, Liang, Han et al. 1998) (Figure 4). HR uses the sister chromatid as a template to repair the break. It repairs in an error-free manner since it requires the presence of an identical or almost identical sequence and is thus mainly activated in S/G2 phases of the cell cycle (Jasin and Rothstein 2013). This pathway consists of two main steps: end resection and strand invasion. Briefly, a 3' ssDNA tail is created during resection and it is able to invade into the double stranded homologous sequence and use it as a primer for the template- based repair synthesis (Wright et al. 2018). These 3' overhangs create a substrate for Rad51 loading that allow the strand invasion and D-loop and Holliday-Junction (HJ) formation. The formation of D-loop is a critical point of the pathway as it can give rise to the three different sub-pathways of HR (Heyer, Ehmsen et al. 2010). First, in BIR, a single-ended DSB invades and the D-loop is transformed in a replication fork, leading to the replication of the whole chromosome (Heyer, Ehmsen et al. 2010). This mechanism is used in ALT-telomere maintenance where telomerase is not present, and I have further discussed it on our review Yilmaz*,Mitrentsi* et al. , *in preparation*. Second, in Synthesis-Dependent Strand Annealing (SDSA), the D-loop is reversed and the newly synthetized strand anneals with the resected strand of the second end, thus avoiding crossovers and reducing genomic rearrangements (Heyer, Ehmsen et al. 2010). Finally, and in contrast to the SDSA, in meiotic recombination crossover generation double Holiday Junction (dHJ) is required, and involves the formation and

resolution/dissolution of Holiday junctions.

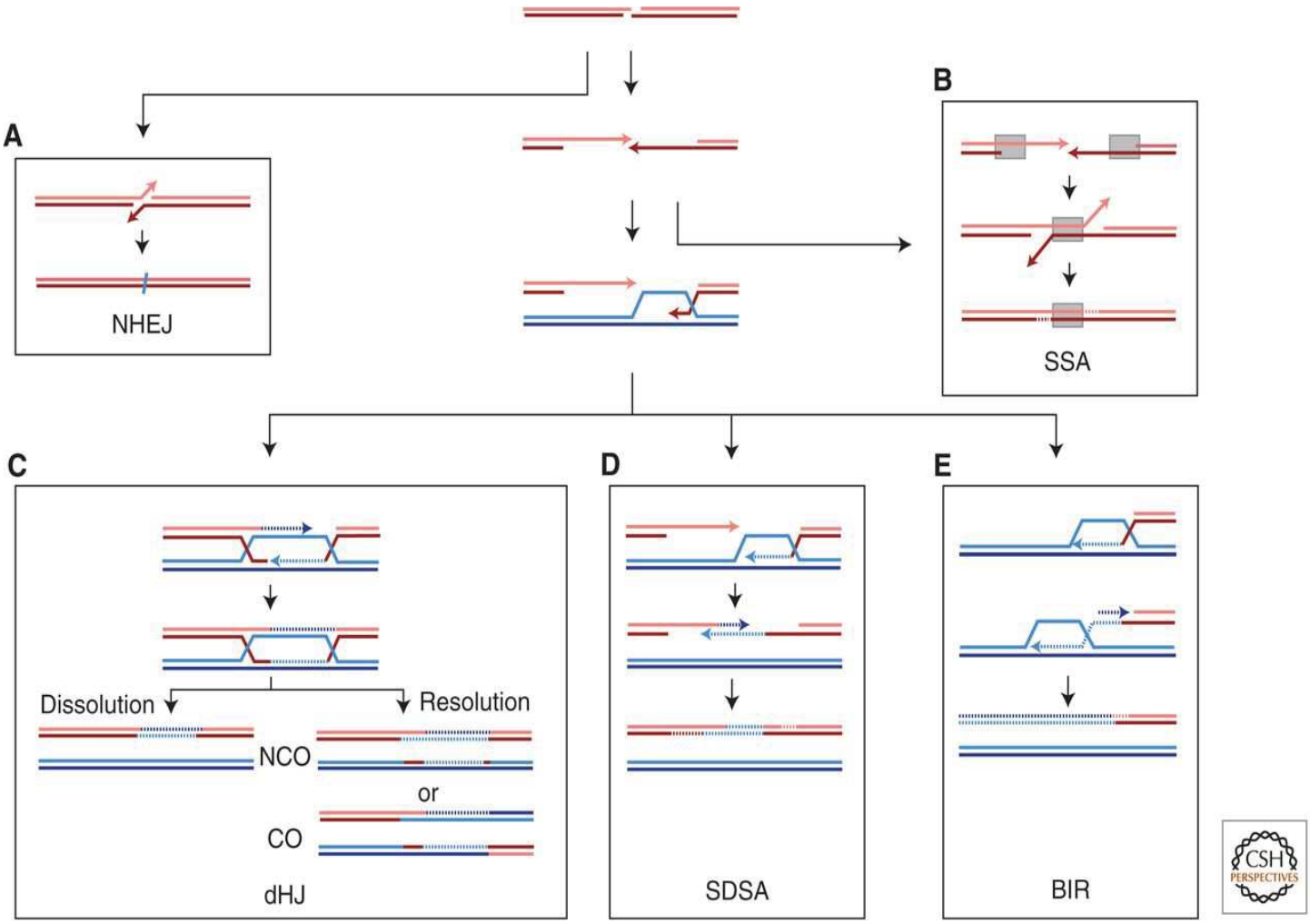

Figure 4. NHEJ, SSA and Subpathways of HR Adapted from Anuja Mehta, and James E. Haber Cold Spring Harb Perspect Biol 2014;6:a016428.

Break recognition and resection

End resection is the first step of HR , and is responsible for the creation of ssDNA overhangs that will later invade the homologous sequence (Figure 5). Resection is initiated after the DSB recognition by the MRN/X complex, together with CtIP/Sae2 protein, both in mammalian and in yeast cells (D'Amours and Jackson 2002, Zhang, Hefferin et al. 2007, Huertas, Cortés-Ledesma et al. 2008, Huertas and Jackson 2009). The interaction between CtIP and Mre11 then promotes the recruitment of RPA and ATR kinase (Sartori, Lukas et al. 2007), but their nucleolytic activity to generate 3' ssDNA overhangs is also stimulated by ATM (Geuting, Reul et al. 2013, Yamane, Robbiani et al. 2013, Bakr, Oing et al. 2015). After the processing of the DNA ends, extensive resection takes place to promote HR. This step requires the activity of BLM, DNA2 helicase/nuclease, EXO1, the MRN complex and RPA (Nimonkar, Genschel et al. 2011). BLM is first recruited by the MRN complex and it interacts with DNA2 in order to participate in resection through their helicase and nuclease activity, respectively (Nimonkar, Genschel et al. 2011, Sturzenegger, Burdova et al. 2014). Another helicase that interacts with DNA2 to promote resection is the WRN(Pinto, Kasaciunaite et al. 2016). Moreover, BLM, MRN and RPA also stimulate the activity of EXO1 to resect DNA ends (Nimonkar et al. 2011). EXD2 is an exonuclease that by interacting with CtIP cooperates with Mre11 during resection (Broderick 2016). BRCA1 also facilitates end resection, through its E3 ubiquitin ligase activity and its tandem C-terminal BRCT domains (Huen, Sy et al. 2010) and as a complex with BRCA1-associated RING domain protein 1 (BARD1) interacts with CtIP and MRN. Last but not least, the ssDNA ends that are produced by resection are protected by the RPA heterotrimer.

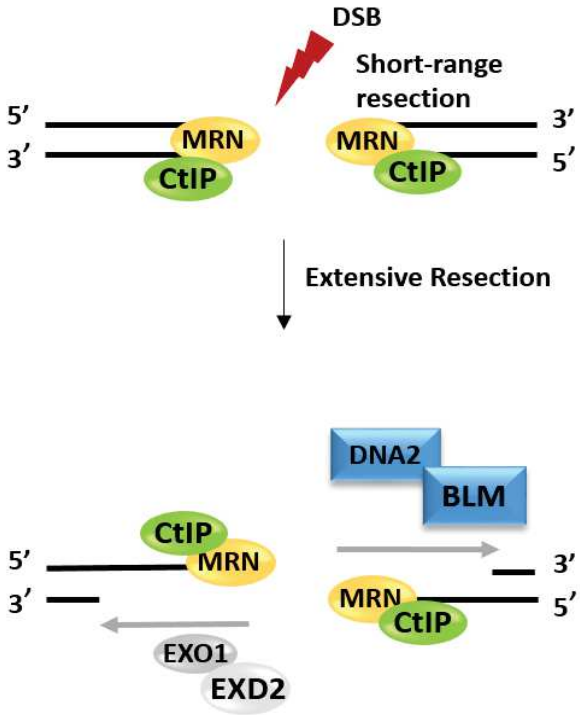

Figure 5: Short-range and extensive resection. At the short-range resection MRN complex and CtIP are needed for the first processing of the two broken DNA ends. After this limited resection, the DNA ends are extensively processed by EXO1, EXD2 and DNA2 nucleases. DNA2 interacts with BLM to promote resection.

Rad51 assembly and Strand invasion

Rad51 is the major player in the second step of HR, strand invasion. The bacterial homologue of Rad51 is the RecA ATPase. Rad51 is involved in HR in both normal cell processes such as meiosis, and after undesirable DNA damage (Shinohara, Ogawa et al. 1992, Chen, Yang et al. 2008). RPA binding at the resected ends prevents the assembly of Rad51 nucleoprotein filaments (San Filippo, Sung et al. 2008). This is totally reversed when BRCA2 directly binds Rad51, enabling its polymerization and filament formation before it finally invades in the homologous sequence template. The BRCA2-Rad51 interaction is promoted by the deubiquitinase UCHL3 (Luo, Li et al. 2016). BRCA2 loading at the sites of breaks is depending on BRCA1-PALB2 interaction (Huen et al. 2010). The proteins responsible for PALB2 loading are MDC1, RNF9, RAP80 and Abraxas (Zhang, Bick et al. 2012). Another player of HR that interacts with PALB2 is RF168 (Luijsterburg, Typas et al. 2017). The whole BRCA1-PALB2-BRCA2 complex recruitment is also dependent on CDK9 (Nepomuceno, Fernandes et al. 2017). Moreover, PALB2 is deubiquitinated specifically in G2 by USP11 and this allows its interaction with the other components of the complex (BRCA2 and BRCA1) in order to load Rad51 (Orthwein, Noordermeer et al. 2015). The stabilization of the D-loop before the transition to the DNA synthesis is promoted by RAD54 recruitment that dissociates Rad51 from the heteroduplex DNA (Heyer, Li et al. 2006). Finally, the D-loop is formed and one of the different HR subpathways can take place (Heyer, Ehmsen et al. 2010), with all of them requiring a step of DNA synthesis (Figure 6).

1.5.2 Non Homologous End Joining (NHEJ)

HR was the first DSB repair pathway that was described and it was initially thought to be unique. However, in 1982 NHEJ was identified, efficiently joining unrelated DNA fragments(Wilson, Berget et al. 1982). This pathway is evolutionary conserved from prokaryotes to higher eukaryotes (Shuman and Glickman 2007, Deriano and Roth 2013) (Figure 6). In contrast to HR, this pathway does not require a homologous sequence as a template, even though microchomologies of one to six complementary bases can appear at the junctions to help align the broken ends (Roth and Wilson 1986). Since the sister chromatid is not necessary,

NHEJ is active throughout the cell cycle, and particularly in G1 (Rothkamm, Krüger et al. 2003). The four main steps of this pathway are: break recognition, DNAPKcs recruitment, end processing and end joining.

Break recognition

When the DNAbreak is recognized by the Ku70-Ku80 heterodimer a protein ring is created around the broken DNA and NHEJ pathway is promoted (Walker, Corpina et al. 2001). Ku functions to keep the two broken ends together, thus limiting DSB mobility, as highlighted by the fact its depletion can increase the translocation frequency since the broken ends are separated and more mobile in the nucleus (Soutoglou, Dorn et al. 2007, Roukos, Voss et al. 2013). Moreover, Ku binding at the DNA allows the recruitment of its interaction partners, including ligase IV- XRCC4 and DNAPKcs.

DNAPKcs recruitment

After formation of the Ku 70-K80 complex at the break, DNAPKcs is recruited at the sites of damage and induces an inward translocation of this dimer by one helical turn (Dynan and Yoo 1998, Yoo and Dynan 1999). This interaction allows the stimulation of the DNAPKcs kinase activity (Singleton, Torres-Arzayus et al. 1999) and it is able to phosphorylate several NHEJ factors such as, Ku70-Ku80(Chan, Ye et al. 1999), Artemis (Ma, Pannicke et al. 2005, Goodarzi, Yu et al. 2006), XRCC4 (Leber, Wise et al. 1998), XLF (Yu, Mahaney et al. 2008) and DNA ligase IV (Wang, Nnakwe et al. 2004). However, none of these phosphorylation events are required for successful NHEJ (Kaneko, Cuyun-Lira et al. 2003, Wang, Nnakwe et al. 2004, Goodarzi, Yu et al. 2006, Yu, Mahaney et al. 2008), likely due to the functional redundancy between the different phosphorylation sites. DNAPKcs is also autophosphorylated (Cui, Yu et al. 2005, Neal, Dang et al. 2011, Neal and Meek 2011), leading to structural changes that allow end-processing enzymes and ligases to access the broken ends and finalize the NHEJ process (Neal et al. 2011).

End processing

DNA ends with complex modifications after damage induction or hairpin structure formation require further processing than the ones that possess a 5’ phosphatte and a 3’ hydroxyl group. DNAPKcs interacts with Artemis nuclease, which is responsible for this processing through its endonuclease activity (Ma, Pannicke et al. 2002). Other factors that are implicated in this

process are Ku80 (Roberts, Strande et al. 2010) and poly-nucleotide kinase/phosphatase (PNKP), which can create correct chemical groups at the broken ends in order for them to ligate (Chappell, Hanakahi et al. 2002). Moreover, factors such as XLF, through interaction with polymerases λ and μ (Akopiants, Zhou et al. 2009), and WRN, through its interaction with Ku and XRCC4 (Bohr, Cooper et al. 2000, Perry, Yannone et al. 2006, Kusumoto, Dawut et al. 2008), also participate in end processing.

End joining

After the recognition and processing of the broken DNA ends, the XRCC4/DNA Ligase IV/XLF complex is responsible for the last step of NHEJ, the ligation of the ends. Binding of XRCC4 allows its interaction with DNA ligase IV and stimulates its activity (Grawunder, Wilm et al. 1997, Wilson, Grawunder et al. 1997, Grawunder, Zimmer et al. 1998). XLF is structurally similar to XRCC4 and it stimulates the ligase activity of the complex (Andres, Modesti et al. 2007, Riballo, Woodbine et al. 2009). PAXX was recently identified as an additional NHEJ factor that belongs to the XRCC3 family (Ochi, Blackford et al. 2015). PAXX interacts with Ku70 to stimulate the activity of XRCC4/DNA ligase IV complex, but only under XLF depletion (Tadi, Tellier-Lebègue et al. 2016, Liu, Shao et al. 2017).

The depletion of each one of these factors has severe outcomes that underline their importance in maintaining genomic stability. For example, XRCC4 or DNA ligase IV depletion in Ku80-/- mice lead to embryonic lethality (Frank, Sekiguchi et al. 1998, Gao, Ferguson et al. 2000). Moreover, depletion of DNA ligase IV causes extensive apoptotic cell death in the embryonic central nervous system, thus leading to lethality (Frank et al. 1998). Massive neuronal apoptosis is also observed after XRCC4 depletion (Gao et al. 200). Finally, even though XLF depletion does not have any phenotype (Liu et al. 2017), its combined depletion with PAXX affects V(D)J recombination (Lescale, Lenden Hasse et al. 2016). These phenotypes reveal the major role of this pathway in the proper development of organisms and in the immune system response.

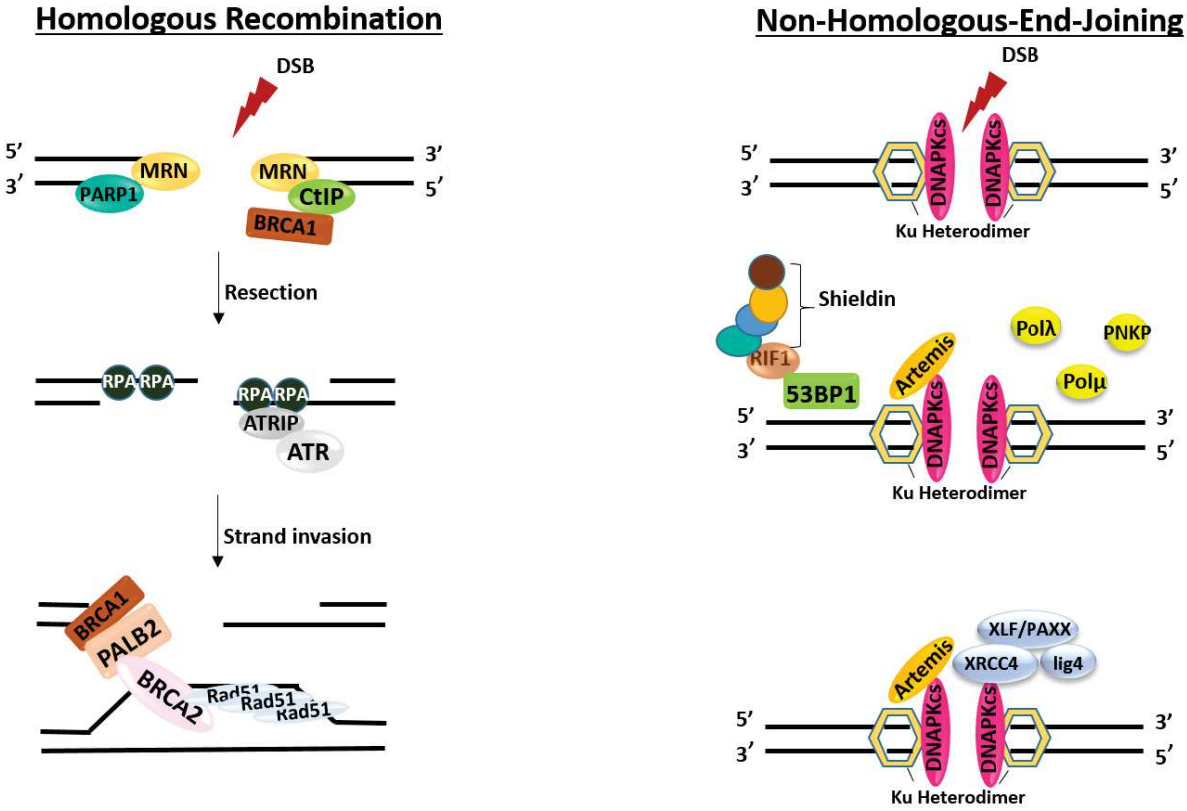

Figure 6 The two main DSB repair pathways: Homologous Recombination and Non-Homologous-End Joining In HR break recognition is mediated by PARP1 and the MRN complex. Then CtIP is recruited to initiate resection. Resected ends are protected by RPA and the ATR kinase is recruited through ATRIP. FInaly BRCA1-PALB2-BRCA1 complex mediates the strand invasion step and allows Rad51 loading. In NHEJ break recognition is mediated by Ku heterodimer and DNAPKcs is subsequently recruited. The DNA ends are processed by different factors including Artemis , PNKP and different polymerases. Finally, the ligation of the two broken ends is mediated by XRCC4-Lig4-XLF/PAXX.

1.5.3 Alternative end Joining (alt-EJ)

Alt-EJ is used as a backup mechanism by the cells when canonical NHEJ is impaired (Figure 7). The activation of this pathway requires the recognition of the broken ends by PARP1 (Audebert et al. 2004, Wang et al. 2006). Next, there is an initial resection step that depends on the nuclease activity of Mre11 (Ma, Kim et al. 2003, Deriano, Stracker et al. 2009, Rass,

Grabarz et al. 2009, Xie, Kwok et al. 2009, Truong, Li et al. 2013), and in the resection activity of CtIP (Boboila, Jankovic et al. 2010, Lee-Theilen, Matthews et al. 2011, Zhang and Jasin 2011). In contrast, BLM and Exo1, promoters of long-range resection, suppress alt-EJ, because extended resection favors HR, and in alt-EJ only microhomology is needed (Truong, Li et al. 2013, Deng, Gibb et al. 2014). After the end processing, Polθ catalyzes overhang extension in both Drosophila and mammalian cells (Chan, Yu et al. 2010, Yu and McVey 2010). Polθ is recruited by PARP1 to promote alt-EJ (Mateos-Gomez, Gong et al. 2015) by inhibiting the Rad51 mediated recombination (Ceccaldi, Liu et al. 2015) and promoting DNA synapse formation and strand annealing (Kent, Chandramouly et al. 2015). Finally, the broken ends are ligated by the XRCC1-Ligase III complex (Simsek, Brunet et al. 2011, Boboila, Oksenych et al. 2012, Dutta, Eckelmann et al. 2017). In contrast to the canonical NHEJ, which protects genome integrity, alt-EJ has been suggested to be the main mechanism responsible for the formation of translocations in mouse cells (Simsek et al., 2011; Zhang and Jasin, 2011).

1.5.4 Single Strand Annealing (SSA)

An additional pathway employed by cells to repair resected DSBs is SSA (Figure 7). In contrast to HR, SSA doesn't require a donor sequence as a template for the repair, and for this reason doesn't involve a strand invasion step nor Rad51 activity (Sung 1997). It is activated mainly in repeated sequences, where it uses resection mediated by CtIP and RPA to reveal and then anneal exposed complementary ends (Bhargava, Onyango et al. 2016). One of the major SSA factors responsible for the annealing of the resected complementary ends is Rad52 DNA binding protein (Symington 2002, Sugawara, Wang et al. 2003). Before the final ligation step, the 3' ssDNA ends that are not homologous are removed by the ERCC1/XLF complex (Motycka, Bessho et al. 2004). SSA is a mutagenic pathway and results in deletion rearrangements between homologous repeats, corresponding to a loss of genetic information.

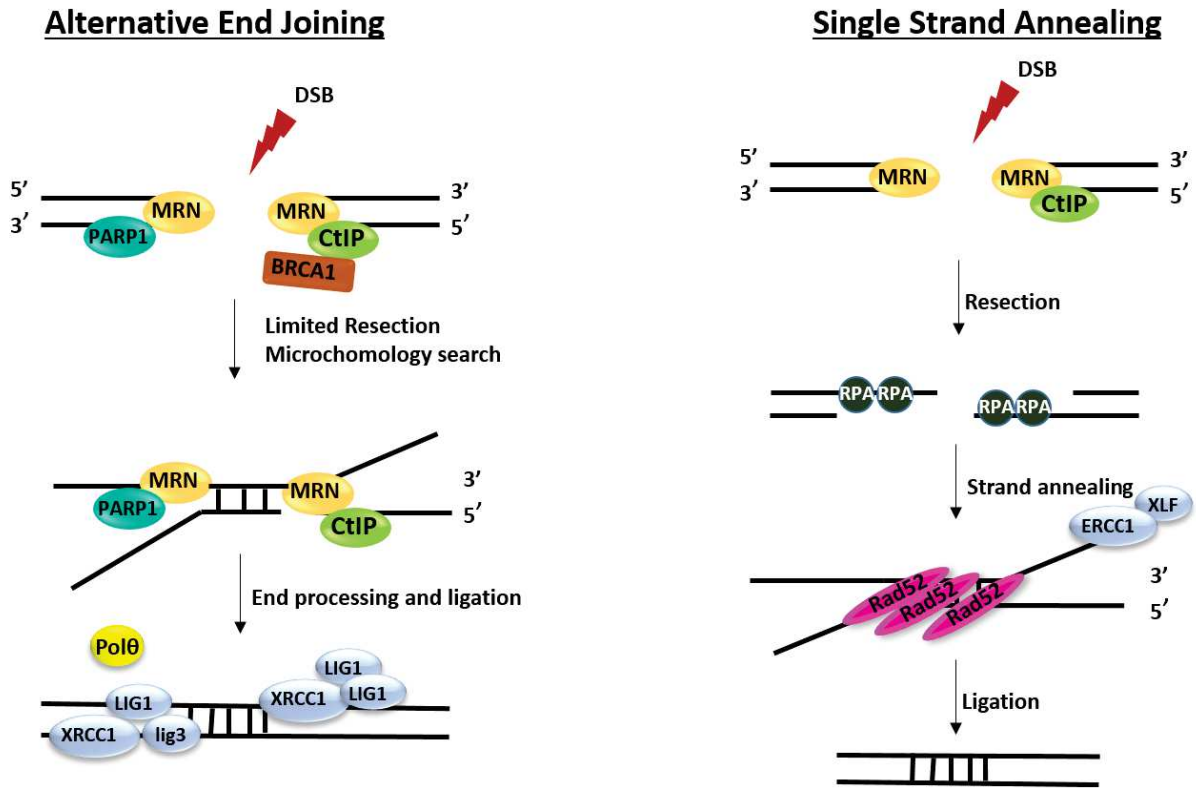

Figure 7 The two alternative pathways for DSB repair : Alternative End Joining and Single Strand Annealing In alt-EJ the break recognition is mediated by PARP1 and the MRN complex. CtIP is then recruited in order to initiate a limited resection that facilitates the microhomology search. Polθ is filling of gaps before the ligation that is mediated by XRCC1-Lig3 complex. In SSA the MRN complex and CtIP are involved in the break recognition. Resection is initiated and the resected ends are bound by RPA. Finally the homologous ssDNA segments are annealed through Rad52 and ligated by the ERCC1-XLF complex

1.6 Regulation of pathway choice

As described above, DSBs can be repaired by four main pathways, NHEJ and HR, the major pathways, and the two alternative pathways alt-EJ and SSA. Alt-EJ is used by the cells when NHEJ is impaired whereas SSA is more specific for DSB repair in repetitive DNA sequences. The choice among these repair pathways is regulated by the cell cycle, the competition of different factors to specifically promote a pathway as well as the chromatin structure.

1.6.1 Role of end resection in DNA repair pathway choice

End resection is a very important step in the choice of repair pathway since it is involved in three different pathways, HR, alt-EJ and SSA. The major difference is the extent of resection that is required for each pathway activation, with Alt-EJ requiring end processing of a small number of base pairs, while HR and SSA need long extensive resections (Ceccaldi, Rondinelli et al. 2016). Different factors have been shown to compete in order to regulate resection and thus DNA repair pathway choice in a cell cycle dependent manner (Figure 8).

The first competition event is that of Ku heterodimer with the MRN complex in the break recognition step. It has been shown in yeast, that Mre11 and Sae2 (the CtIP homologue) mediated resection, is able to reduce the ability of Ku to bind at the broken ends (Zhang, Hefferin et al. 2007). Moreover, end resection and Mre11 recruitment is increased after NHEJ factor depletion (Zhang, Hefferin et al. 2007, Clerici, Mantiero et al. 2008, Zierhut and Diffley 2008). Supporting this notion, Ku-deficient cells are able to start resection in G1 phase of the cell cycle (Clerici et al. 2008, Zierhut and Diffley 2008).

The second competition event is between 53BP1 and BRCA1, which is one of the most studied regulators of resection (Panier and Boulton 2014) (Figure 8). These two factors have been shown by different research groups to be mutually exclusive at sites of DNA damage (Chapman et al. 2012, (Ochs, Karemore et al. 2019). Several studies have reported their roles in balancing pathway regulation, in different phases of the cell cycle. First, 53BP1 was shown to negatively regulate resection in G1 (Bothmer, Robbiani et al. 2010). BRCA1, on the other hand promotes 53BP1 removal in S-phase in order to allow resection (Bunting, Callén et al. 2010). 53BP1 is phosphorylated by ATM in order to interact with other effector proteins (Bothmer, Robbiani et al. 2011), such as RIF, another NHEJ promoting factor that is removed from DSBs in S/G2 in a BRCA1 and CtIP dependent manner (Silverman, Takai et al. 2004, Chapman, Barral et al. 2013, Di Virgilio, Callen et al. 2013, Escribano-Díaz, Orthwein et al. 2013, Feng, Fong et al. 2013). This RIF1 release is achieved through the BRCA1 dependent dephosphorylation of 53BP1 in G2, in order to allow the progression of resection (Isono, Niimi et al. 2017). Another effector protein that recognizes ATM-phosphorylated 53BP1 is PTIP, and its depletion has been shown to partly restore resection in BRCA1 -/- cells (Jowsey, Doherty et al. 2004, Munoz,

Jowsey et al. 2007, Gong, Cho et al. 2009, Yan, Shao et al. 2011, Callen, Di Virgilio et al. 2013).

Several studies have tried to investigate in detail the mechanism and identify additional effectors of 53BP1 that participate in the resection inhibition and promote NHEJ (Figure 8). In 2015, two studies identified the Rev7, or MAD2L2 in mammals, as a downstream factor of 53BP1 and RIF1 (Boersma, Moatti et al. 2015, Xu, Chapman et al. 2015). Rev7 was known to function in translesion DNA synthesis (TLS) as a part of DNA pol ζ (Rosenberg and Corbett 2015), and since it is a very small molecule it was already hypothesized to act together with other proteins in this process. Using CRISPR/Cas9 screenings and proteomics approaches, there were three factors identified to interact with REV7; C20orf196, FAM35A and CTC-534A2 (Dev, Chiang et al. 2018, Gao, Feng et al. 2018, Gupta, Somyajit et al. 2018, Noordermeer, Adam et al. 2018). The whole complex was named SHLD (Shieldin) and the proteins SHLD1, SHLD2 and SHLD3 respectively. SHLD1/2/3 depletion in BRCA1-deficient cells suppresses their sensitivity to PARP inhibitors (Dev, Chiang et al. 2018, Ghezraoui, Oliveira et al. 2018, Gupta, Somyajit et al. 2018, Mirman, Lottersberger et al. 2018, Noordermeer, Adam et al. 2018). Moreover, SHLD1 or 2 knockout enabled mouse embryonic stem cells to survive BRCA1 loss (Noordermeer, Adam et al. 2018). Shieldin's role in promoting NHEJ was also confirmed by the EJ5-GFP reporter assay. The fact that it directly antagonizes resection is confirmed by the fact that its depletion increases the levels of pRPA and RPA foci formation after DSB induction (Dev, Chiang et al. 2018, Findlay, Heath et al. 2018, Gao, Feng et al. 2018, Gupta, Somyajit et al. 2018, Noordermeer, Adam et al. 2018, Tomida, Takata et al. 2018). Camptothecin treatment in Shieldin knockout cells also increased the amount of ssDNA, which is a characteristic extensive DNA end resection (Ghezraoui, Oliveira et al. 2018, Gupta, Somyajit et al. 2018). Recently, CST was identified as a complex that antagonizes end resection, and its recruitment to DSBs was 53BP1 and Shieldin dependent (Mirman et al. 2018). CST is known to interact with the Pola-primase complex to synthesize DNA during telomere replication (Wu, Takai et al. 2012). So possibly its role in this axis is to perfoma a fill-in resection, or to protect the 5' overhangs from the EXO1 and block the access to the resection helicases (Mirman and de Lange 2020).

In addition to the above factors that can affect DNA-end resection, cell cycle stage is important for regulating of the pathways. 53BP1 is correlated with NHEJ in G1 and BRCA1 with resection in S/G2 and those factors are mutually exclusive. CtIP also has a role in this process, as it is phosphorylated by CDK1 in S phase to promote efficient end-resection (Huertas et al. 2008. Huertas and Jackson 2009). Moreover, it interacts with BRCA1 in S/G2 and together they form the CtIP-MRN-BRCA1 complex to allow resection (Greenberg, Sobhian et al. 2006, Chen, Nievera et al. 2008). This complex was also shown to trigger the removal of RIF1 (Escribano-Diaz et al. 2013). The BRCA1 binding specifically in S/G2 phase is also mediated by phosphorylation of its cofactor BACH1 (Dohrn, Salles et al. 2012) and by the BRCA1 A complex that is not enriched in G1 (Hu, Scully et al. 2011). Resection in G2 is also promoted by phosphorylation of DNA1 in yeast and EXO1 in mammalian cells (Chen, Niu et al. 2011, Tomimatsu, Mukherjee et al. 2014). However, in G1 CDK activity is reduced and the impairment of the CtIP and EXO1 phosphorylations decreases end resection and favors NHEJ(Symington and Gautier 2011).

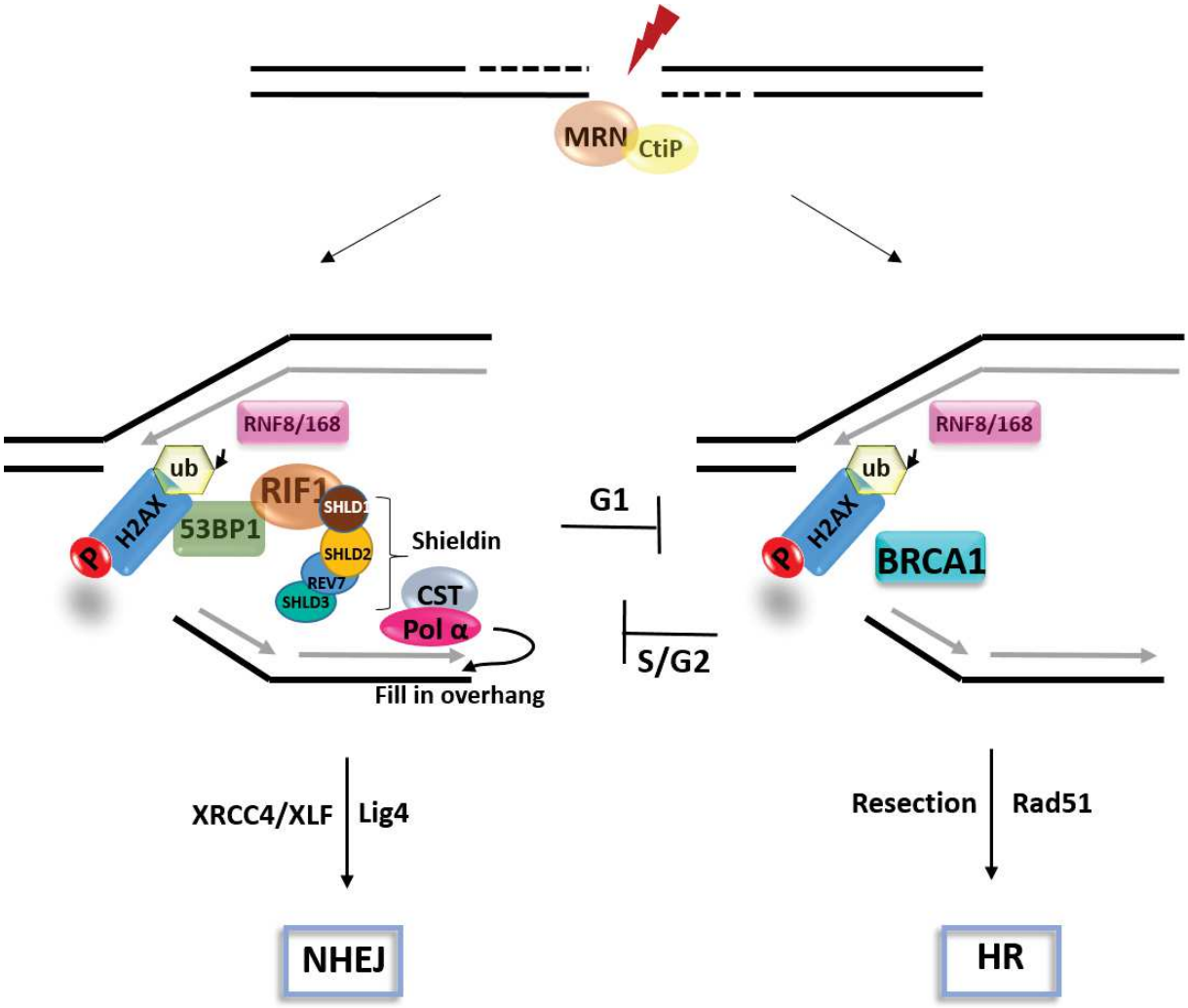

Figure 8: 53BP1 and BRCA1 compete to inhibit and promote resection, respectively 53BP1 is recruited at the sites of Damage by recognizing RNF8/168 ubiquitinated H2A histones and other marks (not displayed in the figure). Then RIF1 is recruited and recruits the Shiledin complex that promotes the inhibition of resection. Finally CST and Polα are recruited to fill in the overhangs and allow NHEJ completion by XRCC4/XLF/lig4. In S/G2 BRCA1 works in competition with 53BP1 to promote resection and HR.

These important phosphorylation events that correlate with cell cycle stage are not the only events that can affect the pathway choice. The interaction of BRCA1 with PALB2-BRCA2 is regulated by the presence of USP11, a de-ubiquitinase that in S/G2 is stabilized and deubiquitinates PALB2 in order to interact with BRCA1, but in G2 it gets degraded thus inhibiting this interaction and the progression of HR (Orthwein et al. 2015).

1.6.2 Role of Rad51 in homology based repaired pathway choice

As already discussed, once resection is accomplished, NHEJ is completely inhibited and one of the three other pathways (Alt-EJ, SSA or HR) can be activated to repair DSBs. Alt-EJ is suppressed after the binding of RPA to resected ends to protect them from degradation (Deng 2014). Rad51 loading is also blocked and this is alleviated by BRCA2 in mammalian cells and Rad52 in yeast, in order for strand-invasion to be promoted in HR (Moynahan, Pierce et al. 2001, Symington 2002, Sugawara, Wang et al. 2003, Esashi, Galkin et al. 2007, Carreira and Kowalczykowski 2011).

Several factors that negatively regulate Rad51 loading are shown to favor alt-EJ and SSA. The yeast helicase Srs2 and the PARI mammalian helicase remove Rad51 nucleofilaments through an ATP-driven process (Krejci, Van Komen et al. 2003, Chiolo, Carotenuto et al. 2005). Depletion of Srs2 led to HR increase and reduced alt-EJ and SSA, thus balancing the regulation of the pathway choice between HR and alt-EJ/SSA (Chiolo et al. 2005, Krejci et al. 2003). Rad51 filaments are also disrupted by the RECQL4 mammalian helicase which in turn promotes SDSA (Islam, Paquet et al. 2012). Two other helicases that disassemble Rad51 filaments are FANCJ and FBH1 (Sommers, Rawtani et al. 2009, Simandlova, Zagelbaum et al. 2013). Finally, one of the major factors promoting alt-EJ by blocking Rad51 loading is Polθ (Mateos-Gomez et al. 2015). Alt-EJ is also facilitated through PARP1 since it implements the displacement of Ku70/Ku80 or RPA (Sallmyr and Tomkinson 2018).

A major competitor of Rad51 in mammalian cells is Rad52, the main factor that promotes SSA (Bennardo et al. 2008). In heterochromatic DSBs, depletion of Rad52 increased the Rad51 localization at the periphery of the domain, where it gets recruited to activate HR (Tsouroula et al. 2016). The SSA pathway is also promoted when the amount of 53BP1 is limited, thus leading to hyper-resection. Hyper resected breaks are unable to load Rad51, which is completely replaced by Rad52 (Ochs, Somyajit et al. 2016). Depletion of Rad51 does not affect cell growth in mammalian cells, but it becomes synthetically lethal with deficiency in BRCA1, PALB2 and BRCA2, all HR factors, suggesting that SSA is activated as a backup pathway when HR is not available (Lok, Carley et al. 2013).

1.7 Double Strand Break repair in the context of highly-structured chromatin

Chromatin environment is very important for the regulation of DDR and repair, and also affects the DNA repair pathway choice. Upon damage induction, chromatin structure is changed, by post–translational modifications, its histone composition and the presence of different chromatin remodelers. A "Prime, Repair, Restore" model suggests that damaged chromatin first needs to become more accessible to enable DNA repair, and after this process is finished it returns at its initial state (Soria, Polo et al. 2012).

1.7.1 Hierarchical organization of chromatin

DNA is packaged inside the cells through different hierarchical folding steps, via the inclusion of multiple chromatin proteins (Bickmore and van Steensel 2013, Bonev and Cavalli 2016) (Figure 9). Nucleosomes are composed of 147 base pairs of DNA wrapped around a histone octamer, which contains a variable combination of the four core histones (H3, H4, H2A and H2B). This represent the first step of the chromatin folding process (Luger, Mäder et al. 1997). The connection of nucleosomes is achieved through linker DNAs, which are small DNA segments bound by the H1 linker family of histones (Hergeth and Schneider 2015). The primary structure of chromatin is a linear arrangement of nucleosomes forming 10 nm fibers, which are further folded in a secondary chromatin structure of 30 nm fibers (Luger, Dechassa et al. 2012). The next step of chromatin folding results from the interaction of *cis* regulatory elements leading to the formation of chromatin loops (Lieberman-Aiden, van Berkum et al. 2009, Rao, Huntley et al. 2014). Chromatin loops have been shown to be parts of Topologically Associated Domains (TADs), megabase-scale domains of chromosomes, in which regions in the same TAD interact (are spatially close) with each other in higher frequencies than with regions located in different TADs (Dixon, Selvaraj et al. 2012, Nora, Lajoie et al. 2012, Sexton, Yaffe et al. 2012). Finally, chromosome territories, correspond to specific regions of the nucleus that are occupied by each chromosome (Lichter, Cremer et al. 1988, Pinkel, Landegent et al. 1988).

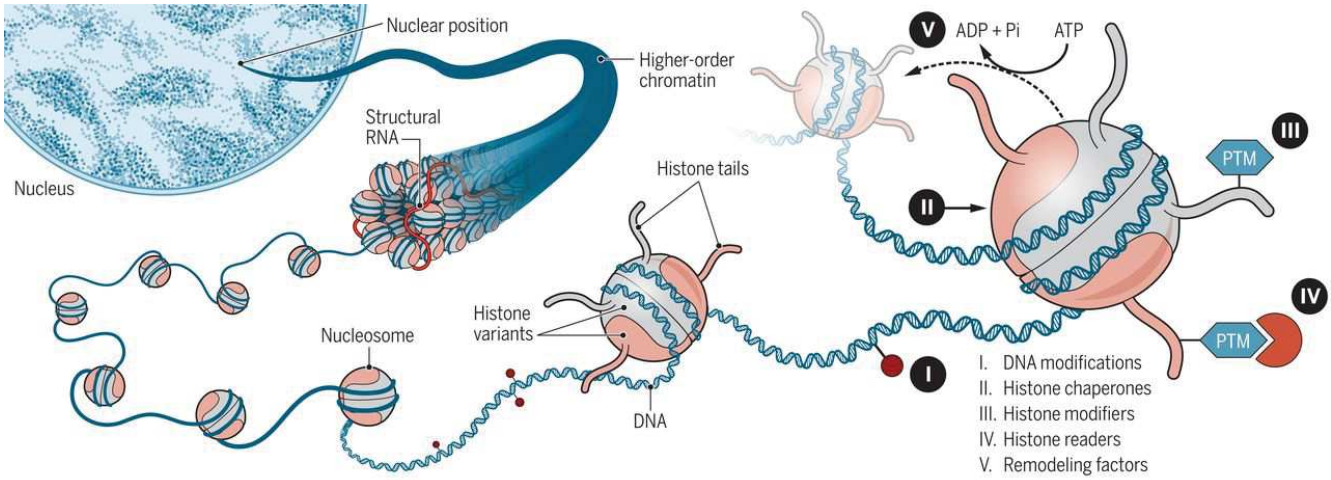

Figure 9: Chromatin plasticity Adapted from Tejas Yadav et al. Science 2018;361:1332-1336.

1.7.2 Global chromatin environments: euchromatin and heterochromatin - Compaction and chromatin marks

Chromatin can be divided in two distinct chromatin environments, euchromatin and heterochromatin, depending on the composition of histone modifications, histone variants, chromatin remodelers, their interacting proteins and their biophysical properties (Fraser and Bickmore 2007, Van Bortle and Corces 2012). Euchromatin represents a more open chromatin structure that is associated with active transcription. In contrast, heterochromatin is highly condensed, gene poor and poorly transcribed (Fraser and Bickmore 2007). Since in euchromatin transcription is active, its specific modifications are related to this process, and include H3K4me1 that is enriched at active enhancers, H3K4me3 that marks the transcriptional start site (TSS) of active genes and H3K36me3 that is enriched in the whole transcribed region (Barski, Cuddapah et al. 2007). Acetylated histones are also a characteristic of euchromatin as they promote its relaxation.

Heterochromatin, on the other hand, is a compact structure with hypoacetylated histones, and is further distinguished into facultative and constitutive heterochromatin. Facultative heterochromatin is characterized by a plasticity, since it represents euchromatic gene-rich domains that have the ability to be heterochromatinized when their expression is not needed,

either in different developmental stages or during differentiation process (Trojer and Reinberg 2007). The main histone modification of facultative heterochromatin is H2K27me3, which is catalyzed by Polycomb Croup (PcG) proteins (Trojer and Reinberg 2007). Conversely, constitutive heterochromatin, which composes 25%-90% of the multicellular eukaryotic genomes (Lander, Linton et al. 2001) (Vicient and Casacuberta 2017), is characterized by highly repetitive sequences that are mostly found in the pericentromeric and telomeric regions of chromosomes (Figure 10). It is further marked by the H3K9me3 histone modification and its binding protein HP1 (James and Elgin 1986, Lachner, O'Carroll et al. 2001, Maison and Almouzni 2004, Bannister and Kouzarides 2011). Mammalian cells have three HP1 isoforms, HP1α, HP1β and HP1γ, while *D. melanogaster* has only HP1α and *S. pombe* has its homologue Swi6 (Maison and Almouzni 2004). Several proteins have been shown to participate in the maintenance of the compacted structure of heterochromatin. For example the methyl transferases Suv3-9 and Suv4-20, which catalyze H3K9me3 and H4K20me2/3 respectively, KAP1, SETDB1 (histone methyltransferase), HDAC1 and HDAC2 (histone deacetylases) and CHD3Mi-2a (CHD nucleosome remodeling factor) (Maison and Almouzni 2014). Depending on the organism, heterochromatin can appear in different locations and form different structures in the nucleus. In human cells, constitutive heterochromatin can be found in perinucleolar regions of the nucleus, in pericentromeric bodies and at the nuclear periphery (Horsley, Hutchings et al. 1996, Minc, Allory et al. 1999). In contrast, in mouse and *D. melanogaster* the heterochromatic domains cluster and aggregate to form large chromocenters that can be visualized as DAPI (a DNA dye) dense regions by microscopy (Mayer, Brero et al. 2005). The pattern of heterochromatin can have different roles, depending on the cell or tissue type, for example in mouse rod photoreceptor nuclei, where the central chromocenter combined with peripheral euchromatin facilitates nocturnal vision (Solovei, Kreysing et al. 2009), or in stem cells were the more dispersed heterochromatin architecture is thought to help the maintenance of epigenetic plasticity (Sridharan, Gonzales-Cope et al. 2013).

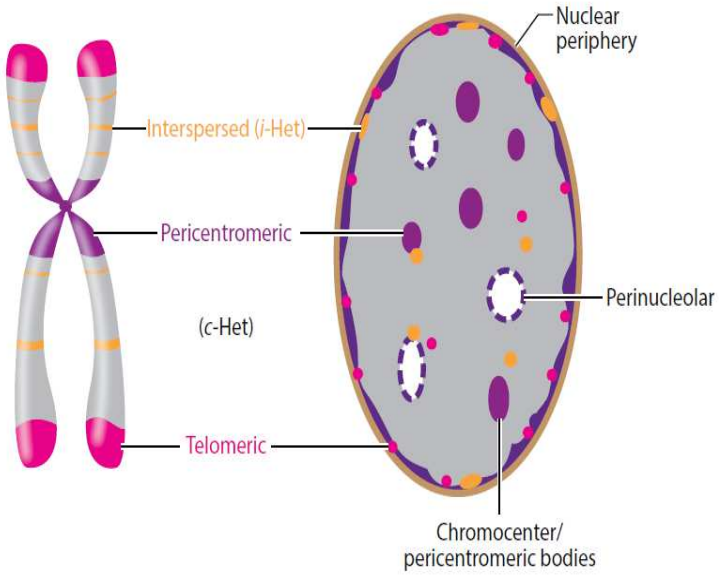

Figure 10: Localization of heterochromatin Adapted from Janssen and Karpen 2018
Repetitive sequences are enriched at pericentromeric and telomeric regions of chromosomes to form constitutive heterochromatin (c-Het). The localization patterns can differ across cell types and are found at perinucleolar domains, as pericentromereric bodies, as chromocenters and at the nuclear periphery.

1.7.3 Enrichment of heterochromatin in repeated DNA sequences

As mentioned above, heterochromatin consists of repetitive sequences that are marked with H3K9me3, a modification catalyzed mainly by SUV3-9 methyltransferases. These repeats are in a form of satellite DNA (short tandem repeats), transposons or ribosomal DNA (rDNA) (Zeller, Padeken et al. 2016). The DNA sequence varies greatly between the different types of repeats, especially in different organisms. In mouse cells, centromeres are composed of tandem minor satellite repeats and the pericentromeres contain major satellite repeats, which are identical in all chromosomes and cluster to form chromocenters. In human cells though, the centromeres are composed of tandem repeats of 177bp α-satellites, but the pericentromeric repeats contain different satellite subfamilies (sat II, sat III, sn5 and β-γ satellite) (Eymery, Callanan et al. 2009). The human pericentromeric repeats differ from the mouse ones since they are not identical in all chromosomes, for example sat III repeats are mainly located on chromosome 9q12 locus whereas sat II repeats are predominantly on chromosome 1 (reviewed in Eymery et al. 2009). Moreover, human telomeres contain tandem TTAGGG repeats to protect the chromosome ends. Heterochromatin is also recruited at the highly transcribed rDNA repeats of the five human acrocentric chromosomes (13, 14, 15, 21 and 22) to transcriptionally

repress almost half of the 300-400 rDNA repeats, when necessary (McStay and Grummt 2008). Finally, transposons, coding DNA sequences related to viruses, are found in heterochromatin, fragmented and predominantly inactive. Human euchromatin also contains repeats that are transposon-related elements and are called LINEs (Lander, Linton et al. 2001).

1.7.4 Establishment and Maintenance of heterochromatin

In yeast and plants it has been proposed that heterochromatin formation depends on non-coding RNA mechanisms, and involves the RNAi silencing components, which recruit SUV39 methyltransferases at the repetitive DNA (reviewed in (Allshire and Madhani 2018)). It is currently unclear if this mechanism is conserved in mammalian cells, even though mouse heterochromatin is sensitive to RNase A treatment (Probst, Okamoto et al. 2010) and SUV39H1/H2 recruitment to pericentromeric repeats is proposed to happen through RNA-dependent mechanisms (Johnson, Yewdell et al. 2017, Shirai, Kawaguchi et al. 2017). Heterochromatin formation in mouse also depends on the binding of other factors, such as Pax3 and its related transcription factors (Bulut-Karslioglu, De La Rosa-Velazquez et al. 2014). Recently though, several research groups proposed that phase separation drives the heterochromatin domain formation (Hinde, Cardarelli et al. 2015, Larson, Elnatan et al. 2017, Strom, Emelyanov et al. 2017). Briefly, heterochromatin and other compartments form membrane-less organelles in the nucleus, which exhibit Liquid-Liquid Phase Separation properties (LLPS). In my review (Yilmaz*, Mitrentsi*, *in preparation*), I discuss in detail the recent findings in the field concerning the physical properties of each compartment (pericentromeric heterochromatin, telomeres, nucleolus) that renders them the properties of liquid droplets.

After its establishment heterochromatin has to be maintained. The major mechanism for the maintenance of heterochromatin is the binding of HP1 to the SUV39 methyltransferases and histone deacetylases (Aagaard, Laible et al. 1999, Motamedi, Hong et al. 2008, Fischer, Cui et al. 2009). Repetitive cycles of deacetylation, methylation, HP1 binding, and finally recruitment of SUV39 ensures the spreading of chromatin. Some cellular processes, including mitosis and DNA replication, disrupt heterochromatin maintenance and its reestablishment is crucial.

During mitotic entry, H3S10 is phosphorylated by Aurora B and HP1 binding to H3K9me3 is blocked (Hirota, Lipp et al. 2005), and it only reassembles in anaphase/telophase (Wurzenberger et al. 2011). Thus, the continuous assembly and disassembly of HP1 during mitotic cell divisions ensures the heterochromatin maintenance. The replication machinery might also disrupt the heterochromatin structure at its passage, but the retention of parental H3K9me3 histones is random on daughter strands behind the replication fork, and CAF-1 complex allows the recruitment of Hp1 for the nucleosome reassembly (Quivy, Roche et al. 2004, Quivy, Gerard et al. 2008).

1.7.5 Heterochromatin and DNA repair

Maintaining the integrity of heterochromatin is highly important as it has been shown that dysfunctional heterochromatin is involved in cancer progression. For example, cancer progression and metastasis are associated with changes in HP1 levels and the distribution of H3K9me2/me3 (De Koning 2009, Dialynas 2008, Feinberg 2016, Rondinelli 2015m Slee 2012, Vad-Nielsen 2016). Changes in heterochromatin can increase susceptibility to DNA damage and thus lead to genomic instability. In the following review (Yilmaz*, Mitrentsi* et al , *In preparation*) we discuss the recent findings on how the genome is maintained in repetitive regions (centromeres, pericentromeres, telomeres, rDNA), as well as the role of phase separation in the formation of these compartments.

How to maintain the integrity of the repetitive genome *In preparation*

Duygu Yilmaz[1,2,3,4,*], Ioanna Mitrentsi [1,2,3,4,*] and Evi Soutoglou [1,2,3,4,5,#]

1. Institut de Génétique et de Biologie Moléculaire et Celullaire, 67404 Illkirch, France
2. Institut National de la Santé et de la Recherche Médicale, U964, 67404 Illkirch, France
3. Centre National de Recherche Scientifique, UMR7104, 67404 Illkirch, France
4. Université de Strasbourg, 67081 Strasbourg, France
5. Current address: Genome Damage and Stability center, Sussex University, School of Life Sciences, University of Sussex, Brighton, BN1 9RH

[*] Equal contribution

[#]Corresponding author E.Soutoglou@sussex.ac.uk

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, hairpin 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., 2016). 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., 2018) and centromeric recruitment of BLM during anaphase to allow proper segregation (Ke et al., 2011), 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., 2010; 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., 2008). Such neocentromere formation associated with chromosomal rearrangements is found in patients with developmental disorders (Burnside et al., 2011; Marshall et al., 2008; 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, an 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. Elegans 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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1.8 Double Strand Break mobility and repair in the compartmentalized nucleus

In addition to chromatin organization through folding and creation of distinct chromatin compartments, further distinct substructures (compartments) are found in the nucleus (Dundr and Misteli 2001) (Figure 11). Briefly, at the periphery of the cell is the nuclear envelope, which consists of two membranes (inner and outer) that are fused together at the nuclear pores. Nuclear pores, on the other hand, are transmembrane complexes that consist of about 30 proteins (nucleoporins), and they allow the transportation of molecules between the nucleus and the cytoplasm (Beck and Hurt 2016). The nuclear lamina is found just inside the nuclear envelope, and it has a role in maintenance of the nuclear shape (van Steensel and Belmont 2017). Another substructure of the nucleus, which has been described in detail in our review (Yilmaz*, Mitrentsi* et al *in preparation*) is the nucleolus, the site of rRNA synthesis. Finally, different nuclear bodies, such as cajal bodies, stress bodies or PML bodies have also been identified in the nucleus and are responsible for/the sites of distinct cellular processes, including mRNA splicing, heat shock response and transcription. The existence of these compartments can affect the DNA repair outcome and DSB pathway choice. Several studies have been published that trying to elucidate insight on whether DSB repair is compartmentalized or if the DSBs are mobile and are moving towards specific compartments to be efficiently repaired (Misteli and Soutoglou 2009, Chiolo, Minoda et al. 2011, Dion and Gasser 2013, Miné-Hattab and Rothstein 2013, Lemaître, Grabarz et al. 2014, Caridi, D'Agostino et al. 2018, Marnef, Finoux et al. 2019, Pinzaru, Kareh et al. 2020) However, many questions still remain open.

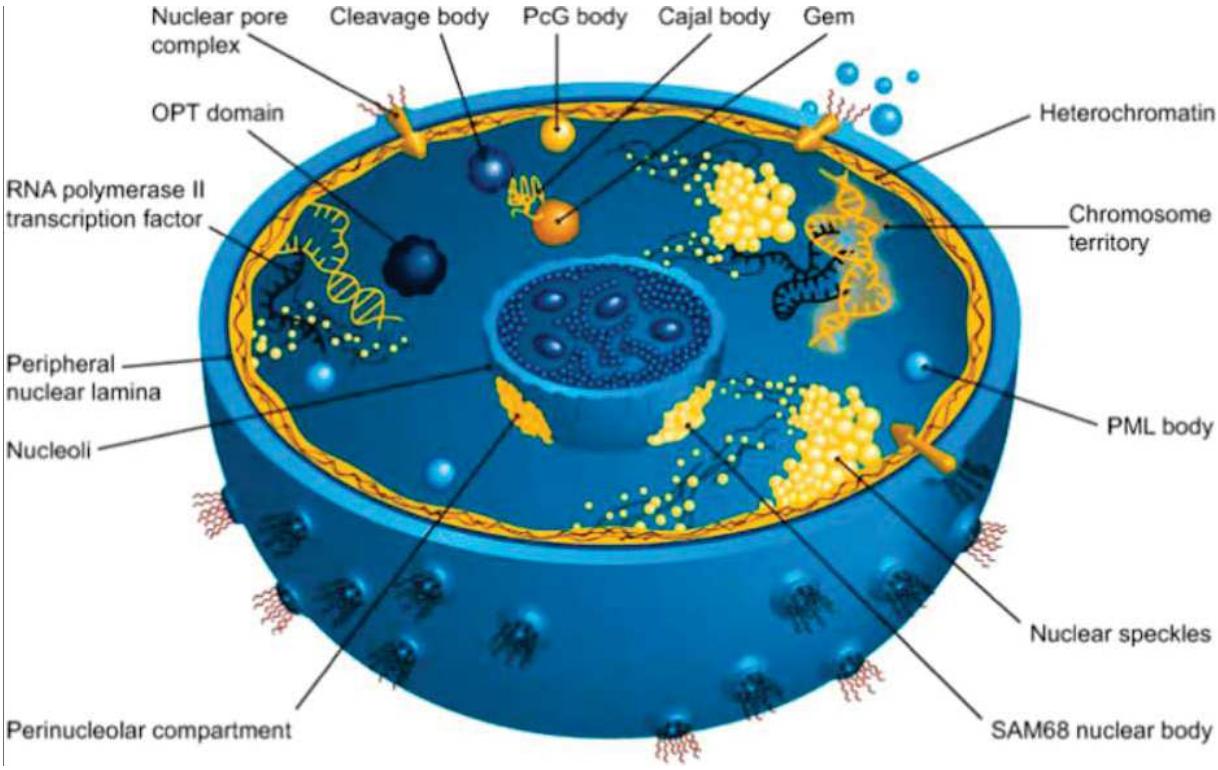

Figure 11 : Nuclear compartments Adapted from Telma Frege and Vladimir N Uversky 2015

In order to address whether DSBs are mobile and the mechanisms underlying this mobility, different methods for the DSB induction have been applied, in several different model organisms. Studies performed in yeast (*S. cerevisiae*), mainly show that chromatin mobility is increased after damage. More specifically, Rad52 foci acts as centers of DNA repair, recruiting more than one DSBs (Lisby, Mortensen et al. 2003), with factors such as Rad51, Rad53, Mec1 and Rad9 enhancing their mobility (Dion, Kalck et al. 2012). Studies on two homologous loci that occupy separated regions in the yeast nucleus reveal that the cut and uncut chromosomes were colocalizing more often after the DSB induction (Miné-Hattab and Rothstein 2012), supporting a model of homology search machinery where the mobility facilitates the search of the homologous sequence and thus the repair by HR. Moreover, in yeast, it has been reported that DSBs are relocating to different nuclear compartments to activate different repair pathways. For example, irreparable DSBs and telomeres move towards the nuclear periphery, either to the inner nuclear membrane (Kalocsay, Hiller et al. 2009, Oza, Jaspersen et al. 2009, Schober, Ferreira et al. 2009) or to the nuclear pores (Nagai, Dubrana et al. 2008)(Kalocsay et al. 2009, (Khadaroo, Teixeira et al. 2009) (Therizols, Fairhead et al. 2006)). The site of

relocation matters. DSB relocation to the inner nuclear membrane restricts recombination events (Oza et al. 2009, Schober et al. 2009), whereas nuclear pores represent a more permissive environment for DSB repair and recombination (Nagai et al. 2008, Therizols et al. 2006). Breaks that are inefficiently repaired at the nuclear membranes may possibly delocalize at the pores in order to complete this process.

Parallel studies in *D. melanogaster* find that heterochromatic DSBs relocate to the nuclear pores in order to be repaired by HR (Chiolo et al. 2011, Ryu et al. 2015), in an actin dependent manner (Caridi et al. 2018). Similarly, DSBs induced in the nucleolar rDNA repeats, are delocalized to the periphery of the nucleolus to be repaired by HR, both in yeast and in human cells (Torres-Rosell, Sunjevaric et al. 2007, Harding, Boiarsky et al. 2015)(Van Sluis and Mac Stay 2015). It was recently reported that rDNA repeats also interact with the nuclear periphery after the damage induction, through nuclear envelope invaginations that are formed in an actin dependent manner (Marnef et al. 2019). Similarly, a recent study showed that replication stress lead to a repositioning of telomeres to the nuclear pore complex in a F-actin polymerization dependent manner (Pinzaru et al. 2020). We discuss in detail the role of actin in the mobility of the DSBs in our review that follows (Mitrentsi, Yilmaz et al. 2020). In mouse cells DSBs induced in centromeres and pericentromeres relocate to the periphery of the heterochromatic domain (chromocenter) to be repaired by HR, but in this case there is no movement to the nuclear pores (Jakob et al. 2011, Tsouroula et al. 2016). Little is known about the mobility of DSBs induced in human pericentromeric heterochromatin, and thus investigation of this aspect was the primary goal of my PhD studies, that is described in the results and discussion sections (2,3 and 4). Unlike in other species, in human cells DSBs at the nuclear periphery that fail to activate HR, do not relocate at the pores, but remain positionally stable and are repaired *in situ* by alt-EJ (Lemaitre et al. 2014). It is clear that HR inhibition within heterochromatic domains that consist of clustered repetitive elements is an evolutionary conserved mechanism in yeast, *D. melanogaster* and mouse cells, but it remains unknown what happens in human pericentromeric heterochromatin that do not form clustered nuclear condensates.

Even though heterochromatic DSBs mobility shares some common features between the different cell models studied, similar studies in which the DSBs are not specifically induced in this region show more controversial results. In human cells, AsiSI-induced DSBs exhibit

increased clustering ability in G1 (Caron, Choudjaye et al. 2015, Aymard, Aguirrebengoa et al. 2017). A characteristic of these breaks is that they correspond to transcriptionally active genes (Aymard, Bugler et al. 2014) and their clustering depends on ATM activity, actin organization and cytoskeleton LINC complex (Aymard et al. 2017, Marnef et al. 2019). Moreover, chromatin domains in which DSBs were induced by γ-irradiation or etoposide treatment showed increased mobility compared to intact chromatin(Krawczyk, Borovski et al. 2012) . In contrast, studies in mammalian cells showed that UV-laser and irradiation-induced DSBs are immobile in mammalian cells (Kruhlak, Celeste et al. 2006, Becker, Durante et al. 2014). Along the same line, Soutoglou et al. (2007), using a LacO/LacR system and live-cell imaging, proved that the two broken ends are positionally stable (Soutoglou, Dorn et al. 2007). This immobility is mediated through Ku70-Ku80 or XRCC4-XLF complex as they bridge and hold together the two ends of the break together (Soutoglou, Dorn et al. 2007, Brouwer, Sitters et al. 2016). This idea is supported by Cho et al. (2014), were they induced FokI endonuclease breaks and validated that their mobility did not increase in human cells (Cho, Dilley et al. 2014). The difference between yeast and mammalian DSBs is clear, and it seems that mammalian DSBs are not able to search the whole nucleus for a homologous template. Indeed, DSBs that pair are more mobile, and they rarely lead to translocations (Roukos et al. 2013).

In conclusion, DSB mobility and repair pathway choice are still an open questions in the field, since the results are contradictory (Figure 12). In yeast, DSBs show increased mobility compared to mammalian cells, but this might be explained by taking into consideration the size of the yeast nucleus, which is only 1μm, and thus represents a very small movement in the ten times bigger mammalian nucleus. In *D. Melanogaster* heterochromatin, rDNA repeats, and telomeres, actin seems to regulate the DSB movement at the nuclear periphery, in contrast to the mouse heterochromatin where the DSBs are only relocating at the periphery of heterochromatic domains. The different methods of DSB induction, differences in the spatial organization of heterochromatin, use of different model organisms and type of analysis could explain the difference between studies. In our following review, how to maintain the genome in nuclear space (Mitrentsi, Yilmaz et al. 2020), we have discussed in detail the recent studies that have revealed that the 3D genome folding, nuclear compartmentalization and cytoskeletal components affect the spatial distributions of DSBs in nuclear space and dictate their repair mode.

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How to maintain the genome in nuclear space

Ioanna Mitrentsi[1,2,3,4,a], Duygu Yilmaz[1,2,3,4,a] and Evi Soutoglou[1,2,3,4,b]

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 address their mode of repair.

[1] Institut de Génétique et de Biologie Moléculaire et Celullaire, 67404, Illkirch, France
[2] Institut National de La Santé et de La Recherche Médicale, U964, 67404, Illkirch, France
[3] Centre National de Recherche Scientifique, UMR7104, 67404, Illkirch, France
[4] Université de Strasbourg, 67081, Strasbourg, France

Corresponding author: Soutoglou, Evi (E.Soutoglou@sussex.ac.uk)
[a] Equal contribution. [b] Current address: Genome Damage and Stability Center, Sussex University, School of Life Sciences, University of Sussex, Brighton, BN1 9RH, UK.

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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.

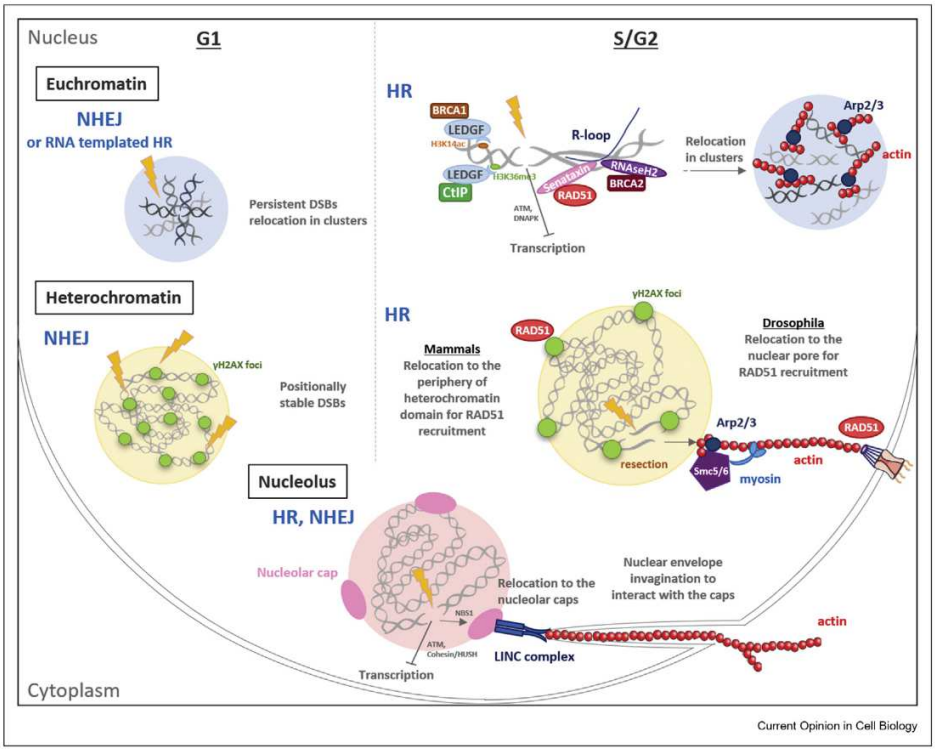
Interplay between transcription and DNA repair

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**].

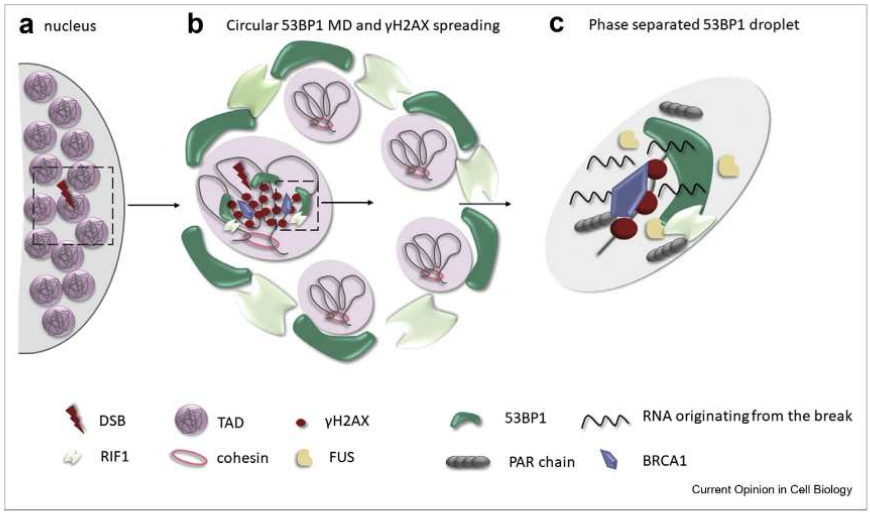
Although the stability of the active genomic regions is in constant jeopardy, DSBs at transcriptionally active loci are mainly repaired by the error-free HR in S/G2 phases of the cell cycle [23] (Figure 1). The mechanism underlying this preference involves the affinity of HR promoting factors such as 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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1.9 Brief Summary

The genome is subjected to a barrage of insults every day, which can lead to DNA lesions. It is very important that the cells repair these lesions because the resulting genomic instability from unrepaired or misrepaired DNA breaks can lead to serious consequences for the organism, including potentially deadly diseases such as cancer, aging and infertility. To combat this, the cell has developed a sophisticated DDR, which utilizes multiple repair pathways, The choice of pathway is dependent on many factors, including the type of damage caused, the stage of the cell cycle, chromatin status and structure, the availability of specific repair factors and where within the nucleus the break resides. The choice of pathway can have consequences for the cell since some are error free, while others are prone to errors, which themselves can lead to genomic instability. Major open areas of research focuses on how important the various sub-comparments of the nucleus are for repair outcomes, and on DSBs mobility, covering questions such as in what instances are DSBs mobile, why are there differences between species, what is the influence of euchromatin versus heterochromatin, and what consequences to the cell/repair process does the movement of DSBs have?

2. Aim of the study

Repetitive DNA is packaged in heterochromatin structures like the centromere that consists of pericentromeric and centromeric heterochromatin. Preserving the centromeric DNA integrity is crucial as unrepaired lesions could lead to the failure of chromosome segregation to daughter cells, further leading to aneuploidy, a hallmark of cancer. As this process is quite challenging, there has been great interest in the DNA repair field over the years in understanding the mechanisms that are used to repair heterochromatic Double Strand Breaks (DSBs).

Previously, our team showed that in mouse pericentromeric HC (chromocenters), CRISPR/Cas9 induced DSBs are positionally stable in G1 and recruit NHEJ factors. In S/G2, DSBs are resected and relocate to the periphery of HC, where they are retained by RAD51 in an actin independent manner (our ubpublished results). On the contrary, in *Drosophila melanogaster,* DSBs move outside of the heterochromatic domain and more specifically at the nuclear pores in order to be repaired by HR, in an actin dependent manner.

Although several studies have set the basis for understanding how DNA repair proceeds in chromatin dense regions, little is known about human heterochromatic DSB repair. The goal of my PhD was to investigate DSB repair within human heterochromatin. Our original aim was to verify if the heterochromatic DSB repair is a conserved mechanism and weather in human cells there is mobility of DSBs correlated to HR pathway activation. To address these questions we have developed a CRISPR/Cas9 system to specifically target and visualize DSBs in human pericentromeric heterochromatin, and my results demonstrate fundamental differences between mouse and human cells' response.

Heterochromatic repeat clustering imposes a physical barrier on homologous recombination to prevent chromosomal translocations.

Ioanna Mitrentsi[1,2,3,4], Adèle Kerjouan[5], Bernardo Reina-San-Martin[1,2,3,4], Elizabeth Hinde[6] and Evi Soutoglou[1,2,3,4,7,#]

1. Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Illkirch, France
2. Institut National de la Santé et de la Recherche Médicale (INSERM), U1258, Illkirch, France
3. Centre National de Recherche Scientifique (CNRS), UMR7104, Illkirch, France
4. Université de Strasbourg, Illkirch, France
5. School of Physics, University of Melbourne, Melbourne, Victoria, Australia
6. Department of Biochemistry and Molecular Biology, Bio21 Institute, University of Melbourne, Melbourne, Victoria, Australia
7. Current address: Genome Damage and Stability Centre, Sussex University, School of Life Sciences, University of Sussex, Brighton, BN1 9RH, UK

[#]Corresponding author E.Soutoglou@sussex.ac.uk

Abstract

Mouse pericentromeric DNA is composed of tandem major satellite repeats, which are heterochromatinized and cluster together to form chromocenters containing homologous sequences from different chromosomes. These clusters are protected from DNA damage and are refractory to DNA repair through homologous recombination (HR). Moreover, double stranded DNA breaks (DSBs) occurring within chromocenters relocate away from the core domain to be repaired. The mechanisms by which pericentric heterochromatin imposes a barrier on HR and the implications of repeat clustering are unknown. Here, we compare the spatial recruitment of HR factors upon DSBs induced specifically in human and mouse pericentromeric heterochromatin, which differ in their capacity to form clusters. We show that while DSBs in human pericentromeric heterochromatin increase the accessibility of the domain by disrupting HP1α dimerization, mouse pericentromeric heterochromatin repeat clustering imposes a physical barrier that requires many layers of decompaction to be accessed. Our results are consistent with a model in which the 3D organization of the different heterochromatic regions dictates the spatial activation of DNA repair pathways and is key to prevent the activation of HR within clustered repeats and the onset of chromosomal translocations.

Introduction

Chromatin compaction plays a key role in the choice of DNA repair pathways (Aymard et al., 2014). Repetitive DNA elements located at centromeric or pericentromeric chromosomal regions are packaged into heterochromatin, which is highly condensed to prevent unscheduled DNA transactions (Guenatri et al., 2004). Double stranded DNA breaks (DSBs) in pericentromeric heterochromatin, in both mouse and *Drosophila* cells, re-locate outside of the core heterochromatin domain, presumably to avoid encountering homologous sequences and illegitimate joining (Chiolo et al., 2011; Jakob et al., 2011). In *Drosophila* cells, heterochromatic DSBs relocate to the nuclear pore (Ryu et al., 2015) in an actin- and myosin-dependent manner (Caridi et al., 2018; Schrank et al., 2018). In mouse pericentromeric heterochromatin (chromocenters), DSBs relocate to the periphery of the domain, where they are retained by homologous recombination (HR) factors such as RAD51, whose access into the core of chromocenters is restricted (Tsouroula et al., 2016). Nevertheless, the mechanism responsible for restricting the access of HR factors into the core domain of pericentromeric heterochromatin is unknown. In human cells, pericentromeric heterochromatin has a different spatial organization. Unlike mouse cells where identical major satellite repeats from many different chromosomes cluster together to form chromocenters, human pericentromeric repeats do not spatially cluster. It is unclear whether DSB relocation is a mechanism conserved in human pericentromeric heterochromatin or whether it has evolved alternative mechanisms to prevent detrimental recombination between repetitive elements located on different chromosomes.

Results and discussion

To address the DSB repair mechanisms in heterochromatin are conserved in human cells, we have generated an experimental system to specifically induce DSBs at human pericentromeric satellite III repeats (SatIII) using CRISPR/Cas9 (**Fig. S1A**). Contrary to mouse cells, human pericentromeric heterochromatin does not correspond to DAPI-dense regions. To visualize satellite III repeats (SatIII), which are mainly located on chromosome 9q12 (**Fig. S1A**), we expressed a Heat Shock Factor 1 (HSF1) mutant lacking the activation domain fused to EGFP (DBD-TRIM-GFP), which constitutively binds to SatIII DNA without activating transcription (Jolly et al., 2002). To induce DSBs at SatIII regions, we expressed Cas9 together with SatIII-specific gRNAs (**Fig. S1A and S1B**). As expected, DBD-TRIM co-localized with γ-H2AX and 53BP1, markers of DNA damage, (**Fig. 1A and S1B**) and was dependent on the catalytic activity of Cas9 (**Fig. S1C and S1D**). The specific induction of DSBs at SatIII DNA was further confirmed by immuno-FISH (**Fig. S1E**). The number of SatIII domains in U2OS cells was variable between individual cells with the majority of them having three to four foci, corresponding to the number of chromosomes 9 contained within each cell (**Fig. S1F**). Cas9-induced DSBs in SatIII resulted in the activation of the DNA Damage Response (DDR), as exemplified by the phosphorylation of ATM ($pATM^{S1981}$) and the recruitment of MDC1 (**Fig. S2A and S2B**). Notably, the approach did not lead to a massive activation of the DDR, such as that observed when cells were treated with the DNA damaging agent neocarzinostatin (NCS), further demonstrating that the number of DSBs induced is limited and that the response is localized and not global (**Fig. S2C**).

To determine whether the localization of DSBs generated at SatIII repeats is regulated by the cell cycle, as is the case in mouse pericentric heterochromatin (Tsouroula et al., 2016), we assessed the spatial distribution of γ-H2AX in relation to the SatIII domains using 3D Structured Illumination Microscopy (3D-SIM). Remarkably, and in contrast to mouse cells where γ-H2AX is excluded from heterochromatin in S/G2, we found that γ-H2AX was induced throughout the SatIII core domain independently of the cell cycle stage (**Fig. 1A-C and S2D**). These results suggest that in human heterochromatin the Cas9-induced DSBs remain positionally stable throughout the cell cycle and reveal a fundamental difference between human and mouse heterochromatic DSBs.

To investigate whether the distinct DSB localization in human versus mouse heterochromatin is due to differences in the DSB repair mechanisms activated, we assessed in high-resolution the distribution of Rad51 in response to Cas9-induced DSBs at SatIII repeats. Surprisingly, and in contrast to what we observed in mouse heterochromatin where RAD51 was at the periphery of the heterochromatic domain promoting DSB relocation (Tsouroula et al., 2016), we found that RAD51 is recruited at the core of the SatIII domains, similar to the γ-H2AX internal pattern (**Fig. 1D**). This was also observed in RPE1 and HeLa cells, excluding the possibility of an artifact due to a specific cell line (**Fig. S2E**).

RPA, which was used as a marker for DNA end-resection was also recruited at the core of the SatIII domains (**Fig. 1E**), suggesting that DSBs are positionally stable and that the SatIII domains are not refractory to homologous recombination (HR). Notably, non-homologous end-joining

(NHEJ)-promoting factors such as 53BP1 and RIF1 were predominantly positioned at the periphery of the SatIII domains (**Fig. 1G, 1I and S3A**), suggesting that in the majority of cells in S/G2 there is a spatial separation of repair pathway factors, where HR factors are recruited at the core domain and NHEJ proteins are restricted to the periphery. Consistent with the competition between 53BP1/RIF1 and BRCA1 (Isono et al., 2017), we found a mutually exclusive recruitment pattern between BRCA1 and RIF1 (**Fig. 1F-I**).

To determine whether these findings can be generalized to additional repetitive elements of the human genome, we induced Cas9-specific DSBs on satellite II repeats (mainly located on chromosome 1) (Vourc'h and Biamonti, 2011), centromeres and telomeres. None of these regions were refractory to RAD51 recruitment (**Fig. S3B-D**), suggesting a general feature of human repetitive elements.

As one of the fundamental differences between mouse and human pericentromeric repeats relies on the extent of clustering between repetitive elements, we hypothesized that the use of HR within clustered repeats could result in aberrant recombination between different chromosomes leading to translocations. Consistent with this, aberrant recombination would not be a risk in human cells since repeats do not cluster. To directly assess the role of clustering in DSB relocation and RAD51 exclusion from mouse heterochromatin, we induced DSBs at major satellite repeats in mouse ES cells depleted of Chromatin Assembly Factor 1 (CAF1), where chromocenter clustering is disrupted throughout the cell cycle (Houlard et al., 2006) **(Fig. S4A-**

S4C). Strikingly, we found that at DSBs induced at the non-clustered major satellite repeats (siCaf1), RAD51 is no longer excluded from the domains (**Fig. 2A-B and S4B**). To further explore the role of clustering, we expressed the multi-AT-hook satellite DNA-binding protein D1 from *Drosophila melanogaster* in mouse cells. As shown before (Jagannathan et al., 2018), in contrast to control cells **(Fig. 2C),** expression of the D1 protein boosted clustering and formed super-chromocenters (**Fig. 2D**), which displayed enhanced RAD51 exclusion upon Cas9-specific DSB induction (**Fig. 2E**). These results suggest that heterochromatin organization within the nucleus has fundamental implications in the spatial distribution of DNA repair pathways. This notion, argues against an active mechanism taking place in DSB-relocation. Indeed, we find that DSB-relocation was independent of an actin-related process, as depletion of the actin-related protein ARP2/3 did not alter DSB distribution in mouse pericentric heterochromatin (**Fig. S4D**), contrary to what has been previously suggested (Caridi et al., 2018; Schrank et al., 2018).

Heterochromatin is amongst the densest nuclear compartments, and it excludes macromolecules in a size dependent manner(Bancaud et al., 2009). To investigate how clustering renders the heterochromatic repeats refractory to Rad51, we explored whether DNA damage changes the accessibility of these domains towards protein diffusion. To this end, we employed a novel approach of fluorescence fluctuation spectroscopy (FFS) called pair correlation function (pCF) analysis, which has the capacity to measure the time it takes an inert protein (mKate2– 26 kDa) to diffuse into fluorescently labelled heterochromatic DSB foci traversed by a confocal line scan (**Fig. S5A**). Specifically, in NIH-3T3 (mouse) and Hela (human) cells co-transfected with mKate2 and EGFP-53BP1 or DBD-TRIM-EGFP, respectively, we acquired confocal line scan data in the

mKate2 channel (**Fig. 3A-B**), and via use of the pCF analysis, spatially cross correlated fluctuations in mKate2 fluorescence intensity outside versus inside EGFP-53BP1 (for NIH-3T3 cells) or DBD-TRIM-EGFP (for Hela cells) labelled DSB foci, in G1 and G2, in the presence or absence of DNA damage (**Fig. 3C-D**). In mouse cells, the heterochromatin domain in G1 and more so G2 was found to pose as an obstacle to free mKate2 diffusion that extended from ~ 5-10 ms and DSB induction did not significantly change the delayed characteristic entry time of mKate2 molecules (**Fig. 3C** and **Fig. S5B**). In human cells, Sat III domains were more accessible on the timescale of the experiment in G1 and but in G2 were found to delay mKate2 diffusion revealing another difference between human and mouse heterochromatin. Interestingly, in the presence of Cas9-induced DSBs in G2, where HR is predominant, mKate2 diffusion into SatIII domain becomes more rapid, with a significant shift in the characteristic entry time to ~ 2-5 ms (**Fig. 3D** and **Fig. S5C**). This suggests that Cas9-induced DSBs could increase the accessibility of the human SatIII domain to HR factors specifically in G2, in contrast to the mouse chromocenters. In cells, in the absence of DNA damage, RAD51 travels with BRCA2 in a nearly 2MDa multimeric complex (Marmorstein et al., 2001) in which BRCA2 oligomerizes and each BRCA2 monomer binds to several RAD51 molecules (Jensen et al., 2010). This multimeric assembly which spans up to several hundred nanometers can scan the nuclear volume and effectively deliver RAD51 to ssDNA generated by DNA damage. To assess the potential of BRCA2 to diffuse in heterochromatic domains, we transiently expressed mCherry-BRCA2 both in mouse and human cells and measured its intensity at the areas of interest (**Fig. 3E-3H**). We showed that compared to the nucleoplasm, BRCA2 intensity is lower in the mouse chromocenters (marked by HP1α), but it increases inside the sat III domains (marked by DBD-TRIM) (**Fig 3E,3F and 3H**), suggesting that it can diffuse more easily

in the human cells' heterochromatin compared to the mouse. This was further supported by the fact that BRCA2 intensity, and thus accessibility on super-clustered chromocenters (marked by D1-GFP) was also decreased (**Fig.3G and 3H**). Therefore, although high-molecular weight proteins (such as different DNA repair proteins) have the potential to access heterochromatin in the time scales observed above, the size of BRCA2-RAD51 complex may render its accessibility more sensitive to the diffusion barriers imposed by clustering.

The formation of silenced and condensed heterochromatin involves the enrichment of Heterochromatin Protein 1 (HP1). We have previously demonstrated that DSBs in mouse pericentromeric heterochromatin lead to an increase in the level of H3K9me3 and all HP1 isoforms, consistent with the persistent barrier observed above (Tsouroula et al., 2016). Interestingly however, DSBs at SatIII repeats in human cells did not show an increase in HP1α or HP1β and resulted in the partial eviction of HP1γ (**Fig. S5D**). These results further support the notion that there are fundamental differences between human and mouse cells and that DSBs in human pericentromeric heterochromatin lead to increased accessibility of the domain. The ability of an inert protein to enter heterochromatin (Strom et al., 2017) and thus the permeability of heterochromatin has been correlated with the density of the HP1α protein at the domain boundary (Hinde et al., 2015; Strom et al., 2017). Indeed, it has been shown that HP1α is not equally partitioned in these domains but that it exists as monomers at the core and as a dimer at the boundary generating a physical barrier (Hinde et al., 2015; Larson et al., 2017; Strom et al., 2017). To determine whether the difference in the diffusion of mKate2 that we observe in human

cells between G1 and G2 in response to DSBs correlates with the presence or absence of HP1α dimers, we employed another FFS-based method called Number and Brightness (NB), which has the capacity to spatially map the oligomeric state of fluorescently tagged HP1α (HP1α-RFP657) in a confocal frame scan (Digman et al., 2008). Specifically, in live NIH-3T3 and Hela cells co-transfected with HP1α-RFP657and EGFP-53BP1 and DBD-TRIM-EGFP, respectively, we acquired confocal frame scan data in the HP1α-RFP657 channel and quantified the fraction of HP1α dimers in mouse chromocenters and human SatIII domains (DBD-TRIM-EGFP) in G1 and G2, in the presence or absence of Cas9-induced DSBs (EGFP-53BP1). Based on an HP1α monomer calibration (HP1α-RFP657) we find that HP1α dimerizes at the boundary of both chromocenters and SatIII domains (**Fig. 4A and 4B**). DSBs at SatIII repeats in G1 or mouse heterochromatin both in G1 and G2 resulted in an increase of the density of HP1α dimers (**Fig. 4A and 4C**). Interestingly, and consistent with the increased diffusion of mKate2, DSBs at human SatIII domains in G2 resulted in a significant decrease in the density of HP1α dimers at the boundary of the domain (**Fig. 4B and 4D**). These results reveal an anti-correlation between HP1α dimerization and protein diffusion at the core of SatIII domains.

To investigate whether HP1α dimers render mouse heterochromatin inaccessible to HR factors, we depleted the three isoforms of HP1 (α, β and γ) (Tsouroula et al., 2016) and complemented cells with HP1α or the Hp1α^{I165E} mutant which cannot dimerize and tested the potential of Rad51 and BRCA1 to diffuse inside the chromocenters. As we have shown before (Tsouroula et al., 2016), simultaneous depletion of all HP1 isoforms decreased the percentage of cells that

recruited Rad51 and BRCA1 at pericentromeric DSBs (**Fig. S6A and S6B**), consistent with the previously described role of HP1 in DNA end-resection (Soria and Almouzni, 2013). Interestingly, this effect was dependent on HP1 dimerization (**Fig. S6A, S5B and S6D)** suggesting that HP1 dimerization is important for DNA repair. Remarkably, however, in those cells in which RAD51 and BRCA1 were recruited, they were restricted to the periphery of the chromocenters in G2 (**Fig. S6A and S6B**). Similar results were obtained in SUV3-9 1 and 2 double knockout MEFs in which HP1α is not bound at the chromocenters due to the lack of H3K9me3 (**Fig. S6C and S6E**). These results are consistent with the observation that on SUV3-9 knockout MEFs, chromocenter accessibility to an inert protein is similar to wildtype MEFs (Erdel et al., 2020) and suggest that in mouse heterochromatin additional mechanisms are at play to establish a diffusion barrier. Nevertheless, depletion of all isoforms of HP1 in human cells resulted in an increase of the accessibility of the SatIII domains to BRCA1 in G1 (**Fig. S7A and S7B**), revealing another fundamental difference between mouse and human heterochromatin.

It has been shown that tethering of a strong transcriptional activator (VPR) to mouse heterochromatin disrupts interactions between its segments and renders the domain accessible to diffusion (Erdel et al., 2020). To investigate the behavior of HR proteins under de-condensed heterochromatin conditions, we induced DSBs at chromocenters while simultaneously tethering VPR with catalytically inactive Cas9 (EGFP-dCas9-VPR) in NIH-3T3 cells. This resulted in chromocenter de-compaction and disappearance of DAPI-dense regions (**Fig. 5A**). Surprisingly, under these conditions Rad51 and BRCA1 were visualized for the first time at the core of the domain (**Fig. 5B-5D**). In accordance with these results, when we measured the capacity of BRCA2

to diffuse in the same conditions, we observed an increased intensity compared to the nucleoplasm (**Fig. S7C**), suggesting that mouse chromocenter clustering makes the domain inaccessible to diffusion and that it requires extreme de-condensation for diffusion to occur. Contrary to this, human SatIII domains, which do not cluster, are more permissive to diffusion and in this case, HP1α dimers are sufficient to establish a diffusion barrier. It appears then that in mouse cells several layers of protection are at play to limit protein diffusion inside chromocenters.

To investigate the functional significance of chromocenter clustering in imposing a barrier to prevent deleterious recombination events between different chromosomes, we forced the entry of RAD51 to the core of chromocenters by tethering the BRC3 domain of BRCA2 via dCas9 (EGFP-dCas9-BRC3), which recruits RAD51 (Tsouroula et al., 2016). This resulted in the recruitment of Rad51 inside the core domain (Tsouroula et al., 2016) and in the subsequent stabilization of the breaks inside the chromocenters (**Fig. S7D**). To determine the functional consequence of the lack of DSB relocation and RAD51 internalization, we scored for chromosomal translocations originating at chromocenters on metaphase spreads by FISH, using probes specific for major satellite repeats and telomeres. Chromosomal translocations originating from the pericentromeric repeats of two different chromosomes would be expected to lead to the formation of metacentric chromosomes, with a single pericentromere and lacking the short arm telomeres. Interestingly, we find that inhibition of DSB relocation led to a five-fold increase in the frequency of translocations (**Fig. 5E and 5F**). This was dependent on RAD51 activity, as translocations were reduced in the presence of a catalytic inhibitor of RAD51 (**Fig. 5F**).

Overall, we provide strong evidence that DSB-relocation and RAD51 exclusion from mouse chromocenters is of fundamental importance to avoid illegitimate recombination between clustered repetitive elements and to prevent the onset of potentially oncogenic chromosomal translocations. Our results are consistent with a model (**Fig. S8**) in which the physical organization of the different heterochromatic regions *per se* dictates the spatial activation of DNA repair pathways. In the case of non-clustered repetitive elements, compaction proteins like HP1 can regulate accessibility of the domain. On the other hand, clustering of heterochromatic repeats, leads to the formation of a more inaccessible domain, which comprises several protection layers to prevent de-compaction, protein diffusion (accessibility) and illegitimate recombination. Consistent with this, DSBs at nucleolar repeats, which cluster together, relocate to the periphery and RAD51 is excluded from the domain (Marnef et al., 2019; van Sluis and McStay, 2015, 2017). Contrary to expectations, we show that this is not achieved through an active mechanism and highlight the fact that the major differences in heterochromatin clustering between mouse and human cells has fundamental implications for genome integrity.

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Figure legends

Figure 1. DSBs in Sat III repeats remain positionally stable throughout the cell cycle and recruit HR factors. Immunofluorescence (IF) super resolution (3D-SIM) analysis of U2OS cells expressing DBD-TRIM-GFP (marker for SatIII domains), Cas9 and gRNA targeting the SatIII repeats in **(A)** asynchronous, **(B)** G1/S synchronized and **(C)** S/G2 synchronized cells stained with DAPI and antibodies specific for γ-H2AX **(A-C)**, Rad51 **(D)**, 53BP1 and RPA **(E)** and BRCA1 **(F)**. Cells in D-G are asynchronous. 100% internal denotes the percentage of SatIII foci have the specified DNA repair protein localized within them, after DSB induction. **(G)** IF confocal analysis of U2OS cells expressing DBD-TRIM-GFP, Cas9 + gRNA targeting the SatIII repeats stained with DAPI and antibodies specific for BRCA1 and RIF1. Quantification of spatial distribution of BRCA1 **(H)** and RIF1 **(I)** on SatIII domains after DSB induction in U2OS cells synchronized in G1/S or S/G2 phases of the cell cycle. Wherever a quantification is not provided, 100% of cells exert the phenotype. Images are representative of 5 experiments with n=50 cells. Data are the mean ± SD of 5 experiments with n=50 cells. Scale bars represent 5 μm.

Figure 2. Repeat clustering excludes Rad51 from heterochromatic DSBs. **(A)** ImmunoFISH confocal analysis of wt E14 cells or cells depleted of Caf1 siRNA expressing Cas9 and a gRNA targeting major satellite repeats, stained with DAPI, a major satellite probe and an antibody specific for Rad51 and **(B)** quantification of Rad51 pattern. **(C-E)** Immunofluorescence (IF) confocal analysis of **(C)** non transfected NIH-3T3 cells stained with DAPI and CENPA, **(D)** NIH-3T3 cells expressing the D1-GFP protein, stained with DAPI and CENPA and **(D)** NIH-3T3 cells

expressing the D1-GFP protein in combination with DSB induction in major satellite repeats stained with DAPI and γH2AX and Rad51 specific antibodies. Wherever a quantification is not provided, 100% of cells exert the phenotype. Images are representative of 3 experiments with n= 50 cells. Scale bars represent 5 μm.

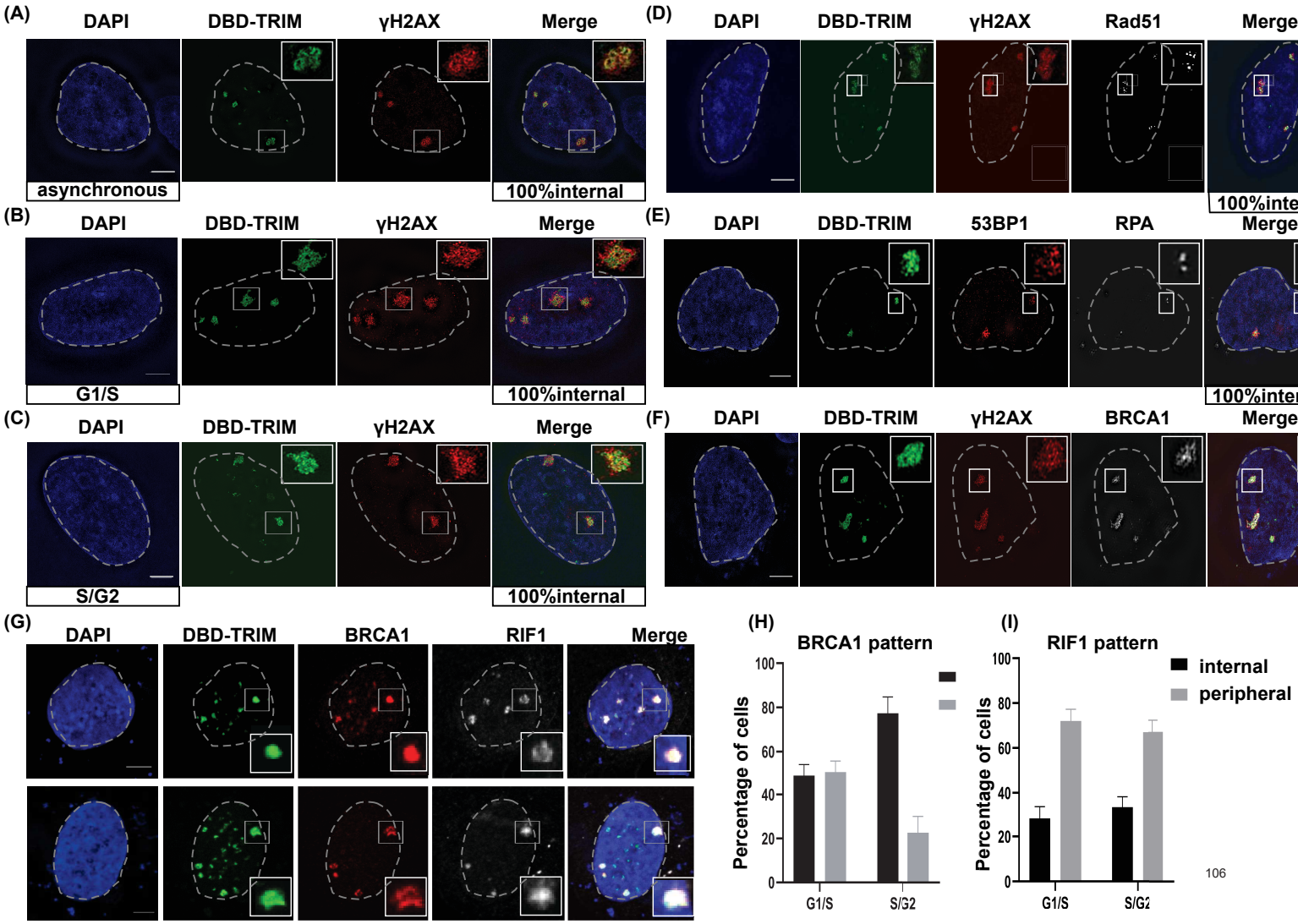
Figure 3. Spatial evolution mKate2 and BRCA2 access into mouse and human heterochromatin foci. **(A)** Two-colour confocal image of a NIH3T3 cell nucleus expressing mKate2 in the presence of Hoechst 33342 (heterochromatin with no DSB) or 16 hours after co-transfection with eGFP-53BP1 and Cas9 (heterochromatin with a DSB in G1 or G2) (top row), with the region of interest, where a line scan (white arrow) was selected for pCF analysis, enlarged (bottom row). Note G1 and G2 were differentiated based on the 53BP1 localization (Tsouroula et al., 2016). Scale bar represents 2μm **(B)** Two-colour confocal image of a Hela cell expressing mKate2 and DBD-TRIM in the absence of Cas9 (heterochromatin with no DSB) or 8 hours after transfection with Cas9 (heterochromatin with DSB) (top row), with the region of interest, where a line scan (white arrow) was selected for pCF analysis, enlarged (bottom row). Note G1 and G2 were isolated by standard cell synchronization procedures (see materials and methods). **(C)** pCF6 (mKate2 accessibility into the structure at a distance of δr = 6 (pCF6) pixels) analysis of mKate2 access into NIH3T3 G1 heterochromatin foci before (grey) versus after (green) DSB induction (left panel) and NIH3T3 G2 heterochromatin foci before (grey) versus after (red) DSB induction (right panel) (N = 3-5 measurements across N = 2-4 cells and 2 biological replicates). Scale bar represents 2 μm. Note in Fig. S5B pCF5 and pCF7 are also presented. **(D)** pCF6 analysis of mKate2 access into HeLa G1 heterochromatin foci before (grey) versus after (green) DSB induction (left panel) and HeLa

G2 heterochromatin foci before (grey) versus after (red) DSB induction (right panel) (N = 5-7 measurements across N = 4-6 cells and 2 biological replicates). Scale bar represents 2 µm. Note in Fig. S5C pCF5 and pCF7 are also presented. **(E-G)** Immunofluorescence (IF) confocal analysis of NIH-3T3 cells expressing HP1α-GFP and mCherry BRCA2 (**E**), HeLa cells expressing DBD-TRIM and mCherryBRCA2 (**F**) and NIH-3T3 cells expressing D1-GFP and mCherryBRCA2 (**G**). (**H**) Intensity quantification of mCherryBRCA2 in heterochromatin area versus the nucleoplasm is represented by a fold change. Data are the mean ± SD of 3 experiments with n=50 cells. Statistical significance was determined by one-way ANOVA (*p<0,05, **p<0,01, ***p<0,001, ****p<0.0001).

Figure 4. Hp1α dimerization in heterochromatin in the absence or presence of DSBs. **(A)** Intensity image of a NIH3T3 cell co-transfected with 53BP1-EGFP and HP1α-RFP657 in the absence or presence of Cas9 after 16 hours, as well as the region of interest from which Number and Brightness (NB) frame scan acquisition was recorded and a merged image between HP1α intensity and the brightness map of HP1α NB data in pseudo-color. Scale bar 5 µm. **(B)** Intensity image of a Hela cell co-transfected with DBD-TRIM, HP1α-RFP657 and +/- Cas9 after 8 hours, the region of interest from which NB frame scan acquisition was recorded and the merged image between HP1α intensity and the brightness map of HP1α NB data in pseudo-colour. Scale bar 5 µm. **(C)** NB quantitation of the percentage of HP1α-RFP657 dimer present in HP1α without Cas9 treatment, after Cas9 in G1 and in G2, in NIH-3T3 cells (N=7 cells, two biological replicates). **(D)** NB quantitation of the percentage of HP1α-RFP657 dimer present in HP1α in G1 and in G2 both before and after cas9 treatment, in HeLa cells (N=25 cells, three biological replicates). Box and whisker plots in (**C, D**) show mean line and all data points.

Figure 5. Repeat clustering imposes a physical barrier to prevent homologous recombination between repeats and chromosomal translocations. Immunofluorescence (IF) confocal analysis of **(A)** NIH3T3 cells expressing dCas9 fused to GFP and VPR (EGFP-dCas9-VPR) + gRNA targeting major satellite repeats stained with DAPI and **(B-D)** NIH-3T3 cells expressing EGFP-dCas9-VPR + gRNA targeting major satellite repeats + Cas9 stained with DAPI and antibodies specific for γ-H2AX, Rad51 and BRCA1. **(E)** Representative confocal image of metaphase spreads in cells expressing dCas9-BRC3-EGFP, after fluorescence *in situ* hybridization (FISH) for major satellite repeats or telomeres (PNA) and stained with DAPI. The insets depict chromosomal translocations. **(F)** Quantification of the number of translocations in cells expressing a gRNA targeting the major satellites together with dCas9 or dCas9-BRC3-EGFP and treated with DMSO or Rad51 inhibitor (Rad51i). Wherever a quantification is not provided, 100% of cells exert the phenotype. Data are the mean ± SD of 5 experiments with n=50 metaphases. For all graphs, statistical significance was determined by t-test (*p<0,05, **p<0,01, ***p<0,001, ****p<0.0001).

Figure 1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γH2AX
Merge
asynchronous
100%internal
(B)
DAPI
DBD-TRIM
γH2AX
Merge
G1/S
100%internal
(C)
DAPI
DBD-TRIM
γH2AX
Merge
S/G2
100%internal
(D)
DAPI
DBD-TRIM
γH2AX
Rad51
Merge
100%internal
(E)
DAPI
DBD-TRIM
53BP1
RPA
Merge
100%internal
(F)
DAPI
DBD-TRIM
γH2AX
BRCA1
Merge
(G)
DAPI
DBD-TRIM
BRCA1
RIF1
Merge
(H)
BRCA1 pattern
Percentage of cells
100
80
60
40
20
0
G1/S
S/G2
(I)
RIF1 pattern
internal
peripheral
Percentage of cells
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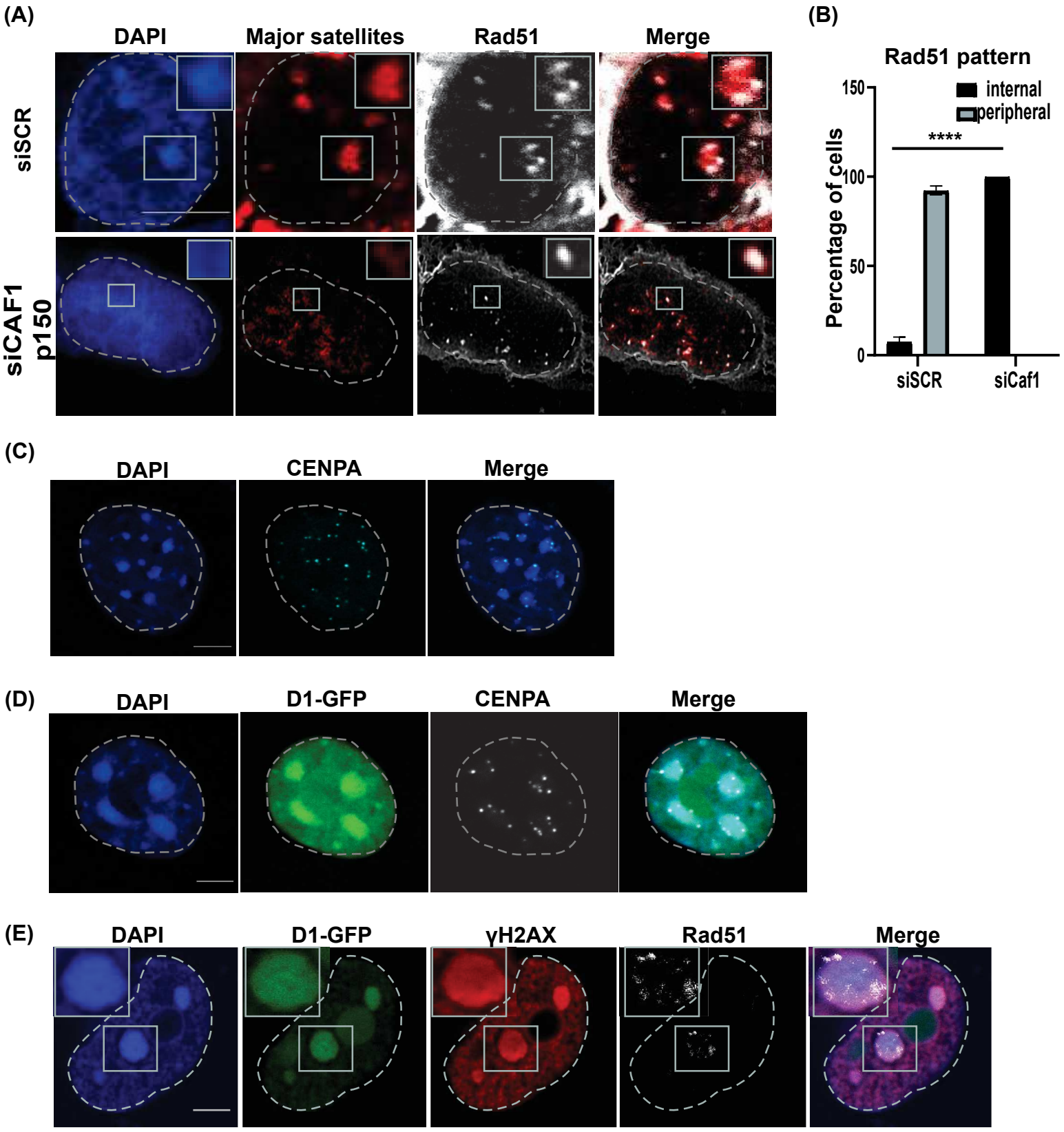
80
60
40
20
0
G1/S
S/G2

Figure 2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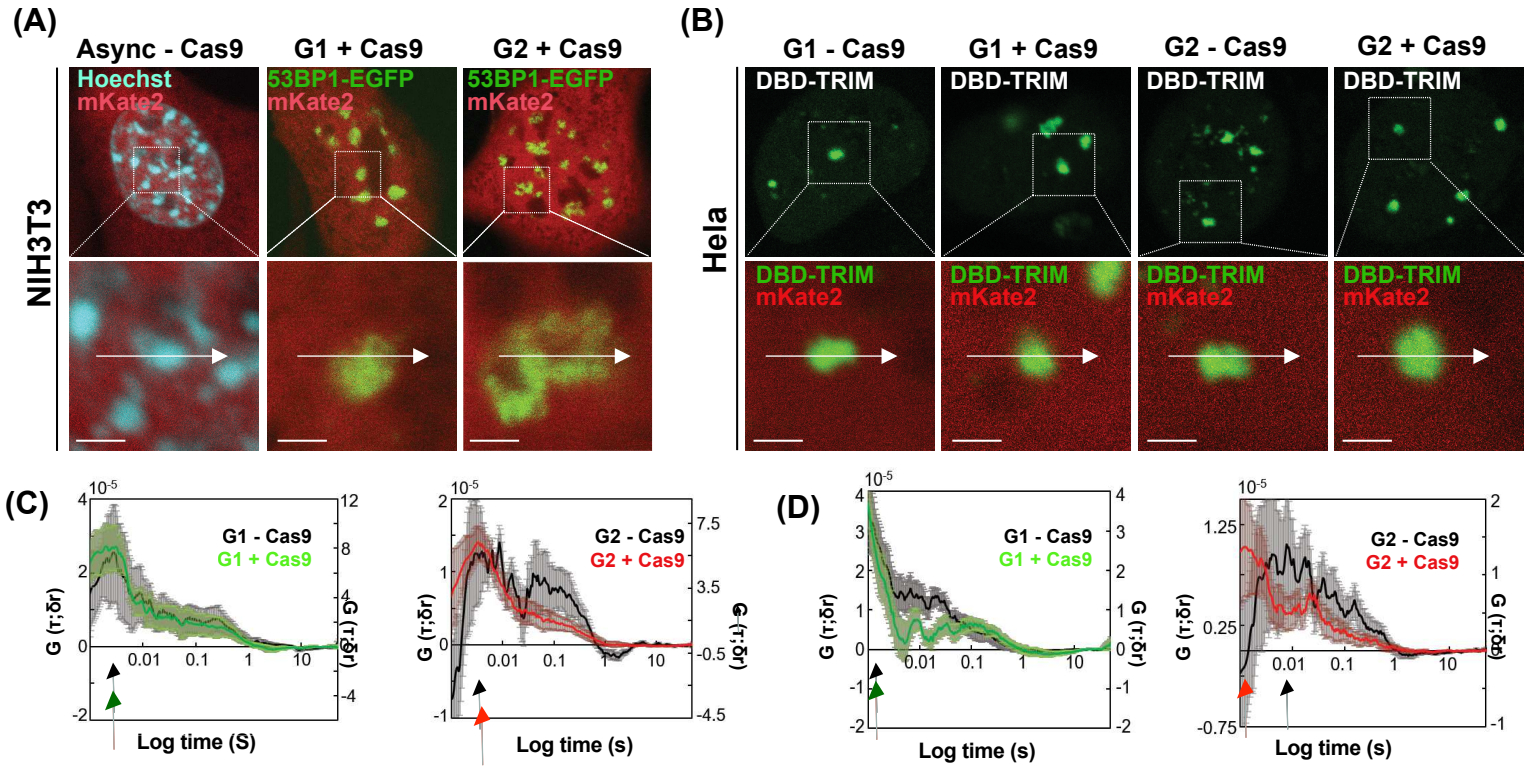
DAPI
Major satellites
Rad51
Merge
siSCR
siCAF1 p150
(B)
Rad51 pattern
internal
peripheral
****
Percentage of cells
150
100
50
0
siSCR
siCaf1
(C)
DAPI
CENPA
Merge
(D)
DAPI
D1-GFP
CENPA
Merge
(E)
DAPI
D1-GFP
γH2AX
Rad51
Merge

Figure 3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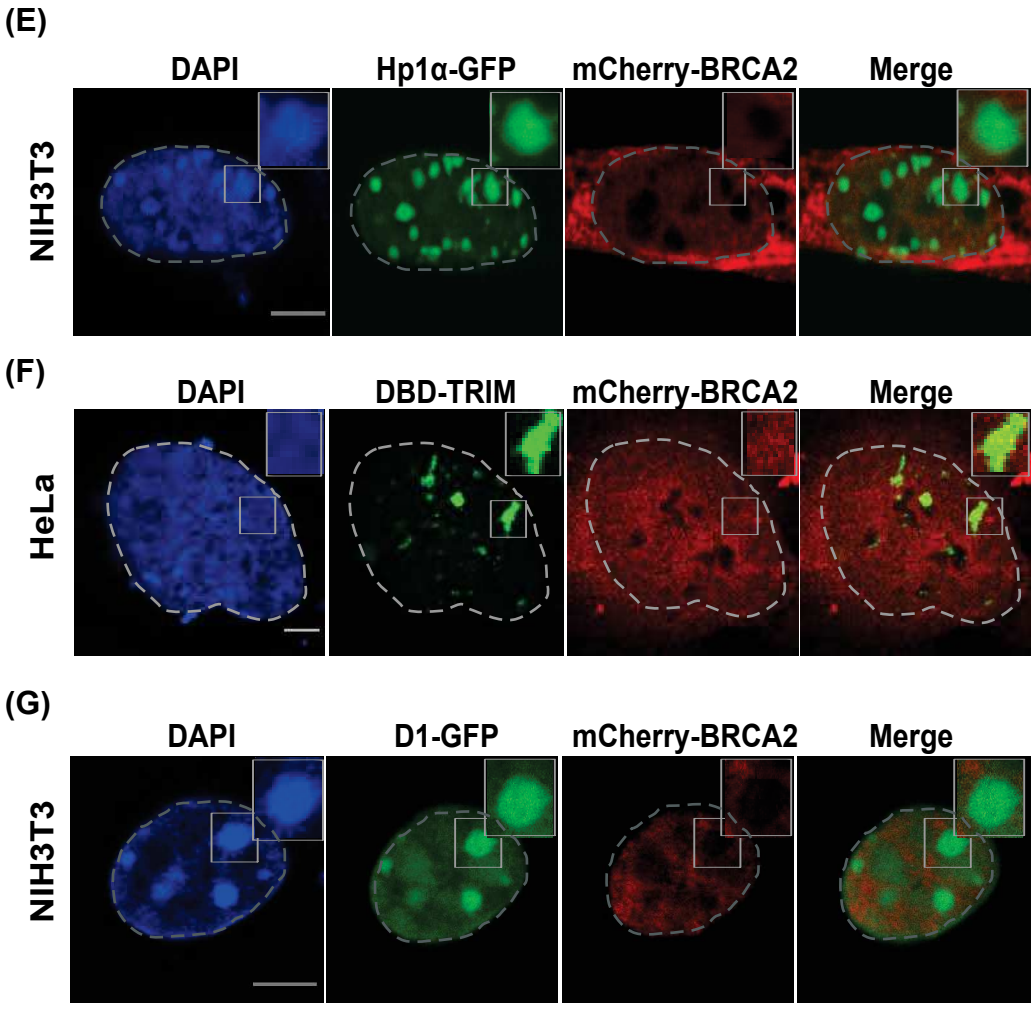
G1 + Cas9
G2 + Cas9
NIH3T3
Hoechst
mKate2
53BP1-EGFP
mKate2
(B)
G1 - Cas9
G1 + Cas9
G2 - Cas9
G2 + Cas9
Hela
DBD-TRIM
DBD-TRIM mKate2

(C)
G1 - Cas9
G1 + Cas9
G2 - Cas9
G2 + Cas9
Log time (S)
Log time (s)
(D)
G1 - Cas9
G1 + Cas9
G2 - Cas9
G2 + Cas9
Log time (s)
Log time (s)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Merge
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mCherry-BRCA2
Merge
HeLa
(G)
DAPI
D1-GFP
mCherry-BRCA2
Merge
NIH3T3

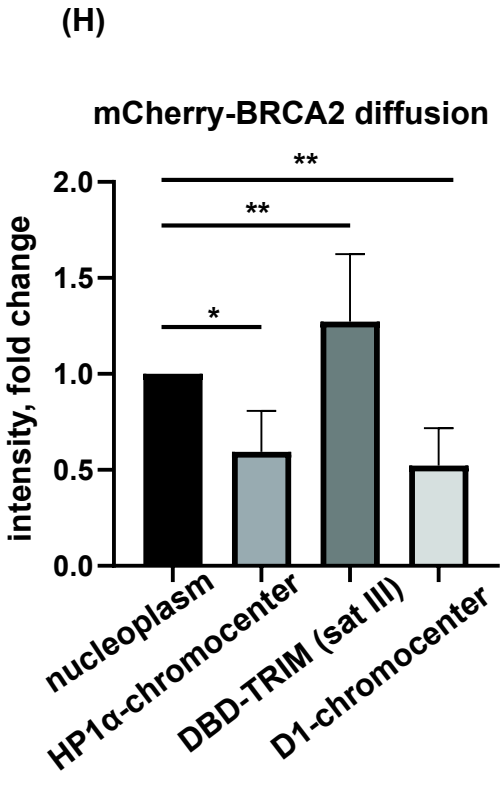
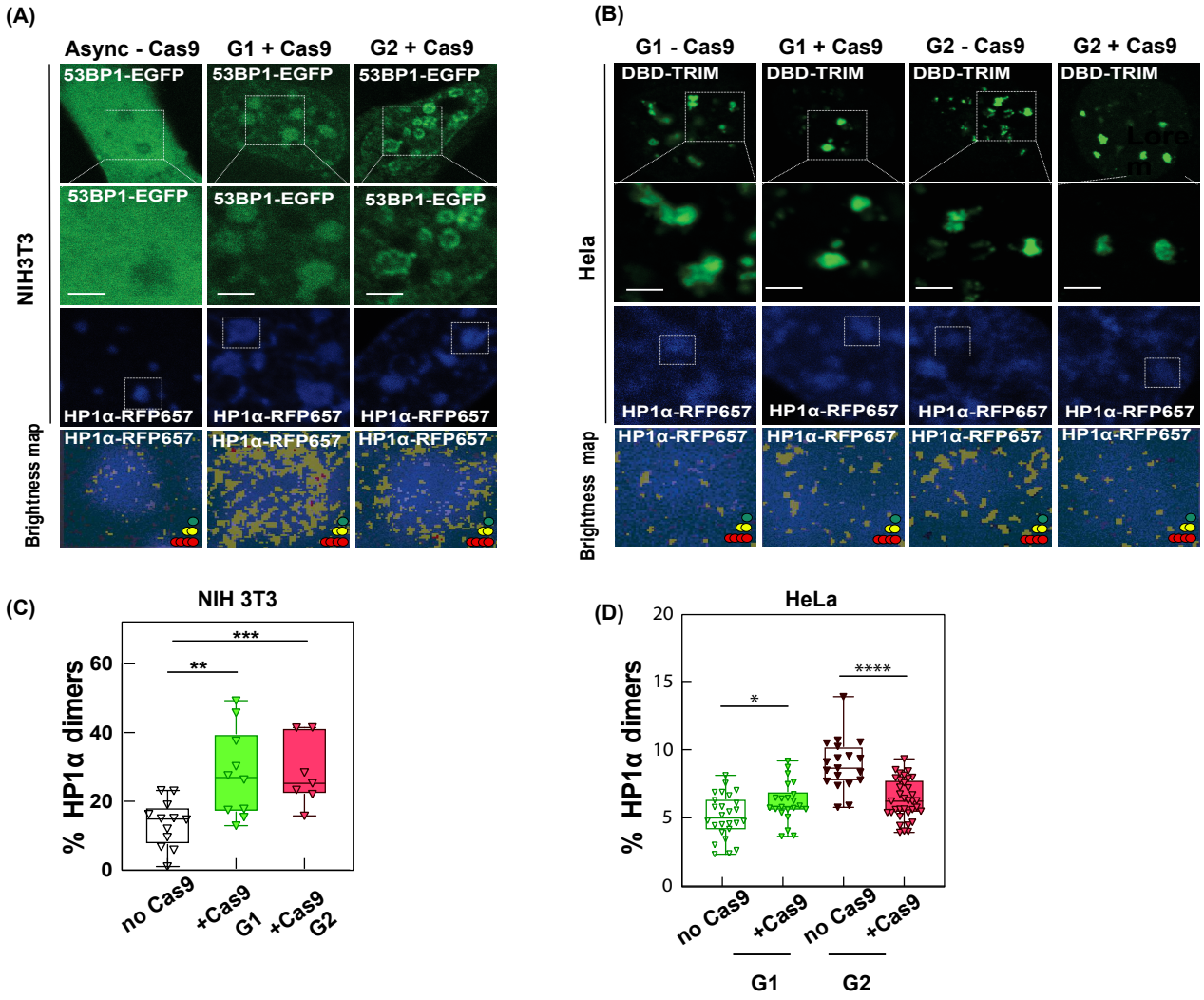

(H)
mCherry-BRCA2 diffusion
intensity, fold change
2.0
1.5
1.0
0.5
0.0
**
**
*
nucleoplasm
HP1α-chromocenter
DBD-TRIM (sat III)
D1-chromocenter

Figure 4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Async - Cas9
G1 + Cas9
G2 + Cas9
NIH3T3
53BP1-EGFP
HP1α-RFP657
Brightness map
(B)
G1 - Cas9
G1 + Cas9
G2 - Cas9
G2 + Cas9
Hela
DBD-TRIM
HP1α-RFP657
Brightness map
(C)
NIH 3T3
% HP1α dimers
0
20
40
60
**
***
no Cas9
+Cas9 G1
+Cas9 G2
(D)
HeLa
% HP1α dimers
0
5
10
15
20
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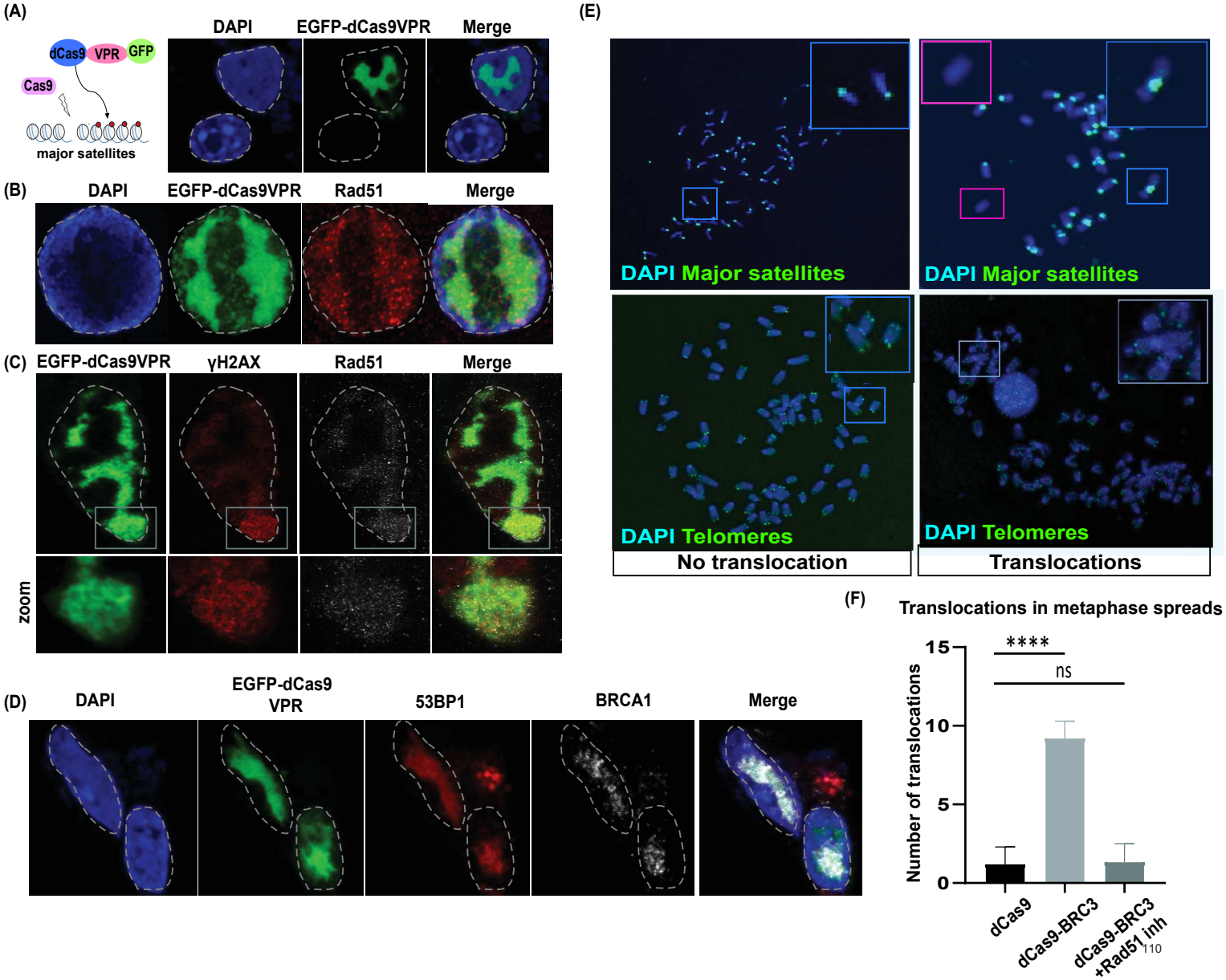
+Cas9
no Cas9
+Cas9
G1
G2

Figure 5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DAPI
EGFP-dCas9VPR
Merge
dCas9
VPR
GFP
Cas9
major satellites
(B)
DAPI
EGFP-dCas9VPR
Rad51
Merge
(C)
EGFP-dCas9VPR
γH2AX
Rad51
Merge
zoom
(D)
DAPI
EGFP-dCas9 VPR
53BP1
BRCA1
Merge
(E)
DAPI Major satellites
DAPI Major satellites
DAPI Telomeres
DAPI Telomeres
No translocation
Translocations
(F)
Translocations in metaphase spreads
Number of translocations
15
10
5
0
****
ns
dCas9
dCas9-BRC3
dCas9-BRC3 +Rad51 inh

SUPPLEMENTARY: Heterochromatic repeat clustering imposes a physical barrier on homologous recombination to prevent chromosomal translocations.

Ioanna Mitrentsi[1,2,3,4], Adèle Kerjouan[5], Bernardo Reina-San-Martin[1,2,3,4], Elizabeth Hinde[6] and Evi Soutoglou[1,2,3,4,7, #]

1. Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Illkirch, France
2. Institut National de la Santé et de la Recherche Médicale (INSERM), U1258, Illkirch, France
3. Centre National de Recherche Scientifique (CNRS), UMR7104, Illkirch, France
4. Université de Strasbourg, Illkirch, France
5. School of Physics, University of Melbourne, Melbourne, Victoria, Australia
6. Department of Biochemistry and Molecular Biology, Bio21 Institute, University of Melbourne, Melbourne, Victoria, Australia
7. Current address: Genome Damage and Stability Centre, Sussex University, School of Life Sciences, University of Sussex, Brighton, BN1 9RH, UK

[#]Corresponding author E.Soutoglou@sussex.ac.uk

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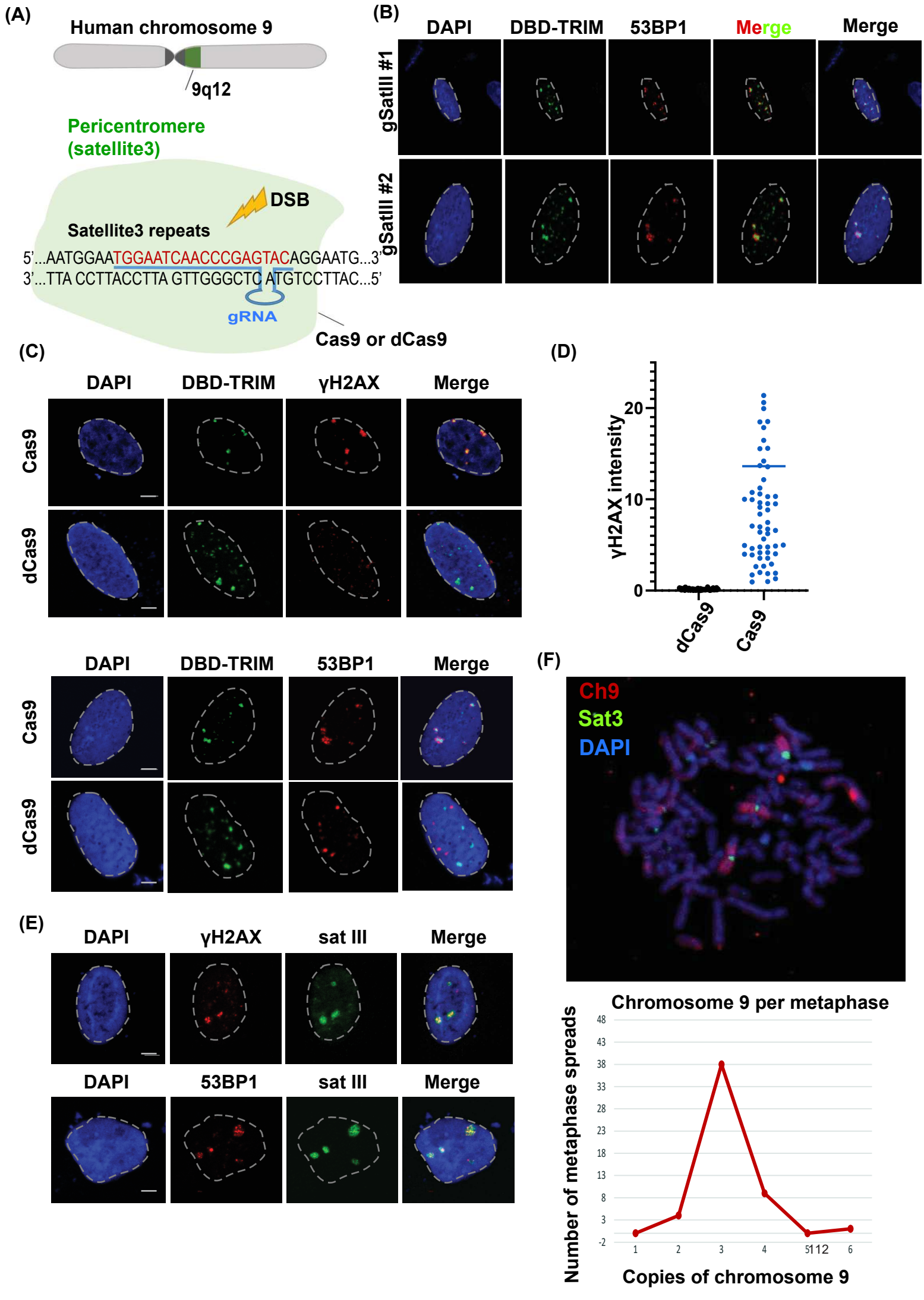

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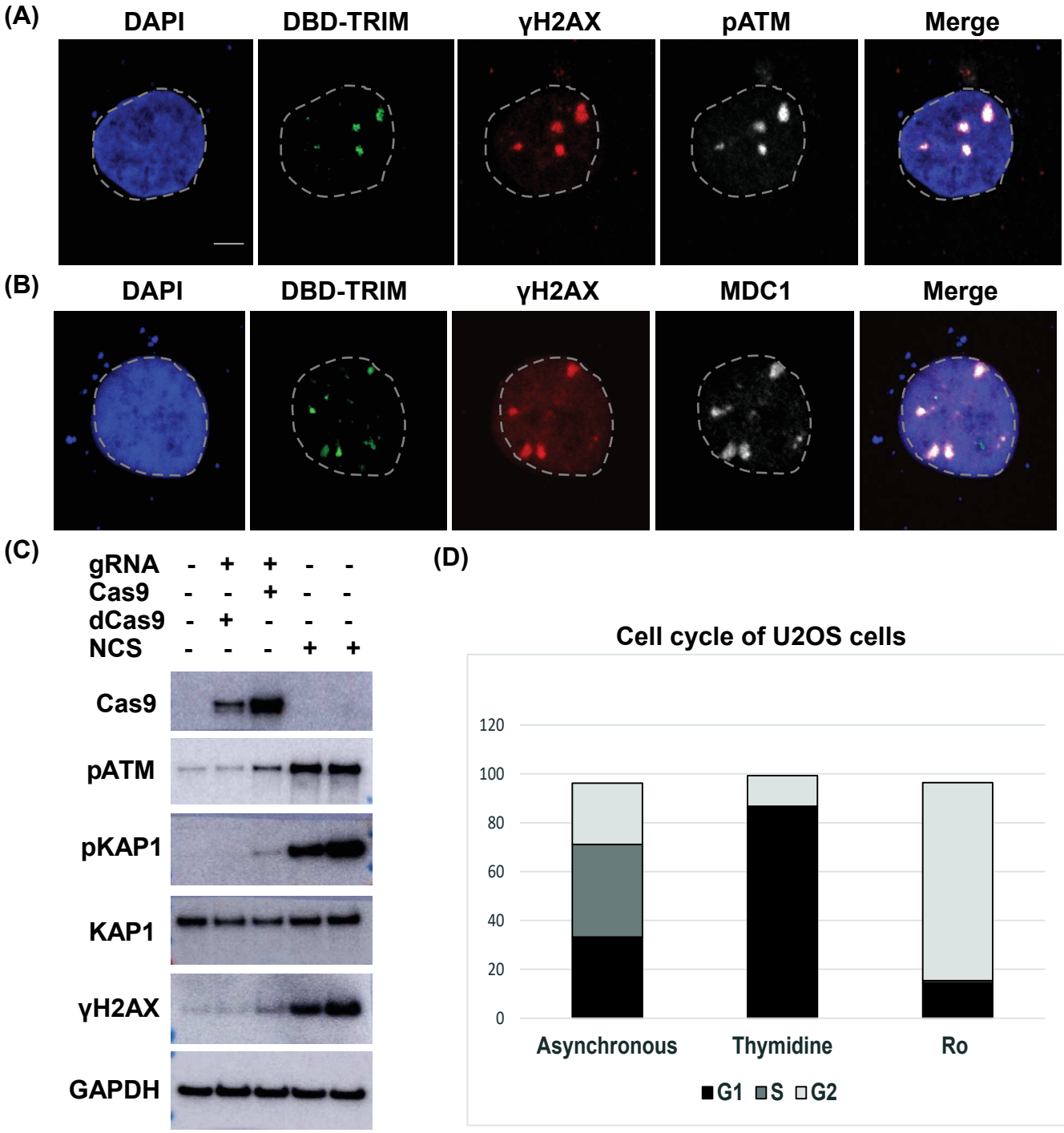

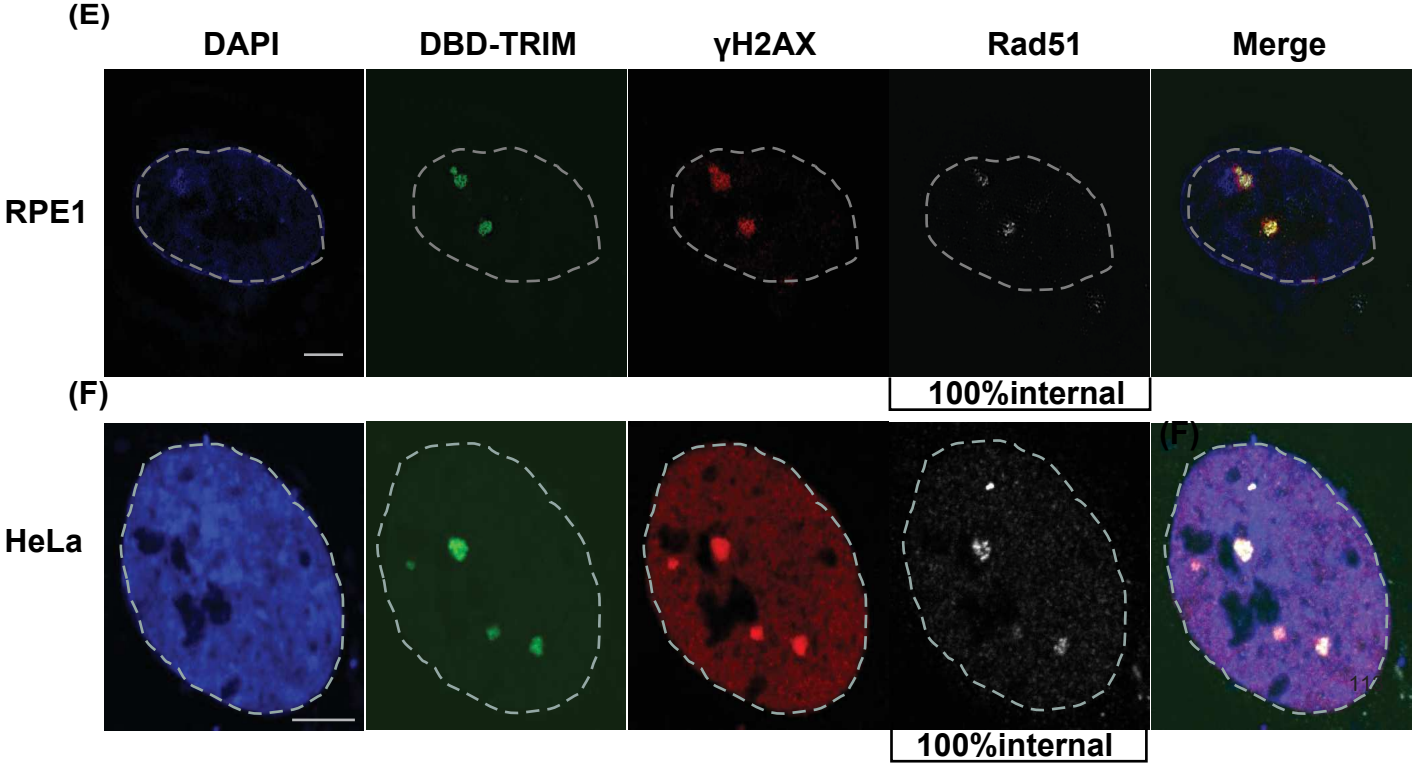

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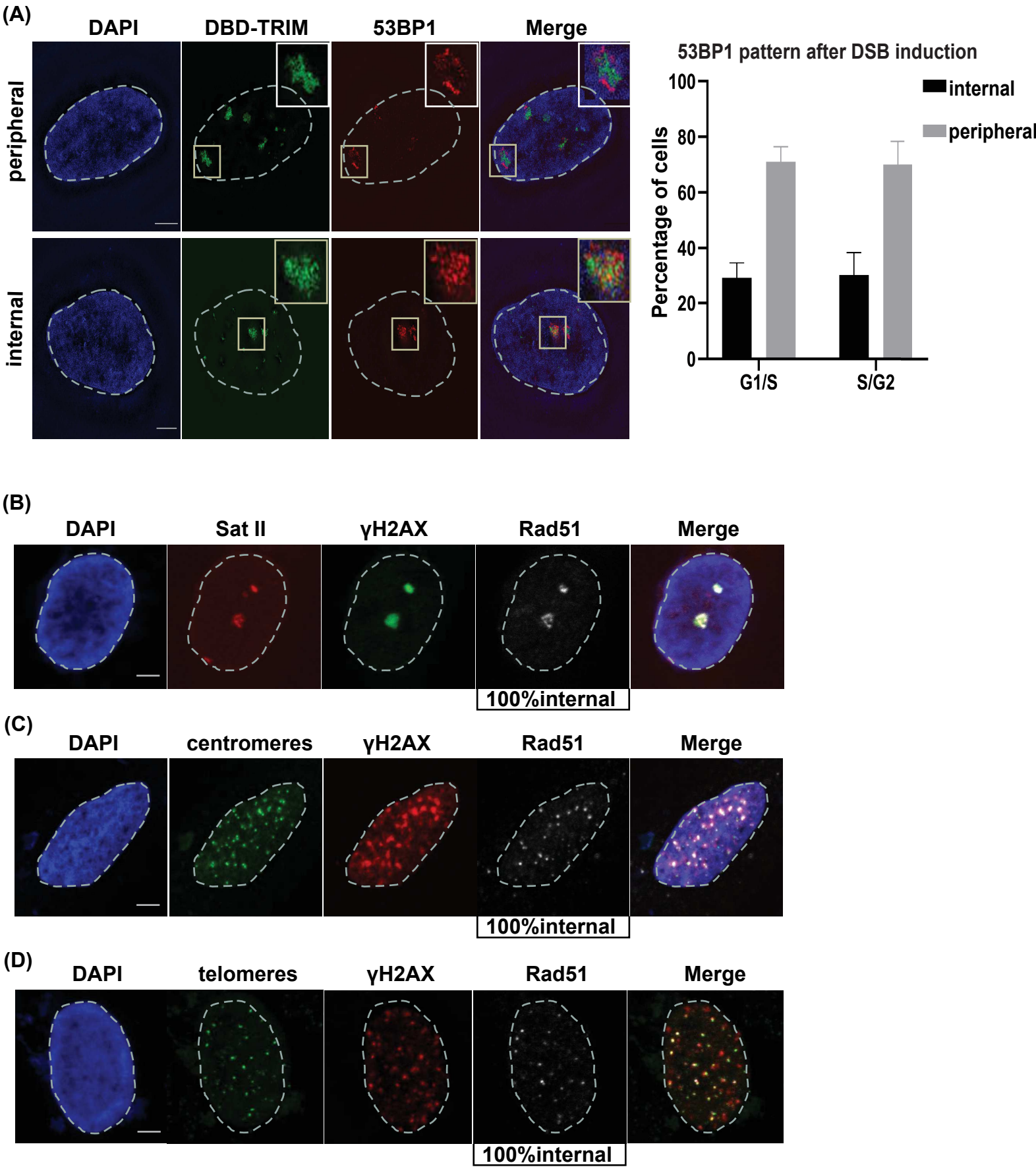

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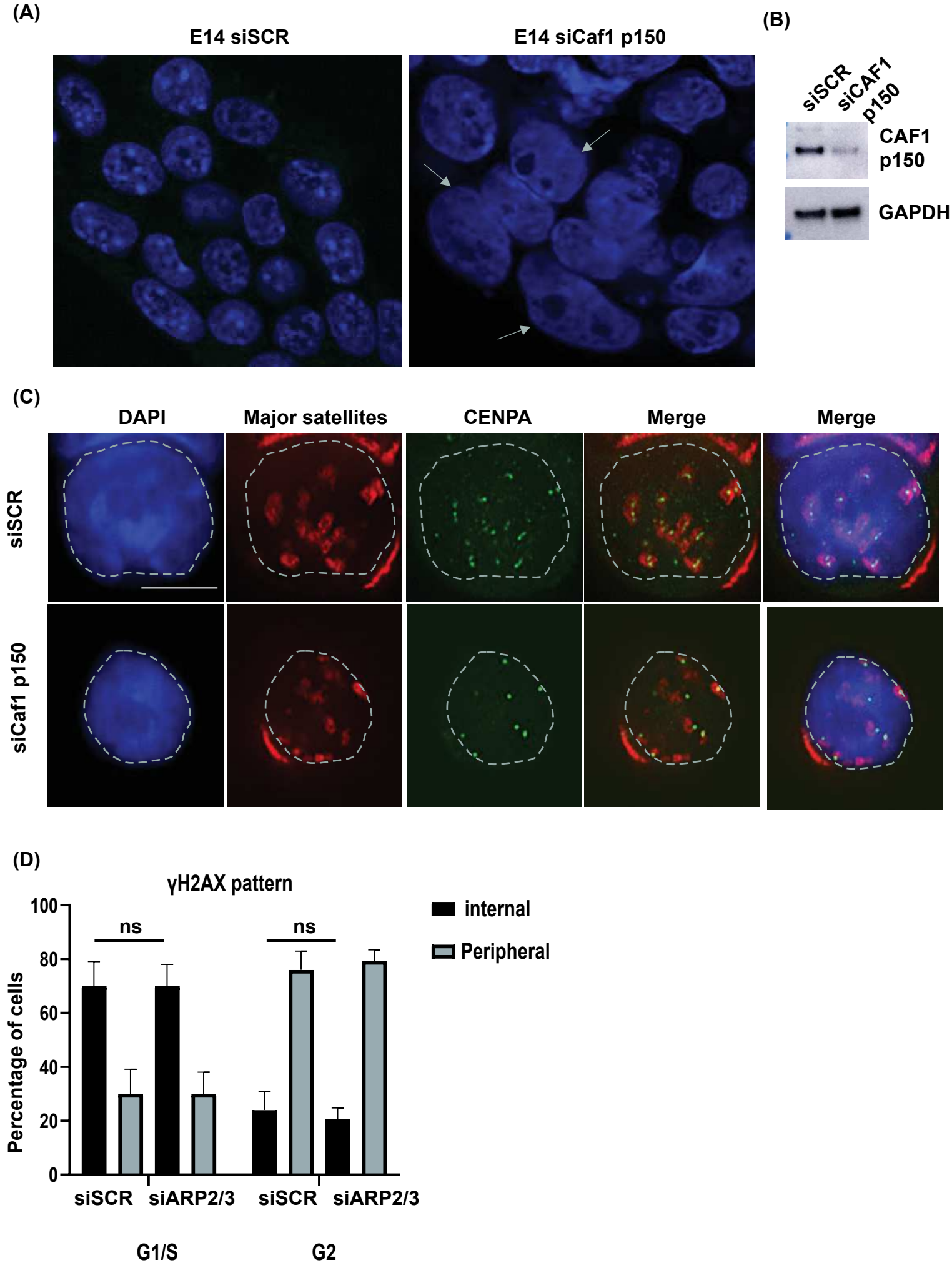

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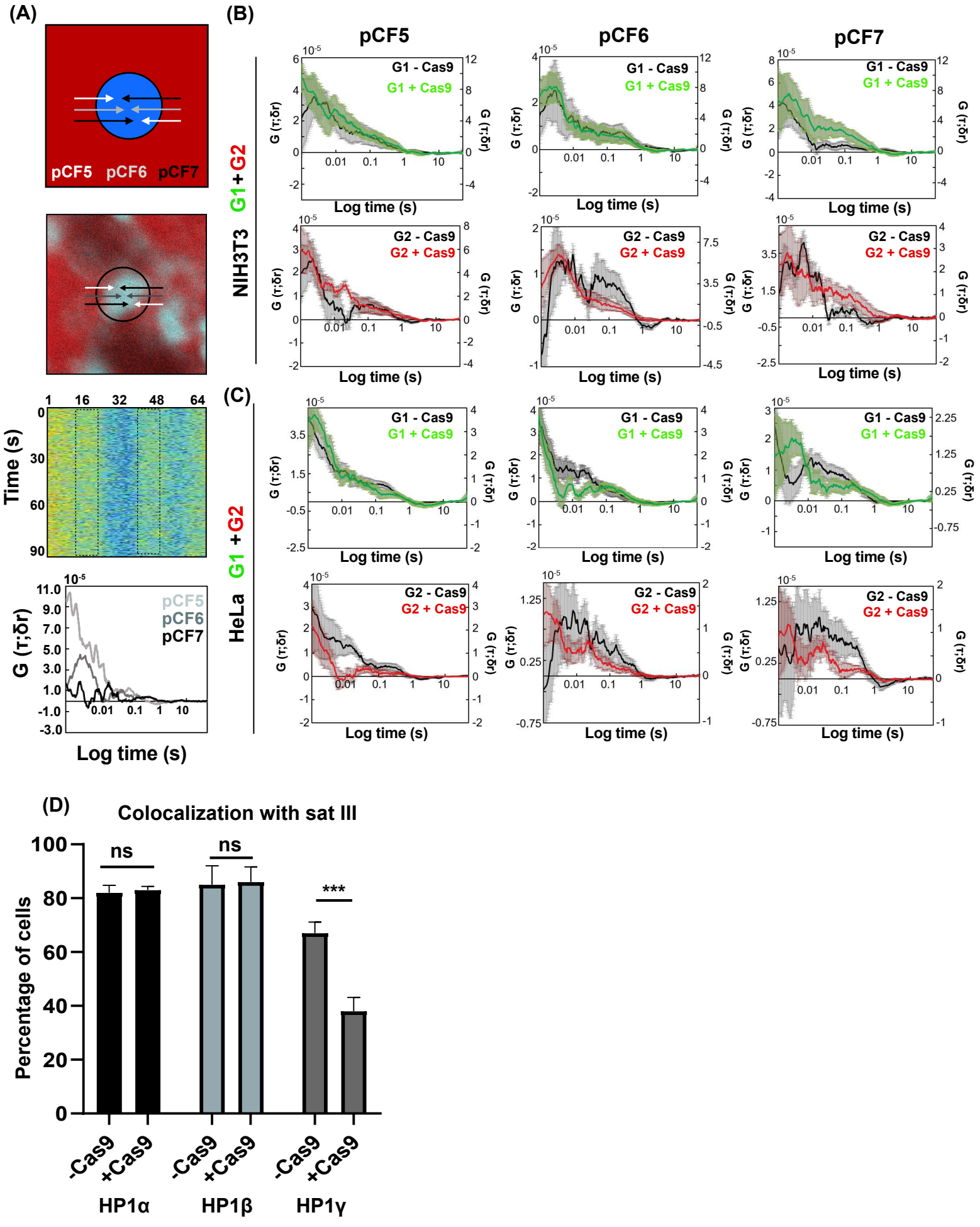

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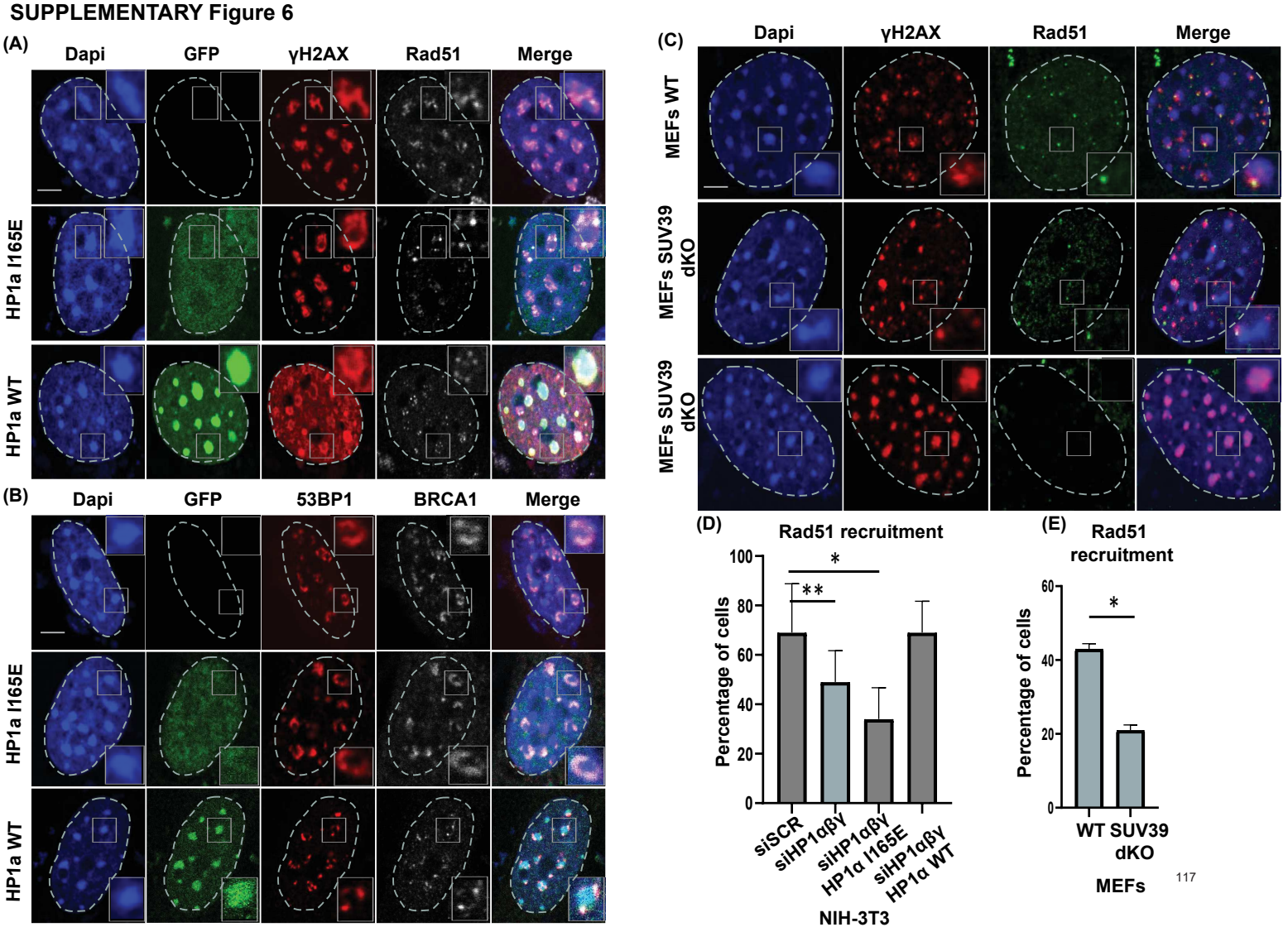

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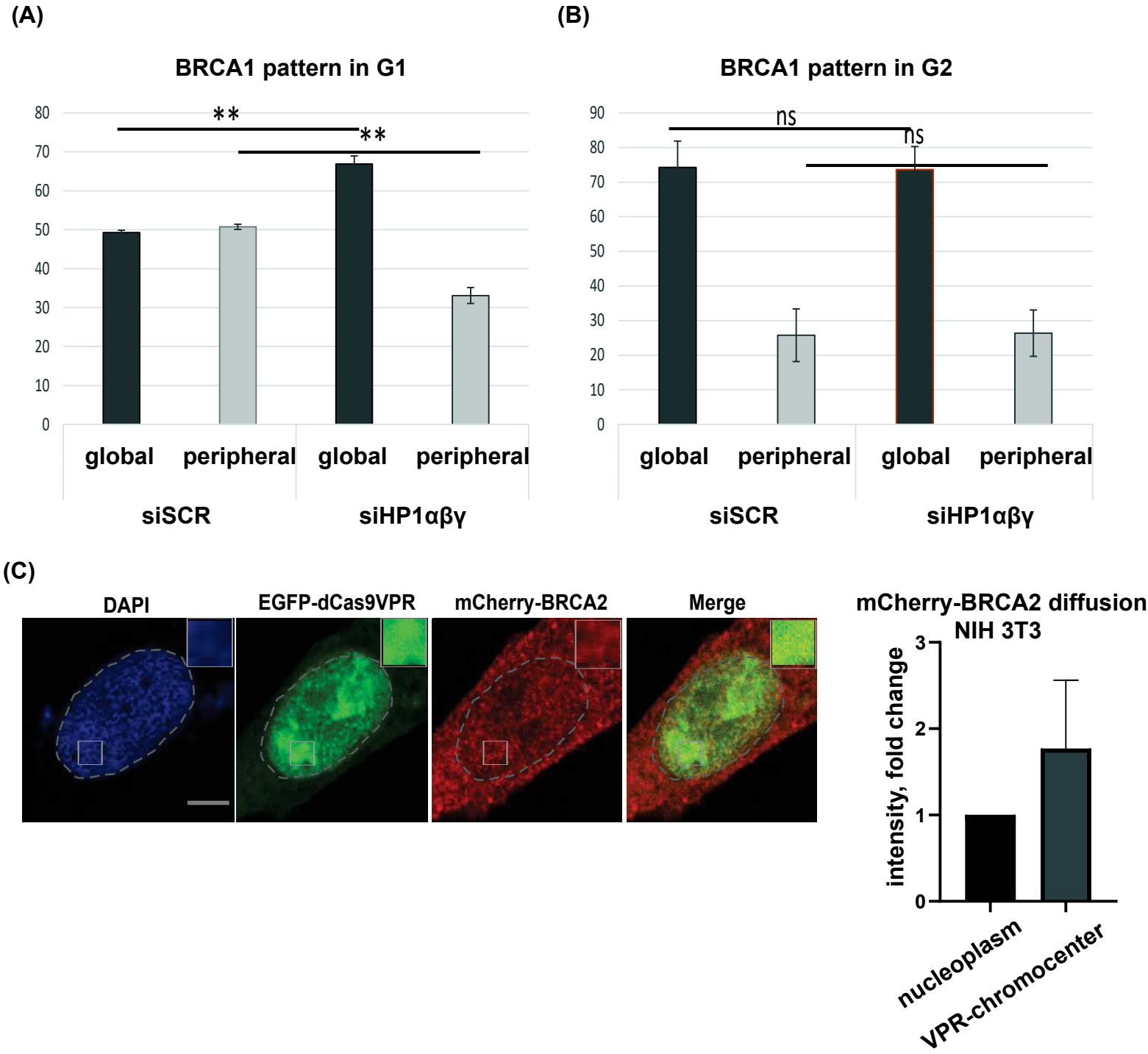

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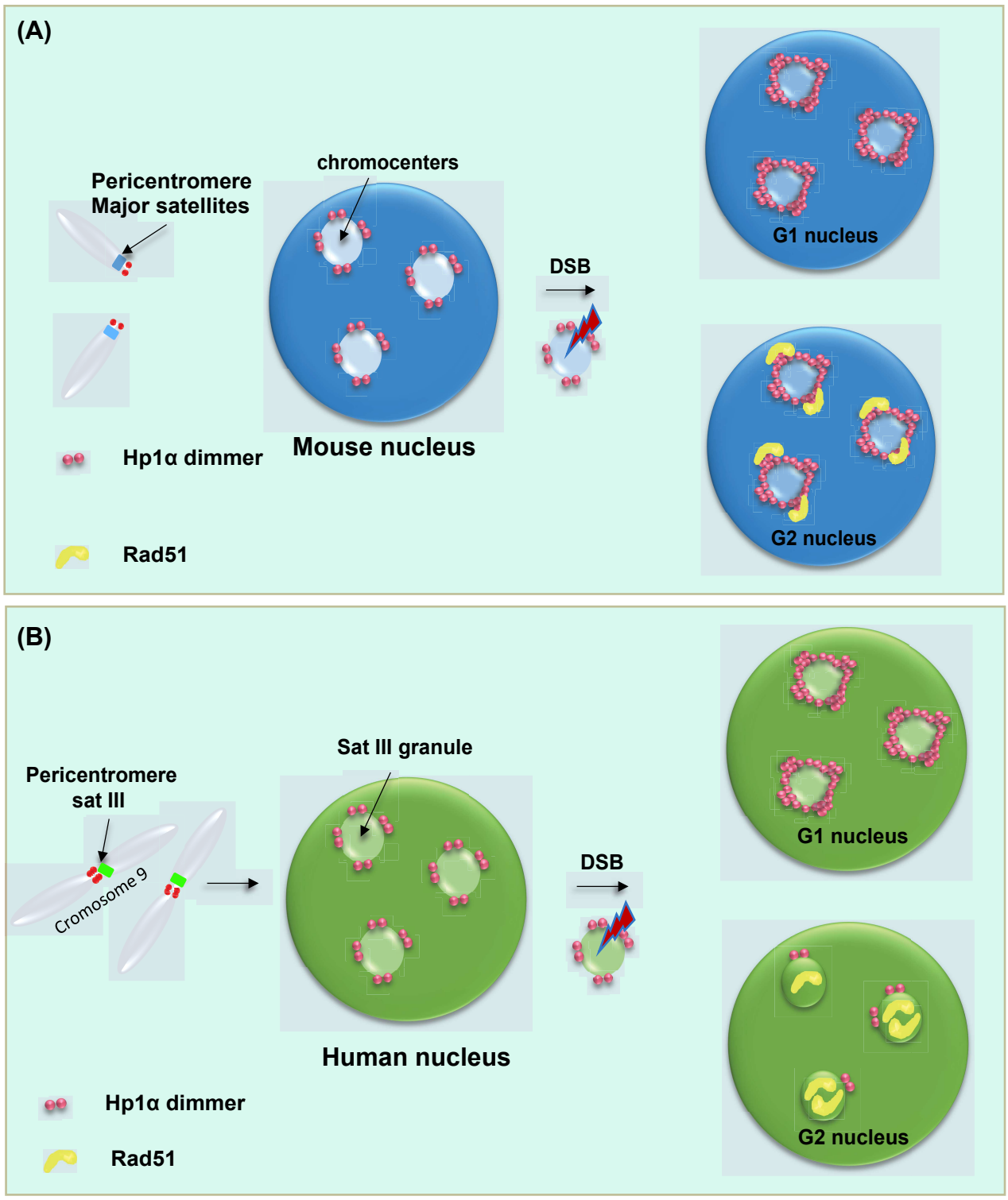

Supplementary Figure Legends

Figure S1. (A) Schematic representation of the experimental system. Immunofluorescence (IF) confocal analysis of **(B)** cells co-expressing DBD-TRIM, Cas9 + two different gRNAs specific for SatIII repeats stained with DAP1 and a 53BP1-specific antibody and **(C)** cells co-expressing DBD-TRIM, a gRNA specific for SatIII repeats and either catalytically inactive dead Cas9 (dCas9) or Cas9 and stained with DAPI and antibodies specific for γH2AX and 53BP1. **(D)** Quantification of γH2AX intensity at sat III domains in cells co-expressing DBD-TRIM, a gRNA specific for SatIII repeats and either catalytically inactive dead Cas9 (dCas9) or Cas9 **(E)** IF combined with Fluorescence *in situ* Hybridization (ImmunoFISH) confocal analysis on U2OS expressing Cas9 + gRNA specific for the SatIII repeats stained with DAPI, a PNA-Sat III probe (green) and antibodies specific for γ-H2AX and 53BP1. **(F)** Representative confocal image of metaphase spreads after FISH with a PNA-SatIII probe (green) and chromosome paint (chromosome 9; red) and quantification of chromosome 9 copies per metaphase in U2OS cells. Data are the mean of 2 experiments (n= 50 cells).

Figure S2. (A-B) Immunofluorescence (IF) confocal analysis of U2OS cells expressing DBD-TRIM + Cas9 + gRNA specific for SatIII repeats stained with DAPI and antibodies specific for □-H2AX, pATM^{S1981} and MDC1. Images are representative of 3 experiments with n=50 cells. **(C)** Western blot analysis for Cas9, γ-H2AX, pATM^{S1981}, KAP1, pKAP1^{S824} and GAPDH in protein extracts prepared from U2OS cells expressing Cas9 + gRNA specific for SatIII repeats or treated with NCS (100 ng/ml and 200 ng/ml). **(D)** Cell cycle analysis by flow cytometry using propidium iodide and EdU in U2OS cells treated or not with double thymidine or RO-3306. **(E)** IF and super resolution

(upper panel) or confocal (lower panel) analysis of RPE1 (upper panel) or HeLa (lower panel) of cells expressing DBD-TRIM-GFP, Cas9 + gRNA targeting SatIII repeats stained with DAPI and antibodies specific for γ-H2AX and Rad51. Wherever a quantification is not provided, 100% of cells exert the phenotype. Scale bars represent 5 µm.

Figure S3. (A) Immunofluorescence (IF) super resolution (3D-SIM) analysis of U2OS cells expressing DBD-TRIM + Cas9 + gRNA targeting SatIII repeats stained with DAPI and a 53BP1-specific antibody. The upper panel represents a peripheral 53BP1 pattern and the lower an internal. Quantification of 53BP1 pattern after DSB induction in either G1/S or S/G2 synchronized U2OS cells is shown on the right. Images are representative of 5 experiments with n=50 cells. Data are the mean ± SD of 5 experiments with n=50 cells. Scale bars represent 5 µm. **(B)** ImmunoFISH confocal analysis or U2OS cells expressing Cas9 + gRNA targeting SatII repeats stained with DAPI, a SatII-specific probe, and antibodies specific for □-H2AX and Rad51. **(C)** IF confocal analysis of U2OS cells expressing Cas9 + gRNA targeting centromeres, stained with DAPI and antibodies specific for centromeres (CREST), γ-H2AX and Rad51. **(D)** ImmunoFISH confocal analysis of U2OS cells expressing Cas9 + gRNA targeting telomeres stained with DAPI, a telomeric-specific probe and antibodies specific for γ-H2AX and Rad51. Wherever a quantification is not provided, 100% of cells exert the phenotype. All images are representative of 3 experiments with n= 50 cells. Scale bars represent 5 µm.

Figure S4. (A) Confocal analysis of E14 cells depleted for Caf1 (right panel) or with a non-targeting control (left panel) stained with DAPI. **(B)** Western blot analysis of CAF1 levels at wt E14 cells and cells depleted of Caf1. **(C)** ImmunoFISH confocal analysis of E14 control (upper panel) or E14 depleted for Caf1 (lower panel) cells expressing Cas9 + gRNA targeting major satellite repeats stained with DAPI, a major satellite-specific probe and a CENP-A specific antibody. Images are representative of 3 experiments with n = 50 cells. Scale bars represent 5 µm. **(D)** Quantification of the □-H2AX pattern at chromocenters of NIH-3T3 cells expressing Cas9 + gRNA specific for the major satellite repeats, either depleted of ARP2/3 (siARP2/3) or with a non-targeting control (siSCR) in G1 or G2 phases of the cell cycle. Data are the mean ± SD of 3 experiments with n=50 cells. For all graphs, statistical significance was determined by t-test (*p<0,05, **p<0,01, ***p<0,001).

Figure S5. (A) Schematic of heterochromatin foci and pCF analysis of mKate2 accessibility into this structure at a distance of δr = 5 (pCF5), 6 (pCF6) and 7 (pCF7) pixels (top panel). Representative example of a heterochromatin foci stained with Hoechst 33342 in a NIH3T3 cell expressing mKate2 that has been selected for pCF analysis. This involves scanning a confocal line scan across this structure rapidly as a function of time, plotting the recorded fluctuations in mKate2 intensity in the form of an intensity carpet and then spatially correlating these fluorescence fluctuations. The resulting pCF5, pCF6 and pCF7 profiles for columns adjacent to the foci that test mKate2 accessibility into the DSB foci are presented. **(B)** pCF5, pCF6 and pCF7 analysis of mKate2 access into NIH3T3 G1 versus G2 heterochromatin foci before and then after DSB induction track the spatial evolution of this transit in mouse cells (N = 3-5 measurements

across N = 2-4 cells and 2 biological replicates). **(C)** pCF5, pCF6 and pCF7 analysis of mKate2 access into HeLa G1 versus G2 heterochromatin foci before and then after DSB induction track the spatial evolution of this transit in human cells (N = 5-7 measurements across N = 4-6 cells and 2 biological replicates). **(D)** Quantification of HP1α, Hp1β or HP1γ colocalization with SaIII repeats in U2OS cells expressing a gRNA specific for SatIII repeats with or without Cas9-specific DSB induction. Data are the mean ± SD of 3 experiments with n=50 cells. Statistical significance was determined by t-test (*p<0,05, **p<0,01, ***p<0,001).

Figure S6. Immunofluorescence (IF) confocal analysis of G2- synchronised NIH-3T3 cells expressing Cas9 + gRNA targeting major satellite repeats, depleted of Hp1αβ (upper panel) and complemented with HP1α^{I165E}-GFP (middle panel) or HP1α-GFP (lower panel) stained with **(A)** DAPI and antibodies specific for γ-H2AX and Rad51 or **(B)** DAPI and antibodies specific for 53BP1 and BRCA1. Images are representative of 3 experiments with n= 50 cells. **(C)** IF confocal analysis of WT MEFs (upper panel) or SUV3/9 dKO MEFs (middle and lower panels) expressing Cas9 + gRNA targeting major satellite repeats stained with DAPI and antibodies specific for γ-H2AX and Rad51. Images are representative of 3 experiments with n=50 cells. Quantification of **(D)** Rad51 recruitment in G2-syncrhonised NIH-3T3 cells expressing Cas9 + gRNA targeting major satellite repeats, depleted of HP1αβγ (siHP1αβγ) and complemented with HP1α^{I165E}-GFP or WT HP1α-GFP and **(E)** Rad51 recruitment in WT MEFs and SUV3/9 dKO MEFs expressing Cas9 + gRNA targeting major satellite repeats. Data are the mean ± SD of 3 experiments with n=50 cells. For all graphs, statistical significance was determined by t-test (*p<0,05, **p<0,01, ***p<0,001). Scale bars represent 5 μm.

Figure S7. Quantification of BRCA1 pattern in U2OS cells expressing Cas9 + gRNA targeting SatIII repeats, depleted of HP1αβγ (siHP1αβγ) or transfected with a non-targeting control cells (siSCR) in **(A)** G1/S and **(B)** S/G2 phase of the cell cycle. Data are the mean ± SD of 3 experiments with n=50 cells. For all graphs, statistical significance was determined by t-test (*p<0,05, **p<0,01, ***p<0,001). **(C)** IF confocal analysis of NIH-3T3 cells expressing EGFP-dCas9-VPR + gRNA targeting major satellites + mCherryBRCA2 and quantification of BRCA2 intensity in heterochromatin area versus the nucleoplasm. Statistical significance was determined by one-way ANOVA (*p<0,05, **p<0,01, ***p<0,001, ****p<0.0001). **(D)** IF confocal analysis of NIH-3T3 cells expressing dCas9 fused to EGFP and BRC3 (dCas9-BRC3-EGFP) + gRNA targeting major satellites, stained with DAPI and antibodies specific for γ-H2AX and Rad51. Images are representative of 6-7 experiments .

Figure S8. Model of the spatial activation of DNA repair pathways in DSBs occurring in mouse and human heterochromatin.

Materials and Methods

Cell culture

NIH-3T3 mouse fibroblasts were maintained in high glucose DMEM supplemented with 10% newborn calf serum and gentamycin (40 mg/ml). U2OS human osteosarcoma cells were maintained in low glucose DMEM supplemented with 10% Fetal calf serum and gentamycin (40 mg/ml). HeLa (human cervical carcinoma) cells were maintained in high glucose DMEM supplemented with 10% fetal calf serum and gentamycin (40 mg/ml). RPE1 (human, retina epithelial) cells were maintained in DMEM/F12 with GLUTAMAX-I, which was supplemented with 10% Fetal calf serum and gentamycin (40 mg/ml). E14 cells (mouse embryonic stem cells) were maintained in high glucose DMEM supplemented with 10% Fetal Calf Serum, MEM Non-essential amino acids, L- Glutamine (200mM), β- mercaptoethanol (50 mM), Leukemia Inhibiting Factor and Penicilin/Streptomycin (40 mg/ml).

Cell treatments

Cells were synchronized in the G2 phase of the cell cycle with the Cdk1 inhibitor IV RO-3306 (217699; Calbiochem; 10 μM). For NIH-3T3 cells, the inhibitor was added 8h before transfection. with the cells fixed 16h after transfection. For the human cell lines (U2OS, HeLa, RPE1), the Cdk1 inhibitor was added 16h before transfection and cells were fixed 8 afterward transfection, thus, in both cases leading to a total of 24h treatment. Cells were arrested in G1/S phase of the cell cycle with a double-thymidine (T1895; Sigma) block: 18h thymidine treatment (2 mM), 9h release, 16h thymidine treatment (2 mM). Cells were then transfected for 8h prior to fixation.

Cell-cycle arrest was confirmed by flow cytometry. Neocarzinostatin (NCS; N9162-100 UG; Sigma) was added (100 or 200 ng/ml), 15 min later medium was replaced and cells were harvested for Western blot analysis 1 h later.

Cell cycle analysis

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

Transfection

Transient transfections were performed with Lipofectamine 2000 (Invitrogen Life Technologies) for NIH-3T3 and E14 cells and JetPei (Polyplus transfection) for U2OS, HeLa and RPE1 cells according to the manufacturer's instructions.

Western Blot analysis

Proteins were fractionated by SDS-PAGE and transferred to Protran Nitrocellulose membranes (Sigma Aldrich) and blotted with the antibodies listed in **Table 1**.

Immunofluorescence – immuno FISH and Confocal and Super-resolution Microscopy

Cells were cultured on coverslips and pre-extracted in 0.1% Triton/1X PBS for 30 s prior to fixation in 4% paraformaldehyde (PFA)/1X PBS for 10 min on ice. After a second fixation step of 4% PFA/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 1h and incubated with primary antibody (in 1% BSA/1X PBS/0.1%Tween) for 1 hr (see **Table 1** for antibodies) and secondary antibody (Alexa Fluor , Thermofisher) for 1 hr (in 1% BSA/1X PBS/0.1%Tween). Cells were counterstained with DAPI (1 mg/ml) and mounted on slides(Mitrentsi and Soutoglou, 2021). For EdU incorporation, the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen) was used. For immuno-FISH, the same protocol was used, except the secondary antibody incubation was followed by a post-fixation in 4% PFA/PBS for 10 min. Cells were washed in 2X SSC for 45 minutes, at 72 °C. This was followed by sequential washes with 70% and absolute ethanol. The coverslips were dried at room temperature, incubated with 0.1N NaOH for 10 minutes and washed with 2X SSC. Then they were washed with 70% ethanol and absolute ethanol. After drying, cells were hybridized with DNA probe (major satellite repeats) for 30sec at 85°C and incubated overnight at 37°C.

The immuno-FISH probe was prepared by nick translation from the major satellite repeats containing plasmid (see **table 2**). Probe DNA (0.3 mg) was mixed with 9 μg of ssDNA and precipitated with 2.50 vol of ethanol and 1/10 vol of 2.5M sodium acetate for 1h at -80°C. After 20 min of centrifugation, the supernatant was discarded, and the pellet was washed with70%ethanol and centrifuged again for 5min.The supernatant was discarded, and the pellet was dried. The pellet was resuspended in hybridization solution (50%formamide, 2X SSC, 10%

dextran sulfate) (20 µl per coverslip) by vortexing for 1h.The probe was denaturated for 5 min at 90°C and pre annealed for at least 30 min at 37°C before hybridization with cells.

The day after hybridization, coverslips were washed twice in 2X SSC for 20 minutes at 42°C. Finally, the cells were incubated with the secondary antibody (AlexaFluor Thermofisher) and Neutravidin-Texas Red (Thermofisher). Cells were counterstained with DAPI (1 mg/ml) and mounted on slides. Cells were observed on a confocal laser scanning microscope (TCS SP8; Leica) using a 633 objective, an OMX BLAZE 3D-structured illumination, super-resolution microscope or on a NIKON inverted confocal spinning disk microscope with a GATAKA Live SR module.

Sat III PNA FISH– immunofluorescence

For sat III staining, FISH was performed before the immunofluorescence. More specifically, cells were fixed in 4% PFA/PBS for 10 minutes in RT, permeabilized for 10 minutes with Triton 0.5%/PBS and washed three times with PBS. Next cells were dehydrated through an ethanol series of 50%, 70% and absolute for 3 minutes each at RT and coverslips were air-dried. Hybridization buffer (70% formamide, 10mM Tris pH 7.2) containing 50nM of PNA sat III probe (see **table 4**) was added on a slide, covered with the coverslip containing the cells and they were denatured on an 80°C hot plate for 3 minutes. This was followed by hybridization for 2h at RT. Coverslips were then washed twice in 70% formamide, 10mM Tris pH7.2 for 15 minutes, then three times in 50mMTris pH7.5/150mM NaCl/ 0.05% Tween for 5 minutes and finally twice with PBS. Subsequently, the cells were blocked in 5% BSA/1X PBS/0.1% Tween for 1h and incubated with primary antibody (in 1% BSA/1X PBS-0.1%Tween) for 1 hr (see **table 1** for antibodies) and

secondary antibody for 1 hr (in 1% BSA/1X PBS-0.1%Tween). Cells were counterstained with DAPI (1 mg/ml) and mounted on slides.

Immuno-FISH sat II

Cells were permeabilized in ice-cold CSK Buffer (10 mM HEPES-KOH, pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2) containing 0.5% Triton X-100 for 5 min, washed in CSK Buffer for 5 min, and then fixed with 2% PFA/2% sucrose for 10 min, on ice. They were then permeabilized in cold 0.4% Triton X-100 / 1X PBS for 10 min on ice. Next cells were blocked in 5% BSA/1X PBS/0.1% Tween for 1h and incubated with primary antibody (in 1% BSA/1X PBS/0.1%Tween) for 1 hr (see **table 1** for antibodies) and secondary antibody for 1 hr (in 1% BSA/1X PBS-0.1%Tween). This was followed by a post-fixation with 2% PFA/2% Sucrose for 10 min on ice and cells were incubated with 4 mg/mL RNase A in 2X SSC for 30 min, at 37 °C. Cells were re-permeabilized with cold 0.7% Triton X-100 / 0.1M HCl for 10 min on ice, and denatured in 2M HCl for 30 minutes at RT. After three quick rinses in cold 1X PBS, cells were incubated with Hybridization Buffer (100 ng/µL salmon sperm DNA, 5X Denhart's solution, 10% dextran sulfate, 5X SSC, 50% formamide) containing 10 ng/µL sat II FISH probe (See **table 4**) overnight in a dark and humid chamber, at 37°C. The next day, coverslips were washed three times in 2X SSC/0.1% Tween for 5 min at RT and blocked with 5% BSA/1X PBS/0.1% Tween for 30 min at RT. Subsequently, cells were incubated with Streptavidin and secondary antibody for 1 hr (in 1% BSA/1X PBS/0.1%Tween). Finally, Cells were counterstained with DAPI (1 mg/ml) and mounted on slides.

Metaphase spreads and Fluorescence in Situ Hybridization (FISH)

Colcemid was added in the cells (0.02 µg/ml, 15210040; Fisher Scientific) for 4 h. Cells were trypsinized, harvested, and the resulting cell pellet was resuspended in a prewarmed 0.06M KCl solution and incubated for 30 min at 37°C. The cell pellet was fixed in an ethanol:glacial acetic acid (3:1) solution, 3 times. The following day, metaphase chromosomes were spread on wet cold glass slides, and air-dried. The spreads were fixed with 4% PFA/PBS for 4 min at 37°C, and treated with RNAse A solution (100 µg/ml in 2X SSC) for 1h at 37°C. Afterwards cells were dehydrated through an ethanol series (70%, 85%, absolute) and air-dried. DNA was denatured at 85°C for 10 minutes after the addition of 0.07µM PNA probe and/or whole chromosome 9 probe (see **table 4**). This was followed by hybridization for 2h at room temperature. Slides were washed twice in SSC/0.1% Tween at 60°C for 10 min. DNA was counterstained with DAPI (1mg.ml) and mounted with a long coverslip.

Plasmid construction

Individual gRNAs (see **table 3**) were cloned into a vector containing the U6 promoter followed by a gRNA scaffold. All plasmids (see **table 2**) were assembled by golden gate cloning (Engler et al., 2009).

Confocal Laser Scanning Microscopy.

All microscopy measurements were performed on an Olympus FV3000 laser scanning microscope coupled to an ISS A320 Fast FLIM box for fluorescence fluctuation data acquisition. A 60X water immersion objective 1.2 NA was used for all experiments and the cells were imaged at 37 degrees

in 5% CO2. For single channel Number and Brightness (NB) fluorescence fluctuation spectroscopy (FFS) measurements HP1 α-RFP657 were excited by a solid-state laser diode operating at 640 nm and the resulting fluorescence signal directed through a 405/488/561 dichroic mirror to a photomultiplier detector (H7422P-40 of Hamamatsu) fitted with an RFP657 500/25 nm bandwidth filter. For the pair correlation function (pCF) FFS measurements mKate2 were excited by solid-state laser diodes operating at 561 nm and the resulting signal was directed through a 405/488/561 dichroic mirror to two internal GaAsP photomultiplier detectors set to collect 600-700 nm.

Microscopy data acquisition. NB FFS measurements of HP1 α-RFP657 involved selecting a region of interest within a NIH-3T3 and Hela cell nucleus at a zoom of 20 ,which for a 256 x 256 pixel frame resulted in a pixel size of 41 nm, and acquiring a frame scan acquisition (n = 100 frames) with the pixel dwell time set to 12.5 µs, which resulted in a line time of 4.313 ms and a frame time of 1.108 s. pCF FFS measurements of mKate2 involved selecting a 5.3 µm line across the middle of 53BP1 or TRIM foci, which for a 64 x1 pixel line resulted in a pixel size of 83 nm, and acquiring a two channel line scan acquisition (n = 100,000 lines) at maximum speed (8 µs pixel dwell time / 1.624 ms line time).

Number and brightness (NB) analysis. The brightness of a fluorescently tagged protein is a readout of that protein's oligomeric state that can be extracted by a moment-based Number and brightness (NB) analysis of an FFS frame scan acquisition, as described in previously published paper (Digman et al., 2008; Digman et al., 2009; Qian and Elson, 1990). In brief, within each pixel

of the frame scan we have an intensity fluctuation that has an average intensity (first moment) and a variance (second moment), where the ratio of these two properties describes the apparent brightness (B) of the molecules that give rise to the intensity fluctuation.

The true molecular brightness (ε) of the molecules is related to the measured apparent brightness (B) by $B = \varepsilon + 1$, where 1 is the brightness contribution of our photon counting detector. Calibration of the apparent brightness of monomeric HP1α(I165E)-RFP657 ($B_{monomer}$ = 1.15) enabled extrapolation of the expected apparent brightness of HP1α-RFP657 dimers (B_{dimer} = 1.30) which in turn enabled definition of brightness cursors to extract and spatially map the fraction of pixels within a given frame scan acquisition that contain these different species. An intensity-based mask was used to quantify HP1α-RFP657 dimers in the nucleoplasm versus within foci. Artefact due to cell movement or photobleaching were subtracted from acquired intensity fluctuations via use of a moving average algorithm. All brightness calculations were carried out in SimFCS from the Laboratory for Fluorescence Dynamics (www.lfd.uci.edu).

Pair correlation function (pCF) analysis. pCF analysis of spatially distinct fluorescence fluctuations acquired along a confocal line scan acquisition can track the evolution of protein transport, as described in previously published papers(Digman and Gratton, 2009; Hinde et al., 2013; Hinde et al., 2016). In brief, in each pixel of a line scan we have an intensity fluctuation that we format into an intensity carpet, where the *x*-coordinate corresponds to the point along the line (pixel) and the *y*-coordinate corresponds to the time of acquisition. The carpet data format

enables temporal cross correlation of pairs of intensity fluctuations separated by a set distance (δr) for allpossible delay times (τ).

The pCF profile that results from correlation of intensity fluctuations reports the time (τ) it takes a population of molecules to translocate a set distance (δr). Thus, given that the FFS line scan measurements presented here were acquired with a pixel size of ~80 nm, the spatial evolution of mKate2 transport was tracked by performing pCF analysis at a distance of δr = 6 (pCF6= 480nm). This distance enabled quantification of the time mKate2 takes to diffuse onto a 53BP1 or TRIM foci.

Artefact due to cell movement or cell bleaching were subtracted from acquired intensity fluctuations via use of a moving average algorithm. All pCF calculations were carried out in SimFCS from the Laboratory for Fluorescence Dynamics (www.lfd.uci.edu).

Statistics and figure preparation. Statistical analysis was performed by using GraphPad Prism software. Figures were prepared by use of Adobe Illustrator, MATLAB, and SimFCS.

Table 1

Antibodies used		
Antibody	**Company (reference)**	**Dilution (Use)***
g-H2AX (H2AX S139)	Abcam (ab22551)	1:1000 (IF & WB)
53BP1	Novus Biologicals (100-304)	1:1000 (IF)
pATM (S1981)	Abcam (ab81292)	1:500 (IF), 1:1000 (WB)
KAP1	Euromedex (1TB1A9)	1:1000 (IF & WB)
MDC1	Made in IGBMC	1:500 (IF)
RAD51	Calbiochem (PC130)	1:100 (IF)
RPA32	Novus Biologicals (600-565)	1:250 (IF)
CREST	Antibodies Incorporated (15-235-F)	1:500 (IF)
mouse CENP-A	Cell Signaling Technology (2048)	1:500 (IF)
BRCA1 (for mouse cells)	Gift from Dr. Andre Nussenzweig	1:200 (IF)
BRCA1 (for human cells)	Santa Cruz (sc-642)	1:100 (IF)
RIF1 (N20)	Santa Cruz (sc55979)	1:100 (IF)
GAPDH	Millipore (MAB374)	1:5000 (WB)
Cas9	Diagenode (C15200203)	1: 1000 (WB)

*****IF: immunofluorescence, WB: Western Blot

Table 2

Plasmids used*	
pCX-5	CMVp-Cas9-EGFP-SV40p-PuroR-pA
pCX-15	CMVp-dCas9-EGFP-SV40-PuroR-pA
pX-86	U6p-gRNA(Ma-Sat#3)-CMVp-Cas9-mCherry-SV40p-HygroR-pA
pG-56	U6p-gRNA (Ma-Sat#3)-gRNA Scaffold
pX-473	U6p-gRNA (satIII #347)-SV40p-Cas9-EGFP-PuroR-pA
pX-474	U6p-gRNA (satIII #348)-SV40p-Cas9-EGFP-PuroR-pA
pX-475	U6p-gRNA (satIII #349)-SV40p-Cas9-EGFP-PuroR-pA
pX-476	U6p-gRNA (satIII #350)-SV40p-Cas9-EGFP-PuroR-pA
pX-479	U6p-gRNA (satIII #349)-SV40p-dCas9-EGFP-PuroR-pA
pX-789	U6p-gRNA(Ma-Sat#3)-SV40p-EGFP-dCas9-hRad51-PuroR-pA
satII-Cas9	U6p-gRNA (satII)-SV40p-Cas9-EGFP-PuroR-pA
hCentromere-Cas9	U6p-gRNA (hCentromere)-SV40p-Cas9-EGFP-PuroR-pA
hTelomere-Cas9	U6p-gRNA (hTelomere)-SV40p-Cas9-EGFP-PuroR-pA
EGFP-dCas9-BRC3	U6p-gRNA(Ma-Sat#3)-SV40p-EGFP-dCas9-BRC3-PuroR-pA
EGFP-dCas9-VPR	Gift from Dr. Fabian Erdel
D1-GFP	Gift from Dr. Yamashita
DBD-TRIM -EGFP	Gift from Dr. Claire Vourch
mKate2	Gift from Dr. David Stroud
HP1a-RFP657 eGFP-53BP1 Major satellite repeats mCherry BRCA2	 Addgene, 60813 Gift from Dr. Maria-Elena Torres Padilla Gift from Dr. Judit Jimenez Sainz

*plasmids are available upon request

Table 3

Guide RNAs used	***Sequence***
g347 (sat III #1)	AATGGAATCAACACGAG
g349 (sat III #2)	TGGAATCAACCCGAGTAC
hCentromeres	GAATCTGCAAGTGGATATT
hTelomeres	Gift from Dr. Bernardo Reina San Martin
g501 (sat II)	CCAGTGTGAGCATCATCGAA

Table 4

Probes for FISH		
Probe	**Company (reference)**	**Sequence**
TelG-Alexa488 PNA	Panagene (F1008)	G-rich probe (repeats of TTAGGG)
Sat III PNA - Alexa488	Panagene	Biotin-O-TTCCATTCCATTCCATTCCA
Sat II	Sigma	Biotin-TCGAGTCCATTCGATGAT-Biotin
Human chromosome 9	Applied Spectral Imaging (FPRPR0166)	

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4. Discussion

CRISPR/Cas9 induced DSBs in human pericentromeric heterochromatin remain positionally stable throughout the cell cycle

In our recently submitted work, we show that heterochromatic DSB repair is not entirely conserved in different species due to the specific spatial arrangement of heterochromatin and its biophysical properties. Previously our team showed that in mouse chromocenters CRISPR/Cas9 induced DSBs are positionally stable in G1 and recruit NHEJ factors, whereas in S/G2, DSBs are resected and relocate to the periphery of heterochromatin, where they are retained by RAD51 (Tsouroula, Furst et al. 2016). Even though there was a lot of progress made in studying those mechanisms in yeast (Lambert, Mizuno et al. 2010), drosophila (Chiolo, Minoda et al. 2011, Caridi, D'Agostino et al. 2018, Schrank, Aparicio et al. 2018) or mouse cells (Tsouroula, Furst et al. 2016), little was known about human heterochromatic DSB repair.

Here, for the first time we have developed a CRISPR/Cas9 system to specifically target and visualize DSBs in human pericentromeric heterochromatin, and our results demonstrated fundamental differences between mouse and human cells' response. In detail, we designed a gRNA targeting sat III pericentromeric regions of human cells and by using confocal and high-resolution imaging techniques we showed that these DSBs are homogenously distributed in the sat III granules and efficiently activate the DDR pathway. Notably, this was not a massive DDR activation as compared to breaks induced by radiomimetic drugs, or Cas9 breaks in mouse heterochromatin. A possible explanation is the limited breaks that we are inducing, as sat III repeats are mainly located on chromosome 9 (Jolly, Konecny et al. 2002), whereas in mouse heterochromatin the breaks are induced at the pericentromeres of all chromosomes.

Since previous data in other model organisms had shown a break relocation that is specific to the cell cycle phase (Tsouroula, Furst et al. 2016), we first wanted to investigate whether their localization would also be altered depending on the different cell cycle phases. Interestingly, after our synchronizations of the cells either in G2 (using Ro-3306 inhibitor) or in G1/S (using double thymidine block), we showed that there was no relocation of the DSBs, which were remaining positionally stable throughout the cell cycle. Thus, we hypothesized that DSB repair is not entirely conserved in different species due to the specific spatial arrangement of heterochromatin, and we focused our interest on human pericentromeric heterochromatin.

Cas9-induced DSBs in human pericentromeric heterochromatin and repair pathway choice

Having shown that the DSBs induced on human sat III pericentromeric regions are positionally stable throughout the cell cycle, we studied the activation of HR and NHEJ pathways. We find that in different human cell lines, HR was activated inside the core domain of sat III domains, revealed by the visualization of characteristic resection and strand invasion steps' factors (RPA, BRCA1 and Rad51). On the other hand, NHEJ promoting factors, like RIF1 and 53BP1 were recruited both inside and outside the satellite III granule, showing a mirror image with the HR factors. In S/G2, RIF1 and 53BP1 exerted a peripheral localization as HR takes place predominantly within the sat III domains. In G1 however, their localization varied and was mutually exclusive with the localization of BRCA1 as reported before (Panier and Boulton 2014). Consistent with the literature, the recruitment of BRCA1 at DSBs is not restricted in S/G2 phases of the cell cycle. BRCA1 was recruited in DSBs at sat III domains in a large fraction of cells in G1. Interestingly, the 50% cells in which BRCA1 is recruited at sat III domains in G1, it is recruited at periphery of the domain. In these cells, NHEJ promoting factors are visualized within the core of the domain suggesting that NHEJ is the predominant pathway in these cells. BRCA1 is however recruited within the satIII domain in the other 50% of G1 cells. It is possible that these are the late G1 cells that prepare to enter S phase and BRCA1 is entering the domain to prepare for end resection. The mechanism that controls the shift of BRCA1 and NHEJ factors localization from the periphery to the core of the Sat III domain remains unknown.

Ochs et al. recently reported the existence of 53BP1 microdomains (53BP1-MDs) that label damaged chromatin by a ring of 53BP1. This event is followed by RIF1 recruitment, to stabilize the 3D topology of the broken site, with resection factors being recruited in a mutually –exclusive way (Ochs, Karemore et al. 2019). Possibly in our system, of Cas9 induced DSBs in sat III granules, the peripheral 53BP1 pattern in combination with a BRCA1 internal pattern is representing this circular organized structure, in order to create a protective environment for resected DNA.

Moreover, since BRCA1 is often participating in other complexes and processes, we cannot exclude the possibility that its peripheral recruitment in G1 occurs with BRCA1 being a part of another complex, for example with Rap80 (Wang, Matsuoka et al. 2007, Hu, Scully et al. 2011).

Even though in mouse chromocenters, DNA end-resection takes place inside the heterochromatic domain, RAD51 is excluded the domain and was shown to stabilize the relocated resected DNA ends at the periphery of the domain. (Tsouroula, Furst et al. 2016). In line with this, tethering of the BRCA2 domain BRC3 to chromocenters by fusing it to dCas9 lead to the recruitment of Rad51 at the core of the mouse HC and the stabilization of the break inside the domain, suggesting that RAD51 localization correlates with the DSB localization. (Tsouroula et al 2016). Rad51 recruitment inside the sat III domain is something unique in the human heterochromatin further supporting the notion that when Rad51 is recruited inside the human heterochromatin, the breaks remain positionally stable.

Clustering is affecting the DSB relocation and Rad51 exclusion from heterochromatin

A major difference between the mouse and human heterochromatic domains is the degree of clustering of HC repeats. Both the D. Melanogaster and mouse heterochromatin domains consist of identical repeats that form clusters. In Drosophila Melanogaster, DSBs are moving to the periphery and finally relocate at the nuclear pores to accomplish HR, in an active and myosin dependent manner(Chiolo, Minoda et al. 2011, Caridi, D'Agostino et al. 2018). These observations are reminiscent of the ones in yeast where persistent DSBs migrate to the nuclear pore(Lambert, Mizuno et al. 2010) . One has to note, that the only case where clustering is observed in human cells, is at the nucleolus. There, rDNA repeats of the five human acrocentric chromosomes cluster together, and when DSBs are induced on these repeats they relocate to the periphery of the domain(van Sluis and McStay 2015, Warmerdam, van den Berg et al. 2016, van Sluis and McStay 2017, Korsholm, Gal et al. 2019, Marnef, Finoux et al. 2019). To further validate the importance of clustering in DSB relocation, we used again the CRISPR/Cas9 system and targeted other types of repetitive elements that do not cluster in the human nucleus. These included sat II repeats, centromeres and telomeres and none of these cases were refractory for Rad51 recruitment, showing that our observation is a general feature of human heterochromatic repeats that do not cluster. We hypothesized that HR within clustered repeats would allow deleterious recombination events between different chromosomes and potentially lead to translocations and this risk is absent in human cells where repeats do not cluster. To directly assess the role of clustering in DSB relocation and RAD51 exclusion from mouse heterochromatin, we used a model system of mouse ES cells

with a specific microcephalin mutation (Δm61) that did not allow the proper formation of chromocenters in G1 and G2 phase of the cell cycle due to chromosomes' premature condensation events, and after DSB induction on major satellites we showed that Rad51 could be recruited inside the pericentromere of these chromosomes. In parallel, when we induced super clustering (Jagannathan, Cummings et al. 2018) of mouse chromocenters on NIH-3T3 cells, Rad51 was further restricted at the periphery of these domains. Both results highlighted the importance of clustering on DSB relocation. Interestingly, and in contrast to what was shown in Drosophila heterochromatic DSBs or in human rDNA repeats (Marnef, Finoux et al. 2019), we showed that relocation in mouse heterochromatin is not dependent on actin related processes.

Biophysical properties of heterochromatin render the domain in accessible to Rad51

Despite the fact that we have identified the role of clustering in the DSB relocation process, the way that Rad51 is restricted from heterochromatic structures of repeats that cluster is still unknown. Previous work from our team showed that in contrast to *drosophila* cells(Chiolo, Minoda et al. 2011, Ryu, Spatola et al. 2015), in mouse cells the compacted state of heterochromatin was not refractory for Rad51 access, as its relaxation did not lead to an increase in Rad51 internal pattern at the chromocenters(Tsouroula, Furst et al. 2016). Moreover, even if depletion of HP1s led to a decrease of Rad51 recruitment, the restriction at the periphery of chromocenters was still observed (Tsouroula, Furst et al. 2016). To investigate further the mechanism underlying Rad51 restriction of the chomocenters we focused our interest on their biophysical properties, and more specifically at the ability of proteins to diffuse in these domains. We compared the ability of an inert protein to diffuse either in mouse chromocenters or in human sat III granules in different phases of the cell cycle, and we showed that in mouse cells the diffusion of protein is decreased after DSB induction, whereas in human cells the diffusion was rapid, especially in G2 were HR pathway is predominant. This was not the case in G1 cells, suggesting that in G2 synchronized human cells, the accessibility of the domain increases to favor the entrance of HR factors, like Rad51.

The reduced accessibility of chromocenters partially explains the exclusion of Rad51 of the domain, but we wanted to identify if there are other barriers related to chromatin, as it is known that chromatin undergoes through different changes in response to DNA damage. Previous studies

in mouse heterocrhomatin showed increased intensity of all three HP1 variants (HP1α , β and γ) after Cas9-iduced DSBs specifically in G2 (Tsouroula, Furst et al. 2016). These observations were in line with other studies that showed recruitment of HP1s after damage ((Baldeyron, Soria et al. 2011) (Luijsterburg, Dinant et al. 2009) (Zarebski, Wiernasz et al. 2009), supporting their possible role in DNA repair and specifically HR. Interestingly however, DSBs at sat III repeats of human cells did not affect HP1α and HP1β, but lead to a partial eviction of Hp1γ, supporting once again that there are fundamental differences between human and mouse heterochromatic DSBs repair.
In parallel, several recent studies have shown that the ability of proteins to diffuse inside heterochromatin is correlated with increased HP1α dimerization at its periphery (Hinde, Cardarelli et al. 2015, Larson, Elnatan et al. 2017, Strom, Emelyanov et al. 2017). Strikingly, we identified an alteration of Hp1α dimerization after the DSB induction. The HP1a dimmers increased at the periphery of the mouse chromocenters in G2 phase of the cell cycle, but not at the human satellite III domains, showing an anti-correlation in the protein diffusion and the HP1a dimerization in G2 human cells. This was also observed in G1 synchronized human cells, where the diffusion was less rapid and this was accompanied by an increase in HP1α dimerization.

In order to investigate in detail the role of HP1a dimerization in Rad51 exclusion from mouse chromocenters, we depleted all three HP1 isoforms and complemented with the HP1a dimer mutant, or WT HP1a, and showed that Rad51 recruitment was decreased but its peripheral localization was not affected. This was also validated in SUV39 KO MEFs, where HP1a is absent from the chromocenters. Recently, Erdel et al have reported that in WT MEFs and SUV39 KO MEFs the accessibility of the chromocenters, and thus the ability of proteins to diffuse is the same (Erdel, Rademacher et al. 2020) . Possibly, the increase of Hp1a dimerization at the periphery of the domain is further restricting protein diffusion but the presence of HP1s is not sufficient as a mechanism to impose the entire HC barrier and other mechanisms are in place. In order to render the choromocenter domain more accessible to protein diffusion, we tethered VPR (Erdel, Rademacher et al. 2020), a strong transcriptional activator, to major satellite repeats and disrupted the interaction between heterochromatin segments. Surprisingly, the chromocenters were de-compacted and both Rad51 and BRCA1 were recruited inside the core of the domain.
Together these data suggest that clustering or repeats in mouse chromocenters makes the domain

less accessible to protein diffusion, which is further enhanced by HP1a dimerization, but still requires a strong de-condesation for diffusion to occur. In human cells though, were there is no clustering of repeats, the diffusion is easier and HP1 dimerization is not needed. Hp1a seems to have a role in the regulation of the HR at the periphery of the chromocenters, but it still remains unknown how this is achieved and which is the driving force of the dimerization. Two scenarios can be envisaged. In the first HP1 dimers are generated to block RAD51 diffusion within the domain inducing a spatial separation of DNA end-resection and strand invasion forcing the ends to relocate to be meet RAD51 and get stabilized at the periphery. Another scenario is that HP1 dimerization is a mere result of the DBS relocation and when the broken chromatin is relocating from the core domain to the periphery to be repaired by HR, the HP1a molecules from the internal part find the outer HP1 and create dimmers inducing further local condensation. It was previously reported that HP1 regulates DNA end resection (Soria and Almouzni 2013). It is therefore possible that lack of HP1 reduces DNA end-resection and subsequently the DNA end resection-dependent break relocation leading to the observed reduction of RAD51 recruitment at the periphery of mouse HC. Since in the absence of HP1s, only the recruitment Rad51 and not its localization is affected, it is possible that the HP1 dimerization is a result of DSB relocation and that the diffusion barrier in mouse HC is imposed by other mechanisms.

HP1a dimerization is shown by several research groups to be the driving force for the heterochromatin domain formation, through phase separation. Briefly, chromocenters among other nuclear compartments have been shown to behave like membrane-less organelles in the nucleus, through liquid liquid phase separation (LLPS) (Hinde, Cardarelli et al. 2015, Banani, Lee et al. 2017, Larson, Elnatan et al. 2017, Strom, Emelyanov et al. 2017). Maybe the increase of the dimerization is further enhancing this biophysical property of the domain after the DSB induction in order to dictate the positioning of HR activation. Another possible explanation is that Rad51 foci have themselves LLPS properties, as it has already been reported for 53BP1 and Rad52 DNA repair foci (Kilic, Lezaja et al. 2019, Pessina, Giavazzi et al. 2019, Oshidari, Huang et al. 2020). If this is the case, maybe Rad51 and chromocenters difference in their liquid properties does not allow their proper mixing.

Clustering is imposing a physical barrier to prevent chromosomal translocations due to deleterious recombination events

As mentioned previously, we hypothesized that the correlation of heterochromatic repeat clustering with the relocation of DSBs, reveals a mechanism to prevent HR between identical sequences of different chromosomes that would lead to illegitimate recombination. To this end, we fused dCas9 with the BRC3 domain of BRCA2 that is responsible for its interaction with Rad51, or with Rad51 itself and after co-expressing them with gRNAs specific for major satellite repeats of chromocenters we inhibited the DSB relocation, as shown previously (Tsouroula, Furst et al. 2016). Interestingly, inhibition of the DSB relocation led to an increase in the number of translocations originating from these repeats. By inhibiting Rad51 catalytic activity, we showed that indeed the translocations are mediated by HR. It is known that alternative pathways as alt-EJ are more involved in the formation of translocations. In our case though, the tethering is performed in cells without induction, to access the impact of physiological endogenous breaks generated by replication stress that occurs after tethering of dCas9 in the chromocenters, thus HR pathway is more expected to be used. Therefore DSB relocation seems to be essential for avoiding translocation events, and the cells have developed mechanisms to ensure it whenever there is clustering of repetitive elements, probably by taking advantage of their biophysical properties.

5. Perspectives

Clustering and Rad51 exclusion from heterochromatin

Our data strongly support the correlation between heterochromatic repeat clustering and Double Strand Break relocation. We have shown that clustering is sensed by the cells by reducing the accessibility of protein diffusion inside the chromocenters, but how is clustering of repeats maintained and conserved in several species still remains unknown. Some studies have identified proteins like HMGA1 (Jagannathan, Cummings et al. 2018) or ChRO1 RNA (Park, Lee et al. 2018)to be involved in the chromocenter formation through clustering, but depletion of neither of them resulted in the complete disappearance of chromocenters. It would be interesting therefore to identify new factors that are promoting clustering of repeats and test their potential to dictate Rad51 positioning. Tethering the VPR transcriptional activator at the major satellite repeats lead to a substantial de-compaction of the chromocenters, suggesting that it could be used as a tool to identify proteins that have a role in this process. To address this, we could employ the BioID technology (Fairhead and Howarth 2015)to identify the proteins that bind the clustered repeats and compare them with proteins that bind to the repeats when they are de-compacted and non-clustered. To this end, we will tether the biotin ligase BirA* to the chromocenters by fusing it to dCas9 or dCas9-VPR and coexpressing it in mouse cells together with the gRNA targeting major satellites. These proteins can be affinity purified and can be submitted for mass spectrometry analysis. Possibly some factors that are essential for clustering and chromocenter formation will not be present in the VPR condition and we would ideally identify new factors involved in clustering of heteochromatic repeats. Then, these candidates can be tested by immunofluorescence experiments in order to identify if they affect Rad51 exclusion.

A second way to identify proteins that are important in Rad51 exclusion from heterochromatin, would be to engineer a tagged Rad51 knock-in in 3T3 cells. Inserting a flag-EGFP tag would allow us to isolate proteins that interact with Rad51 by immunoprecipitation and test their potential to regulate its localization.

Role of HP1α dimerization in Homologous Recombination

We have showed that HP1α depletion decreases both Rad51 and BRCA1 recruitment and this

phenotype is only rescued when we complement the cells with the WT HP1α and not with the HP1α I165E dimer mutant. As discussed previously, the dimerization of HP1α might be an outcome of the DSB relocation but it can also be directly involved in the HR process. In order to asses this question, we are planning to deplete all three isoforms of HP1α and complement the cells as previously with either WT or I165E HP1α, and study the recruitment of other HR factors, like BRCA2, or resection factors like RPA, CtIP and PalB2 after DSB induction. Moreover, the same experiments can be performed in cells that have stably integrated the DRGFP cassette used to access gene conversion.

In case resection is also dependent on HP1α dimerization, we would like to explore the possibility of its involvement in pathways like SSA, by checking Rad52 recruitment. We can also use fluorescent tagged constructs of those proteins and by confocal microscopy and FRET identify if any of them has an affinity and directly interacts with the HP1α dimer and not monomer.

Role of phase separation in Rad51 exclusion of heterochromatin

As explained in the introduction and discussion parts, HP1α dimerization is related to the ability of chromocenters to behave like membrane-less organelles in the nucleus, by phase separation. So possibly, an increased dimerization of HP1α at the periphery of chromocenters after the DSB is a required step to achieve a stronger barrier through LLPS. Our preliminary data (fig P1) suggest that disruption of phase separation by hexanediol increases the Rad51 recruitment in the core domain of chromocenters.

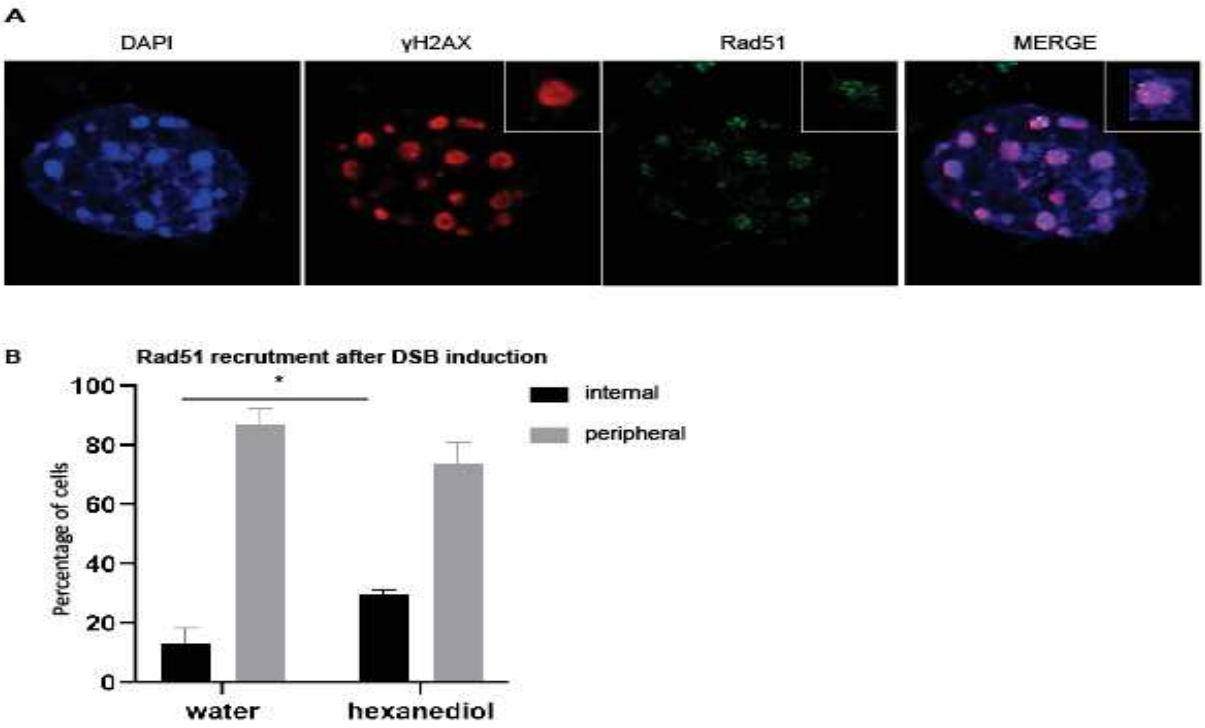

Figure P1: **A**. Confocal immunofluorescence analysis of 3T3 cells after Cas9 specific DSB induction at major satellites and treatment with Hexanediol, stained with Dapi, and γH2AX or Rad51 specific antibodies **B**. Quantification of Rad51 pattern after DSB induction and treatment with water or Hexanediol

However, hexanediol is a drug that is not well characterized despite the fact that it is shown that it interrupts hydrophobic interactions. It is possible that it affects other cellular processes, or even the accessibility of the chromocenter domain itself, and thus further results are needed to validate the role of phase separation in Rad51 localization. A potential treatment to use is a drug that showed similar results with hexanediol treatment and dissolved 53BP1 foci, the Ammonium acetate (NH_4OAc) (Pessina, Giavazzi et al. 2019). NH_4OAc can target RNA foci, but since RNA has been shown to be involved in phase separation, it can possibly affect mouse chromocenters too. Moreover, in the same study, the authors observed a fast and homogenous recovery of 53BP1 after FRAP, which is a characteristic of liquid compartments (Pessina, Giavazzi et al. 2019). Thus it would be interesting to use these techniques and study the behavior of HP1α before and after DSB induction.

Human sat III granules are not shown to be phase separated, and we would like to transform them in liquid droplets and test whether this would lead to Rad51 exclusion. In order to achieve that, we are going to use a recently published technique that is called Cas-Drop (Shin, Chang et al. 2019). This is a CRISPR-Cas9-based optogenetic technology, which can induce localized condensation of liquid droplets at specific genomic loci. The system allows local optogenetic molecular assembly of any known IDR domains or proteins and local induction of phase separation. To this end, dCas9 fused to Sun-Tag (ST) will be co-expressed with a cognate for ST fused to an optogenetic dimerization protein (iLID) and an IDR-containing protein (HP1, FUS or BRD4). In the presence of gRNA against sat III repeats, the fusion proteins can self-assemble on Sat3 and blue light exposure will cause formation of IDR oligomers through dCas9-ST-iLID scaffolds and sat3 granules will be converted to liquid condensates. We can then test whether the artificially induced phase separation of sat3 granules is able to exclude Rad51 from these domains.

Spatial regulation of NHEJ and HR in the active and inactive chromatin states on the exact same locus

One of the special features of human sat III repeats, is their ability to be converted from silent, to actively transcribed regions after heat shock. Indeed, our preliminary data have validated their ability to recruit transcription factors, such as Heat Shock Factor 1 (HSF1) and RNA pol II, after Heat shock. We have already established that we can activate the transcription at 42 deegres for 1h (Figure P2).

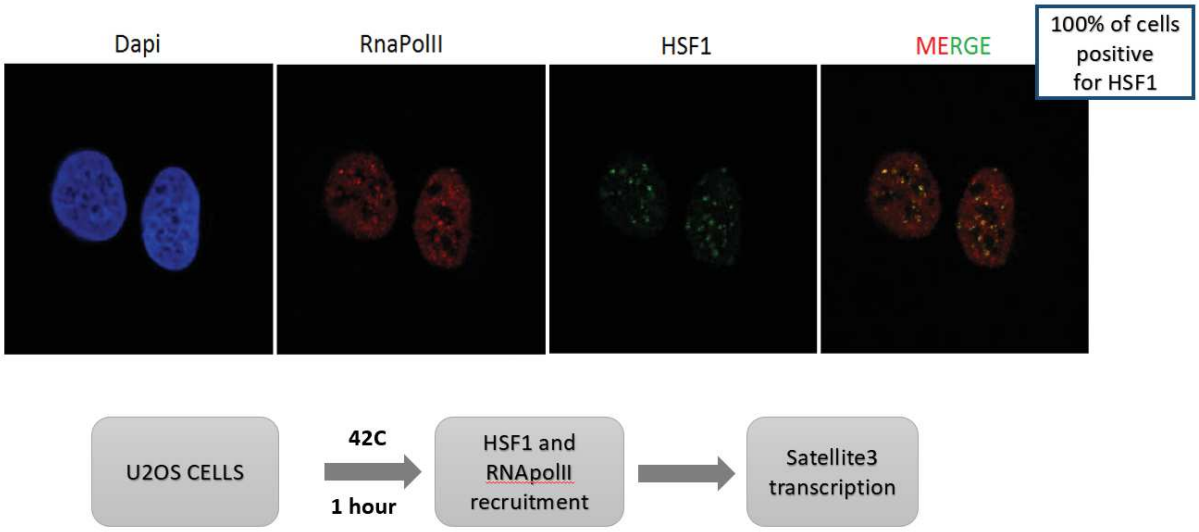

Figure P2: Confocal immunofluorescence analysis of U2OS cells after 1 hour of heat shock at 42C, stained with Dapi, and RnaPol II or HSF1 specific antibodies

This characteristic of the sat III repeats will allow us to study the DSB repair before and after heat shock, and compare the spatial regulation of NHEJ and HR in the active and inactive chromatin states on the exact same locus. Moreover, we have designed in vitro transcribed gRNAs for sat III repeats, in order to instantly induce DSBs before or after heat shock and compare the two situation. First, we will use immunofluorescence to visualize major factors that participate in HR or NHEJ and compare their localization and recruitment to test if the balance between the pathways is altered. If this is the case, we would like to determine if it happens due to changes in chromatin modifications or due to the presence of RNA per se. In order to assess this question we will deplete the sat III RNA either by siRNA or gapmeres.

A major question to address is whether the transcription would affect the DSB or the DSBs will affect the transcription and what is the contribution of repair factors in this process. That can be answered by quantifying sat III RNA either by RT-PCR or RNA FISH, in the presence of heat shock before and after DSB induction.

Finally we would like to identify the specific factors that are involved in the DNA repair process before or after transcription activation, by using a mass spectrometry approach. For this, we have

have fused the APEX or BirA*,to Cas9, in order to achieve peroxidase-catalyzed proximity biotinylation (Han, Udeshi et al. 2017) of proteins after the DSB induction either before or after heat shock. Then we will isolate the biotinylated proteins and submit them to identification by mass spectrometry. That will allow us to identify new factors that are involved in DSB repair and are specific for inactive or transcriptionally active regions.

6. Thesis summary in French

Les dommages à l'ADN sont un événement très fréquent au cours du cycle cellulaire qui peut provoquer des changements dans la structure du matériel génétique et affecter le bon fonctionnement du mécanisme de réplication de la cellule. Différentes sources de dommages, soit endogènes, comme les processus métaboliques normaux de la cellule, soit exogènes, qui sont causés par des agents externes, attaquent constamment l'AND. Les cassures double brin de l'ADN (DSB), qui coupent les deux brins de l'ADN, sont les dommages les moins fréquents mais les plus toxiques car ils sont à l'origine d'instabilité génomique, de translocations chromosomiques et du cancer. Les DSB peuvent activer la voie de réponse aux dommages à l'ADN (DDR), une cascade de signalisation qui permet l'activation des points de contrôle après la reconnaissance de la cassure, ainsi que l'interruption de la progression du cycle cellulaire, laissant le temps à la cellule de réparer les cassures avant de se diviser. Les cellules réparent les DSBs en utilisant deux voies principales: la recombinaison homologue (HR) et la jonction d'extrémités non-homologues (NHEJ). NHEJ, qui est actif tout au long du cycle cellulaire, rejoint les extrémités libres cassées par re-ligature, et c'est un mécanisme potentiellement sujet aux erreurs. D'autre part, HR, qui nécessite une séquence identique comme modèle, est activé dans les phases S / G2 du cycle cellulaire en tirant parti des informations codées par la matrice homologue de la chromatide sœur pour réparer le DSB de manière non erronée. Cette voie se compose de deux étapes principales. La résection terminale de l'ADN est la première, qui a lieu après la reconnaissance de rupture pour générer des extrémités d'ADN simple brin 3 'qui sont liés par la protéine de réplication A (RPA). Cela facilite le chargement de Rad51, ce qui favorise l'invasion des brins qui conduit à la formation d'une boucle en D et d'une jonction de Holliday. Enfin, le modèle homologue est copié et les informations génétiques affectées par le DSB sont restaurées. Un autre facteur important est BRCA1 qui favorise à la fois la résection de l'extrémité de l'ADN et le chargement de Rad51. Au niveau de la résection, BRCA1 est en concurrence avec 53BP1 et RIF1 qui inhibent le processus, dirigeant la réparation vers la NHEJ. Malgré leur concurrence, la voie qui est activée conduit finalement à l'activation des points de contrôle et à l'arrêt du cycle cellulaire. Si le processus de réparation n'est pas terminé efficacement, la voie apoptoti que ou de sénescence sont activées.

L'ADN répétitif est emballé dans des structures d'hétérochromatine (HC) comme le centromère qui se compose de HC péricentromérique et centromérique. La préservation de l'intégrité de l'ADN

centromérique est cruciale car des lésions non réparées pourraient conduire à l'échec de la ségrégation chromosomique des cellules filles, conduisant en outre à l'aneuploïdie, une caractéristique du cancer. Comme ce processus est assez difficile, il y a eu un grand intérêt dans le domaine de la réparation de l'ADN au fil des ans pour comprendre les mécanismes qui sont utilisés pour réparer les HC DSB.
Auparavant, notre équipe a montré que dans les HC péricentromèriques de souris (chromocentres), les DSB induits par CRISPR / Cas9 sont stables en position G1 et recrutent des facteurs NHEJ. Dans S / G2, les DSB sont réséqués et déplacés vers la périphérie de HC, où ils sont retenus par RAD51 de manière indépendante de l'actine (nos résultats non publiés). Même si de nombreux progrès ont été réalisés dans l'étude de ces mécanismes chez la drosophile ou les cellules de souris, on en savait peu sur la réparation hétérochromatique du DSB chez l'Homme. Le but de ma thèse était de développer un système CRISPR / Cas9 pour cibler et visualiser spécifiquement les DSB dans l'hétérochromatine péricentromérique humaine, et mes résultats ont démontré des différences fondamentales entre la réponse des cellules de souris et humaines.

Plus spécifiquement, nous avons généré des DSB spécifiques au niveau des régions péricentromères sat III des cellules humaines, qui ont été visualisées par immuno-FISH et après imagerie de super résolution, nous avons montré que ces DSB sont capables d'activer une signalisation DDR appropriée. Fait intéressant, dans trois lignées cellulaires humaines différentes, HR a été activée à l'intérieur du domaine central des granules sat III, comme le révèle la visualisation des facteurs caractéristiques des étapes de résection et d'invasion de brin (RPA et Rad51). Les deux RPA et Rad51 ont montré un modèle interne dans 100% des cellules, quand ils ont été synchronisés en phase S / G2 du cycle cellulaire, où HR est normalement actif. D'autre part, les facteurs NHEJ ont montré des modèles à la fois internes et périphériques, respectivement dans les phases G1 ou G2 du cycle cellulaire. Compte tenu de la concurrence entre les deux voies, nous avons marqué des facteurs NHEJ et HR et observé une image miroir dans leur chargement après la génération du DSB :
Cette découverte suggère que les différences dans les structures HC de différents organismes, ont un rôle crucial dans la position DSB et l'activation HR. La principale différence est l'absence de regroupement dans la HC péricentromérique humaine qui pourrait protéger les cellules d'une recombinaison délétère entre des séquences identiques et de l'activation de voies mutagènes. Pour

valider l'importance du regroupement dans la relocalisation DSB, nous avons utilisé un système modèle de cellules ES de souris avec une mutation de microcéphaline spécifique qui ne permettait pas la formation appropriée de chromocentres en phase G1 et G2 du cycle cellulaire en raison d'événements de condensation prématurée des chromosomes et montré que Rad51 pourrait être recruté à l'intérieur du péricentromère de ces chromosomes.

Ensuite, nous avons émis l'hypothèse que la corrélation du regroupement des répétitions HC avec la relocalisation des DSB, révèle un mécanisme pour empêcher HR entre des séquences répétitives identiques de différents chromosomes qui conduirait à une recombinaison illégitime. À cette fin, nous avons inhibé la relocalisation DSB en attachant Rad51 à l'intérieur des chromocentres et nous avons observé une augmentation des translocations provenant de ces répétitions.

Malgré le fait que nous ayons identifié l'importance du regroupement dans le processus de relocalisation DSB, la façon dont Rad51 est exclu des structures hétérochromatiques de répétitions de ce regroupement est encore inconnue. Pour étudier plus en détail le mécanisme sous-jacent à l'exclusion de Rad51 des chomocentres et la détection de l'agrégation, nous avons concentré notre intérêt sur leurs propriétés biophysiques. Récemment, différents groupes de recherche ont montré que les chromocentres sont des organites sans membrane avec des propriétés de séparation de phase liquide liquide (LLPS). Nous avons inhibé la LLPS et montré que cela pouvait augmenter l'accès de Rad51 au cœur du domaine HC. Puisqu'il a été proposé que la LLPS des chromocentres se produise via la dimérisation de Hp1α à la périphérie des chromocentres, nous avons émis l'hypothèse que cette dimérisation pourrait être encore renforcée lorsqu'un DSB est généré, conduisant à l'exclusion de Rad51. Tout d'abord, nous avons montré que l'épuisement des HP1 conduisait à une diminution du chargement de Rad51, mais l'exclusion à la périphérie des chromocentres était toujours observée. Enfin, nous avons montré par analyse fluorescence correlation spectroscopy (FCS) qu'il existe effectivement une augmentation de la dimérisation de HP1α après la génération du DSB à la périphérie du domaine hétérochromatique dans les cellules de souris, ce qui n'était pas le cas pour les cellules humaines. Nous avons donc émis l'hypothèse que l'augmentation spécifique du dimère Hp1α à la périphérie du domaine HC de la souris constitue une barrière à la diffusion des protéines HR. De plus, nous avons obtenu des résultats sur les MEF SUV39 KO sur lesquels les HP1 ne sont pas présents dans les chromocentres qui indiquent que la formation de dimères HP1α est pertinente avec la relocalisation du DSB. Le texte de ce projet est en préparation, et nous avons réussi à montrer la différence entre les domaines HC

de la souris et de l'homme dans la façon dont ils sont réparés et à éclairer le domaine de la chromatine et de la réparation de l'ADN. Après une comparaison détaillée entre la réparation du HC DSB chez la souris et l'homme, nous avons expliqué en profondeur la nécessité de déplacer le DSB lorsque les répétitions HC se regroupent. Cet événement se produit afin d'éviter la formation de translocations. Nous avons également corrélé la relocalisation DSB avec la formation de dimères HP1α à la périphérie du domaine HC dans les cellules de souris.

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Ioanna MITRENTSI

Double Strand break repair in human heterochromatin

Résumé

Les dommages à l'ADN sont un événement très fréquent au cours du cycle cellulaire qui peut provoquer des changements dans la structure du matériel génétique et affecter le bon fonctionnement du mécanisme de réplication de la cellule. L'ADN répétitif est emballé dans des structures d'hétérochromatine (HC) comme le centromère qui se compose de HC péricentromérique et centromérique. La préservation de l'intégrité de l'ADN centromérique est cruciale car des lésions non réparées pourraient conduire à l'échec de la ségrégation chromosomique des cellules filles, conduisant en outre à l'aneuploïdie, une caractéristique du cancer. Comme ce processus est assez difficile, il y a eu un grand intérêt dans le domaine de la réparation de l'ADN au fil des ans pour comprendre les mécanismes qui sont utilisés pour réparer les HC DSB. Auparavant, notre équipe a montré que dans les HC péricentromèriques de souris (chromocentres), les DSB induits par CRISPR / Cas9 sont stables en position G1 et recrutent des facteurs NHEJ. Dans S / G2, les DSB sont réséqués et déplacés vers la périphérie de HC, où ils sont retenus par RAD51 de manière indépendante de l'actine (nos résultats non publiés). Même si de nombreux progrès ont été réalisés dans l'étude de ces mécanismes chez la drosophile ou les cellules de souris, on en savait peu sur la réparation hétérochromatique du DSB chez l'Homme. Le but de ma thèse était de développer un système CRISPR / Cas9 pour cibler et visualiser spécifiquement les DSB dans l'hétérochromatine péricentromérique humaine, et mes résultats ont démontré des différences fondamentales entre la réponse des cellules de souris et humaines

Mots clés: Hétérochromatine, CRISPR/ Cas9, HR, RAD51

Resume en Anglais

Various types of agents constantly assault DNA. DNA double-strand breaks (DSBs) are the most toxic lesions because their unfaithful repair leads to chromosomal translocations, and cancer. Heterochromatin (HC), which is highly condensed and restricts DNA transactions, makes the DSB repair a challenging process. Previously it was shown that DNA repair in mouse pericentromeric HC is spatially and temporarily uncoupled. In G1, DSBs are positionally stable and recruit NHEJ factors. In S/G2, DSBs are resected and relocate to the periphery of HC where they are retained by RAD51. Here we have developed a CRISPR/Cas9 system to induce DSB in the satellite3 pericentric HC regions in human cells. Interestingly, in this case HR can be activated inside the core domain of the sat3, demonstrating fundamental differences between mouse and human HC DSB repair. Inhibiting the relocation of the DSB on mouse cells was able to lead at the formation of translocations originating from HC satellite repeats.

Keywords: heterochromatin, CRISPR/Cas9, HR, Rad51