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Double-strand breaks repair at the nuclear periphery

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Abbreviations

μSSA .....micro single strand annealing (=alt-EJ)
3C .....Chromosome conformation capture
6-4PPs .....Pyrimidine 6-4 pyrimidone photoproducts
8-oxo-G.....8-hydroxyguanine
9-1-1 .....Rad9-Rad1-Hus1 complex
alt-EJ .....Alternative end-joining
altNHEJ .....Alternative non-homologous end-joining (=alt-EJ)
aNHEJ .....Aternative non homologous end-joining (=alt-EJ)
ATLD.....Ataxia telangiectasia-like disorder
ATM.....Ataxia telangiectasia mutated
ATR.....ATM and Rad3-related
ATRIP .....ATR interacting protein
BAF .....Barrier to autointegration factor
BER .....Base excision repair
BIR .....Break-induced replication
bNHEJ .....Backup NHEJ (=alt-EJ)
Chk1.....Chekpoint kinase 1
Chk2.....Checkpoint kinase 2
CPDs.....Cyclobutane pyrimidine dimers
DamID.....DNA adenine methyltransferase identification
DDR.....DNA damage response
dHJ.....Double holiday junction
DNA .....Deoxyribonucleic acid
DNAPK .....DNA-dependent kinase
DNAPKcs.....DNAPK catalytic subunit
DSBs.....Double strand breaks
dsDNA .....Double stranded DNA
EMD.....Emerin
FA.....Fanconi anemia
FG.....Phenylalanine-Glycine
FISH.....Fluorescence in situ hybridization
HP1 .....Heterochromatin protein 1
HR.....Homologous recombination
INM .....Inner nuclear membrane
LADs.....Lamina associated domains
LBR .....Lamin B receptor
me2 .....Dimethylation
me3 .....Trimethylation
MEPS .....minimal efficient processing segment
MMEJ.....Microhomology-mediated end joining (=alt-EJ)
MMR.....Mismatch repair
MMS .....Methylmethanesulfonate
NBS .....Nijmegen breakage syndrome
NCS.....Neocarzinostatin
NER .....Nucleotide excision repair
NHEJ .....Non-homologous end-joining

NPC .....Nuclear pore complex
Nups .....Nucleoporins
PAR.....Poly ADP ribosylation
PARP .....Poly ADP ribose polymerases
PARPi .....PARP inhibitor
PBZ .....PAR-binding zing finger
PIKK .....Phosphatidylinositol 3-kinase related kinase
PRC2.....Polycomb repressive complex 2
PTM .....Post-translational modification
RNF168.....Ring finger protein 168
RNF8.....Ring finger protein 8
ROS.....Reactive oxygen species
RPA.....Replication protein A
SDSA.....Synthesis-dependent strand annealing
SSA.....Single-strand annealing
SSBs.....Single strand breaks
ssDNA .....Single strand DNA
TopBP1 .....DNA Topoisomerase II binding protein
γH2AX.....Phosphorylated H2AX

Introduction

I. DNA lesions: from physiology to pathology

Deoxyribonucleic acid (DNA) is the molecule that encodes the genetic information. The preservation of its integrity is therefore necessary for development and proper functioning of all living-organisms. Its structure *per se* is appropriate for biological information storage. For example, the existence of two strands that contain the same genetic information constitutes a protection against genetic information loss or modification. However, evolution demonstrates the plasticity of the DNA molecule that can be mutated in consequence of various DNA lesions. The different types of DNA lesions include single strand breaks (SSBs), base modification or loss and double strand breaks (DSBs). They are caused by different sources, endogenous or exogenous that are summarized below.

1. Causes of DNA lesions

The total number of DNA lesions that a cell experience per day is estimated at 10^5 (Lindahl 1993). These DNA lesions are the consequences of endogenous and exogenous sources, each of them being responsible for different kind of lesions. Given the toxicity of these lesions, exogenous induction of lesions by chemotherapy or radiotherapy is a fundamental therapeutic approach in the treatment of cancer.

a) Endogenous sources

- Hydrolysis

The intrinsic chemical properties of the DNA make it prone to hydrolysis under physiological conditions. This hydrolysis leads to depurination of the DNA (Guanine or Adenine loss) or to deamination that triggers for example the conversion of cytosine into uracile base. The base excision repair pathway ensures subsequent repair. The turnover of purine bases due to hydrolysis and subsequent repair in mammalian cells is estimated to 2000-10000 per day (Lindahl 1993).

- By-products of cellular metabolism

The oxidative respiration produces reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and hydroxyl radicals. These ROS are a major cause of DNA lesions, generating for example 8-hydroxyguanine (8-oxo-G), which base-pairs preferentially with adenine instead of cytosine and therefore generates mutations upon replication (Kasai and Nishimura 1984). The number of oxidative DNA damage per cell per day in human cells is estimated at approximately 10.000 (Ames, Shigenaga, and Gold 1993).

- Replication stress

Replication stress is defined as the slowing or stalling of replication fork progression and/or DNA synthesis (for review (Zeman and Cimprich 2014)). It usually results in the formation of long stretches of single-stranded DNA (ssDNA) that activates the replication stress response pathway. In order to give cells time for resolution of the stress, the stress response pathway inhibits cell cycle progression and suppresses late origin firing. In addition, it allows the stabilization and restart of the fork. If replication stress persists or if replication stress response components are lost, fork fails to restart and collapses. Fork collapse leads to the formation of double stranded DNA breaks (DSBs). Various sources of replication stress were identified, such as unrepaired DNA lesions, misincorporation of ribonucleotides, some DNA sequences that form secondary DNA structures and that are intrinsically challenging for replication machinery, collision between transcription and replication, nucleotide depletions or common fragile sites. Collapsed forks are considered as the major source of endogenous DSBs.

- Telomeres

Telomeres are the ends of chromosomes and therefore resemble one half of a DSB. They are composed of TTAGGG repeats (Shampay, Szostak, and Blackburn 1984) and a 3' ssDNA overhang that form a tail, which is able to pair with CCCTAA

repeats of the duplex telomeric repeat array forming a structure called t-loop (Griffith et al. 1999). The t-loop structure and the binding of specific "capping" proteins to telomeres protect them from recognition by the DNA repair pathways (de Lange 2002). However, in most mammalian cells, the replication of telomeres cannot be completed and they are shortened at each cell division. Telomere shortening causes telomere capping defects and their subsequent recognition by DNA repair pathways. When de-protected, telomeres are considered as DSBs and can fuse through the non-homologous end joining (NHEJ) pathway, therefore leading to major chromosome rearrangements (Celli and de Lange 2005; Celli, Denchi, and de Lange 2006).

- Programmed breaks

DNA lesions can also be programmed by the cells and are necessary for their proper functioning. In that case, cells use specific nucleases to provoke DNA lesions in a controlled manner. Such programmed lesions are for example initiated by the protein Spo11 during meiosis or by the proteins RAG1/2 and AID/UNG during the creation of the immune system repertoire by V(D)J recombination, class-switching and somatic hypermutation.

b) Exogenous sources

- UV component of the sunlight

Among the different groups of UV radiation, UV-B is the most harmful to the DNA. Indeed, the DNA does not absorb UV-A and UV-C is absorbed by oxygen and ozone in the Earth's atmosphere. UV-B leads to three major classes of DNA lesions: cyclobutane pyrimidine dimers (CPDs), pyrimidine 6-4 pyrimidone photoproducts (6-4PPs) and their Dewar isomers. If not repaired, these lesions cause structural distortions and can affect DNA replication or transcription (Lindahl 1993). A single day of exposition in the sun may induce up to 100,000 UV photoproducts in each keratinocyte, therefore enhancing considerably the number of DNA lesions in the cell (Garinis et al. 2008).

- Ionizing irradiation

Ionizing radiation causes different types of lesions either by directly acting on DNA or by forming oxygen reactive species. Among the different lesions provoked by ionizing radiation, DSBs are the most dangerous (Ward 1988). Ionizing radiation results from radioactive decay of naturally radioactive compounds, medical (cancer radiotherapy for example) or historical exposures.

- Genotoxic chemicals

Tobacco products are well-known genotoxic chemicals that can cause various cancers due to the DNA damages that they produce. Other DNA-damaging agents can be absorbed through the environment, for example when they contaminate foods. However, such chemicals can also be used to treat cancer. Therefore, radiomimetic drugs (drugs that mimic the effects of ionizing radiation) are used as cancer chemotherapy. Bleomycin is one of those radiomimetic drugs used in cancer treatments. Additional radiomimetic drugs, such as neocarzinostatin (NCS) or phleomycin are not used in clinic but are used for research purposes. In addition to the radiomimetic drugs, DNA topoisomerase inhibitors, such as camptothecin or etoposide and alkylants agents such as methyl methanesulfonate (MMS) are other gentoxic agents that are used in cancer therapy.

2. Different types of DNA lesions, different repair mechanisms

To avoid genomic instability, cells evolved several repair pathways that are specialized for a specific type of lesion. The major repair pathways for single stranded damages are mismatch repair (MMR) that repair erroneous insertion, deletion, and mis-incorporation of bases that induce base mismatch, base excision repair (BER) that removes small non-helix distorting base lesions and nucleotide excision repair (NER) that removes bulky adducts such as 6-4PPs or CPDs. On the other hand, specific mechanisms such as homologous recombination (HR) and non-homologous end joining (NHEJ) evolved to repair DSBs. These mechanisms will be described in more details in the part III of the introduction.

The different types of lesions, their causes and their associated repair mechanisms are summarized in figure 1 (Rastogi et al. 2010).

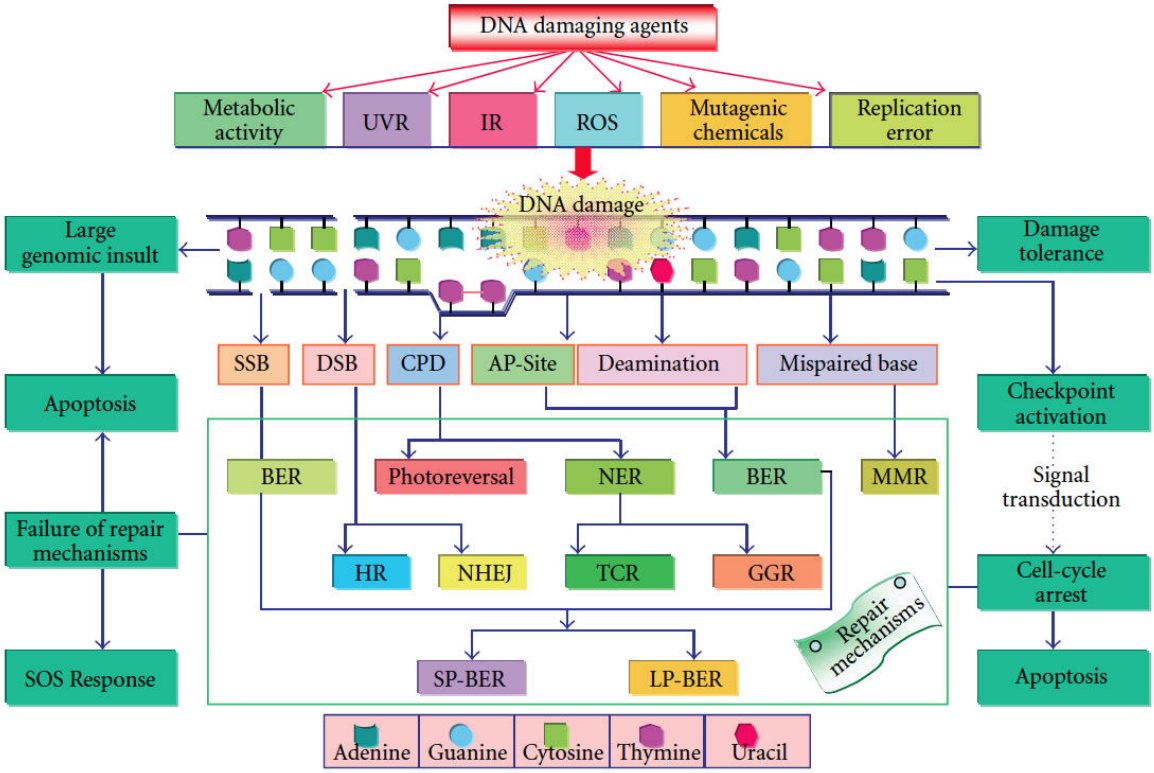


Figure 1- DNA repair mechanisms (Rastogi et al. 2010)

Genomic lesions produced by various DNA damaging agents trigger several specific repair machinery to conserve the genomic integrity. In case of severe damage and/or failure of repair mechanisms, cells undergo apoptosis or induce a complex series of phenotypic changes, that is, SOS response. Sometimes the potentiality of lesions in the genome is mitigated by a phenomenon known as damage tolerance, during which DNA lesions are recognized by certain repair machinery, allowing the cells to undergo normal replication and gene expression. The cellular response to DNA damage may activate cell-cycle checkpoint by means of a network of signaling pathway that gives the cell extra time to repair the genomic lesions or may induce cell suicide response/programmed cell death.

3. Physiological roles of DNA lesions

As mentioned earlier, some DNA lesions are programmed by the cells and are necessary for their proper functioning. In this cases, they are used to induce genetic variability, for example during meïosis, during V(D)J recombination, class switch recombination or somatic hypermutation.

a) DSBs in meïosis

Meïosis ensures the proper segregation of chromosomes during sexual reproduction in eukaryotes. During the first meiotic prophase hundreds of DSBs are formed. These DSBs are necessary for a proper segregation of chromosomes in the first meiotic division. Indeed, their repair by homologous recombination promotes chromosome pairing. These different events are highly regulated at different levels (reviewed in (Borde and de Massy 2013)). First, the induction of DSBs by the Spo11 enzyme does not occur randomly in the genome but rather on "recombination hotspots", which localization is regulated by multiple mechanisms. Among them, chromosome structure and organization seems to be a key parameter in the targeting of Spo11. In yeast for example, hotspots are generally nucleosome depleted whereas in mouse cells they are enriched in nucleosome but associated with the H3K4me3 histone mark (Lichten and de Massy 2011; Pan et al. 2011; Smagulova et al. 2011). Second, the repair mechanism itself is highly regulated, promoting the use of the homologous chromosome instead of sister chromatid as a template for repair and producing recombination intermediates leading to gene conversion with reciprocal exchanges (crossovers) or without reciprocal exchanges. Proper chromosome pairing seems to necessitate at least one crossover per chromosome pair and the formation of crossovers is tightly regulated (reviewed in (Baudat and de Massy 2007), (Hyppa and Smith 2010; Rosu, Libuda, and Villeneuve 2011)).

b) DNA lesions in immune repertoire establishment

To ensure recognition of "non-self" molecules, the immune system developed several strategies. One of them consists in the establishment of an important immune repertoire, which is defined as the number of different immunoglobulins (B cell receptors and antibodies) and T cell receptors that the immune system produces. This variation allows the recognition of a big number of antigens. Immunoglobulins and T cell receptors are constituted by a so-called variable part that binds to the antigen and a constant part that activates the immune response. These two parts are subjected to changes that are initiated by DNA lesions and that allow variability of

the immune repertoire. Two major enzymatic complexes are responsible for the targeted induction of DNA lesions in immunnoglobulins and T cell receptors: RAG1/RAG2 that is responsible for V(D)J recombination of the variable region and AID that is responsible for class switch recombination of the constant region and somatic hypermutation of the variable region (reviewed in (Alt et al. 2013)). Figure 2 shows the sequential events leading to the establishment of the immune repertoire.

- RAG1/RAG2 induced DSBs are necessary for V(D)J recombination

The N-terminal variable region of immunoglobulins and T cell receptors is encoded by V, D and J gene segments. V(D)J recombination is ensuring the diversity of B cell and T cell receptors by assembling the numerous V, D and J segments in different combinations (Davis and Bjorkman 1988). The initiation of V(D)J recombination is ensured by the lymphocyte-specific endonucleases RAG1 and RAG2 that induce DSBs next to the target V, D and J segments (Schatz, Oettinger, and Baltimore 1989; Oettinger et al. 1990). These segments are consequently fused through the NHEJ DSB repair pathway (Taccioli et al. 1993).

- AID induced lesions are necessary for class switching and somatic hypermutation

Upon antigen activation of B cells, two additional somatic alterations take place in these cells, ensuring variability of B cell receptors and antibodies. These two mechanisms -class switching and somatic hypermutation- are mediated by the enzyme AID. AID is a cytidine deaminase that is targeted to DNA in a transcription-dependent manner (reviewed in (Pavri and Nussenzweig 2011)).

Somatic hypermutation affects the variable part, within the V region, of immunoglobulins and ensures the production of higher affinity antibodies (McKean et al. 1984). AID generates cytidine deamination lesions that are processed by base excision repair or mismatch repair mechanisms, leading to mutations.

In the case of class-switching, the constant region of immunoglobulins are recombined, modifying their function without affecting their affinity with antigens. This mechanism increases the efficiency of the immune response. AID is specifically

targeted to the transcribed S (Switch) regions that exhibit single stranded DNA. Under AID action, cytidines are converted in uraciles that are recognized by the UNG (uracile DNA glycosylase) enzyme, thus creating an abasic site. These lesions are further converted in nicks, which are forming DSBs when positioned in front of each other (Wuerffel et al. 1997; Petersen et al. 2001). DSBs in a donor S region and in an acceptor S region are joined through NHEJ (reviewed in (Pavri and Nussenzweig 2011)).

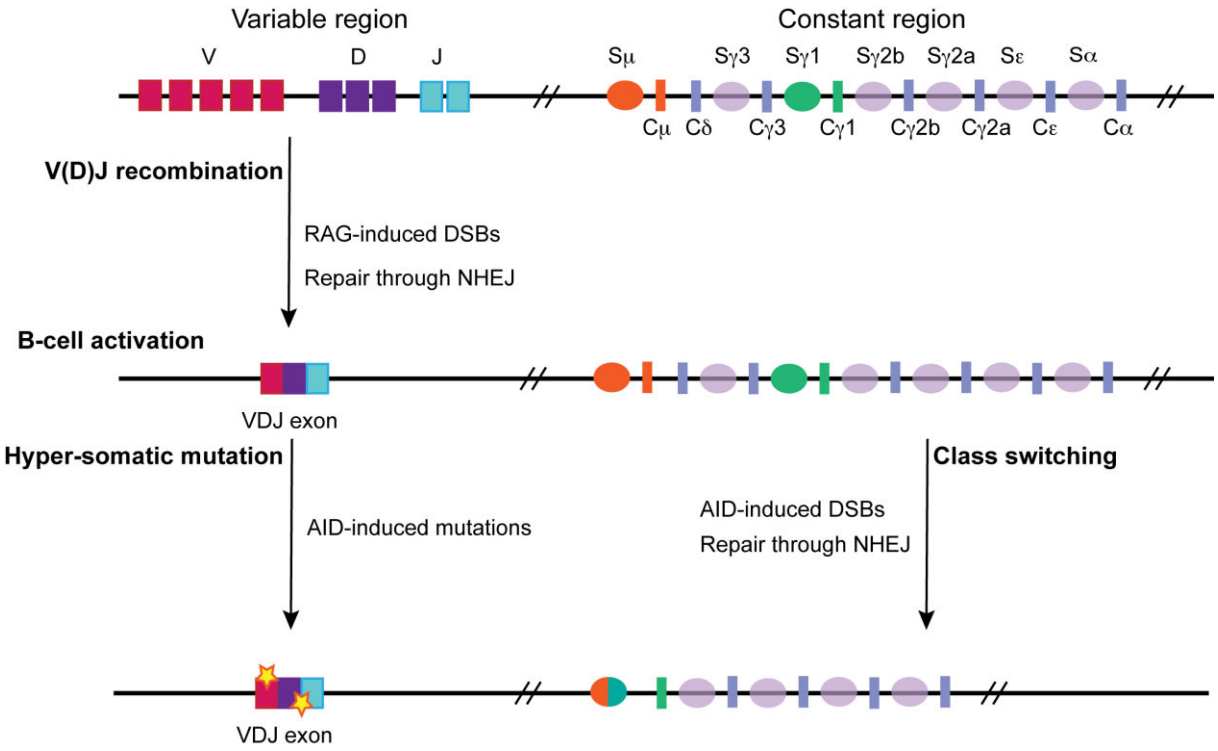


Figure 2- Establishment of the immune repertoire

V(D)J recombination ensures recombination within the variable regions of B and T cell receptors. DSBs are induced by RAG1/RAG2 and repaired through NHEJ, allowing the fusion of V, D and J segments. Upon B cell activation, AID induces somatic hyper-mutations in the variable region whereas it allows the formation of DSBs and class-switching within the constant region of B cell receptor and antibodies.

4. DNA lesions in ageing and pathology

Mutations in the DNA repair genes are associated with various diseases. Most of them can be classified in two categories: the syndromes with phenotypes resembling accelerated ageing (progeria) or cancer. It is therefore commonly admitted that on

one hand DNA lesions participate to ageing and that on the other hand they can lead to cancer when not repaired properly. The different types of DNA lesions can have different outcomes. For example, some of the lesions are highly mutagenic and favor cancer development whereas others are cytotoxic and induce senescence or cell death, which could be responsible for ageing (reviewed in (Garinis et al. 2008)). In addition to ageing and cancer, DNA lesions can be responsible for neurodegenrative disorders, immune deficiencies and infertility.

a) DNA lesions and ageing

Ageing is thought to be the consequence of stochastic damage accumulation of various macromolecules (Kirkwood 2005). Among them, DNA damage accumulation might be partially responsible for ageing. Indeed, DNA lesions are shown to accumulate with age in the nuclear and mitochondrial genome (Sedelnikova et al. 2004), whereas DNA-repair capacity over time might decrease. This accumulation of damage induces cell senescence and apoptosis -two mechanisms that are prone to ageing- through the DNA damage response pathway. Of particular interest, telomeres shortening at each cell division, the subsequent final loose of telomeric protection and their recognition as DSBs trigger chromosomal fusions that activate the DNA damage response pathway, which leads to senescence or apoptosis (d' Adda di Fagagna, Teo, and Jackson 2004; Longhese 2008).

b) DNA lesions and Cancer

Genome instability is a hallmark of cancer cells (Stratton, Campbell, and Futreal 2009). The genetic alterations found in tumors can be classified in 4 categories: subtle sequence changes, alterations in chromosome numbers, chromosome translocations, gene amplifications (Lengauer, Kinzler, and Vogelstein 1998).

- Subtle sequence changes

Single point mutations, insertions and deletions are often present in cancer cells. These types of lesions are usually repaired by nucleotide excision repair (NER) and mismatch repair (MMR). It is therefore not surprising that patients lacking functional NER proteins are particularly sensitive to skin cancer following UV exposure.

Additionally, mismatch repair defects cause microsatellite instability that predisposes to colorectal and endometrial carcinomas (Umar et al. 1994). Dysfunction of the MMR pathway is thought to be responsible for the accumulation of mutations in oncogenes and tumour-suppressor genes resulting in tumorigenesis (Lengauer, Kinzler, and Vogelstein 1998).

- Alteration in chromosome number

More than 90% of all solid tumor cells are aneuploid (B. R. Williams and Amon 2009). The causes of aneuploidy are still under investigation. The major cause for alteration of chromosome number is an abnormal mitosis and some key parameters in the maintenance of ploidy are therefore chromosome condensation, sister-chromatid cohesion, kinetochore structure, microtubule dynamics, and proper activation of the spindle checkpoint.

- Chromosome translocations

Chromosome translocation is defined as a fusion between two different chromosomes. They arise from DSBs that are aberrantly rejoined. They can result in the expression of fusion genes, or deregulation of genes, both cases having the potential to trigger tumorigenesis (figure 3 and (Roukos and Misteli 2014)). Indeed, they are considered as causal in ≈ 20% of cancers (Mitelman, Johansson, and Mertens 2007).

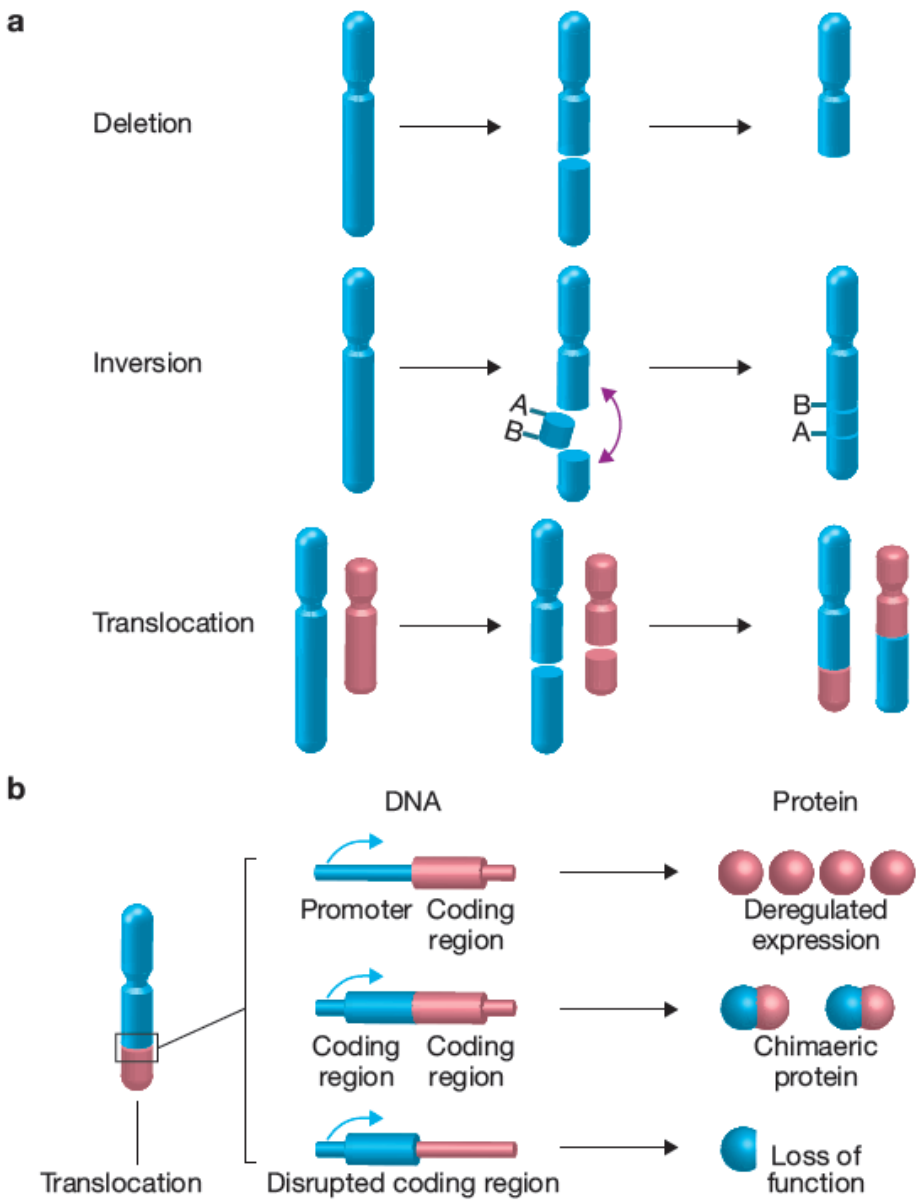


Figure 3- Consequences of chromosome rearrangement (Roukos and Misteli 2014)

(a) Chromosome breakage may lead to loss of genetic material (deletion). When two breaks occur in the same chromosome, the resulting piece of chromosome can be inversed and re-inserted into the chromosome, leading to the formation of an inversion. Genomic material can also be transferred and join to a different chromosome, resulting in the formation of chromosome translocation.
(b) A translocation may provide a proliferative or survival advantage to the cell by generating a chimaeric fusion protein with oncogenic potential, through disruption of a tumor suppressor gene or by fusion of a tumor-promoting gene to a strong transcriptional promoter.

The exact mechanisms underlying chromosome translocations are still under investigation. However some key parameters were identified. One of them is the timing the breaks are repaired. Indeed, persistent DSBs are supposed to be more

prone to chromosome translocations (Roukos 2014). Additionally, spatial genome organization and the choice of DNA repair pathway used are regulating the frequency of translocations. One of the important questions in the establishment of translocation is how chromosomes find their translocation partner. Although in yeast, broken chromosomes seem to be able to move and scan the entire nucleus to allow homologous recombination (Miné-Hattab and Rothstein 2012), in mammals DSBs are positionally stable and DSBs ends are maintained in a close proximity (Kruhlak et al. 2006; Soutoglou et al. 2007; Jakob et al. 2009). This stability implies that translocations partners should be in proximity (Soutoglou et al. 2007; Roukos et al. 2013). In addition to mobility, transcription might be one of the regulatory parameters in the formation of translocations. For example, the MYC gene and its frequent translocation partners IGH, IGK and IGL are thought to share a transcription factory (Osborne et al. 2007). Finally, DNA repair pathway choice determines the frequency of translocations. Indeed, the NHEJ factors have a protective role against translocations (Ferguson et al. 2000; M. J. Difilippantonio et al. 2000), whereas the alternative end-joining pathway (alt-EJ) seems to be prone to translocations. Homologous-recombination-based pathways have also been implicated in translocations. Especially, the alternative homologous repair pathway single-strand-annealing (SSA) can form translocations (Elliott, Richardson, and Jasin 2005). Therefore, a proper balance between the different DSB repair pathways appears to be crucial to avoid genomic instability.

- Gene amplifications

Gene amplification is a copy number increase of a restricted region of a chromosome arm (Albertson 2006). An example of gene amplification occurring in cancer is the amplification of N-myc in ≈30% of advanced neuroblastomas (Seeger et al. 1985). Gene amplification seems to be initiated by DSBs in cells lacking proper checkpoint activation (Chernova et al. 1998; Kuo et al. 1994; Paulson et al. 1998; Pipiras et al. 1998), however the exact mechanism leading to gene amplification is still unknown.

c) DNA lesions and Neurodegenerative disorders

Neurons exhibit high mitochondrial respiration that creates numerous ROS therefore exposing nuclear and mitochondrial DNA to an important number of lesions (Weissman et al. 2007). The accumulation of these lesions is associated with various neurodegenerative disorders, including Alzheimer's and Parkinson's diseases (Kulkarni and Wilson 2008). Consistently with an involvement of DNA lesions in neurodegenerative disorders, defects in BER or single-strand break repair pathways are associated with neuronal dysfunction and degeneration (Rass, Ahel, and West 2007; Caldecott 2008). Several reasons render nervous system particularly sensitive to DNA lesions: first it has a limited capacity of cell replacement in adulthood , eventually leading to accumulation of damage and second DSB repair might occur only through the error-prone NHEJ pathway and not through the more accurate HR since cells are blocked in the G0 phase of the cell cycle (Rass, Ahel, and West 2007).

d) DNA lesions and Immune deficiencies and infertility

As seen earlier, DNA lesions are required for several physiological processes, including establishment of the immune repertoire and meïosis. Therefore, defects in DSB repair pathways or DNA damage response can be responsible for immune deficiencies or infertility (Matzuk and Lamb 2008).

5. DSBs are the most dangerous DNA lesions

Among the different types of DNA lesions listed above, DSBs are the less frequent ones. However, any kind of DNA lesions can be converted in DSBs during their repair or during replication. In contrary to single-stranded lesions, DSBs do not have a direct template for their repair, since both strands are affected, and are therefore thought to be the most difficult to repair. The consequences of a defective DSB repair leads to major genomic rearrangement such as chromosome translocations and DSBs are described as the most dangerous type of DNA lesions. **My PhD project is focusing on this specific type of lesions and the study of the influence of nuclear architecture on DSB repair**. Several repair pathways have evolved to cope with DSBs, the major ones being NHEJ and HR. Additionally to the repair pathways, a specific signaling pathway, the DNA damage response (DDR) is responsible for the

recognition of DSBs and for the coordination between their repair and the progression of the cell cycle. These different pathways will be described in details below (see sections II and III).

II. DSB signaling: the DNA damage response (DDR)

DSBs can lead to genomic instability. In particular, DSBs can impair replication and if they persist through mitosis they lead to chromosome segregation defects. To avoid those gross genomic rearrangements, cells evolved several checkpoints to prevent cells from starting replication (the G1/S checkpoint), from replication progression (intra-S checkpoint) or from entering in mitosis (G2/M checkpoint) if they contain damaged DNA (reviewed in (Zhou and Elledge 2000)). The existence of these checkpoints implies coordination between DNA repair and cell cycle progression. The DDR is a complex signaling network that is responsible for this coordination. On one side, it promotes DNA repair by participating to the sensing of the break and on the other side it allows the pausing of the cell cycle until breaks are repaired. Alternatively, it can also promote senescence or apoptosis. Upon sensing of the breaks, the major transducers of the DDR -the kinases ATM, ATR and DNAPK- are recruited and activated. This activation allows the phosphorylation of numerous targets, allowing the amplification of the signal and finally leading to cell cycle control and DNA repair. This complex network of pathways is therefore central to the preservation of genome integrity.

1. DSBs sensing

Several sensors have been implicated in the recognition of DSBs: PARP, Ku70/80, MRN and with DSB processing RPA. These different sensors are not only implicated in the signaling of the breaks but also in their repair by the different pathways that will be described in more details in part III. It remains unclear whether they act independently or not and how the regulation between these different sensors is made.

a) DSB recognition by PARP

Poly ADP ribosylation (PAR) is a post-translational modification of proteins that is used as a signal transducer in various signaling pathways (reviewed in (Schreiber et al. 2006)). PARylation consists in the modification of Glu, Lys or Asp residues of acceptor proteins by Poly-ADP-ribose polymerases (PARP) that are able to attach covalently linear or multibranched polymers of PAR units. PARP inhibition leads to cellular sensitivity to DNA damaging agents (Haince et al. 2007) therefore indicating a role for PARylation in DNA damage signaling and repair. Furthermore, PARP1 and PARP3 rapidly accumulate and are activated upon DSB formation (Haince et al. 2008; Boehler et al. 2011). The recruitment of PARP is considered as one of the earliest event of the DDR. Structurally, two zinc finger domains of PARP1 (Zn1 and Zn2, see PARP1 domain architecture in figure 4) where involved in DNA damage detection and the binding of PARP1 to damaged DNA was shown to induce a conformational change that allows the activation of the enzymatic activity (Langelier et al. 2012).

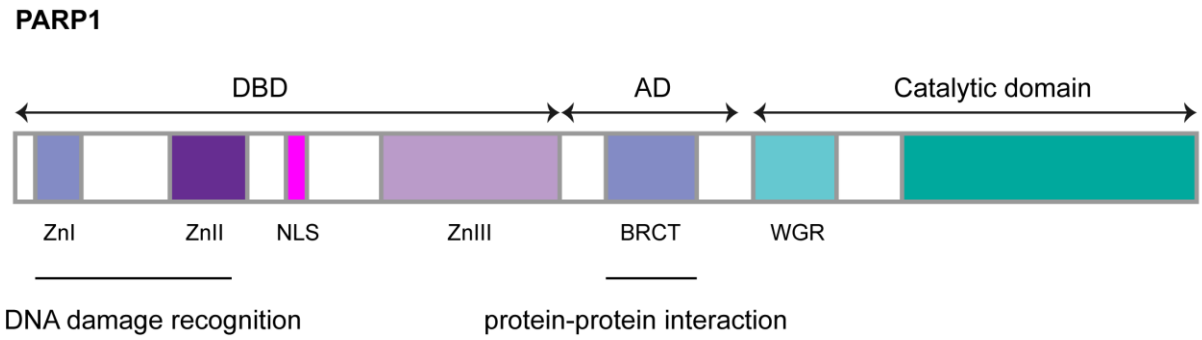


Figure 4- PARP1 domains architecture

Schematic representation of PARP1 domains. DBD=DNA binding domain, AD=auto-modification domain

In absence of PARP1, the phosporylation of ATM substrates is delayed, therefore indicating that PARP1 facilitates ATM activation (Haince et al. 2007). Additionally, PARP1 mediates the initial accumulation of the MRN complex, which in turns allows also the accumulation of ATM (Haince et al. 2008). Furthermore, PAR structures act as platforms allowing the recruitment of additional repair factors. Therefore it plays a role not only in the recognition of the DSB but also in the whole DDR pathway as

well as in DNA repair. Three PAR binding motifs have been described: the macrodomain, the PAR-binding zinc finger (PBZ) domain and an 8 amino-acid basic residue-rich cluster (Kleine and Lüscher 2009). Among the proteins containing a macrodomain, macroH2A1.1 and the chromatin remodeler ALC1 are shown to be recruited to DNA damage and participate in chromatin reorganization, especially by nucleosome sliding (Ahel et al. 2009; Gottschalk et al. 2009; Timinszky et al. 2009). Several DDR factors contain the acid basic residue-rich cluster including p53, XRCC1, LIG3, MRE11 and ATM and PBZ motif is found in the protein APLF that facilitates repair by NHEJ (Gagné et al. 2008; Rulten et al. 2011). Interestingly different PARP enzymes can have different outcomes. Indeed, PARP1 is supposed to promote repair by homologous recombination and alt-EJ, essentially by inhibiting Ku70/80 binding and promoting MRE11 binding (Haince et al. 2008; Hochegger et al. 2006) whereas PARP3 facilitates NHEJ on one hand via the recruitment of the histone chaperone APLF that in turn accelerates the XRCC4/DNA ligase IV-mediated ligation (Rulten et al. 2011) and on the other hand, in a coordinated action with Ku80, protects the DNA end from extensive resection by Mre11/CtIP (Beck et al. 2014).

b) DSB recognition by Ku70/Ku80

The Ku heterodimer (Ku70 and Ku80) has a toroidal (donut-like) structure (figure 5 and (Walker, Corpina, and Goldberg 2001)) and binds around the double stranded DNA ends with a very strong affinity. Indeed, *in vitro* studies demonstrate that Ku binds with high affinity to duplex DNA ends, independent of the end sequence or precise structure, but binds with low affinity to circularized DNA (Mimori and Hardin 1986; Paillard and Strauss 1991; Falzon, Fewell, and Kuff 1993). Ku localizes within seconds to DNA where it loads and activate the catalytic subunit of DNA-PK (DNAPKcs) to initiate repair by NHEJ ((Drouet et al. 2005) and partIII).

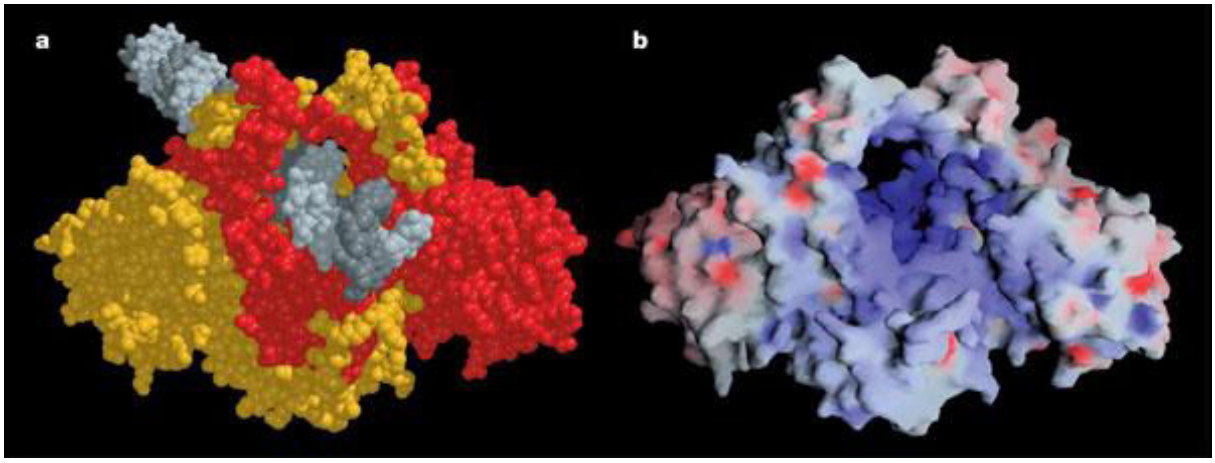


Figure 5- Ku70/80 heterodimer binding to double stranded DNA ends (Walker, Corpina, and Goldberg 2001)

(a) Space-filling model showing Ku bound to DNA. The model was prepared by fitting a 32-bp B DNA to the crystallographically observed duplex. DNA extends towards the viewer to the +11 level. Ku70 is coloured red and Ku80 orange. DNA is shown with one light grey and one dark grey strand.
(b) Molecular surface representation of Ku is coloured according to electrostatic potential, calculated using the program GRASP[44]. Negative potential is coloured red and positive potential blue.

c) DSB recognition by the MRN complex

The MRN complex is composed of the proteins Mre11, Rad50 and Nbs1 that associate in a (Mre11)2/(Rad50)2/(NBS1)1 stoechiometry (Dolganov et al. 1996; Hopfner et al. 2001). This complex is involved in various steps of the DDR and DSBs repair by both NHEJ and HR. The absence of one of its components triggers early embryonic lethality (Xiao and Weaver 1997; Luo et al. 1999; Zhu et al. 2001). Furthermore, hypomorphic mutations in the human Mre11 and Nbs1 genes cause ataxia telangiectasia like disorder (ATLD) and Nijmegen breakage syndrome (NBS), respectively, and result in genome instability (Taylor, Groom, and Byrd 2004; *Archives of Disease in Childhood* 2000). It is considered as one of the first player in the recognition of the DSB and Mre11-Rad50 bind directly to double-stranded DNA ends (de Jager et al. 2001). Its binding to DSBs triggers the activation and recruitment of ATM.

-Mre11

Mre11 can directly bind to the DNA via its DNA binding domains, to Rad50 and to Nbs1. It forms a U-shaped dimer and its dimerization is necessary for its DNA binding activity (R. S. Williams et al. 2008). Mre11 dimers can form two types of complexes with DNA -synaptic DNA complex and branched DNA complex-, depending on the DNA structure. Synaptic DNA complexes are formed between Mre11 and double-stranded DNA ends, whereas branched DNA complexes are formed between Mre11 and oligonucleotides containing both double and single stranded DNA oligonucleotides that resemble a stalled replication fork (figure 6, (R. S. Williams et al. 2008; Rupnik, Lowndes, and Grenon 2010)).

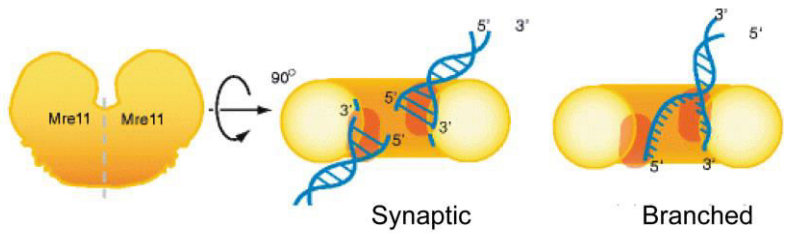


Figure 6- Mre11 structure (Rupnik, Lowndes, and Grenon 2010)

The Mre11 dimer can interact with two dsDNA ends forming a "synaptic" complex or a single ssDNA/dsDNA end forming a "branched" complex.

Additionally to its DNA binding activity, Mre11 also has endonuclease and exonuclease activities (Furuse et al. 1998; T. T. Paull and Gellert 1998; Trujillo et al. 1998; Trujillo and Sung 2001). The endonuclease activity allows the opening of the DNA double helix, whereas the exonuclease activity is involved in the initiation of resection that is necessary for HR. NBS1 presence is necessary for the nuclease activity of Mre11 and Rad50 can stimulate this activity (T. T. Paull and Gellert 1998).

-Rad50

Rad50 can also bind directly to the DNA. It is a member of the SMC (structural maintenance of chromosome) family of ATPases that are involved in chromosome organization, chromosome condensation, sister-chromatid cohesion and DNA repair.

Rad50 contains Walker A and Walker B motifs that are responsible for the ATP and nucleotide binding. These two domains are separated by two coiled-coil regions that mediate intramolecular interactions (de Jager et al. 2001; Hopfner et al. 2001). These coiled-coil regions allow the bridging of DNA ends and possibly sister-chromatids. The ATPase activity of Rad50 is necessary for its functions, including DNA binding and stimulation of Mre11 nuclease activity, and mutations in the Walker A motif exhibit phenotypes equivalent to Rad50 deletion (Alani, Padmore, and Kleckner 1990; Bhaskara et al. 2007; Chen et al. 2005). The presence or absence of ATP modulates the conformation of Rad50, switching from an open structure, in absence of ATP to a closed structure, in presence of ATP ((Lim et al. 2011; Lammens et al. 2011; Möckel et al. 2012; Tanya T. Paull and Deshpande 2014) and figure 7). The slow rate of ATPase activity of Rad50 suggests that the closed conformation is predominant (Bhaskara et al. 2007). The closed state seems to promote end specific DNA binding of MRN, the tethering of both ends together and the ATM activation. Indeed, stable binding of MRN to DNA fragments is supported by non-hydrolysable analogs of ATP (Lee et al. 2003). Furthermore FRET analysis demonstrate that DNA unwinding by MRN is an ATP-dependent reaction and a Rad50 catalytic domain mutant, deficient in the ATP-dependent opening of DNA is impaired in DNA end resection (Cannon et al. 2013). However, Mre11 nuclease activity sites are occluded in the closed conformation (Lim et al. 2011; Möckel et al. 2012) and stabilization of the closed state results in loss of Mre11 nuclease activity (Deshpande et al. 2014), therefore suggesting that ATP hydrolysis is necessary for Mre11 nuclease activity when bound to Rad50.

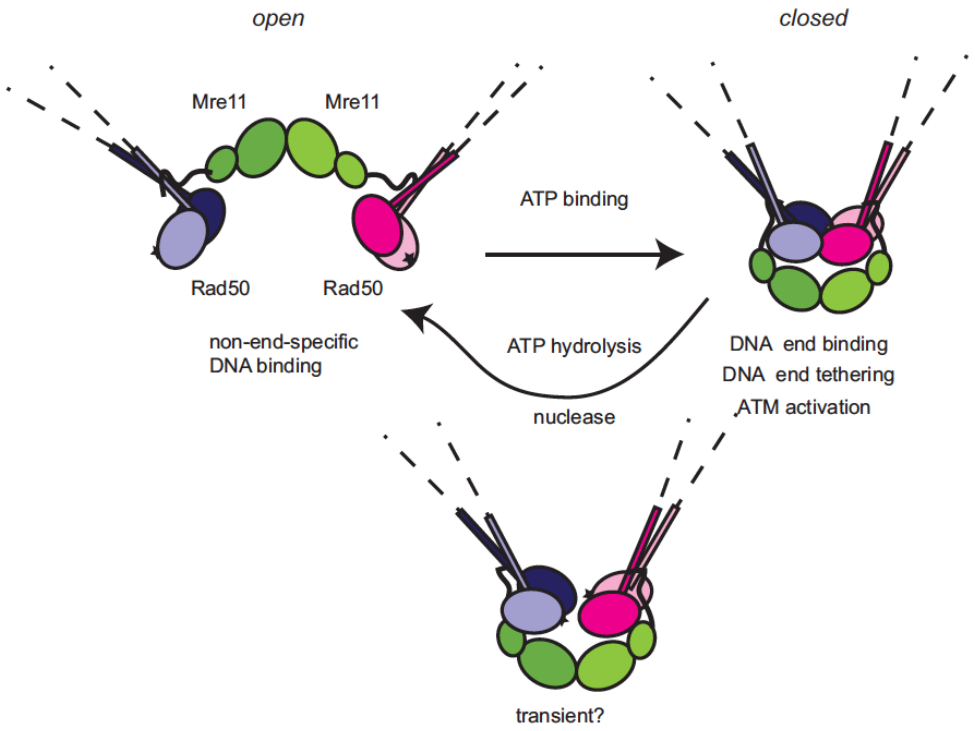


Figure 7- ATP-induced conformational changes in the MRN complex (Tanya T. Paull and Deshpande 2014)

Mre11 binds Rad50 at the base of coiled coils. In the ATP unbound form, the structure is "open", with Mre11 nuclease active sites accessible. ATP binding sites are shown as stars. In this state, the complex can engage DNA in a non-end specific manner. Binding of ATP brings the ATPase domains together forming a "closed" state. This form promotes end specific DNA binding and DNA tethering by MR/MRN complex and ATM checkpoint activation. Although this form blocks the nuclease site, ATP hydrolysis followed by separation of the ATPase domains is required for nuclease activity of Mre11, likely through a transient intermediate, although the structure of this theoretical conformation is unknown.

-Nbs1

The Nbs1 component of MRN regulates the activity of Mre11 and Rad50 and is responsible for localizing the Mre11/Rad50 complex to the nucleus (Desai-Mehta, Cerosaletti, and Concannon 2001). Nbs1 interacts directly with Mre11 and is proposed to stabilize the ATP-bound form of the Mre11/Rad50 complex and to be required for the ATP-dependent functions of the complex. Nbs1 contains a C-terminal ATM-binding region that is critical for ATM activation upon DSBs (Falck,

Coates, and Jackson 2005). Nbs1 is also involved in amplification of the DNA damage signaling. Indeed, besides its ability to directly bind to DNA ends independently of the MDC1 protein (Lukas et al. 2004), MRN is also recruited to DSBs via the interaction of Nbs1 and MDC1 (Chapman and Jackson 2008), following the activation of ATM, the phosphorylation of H2AX and the subsequent recruitment of MDC1 (see part II).

d) DSB recognition by RPA:

Replication protein A (RPA) is a three subunits protein complex, formed by a 70kDa (RPA1), a 32kDa (RPA2) and a 14kDa (RPA3) subunits. It is the major single-stranded-DNA binding-protein in eukaryotes and was initially described as a replication factor (Wold and Kelly 1988; Fairman and Stillman 1988). It allows the protection of single-stranded DNA and avoids the formation of secondary structures. It also plays an important role in DNA repair and DNA damage response activation. In particular, RPA associates with stalled replication forks and promotes their restart by recruiting helicases and translocases (reviewed in(Oakley and Patrick 2010)). In case of collapsed forks, RPA allows the recruitment of the ATR-interacting protein (ATRIP) that is in turn responsible for ATR recruitment (Zou and Elledge 2003) and initiation of Chk1-mediated DNA damage response. Additionally, RPA binds to resected DNA ends and participates in HR repair as a nucleation point for recombination proteins (see part III).

2. Signal transduction: the PIKK kinases ATM, ATR, DNAPK

As seen earlier, the different break sensors allow the recruitment and activation of different kinases: ATM, ATR and DNAPK. These three kinases are part of the phosphatidylinositol 3-kinase related kinase (PIKK) protein kinases family and trigger the phosphorylation of various DNA damage response mediators that allow amplification of the DNA damage response (for example the histone variant H2AX) and direct regulation of cell cycle progression, apoptosis or senescence (for example the checkpoint kinases Chk1 and Chk2).

a) ATM signaling

Ataxia telangiectasia mutated (ATM) is a large 350kDa protein containing a PI3K signature motif in its C-terminal. It is a protein kinase that triggers the phosphorylation of Ser or Thr residues followed by Glu (S/T-Q motif). Due to its important size, its structure is not resolved yet. ATM is subjected to various post-translational modifications, including auto-phosphorylations that arise in various cellular contexts and participate in the regulation of its activity (figure 8 and (Shiloh and Ziv 2013)). Mutations in ATM are responsible for the genomic instability disorder Ataxia-telangiectasia. ATM is considered as the major transducer of DDR, however patients cells, displaying mutations in ATM, still show a partial DDR activation, suggesting a cooperation with other kinases such as ATR and DNAPK (Tomimatsu, Mukherjee, and Burma 2009).

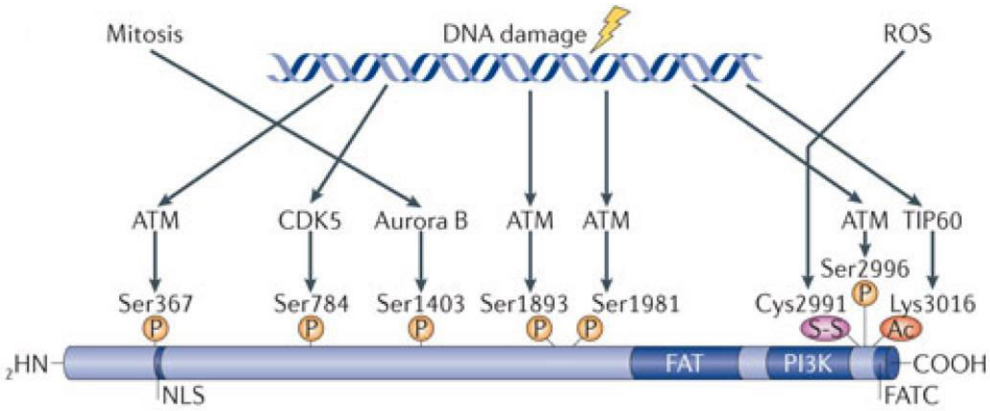


Figure 8- Schematic representation of ATM (Shiloh and Ziv 2013)

Schematic representation of ATM depicting its major domains. The sites of PTMs associated with ATM activation in various contexts and the proteins responsible for these modifications, including ATM itself, are indicated. Ac, acetylation; FATC, FAT carboxy-terminal; NLS, nuclear localization sequence; P, phosphorylation; ROS, reactive oxygen species; S-S, disulphide bridge

-ATM recruitment and retention at DSBs sites

Upon DSBs induction, although its total protein amount is not modified (K. D. Brown et al. 1997), a fraction of ATM is rapidly relocated at sites of DSBs where it persists for several hours (Andegeko et al. 2001). As mentioned earlier, the MRN

complex is a major actor in the recruitment of ATM. Indeed, direct interaction of Nbs1 and ATM is necessary for ATM recruitment and retention (Falck, Coates, and Jackson 2005; S. Difilippantonio and Nussenzweig 2007). This interaction is further regulated by K63-linked ubiquitination of Nbs1 by the Skp2 E3 ubiquitin ligase (Wu et al. 2012). The retention of ATM at DSBs sites is partially mediated by positive feedback loops, including the recruitment of the MDC1 protein. Indeed, MDC1 directly interacts with ATM and is able to recruit it to DSBs sites. ATM phosphorylates the histone variant H2AX that will recruit the MDC1 protein that in turn recruits additional ATM proteins (Lou et al. 2006). This positive feedback loop participates in the spreading of the signal along the DNA, around the break site (Savic et al. 2009). Additional downstream factors of the DDR can also modulate the retention of ATM, as it was proposed for 53BP1 or BRCA1 (Lee et al. 2010).

-ATM activation

ATM activation upon DNA damage was firstly described in vitro as a modest enhancement of its kinase activity when cells were pre-treated with damaging agents or irradiated (Canman et al. 1998; Banin et al. 1998). In vivo, ATM switches from a quiescent state to a potent active form that phosphorylates multiple targets upon DNA damage (Bakkenist and Kastan 2003; Lee and Paull 2004; Lee and Paull 2005). Its activation is controled by several mechanisms. ATM can be present in two different forms in the cells: as an inactive homodimeric complex or as an active monomere. The dissociation of the dimer is necessary for its activation (Bakkenist and Kastan 2003; Lee and Paull 2005; Dupré, Boyer-Chatenet, and Gautier 2006). Additionally, ATM post-translational modifications (PTMs) were also shown to be necessary for ATM activation. The first PTM described in activated ATM was its autophosphorylation on Ser1981 (Bakkenist and Kastan 2003). Three additional autophosphorylation sites were associated with the active form of ATM (Bensimon et al. 2010; Kozlov et al. 2006; Kozlov et al. 2011). Furthermore, ATM is acetylated by Tip60 on Lys3016 and this acetylation is necessary for its activation (Sun et al. 2005; Sun et al. 2007). The PTMs of ATM directly modulate ATM activity, they are required for its proper function in DDR and its retention at DSBs sites but they are not necessary for its initial recruitment.

Several parameters were involved in the intiation of ATM activation such as modulation of chromatin condensation (Bakkenist and Kastan 2003) or direct interaction of ATM with broken DNA (You et al. 2007). The MRN complex is also involved in the activation of ATM. On one hand by recruiting ATM to DSBs sites it allows the concentration of broken ends at the vicinity of ATM, which could be sufficient to dissociate ATM dimers. In line with this hypothesis, increasing the concentration of DNA damage bypasses the need of MRN for the dissociation of ATM dimers (Dupré, Boyer-Chatenet, and Gautier 2006). On the other hand MRN also seems to be directly involved in the establishment of ATM PTMs since Nbs1 is sufficient to induce ATM autophosphorylation in vitro, even in absence of DNA (Dupré, Boyer-Chatenet, and Gautier 2006).

-ATM targets

Several proteomics studies demonstrate that ATM possesses an extensive range of substrates ((Shuhei Matsuoka et al. 2007; Mu et al. 2007; Stokes et al. 2007; Bensimon et al. 2010; Shiloh and Ziv 2013) and figure 9). This important number of targets led to the conclusion that ATM is a "promiscuous" kinase that phosphorylates all the proteins brought in proximity.

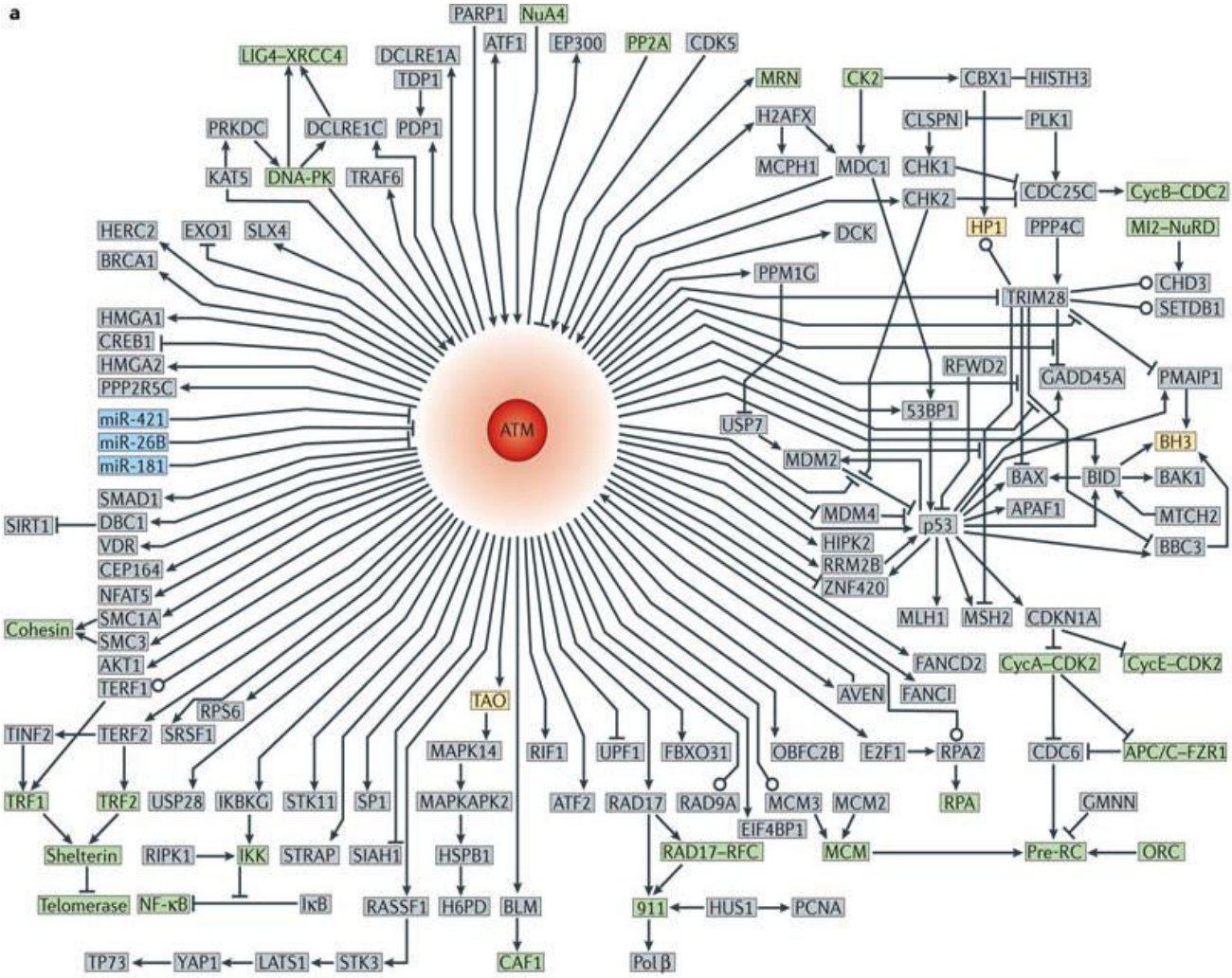


Figure 9- ATM functional interactions (Shiloh and Ziv 2013)

Map of ATM functional interactions, each of which has been thoroughly documented in at least one publication. The map is based on information collected from the SPIKE database of signalling pathways. In most cases, proteins that functionally interact with ATM are shown for each pathway, most of which are ATM substrates. Proteins are depicted in grey, microRNAs (miRNAs) in blue, protein complexes in green and protein families in yellow. Arrows correspond to activation, T-shaped edges to inhibition, and open circles denote regulations the effect of which is still unclear.

In addition to the direct phosphorylation of various targets, ATM also phosphorylates protein kinases and therefore modulates their activity. Among these protein kinases, the checkpoint kinase 2 (Chk2) is a direct target of ATM and allows the regulation of cell cycle progression (S. Matsuoka et al. 2000). Chk2 phosphorylation is necessary for its activation that allows the maintenance of G2 arrest following irradiation. It acts through two different pathways: on one hand it phosphorylates p53 and allows its activation (Hirao et al. 2000) and on the other

hand it was shown *in vitro* to phosphorylate the phosphatase Cdc25C which might lead to its inactivation and translocation in the cytoplasm (Ahn and Prives 2002). When Cdc25C is active it allows the de-phosphorylation and activation of the Cdc2 kinase that promotes cell cycle progression. When Cdc25C is inactive, Cdc2 remains phosphorylated and the cell cycle is arrested. ATM also phosphorylates directly p53 suggesting that Chk2 and ATM might synergize to ensure p53 activation (figure 10).

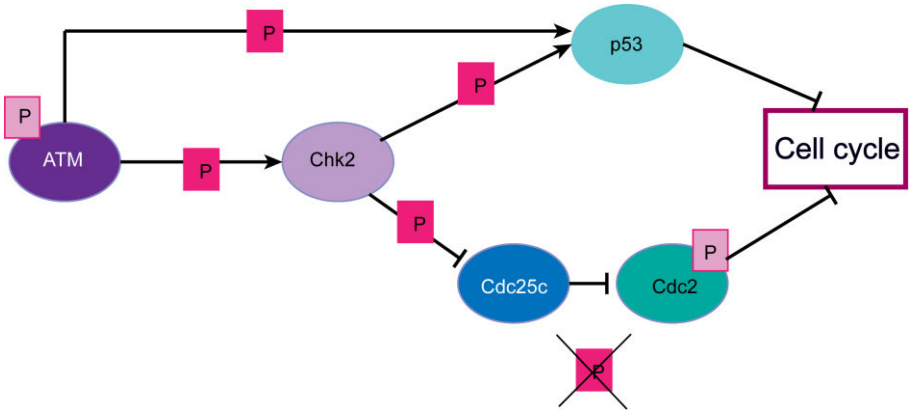


Figure 10- Schematic representation of cell cycle checkpoint activation via the ATM-Chk2 pathway

Phosphorylated ATM can phosphorylate Chk2 and p53. Chk2 phosphorylation leads to the phosphorylation and inactivation (t-shape arrow) of cdc25c that dephosphorylate and inactivate Cdc2 phosphorylated. Chk2 also phosphorylates directly p53. These events lead to cell cycle arrest. Arrows represent activation, t-shape arrows represent inactivation, P means phosphorylation.

b) ATR signaling

While ATM is strictly activated by DSBs, ATM and Rad3-related (ATR) kinase responds to a much broader spectrum of DNA damage, including DSBs and damages that interfere with replication (reviewed in (Zou 2007; Cimprich and Cortez 2008)). ATR function is absolutely essential for development and ATR knock-out mice dye at early stage of development (E. J. Brown and Baltimore 2000; de Klein et al. 2000). However, hypomorphic mutations of ATR, that cause reduced ATR function were identified in patients with the rare Seckel Syndrome, which is characterized by microcephaly and growth retardation (O'Driscoll et al. 2003).

-ATR recruitment

ATR is recruited at ssDNA and junctions between ssDNA and double-stranded DNA (dsDNA) (Zou 2007). Indeed, circular ssDNA that is annealed with primers, therefore forming ssDNA-dsDNA junctions, are sufficient to activate ATR mediated checkpoint in *Xenopus laevis* egg extracts (MacDougall et al. 2007). The major causes of ssDNA are DNA damage and their repair mechanisms, mis-coordination between DNA polymerase and helicase during DNA replication, and DSB resection. In all of these situations, the RPA protein is recruited and allows the accumulation of ATR interacting protein (ATRIP) via direct interaction. ATRIP and ATR interaction therefore allows the accumulation of ATR at sites of damage ((Zou and Elledge 2003)and figure 11). The stabilities of ATR and ATRIP are linked and their association does not appear to be regulated. Furthermore, ATR and ATRIP loss result in the exact same phenotype, regardless of the organism studied. These observations suggest that ATRIP is an obligate subunit of ATR (Cimprich and Cortez 2008).

-ATR activation

The recruitment of ATR to the damaged DNA by RPA-ATRIP is not sufficient for its activation. Indeed, ATR activation depends on its colocalization with the ring-shaped complex Rad9-Rad1-Hus1 (9-1-1), which structure and sequence are related with the replication protein PCNA. 9-1-1 is loaded to DNA ends that are adjacent to a stretch of RPA-coated ssDNA, in a mechanism dependent on the recruitment of the Rad17 protein (Zou, Liu, and Elledge 2003). The recruitments of Rad17 and ATR are independent and the colocalization of 9-1-1 and ATR is enough to activate ATR-mediated checkpoint in *S.Cerevisiae, in vitro,* suggesting that the role of Rad17 is to promote their colocalization (Bonilla, Melo, and Toczyski 2008).

In other species however, additional activation mechanisms were identified. Notably, the DNA topoisomerase II binding protein (TopBP1) is a major activator of ATR and can stimulate ATR activity even in absence of DNA (Kumagai et al. 2006). TopBP1 mutant that cannot interact with ATR induces decreased checkpoint activation (Kumagai et al. 2006). The 9-1-1 complex is responsible for TopBP1 accumulation at

damage sites via its interaction with the Cterminal tail of Rad9, which is phopsphorylated at residue Ser387. This phosphorylation event creates a recognition site for BRCT domains I and II of TopBP1, thereby recruiting TopBP1 to ATR. TopBP1 contains an ATR activation domain that interacts with and activates ATR-ATRIP complexes *in vitro* (Kumagai et al. 2006) (figure 11).

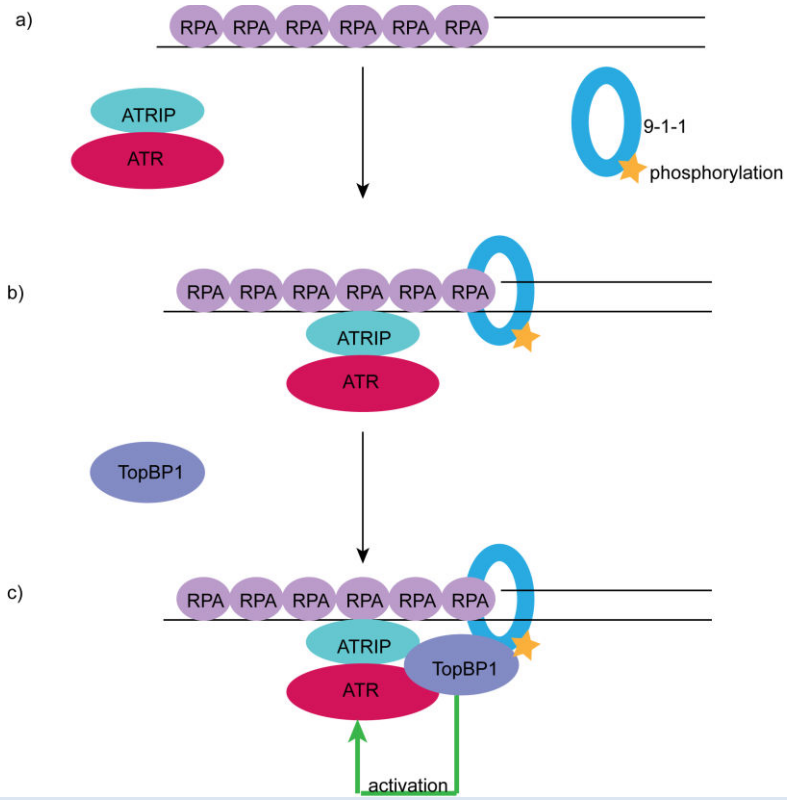


Figure 11- Schematic representation of ATR activation

(a) RPA coats ssDNA (b) ATRIP-ATR recruitment at RPA-coated ssDNA and independent recruitment of 9-1-1 complex at ds-ssDNA junction (c) TopBP1 is recruited through the 9-1-1 complex and binds to ATRIP, allowing the activation of ATR.

Another interesting activation mechanism of ATR involves interplay between ATM and ATR. Indeed, ATR responds directly to interference with DNA replication but is also activated upon DSBs. This activation depends on ATM activity (Jazayeri et al. 2006; Myers and Cortez 2006). In this case, ATR is activated more slowly than ATM and predominantly during the S and G2 phase of the cell cycle (Jazayeri et al. 2006). The dependence of ATR activation on ATM was attributed to its role in resection that mainly happens during the S and G2 phases of the cell cycle. Consistently, ATR

activation upon DSB induction was shown to necessitate resection factors (Jazayeri et al. 2006).

-ATR targets

ATM and ATR share the same consensus sites of phosphorylation and proteomics approaches mainly identified common targets (Shuhei Matsuoka et al. 2007; Mu et al. 2007; Stokes et al. 2007) but some specific ATR targets were identified by using ATR-deficient cells from Seckel patients. Among the different targets of ATR, the proteins involved in its recruitment such as RPA, ATRIP, Rad17, 9-1-1, TopBP1 are shown to be phosphorylated, suggesting that, as ATM, ATR might be a proximal kinase. In addition to these proteins, ATR phosphorylates the checkpoint kinase 1 (Chk1) on its S317 and S345 therefore allowing checkpoints activation ((Bartek and Lukas 2003)and figure 12). Once Chk1 is activated by ATR phosphorylation, it is released from chromatin and phosphorylates its targets (Smits, Reaper, and Jackson 2006): the Cdc25 phosphatases that regulate cell cycle transitions (Furnari, Rhind, and Russell 1997). The phosphorylation of Chk1 by ATR is regulated by the Claspin protein, which brings Chk1 and ATR together. Claspin and Chk1 interacts in a damage-dependent manner, and this interaction requires ATR dependent phosphorylation of Claspin (Kumagai and Dunphy 2003). However the responsible kinase for Claspin might not be ATR itself since the modified serines are not part of a consensus ATR phosphorylation site.

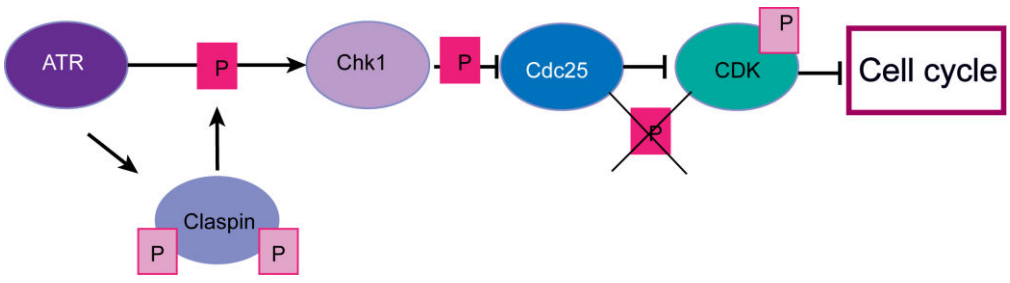


Figure 12- Schematic representation of cell cycle checkpoint activation via the ATR-Chk1 pathway

Chk1 phosphorylation by ATR depends on phosphorylated Claspin. Chk1 phosphorylation leads to the phosphorylation and inactivation (t-shape arrow) of Cdc25 phosphatases that dephosphorylate and inactivate CDK phosphorylated. These events lead to cell cycle arrest. Arrows represent activation, t-shape arrows represent inactivation, P means phosphorylation.

c) DNAPK signaling

DNAPK is a serine/threonine kinase form the PI3K-like family, composed by three proteins: Ku70, Ku80 and the DNAPK catalytic subunit (DNAPKcs). Interestingly, DNA-PK is the most abundant PIKK in human cells, at significantly lower levels in rodent cells, and entirely absent from nematodes, flies and yeast (Anderson and Lees-Miller 1992; Yang et al. 2003).

DNAPK plays a major role in DSB repair by NHEJ. Indeed, Ku70/80 can directly recognize double-stranded DNA ends and induces the recruitment of DNAPKcs to DSBs, which triggers the stabilization and tethering of the two DNA ends that finally allows repair by ligation (for more details see section III of the introduction). However, besides its role in NHEJ, DNAPKcs has also been implicated in DDR. Indeed, similarly to ATM and ATR, DNAPKcs can phosphorylate H2AX (Stiff et al. 2004), therefore triggering activation of DDR signaling.

Another relevant target of DNAPKcs in DDR is the RPA32 subunit of RPA. Indeed, both ATR and DNAPKcs can phosphorylate RPA32 upon replication stress (Shao et al. 1999; Block, Yu, and Lees-Miller 2004), whereas ATM and DNAPKcs can phosphorylate it upon ionizing radiation (Wang et al. 2001). This phosphorylation of RPA32 by DNAPKcs was shown to participate in the G2/M and intra-S checkpoints (Liaw, Lee, and Myung 2011; Liu et al. 2012).

Furthermore, a recent study implicated DNAPKcs in the cytoplasmic response to DNA damage. In this study, the authors showed that following DNA damage, DNAPKcs phosphorylates the GOLPH3 protein. This phosphorylation leads to Golgi dispersal (Farber-Katz et al. 2014). Consistantly with a role for DNAPKcs in integrating DNA damage signaling and cytoplasmic response a recent study identified several cytoskeleton proteins as targets of DNAPKcs. Interestingly, the phosphorylation of the intermediate filament vimentin was associated with lower cellular adhesion and increased migration (Kotula et al. 2013). DNA-PK has been reported to be overexpressed in various metastatic tumors (Hsu, Zhang, and Chen 2012); however whether it plays a role in the metastatic process remains unclear. This

study provides a mechanism by which DNAPK could participate in the metastatic process.

3. Signal amplification, spreading and formation of DNA repair foci

One of the early events of the DDR is the phosphorylation of the histone variant H2AX on its serine 139 (Rogakou et al. 1998) by the PIKK kinases (phosphorylated H2AX is called γH2AX) in close proximity to the break. This phosphorylation initiates the DDR signaling cascade. Indeed, it allows the recruitment of the mediator protein MDC1, which acts as a platform to recruit additional MRN-ATM complexes that can phosphorylate H2AX on adjacent nucleosomes. This activating loop triggers the amplification of the signal by spreading γH2AX for more than 2Mb around the break site (Rogakou et al. 1999). The spreading of DDR factors allows their visualization by microscopy as foci that were named IRIF (irradiation induced foci, figure 13 and Kinner et al. 2008; Nagy and Soutoglou 2009).

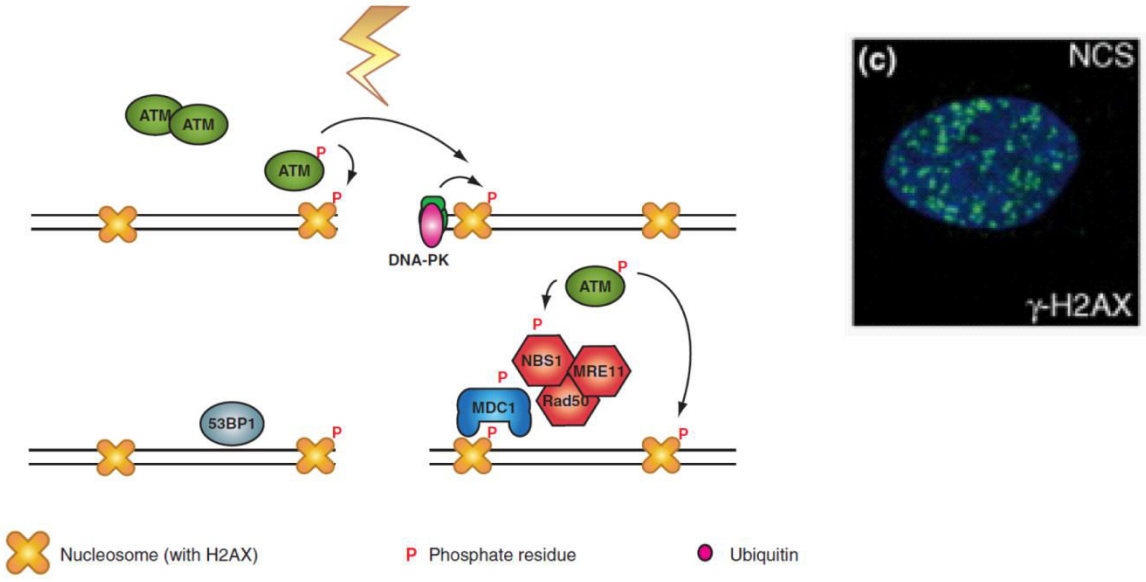


Figure 13- γH2AX spreading

Left panel (adapted from Kinner et al., 2008): Following the initial phosphorylation of H2AX by ATM, DNA-PK or ATR, a nucleation reaction is initiated starting with the recruitment of MDC1 and continuing with that of the MRN complex to further activate ATM. This generates a feedback loop that leads to further phosphorylation of H2AX on the adjacent nucleosomes.

Right panel (adapted from Nagy and Soutoglou 2009): Upon treatment of HeLa cells with the radiomimetic drug NCS, γH2AX foci can be observed by microscopy (γH2AX green, DAPi blue)

Additionally to its role in the spreading of γH2AX, MDC1 also triggers the recruitment of additional downstream factors, such as the ubiquitin ligase ring finger protein 8 (RNF8). RNF8 recruitment to DSBs triggers the recruitment of an additional ubiquitin ligase, ring finger protein 168 (RNF168). RNF8 and RNF168 subsequently ubiquitinate H2A and H2AX, which leads to the recruitment of downstream effectors such as BRCA1 and 53BP1, two proteins involved in DSB repair (figure 14 and Bartocci and Denchi 2013).

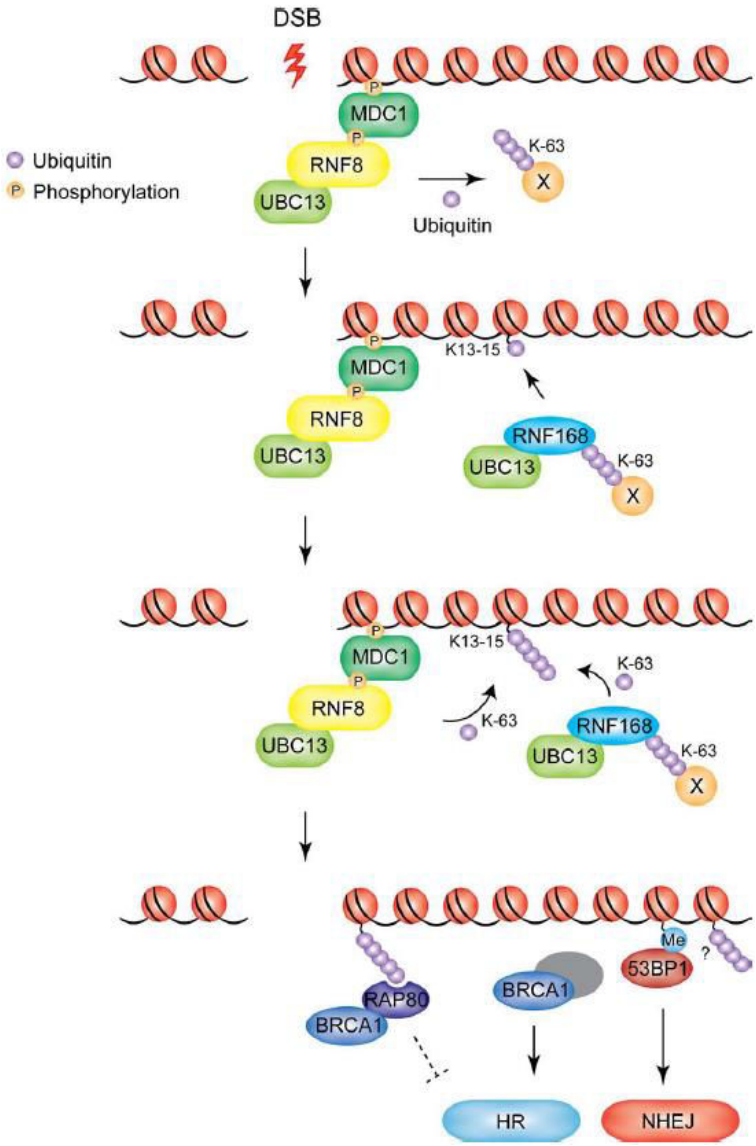


Figure 14- Model of RNF8/RNF168-mediated ubiquitylation at DSBs (from Bartocci and Denchi 2013)

RNF8 is recruited to DSBs through its interaction with MDC1. Chromatin-bound RNF8 cooperates with the E2 UBC13 to ubiquitylate an unknown non nucleosomal target in the vicinity of the damaged chromatin(X). Ubiquitylated target-X is recognized by RNF168, which catalyzes monoubiquitylation of K13-15 on H2A-type histones. RNF8 and RNF168 work in concert to extend the ubiquitin chains on H2A-type histones. BRCA1 and 53BP1 are recruited as downstream effectors. BRCA1 accumulates at DSBs in an RNF8/RNF168-dependent manner, through RAP80, which binds to the K63 linked ubiquitin chains deposited by RNF8/RNF168.The RAP80–BRCA1 complex is thought to inhibit excessive HR, while BRCA1 in complex with several other DNA damage response proteins is known to primarily promote DNA repair by HR. 53BP1 accumulation at DSBs depends on RNF8/RNF168 mediated modifications to the chromatin surrounding the DNA lesion. 53BP1 promotes DNA repair by NHEJ (Me, methylation of H4K20)

4. DDR outcomes: Cell cycle arrest, senescence and apoptosis

As mentioned earlier, DDR signaling through the ATM and ATR kinases leads to the activation of the checkpoint kinases Chk2 and Chk1 respectively. Both pathways trigger checkpoint activation that can lead to three different outcomes: cell cycle arrest, senescence or apoptosis (figure 15 and d' Adda di Fagagna 2008).

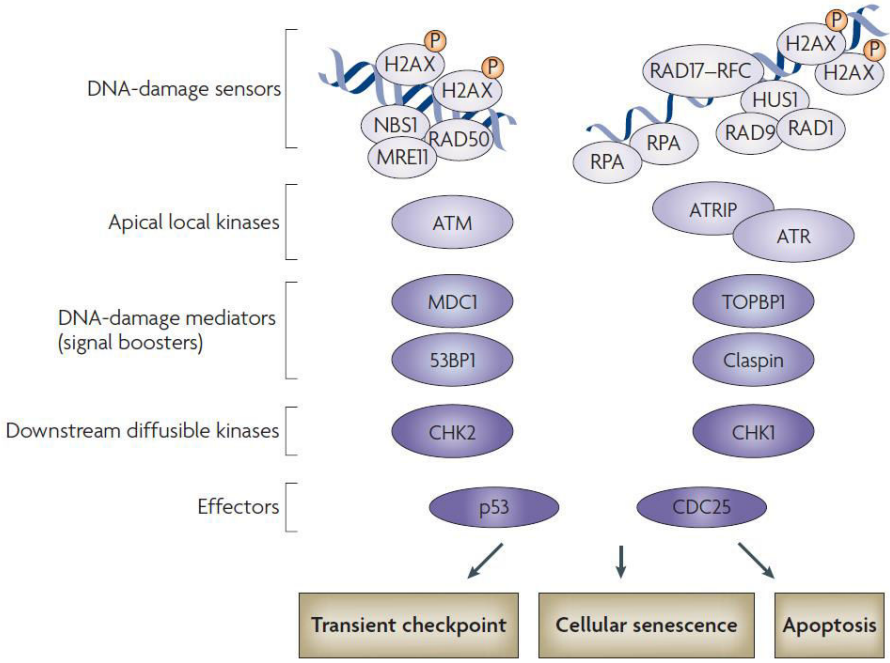


Figure 15- DDR outcomes (from d'Adda di Fagagna 2008)

ATM activation during the DDR, allows the activation of Chk2, whereas ATR signaling allows the activation of Chk1. Both ways lead to the activation of transient checkpoints until breaks are repaired, or cellular senescence or apoptosis if the breaks cannot be repaired.

a) Cell cycle arrest

The major outcome of the DDR is cell cycle arrest that gives cells time to repair the breaks. Depending on the cell cycle phase in which the breaks occur, the mechanism that induce cell cycle arrest is different. Arrest in G1 is mainly mediated by the p53 protein. Upon phosphorylation of p53 on its serine 15 by ATM or ATR, p53 interaction with the ubiquitin ligase MDM2 is disrupted, therefore avoiding ubiquitination and proteasomal degradation of p53 (Shieh et al. 1997). As a

consequence of its stabilization, p53 can activate p21 transcription that leads to CDK inhibition and subsequent G1/S transition block (Harper et al. 1993).

Arrest in S phase is mainly mediated by progressive slowing down of replication fork progression and decreased activation of replication origins (Grallert and Boye 2008; Seiler et al. 2007). MRE11 interaction with RPA is necessary to allow the intra-S checkpoint activation (Olson et al. 2007).

In G2, cell cycle arrest is due to the inhibition of the CDK1-Cyclin B complex, in response to the inhibition of the Cdc25 phosphatases (as seen in figure 12) (O'Connell, Walworth, and Carr 2000).

b) Senescence

Senescence is defined as the irreversible condition in which damaged cells remain alive but are unable to proliferate (Campisi and d' Adda di Fagagna 2007). Two different types of senescence were described: replicative senescence that is mainly due to shortening of telomeres (Harley, Futcher, and Greider 1990) and oncogene-induced senescence, a tumour suppressive mechanism that impedes the proliferation of a cell that expresses high levels of an aggressive oncogene (Serrano et al. 1997; Prieur and Peeper 2008).

In both cases, senescence is induced by DDR activation. Indeed, when telomeres shorten below a threshold length (which is still unknown), DDR is activated and DDR foci are visible in proximity of telomeric DNA (Takai, Smogorzewska, and de Lange 2003; Herbig et al. 2004; d' Adda di Fagagna et al. 2003). Senescence is not determined by the average telomere length but rather by the presence of a few telomeres that are sufficiently short to trigger the DDR. Oncogene-induced senescence has been proposed to result from altered DNA replication, which can activate an ATR-dependent checkpoint (Herbig et al. 2004; Hemann et al. 2001).

The persistent activation of DDR in these cells triggers p53 phosphorylation, activation and stabilization (Turenne et al. 2001), which leads to the transcription of

p21, a cyclin-dependent kinase inhibitor (Deng et al. 1995) and results in stable cell-cycle arrest.

c) Apoptosis

Another possible response to DDR activation is programmed cell death, also named apoptosis. Similarly to senescence, apoptosis is mediated by the p53 protein, but instead of activating the transcription of p21 that would lead to cell cycle arrest it induces BH3-only proteins. How the regulation between the p21 and the BH3-only p53 responses is balanced is poorly understood.

BH3-only proteins such as PUMA, BAX and BAK are potent activators of apoptosis. In response to the subsequent apoptotic signals, the caspases proteases are activated and trigger cell death (Reinhardt and Schumacher 2012).

III. Major DSB repair pathways

The two main pathways to repair DSBs are the non-homologous end-joining (NHEJ) and the homologous recombination (HR). Although being sometimes considered as an error-prone pathway, NHEJ allows faithful rejoining of broken ends, unless they cannot be simply rejoined and need additional processing. HR is an error-free mechanism that occurs during the S and G2 phases of the cell cycle. Indeed, it uses the homologous sister chromatid as a template for the repair. Its strict cell cycle regulation is therefore necessary to maintain genomic stability. A third repair pathway, the alternative end-joining (alt-EJ) has been identified more recently. This less well defined mechanism is error-prone and its use seems to lead to genomic rearrangement such as chromosomal translocations. In this section, I will describe these three DSB repair mechanisms and I will describe their regulation and interplay in the next section. A fourth DSB repair mechanism, single-strand annealing (SSA), that occurs between tandem repeated sequences and necessary leads to loss of one repeat, has been described. For more details concerning this pathway, see (Pâques and Haber, 1999).

1. HR

HR is the major mechanism used to repair breaks induced physiologically during meiosis or accidentally during replication. HR uses homologous sequence –mainly the homologous sister chromatid in mammals- as a template for repair. It consists in the exchange of DNA molecules that exhibit sequence homology. The required homology does not necessary need to be perfect, however below a certain length of perfect homology (called MEPS: minimal efficient processing segment), the efficiency of recombination drops dramatically. In mammals, the MEPS has been estimated between 200 and 250 nucleotides (Liskay et al., 1987; Lopez et al., 1992). The molecular mechanism of HR consists in a first step of resection that generates a 3'tail, which can invade the double-stranded DNA homologous sequence, therefore forming a heteroduplex, leading to strand exchange. When the homology is not perfect, the heteroduplex contains mismatches that can be repaired by the mismatch repair pathway and the genetic information may be modified, leading to gene conversion (figure 16). In some cases, invasion of the homologous strand leads to the

formation of cruciform structures called Holiday junctions. Their resolution can lead to a reciprocal exchange of the adjacent structure, named crossover (figure 16).

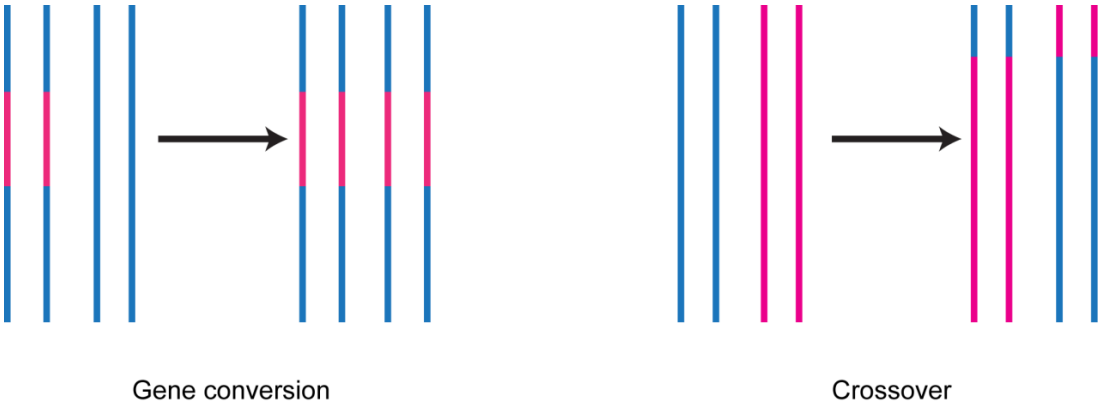


Figure 16- Gene conversion and crossovers

Gene conversion (left panel): non reciprocal transfer of genetic material. Crossover (right panel): reciprocal exchange of genetic material.

Three main subpathways of HR were identified: break-induced replication (BIR), synthesis-dependent strand annealing (SDSA), double holiday junction (dHJ) (Pâques and Haber, 1999). Whereas dHJ involves the resolution of holiday junctions, BIR and SDSA don't. BIR occurs in the absence of a second end. In that case the entire chromosome is replicated (figure 17 and Heyer et al., 2010). This mechanism is underlying the ALT system that allows telomere maintenance in the absence of telomerase (Dunham et al., 2000). During, SDSA the D-loop formed by the heteroduplex is reversed, leading to the annealing of the newly synthesized strand with the resected strand of the second end (figure 17 and Heyer et al., 2010). SDSA seems to be the major HR subpathway in somatic cells. By avoiding crossovers, it reduces potential for genomic rearrangements. On the contrary, generation of crossovers is the purpose of meiotic recombination that therefore mainly uses dHJ, a mechanism that involves formation and resolution or dissolution of Holiday junctions (figure 17 and Heyer et al., 2010).

All the three subpathways are initiated by common steps: resection, invasion and exchange of homologous DNA strand. The molecular mechanisms underlying these steps will be described below.

a) End resection

End resection produces single strand DNA that is necessary for the invasion of the homologous sequence. This step is a key determinant in the choice of the repair pathway to be used since NHEJ is unable to repair resected breaks (see part IV). Studies in yeast showed that resection occurs in two steps. A first limited resection is ensured by the MRX complex and the Sae2 protein (CtIP functional homolog). The single-stranded DNA formed is a preferred substrate for the exonuclease Exo1 and the Sgs1/endonuclease DNA2 complex that ensure the second, long-range step of resection (Mimitou and Symington, 2009a, 2009b). In mammalian cells, a similar model is proposed. Indeed, the MRN complex in association with CtIP, that stimulates the endonuclease activity of MRE11, allows the initiation of resection (Sartori et al., 2007), whereas helicases such as BLM and nucleases such as Exo1 (Bolderson et al., 2010) and DNA2 allow the second step of resection (Nimonkar et al., 2011).

The single-strand DNA produced by resection is protected by the RPA heterotrimer. In addition to its protective role RPA also seems to be involved in the resection process. Indeed, RPA was proposed to regulate the directionality of resection by DNA2 (Nimonkar et al., 2011). RPA is subsequently phosphorylated on its RPA2 subunit by ATR, ATM and DNAPK. This phosphorylation is necessary for HR and might allow the recruitment of additional HR factors such as PALB2 (Murphy et al., 2014) or Rad51 (Shi et al., 2010) but its exact role remains unclear (Oakley and Patrick, 2010).

b) Strand invasion and exchange

Following RPA phosphorylation, the BRCA complex, formed by BRCA1-PALB2-BRCA2 proteins allows the recruitment of the Rad51 protein. BRCA1 is recruited to DSBs via its interaction with MRN. PALB2 is the protein linking BRCA1 and BRCA2, which can directly bind Rad51. Rad51 polymerizes and forms a filament that can invade the homologous sequence, therefore forming a displacement loop (D-loop). The Rad54 protein that is subsequently recruited allows the stabilization of Rad51 filament and of the D-loop. Rad54 also promotes the transition from DNA-strand

invasion to DNA synthesis by dissociating Rad51 from heteroduplex DNA (Heyer et al., 2006). Upon D-loop formation, the different HR subpathways can take place (figure 17 and Heyer et al., 2010), all of them including a step of DNA synthesis. In the case of dHJ, the Holiday junctions are resolved thanks to endonucleases or dissolved by BLM topoisomerase 3 and cofactors.

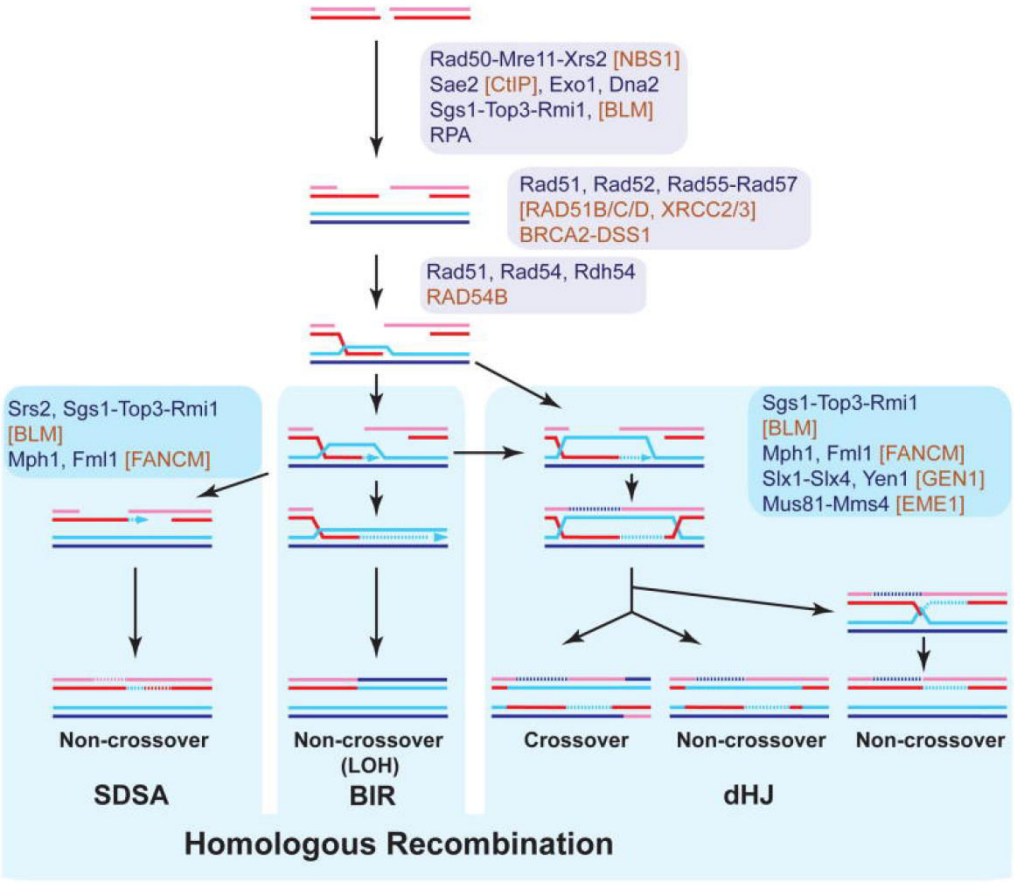


Figure 17- Homologous recombination subpathways (adapted from Heyer, 2010)

Protein names refer to the budding yeast *S. cerevisiae* (black). Where different in human, names (brown) are given in brackets. For proteins without yeast homolog brackets for human proteins are omitted. Broken lines indicate new DNA synthesis and stretches of hDNA that upon MMR can lead to gene conversion.

2. NHEJ

HR was the first DSB repair pathway described and was initially considered as the only efficient and safe mechanism to repair DSBs. This idea was reinforced by the fact that laboratory strains of *E. Coli* lacked the capacity to efficiently join broken DNA ends by ligation (Malyarchuk et al., 2007). Therefore, the discovery that mammalian cells were able to efficiently join unrelated DNA fragments end-to-end

using the now-called NHEJ repair pathway was surprising (Wilson et al., 1982). This repair pathway does not require sequence homology, although microhomologies of one to six complementary bases can appear at the junctions and may help to align the ends (Roth and Wilson, 1986). NHEJ is active during all phases of the cell cycle and is now considered as the predominant pathway for repairing DSBs in mammalian cells (Rothkamm et al., 2003).

NHEJ might seem an easy repair mechanism since it simply joins DNA ends by ligation and does not need to search for a homologous repair template. Accordingly to this idea, NHEJ is faster than HR (Mao et al., 2008). However, in some situations, NHEJ involves more sophisticated mechanisms. Indeed, when DNA ends are not ligatable, for example if they are chemically modified or if they are not blunt, NHEJ proceed to an additional processing step to finally allow the ligation. Additionally, NHEJ allows the maintenance of the two broken ends in close proximity to avoid inappropriate joining to other ends that may co-exist.

a) DNA end tethering by the Ku70/Ku80 complex

NHEJ is initiated by the binding of the Ku70/Ku80 complex (Ku complex). As mentioned in the part II of the introduction, the Ku complex has a very high affinity for DNA ends, which allows its rapid recruitment to DSBs. Binding of Ku to the DNA allows the recruitment of its interacting partners such as DNAPKcs and ligaseIV-XRCC4. This recruitment might depend on a conformational change of the Ku complex, when bound to DNA. Indeed, Ku forms stable complex with DNAPKcs (Yaneva et al., 1997) and with XRCC4-ligaseIV (Nick McElhinny et al., 2000) only in presence of DNA.

Besides its role in recruiting additional NHEJ factors, Ku has a key role in the maintenance of genome stability. Indeed, it protects DNA from nucleases digestion (Foster et al., 2011) and allows tethering of DNA ends. Indeed, observation of DSBs in live cell microscopy showed that the two DNA ends do not separate (Soutoglou et al., 2007). Ku is necessary to hold the ends together since its depletion leads to separation of the broken ends (Soutoglou et al., 2007) that might increase the translocations frequency. Additionally, Ku was shown to limit DSB mobility, which

constitute a second protective mechanism against translocations (Roukos et al., 2013, discussed more in detail in the discussion part of this manuscript).

The exact stoechiometry of Ku association to the DNA remains unknown since Ku can translocate along the DNA allowing multiple Ku complexes to be loaded (Downs and Jackson, 2004). Furthermore, the Ku-DNA complex might be stable even after ligation and the mechanisms underlying release of Ku from the DNA -that seem to involve ubiquitin-mediated degradation (Postow et al., 2008)- is still largely unknown.

b) DNAPKcs recruitment

DNAPKcs is recruited to DSBs by the C-terminal domain of Ku80 (Singleton et al., 1999). Subsequently to DNAPKcs recruitment, Ku translocates inward, allowing DNAPKcs to contact an approximately 10bp-long DNA region at both termini (Yoo and Dynan, 1999). The two molecules of DNAPKcs bound to opposing sides of the DSB can interact, contributing to synapsis of the broken DNA ends (DeFazio et al., 2002). The formation of DNAPKcs-Ku-DNA complex (called the synaptic complex) allows the activation of the kinase activity of DNAPKcs (Hammarsten and Chu, 1998; Yaneva et al., 1997), which is necessary for repair by NHEJ (Kienker et al., 2000; Kurimasa et al., 1999). Phosphorylation targets of DNAPKcs include several NHEJ factors, such as Ku70, Ku80 (Chan et al., 1999), Artemis (Goodarzi et al., 2006; Ma et al., 2005), XRCC4 (Leber et al., 1998), XLF (Yu et al., 2008), LigaseIV (Wang et al., 2004). However, none of these phosphorylation events is required for successfull NHEJ (Douglas et al., 2005; Goodarzi et al., 2006; Wang et al., 2004; Yu et al., 2003, 2008). A possible explanation is a functional redundancy between the different phosphorylation sites. Consistantly, phosphorylations of XRCC4 and XLF by DNAPKcs are functionally redundant and promote XRCC4-XLF complex dissociation (Roy et al., 2012).

Additionally, DNAPKcs autophosphorylation seems to play major roles in NHEJ (Chan et al., 2002; Chen et al., 2005; Cui et al., 2005; Ding et al., 2003; Douglas et al., 2002; Meek et al., 2007; Soubeyrand et al., 2003). Indeed, phosphorylations of two clusters of residues named ABCDE and PQR regulate the accessibility of DNA ends

to processing enzymes and ligases (Dobbs et al., 2010). Interestingly, phosphorylation of the ABCDE cluster promotes access to DNA ends whereas phosphorylation of the PQR cluster inhibits access (Neal and Meek, 2011). The different autophosphorylation sites of DNAPKcs and their role in the regulation of DSB repair are represented in figure 18 (Neal and Meek, 2011).

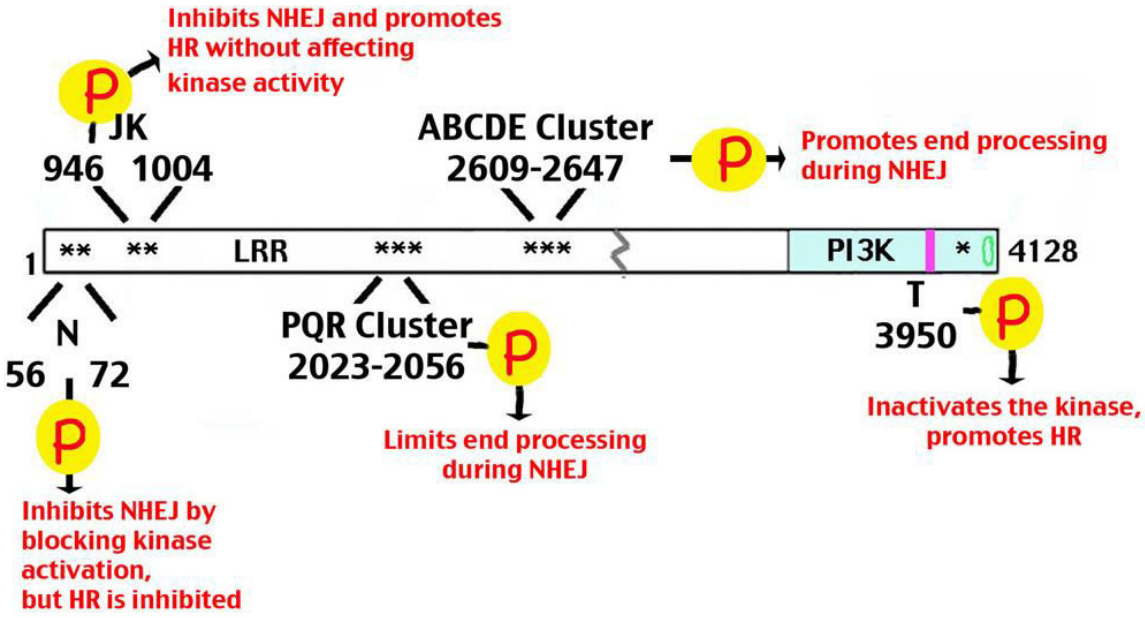


Figure 18- DNAPK's autophosphorylation is functionally complex (Neal and Meek, 2011)

DNAPK is phophorylated on numerous sites (likely more than 40) in vitro and in vivo. Phosphorylation at T in the activation loop of the kinase domain inactivates the kinase; thus blocking NHEJ and promoting HR. JK phosphorylation also impedes NHEJ while promoting HR; however, JK phoshorylation does not affect enzymatic activity. Phosphorylation of N impedes (but does not block) kinase activation, thus inhibiting NHEJ. However, (unlike JK and T phosphorylation) N phosphorylation does not promote HR. Phosphorylation of sites within either of the two major clusters (ABCDE and PQR) enhances NHEJ by reciprocal regulation of end processing. None of the ABCDE or PQR sites alter enzymatic activity or mediate autophosphorylation induced kinase dissociation

c) End processing

In theory, the two ends of a DSB that are maintained in close proximity within the synaptic complex can be directly ligated. However, more complex DNA ends, that are chemically modified (as it is often the case for damage caused by radiation) or have a specific structure, such as the hairpin coding ends that are produced during V(D)J recombination necessitate additional processing (Deriano and Roth, 2013). Several enzymes have been implicated in end processing, including the

polynucleotide kinase phosphatase (PNKP), that can phosphorylate 5' end and dephosphorylate 3' end, therefore providing the chemical end groups required for ligation (Chappell et al., 2002), some polymerases that can fill in the gaps at or near the site of a DSB and allow nucleotide addition during resolution of V(D)J recombination intermediates (Ramsden, 2011) or the nuclease Artemis. Artemis is an endonuclease that was identified as a gene mutated in certain radiosensitive severe combined immunodeficiencies (Moshous et al., 2001). The endonuclease activity of Artemis is stimulated by DNAPK activity and carries out hairpin opening (Ma et al., 2002). Its activity is therefore necessary during V(D)J recombination (Rooney et al., 2004). Furthermore, Artemis is required for removing ssDNA overhangs containing damaged nucleotides (Kurosawa and Adachi, 2010). Interestingly, Ku itself also seems to participate in the end processing (Strande et al., 2012).

d) End-joining

The final step of NHEJ consists in the joining of DNA ends by Ligase IV that is recruited within a complex including XRCC4 and XLF proteins. Binding of XRCC4 stabilizes and stimulates Ligase IV activity (Bryans et al., 1999; Grawunder et al., 1997). XLF stimulates ligation of noncohesive DNA ends (Akopiants et al., 2009; Riballo et al., 2009) and might participate in the alignment of the DNA ends prior ligation (Akopiants et al., 2009). Ligase IV, XLF and XRCC4 can interact directly with the Ku complex, but whether DNAPKcs is also required for the recruitment of these proteins remains unclear (Mahaney et al., 2009). Therefore, whether DNAPKcs and Ligase IV-XRCC4-XLF are recruited independently or sequentially is an open question.

e) Uses and targeting of NHEJ in therapy strategies

Due to the processing of DNA ends, NHEJ has often been referred to as an error-prone pathway. However, it usually restores chromosome integrity without leading to chromosome rearrangements and the mutations induced by NHEJ are usually minor compared to those formed by the alt-EJ pathway. Therefore, NHEJ is not considered as a particularly error-prone pathway anymore (Deriano and Roth, 2013 and figure19). However its non-conservative nature is now used in gene therapy

strategies in which the propensy of NHEJ to make slight modifications at the junction is used to inactivate target genes cleaved by sequence specific nucleases (Perez et al., 2008).

On the other hand, the knowledge on NHEJ mechanisms allows to develop new therapeutic strategies against cancer. Indeed, genetic analyses of 489 ovarian tumors revealed defects in HR in half of them (Cancer Genome Atlas Research Network, 2011), suggesting that these cells employ NHEJ exclusively to repair DSBs. This suggests that inhibition of NHEJ in combination with classical radiotherapy or chemiotherapy could selectively kill cancer cells. Accordingly, DNA ligase IV inhibitor impedes tumor progression in mouse cancer models (Srivastava et al., 2012).

3. Alt-EJ

Genetic alterations of some of the NHEJ components does not lead to a total impairment of DSB end-joining (Kabotyanski et al., 1998; Liang and Jasin, 1996) or to complete loss of V(D)J recombination junctions formation (Blackwell et al., 1989; Bogue et al., 1997, 1998; Malynn et al., 1988), therefore suggesting the existence of an alternative end-joining repair pathway. Several names were given to this alternative end-joining mechanism: alternative NHEJ (altNHEJ, alt-EJ, aNHEJ), microhomology-mediated end joining (MMEJ), backup NHEJ (bNHEJ), micro-single strand annealing (μSSA). For clarity, I will use alternative end-joining (alt-EJ) throughout the entire manuscript.

Analyses of junctions formed by alt-EJ allowed the description of some of its features. Indeed, alt-EJ often leads to large deletions, presence of microhomologies or occasional insertions of large DNA segments (Deriano and Roth, 2013). In accordance with these characteristics, the proposed mechanism for alt-EJ involves a first step of short-range resection. The resection would allow uncovering microhomologies that could hybridize and promote the final ligation step (figure 19, right pannel and (Deriano and Roth, 2013)). The exact proteins mediating this mechanism are probably not all known yet, however some actors of this pathway were recently identified. The recognition step seems to be ensured by PARP1 (Audebert et al., 2004)

that competes with Ku binding to the DNA (Wang et al., 2006). Consistently, PARP1 was shown to favor the use of microhomologies for repair of breaks induced during class-switching (Robert et al., 2009). The resection step of alt-EJ seems to be mediated both by CtIP (Zhang and Jasin, 2011) and MRN (Dinkelmann et al., 2009; Rass et al., 2009; Xie et al., 2009; Zhuang et al., 2009). Two different ligases were proposed to be responsible for the ligation step during alt-EJ: the ligase I and the XRCC1-ligase III complex. Ligase III depletion leads to decrease of translocations formation by alt-EJ whereas depletion of ligase I does not modify translocation frequency. However, ligase I depletion in ligase III deficient-cells further decrease translocations frequency by alt-EJ, therefore suggesting that ligase III is the major ligase of alt-EJ and ligase I constitutes a backup ligase (Simsek et al., 2011).

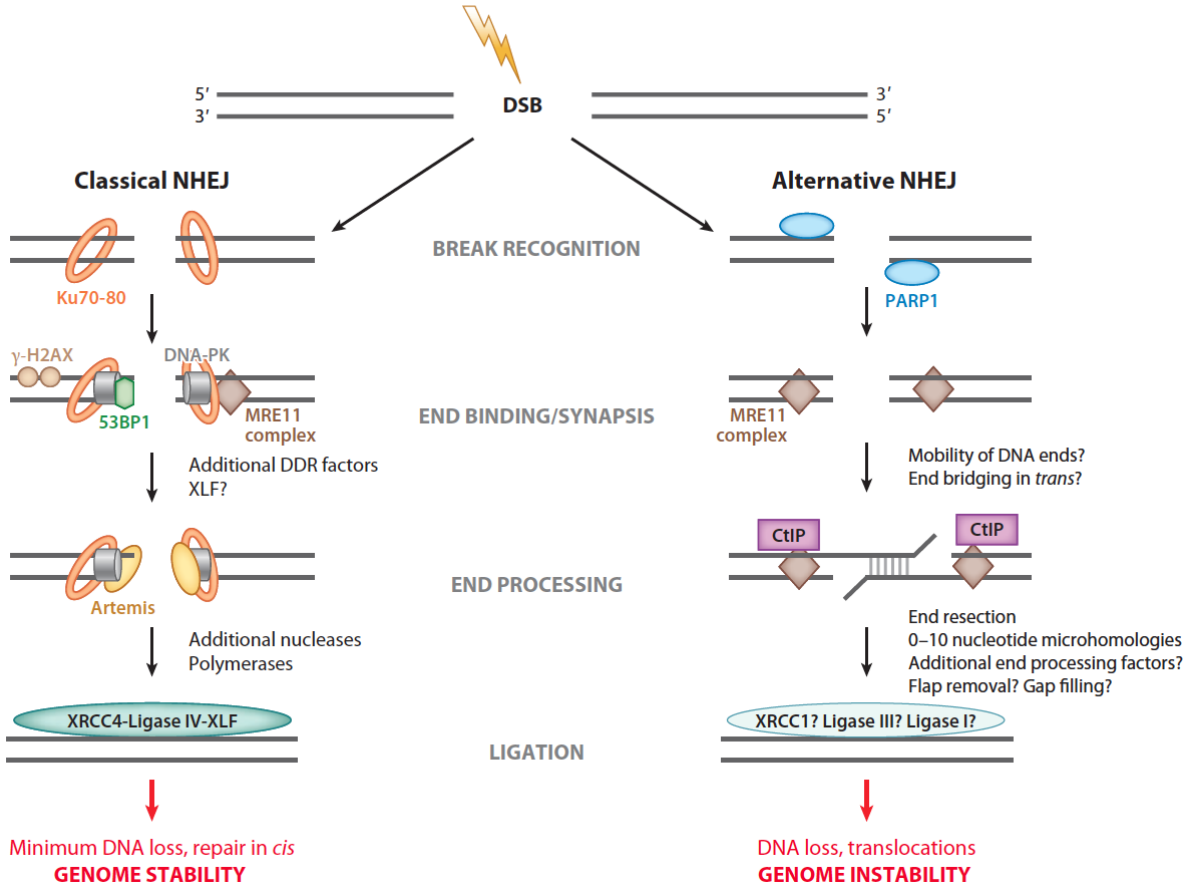


Figure 19- NHEJ and alt-EJ pathways (Deriano and Roth, 2013)

NHEJ (left pannel), involves the recognition of DSBs by the Ku complex, the formation of a synapsis that brings the ends in close proximity, notably thanks to DNAPKcs, processing of the ends by Artemis, other nucleases and polymerases and ligation by XRCC4-LigaseIV-XLF complex. Although not being entirely conservative when DNA ends need processing, NHEJ allows genome stability.

Alt-EJ (right pannel), involves the recognition of the break by PARP1, resection by CtIP and MRN and ligation by XRCC1-LigaseIII or LigaseI. In comparison to NHEJ, alt-EJ induces major genome instability, is involved in DNA loss and translocations.

4. Contribution of the different repair pathways in the formation of translocations

Translocations formation implies aberrant repair of multiple DSBs in different chromosomes that are joined together. Understanding which repair mechanism mediates this aberrant repair is therefore key in the study of tumorigenesis. HR does not seem to be at the origin of chromosomal translocations because it only rarely form crossovers outside of meïosis (Weinstock et al., 2006). On the contrary, NHEJ and alt-EJ were both implicated in the formation of translocations. Indeed, breakpoint junctions usually do not occur within long stretches of homology between two chromosomes but rather at sites of microhomology or without any homology (Weinstock et al., 2006). Translocations junctions also display other features usually associated with NHEJ or alt-EJ such as end modifications and deletions (Weinstock et al., 2006).

a) Alt-EJ in the formation of translocations

Several studies in mouse cells suggested that alt-EJ is the major repair mechanism leading to translocations. Indeed, translocation frequency increases upon Ku depletion (Guirouilh-Barbat et al., 2004; Weinstock et al., 2007). Accordingly the classical NHEJ pathway seems to have a protective role against translocations and cells deficient for XRCC4-LigaseIV showed an increased rate of translocations (Simsek and Jasin, 2010), whereas cells depleted for CtIP (Zhang and Jasin, 2011) or for ligase I and III (Simsek et al., 2011) had a decreased translocations frequency. Furthermore, analyses of human tumours also revealed several features of alt-EJ such as microhomologies use and resection (Stephens et al., 2009; Zhang and Rowley, 2006), further pointing to a role for alt-EJ in the formation of translocations in the context of tumorigenesis.

This observation questions the physiological relevance of alt-EJ. Indeed, on one hand alt-EJ was described as a backup pathway, that is used only when NHEJ is not functional and on the other hand it seems to be at the origin of numerous translocations that can lead to tumorigenesis (eventually in cells that are NHEJ-proficient), therefore raising the question whether cancer cells use alt-EJ instead of NHEJ or whether alt-EJ can be used in physiological conditions, even when NHEJ is

functional. Therefore, understanding how the choice between the different repair pathways is made is crucial and I describe the regulation of DSB repair pathway choice in the part IV of the introduction. Notably, a part of my PhD project demonstrated that nuclear position can influence DNA repair pathway choice and these results will be presented in the second part of the results section.

b) NHEJ in the formation of translocations

Although alt-EJ is commonly accepted as the main repair pathway involved in the formation of chromosomal translocations in mouse cells (Yan et al., 2007), a recent study involved NHEJ in the formation of chromosomal translocations in human cells (Ghezraoui et al., 2014). Indeed, translocations rates decreased in cells deficient for ligase IV or XRCC4, therefore suggesting that NHEJ is involved in the formation of translocations. Furthermore in absence of ligase IV or XRCC4, translocation junctions had significantly longer deletions and more microhomology, suggesting that, unlike wild-type cells, deficient cells form translocations by alt-EJ (Ghezraoui et al., 2014). However, whether NHEJ is the only mechanism involved in translocations during tumorigenesis in human cancer or if both alt-EJ and NHEJ can form translocations remain to be investigated. Accordingly, some human tumors display features of alt-EJ (Stephens et al., 2009; Zhang and Rowley, 2006).

IV. Regulation of DNA repair pathway choice

In mammalian cells, several pathways can repair DSBs, including the two major pathways NHEJ and HR and the more recently discovered and highly mutagenic alt-EJ pathway. NHEJ is often seen as an error-prone mechanism since it can involve processing of the ends, leading to small deletions or insertions (Lieber, 2010). On the other hand, HR is seen as an error-free mechanism since it uses the homologous sister chromatid as a template for the repair. However, to maintain genomic stability, a proper regulation between these pathways is needed. The first key point is the regulation of pathway choice during cell cycle progression. Indeed, the use of HR outside of the S/G2 phase of the cell cycle, when the sister chromatid is absent can be deleterious. It can trigger loss of heterozygocity if the repair template used is the homologous chromosome and was proposed to trigger genomic rearrangement if used between repetitive sequences (Chiolo et al., 2011). Besides this cell cycle regulation, additional regulatory mechanisms might be used to proceed to the DNA repair pathway choice during S or G2 phase of the cell cycle. Indeed, the three repair mechanisms can be used during these phases and NHEJ is even thought to be the major pathway repairing DSBs, even in G2 (Shibata et al., 2011). However, breaks occurring during replication generate one-ended breaks that cannot be repaired by NHEJ, unless forming translocations (figure 20, Brandsma and Gent, 2012). Furthermore, the inappropriate use of NHEJ during S phase may lead to chromosomal abnormalities and repair defects of the cancer predisposition syndrome Fanconi anemia (FA). Indeed, depletion of some of the NHEJ repair factors rescues chromosomal abnormalities features associated with FA in human, chicken or *C.elegans* cell lines (Adamo et al., 2010; Pace et al., 2010). Therefore, unraveling the exact mechanisms triggering DSB repair pathway choice is key to a better understanding of the mechanisms allowing the maintenance of genomic stability. I will be presenting below the mechanisms allowing regulation of DSB repair pathway choice.

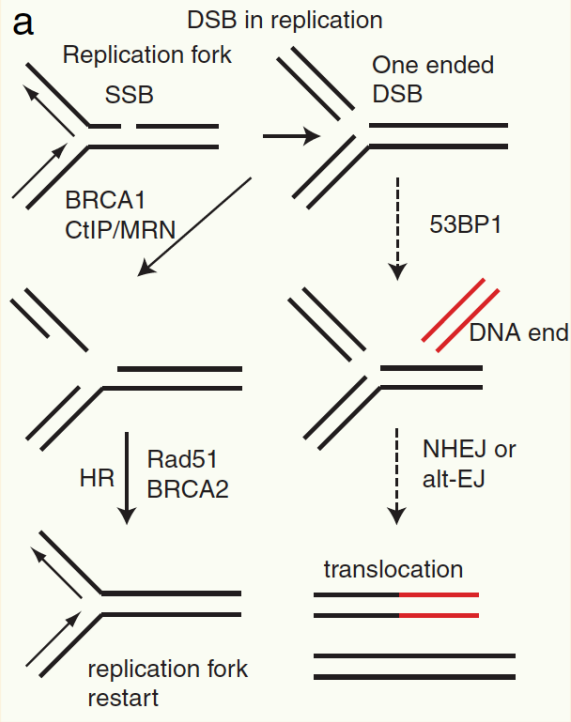


Figure 20- Repair of one-ended DSBs (adapted from Brandsma and Gent, 2012)

Repair of replication-associated breaks requires HR. 53BP1 blocks resection of the one-ended break in BRCA1 deficient cells, preventing repair via HR. The breaks are either left unrepaired or repaired via NHEJ using other random DNA ends, which leads to chromosomal rearrangements and genomic instability. In the absence of 53BP1, resection of the DNA ends can take place, allowing faithful repair via HR

1. Competition between NHEJ and HR factors for break recognition

One of the first steps of HR is end resection. Once resection is initiated Ku is unable to bind to DNA ends, NHEJ is inhibited and cells are committed to HR. Therefore, regulation of resection initiation is a key parameter in the regulation of DNA repair pathway choice.

Both the MRN complex and the Ku70/Ku80 complex can bind directly to the DSB ends. However, their bindings have different outcomes. Indeed, while Mre11 has a nuclease activity that seems necessary for the initiation of resection, Ku70/Ku80 binding helps maintaining the ends in close proximity and protect them from resection. Moreover, Ku70/80 loss increases HR levels in mice cells (Bunting et al., 2012; Pierce et al., 2001) and, interestingly, Ku70 is down-regulated during meiosis and this down-regulation was proposed to favor repair by HR (Goedecke et al., 1999). These observations further point to an inhibitory role of Ku70/Ku80 for HR. However, the interplay between the two complexes is not very clear and it is unknown whether they are competing for the binding of DNA ends or whether they are both present.

2. Cell cycle regulation

The initiation of resection is also controlled via cell cycle regulation. Indeed, in yeast, CDK activity of the Clb-CDK complex -which promotes the entry in S-phase- is required for efficient end-resection (Aylon et al., 2004). In yeast, this effect is mediated by the Sae2 protein -the homologue of CtIP-, which allows efficient resection when phosphorylated by CDK (Huertas et al., 2008). Although Sae2 and CtIP homology is very limited, the residue phoshorylated by CDK is conserved (Ser267 in Sae2 is equivalent to Thr847 in CtIP) and mutations of this residue into non-phosphorylable form impairs end resection (Huertas and Jackson, 2009). CDK phosphorylates another site on CtIP, the Ser327. This phosphorylation facilitates the binding of CtIP to BRCA1 and MRN during the S/G2 phase of the cell cycle, which allows its recruitment to DSBs and therefore promotes resection (Chen et al., 2008; Yu and Chen, 2004; Yun and Hiom, 2009). The regulation of HR by CtIP phosphorylation during the progression of the cell cycle is schematized in figure 21. Indeed, BRCA1 exists in different complexes that modulate resection efficiency. Besides its interaction with CtIP, that activates resection; BRCA1 is also found in a complex with BACH1. The binding of BACH1 with BRCA1 is regulated during the cell cycle by the phoshorylation of BACH1 on its Ser990. This phosphorylation allows the binding of BACH1 to BRCA1 during the S/G2 phases of the cell cycle and promotes resection (Dohrn et al., 2012). On the other hand, the complex formed by BRCA1, Abraxas/CCDC98 and RAP80 is limiting the access of nucleases to DNA ends and therefore inhibits excessive resection. Interestingly, this complex is not enriched during the G1 phase of the cell cycle and might act efficiently during the S/G2 phase of the cell cycle (Hu et al., 2011), therefore arguing against a role of this complex in the choice between HR and NHEJ but in favor of a regulation of the extent of resection.

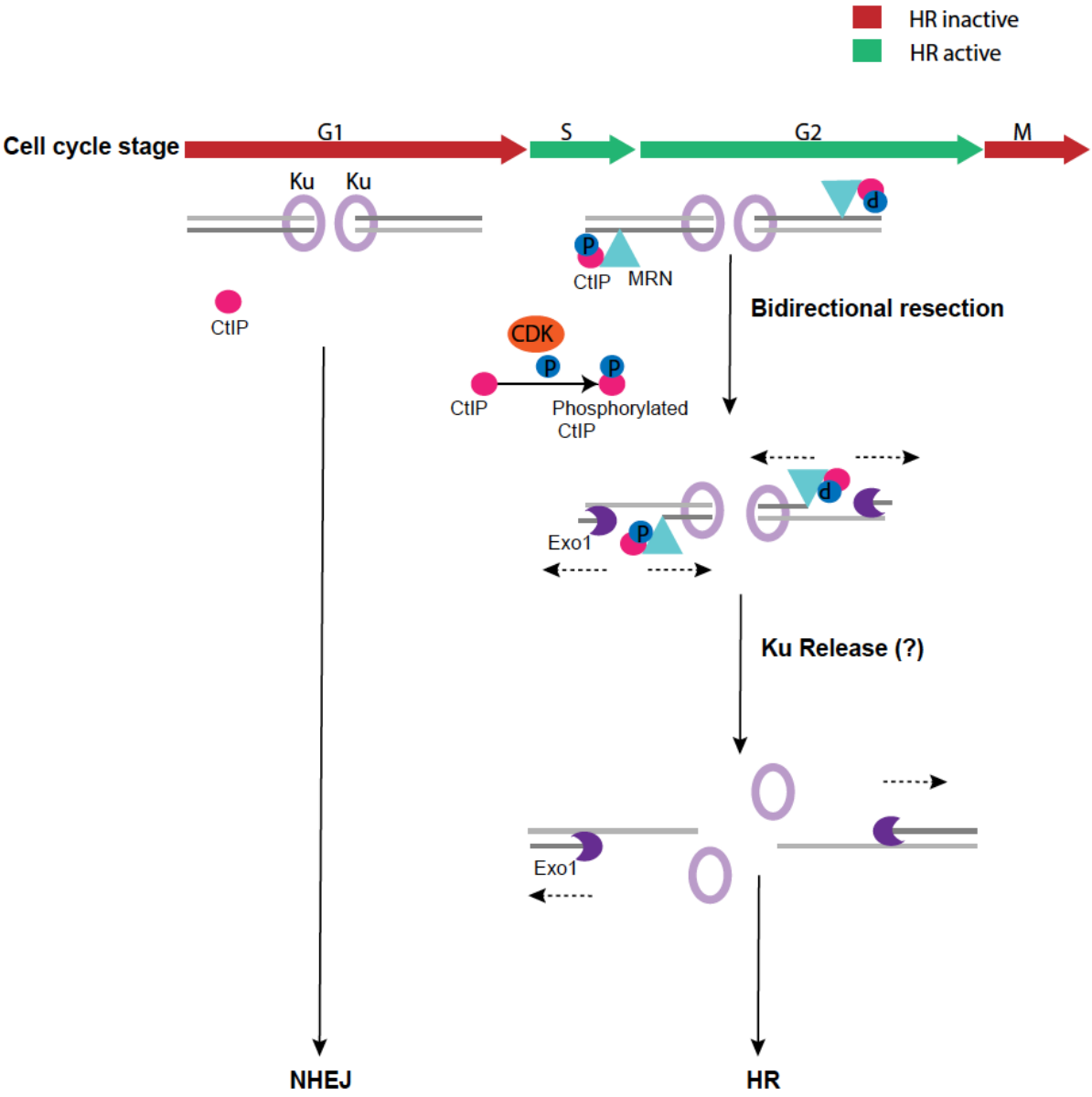


Figure 21- Regulation of DNA repair pathway choice during cell cycle progression by phosphorylation of CtIP

During the G1 phase of the cell cycle, the Ku heterodimer binds to DNA ends and promotes repair by NHEJ. During the S or G2 phase of the cell cycle, CtIP is phosphorylated by CDK and recruited to DSBs via its interaction with MRN and BRCA1 (not represented here). The association of phosphorylated CtIP and MRN allows the initiation of resection by Mre11 and Exo1, therefore triggering repair by HR.

3. Competition between 53BP1 and BRCA1

a) Recruitment and mutual exclusion of 53BP1 and BRCA1

53BP1 and BRCA1 are both recruited at DSBs sites and they share common mechanisms of recruitment, which depends on γH2AX, MDC1, RNF8 and RNF168. However, super-resolution microscopy images show that these two factors do not colocalize within repair foci, suggesting that they are mutually exclusive (Chapman et al., 2012). Moreover they are dedicated to opposite functions. 53BP1 on one hand promotes NHEJ and inhibits resection, as demonstrated by its requirement for NHEJ repair during class switching at immunoglobulin loci (Manis et al., 2004; Ward et al., 2004) and for fusions of deprotected telomeres (Dimitrova et al., 2008). On the other hand BRCA1 is part of the HR pathway and its loss induces chromosome aberrations arising from aberrant NHEJ during S phase. The use of PARP inhibitor (PARPi) in the *Brca1-/-* cells reveals these chromosome aberrations by inhibiting the repair of ssDNA breaks that are converted in DSBs and is therefore used in clinical trials to selectively kill HR-deficient cells (Jackson and Bartek, 2009). Further evidence of the antagonism of BRCA1 and 53BP1 came from the fact that 53BP1 depletion is able to rescue the chromosomal rearrangements induced by PARP inhibition in *Brca1-/-* cells (Bouwman et al., 2010; Bunting et al., 2010). Therefore a major part of BRCA1 function might be to counterbalance 53BP1 activity. Understanding how the recruitment of these two factors is mediated and regulated is key to elucidate how the choice between HR and NHEJ is made in S/G2 phases of the cell cycle. The localization of 53BP1 to DSBs is mediated by the recognition of H4K20me1 and H4K20me2 by its tudor domain (Botuyan et al., 2006; Oda et al., 2010). However, this modification is highly abundant and found in ≈ 80% of total H4 in human cells (Pesavento et al., 2008). Therefore, it was postulated that the pre-existence of H4K20me1/H4K20me2 was sufficient to induce 53BP1 recruitment and that therefore this modification would not play a role in regulating the recruitment of 53BP1. However, recent studies demonstrated that H4K20 is *de novo* methylated by the concerted actions of the methyltransferases PR-Set7 and Suv4-20 (Tuzon et al., 2014) or by the methyltransferase SET8 (Dulev et al., 2014) at DSB sites and propose that this mechanism is implicated in the regulation of 53BP1 recruitment and

therefore on DSB repair pathway choice . Additionally, the binding of 53BP1 to H4K20 methylated is regulated by a second modification of the histone H4. Indeed, H4K16ac appeared to reduce this binding and the modulation of the acetylation of histone H4 by the acetyl-transferase Tip60 or by the histone deacetylases HDAC1 and HDAC2 was therefore proposed to be determinant in the competition between BRCA1 and 53BP1 binding at DSBs (Hsiao and Mizzen, 2013; Tang et al., 2013) (figure 22 and Panier and Boulton, 2014). Interestingly the histone H4 acetyl-transferase MOF has been shown to undergo ATM-dependent phosphorylation and this phosphorylation is necessary to promote 53BP1 dissociation from breaks in S/G2 phases of the cell cycle and for the subsequent recruitment of BRCA1 and HR factors (Gupta et al., 2014). The role of phosphorylated MOF does not seem to be mediated by H4K16 acetylation (Gupta et al., 2014), however whether it can affect the acetylation of another amino acid or if its role is independent from its acetyltransferase activity remains to be investigated.

b) 53BP1 effectors in the inhibition of resection

The binding of 53BP1 to chromatin is necessary for its function in promoting NHEJ (Bothmer et al., 2011), however it does not possess any enzymatic activity, suggesting that its action might be indirect and mediated by other effectors. In line with this hypothesis, a mutant 53BP1 allelle in which all ATM phosphorylation sites were changed to alanine ($53BP1^{28A}$) failed to rescue 53BP1 deficiency (Bothmer et al., 2011), suggesting that it might act through phosphorylation-dependent protein interactions.

-RIF1

RIF1 -a protein originally identified for its role as a regulator of telomere length in *S.*Cerevisiae (Hardy et al., 1992)- was the first 53BP1 effector identified (Chapman et al., 2013; Escribano-Díaz et al., 2013; Feng et al., 2013; Di Virgilio et al., 2013; Zimmermann et al., 2013). Its recruitment to DSBs is mediated by 53BP1 (Silverman et al., 2004) and is necessary for NHEJ during class-switching (Chapman et al., 2013; Escribano-Díaz et al., 2013; Di Virgilio et al., 2013) and for fusion of uncapped

telomeres (Chapman et al., 2013; Zimmermann et al., 2013). Furthermore, RIF1 loss leads to increased resection during class-switching events (Chapman et al., 2013; Di Virgilio et al., 2013), further pointing to a role for RIF1 in the inhibition of resection and promotion of NHEJ. *Rif1-/-* cells are sensitive to irradiation and to bleomycin-induced breaks and no additional sensitivity was observed in *53bp1-/- Rif1-/-* double knock out cells (Chapman et al., 2013; Escribano-Díaz et al., 2013). Similarly, resection was increased equally in *Rif1-/-* cells and *Rif1-/- 53bp1-/-* double knock out cells (Chapman et al., 2013). Therefore, 53BP1 and RIF1 seem to act in the same pathway to promote NHEJ and inhibit resection (figure 22 and Panier and Boulton, 2014).

BRCA1 was shown to antagonize the action of 53BP1-RIF1 during the S/G2 phases of the cell cycle. Indeed, 53BP1-RIF1 dissociate from DSB foci at the entry of S phase. This dissociation is mediated by the concerted action of BRCA1 and CtIP, when phosphorylated by CDK (Chapman et al., 2013; Escribano-Díaz et al., 2013). Moreover, similarly to 53BP1 depletion, RIF1 depletion rescued the chromosomal abnormalities due to abberant NHEJ in *Brca1-/-* cells treated with PARPi (Chapman et al., 2013; Escribano-Díaz et al., 2013). All together, these different observations lead to the conclusion that 53BP1-RIF1 and BRCA1-CtIP are part of a regulatory circuit allowing the fine tuning of resection throughout the cell cycle (figure 22 and Panier and Boulton, 2014).

However, RIF1 depletion has a milder effect than 53BP1 depletion on the fusion of deprotected telomeres (Zimmermann et al., 2013) and only partially rescues chromosomal abnormalities due to abberant NHEJ in *Brca1-/-* cells treated with PARPi (Escribano-Díaz et al., 2013; Feng et al., 2013; Zimmermann et al., 2013). This indicates that an additional 53BP1-dependent mechanism might regulate the resection inhibition. The fact that $53BP1^{28A}$ on the other hand is as defective as complete loss of 53BP1 suggests that this mechanism is mediated by another phosphorylation-dependent protein interaction.

-PTIP

A 53BP1 phosphomutant (53BP1^{8A}), in which the eight most N-terminal S/TQ phosphorylation sites were mutated, mimics 53BP1 deficiency and restores genome stability in *Brca1-/-* cells but behaves like wild-type in class-switching. The analysis of the 53BP1^{8A} mutant lead to the identification of a new effector of 53BP1, PTIP (Callen et al., 2013). Indeed, 53BP1^{8A} recruits RIF1 properly but fails to recruit PTIP and PTIP depletion mimics 53BP1^{8A}. PTIP has been previously implicated in DDR (Gong et al., 2009), HR (Wang et al., 2010) and NHEJ (Callen et al., 2012) but its exact role and mechanism of action remained unknown. Fusion of uncapped telomeres was reduced in PTIP deficient cells and PTIP depletion enhanced resection and rescued the chromosomal abnormalities due to abberant NHEJ in *Brca1-/-* cells treated with PARPi. However, PTIP depletion did not impair class switching (Callen et al., 2013). All together, these results point to a role for PTIP as an effector of 53BP1 to inhibit resection independently of RIF1 (figure 22 and Panier and Boulton, 2014). Whether RIF1 and PTIP compete for the binding of 53BP1 or are bind simultaneously, as well as an eventual cell cyle regulation of 53BP1-PTIP binding remains to be investigated. Moreover, the exact interplay between 53BP1 and PTIP remains unclear. Indeed, while one study reported that the accumulation of PTIP at DSBs was decreased in absence of 53BP1 (Callen et al., 2013), another study indicated that PTIP accumulated independently of 53BP1 (Jowsey et al., 2004) and a third one showed that conditional knock-out of PTIP impairs recruitment of 53BP1 (Wu et al., 2009). Interestingly, these studies revealed the existence of two different pathways for regulating resection in physiological (class switching) or pathological (*Brca1-/-*, uncapped telomeres) situations.

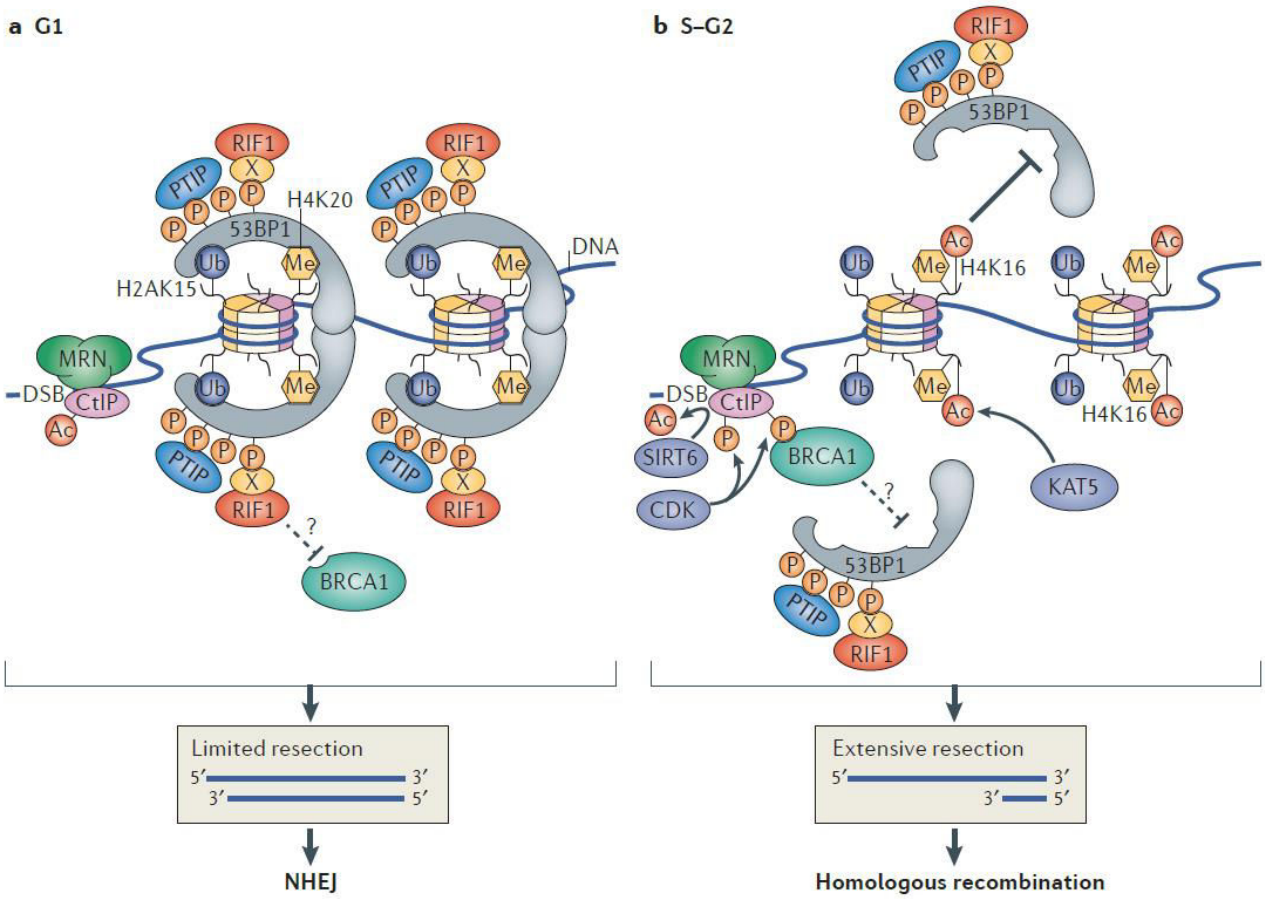


Figure 22- Antagonistic relationship of 53BP1 and BRCA1 during DSB repair pathway choice. (Panier and Boulton 2014)

a) During G1, oligomerized p53-binding protein (53BP1) binds to ubiquitylated Lys15 of histone 2 (H2AK15ub) and dimethylated H4K20 (H4K20me2) and recruits its effector proteins RIF1 (RAP1-interacting factor 1) and PTIP (PAX transactivation activation domain-interacting protein), both of which interact with ataxia-telangiectasia mutated (ATM)-phosphorylated Ser/Thr-Gln (S/T-Q) motifs in the 53BP1 amino terminus. Whether RIF1 binds to phosphorylated 53BP1 directly or via an adaptor protein (denoted as 'X') is unclear. In addition, it is currently not known whether the effector proteins bind to 53BP1 simultaneously (as depicted), or whether they associate with distinct 53BP1 molecules. Chromatin-bound 53BP1–RIF1 prevents the association of breast cancer 1 (BRCA1) with MRE11–RAD50–NBS1 (MRN) complex-bound CtBP-interacting protein (CtIP; through an unknown mechanism, indicated by a question mark). How PTIP inhibits end-resection in G1 is not known. The net outcome of this inhibition is to limit DNA double-strand break (DSB) end-resection, which enables non-homologous end-joining (NHEJ)-mediated repair and prevents homologous recombination.
b) As cells enter S phase, CtIP is phosphorylated in a cyclin-dependent kinase (CDK)-dependent manner, which promotes its binding to BRCA1 and prevents the chromatin association of 53BP1–RIF1, and possibly also the association of 53BP1–PTIP, through unknown mechanisms (indicated by a question mark). The Lys acetyltransferase 5 (KAT5)-mediated acetylation of H4K16 (H4K16ac) further reduces 53BP1 binding to the H4K20me2 mark. In addition, the end-resection activity of CtIP is upregulated by the deacetylase sirtuin 6 (SIRT6), which removes an inhibitory acetylation mark on CtIP. Together, these events enable the extensive 3 to 5´resection of the DNA end, which commits cells to homologous recombination-directed DSB repair.

4. Regulation of DSB repair pathway choice by transcription

Recently, transcription was identified as a new parameter regulating DSB repair pathway choice in mammalian cells. Indeed, actively transcribed genes were shown to be prone to HR whereas repressed genes are prone to NHEJ (Aymard et al., 2014). More specifically, the tri-methylation of H3K36 (H3K36me3) -a histone mark that is usually associated with gene activation- seemed to act as a platform allowing the recruitment of HR factors in S/G2 phase of the cell cycle whereas breaks induced in inactive genes, that are not associated with H3K36me3 recruited NHEJ factors. Interestingly, H3K36me3 was not acquired *de novo* upon DSB induction but instead the pre-existing mark was able to regulate DNA repair pathway choice and the same gene could be repaired either by NHEJ when turned off or by HR when turned on (Aymard et al., 2014). These results were reminiscent of the fact that LEDGF, a chromatin binding protein that was known to bind H3K36me3, was shown to promote resection and HR by recruiting CtIP (Daugaard et al., 2012). Indeed, LEDGF loss does not modify H3K36me3 levels but decreased RAD51 recruitment, further pointing to a role for LEGDF binding to H3K36me3 in the targeting of HR (Aymard et al., 2014). Further confirmation of the direct implication of H3K36me3 in the promotion of HR came from the fact that SETD2, the histone methyl-transferase responsible for the tri-methylation of H3K36 promotes HR (Pfister et al., 2014).

Interestingly, H3K36me2, which is also associated with active transcription, was shown to be induced at DSBs sites and to promote NHEJ and inhibit HR (Fnu et al., 2011). However, whether pre-existing H3K36me2 could play a role in the regulation of DNA repair pathway choice has not been investigated.

Additionally in yeast, that do not express LEGDF, H3K36 methylation was shown to promote NHEJ and to inhibit HR (Pai et al., 2014). In that case, loss of Set2, the enzyme responsible for H3K36 methylation in yeast- was proposed to increase chromatin accessibility which would in turn promote resection and HR (Jha and Strahl, 2014). This result therefore suggests that chromatin compaction state is another parameter influencing DSB repair pathway choice. The influence of

chromatin compaction as well as the role of nuclear architecture on DSB repair pathway choice will be presented in the next section of the introduction.

5. Competition between alt-EJ and other repair mechanisms

a) Competition between alt-EJ and NHEJ

The alt-EJ repair mechanism was revealed in NHEJ-deficient cells. Therefore, the competition between these two pathways seems to favor the use of NHEJ. The first step of alt-EJ is end-resection mediated by MRN and CtIP. Therefore the mechanisms (described earlier) regulating resection that apply in the competition between HR and NHEJ might apply as well in the competition between NHEJ and alt-EJ. Ku was shown to inhibit alt-EJ and cells depleted for Ku display increased alt-EJ efficiency. Furthermore, XRCC4 depletion also leads to increased use of alt-EJ, but less efficiently than in the absence of Ku. Indeed, in that case, Ku can still bind to DNA ends and partially inhibits alt-EJ (Guirouilh-Barbat et al., 2007; Schulte-Uentrop et al., 2008). A proposed mechanism for the inhibitory role of Ku on alt-EJ is a competition with PARP binding. Indeed, *in vitro* experiments showed that important concentration of PARP1 can disrupt Ku binding to the DNA and reversely (Wang et al., 2006).

b) Competition between alt-EJ and HR

The alt-EJ and the HR pathways share a common step of resection. Therefore, specific mechanisms might regulate the balance between these two pathways. Consistantly with this hypothesis, a recent study showed that RPA coating of ssDNA produced during resection prevents the use of alt-EJ mechanism and favor the use of HR (Deng et al., 2014). Indeed, RPA removes secondary structures from ssDNA and might prevent annealing of microhomologies (Deng et al., 2014), a step that was proposed to promote ligation by alt-EJ (Robert et al., 2009). Consistantly with a competition between alt-EJ and HR, rad51 and rad52 yeast mutants showed increased alt-EJ efficiency (Deng et al., 2014). Additionally to this observation in yeast, my work also showed that alt-EJ might substitute HR for the repair of breaks induced at the nuclear lamina. These data will be presented in the second part of the results section.

V. Nuclear architecture regulates DNA repair pathway choice

1. 3D-organization of the genome

a) Chromatin structure

The distinction between euchromatin and heterochromatin was made by the observation that chromatin had distinct appearance in certain regions of the same nucleus (work from Heitz 1928, described in Passarge, 1979), prior to the discovery of the DNA double helix and the nucleosome. Characterization of the biochemical components of chromatin has allowed the establishment of the current model of chromatin organization (figure 23 and Probst et al., 2009), which is based on the folding of DNA into structures of increasing complexity, compaction and size (Hubner et al., 2013). These different levels of chromatin organization are subjected to variations and therefore chromatin carries information in addition to the one stored in DNA sequence.

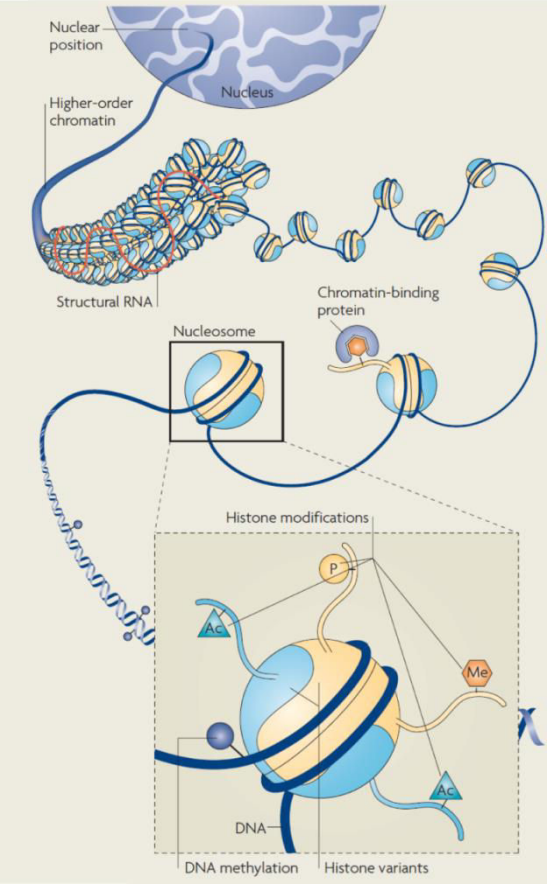


Figure 23- Chromatin structure (adapted from Probst et al., 2009)

The nucleosome is the basic unit of chromatin. Histone modifications or histone variants incorporation within the nucleosome modulate chromatin function. The DNA organized into nucleosome is further compacted, forming higher-order chromatin that is positioned within the nucleus in a non-random fashion. All these levels of organization contribute to the regulation of DNA-related functions.

- The nucleosome

The nucleosome is the basic unit of chromatin. It is composed of a histone octamere folded by ≈147bp of DNA. The octamere is composed by 4 different core histones: H3, H4, H2A, H2B (Kornberg, 1974). The histones H3 and H4 form a tetramer, flanked by two H2A-H2B dimers (Eickbush and Moudrianakis, 1978; Kornberg and Thomas, 1974; Luger et al., 1997 and figure 24). These core histones are small, highly conserved proteins that share a similar structure comprising a globular domain and unfolded N- and C-termini. The globular domain, which contains the characteristic histone-fold motif ensures the cohesion of the octamer and the wrapping of DNA (Arents and Moudrianakis, 1995). The termini of each histone molecule extend out and their residues can participate in interactions with DNA or be modified. Two nucleosomes are connected via internucleosomal DNA whose length varies according to cell type and organism (Kornberg, 1977). The succession of nucleosomes and internucleosomal DNA forms a 10 nm fiber that appears like beads-on-a-string by EM and provides the first level of DNA compaction (Olins and Olins, 1974).

Modulation of nucleosomes composition by incorporation of histone variants or modifications of the histone tails participate in the regulation of all DNA-associated functions such as transcription, replication or repair.

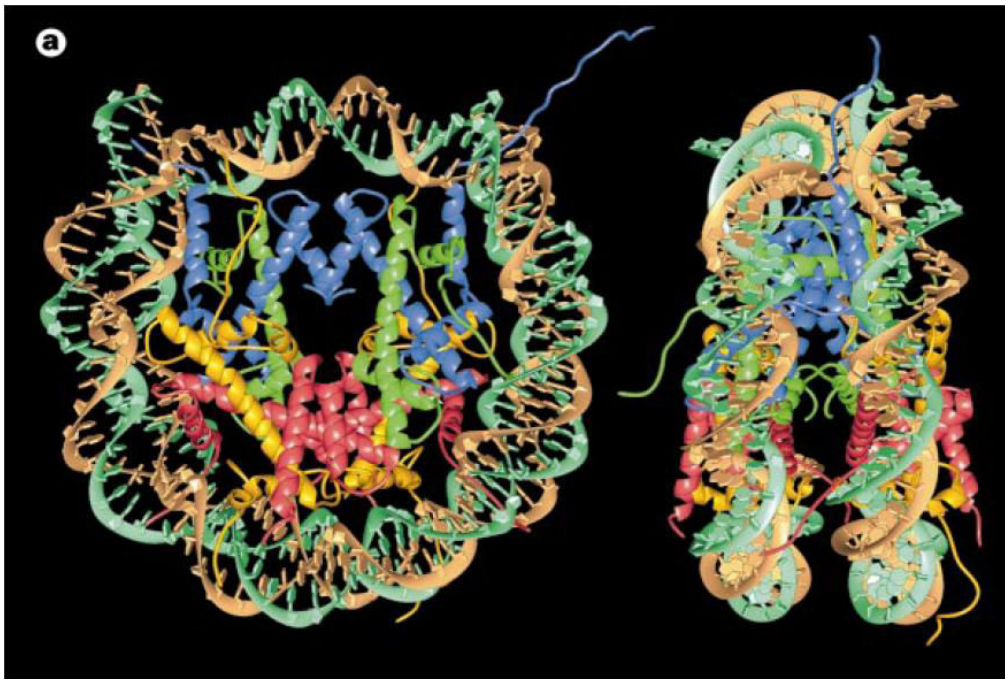


Figure 24- Structure of the nucleosome core particle (Luger et al., 1997)

Ribbon traces for the 146-bp DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (blue: H3; green: H4; yellow: H2A; red: H2B.The views are down the DNA superhelix axis for the left particle and perpendicular to it for the right particle. For both particles, the pseudo-two fold axis is aligned vertically with the DNA centre at the top.

- Histone post-translational modifications

As mentioned above, the residues located in the histone tails can be modified by various post-translational modifications (PTMs). Among the different histone PTMs, phosphorylation, acetylation and methylation are the most extensively studied and their dynamic regulation is key for proper DNA metabolism including DSB repair (Rothbart and Strahl, 2014).

Acetylation of lysine residues is catalyzed by histone acetyl-transferases (HATs) and can affect both soluble and chromatin-bound histones. Acetylation of soluble histones soon after their synthesis is important for their deposition and is erased once they are incorporated into chromatin (Campos et al., 2010). In chromatin, acetylation of histones results in neutralization of the positive charge of lysines and destabilizes their interaction with DNA. Histone acetylation is therefore generally associated with chromatin opening and gene activation by allowing an increased accessibility of

chromatin for the transcription machinery. On the contrary, histone deacetylases (HDACs) are often involved in transcriptional repression (for review, see MacDonald and Howe, 2009).

Phosphorylations of serines, threonines, tyrosines are involved in the DDR, transcription and chromatin compaction (for review, see Rossetto et al., 2012). The phosphate group brings an additional negative charge that can alter the nucleosome-DNA binding, or regulate recognition by histone binding partners. For example, the phosphorylation of the histone variant H2AX during the DDR serves as a binding platform for downstream signaling factors or repair factors.

Lysines and arginines can also be methylated by histone methyltransferases and carry up to three methyl groups. The removal of methyl groups is ensured by demethylases. Histone methylation plays important roles in transcription regulation and heterochromatin establishment and maintenance. For example, trimethylation of lysine 4 of H3 (H3K4me3) and H3K36me3 are associated with active transcription whereas H3K9me3, H4K20me3 and H3K27me3 are hallmarks of transcriptional repression in heterochromatin (for review, see Bannister and Kouzarides, 2011).

The different histone PTMs are not independent and they are involved in complex crosstalk within the same histone tail, nucleosome or between nucleosomes (Latham and Dent, 2007; Rothbart and Strahl, 2014). In particular, certain marks are frequently found together at a particular genomic region under specific circumstances (Schübeler et al., 2004). This observation is in agreement with the histone code hypothesis, which proposed that PTMs are recognized in a combinatorial manner and leads to a context-dependent outcome (Jenuwein and Allis, 2001). However, this hypothesis is still debated and the effect of PTMs on gene expression is often unpredictable (Rando, 2012).

- Histone variants

All histones with the exception of H4 exist as variants of different protein sequences (Franklin and Zweidler, 1977). The different variants are encoded by paralogous genes or alternative spliced isoforms (Talbert et al., 2012). The replicative histones

H2A, H3.1 and H3.2, whose expression picks in S phase of the cell cycle and constitute the main supply of histones during replication are considered as canonic histones. They are deposited in a DNA synthesis-dependent manner. On the other hand, replacement histones (variants) are deposited in a DNA synthesis-independent manner. Despite the existence of different variants, the overall structure of the nucleosome is the same (Bönisch et al., 2012; Kurumizaka et al., 2013). However, the stability of these nucleosomes might vary, as it was suggested by *in vitro* studies (Andrews and Luger, 2011; Mariño-Ramírez et al., 2005). Additionally to an eventual role in nucleosome stability, the different variants have different interacting partners and they can carry specific PTMs. Therefore, they directly participate in the regulation of chromatin functions. For example, the phosphorylation of the histone variant H2AX is key for a proper DNA damage signaling.

b) Higher-order chromatin structure

Association of DNA with nucleosomes does not provide the full level of compaction necessary to fit the genome in the nucleus, but is the basis for folding higher-order structures (Woodcock and Ghosh, 2010).

- Folding of the 10nm fiber

The linker histone H1, which binds the internucleosomal DNA, is necessary for higher-order chromatin structure (Harshman et al., 2013; McGhee and Felsenfeld, 1980). H1 can bind ≈20bp of DNA at the entry and the exit of the nucleosome. Binding of histone H1 allows stabilization of the nucleosome and H1-mediated interactions of adjacent nucleosomes were proposed to participate in the formation of higher-order chromatin structure (Meyer et al., 2011). *In vitro* data allowed the establishment of different models of folding of the 10nm fiber into 30nm fiber have been proposed (solenoid and zig-zag models), however their relevance *in vivo* is still debated (Razin and Gavrilov, 2014).

- Heterochromatin

The distinction between euchromatin and heterochromatin originated from observations in electron microscopy. Indeed, heterochromatin, in contrast to

euchromatin, appeared as regions refractory to electrons (in black). Heterochromatin and euchromatin distinguish two different states of compaction (with heterochromatin being more compacted) and transcriptional activity (with heterochromatin being silent). Heterochromatin can be further divided into facultative and constitutive. Facultative heterochromatin is more plastic and can undergo transitions between open and compact according to cell state (for example during development). A common mark of facultative heterochromatin is H3K27me3, which is catalyzed by the polycomb repressive complex 2 (PRC2) (Trojer and Reinberg, 2007). Constitutive heterochromatin on the contrary is always compact and tends to be enriched in repetitive, gene-poor and late replicating DNA sequences. Constitutive heterochromatin is usually associated with H3K9me3, which allows the recruitment of the three heterochromatin protein 1 paralogs (HP1α,β,γ) via their chromodomain (Bannister et al., 2001; Lachner et al., 2001). HP1 can recruit histone methyltransferases that will catalyze new H3K9 methylation and therefore allows propagation and maintenance of heterochromatin (Aagaard et al., 1999).

- Mitotic chromosome condensation

Mitotic chromosomes attain a 10,000-fold chromatin compaction but their precise organization is not known yet (Nishino et al., 2012). However, several factors involved in this compaction have been identified such as the topoisomerase II, the cohesin and condensin protein complexes (Woodcock and Ghosh, 2010).

c) Chromosome territories and non-random positioning

Besidesw the different compaction states of chromatin, the organization of the different loci within the 3D space of the nucleus constitutes another level of regulation of genome functions. Indeed, the position of a specific locus within the nucleus is not random and several rules govern this position. Some of the features of chromatin organization within the nucleus can be explained by basic polymer physics rules, such as segregation of chromosomes into chromosomes territories, whereas others are the result of local compaction, long-range interactions or association with nuclear structures such as the nuclear lamina or the nucleolus (van Steensel, 2011). The combination of fluorescence in situ hybridization (FISH), which

allows to visualize the position of chromosomes, chromatin domains and individual genes, and techniques derived from chromosome conformation capture (3C), enables to assess the 3D-folding and spatial organization of chromosomes in the nucleus, either in single cells or in a population of cells. These observations are summarized below.

- Chromosome territories

FISH with chromosome paints revealed the existence of chromosomal territories, i.e the fact that each interphase chromosome occupies a portion of the nucleus (Stack et al., 1977). This observation is confirmed by Hi-C analyses of all metazoan genomes (Kalhor et al., 2012; Lieberman-Aiden et al., 2009; Sexton et al., 2012; Zhang et al., 2012). Indeed, most of the captured cross-linked interactions are in *cis* rather than in *trans*. However in *trans* associations, although being less frequent, are also captured (Hakim et al., 2011; Kalhor et al., 2012; Lieberman-Aiden et al., 2009; Simonis et al., 2006; Yaffe and Tanay, 2011). The regions that associate in *trans* are usually gene-rich and transcriptionally active. These regions were hypothesized to loop out of their chromosome territories (Müller et al., 2010). Consistently, gene-dense chromosomal regions decorate the outside of their own chromosome territories (Boyle et al., 2011). The functional consequences of looping out are unclear however it was hypothesized to favor gene activation by allowing genes to interact with transcription factories or nuclear speckles (Brown et al., 2008; Schoenfelder et al., 2010; Sutherland and Bickmore, 2009).

- Radial chromosome organization

Chromosomes are not randomly positioned but have instead a preferred position relative to the nuclear periphery or interior. In the majority of the cell lines, FISH and Hi-C analyses showed that gene-dense chromosomes tend to position in the nuclear interior whereas gene-poor chromosomes are positioned closer to the nuclear periphery (Bolzer et al., 2005; Boyle et al., 2001; Cremer et al., 2001; Kalhor et al., 2012; Küpper et al., 2007). Additionally, rDNA-containing chromosomes cluster close to the nucleoli. However, a given chromosome does not seem to consistently have a

specific neighbor (Kalhor et al., 2012). Therefore, the position of a chromosome or gene is neither random nor fixed.

Within a specific chromosome, chromatin regions are also polarized, with gene-poor regions preferentially oriented toward the nuclear periphery whereas gene-rich regions are oriented toward the nuclear interior (Boyle et al., 2011; Küpper et al., 2007). Furthermore, DNA adenine methyltranferase identification (DamID) allowed the identification of regions directly associated with the nuclear lamina (lamina associated domains-LADs) (Guelen et al., 2008). These LADs are generally gene poor and associated with low levels of gene expression. The nuclear lamina therefore constitutes a nuclear structure able to directly bind specific regions of the genome and participates in the global 3D organization of the genome, which role in gene regulation was extensively studied (see part V.1.e). A second important nuclear structure that binds specific genomic regions is the nucleolus. Interestingly, in addition to rDNA loci, regions that co-purify with the nucleolus substantially overlap with LADs (van Koningsbruggen et al., 2010; Németh et al., 2010). Therefore the nuclear lamina and the nucleolus might provide two alternative locations where the same repressive genomic domains can partition.

d) Nuclear compartments

In contrary to cytosolic compartments, nuclear compartments are not delimited by membranes but are rather defined by a specific subset of proteins associated to a specific function. These compartments can be morphologically identified by light and electron microscopy. The best-studied nuclear compartments are the nuclear lamina, the nuclear pore complexes, the nucleolus, the splicing factors compartments, the Cajal bodies, the PML bodies (for review see Dundr and Misteli, 2001). A description of the different nuclear bodies is provided in the figure 25 (Spector, 2006).

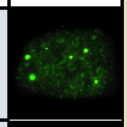
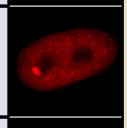
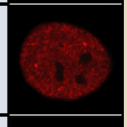
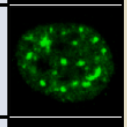
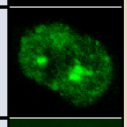
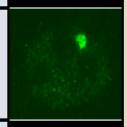
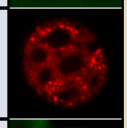
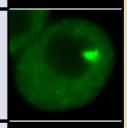
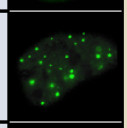
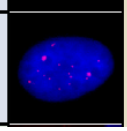
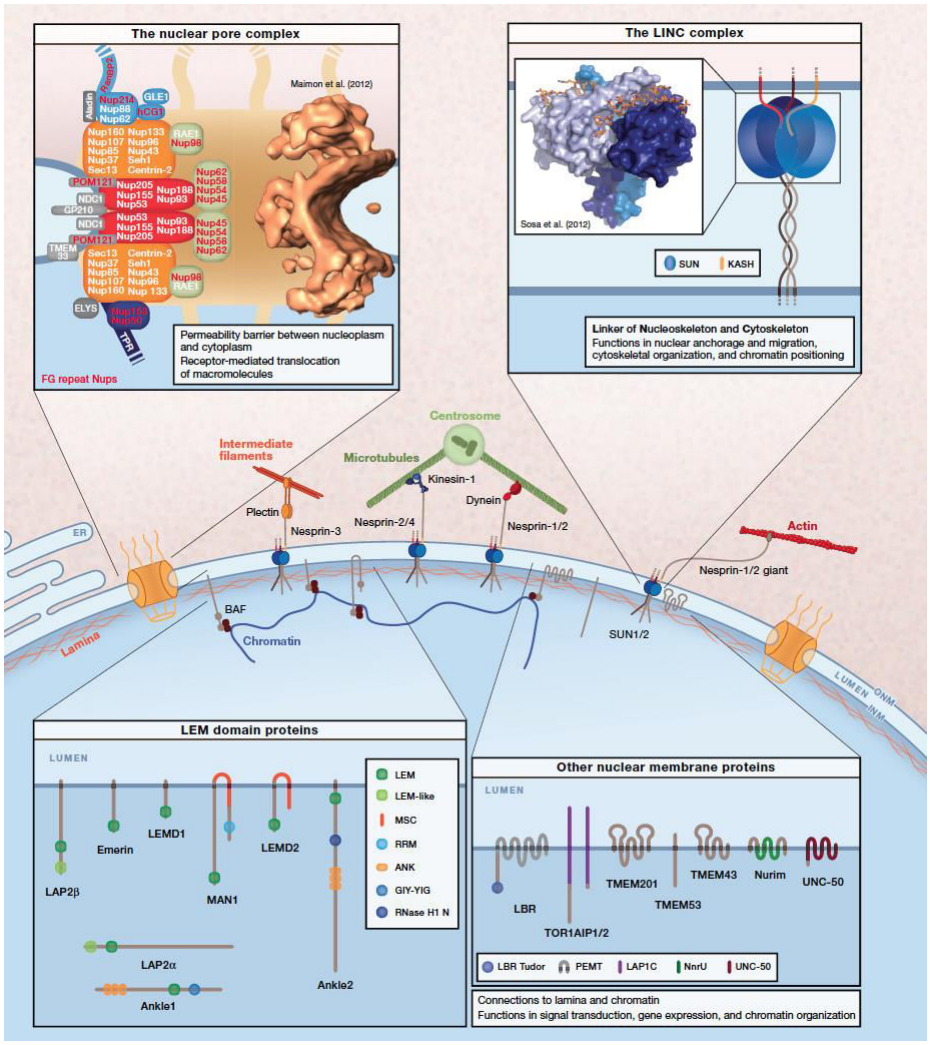
	Body Name	Number/ Cell	Typical Size and Shape	Marker Protein	Description	Image
Nuclear Bodies	**Cajal Body**	0–6	0.1–2.0 μm; round	Coilin	Involved in snRNP and snoRNP biogenesis and posttranscriptional modification of newly assembled spliceosomal snRNAs.	
	Clastosome	0–3	0.2–1.2 μm; irregular	20S core catalytic component of proteasome	Contains ubiquitin conjugates, the proteolytically active 20S core and 19S regulatory complexes of the 26S proteasome, and protein substrates of the proteasome.	
	Cleavage Body	1–4	0.2–1.0 μm; round	CstF 64 kDa	Contains several factors involved in 3′ cleavage of mRNAs. ~20% contain newly synthesized RNA. Some cleavage bodies localize adjacent to Cajal and PML bodies.	
	Nuclear Speckle or Interchromatin Granule Cluster	25–50	0.8–1.8 μm; irregular	SC35, SF2/ASF	Contains proteins for pre-mRNA processing. Involved in the storage, assembly, and/or modification of pre-mRNA splicing factors.	
	Nuclear Stress Body	2–10	0.3–3.0 μm; irregular	HSF1	Induced by heat shock response. Associates with satellite III repeats on human chromosome 9q12 and other pericentromeric regions; recruits various RNA-binding proteins.	
	OPT Domain	1–3	1.0–1.5 μm; round	PTF	Contains several transcription factors (Oct1/PTF) and RNA transcripts; predominant in late G1 cells. Often localizes close to nucleolus.	
	Paraspeckle	10–20	0.5 μm; round	p54^{nrb}, PSP1	Contains several RNA-binding proteins and nuclear-retained CTN-RNA.	
	Perinucleolar Compartment	1–4	0.3–1.0 μm; cap	hnRNPI (PTB)	Cap on surface of nucleolus; found mainly in transformed cells. Contains RNA pol III transcripts and several RNA-binding proteins.	
	PML Body	10–30	0.3–1.0 μm; round	PML	Suggested to play a role in aspects of transcriptional regulation and/or nuclear protein sequestration.	
	Polycomb Body	12–16	0.3–1.0 μm; round/ irregular	Bmi1, Pc2	Contains silencing proteins associated with Polycomb repressive complex 1; associates with heterochromatin.	

Figure 25- Nuclear bodies (adapted from Spector 2006)

List and description of the different nuclear bodies

My work focused on the compartments constituting the nuclear periphery: the nuclear lamina and the nuclear pore complexes (NPCs) that are described below. The nuclear periphery organization is represented in figure 26 (Rothballer and Kutay, 2012a).



The Nuclear Envelope
The nuclear envelope (NE) separates the nucleoplasm from the cytoplasm in eukaryotic cells, generating the spatial and functional compartmentalization that is essential for the maintenance and processing of genetic information. As a central structural and functional element of the cell, the NE plays key roles in cytoskeletal organization, chromatin positioning, signal transduction, and gene expression. Mutations in various genes of NE proteins cause inherited diseases referred to as nuclear envelopathies, or laminopathies.

Nuclear Pore Complexes
Nuclear pore complexes (NPCs) are ~100 MDa protein assemblies with a ring-shaped structure of 8-fold rotational symmetry. The ~30 different protein constituents of NPCs are called nucleoporins (Nups). NPCs form selective passageways between nucleoplasm and cytoplasm. Whereas small molecules diffuse through NPCs freely, macromolecules larger than 30 kDa are restricted in diffusion. Most proteins and RNAs translocate through NPCs by help of shuttling transport receptors that interact with FG-repeat-containing Nups.

Linker of Nucleoskeleton and Cytoskeleton Complexes
Linker of nucleoskeleton and cytoskeleton (LINC) complexes establish the physical connection between the nucleus and the cytoskeleton. LINC complexes bridge the NE by association of SUN (Sad1/UNC-84) and KASH (Klarsicht/ANC-1/SYNE homology) domain proteins residing in the inner and outer nuclear membrane (INM and ONM), respectively. On the cytoplasmic face of the NE, KASH proteins interact with cytoskeletal components, whereas SUN proteins bind to the nuclear lamina and chromatin in the nuclear interior. Allowing for force transduction, LINC complexes function in processes like nuclear migration and anchorage and meiotic chromosome pairing.

Nuclear Membrane Proteins
Most specific nuclear membrane proteins reside in the INM, whereas ONM protein composition largely overlaps with that of the endoplasmic reticulum (ER). INM proteins interact with the nuclear lamina and chromatin and regulate chromatin organization and gene expression. LEM (LAP2/Emerin/MAN1) domain proteins are a well-studied family of INM proteins implicated in chromatin tethering to the nuclear periphery linked to transcriptional silencing. Interaction between the LEM domain and DNA is mediated by the chromatin protein BAF. Proteomic approaches have identified more than 80 novel nuclear membrane proteins, most of which remain to be functionally characterized.

Figure 26- Nuclear envelope (Rothballer and Kutay 2012)

Description of the organization of the nuclear envelope containing the nuclear lamina and the nuclear pores

- Nuclear lamina

The nuclear lamina or inner nuclear membrane (INM) is the scaffolding structure of the nuclear periphery. It is constituted by the type V intermediate filaments proteins

lamin A/C and lamin B that assembles in a meshwork of 10nm filaments underneath the INM. A- and B-type lamins are related, but have different sequences and biochemical properties (Stuurman et al., 1998). Humans have three lamin genes: LMNA that encodes four alternative splicing isoforms including lamin A and lamin C and two genes that encode for B-type lamins (LMNB1 and LMNB2). Lamin B is expressed in all somatic cells and is tightly bound to the INM via a stable C-terminal farnesyl modification, whereas A-type lamins are expressed only in differentiated cells and are present in the INM as well as the nucleoplasm. Several integral membrane proteins associate to the lamins, including for example the lamin B receptor (LBR), which together with lamin A was shown to be necessary for the establishment of heterochromatin at the nuclear periphery (Solovei et al., 2013). Additionally, LBR binds directly to HP1, suggesting that it might play a role in the regulation of gene expression at the nuclear lamina (Ye et al., 1997). Another transmembrane protein of the nuclear lamina that might be involved in regulation of gene expression is RFBP, a protein that interacts directly with a potential chromatin remodeler related to the SWI/SNF chromatin remodelers (Mansharamani et al., 2001). LAP2, emerin (EMD; whose loss-of-function is responsible for the Emery-Dreifuss muscular distrophy) (fore review on emerin see Berk et al., 2013) and MAN1 are transmembrane proteins that belong to the LEM domain family of proteins (Lin et al., 2000). The LEM domain is a 43-residue motif that mediates binding to a protein named Barrier to autointegration factor (BAF) (Furukawa, 1999). BAF is involved in higher-order chromatin organization, transcription regulation and nuclear envelope assembly (Margalit et al., 2007). Additional evidence for a role of transmembrane protein from the nuclear lamina in the regulation of gene expression is the binding of EMD and LAP2β to the histone deacetylase HDAC3 (Demmerle et al., 2012; Nili et al., 2001). Complete list of the different proteins of the nuclear lamina is provided in figure 27 (Rothballer and Kutay, 2012b). Therefore, besides the structural role of the nuclear lamina to maintain nuclear shape, additional functions are emerging, including spatial organization of nuclear pores within the nuclear membrane, anchoring of heterochromatin or regulation of transcription (see part V.1.e) (Liu et al., 2000).

		H. sapiens	D. melanogaster		C. elegans	S. pombe		S. cerevisiae
Lamins[4]	A-type	Lamin-A Lamin-C Lamin-C2 Lamin-AΔ10	Lamin-C (pG-IF)	CG10119	–	–	–	–
	B-type	Lamin-B1 Lamin-B2 Lamin-B3	Lamin Dm0	CG6944	Lamin-1 (Ce-lamin, CeLam-1)	–	–	–
SUN domain proteins		SUN1 (UNC84A) SUN2 (UNC84B, Rab51P) SUN3 (SUNC1) SPAG4 (SUN4) SUN5 (SPAG4L, TSARG4)	Klaroid Giacomo	CG18584 CG6589	UNC-84 SUN-1(Matefin)	Sad1	SPBC12D12.01	Mps3 (Nep98)
KASH domain proteins		Nesprin-1[5] (Syne-1, Enaptin) Nesprin-2[6] (Syne-2, Nuance) Nesprin-3 Nesprin-4	MSP-300 Klarsicht	CG18251 CG17046	ANC-1 ZYG-12 UNC-83 KDP-1	Kms1 Kms2	SPAC3A11.05c SPBC947.12	Mps2 Csm4
LEM/HeH domain proteins[4,7]	Group 1	Emerin LAP2[8] LEMD1 (CT50, LEMP-1)	Otefin Bocksbeutel tbd	CG5581 CG9424 CG3748	Emerin homolog 1 (Ce-emerin)	–	–	–
	Group 2	LEMD2 (LEM2, NET25) MAN1 (LEMD3)	MAN1	CG3167	LEM-2 (Ce-MAN1)	Mug61 (Man1) Lem2	SPAC14C4.05c SPAC18G6.10	Src1 (Heh1) Heh2
	Group 3	Ankle1 (LEM3, ANKRD41) Ankle2 (LEM4)	tbd tbd	CG8679 CG8465	LEM-3 Y55F3BR.8 (LEM-4L)	–	–	–

Figure 27- List of nuclear lamina proteins (adapted from Rothballer and Kutay 2012b)

List of nuclear lamina proteins and their homologues in different species

- Nuclear pore complexes

Nuclear pore complexes (NPCs) are large transmembrane complexes, consisting of about 30 different proteins called nucleoporins (Nups). The list of all nucleoporins as well as their homologues in different species is provided in figure 28.

	H. sapiens	D. melanogaster		C. elegans	S. pombe		S. cerevisiae
Cytoplasmic filaments	RanBP2 (Nup358)	Nup358	CG11856	NPP-9	–	–	–
Cytoplasmic ring and associated factors	Nup214 (CAN)	DNup214	CG3820	NPP-14	Nup146	SPAC23D3.06c	Nup159
	Nup88	Nup88 (Mbo)	CG6819	–	Nup82	SPBC13A2.02	Nup82
	GLE1	GLE1	CG14749	–	Gle1	SPBC31E1.05	Gle1
	hCG1 (NUP2L1, NLP-1)	tbd	CG18789	–	Amo1	SPBC15D4.10c	Nup42 (Rip1)
Nup 98 complex	Nup98	Nup98	CG10198	Npp-10N	Nup189N	SPAC1486.05	Nup145N, Nup100, Nup116
	RAE1 (GLE2)	Rae1	CG9862	NPP-17	Rae1	SPBC16A3.05	Gle2 (Nup40)
Outer NPC scaffold (Nup107-160 complex)	Nup160	Nup160	CG4738	NPP-6	Nup120	SPBC3B9.16c	Nup120
	Nup133	Nup133	CG6958	NPP-15	Nup132, Nup131	SPAC1805.04, SPBP35G2.06c	Nup133
	Nup107	Nup107	CG6743	NPP-5	Nup107	SPBC428.01c	Nup84
	Nup96	Nup96	CG10198	NPP-10C	Nup189C	SPAC1486.05	Nup145C
	Nup85 (PCNT1)	Nup75	CG5733	NPP-2	Nup-85	SPBC17G9.04c	Nup85
	Seh1	Nup44A	CG8722	NPP-18	Seh1	SPAC15F9.02	Seh1
	Sec13	Sec13	CG6773	Npp-20	Sec13	SPBC215.15	Sec13
	Nup37	tbd	CG11875	–	tbd	SPAC4F10.18	–
	Nup43	Nup43	CG7671	C09G9.2	–	–	–
	Centrin-2[1]	tbd	CG17493[1], CG31802[1]	R08D7.5[1]	Cdc31[1]	SPCC1682.04	Cdc31[1]
Central NPC scaffold (Nup53-93 complex)	Nup205	tbd	CG11943	NPP-3	Nup186	SPCC290.03c	Nup192
	Nup188	tbd	CG8771	–	Nup184	SPAP27G11.10c	Nup188
	Nup155	Nup154	CG4579	NPP-8	tbd	SPAC890.06	Nup170, Nup157
	Nup93	tbd	CG7262	NPP-13	Nup97, Npp106	SPCC1620.11, SPCC1739.14	Nic96
	Nup53(Nup35, MP44)	tbd	CG6540	NPP-19	Nup40	SPAC19E9.01c	Nup53, Nup59
Nup62 complex	Nup62	Nup62	CG6251	NPP-11	Nsp1	SPAC26A3.15c	Nsp1
	Nup58/Nup45[2]	Nup58	CG7360	NPP-4	Nup45	SPAC22G7.09c	Nup49
	Nup54	Nup54	CG8831	NPP-1	Nup44	SOBC19G7.15	Nup57
Nuclear ring and associated factors	Nup153	Nup153	CG4453	NPP-7	Nup124	SPAC30D11.04c	Nup1[3]
	Nup50	Nup50	CG2158	NPP-16	Nup61	SPCC18B5.07c	Nup2
	–	–	–	–	Nup60	SPCC285.13c	Nup60
Nuclear basket	TPR	Mtor	CG8274	NPP-21	Nup211	SPCC162.08c	Mlp1, Mlp2
Transmembrane nucleoporins	NDC1	Ndc1	CG5857	NPP-22	Cut11	SPAC24C9.01	Ndc1
	POM121	–	–	–	–	–	–
	GP210	Gp210	CG7897	NPP-12	–	–	–
	TMEM33	Kr-h2	CG9159	Y37D8A.17	Tts1	SPBC1539.04	Pom33
	–	–	–	–	Po152	SPBC29A10.07	Pom152
	–	–	–	–	Mug31	SPAC1002.02	Pom34
Other nucleoporins	ELYS	Elys homolog	CG14215	MEL-28	–	–	–
	Aladin (AAAS)	tbd	CG16892	–	–	–	–
		tbd	CG13137				

Figure 28- List of nuclear pore proteins (adapted from Rothballer and Kutay 2012b)

List of nuclear pore proteins and their homologues in different species

Each nucleoporin exists in multiple copies, resulting in 500-1000 protein molecules in the fully assembled NPC (Cronshaw et al., 2002). They form a ring-shaped structure of 8-fold rotational symmetry. NPCs consist of a membrane-embedded scaffold build around a central transport channel, a cytoplasmic ring, a nuclear ring and eight filaments attached to each ring. The nuclear filaments are connected to a distal nuclear ring to form the nuclear basket of the NPC. The organization of the different nucleoporins within NPC is represented in figure 29 (D'Angelo and Hetzer, 2008). NPCs mediate transport of macromolecules to and from the nucleus and generate a diffusion barrier that allows diffusion of molecules of up to 40 kDa, whereas larger cargoes require active translocation by transport receptors (Ma et al., 2012). The diffusion barrier is formed by unfolded nucleoporins segments that contain numerous phenylalanine-glycine (FG) repeats. Nups carrying 4 to 48 FG repeats fill the central channel of the NPC and form a meshwork determining the pore

permeability limit (D'Angelo and Hetzer, 2008). Transport of molecules bigger than 40kDa necessitates their binding to transport receptors. FG repeats serve as docking sites for transport receptors and the complex between cargo and transport receptor can then move through the diffusion barrier of the pore to finally reach their destination compartment where cargo is released (Raices and D'Angelo, 2012). Besides their role in nucleocytoplasmic transport, nuclear pores are also involved in mitosis, chromatin organization, regulation of gene expression (see part V.1.e) and DNA repair (see part V.3.b).

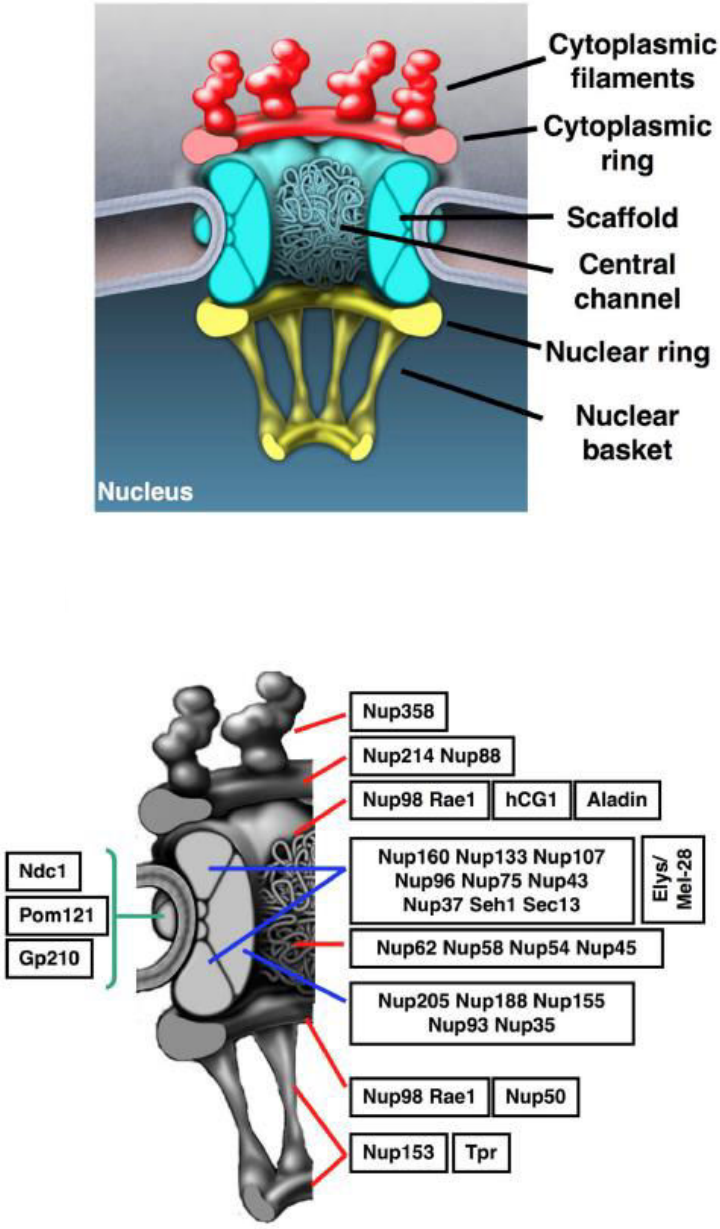


Figure 29- Nuclear pore complex structure and composition (D'angelo and Hetzer, 2008)

Upper panel: Schematic illustration of the NPC structure

Lower panel: Predicted localization of subcomplexes and nucleoporins within the NPC. The members of the Nup214 complex (Nup214, Nup88), Nup98 complex (Nup98, Rae1), Nup107–160 complex (Nup160, Nup133, Nup107, Nup96, Nup75, Nup43, Nup37, Sec13, Seh1), Nup62 complex (Nup62, Nup58, Nup54, Nup45), and Nup93– 205 complex (Nup205, Nup188, Nup155, Nup93, Nup35) are enclosed in the same box. Green lines show the location of the three transmembrane nucleoporins, red lines the location of peripheral components and blue lines indicate the location of scaffold subcomplexes.

e) 3D-organization of the genome participates in the regulation of DNA functions- Example of the role of the nuclear periphery in the regulation of gene expression

Mapping of genome interactions with laminB1 by DamID identified over 1000 lamina-associated domains (LADs) (Guelen et al., 2008). They correspond to large blocks of heterochromatin associated with the nuclear periphery that were firstly observed by electron microscopy (Akhtar and Gasser, 2007; Belmont et al., 1993). Most genes in LADs are transcriptionally silent and associated with repressive histone marks (Guelen et al., 2008; Pickersgill et al., 2006; Wen et al., 2009) and the NL itself seems to directly contribute to gene repression. Indeed, genes associated with the NL in flies lacking one of the lamins are derepressed (Shevelyov et al., 2009) whereas artificial tethering of certain genes to the NL can lead to gene repression (Dialynas et al., 2010; Finlan et al., 2008; Kumaran and Spector, 2008; Reddy et al., 2008).

NPCs perforate the nuclear envelope and are therefore adjacent to the NL. However, these two compartments have distinct properties and no heterochromatin is seen at the vicinity of NPCs as revealed by electron microscopy (Akhtar and Gasser, 2007) or super resolution microscopy (Schermelleh et al., 2008). The gene gating hypothesis (Blobel, 1985) predicted that active genes would associate preferentially with NPCs, to allow coupling between transcription and translocation of mRNA in the cytoplasm. In line with this hypothesis, in yeast genes interacting with the NPCs are active and this interaction is important for gene expression (Brickner and Walter, 2004; Casolari et al., 2004; Taddei et al., 2006). However, in Drosophila mapping of genome interactions with the nucleoporins Nup98, Nup50 and Nup62 by DamID evidenced two different interaction pools: one at the NPC and the other in the nucleoplasm. The NPC bound pool and the nucleoplasmic pool account for 20% and 80% respectively of Nup98 interactions. The interacting genes in the nucleoplasmic pool -and not in the NPC bound pool- displayed higher transcriptional activity than average and were associated with active histone marks, such as H3K4me2 and H4K16ac. Furthermore, Nup98 is directly involved in transcriptional activation since its depletion leads to down-regulation of the interacting genes and its overexpression induces up-regulation of the same genes (Kalverda et al., 2010). How the

nucleoporins regulate transcription remains poorly understood. However, the FG repeats domain, found in several nucleoporins, including Nup98, might play a direct role in this process. Indeed, Nup98 FG repeats domain can recruit histone acetylases or deacetylases (Bai et al., 2006; Kasper et al., 1999; Wang et al., 2007). Additionally, the FG repeats of Nup98 are fused with various proteins in several translocations of acute myeloid leukemia and they are thought to inappropriately trigger gene activation that could be at the origin of tumorigenesis (Franks and Hetzer, 2013). Therefore, nucleoporins and especially the FG-repeats seem to play an important role in the regulation of gene expression.

Given these roles for the nuclear lamina and the NPCs in the regulation of transcription, their involvement in DSB repair is a long-lasting subject of interest. Studies conducted in yeast will be presented in part V.3.b) of this manuscript.

2. Double strand break (DSB) repair in heterochromatin and heterochromatin proteins in DSB repair

Higher-order chromatin structure has been proposed to regulate DDR and DSB repair. Different studies showed that on one hand, chromatin compaction is considered as a physical barrier that has to be alleviated to allow proper repair and on the other hand, heterochromatin-associated proteins were shown to have an active role in DSB repair. In the attached review, I summarized these different studies and discussed the role of chromatin compaction in DSB repair (Lemaître and Soutoglou, 2014).

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Double strand break (DSB) repair in heterochromatin and heterochromatin proteins in DSB repair

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ABSTRACT

Chromosomal translocations are a hallmark of cancer cells and they represent a major cause of tumorigenesis. To avoid chromosomal translocations, faithful repair of DNA double strand breaks (DSBs) has to be ensured in the context of high ordered chromatin structure. However, chromatin compaction is proposed to represent a barrier for DSB repair. Here we review the different mechanisms cells use to alleviate the heterochromatic barrier for DNA repair. At the same time, we discuss the activating role of heterochromatin-associated proteins in this process, therefore proposing that chromatin structure, more than being a simple barrier, is a key modulator of DNA repair.

1. Introduction

DNA lesions and mutations although being the major motor of evolution and necessary for several cellular processes such as immune system diversification or meiosis, can threaten cell viability and genome stability because they can lead to chromosomal rearrangements [22]. DNA breaks can be induced by endogenous sources that are byproducts of our own metabolism or by exposure to damaging agents, UV light and irradiation [22].

Double strand breaks (DSBs) are among the most deleterious lesions since they affect both strands of DNA. To cope with damage, the appearance of a DSB activates the DNA Damage Response (DDR) – a complex network of processes that allows recognition of the break and the activation of checkpoints, allowing the coordination between cell cycle progression and DNA repair [36].

The early step of the signaling cascade involves sensing the break by the ATM kinase (Ataxia Telangiectasia Mutated), which initiates a megabase-wide spreading of a phosphorylated form of the histone variant H2AX at serine 139, around the lesion – γH2AX, which is considered the major transducer of the signaling cascade. These early events are in turn responsible for the subsequent recruitment of repair factors and the initiation of the repair mechanisms [36].

Two major mechanisms repair DSBs: Homologous Recombination (HR) and Non-homologous End Joining (NHEJ). HR takes place in the replicative and post-replicative stages of the cell cycle (S/G2), when sister chromatids are present and allow faithful repair [20]. However, the use of HR outside of the S or G2 phases of the cell cycle or between repetitive sequences can lead to major recombination events [20]. NHEJ on the other hand does not require the presence of an undamaged template in order to repair. It is a flexible but conservative mechanism that enables a direct rejoining of broken DNA ends and is thus active throughout the whole cell cycle. In few instances it involves processing of the DNA ends and thus can be an error-prone mechanism [17]. Recently, a third pathway has been described, called Alternative End Joining (A-EJ), which is highly mutagenic and can be revealed in the absence of key NHEJ factors [12].

Repair by these two pathways must be very tightly regulated in time and space to avoid deleterious chromosomal rearrangements. Increasing evidence suggests that chromatin and its compaction state plays a role in the regulation of DDR and DSB repair. Chromatin is the complex between DNA and its associated proteins. The fundamental unit of chromatin is the nucleosome which consists of ~200 bp of DNA wrapped around an octamer of core histones [25]. Interactions between individual nucleosomes mediated by numerous non-histone proteins lead to the formation of higher order chromatin structure that can have various compaction states.

Heterochromatin, in opposition to euchromatin, was originally described as densely stained regions of the nucleus and corresponds to a highly compacted form of chromatin [34]. Historically, it is considered transcriptionally inactive and rich in repetitive sequences whereas euchromatin is more gene-rich and transcriptionally active. Histone post-translational modifications (PTMs) are proposed to have a main role in defining a chromatin state, and

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specific histone marks are more enriched in heterochromatin such as the trimethylation of the histone H3 on lysine 9 (H3K9me3) whereas others are more enriched in euchromatin, such as histone acetylation that is generally depleted from heterochromatin [26]. The histone marks can be read and bound by specific non-histone proteins that can alter the overall structure of chromatin. Among these proteins, the heterochromatin protein 1 (HP1) proteins are key factors in the establishment and maintenance of heterochromatin. HP1 was initially discovered in drosophila as a protein involved in gene silencing [14]. It directly interacts with H3K9me3 and this binding is necessary for the maintenance of heterochromatin state [34]. It also interacts with the co-repressor KRAB-domain associated protein 1 (KAP1) which is also involved in the regulation of heterochromatin structure through its binding with the histone methyl-transferase SETDB1, the histone deacetylases HDAC1 and HDAC2 and the nucleosome remodeling factor CHD3 [21].

The high compaction of chromatin in heterochromatin is hypothesized to constitute a barrier for DNA repair and DSBs that are formed in heterochromatin are processed slower than in euchromatin [19,32]. On the other hand, recent findings show that heterochromatin-associated proteins play a positive role in DNA repair [45]. Here, we discuss this paradox and we review the recent literature that describes how DNA repair occurs within heterochromatin, and how certain proteins that have repressive roles in heterochromatin possess active roles in DNA repair, proposing thus that more than being a simple barrier, chromatin and its components are key regulators of DSB repair.

2. Ways to alleviate chromatin compaction for efficient DDR and DNA repair

Cells have evolved several mechanisms to allow DNA repair in the context of chromatin and especially in the highly condensed form of chromatin, the heterochromatin. In 2007, Cowell et al. described that cells exposed to ionizing irradiation (IR) depicted only a few number of foci of the early marker of DDR activation, γH2AX, within heterochromatin, suggesting that heterochromatin was less sensitive to IR [11].

However, a recent study performed in drosophila cells showed that the initial formation of γH2Av (the drosophila homologue of H2AX) was equivalent in heterochromatin and euchromatin but that the number of γH2Av foci remaining in heterochromatin 1 h after IR was lower than in euchromatin. By following cells expressing fluorescently tagged versions of HP1a and DSB repair proteins with live cell imaging, Chiolo et al. further showed that the heterochromatic DSB foci relocate at the periphery of the heterochromatin domains [9]. Similar relocation was also observed in mammalian cells upon single ion microirradiation [23].

The increased motion of heterochromatic DSBs is quite unique since repair foci were shown to exert very limited mobility in mammalian cells [46]. On the other hand, unprotected telomeres, which resemble DSBs, were also shown to be mobilized in mouse cells [13], suggesting that motion of DSBs might occur specifically in heterochromatinized nuclear domains. It was proposed that relocation is a mechanism to avoid recombination between repetitive sequences. Although this relocation is dispensable for the first steps of DDR or DNA repair and the recruitment of early factors happens within the heterochromatin [9,23], the late steps of HR are only effective outside the heterochromatin domains. Indeed, RAD51 was shown to be recruited only after the relocation and showed mutual exclusivity with HP1a [9]. Interestingly, the relocation of repair foci outside of heterochromatin requires the activity of the ATR kinase and functional resection [9].

Additional heterochromatin associated factors were shown to be important for this mechanism. Particularly, the SUMO ligase complex Smc5/6 is necessary for the relocalization of heterochromatic DSBs and for the inhibition of Rad51 recruitment within heterochromatin [9]. Nevertheless, the exact mechanism by which DSBs are mobilized remains to be elucidated. Strikingly, an expansion of the heterochromatin domain was observed in parallel to the relocation of the break, suggesting a local decompaction of the compartment [9]. A possible hypothesis is that this alteration of the heterochromatin domain allows the increase of DSB mobility.

Chromatin structure alterations are thought to influence the strength of DDR. In response to DNA damage, chromatin undergoes global decondensation, a process that has been proposed to facilitate genome surveillance by enhancing access of DDR proteins to sites of damage [27]. In line with this idea, when DNA lesions occur at embryonic stem cells (ESCs) from transgenic mice with reduced amounts of the linker histone H1, and thus less compacted chromatin, the strength of the DDR signal that is generated at each break site is enhanced, suggesting that DDR is amplified in the context of open chromatin [37]. The enhanced DDR upon chromatin decondensation is achieved by over activation of the major driver of DDR, ATM [4]. Indeed, ATM becomes rapidly activated in response to changes in chromatin structure, upon exposure of cells to mild hypotonic buffers, treatment with the histone deacetylase (HDAC) inhibitor TSA, depletion of HP1, all conditions that lead to chromatin decondensation [4,24].

On the other hand, the mechanisms by which chromatin decompaction occurs after DSB induction seem to involve the activity of ATM. Indeed, ATM phosphorylates KAP1 on its serine 824 and this phosphorylation is necessary for a proper DNA repair in heterochromatin [19,40]. The phosphorylation of KAP1 induces chromatin decompaction by several mechanisms, including the release of the histone deacetylase CHD3 [18].

Furthermore, the sumoylation of KAP1 is regulated upon DNA damage, leading to chromatin decompaction. Indeed, the corepressive activity of KAP1 is dependent on its sumoylation that allows the binding of CHD3 and the histone methyl-transferase SETDB1. Upon DSB induction, the desumoylase SENP7 desumoylates KAP1, leading to CHD3 release from chromatin [15]. SENP7 was subsequently shown to be necessary for a proper DSB repair by homologous recombination [15]. Collectively, this data show a tight regulation of the balance between phosphorylation and sumoylation of KAP1 during DDR, allowing a modification of chromatin state that is necessary for subsequent DNA repair. Interestingly, different phosphatases like PP4C and PP1alpha and beta (PP1a and b) have been reported to interact and dephosphorylate KAP1 [30,31,28]. Dephosphorylation of KAP1 by PP1a and PP1b was also reported to stimulate KAP1 sumoylation [30]. These phosphatases could then be involved in the restoration of chromatin state after repair since prolonged KAP1 phosphorylation at S843 was shown to delay chromatin restoration after DSB repair [31,28].

Furthermore, KAP1 mediates chromatin decompaction upon DNA damage through the disruption of its interaction with HP1β. In fact, HP1β was shown to be rapidly mobilized and released from heterochromatin upon DNA damage [2] and this mobilization is mediated by the phosphorylation of HP1β by the casein kinase 2 (CK2) and by the phosphorylation of KAP1 by the checkpoint kinase Chk2 [7]. Indeed, additionally to its phosphorylation by ATM, KAP1 is phosphorylated by Chk2 on serine 473 that is located in the HP1 binding motif of KAP1 [7,6,8,50]. This phosphorylation is necessary for the release of HP1β from chromatin and for subsequent DNA repair within heterochromatin [7].

Another important player for chromatin decompaction upon DSB induction is the histone acetyl-transferase Tip60. Tip60 is recruited to DSBs by the MRN complex, leading to the acetylation of histone H4, which induces a subsequent chromatin decompaction.

This chromatin decompaction is necessary for HR [38]. Interestingly, Tip60 also binds to H3K9me3, thus competing with HP1. The release of HP1β from chromatin and the subsequent binding of Tip60 to H3K9me3 allow the activation of its acetyltransferase activity [47].

The mechanisms described above are proposed to act mainly in heterochromatin since HP1 proteins KAP1 and H3K9me3 are enriched in these regions. However they may also occur in euchromatin since chromatin alterations upon DNA damage are global and not only localized at heterochromatin regions. Further studies are needed to understand which are the mechanisms that are specific to heterochromatin or shared with euchromatin.

3. Active role of heterochromatin proteins in DNA repair

Despite the necessity for heterochromatin proteins to leave from the site of DSBs, some of them, including the HP1 proteins, are described to have an active role in DNA repair. A first indication for an active role of HP1 proteins in DNA repair came from the observation that all three HP1 isoforms (HP1α, HP1β, HP1γ) are recruited to DNA lesions [33,51,5].

The HP1 recruitment was sensitive to temperature, indicative of an active mechanism rather than a passive process that depends on diffusion [51]. The association of HP1α with DNA lesions was shown to be transient suggesting that it is important for setting up the environment for the first steps of DNA repair [5]. It occurs in euchromatin as well as in heterochromatin at a similar time scale [51,5], suggesting a general role for HP1, regardless the compaction state of chromatin.

In line with this observation, the recruitment requires the chromoshadow domain (CSD) of HP1 and is thus independent of H3K9me3 binding by the chromodomain. The CSD mediates the interaction of HP1 with several proteins, through their PxVxL motif, including the largest subunit of the histone chaperone chromatin assembly factor 1, p150 CAF1 [39,49]. This binding to p150 CAF1 is necessary for the recruitment of HP1 to DNA damage [5]. Interestingly, neither the other subunit of CAF1, p60, nor the usual determinants for the stable accumulation of HP1 with constitutive heterochromatin such as H3K9me3, Suv39-1,2 or non-coding RNAs were required for this recruitment [5].

The recruitment of HP1 proteins to DNA damage was shown to have an active role in DNA repair. Indeed, U2OS cells depleted for HP1α showed persistent γH2AX foci at late time points after irradiation that is indicative of persistent and unrepaired DNA lesions [5]. Similarly, Lee et al. also showed persistence of γH2AX foci after irradiation in MCF7 cells depleted for HP1α, β or γ [29]. Furthermore, cells depleted for HP1 proteins showed an increased level of apoptosis after irradiation [29]. These data suggest that HP1 proteins are necessary for a proper DDR and DNA repair.

In contrary, a simultaneous depletion of the three HP1 isoforms showed an increased repair of lesions occurring at heterochromatin in ATM-inhibited cells [19]. Different explanations can be given to this apparent discrepancy. First, it is possible that the different HP1 isoforms have dissimilar roles in DNA repair and depletion of all three, masks a potential unique role of an individual isoform. A second explanation could be that Goodarzi et al., performed their experiments in G0 arrested cells, where NHEJ is the predominant mechanism for DNA repair. Under these conditions, a specific effect on HR cannot be visualized and a possibility is that the loss of HP1 impacts more HR than NHEJ.

Indeed, it was shown that depletion of HP1 proteins does not have an impact in NHEJ as evaluated by the use of IsceI-based reporter assay [29] whereas it markedly decreases HR [5,44,29]. Nevertheless, different groups demonstrate different results as for the impact of each HP1 isoform in HR. Although Lee et al., showed a decreased efficiency of HR upon the deletion of all three isoforms, Soria et al. showed similar results for HP1α and HP1β but reported a stimulation of HR by the depletion of HP1γ [44,29].

Lee et al. propose that the interaction of HP1 with BRCA1 is necessary for an efficient HR and show that upon DNA damage induction the deletion of all 3 HP1 isoforms leads to an impaired BRCA1 foci formation. Furthermore, they show that the number and size of 53BP1 foci is increased upon HP1 depletion [29]. They therefore conclude that the increased recruitment of 53BP1 and the decreased recruitment of BRCA1 leads to a mysregulation of the balance between HR and NHEJ, a defective HR and a defective G2/M checkpoint [29].

The defect in HR upon downregulation of HP1α and β observed by Baldeyron et al. and Soria et al. is not explained by an imbalance in the two major DNA repair pathways provoked by 53BP1 foci increase. On the contrary, these studies report a decrease of 53BP1 recruitment in DNA damage sites upon depletion of HP1α [5,29]. The same group showed that down regulation of HP1α and β affect DNA end resection visualized by a reduced level of RPA foci formation and RPA32 phosphorylation and a decreased Rad51 recruitment [5,44]. The role of HP1α and HP1β in DNA end resection is downstream or independent of CtIP since the recruitment of this protein was not affected [44]. Interestingly, Soria et al. report an intriguing difference between the different HP1 isoforms in homologous recombination. They report an inhibitory role of HP1γ in HR since its depletion was leading to an increased HR efficiency [44].

It is not clear why depletion of HP1γ in the two different studies yielded in different phenotypes as to HR. One possible explanation is that the degree of knock down is not the same in the two studies. Dramatic downregulation of HP1γ might alter the protein levels of the other isoforms resulting in defects in HR. Although the cell type used in both studies is the same, the integration site of the DRGFP cassette into the genome might be different and therefore the chromatin context that the I-SceI break is induced might differ. Moreover, the use of different siRNAs in the different studies might be also the origin of the different experimental outcomes.

Although not supported by Lee et al., the hypothesis of different roles for the different isoforms of HP1 cannot be excluded and other data are in agreement with this hypothesis. For example, in nematodes, mutants lacking the HPL-1 isoform are less sensitive to irradiation than wild-type animals whereas mutants lacking the HPL-2 isoform depict a high irradiation sensitivity [33]. Interestingly, the HPL-1 isoform displays increased homology with HP1γ whereas HPL-2 is more similar to HP1β [52]. It is thus tempting to speculate that the different isoforms of HP1 have a conserved opposite role in HR. Furthermore, neuronal cells derived from HP1β deficient mice but not from HP1α deficient mice are subjected to genomic instability [1].

HP1 proteins are not the only heterochromatin-associated proteins that were shown to play an active role in DNA repair. For example, the KRAB associated corepressor KAP1 was also shown to associate with DSBs [53]. This recruitment is also mediated by p150CAF1 and the depletion of HP1α or KAP1 reciprocally affects their accumulation at damage sites [5]. We can thus speculate that the two proteins act together in the same pathway and that Kap1 could have a similar bimodal behavior than HP1. The role of Kap1 on HR and whether its interaction with HP1 after DNA damage is specific to one HP1 isoform remains to be investigated.

Except from their active role in HR, heterochromatin-associated proteins might also act in ensuring the coordination between transcription and repair. Indeed, the transcription is arrested during DNA repair [43,41,42,10], probably as a means to avoid incorrect transcript production. It was shown that polycomb proteins that are marking facultative heterochromatin that was not the focus of this review and they are known to be involved in gene silencing, are recruited to DSB sites, leading to an increase of the repressive

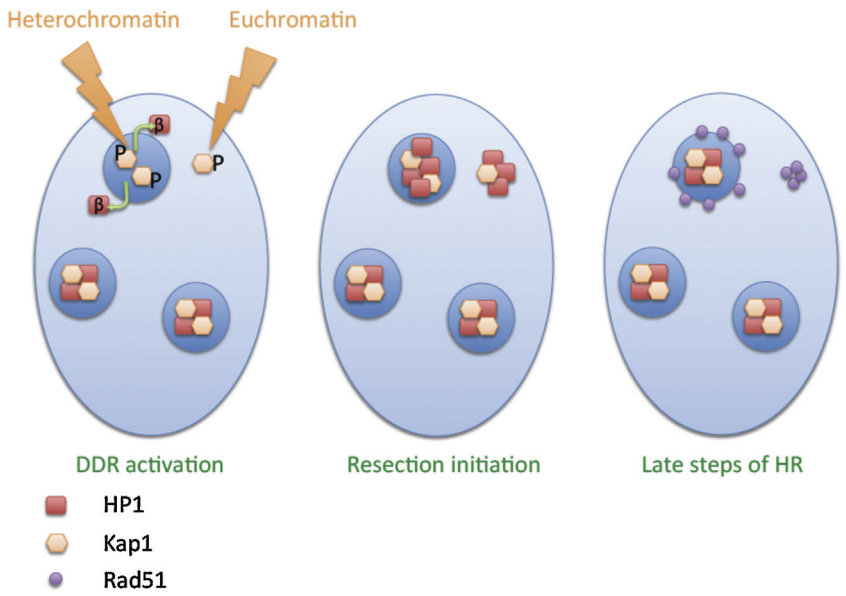

Fig. 1. Model for a differential involvement of HP1 proteins in euchromatic and heterochromatic DSB repair. Heterochromatin is a barrier for the activation of DNA damage response. Therefore, upon DNA damage, the phosphorylation of KAP1 leads to HP1β release from heterochromatin, and the release of additional heterochromatin factors such as CHD3 (left panel). The mobilization of HP1 in heterochromatin is followed by its accumulation in euchromatin as well as in heterochromatin. This step is necessary for the initiation of DNA end resection (middle panel). In heterochromatin HP1 might be retained via its interaction with H3K9me3 whereas the association with damaged euchromatin through the CSD domain is transient. In heterochromatin, Rad51 recruitment is inhibited by HP1 presence and the breaks are relocalizing outside of the heterochromatin compartment to proceed to the latest steps of HR. The release of HP1s from euchromatin allows the recruitment of Rad51 and breaks do not need to be mobilized (right panel).

histone mark H3K27me3 [10]. The recruitment of polycomb proteins was also accompanied by a lack of nascent mRNA, suggesting the formation of trancriptionally inactive heterochromatin at the site of damage [10].

4. Conclusion and perspective

High ordered chromatin structure poses a barrier to DNA repair. However, cells evolved several mechanisms to overcome this barrier and to achieve efficient DSB repair. Upon DNA damage, the chromatin is subjected to major rearrangement that must be reversed once repair is accomplished. How the restoration of the initial chromatin state is achieved is still a question. Notably, heterochromatin proteins such as KAP1 and HP1 might play an important role in this restoration of chromatin state after repair completion. It would be therefore interesting to understand whether the chromatin state is entirely restored after repair and whether the induction of DSBs leads to irreversible epigenetic alterations with consequences in gene expression and 3D genome organization.

If HP1 proteins need to leave and accumulate at the site of damage which event precedes the other? A model that can reconcile these opposite behaviors of HP1 is that persistent recruitment occurs after a first rapid dispersal, pointing to a bimodal behavior of HP1 upon damage [3]. Moreover, one can envisage different modes of HP1 action at lesions occurring at different genomic locations. At sites that the HP1s normally reside like constitutive heterochromatin, an initial release of HP1 is occurring that is concomitant with the KAP1 phosphorylation to initiate DDR (Fig. 1). Subsequently, HP1 is recruited to DNA lesions both in euchromatin and heterochromatin to initiate resection. The recruitment of HP1 in euchromatin is transient and HP1s are leaving the site of damage to allow the latest steps of HR, like the binding of the recombination protein RAD51 (Fig. 1). The presence of HP1 at the chromocenters poses a barrier to the latest steps of HR, to avoid recombination between repetitive sequences. Instead, breaks that will associate with RAD51 relocate at the periphery of heterochromatin domain (Fig. 1). Additionally, the restoration of heterochromatin inhibits uses of unfaithful pathways at resected breaks in G2 [16].

Overall, HR seems to be more sensitive to chromatin compaction than NHEJ. Interestingly, recent data demonstrate that recruitment of histone deacetylase 1 and 2 (HDAC1, HDAC2) upon DSB formation facilitates NHEJ by preventing the sliding of KU70/KU80 away from breaks [35]. Although HDAC recruitment appears contradictory, to the observed H4 hyper-acetylation induced by Tip60 recruitment, the authors report a bi-phasic response at the damage site, corresponding to a rapid deacetylation, which is necessary for NHEJ, followed by subsequent hyperacetylation, which is essential for HR [35]. In line with this observation, Tip60 dependent H4 acetylation was shown to be a key determinant of this balance by regulating 53BP1 binding to H4K20me2 at the sites of damage [48].

Thus, more than being a simple obstacle to DNA repair, chromatin compaction could be a major regulator in the repair pathway choice.

Conflict of interest statement

The authors declare no conflict of interest.

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3. Influence of nuclear compartments in DSB repair

Several DNA-associated functions, such as transcription or mRNA splicing, seem to be compartmentalized and nuclear compartments participate in the optimization of these functions. Whether DNA repair is compartmentalized in mammalian cells remains debated. Indeed, DSBs are repaired individually and do not migrate to be repaired in a specific DSB repair compartment. However, possible differences in DSB repair efficiency or pathway used in different nuclear compartments cannot be excluded and different nuclear compartments seem to be involved in DSB repair. Although, I will only present here the 53BP1 nuclear bodies (OPT domains) and the role of nuclear lamina and nuclear pores in DSB repair, PML bodies and nucleolus also seem to have important roles in DSB repair.

a) DNA repair centres and 53BP1 nuclear bodies

In the yeast *S.Cerevisiae*, several DSBs can migrate to be repaired together in a same repair centre (Lisby et al., 2003). On the other hand, in mammalian cells the mobility of DSBs is limited and individual DSBs seem to be repaired independently (Soutoglou et al., 2007). DSBs mobility in mammalian cells will be discussed in more details in the discussion part of this manuscript. However, in G1 cells large nuclear bodies containing 53BP1 were observed (Harrigan et al., 2011; Lukas et al., 2011), therefore raising the question whether they could constitute repair centres. The characterization of these nuclear bodies demonstrated that they correspond to the previously identified OPT domains (Harrigan et al., 2011) and that they might arise from DNA lesions occuring during mitosis at incompletely replicated loci (Harrigan et al., 2011; Lukas et al., 2011). Consistent with this model, common fragile sites -that are often broken due to under-replication- are found associated with these 53BP1 nuclear bodies (Harrigan et al., 2011; Lukas et al., 2011). Whether the same 53BP1 nuclear body can contain several DNA lesions remains to be investigated. Similarly, the exact role of these 53BP1 nuclear bodies is not known yet. They were proposed to sequester the DNA lesions until the next S phase to allow replication to be completed and to promote repair. 53BP1 was proposed to protect the DNA ends from further resection until S phase (Harrigan et al., 2011; Lukas et al., 2011).

b) DSB repair at the nuclear periphery and role of the nuclear periphery's proteins in DSB repair

To study the role of the nuclear periphery on DSB repair, two different questions can be asked: whether the proteins of the nuclear periphery play a role in DSB repair, independently of the position where it happens in the nucleus or whether the position of a DSB at the nuclear periphery influences its repair. These two aspects will be developed separately in the next paragraphs.

- DSB repair at the nuclear periphery

Studies in yeast showed that persistent DSBs or telomeres are anchored at the nuclear periphery, both at the inner nuclear membrane (Kalocsay et al., 2009; Oza et al., 2009; Schober et al., 2009) and at the nuclear pores (Kalocsay et al., 2009; Khadaroo et al., 2009; Nagai et al., 2008; Therizols et al., 2006).

Anchoring at the inner nuclear membrane is mediated by association with the integral membrane protein Mps3 (Kalocsay et al., 2009; Oza et al., 2009; Schober et al., 2009). Mps3 binds to the Est1 telomerase subunit, which allows the binding of telomeres (via Ku complex) (Schober et al., 2009) and of persistent DSBs that also recruit the telomerase (Oza et al., 2009). Recruitment of DSBs at the inner nuclear membrane was shown to delay repair by recombination and recruitment of telomeres protects telomeric repeats from recombination. However, in both cases, anchoring at the periphery is necessary to maintain genome stability as the loss of Mps3 leads to gross chromosomal rearrangements (Oza et al., 2009; Schober et al., 2009). Therefore, anchoring at the inner nuclear membrane was proposed to enable the irreparable breaks and the telomeres to be repaired by alternative pathways (Oza and Peterson, 2010).

Anchoring at the nuclear pore is mediated by the nucleoporin Nup84 (Kalocsay et al., 2009; Khadaroo et al., 2009; Nagai et al., 2008; Therizols et al., 2006) and the SUMO-regulated Slx5/8 ubiquitin ligase complex (Nagai et al., 2008). Anchoring of telomeres to the nuclear pores was shown to be necessary for the repair of DSBs arising in the subtelomeric region (Therizols et al., 2006) and was proposed to allow

recombination of persistent DSBs in a SUMO-dependent pathway (Nagai et al., 2008).

Whether these two anchorage sites and repair pathways are linked or independent is still under investigation. Indeed, Oza et al., showed that mutation of Mps3 rescued genome instability observed in the absence of Slx5 therefore suggesting a link between anchoring at the inner nuclear membrane and at the nuclear pores (Oza et al., 2009). A potential model would be that persistent DSBs or telomeres are first anchored to the inner nuclear membrane and further relocalized at nuclear pores in case they cannot be repaired (Oza and Peterson, 2010). Consistent with this model, eroded telomeres were proposed to relocalize from their initial position at the inner nuclear membrane to the nuclear pores (Khadaroo et al., 2009). On the other hand, recent work by the Gasser group showed that relocation of persistent DSBs to the nuclear periphery involves different mechanisms (Horigome et al., 2014). Whereas the SWR-C remodeling complex is necessary for relocation of persistent DSBs at the nuclear periphery, both at the nuclear pores and at the inner nuclear membrane, INO80 was shown to be necessary only for relocation at nuclear pores (Horigome et al., 2014). Furthermore, breaks relocated at the two compartments are repaired by different pathways. Indeed, breaks located at the nuclear pores are repaired by BIR or microhomology mediated error-prone mechanisms whereas breaks associated with the inner nuclear membrane are repaired by the error free HR pathway (Horigome et al., 2014). These data argue in favor of independent mechanisms of anchoring and repair at the nuclear periphery.

In mammalian cells, DSBs positional stability (Soutoglou et al., 2007) suggests that relocation of persistent breaks might not be conserved. However, whether the periphery of mammalian cells constitutes an environment that is permissive to alternative repair pathways remained to be investigated and evaluation of repair efficiency and mechanisms at the inner nuclear membrane and at the nuclear pores was not investigated. I investigated these questions during my PhD studies and I present them in the second part of the results section.

- Role of the proteins from the nuclear periphery in DNA repair

Lamin A involvement on genome stability

Mutations and reduced expression of lamins are associated with several degenerative disorders, premature ageing syndromes (including the Hutchinson-Gilford progeria syndrome-HGPS) and cancer, three features that are often associated with increased genomic instability. Indeed, fibroblasts from HGPS patients, and from the *Zmpste*$^{-/-}$ mouse model of progeria showed increased DNA damage and increased sensitivity to damaging agents (Liu et al., 2005; Varela et al., 2005) and *Zmpste*$^{-/-}$ mice exhibit increased sensitivity to ionizing radiation (Liu et al., 2005). Similarly, fibroblasts from laminopathy mandilbuloacral dysplasia type A (MADA) patients as well as *Lmna*$^{-/-}$ MEFs showed increased genomic instability and increased unrepaired DSBs, as visualized by γH2AX foci (Gonzalez-Suarez et al., 2009; di Masi et al., 2008). Accordingly, comet assays upon γ-irradiation showed reduced repair efficiency (Redwood et al., 2011). Comet assay usually shows a biphasic mode of repair with a fast phase of repair that is supposed to be repair by NHEJ and a slow component of repair that is supposed to be repair by HR or alternative pathways. *Lmna*$^{-/-}$ MEFs exerted a profound defect in the fast phase of repair, suggesting a defect of NHEJ (Gonzalez-Suarez et al., 2011). On the other hand, the use of HR substrate showed 40% decreased of HR efficiency in cells depleted for Lamin A compared to control cells (Redwood et al., 2011). These results suggest that Lamin A is involved both in NHEJ and HR. One of the possible cause of the NHEJ defect observed is a defect in 53BP1 foci formation that was observed both in progeria cells (Liu et al., 2005) and in *Lmna*$^{-/-}$ MEFs (Redwood et al., 2011). Further investigation of 53BP1 foci formation in *Lmna*$^{-/-}$ MEFs showed that the observed deficiency was not due to a recruitment defect but to a global decrease of 53BP1 protein level (Redwood et al., 2011). Indeed, Lamin A was subsequently identified as a regulator of 53BP1 stability (Gonzalez-Suarez et al., 2011). Additional mechanisms to explain NHEJ deficiency in Lamin A defective cells involved the absence of DNAPK in progeria cells (Liu et al., 2011) or decreased level of the histone acetyl transferase MOF (Krishnan et al., 2011; Pegoraro et al., 2009), which was already

identified as a necessary protein to allow the recruitment of 53BP1 and BRCA1 (Li et al., 2010). On its side, the decreased HR efficiency observed in absence of wild-type Lamin A was attributed to transcriptional repression of BRCA1 and Rad51 genes (Redwood et al., 2011), therefore leading to decreased recruitment of Rad51 at DSBs upon irradiation of progeria cells (Liu et al., 2005). The role of Lamin A in promoting NHEJ and HR is summarized in the figure 30 (Redwood et al., 2011).

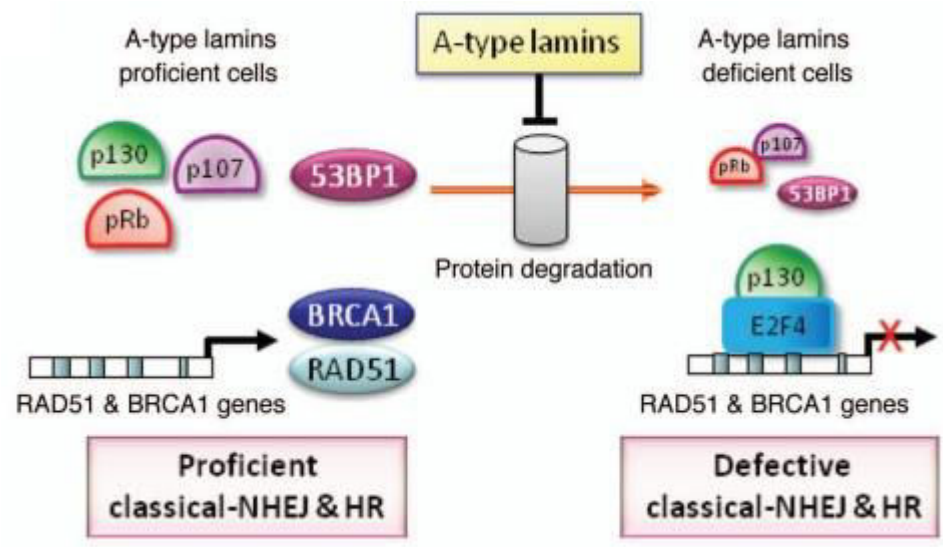

Figure 30- Role of A-type lamins in DSB repair (Redwood et al., 2011)

A-type lamins play a role in the stabilization of the pocket family proteins pRb and p107, as well as 53BP1, in part by preventing their degradation by the proteasome. By stabilizing 53BP1, A-type lamins promote classical-NHEJ. In addition, A-type lamins regulate transcriptionally two key factors in HR, RAD51 and BRCA1. Loss of A-type lamins leads to increased formation of p130/E2F4 complexes, which in turn can bind the RAD51 and BRCA1 gene promoters and inhibit their transcription. Loss of A-type lamins leads to defects in the two major mechanisms of DNA DSBs repair (NHEJ and HR), increased genomic instability and radiation sensitivity.

Nucleoporins involvement

The first evidence of nucleoporins involvement in DNA repair came from a genome-wide screen in yeast, in which five core nucleoporins -Nup84, Nup120, Nup133, Nup170, Nup188- were implicated in the repair of ionizing radiation damage (Bennett et al., 2001). Additionally, Mlp1 and Mlp2, two nucleoporins from the nuclear basket were also identified as regulators of DSB repair. Indeed, deletion of both proteins or of Nup60, which allows their anchoring to NPCs leads to accumulation of Rad52 foci, a marker of DSB (Palancade et al., 2007). The

mechanisms by which nucleoporins participate in DSB repair seem sumoylation-dependent. The Nup84 complex and Nup60 are required for the recruitment of the SUMO protease Ulp1 to NPCs (Zhao et al., 2004) and depletion of these nucleoporins result in displacement of Ulp1 from the NPCs and affects cellular sumoylation patterns (Palancade et al., 2007). Ulp1 mutants that cannot localize to the NPCs show similar phenotypes than nucleoporins mutants regarding DSB repair whereas Ulp1 overexpression can partially rescue this phenotype (Palancade et al., 2007), suggesting that the role of nucleoporins in DSB repair is mediated by their function in the recruitment of Ulp1 at NPCs. A proposed target of this regulatory mechanism is yKu70, which shows decreased sumoylation levels in nucleoporins mutants (Palancade et al., 2007). However, a big number of proteins are sumoylated during DDR and DSB repair (Dou et al., 2011) and other targets of Ulp1 might be important for DSB repair. In mammalian cells, the presence of NPC-associated SUMO-regulating proteins is conserved (Palancade and Doye, 2008; Zhang et al., 2002), however no role for nucleoporins in DSB repair was described. I'm addressing this question in the first part of the results section.

Thesis objectives

When they are not properly repaired, DSBs can be at the origin of major genomic rearrangement and trigger tumorigenesis (Jackson and Bartek 2009). Cells evolved different repair mechanisms, such as NHEJ, HR or alt-EJ. To avoid genomic rearrangement, cells have to choose the right repair pathway to use.

In yeast cells, DSBs are mobile and several DSBs can migrate to be repaired in a common repair centre (Lisby, Mortensen, and Rothstein 2003). On the contrary, in mammalian cells DSBs are positionally stable (Soutoglou et al. 2007). This positional stability might challenge DSB repair that has to be efficient in all nuclear contexts. Indeed, the mammalian nucleus is highly heterogeneous and encompasses various compartments that have different protein content and that are associated with different types of chromatin.

The objective of my thesis entitled "Nuclear architecture and DNA repair: double-strand breaks repair at the nuclear periphery" is to understand how DSB repair is organized in the different nuclear compartments and what are the strategies cells use to allow efficient repair in any nuclear context. We focused our interest on the nuclear periphery –composed by the nuclear pores and the nuclear lamina-, which was already shown to play major role in the regulation of gene expression. We used two different strategies to understand on one hand the participation of nucleoporins in DSB repair and on the other hand the influence of nuclear positioning on DSB repair:

A. We assessed the role of the nucleoporin Nup153 in DSB repair and in DSB repair pathway choice. This work was published in the journal Oncogene and is inserted in this manuscript as the first part of the results section.

B. We developed a cellular system to induce a DSB specifically at the nuclear lamina, the nuclear pores or the inner nucleus and followed their fate. This work was published in the journal Genes and Development and is inserted in this manuscript as the second part of the results section.

Results

I. The nucleoporin 153, a novel factor in double-strand break repair and DNA damage response

1. Research article

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SHORT COMMUNICATION

The nucleoporin 153, a novel factor in double-strand break repair and DNA damage response

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DNA repair is essential in maintaining genome integrity and defects in different steps of the process have been linked to cancer and aging. It is a long lasting question how DNA repair is spatially and temporarily organized in the highly compartmentalized nucleus and whether the diverse nuclear compartments regulate differently the efficiency of repair. Increasing evidence suggest the involvement of nuclear pore complexes in repair of double-strand breaks (DSBs) in yeast. Here, we show that the human nucleoporin 153 (NUP153) has a role in repair of DSBs and in the activation of DNA damage checkpoints. We explore the mechanism of action of NUP153 and we propose its potential as a novel therapeutic target in cancers.

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Keywords: DNA repair; nuclear pore; 53BP1

INTRODUCTION

DNA double-strand breaks (DSBs) are particularly dangerous as their inefficient or inaccurate repair can result in mutations and chromosomal translocations that may induce cancer.[1] DSBs can be repaired by one of two major pathways: homology-based repair (homologous recombination (HR)) using the intact chromatid as a template present in proximity in S and G2 phases of the cell cycle, or direct joining across the break site (non-homologous end joining (NHEJ)).[2]

The coordination between cell cycle progression and DSB repair (DSBR) is regulated by the DNA damage response (DDR) signalling pathway, which activates the cell cycle checkpoints in the presence of DNA breaks.[3] This pathway is initiated by the recruitment of the MRN (MRE11 – RAD50 – NBS1) sensor complex to sites of damage. The recruitment of MRN subsequently activates the ATM kinase, which associates with DSBs and phosphorylates the histone variant H2AX (γ-H2AX).[2] MDC1 can then bind to γH2AX and recruit new MRN and ATM proteins, leading to spreading of the repair machinery along the chromosome. MDC1 also recruits ubiquitin ligases, such as RNF8 and RNF168, which facilitate the recruitment of the downstream factors 53BP1 and BRCA1.[2] When the DNA is resected to single-stranded DNA, it is recognized by replication protein A, which results in the recruitment of ATR.[2] Both the ATM and the ATR dependent branches of the pathway lead to the activation of the checkpoint kinases, CHK1 and CHK2, which stall damaged cells in their cell cycle until the lesions are resolved.[3]

DNA repair, like all DNA-dependent processes, occur in the highly compartmentalized nucleus. Most nuclear events do not occur ubiquitously, but are limited to defined sites.[2] Several studies in yeast have shown that dedicated DNA repair centres exist as preferential sites of repair.[2,4] Furthermore, persistent DSBs in yeast migrate from their internal nuclear positions to the nuclear periphery, where they associate with nuclear pores.[5,6] This sequestration to the nuclear periphery was shown to require certain components of the yeast nuclear pore complex, like NUP84 and the nucleoporin NUP60, located in the basket of the pore.[5,6] Additional studies revealed that depletion of representative members of the NUP84 or NUP60 complex leads to synthetic lethality when combined with genes that are required for DSBR through HR.[4] Moreover, mutants of the NUP84 complex are highly sensitive to DNA-damaging treatments.[7] A more recent study has shown that key nucleoporins are phosphorylated upon DNA damage and act to neutralize the topological tension generated at nuclear pore tethered genes that is inhibitory to origin firing after replication stress.[8]

On the contrary, in mammalian cells, each DSB is repaired individually in the absence of nuclear repair centers.[9] Furthermore, DSBs do not move towards the nuclear periphery, as their motion seems to be very limited in the mammalian nucleus.[9] However, the evolutionary conserved role of nucleoporins in gene regulation raises the question whether nucleoporins have a conserved role in mammalian DSBR. We therefore explored the role of the Nucleoporin 153 (NUP153), a component of the nuclear basket of the mammalian nuclear pore in DSBR. We show that NUP153 is essential for proper activation of the DNA damage checkpoints and regulates the choice between NHEJ and HR. These functions can be partially explained by the role of NUP153 in promoting 53BP1 nuclear localization. Our results will set up the basis of investigation of the role of nuclear pore in DNA repair in mammals and can lead to potential therapeutic innovations.

RESULTS AND DISCUSSION

One of the hallmarks of defective DSBR and DDR is hypersensitivity to DNA damaging agents. To address whether the NUP153 has a role in repair of DSBs, we analysed the effect of its depletion in clonogenic survival of U2OS cells following exposure to genotoxic stress. RNAi-mediated downregulation of NUP153 led

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to an increased sensitivity to the radiomimetic drug phleomycin compared with control cells (Figure 1a). The efficiency of the NUP153 silencing was verified by RT–qPCR (Supplementary Figure 1) and western blot (Figure 1b). One possible explanation for the hypersensitivity to DNA damage upon depletion of NUP153 is the deregulation of cell cycle checkpoints resulting in mitotic progression with unrepaired DSBs. To test this hypothesis, we investigated whether the downregulation of NUP153 affects the activation of checkpoints after treatment with the radiomimetic drug Neocarzinostatin (NCS). Indeed, we observed compromised phosphorylation of ATM, CHK1 and CHK2 kinases and p53 (Figure 1b). In line with this observation, the NUP153 depleted cells didn't properly activate the G2/M check point. Although, 31% of cells treated with si-scramble arrest in G2/M, 8 h after treatment with phleomycin, only 26% of siNUP153 cells exert similar arrest (Supplementary Figure 2). These results support the idea that NUP153 promotes DNA damage checkpoints.

To investigate the possibility that the hypersensitivity to DNA damaging agents stems also from persistent DSBs and defective DNA repair, we sought to directly test whether NUP153 facilitates DNA repair through a specific pathway. To this end, we utilized cell lines that contain stably integrated reporters to assess the rates of HR (DR-GFP[10,11]) and NHEJ.[12–14] For NHEJ, we used cell lines containing two types of NHEJ-reporter substrates; the pCOH-CD4 that permits analysis of the NHEJ of two distal ends (separated by 3.2 kb),[12,13] and a GFP-based substrate,[14] to measure the NHEJ on closely adjacent ends, separated by only 34 bp.[14]

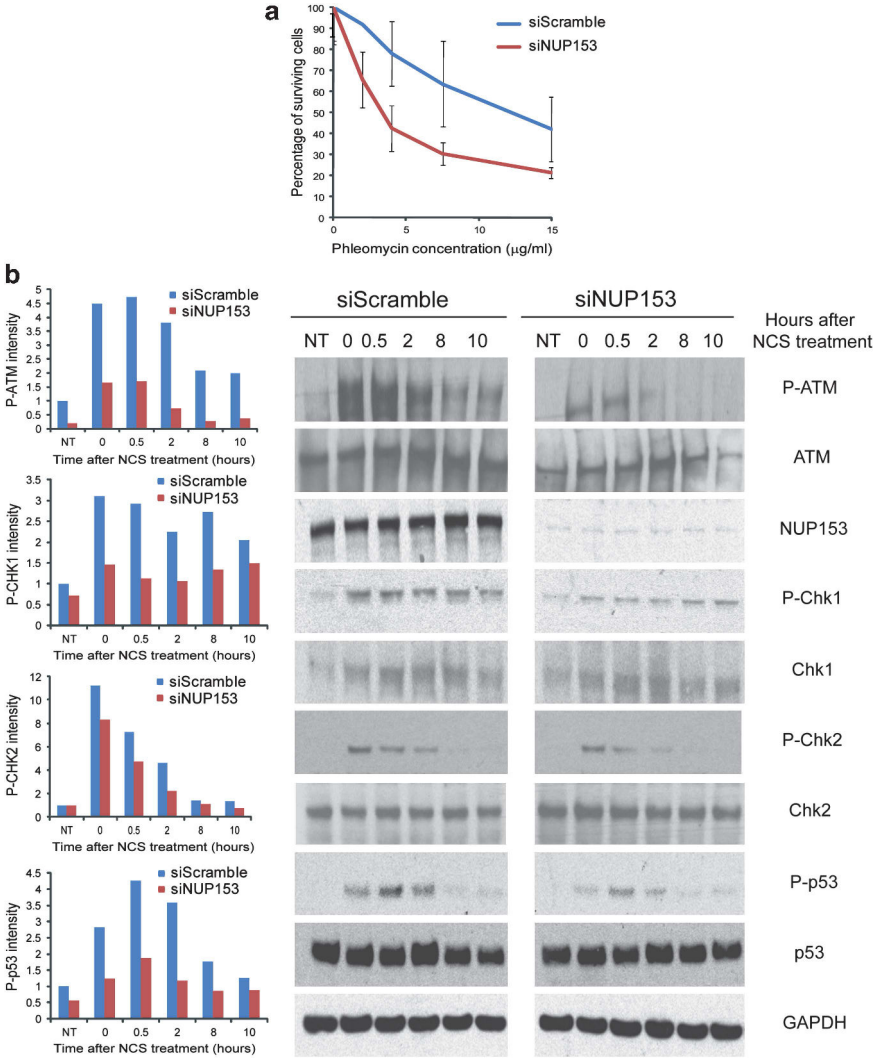

Figure 1. NUP153 promotes survival and is required for proper activation of DNA damage checkpoints. (**a**) Clonogenic survival in U2OS cells treated with the indicated siRNAs, following exposure to increasing concentrations of the radiomimetic drug phleomycin. NUP153-depleted cells exhibit hypersensitivity to phleomycin. This is one representative experiment out of three repetitions and s.d.s represent the errors from three internal triplicates of the depicted experiment. U2OS cells were transfected with scramble and NUP153-specific siRNAs, using Oligofectamine (Invitrogen, Grand Island, NY, USA) and 48 h after transfection, cells were counted and seeded in triplicates in 6-well plates (500 cells per well). The day after, cells were treated with 0-2-4-7.5-15-30 μg/ml of Phleomycin (Sigma, St Louis, MO, USA). Cells were then cultured for 11 days. Colonies were stained with 0.1% crystal violet. The coloration was dissolved in 20% acetic acid and the absorbance at 590 nm was measured by spectrophotometer. (**b**) NUP153-depleted cells exhibit decreased checkpoint activation as monitored by WB. Whole-cell extracts were prepared from non-treated (NT) cells or cells harvested at the indicated times after release from a 15 min NCS-treatment (50 ng/ml), 72 h post transfection with the indicated siRNAs. Equal loading was controlled using the GAPDH antibody and equal expression of ATM, CHK1, CHK2 and p53 was ensured using the respective antibodies (Supplementary Figure 7). The knock down of NUP153 was monitored using the NUP153 antibody. Signal intensities were measured using Image J, NIH, Bethesda, MD, USA.

Interestingly, upon depletion of NUP153, we observed a significant drop in efficiency of NHEJ with both substrates compared with the scramble siRNA (2 fold for the CD4 and 5 fold for the GFP) (Figure 2a). Concomitantly, we observed a more than two fold increase in the rate of HR (Figure 2b). We used downregulation of RAD51 as a control and expectedly observed defective HR. Cell cycle analysis shows that the changes in NHEJ and HR rates cannot be explained by alterations in cell cycle profile upon depletion of NUP153 (Supplementary Figure 2). These results suggest that NUP153 has a role in the balance between NHEJ and HR.

To investigate whether the increase in HR was accompanied by increased resection, we visualized and quantified replication protein A and BRCA1 foci at a single DSB to mimic the break induced in the NHEJ and HR assays. We utilized U2OS cells with stably integrated I-SceI site harboring lacO operator repeats that can be visualized by GFP-lacR (lac repressor). As expected, we found that depletion of NUP153 leads to more than 2-fold increase in the number of replication protein A and BRCA1 foci in U2OS cells after expression of I-SceI (Figures 2c and d). DSB induction was verified by the phosphorylation of H2AX at the lacO array (Supplementary Figures 3A and B). Our results therefore suggest that NUP153 might inhibit HR by blocking resection of DSBs.

To understand the mechanism underlying the involvement of NUP153 in DDR, we first tested whether NUP153 is recruited to DSBs. We were unable to detect any accumulation of GFP-NUP153 at laser-induced breaks (Figure 3a). We then asked whether NUP153 regulates the localization and the ability of known DDR factors to form IRIF upon Neocarzinostatin treatment in U2OS cells. Whereas depletion of NUP153 did not affect γH2AX (Figure 3b) and MDC1 foci (Supplementary Figure 4), it significantly inhibited focal accumulation of 53BP1 quantified by high throughput imaging (Figure 3c). The results were validated by three independent siRNAs targeting distinct regions of NUP153 mRNA ranging from 30–50% decrease in the number of 53BP1 foci (Figure 3c). High-resolution imaging showed that NUP153 siRNA treated cells exerted massive and selective mislocalization of 53BP1 to the cytoplasm (Figure 3b, quantification in Figure 3d). The remaining nuclear 53BP1 either did not accumulate in foci or formed foci localized to the nuclear periphery (Figure 3b). Importantly, depletion of NUP153 did not affect the global levels of 53BP1 (Supplementary Figure 5). To test whether the 53BP1 cytoplasmic localization in the absence of NUP153 is because of an accelerated export or defective nuclear retention, we repeated our experiments in the presence of leptomycin B that selectively inhibits CRM1-dependent nuclear export.[15] We observed no difference in the localization of 53BP1 or the foci formation (data not shown), suggesting that the presence of 53BP1 in the cytoplasm is due to an import defect or accelerated export through a CRM1-independent pathway. Indeed, previous work showed that depletion of NUP153 leads to import impairment of selective proteins.[16] Moreover, while we were conducting this study, Moudry *et al*.[17] confirmed the mislocalization of 53BP1 in NUP153 depleted cells and showed the requirement of NUP153 for 53BP1 nuclear import.

Recent studies have provided important mechanistic insights about how deficiency in 53BP1 restores HR levels in BRCA1-deficient cells by regulating the choice between HR and NHEJ.[18,19] These studies have placed 53BP1 as a top candidate for pharmacological targeting for future breast cancer therapies. Therefore, we sought to understand whether the impairment of 53BP1 is sufficient to explain the NUP153-deficient phenotype in our DNA repair assays, to suggest NUP153 as a potential candidate for targeted cancer therapy. To this end, we performed the survival assay in cells depleted for 53BP1 or for a combination of NUP153 and 53BP1. Indeed, 53BP1 knock down recapitulated the sensitivity to phleomycin treatment and the decreased survival (Figure 4a). Interestingly, combined 53BP1 and NUP153 depletion did not result in an additive survival defect suggesting that the radiosensitivity observed in NUP153 depleted cells is owing to the impairment in 53BP1 localization (Figure 4a). The efficiency of 53BP1 knock down was monitored by RT–qPCR and WB (Supplementary Figure 6). Moreover, 53BP1 was shown to promote ATM activity,[20–22] and its depletion leads to checkpoint activation defects[23] pointing to similar dependency on 53BP1 for activation of the checkpoints.

We then assessed whether the increase in HR at NUP153 depleted cells was mediated by the 53BP1 defect. We observed a moderate (1.3 fold) increase of HR upon depletion of 53BP1 using siRNA (Figure 4b). This effect was similar to that observed by Xie *et al*.[14] in a previous study. However, this increase was smaller than the one observed in NUP153 depleted cells, and the combinatorial depletion of 53BP1 and NUP153 phenocopied the HR efficiency in cells depleted for NUP153 alone (Figure 4b).

Moreover, Guirouilh-Barbat *et al*. observed that 53BP1 silencing leads to a significant decrease in the frequency of end-joining, monitored with the GFP-based substrate, but has no impact on NHEJ frequencies, monitored with the CD4-based substrate (Bernard Lopez personal communication). This observation is different from our results that show that silencing of NUP153 leads to a decrease in NHEJ efficiency in both substrates (Figure 2a), suggesting that NUP153 promotes NHEJ through a pathway that does not involve only 53BP1. Additionally, Guirouilh-Barbat *et al*. showed a decrease in NHEJ accuracy upon 53BP1 depletion (Bernard Lopez personal communication). To test whether NUP153 depletion recapitulates these results, we analyzed repair junctions on the pCOH-CD4 substrate after the silencing of NUP153. Surprisingly, although NUP153 depletion affects the efficiency of NHEJ, it does not promote inaccurate repair (Figure 4c) further pointing to a role of NUP153 in NHEJ independent from 53BP1.

Taken together, our results suggest that in assays where a large amount of DSBs is induced the depletion of NUP153 phenocopies the depletion of 53BP1. On the other hand, when a single DSB is induced, the NUP153 depletion has a stronger and/or divergent phenotype. A possible explanation for this phenomenon is that the amount of protein remaining in the nucleus upon depletion of NUP153, is limited and there is active competition between the breaks for focal accumulation of 53BP1. On the other hand, when one or limited breaks are induced as it is the case with the I-SceI break at the HR and NHEJ assays, there is enough 53BP1 protein to form a repair focus. To test this hypothesis, we used the LacO-I-SceI cell line, where a break is induced at a single locus in the nucleus. Interestingly, we detected a normal recruitment of 53BP1 to I-SceI breaks upon depletion of NUP153 (Figures 4d and e). This finding is in agreement with the observation that the recruitment of 53BP1 at endogenous foci is not impaired upon depletion of NUP153 (Figure 3c–Neocarzinostatin condition).

The stronger effect on HR efficiency observed upon depletion of NUP153 could be explained by the loss of a potential 53BP1 modification and that the unmodified 53BP1 accumulates at the single DSB, acting as dominant negative. An alternative explanation could be that NUP153 has a role in addition to the regulation of nuclear import of 53BP1. It could promote the nuclear accumulation of a NHEJ factor and/or its recruitment to DSBs. However, silencing of classical NHEJ factors that affect the efficiency of end ligation affect the fidelity of repair as well.[13] An alternative scenario could be that NUP153 negatively regulates a protein that promotes HR. Furthermore, we can imagine that the nuclear soluble fraction of 53BP1 has a role in the regulation of the repair pathways, by sequestering certain factors away from the break. Therefore, we can speculate that upon depletion of NUP153, even if 53BP1 has still the ability to bind to DSBs, the absence of its soluble pool can impair DSBR.

Here, we describe a novel role of NUP153 in DDR and DNA repair. TPR, the binding partner of NUP153 at the nuclear basket, is phosphorylated upon DNA damage by ATM/ATR and is involved in the proper activation of G2/M and intra S check point.[24]

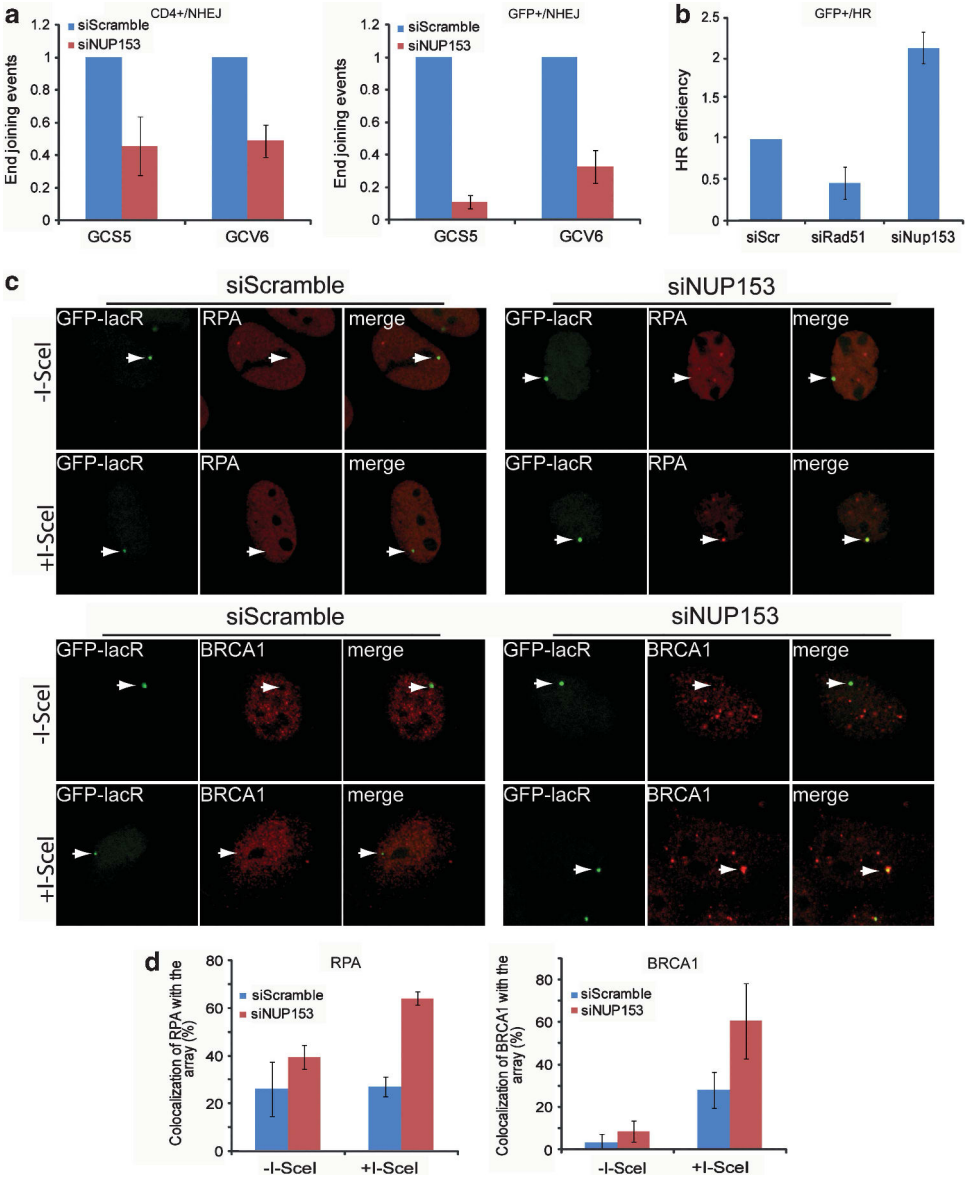

Figure 2. NUP153 regulates the balance between NHEJ and HR. (**a**) NHEJ efficiencies in GC92 cells treated with the indicated siRNAs. The numbers represent values of NHEJ efficiency relative to the control. Two independent GC92 clones (GCS5 and GCV6) each bearing both the CD4-based and GFP-based substrates were used. Values represent the means and s.d of four independent experiments. The cells were first transfected with the indicated siRNAs using interferin (Polyplus, Illkirch, France) and 48 h after, they were transfected with HA-I-SceI expression vector (pCBASce) using jetPei (Polyplus). The GFP and CD4 frequencies were measured by FACS 3 days after the HA-I-SceI plasmid transfection. (**b**) HR efficiency in U2OS cells transfected with the indicated siRNAs. The numbers represent a fold increase of HR efficiency compared with the control. U2OS cells containing the HR reporter DR-GFP, were transfected with the indicated siRNAs and 48 h later transfected with an HA–I–SceI expression vector (pCBASce). GFP intensity was measured by FACS. The mean ± s.d.s of three experiments is shown. (**c**) Immunofluorescence staining of replication protein A (RPA) (red) or BRCA1 (red) at an I-SceI-induced break in U2OS19ptight13 GFPlacR cells transfected with scramble or NUP153 siRNA. The locus where the break is induced is visualized with the GFPlacR (green spot). The pictures represent the phenotype observed in the majority of the cells. U2OS19ptight13 GFPlacR cells were generated as follows: U2OS cells were transfected with a plasmid that contains an I-SceI recognition site flanked by 256 copies of the lac operator (lacO) on one side and by 96 copies of the tetracycline response element on the other side (tetO),[9,26] and stable clones were selected (the clone used is called U2OS19). To obtain the U2OS19 ptight 13 cells, U2OS19 cells were transfected using Fugene 6 with pWHE320-HA-IsceI (that encodes for HA-IsceI under a tet inducible promoter) and pWHEI46 (that encodes for the tet activator) at a ratio of 8:2. The cells were clonal selected with 800 μg/ml G418. Expression of HA-IsceI was obvious 14 h after Doxycyclin (Dox) treatment. The U2OS19 ptight13 GFPlacR cell line that stably expresses the lac repressor (lacR) fused to GFP, was generated by retroviral infection of MSCV-GFP-lacR plasmid.[27] The cells were FACS sorted and GFP positive cells were retained in red phenol free medium, 10% charcoal treated fetal calf serum, 800 μg/ml G418, 2 mM IPTG to avoid the permanent binding of lac repressor to the lacO.[27] For the experiment, U2OS19ptight13 GFPlacR cells were transfected with the indicated siRNAs using oligofectamine, in absence of IPTG. Cells were harvested 72 h after the transfection and 14 h after Dox treatment. (**d**) Quantitative analysis of RPA (left panel) and BRCA1 (right panel) recruitment at the lacO array before and after cutting with I-SceI, in control cells (blue bars) and cells transfected with NUP153 siRNA (red bars). Mean values of two independent experiments are shown (number of cells counted $N = 100$).

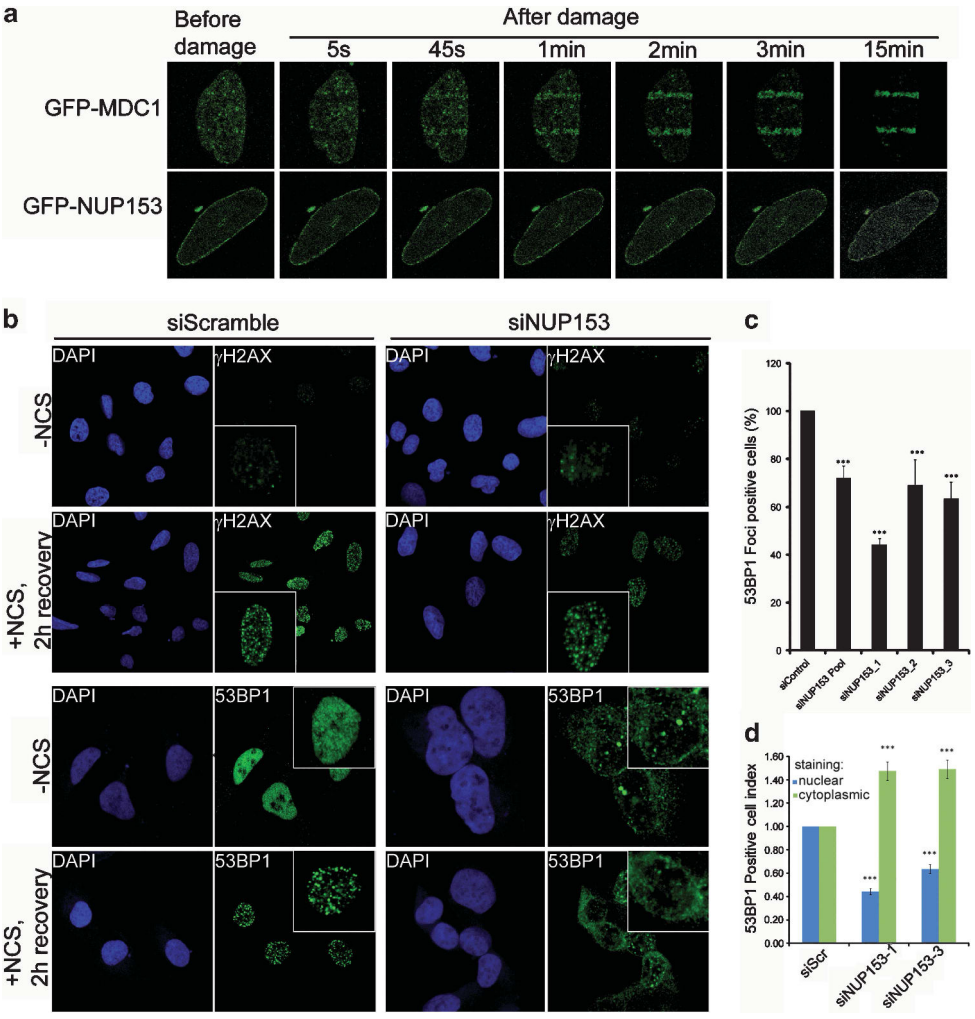

Figure 3. NUP153 promotes nuclear accumulation of 53BP1 and IRIF foci formation. (**a**) U2OS cells expressing GFP-NUP153 or GFP-MDC1 were subjected to laser micro-irradiation using a 800-nm laser and subsequent real time recording of protein assembly at the damaged area. Although MDC1 accumulates efficiently at the sites of damage, recruitment of NUP153 was not detected using the same conditions. U2OS cells were transienlty transfected with 2 μg of the indicated plasmids using Fugene 6 according to the manufacturers' recommendations. (**b**) Immunofluorescence analysis of γH2AX (green) and 53BP1 (green) in control cells and cells treated with siRNA targeting NUP153 at non-treated (-NCS) conditions or 2 h after treatment with the radiomimetic drug NCS (+NCS 2 h). Cells grown on coverslips were fixed with 4% paraformaldehyde, washed with 1XPBS, permeabilized with 0.5% Triton X-100/PBS and blocked with 5% BSA/PBS before incubation with primary antibodies for 1 h in RT. After three washes with 1XPBS, cells were stained with Alexa488--conjugated secondary antibodies (Invitrogen). The nuclei were counterstained with DAPI and the samples were mounted in Prolong Gold (Invitrogen). (**c**) Quantification of 53BP1 foci 2 h after NCS treatment in control cells and cells treated with a pool of four siRNAs or three individual siRNAs targeting NUP153. Cells seeded at 96-well plated were trasnfected with the indicated siRNAs and stained with the indicated antibodies. High content analysis was performed using the InCELL1000 Analyzer workstation and the InCELL Analyzer software for image data processing (GE LifeSciences, Munich, Germany). To quantify the distance from the negative control, we determined the percents of control that reflects the deviation from the negative control. After multiple testing corrections, the *P*-values were determined. ***$P<0.0001$. (**d**) Quantification of 53BP1 intensity in the nucleus and cytoplasm of control cells and cells treated with NUP153 siRNAs. The effect of gene silencing on 53BP1 nuclear and cytoplasmic localization was investigated by immunofluorescence as described above. Image data-processing protocols (InCELL Analyzer software) were specifically developed to quantify 53BP1 foci in the nucleus and cytoplasm. ***$P<0.0001$.

One interesting aspect for further investigation is whether these factors have distinct or overlapping roles in DDR and whether the overlapping roles are mediated through their interaction. In yeast, the nucleoporin complexes NUP84 (hNUP107) and NUP60 (hNUP153) protect against genomic instability through maintenance of proper levels of the sumo protease Ulp1 at NPCs, and through appropriate sumoylation of several proteins, including yKu.[25] It is tempting to speculate that a similar mechanism is conserved in mammals. Furthermore, it would be very interesting to investigate whether the role of NUP153 in DDR is unique or if other mammalian nucleoporins have similar role.

We show here that NUP153 regulates the choice between NHEJ and HR. This observation positions NUP153 as a candidate gene whose reduced expression could promote synthetic lethality in tumor cells that bear mutations in HR factors, like *BRCA1* and *BRCA2*. However, as NUP153 promotes 53BP1 IRIF foci formation after DNA damage, impairment of NUP153 in BRCA1 cancer cells could mimic the phenotype of 53BP1 depletion, rescuing lethality and conferring resistance to PARP inhibition.[18,19] It will be consequently very interesting to exploit in the future the potential of NUP153 as a therapeutic target in certain cancers.

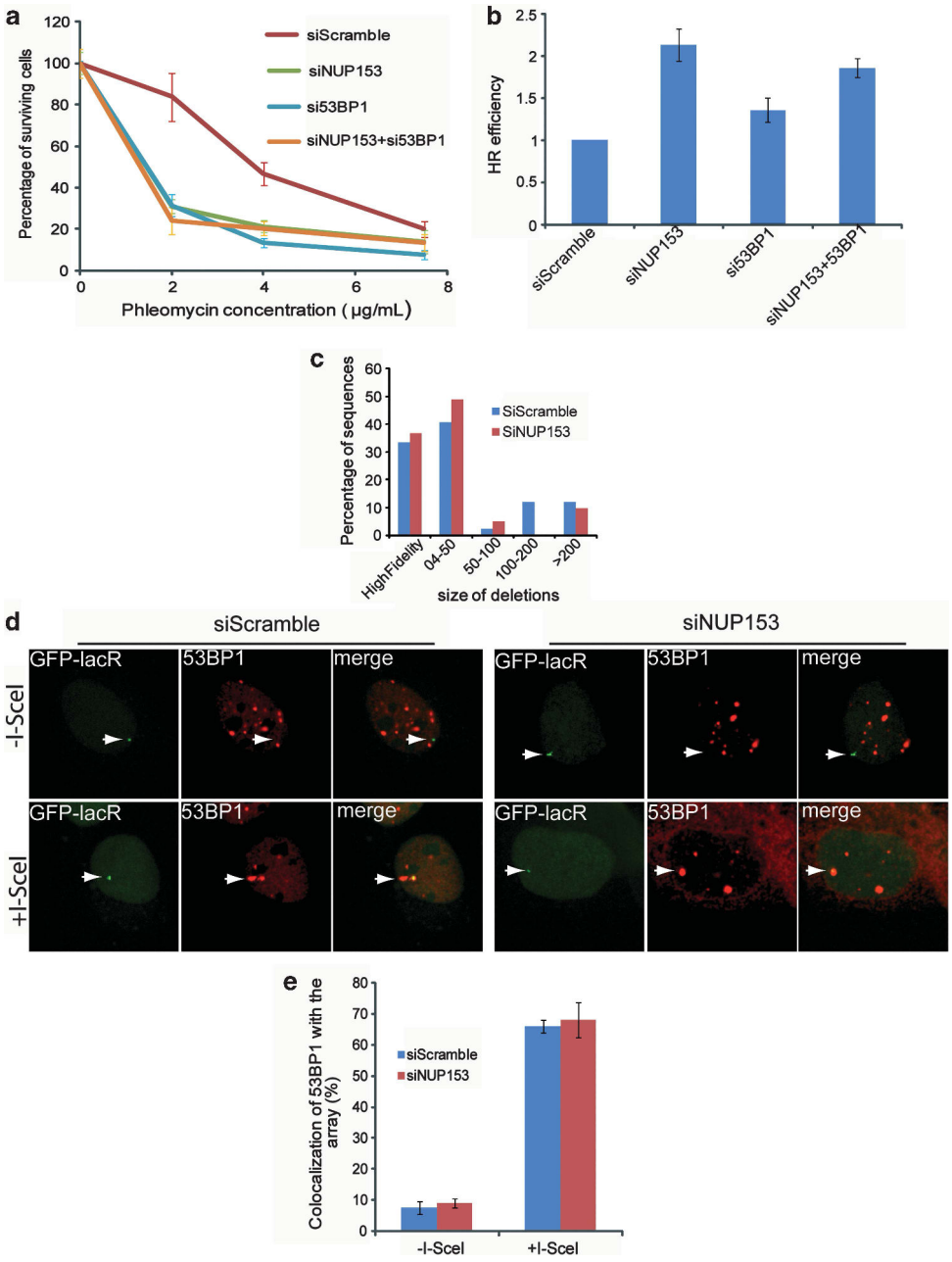

Figure 4. The involvement of NUP153 in DSBR can be partially explained by the impairment of 53BP1 localization. (**a**) Clonogenic survival in U2OS cells treated with the indicated siRNAs, following exposure to increasing concentrations of phleomycin. (**b**) HR efficiency in U2OS cells transfected with the indicated siRNAs. The numbers represent a fold increase of HR efficiency compared with the control. The mean ± s.d.s of three experiments is shown. (**c**) Deletion size distribution in si-Scramble or siNUP153 condition. The junction sequences were amplified by PCR of genomic DNA using the primers CMV-5 (5′-ATTATGCCCAGTACATGACCTTATG-3′) and CD4-int (5′-GCTGCCCCAGAATCTTCCTCT-3′). The PCR products were cloned into the pGEM-T vector (Promega, Madison, WI, USA) and sequenced (GATC). (**d**) Immunofluorescence staining of 53BP1 (red) at an I-SceI induced break in U2OS19 ptight13 GFPlacR cells transfected with scramble or NUP153 siRNA. The locus that the break is induced is visualized with the GFPlacR (green). (**e**) Quantitative analysis of 53BP1 recruitment at the lacO array before and after cutting with I-SceI, in control cells (blue bars) and cells transfected with NUP153 siRNA (red bars). Mean values of two independent experiments are shown (number of cells counted $N = 100$).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Supplementary figure legends

Supplementary figure 1: Validation of NUP153 silencing in U2OS cells by siRNA

Quantitative RT-PCR analysis of NUP153 expression levels in U2OS cells treated with siRNA that targets a scramble sequence and the NUP153 sequence. mRNA values are normalized to cyclophylin B and to the mRNA levels of each gene at the scramble condition. Total cellular RNA was purified from U2OS cells using RNeasy kit total RNA purification kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized with the RT-PCR kit (Qiagen) according to the manufacturer's instructions. qRT-PCR analysis was performed using specific primers and each reaction contained 20 μmol total RNA template and 1 pmol of each primer. Reactions were carried out using a Roche Lightcycler 480 II system for 50 cycles. The purity of the PCR products was determined by melt curve analysis.

Supplementary figure 2: Depletion of NUP153 leads to a decrease in G2/M arrest

Cell cycle profile after Propidium iodide staining in control U2OS cells and cells depleted for NUP153 by siRNA, without treatment or 8h after phleomycin treatment. U2OS cells were transfected with non targeting siRNA (scramble) or NUP153 siRNA using lipofectamine 2000 (invitrogen). 48h after transfection, cells were treated with 50⌈g/ml of phleomycin for 1h. 8h after treatment, they were fixed in ice-cold ethanol overnight, then treated with 100μg/ml of RNAse A for 30 min at 37°C. They were then stained with 40μg/ml propidium iodide for 30 min. Cell cycle analysis was performed by FACS.

Supplementary figure 3: Depletion of NUP153 does not alter H2AX phosphorylation at I-SceI induced DSBs.

A. Immunofluorescence staining of γH2AX (red) at an I-SceI induced break in U2OS19ptight13 GFPlacR cells transfected with scramble or NUP153 siRNA. The locus that the break is induced is visualized with the GFPlacR (green spot).

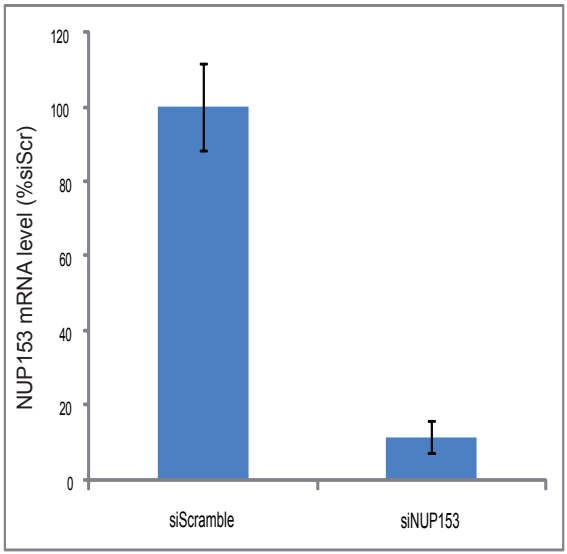
B. Quantitative analysis of γH2AX at the lacO array before and after cutting with I-SceI, in control cells (blue bars) and cells transfected with NUP153 siRNA (red bars). Mean values of 2 independent experiments are shown (number of cells counted N=100).

Supplementary figure 4: NUP153 depletion does not affect MDC1 foci formation upon NCS induced DNA damage. Immunofluorescence analysis of MDC1 (green), in control U2OS cells and cells treated with siRNA targeting NUP153 at non-treated (-NCS) conditions or 2h after NCS treatment (+NCS 2h).

Supplementary figure 5: NUP153 depletion does not alter the total protein level of 53BP1. Western blot analysis of 53BP1 protein levels in control U2OS cells and cells treated with siRNA that depleted NUP153

Supplementary figure 6: Validation of 53BP1 silencing in U2OS cells by siRNA. A. Quantitative RT-PCR analysis of 53BP1 expression levels in U2OS cells treated with siRNA that targets a scramble sequence and the 53BP1 sequence. mRNA values are normalized to cyclophylin B and to the mRNA levels of each gene at the scramble condition. **B.** Western blot analysis of 53BP1 protein levels in control U2OS cells and cells treated with siRNA that depleted 53BP1.

Supplementary figure 7: Antibodies and siRNA references tables


120
100
80
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40
20
0
NUP153 mRNA level (%siScr)
siScramble
siNUP153

Figure S1

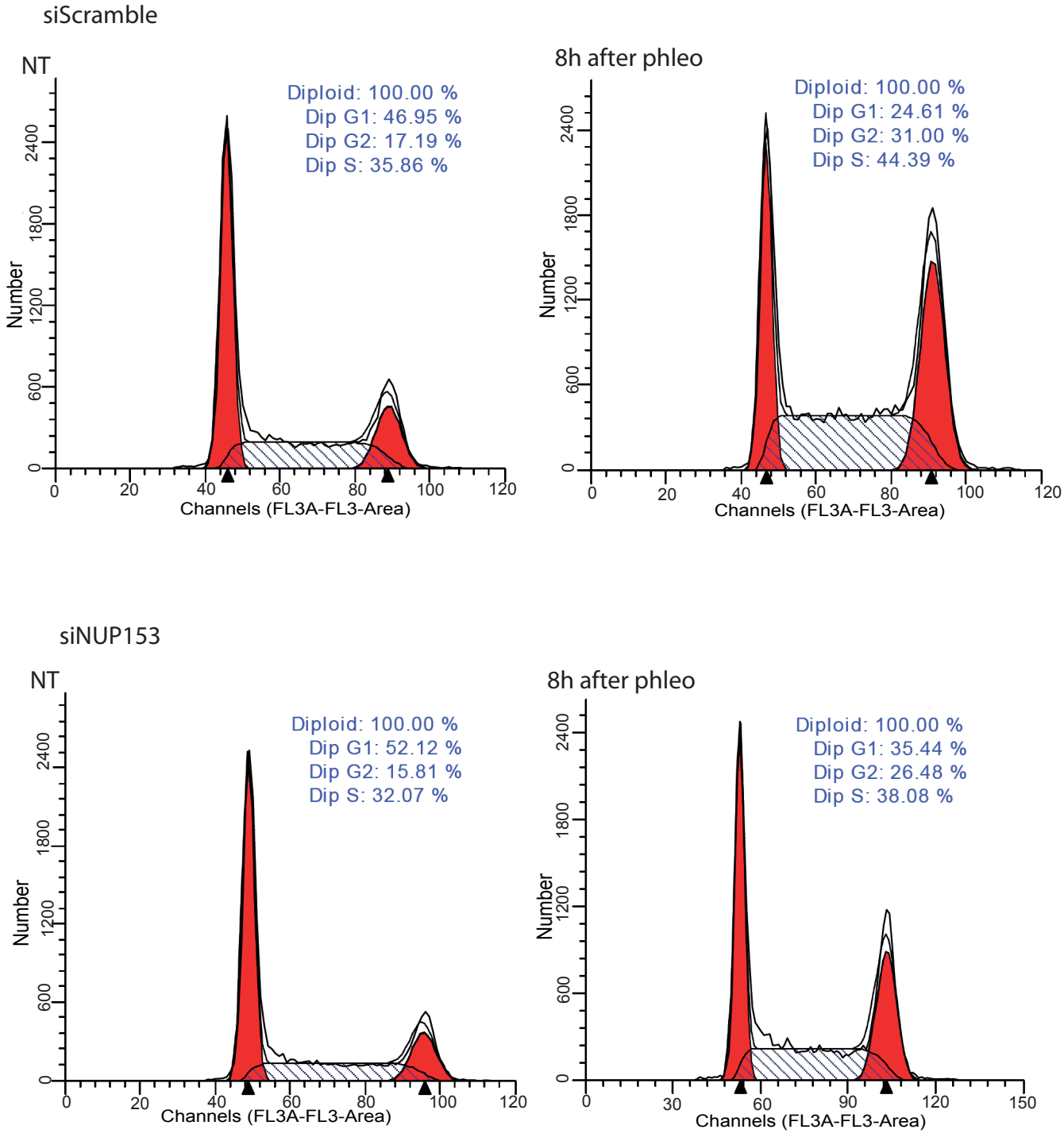
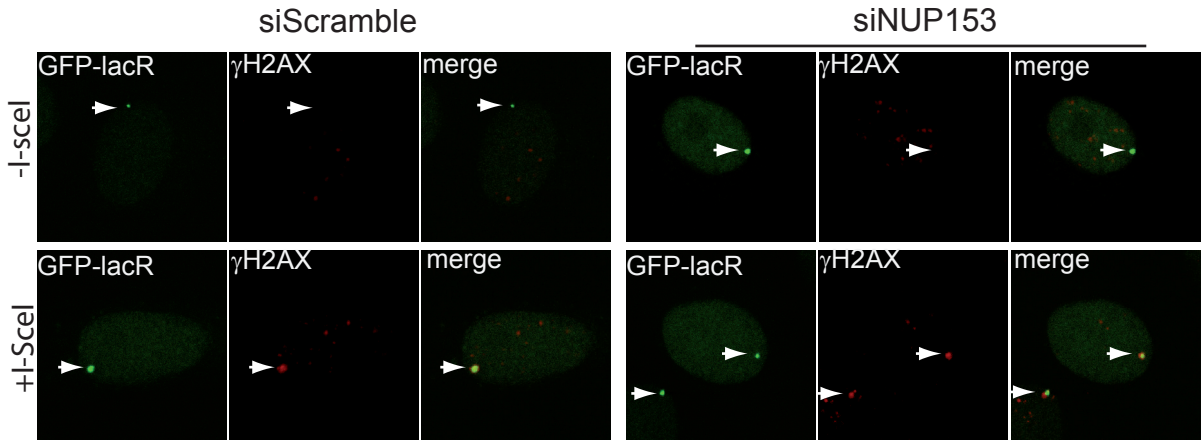
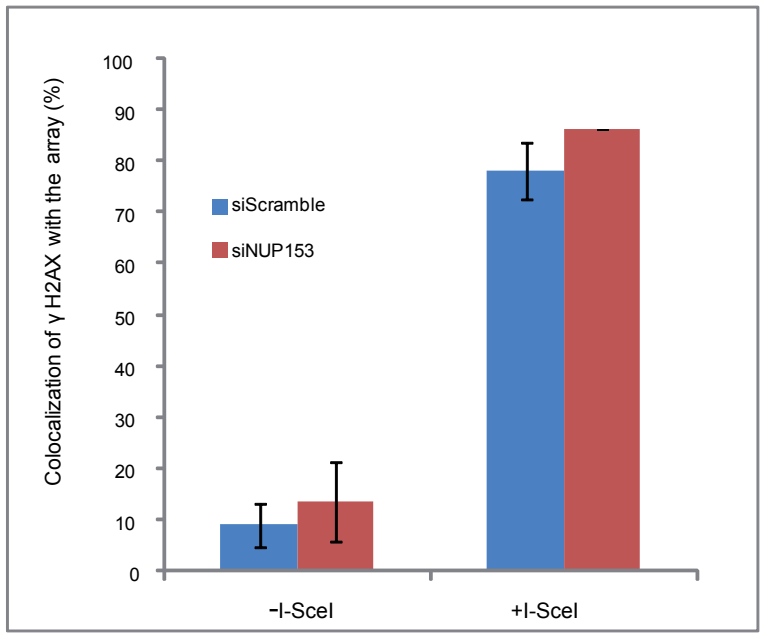

siScramble
NT
Diploid: 100.00 %
Dip G1: 46.95 %
Dip G2: 17.19 %
Dip S: 35.86 %
Number
Channels (FL3A-FL3-Area)
8h after phleo
Diploid: 100.00 %
Dip G1: 24.61 %
Dip G2: 31.00 %
Dip S: 44.39 %
Number
Channels (FL3A-FL3-Area)
siNUP153
NT
Diploid: 100.00 %
Dip G1: 52.12 %
Dip G2: 15.81 %
Dip S: 32.07 %
Number
Channels (FL3A-FL3-Area)
8h after phleo
Diploid: 100.00 %
Dip G1: 35.44 %
Dip G2: 26.48 %
Dip S: 38.08 %
Number
Channels (FL3A-FL3-Area)

Figure S2

A


siScramble
siNUP153
GFP-lacR
γH2AX
merge
-I-scel
+I-Scel

B


Colocalization of γ H2AX with the array (%)
100
90
80
70
60
50
40
30
20
10
0
siScramble
siNUP153
-I-SceI
+I-SceI

FigureS3

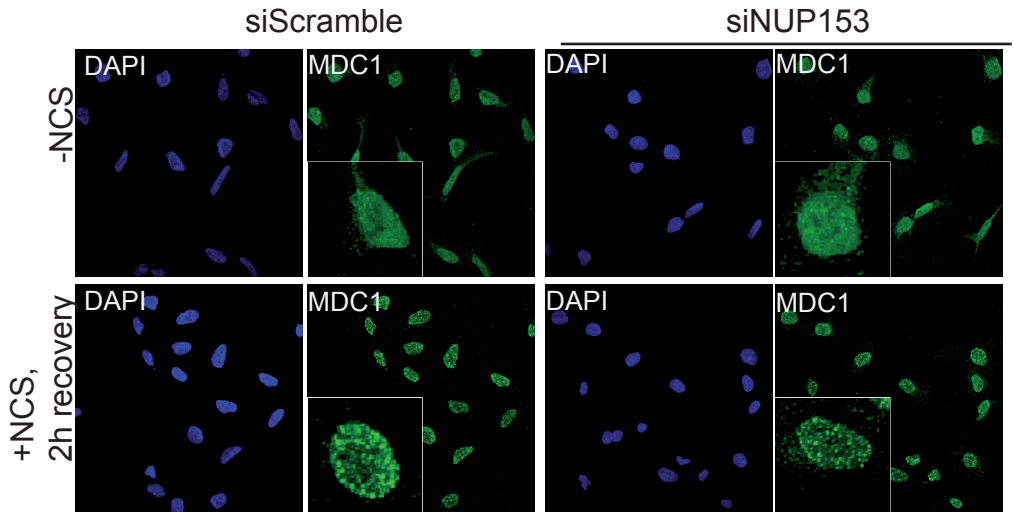

siScramble
siNUP153
-NCS
+NCS, 2h recovery
DAPI
MDC1
DAPI
MDC1
DAPI
MDC1
DAPI
MDC1

Figure S4

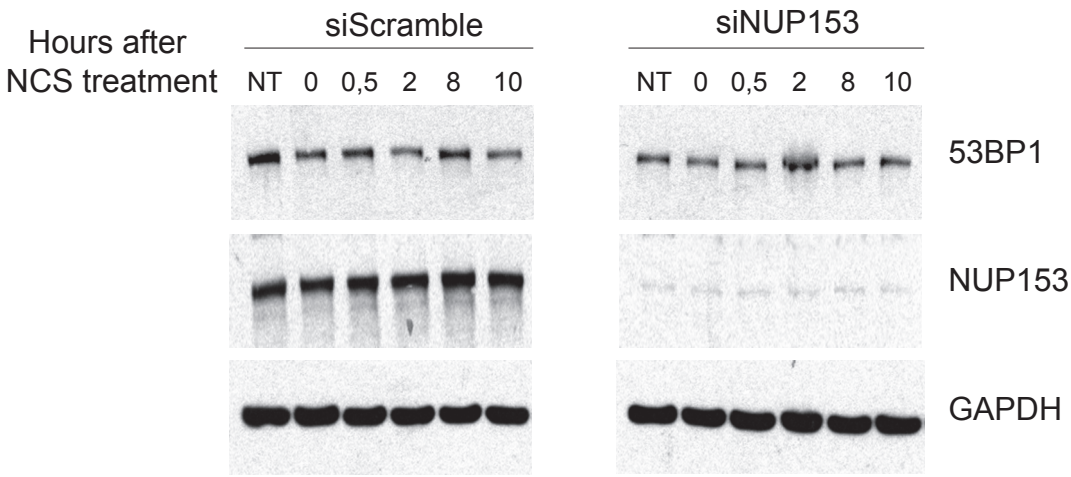
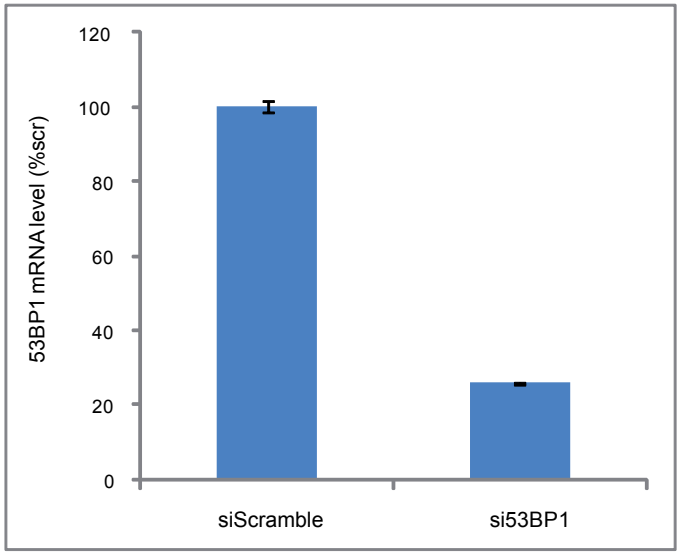

Hours after
NCS treatment
siScramble
siNUP153
NT 0 0,5 2 8 10
NT 0 0,5 2 8 10
53BP1
NUP153
GAPDH

Figure S5

A


120
100
80
60
40
20
0
53BP1 mRNA level (%scr)
siScramble
si53BP1

B

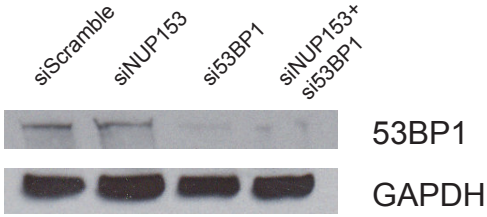

siScramble
siNUP153
si53BP1
siNUP153+
si53BP1
53BP1
GAPDH

Figure S6

Antibody for	Supplier	Reference	Use
ATM	Novus	NB100-104	WB
P-ATM	Cell Signalling	S1981	WB
53BP1	Novus	NB100-304	IF,WB
BRCA1	Calbiochem	OP92 and OP93	IF
CHK1	Abcam	ab47574	WB
P-CHK1	Cell Signalling	2348L	WB
CHK2	Upstate	05-649	WB
P-CHK2	Cell Signalling	2661	WB
GAPDH	Millipore	MAB374	WB
γH2AX	Abcam	ab22551	IF
MDC1	Sigma	MDC1-50	IF
NUP153	Abcam	ab24700-100	WB
p53	Santacruz	doi1	WB
P-p53	Cell Signalling	9284L	WB
RPA	NeoMarkers	MS-691-P1	IF

siRNA for	Supplier	Reference
NUP153	Dharmacon	D-005283-01-0005
Scramble	Dharmacon	D-001810-10-20
53BP1	Dharmacon	D-003548-04

FigureS7

2. Discussion and perspectives

My results identify the nucleoporin Nup153 as a new actor in the choice between NHEJ and HR. Although not being directly recruited at DSBs, it seems to play important roles in DDR and DSB repair. Indeed, its depletion leads to deregulation of the balance between NHEJ and HR, with an increased HR and a decreased NHEJ, defective checkpoints and decreased survival in presence of radiomimetic drugs.

Some of the effects observed can be attributed to a regulation of 53BP1 nuclear amount by Nup153. Indeed, Nup153 depletion leads to a loss of 53BP1 nuclear pool and its relocalization in the cytoplasm. Although the remaining amount of 53BP1 in the nucleus is sufficient to accumulate to a limited number of breaks, when confronted to a big number of breaks, 53BP1 quantity is not sufficient. The limited amount of 53BP1 in the nucleus could therefore explain the decreased survival and impaired checkpoint activation in Nup153 depleted cells. However, the effect on the deregulation of NHEJ-HR balance cannot be attributed only to 53BP1 mislocalization. Indeed, the effects observed upon Nup153 depletion are different than the one observed upon 53BP1 depletion: HR efficiency shows a greater increase in Nup153 depleted cells than in 53BP1 depleted cells and NHEJ fidelity, that is strongly affected in 53BP1 depleted cells is not affected in Nup153 depleted cells.

These results are a first step towards understanding the role of nucleoporins in the maintenance of genome stability in mammalian cells. However they raise several questions: what is the exact mechanism by which Nup153 controls the balance between NHEJ and HR? Do other nucleoporins have similar roles? What are the putative roles of Nup153 in cancer therapeutic approaches? I will discuss these questions below.

a) Mechanisms by which Nup153 regulates the balance between NHEJ and HR

- Regulation of 53BP1 localization

While we were conducting our study, another group identified Nup153 as a regulator of DNA repair (Moudry et al., 2012). They confirmed that 53BP1 nuclear localization depends on Nup153. They showed that the C-terminal part of Nup153 is

required for 53BP1 nuclear import, and that 53BP1 import depends on the Nup153-importin β interplay (Moudry et al., 2012).

However, our results indicate that regulation of 53BP1 nuclear import by Nup153 is not the only way Nup153 regulates DNA repair but that it might in fact regulate several DNA repair components. In yeast, Nup153 ortholog is involved in the regulation of SUMOylation during the response to DNA damage (Palancade et al., 2007). It would therefore be interesting to study the effect of Nup153 on SUMOylation.

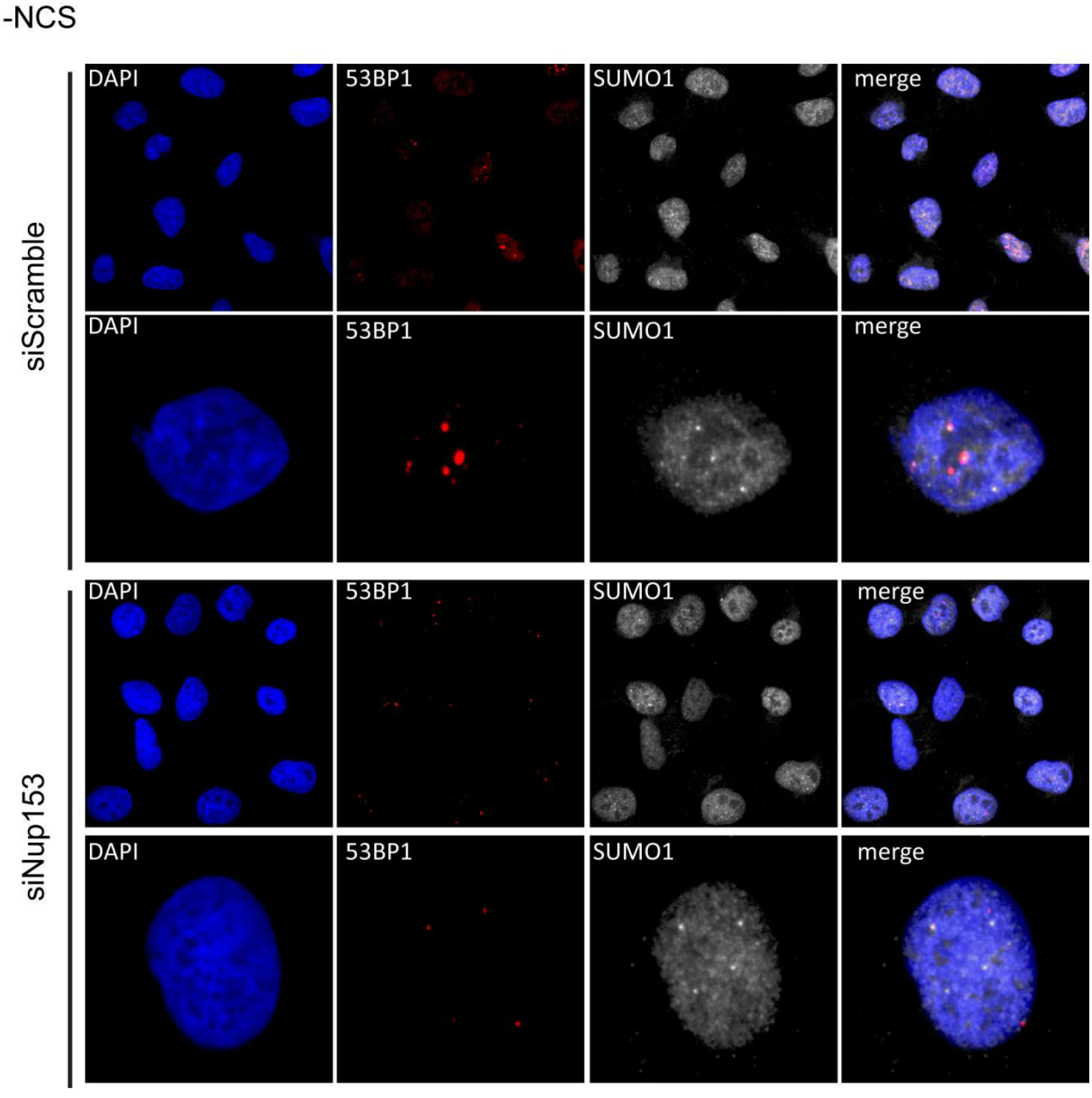
- Involvement of SUMO pathway

Nup60, the yeast ortholog of Nup153 is essential for proper DNA repair (Palancade et al., 2007). Its role is to ensure proper localization of the Ulp1 SUMO protease at the nuclear envelope. The proper localization of Ulp1 at the nuclear envelope is necessary for regulation of SUMOylation of various DSB repair proteins including yKu70 (Palancade et al., 2007).

Interestingly, Nup153 interacts with the SUMO proteases SENP1 and SENP2 (Chow et al., 2012) and allows their retention at the nuclear envelope. An appealing hypothesis is that Nup153 regulates DSB repair by a mechanism similar to the one used by Nup60 in yeast. Indeed, several DNA repair proteins are SUMOylated upon DSB induction, including BRCA1, 53BP1, Ku and BLM. Therefore, by retaining proper localization of SUMO proteases at the nuclear periphery, Nup153 might regulate the levels of SUMO modifications in the nucleoplasm. Interestingly SENP1 level is decreased upon Nup153 depletion (Chow et al., 2012). The observed decrease of SENP1 level might compensate for the loss of its attachement to the nuclear envelope (that would lead to an increased amount within the nucleoplasm) and constitute a protective mechanism against deregulation of SUMOylation levels in the nucleoplasm. However, SENP2 level is not decreased (Chow et al., 2012) and Nup153 depletion might therefore lead to mislocalization of SENP2 in the nucleoplasm and eventually lead to SUMOylation defects. We started testing this hypothesis and I'm

presenting our results below. Some of these results are very preliminary and their interpretation has to be done with caution.

To test whether NUP153 depletion leads to SUMOylation defects at DSBs, we treated U2OS cells with the radiomimetic drug NCS and observed SUMO1 pattern by immunofluorescence. In cells treated with a scrambled siRNA, SUMO1 was accumulating in discrete foci of strong intensity upon NCS treatment. On the contrary, cells treated with siNup153 did not show any SUMO1 foci accumulation upon NCS treatment, but rather displayed a cytoplasmic staining, suggesting a deregulation of the DSB-related SUMO pathway (figure 31).



+NCS, 2h release

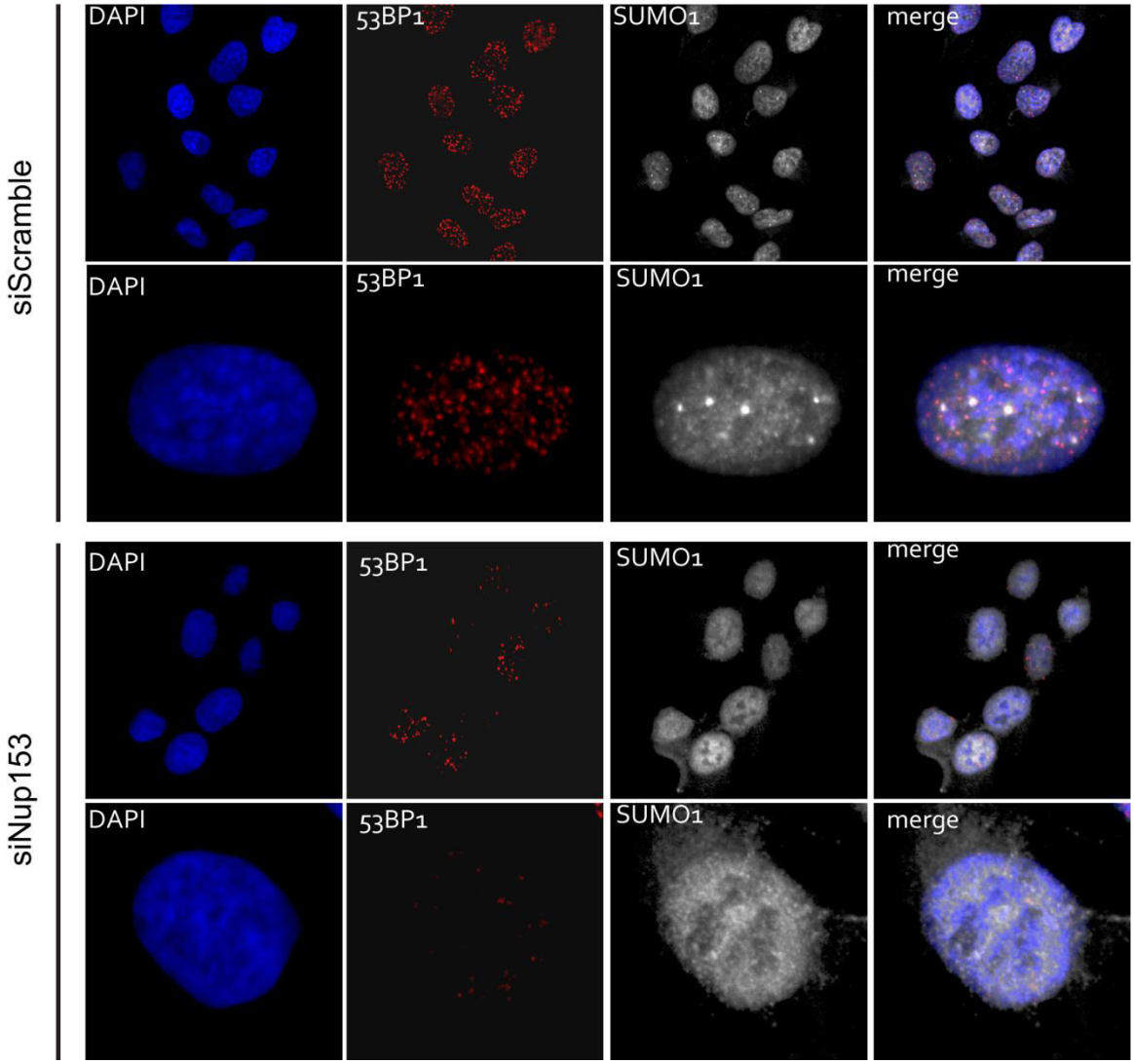


Figure 31- Nup153 promotes SUMO1 accumulation at NCS induced DSBs

Immunofluorescence analysis of 53BP1 (red) and SUMO1 (gray) in control cells and cells treated with siRNA targeting Nup153 at non treated (-NCS) condition or 2h after treatment with NCS (+NCS, 2h release)

We further analyzed the recruitment of SUMO1 at I-SceI induced DSBs in U2OS cells with stably integrated I-SceI restriction site flanked by lacO repeats. In comparison with control cells, cells treated with siNup153 displayed decreased recruitment of SUMO1 at the lacO array upon I-SceI expression (figure 32), therefore confirming that Nup153 depletion leads to SUMOylation deregulation at DSBs sites.

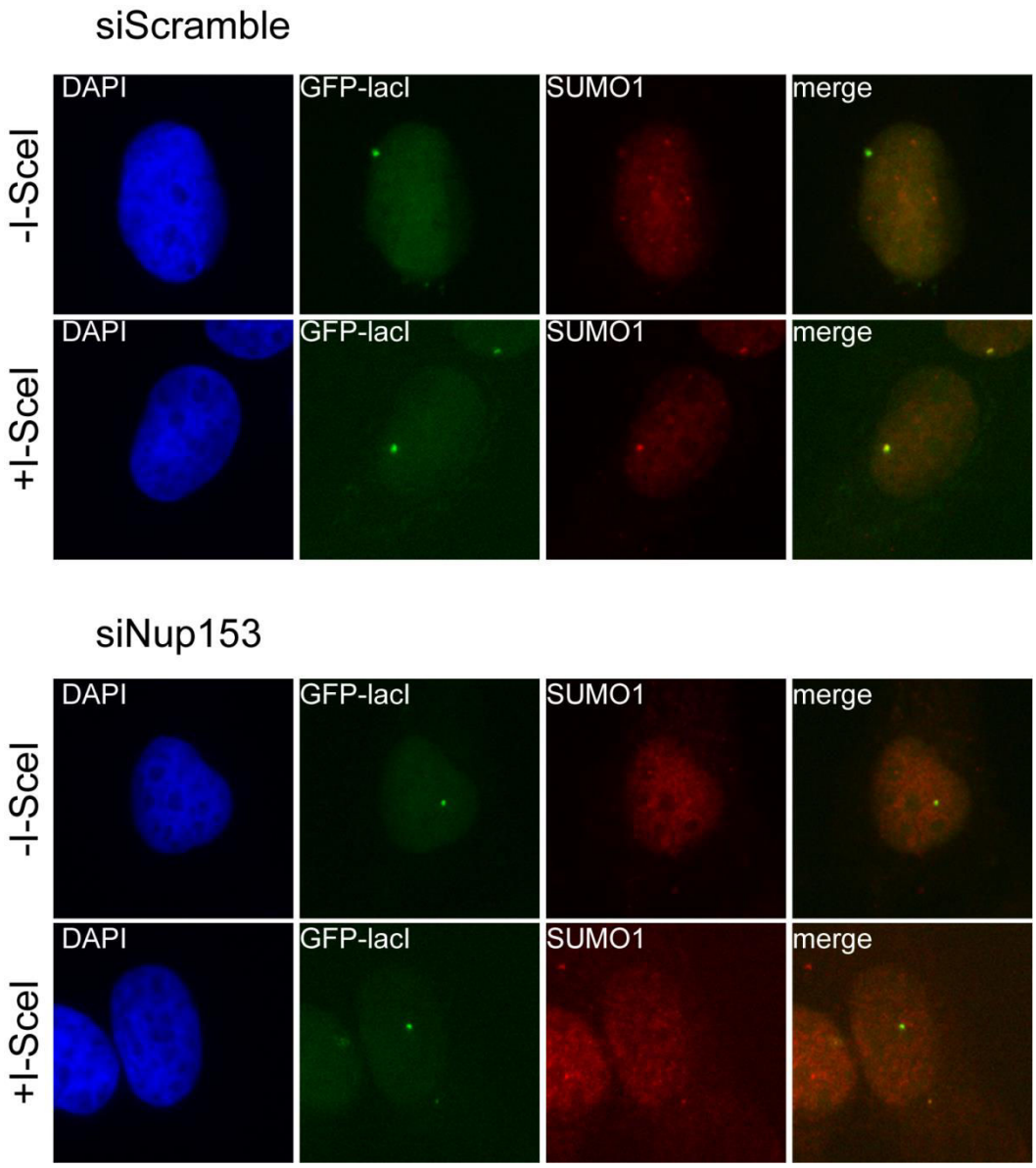


Figure 32- Nup153 promotes SUMO1 accumulation at I-SceI induced breaks

Upper panel: Immunofluorescence analysis of SUMO1 (red) in U2OS cells with stably integrated I-SceI restriction site flanked by lacO repeats and expressing GFP-lacI fusion protein (green). Cells were treated with indicated siRNAs.

Lower panel: Quantification of the colocalization of SUMO1 with the lacO array in three independent experiments.

To test whether the SUMOylation defect observed in Nup153 depleted cells is mediated by SENP2 mislocalization within the nucleoplasm we tested whether SENP2 depletion in Nup153 depleted cells rescues the NUP153 depletion effects. We performed a first survival assay in U2OS cells depleted for SENP2 or SENP2 and Nup153 in combination. This experiment was performed only once and needs repetition. However, in comparison with control cells, we observed a decreased survival in SENP2 depleted cells, whereas cells depleted for SENP2 and Nup153 had a similar survival rate than control cells (figure 33). This result suggests that a fine tuning of SENP2 quantity in the nucleoplasm is necessary for proper DNA repair and that Nup153 is involved in this regulation.

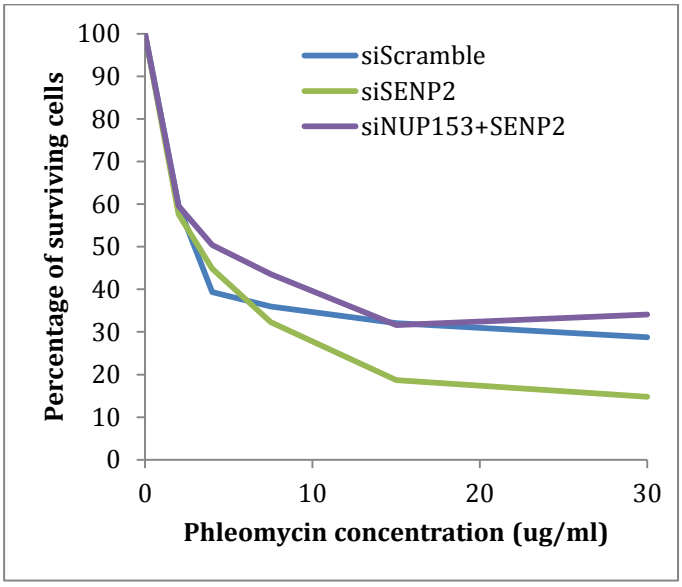


Figure 33- Co-depletion of Nup153 and SENP2 rescues survival defect

Clonogenic survival assay in U2OS cells treated with the indicated siRNAs, following exposure to increasing concentration of the radiomimetic drug phleomycin.

To test whether SENP2 is involved in the choice between HR and NHEJ, we performed a first HR assay in cells depleted for SENP2 or Nup153 and SENP2. Our preliminary results indicate that SENP2 depletion leads to a decreased HR efficiency (in contrary to Nup153 depletion) and that Nup153 depletion in SENP2 depleted cells rescues HR efficiency (figure 34).

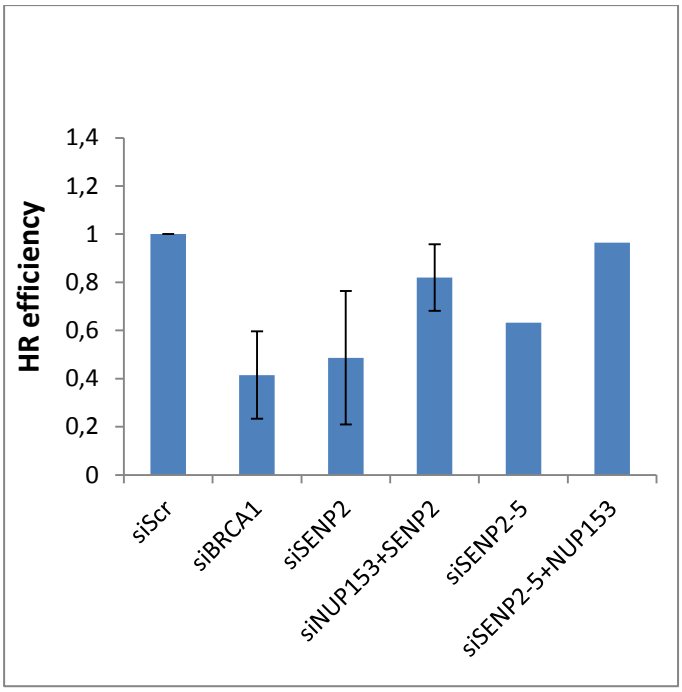


Figure 34- Co-depletion of Nup153 and SENP2 rescues HR defect

HR efficiency in U2OS cells transfected with the indicated siRNAs. The numbers represent a fold-increase of HR efficiency compared with the control.

Taken together these preliminary results indicate that Nup153 participates in the spatial organization of the SUMO machinery within the nucleus and in particular regulates the localization of the SENP2 SUMO protease. This regulation is necessary for proper DNA repair.

DNA damage triggers a wave of SUMOylation of multiple DNA repair proteins and was proposed to act as a glue to stabilize interactions between different proteins of the HR pathway in yeast (Psakhye and Jentsch, 2012). Further studies of the Nup153-SENP2 regulatory pathway in mammals would therefore be very interesting to dissect the SUMOylation response to DSBs in mammalian cells.

As perspectives, we would like to first confirm the results presented here, and characterize the role of SENP2 in DSB repair by performing several experiments in SENP2 depleted cells, including the study of checkpoints activation and NHEJ efficiency. We would like to assess the localization of SENP2 in Nup153 depleted cells and in presence of damage. Finally, we would like to identify the DNA repair SUMOylated proteins that are regulated by SENP2 and Nup153. In particular, we will test the SUMOylation level of the proteins that are known to be SUMOylated upon damage (BRCA1, 53BP1, Ku and BLM). We will confirm that they are indeed SUMOylated upon DNA damage and test whether this DNA damage-induced

SUMOylation is impaired in Nup153, SENP2 or Nup153 and SENP2 depletion conditions.

b) Roles of other nucleoporins in the maintenance of genome stability in mammals

To test whether other nucleoporins have a similar role than Nup153 in the regulation of DSB repair, we are currently performing an siRNA screen against the different nucleoporins in collaboration with the high-throughput screening facility of the IGBMC, using a library of 58 nuclear pore-related proteins. Cells depleted for the different nucleoporins siRNAs are treated with NCS and stained for 53BP1 two hours after treatment (figure 35). Images are acquired with the InCELL1000 analyzer microscope and 53BP1 foci formation is analyzed with the Multi Target Analyzer from GE Healthcare. In these conditions cells depleted for Nup153 show decreased 53BP1 foci formation. Preliminary results indicate that only two nucleoporins (Nup107 and Nup205) display a similar phenotype than Nup153. This result indicates that the phenotypes observed upon Nup153 depletion are specific to this nucleoporin and is not a general effect of NPCs depletion.

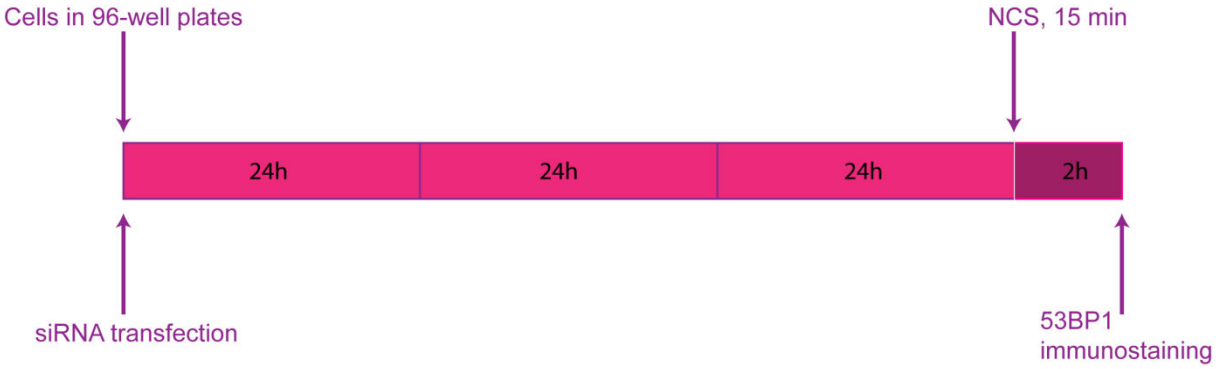


Figure 35- SiRNA screen

U2OS cells are transfected with siRNAs in 96 wells plate, treated with NCS for 15 min, released for 2h and immunostained for 53BP1.

c) Putative roles of Nup153 in cancer therapeutic approaches

Nup153 was recently identified as a gene amplified in a pancreatic cancer cell line (Shain et al., 2013). Furthermore, the composition of NPCs seems to be variable between different cell types (Raices and D'Angelo, 2012) and changes in nuclear pore

composition might even be involved in the differentiation process (D'Angelo et al., 2012). These results suggest that Nup153 levels might vary depending on cell types or cellular context.

In an effort to specifically target cancer cells with higher efficiency, personalized therapeutic approaches are currently under development. These strategies consist in the identification of specific genetic markers in the tumour of the patient to personally adapt its treatment. A classical way to eliminate cancer cells is to induce DNA lesions that will not be repaired and will ultimately kill the cancerous cells. One of the challenges of personalized therapy is to identify differences between cancer cells and normal cells in order to specifically target the cancer cells with minimal side effects. Given the dramatic change in the balance between HR and NHEJ observed upon Nup153 depletion, determination of Nup153 expression level would be interesting to choose drugs that specifically inhibit HR or NHEJ pathways during chemotherapy.

Additionally, Nup153 was recently shown to be required for cell migration in tumor cells (Zhou and Panté, 2010). Therefore, cancer therapies would probably benefit from concerted research on Nup153 expression in cancer cells on one hand and further studies of Nup153 role in DSB repair on the other hand.

II. Nuclear position dictates DNA repair pathway choice

1. Research article

Nuclear position dictates DNA repair pathway choice

Charlène Lemaître, Anastazja Grabarz, Katerina Tsouroula, et al.

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Nuclear position dictates DNA repair pathway choice

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Faithful DNA repair is essential to avoid chromosomal rearrangements and promote genome integrity. Nuclear organization has emerged as a key parameter in the formation of chromosomal translocations, yet little is known as to whether DNA repair can efficiently occur throughout the nucleus and whether it is affected by the location of the lesion. Here, we induce DNA double-strand breaks (DSBs) at different nuclear compartments and follow their fate. We demonstrate that DSBs induced at the nuclear membrane (but not at nuclear pores or nuclear interior) fail to rapidly activate the DNA damage response (DDR) and repair by homologous recombination (HR). Real-time and superresolution imaging reveal that DNA DSBs within lamina-associated domains do not migrate to more permissive environments for HR, like the nuclear pores or the nuclear interior, but instead are repaired in situ by alternative end-joining. Our results are consistent with a model in which nuclear position dictates the choice of DNA repair pathway, thus revealing a new level of regulation in DSB repair controlled by spatial organization of DNA within the nucleus.

[*Keywords*: alternative end-joining; DNA repair; nuclear lamina; nuclear organization]

Supplemental material is available for this article.

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Cells continuously experience stress and damage from exogenous sources, such as UV light or irradiation, and endogenous sources, such as oxidative by-products of cellular metabolism (Jackson and Bartek 2009). To avoid subsequent genomic instability, several pathways evolved to detect DNA damage, signal its presence, and mediate its repair (Misteli and Soutoglou 2009). The two main pathways for double-strand break (DSB) repair are homologous recombination (HR) and nonhomologous end-joining (NHEJ) (Chapman et al. 2012).

DNA repair occurs in the highly compartmentalized nucleus, and emerging evidence suggests an important role of nuclear organization in the maintenance of genome integrity (Misteli and Soutoglou 2009). Observations in yeast suggest that distinct, dedicated DNA repair centers exist as preferential sites of repair (Lisby et al. 2003). Further evidence for spatially restricted repair in yeast comes from the observation that persistent DSBs migrate from their internal nuclear positions to the nuclear periphery, where they associate with nuclear pores (Therizols et al. 2006; Nagai et al. 2008; Oza et al. 2009). In mammalian cells, multiple DSBs on several chromosomes are repaired individually and do not meet on shared repair centers or move toward the nuclear periphery (Soutoglou et al. 2007). In line with these observations, spatial proximity of DSBs in the nucleus is a key parameter that affects the frequency of formation of chromosomal translocations in mammals (Roukos et al. 2013; Roukos and Misteli 2014). Therefore, in mammals, although nuclear organization has emerged as a key parameter in the formation of chromosomal translocations (for review, see Roukos and Misteli 2014), very little is known about how nuclear compartmentalization contributes to genome stability and whether DNA repair occurs throughout the nucleus with the same robustness and accuracy.

[7]**These two authors contributed equally to this work.**
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Here, we used an inducible system to create temporally and spatially defined DSBs in chromatin within different nuclear compartments and followed their fate. We show that the presence of heterochromatin at the nuclear lamina delays DNA damage response (DDR) and impairs HR. We further used live-cell imaging and superresolution microscopy to probe the spatial dynamics of these DSBs. We show that, contrary to what was observed in yeast, DNA DSBs within lamina-associated domains (LADs) do not migrate to more permissive environments for HR, like the nuclear pores or the nuclear interior. Instead, they are repaired in situ by NHEJ or alternative end-joining (A-EJ). Our data reveal a new level of regulation in DSB repair pathway choice controlled by spatial organization of DNA in the nucleus.

Results

To investigate the impact of nuclear compartmentalization on DNA repair, we induced DSBs in chromatin associated with the inner nuclear membrane and then tested the consequences of nuclear position in DDR kinetics and DNA repair efficiency. We generated I-U2OS19 cells that contain a stably integrated I-SceI restriction site flanked by 256 repeats of the lac operator DNA sequences (lacO) (Supplemental Fig. S1A). This cell line was also engineered to express the I-SceI endonuclease under the control of a doxycycline (Dox)-inducible promoter (pTRE-tight), allowing us to temporally control the induction of a DSB at the lacO/I-SceI locus (Supplemental Fig. S1A). Stable expression of the GFP lac repressor (lacI) enables the visualization of the lacO/I-SceI locus in the nucleus. We induced specific tethering of the lacO locus at the inner nuclear membrane by the expression of an Emerin C-terminal deletion (ΔEMD), which localizes at the nuclear lamina, fused to GFP-lacI (GFP-lacI-ΔEMD) (Supplemental Fig. S1A) as described in Reddy et al. (2008).

Consistent with previous results (Reddy et al. 2008), ΔEMD is sufficient to target the GFP-lacI-ΔEMD fusion protein to the nuclear membrane and relocate the lacO/I-SceI-containing chromosome at the nuclear lamina after one mitotic cycle (Supplemental Fig. S1B,C). Indeed, in cells expressing GFP-lacI-ΔEMD, we observed 70% of colocalization of the lacO array with laminB by immuno-FISH in the absence or presence of I-SceI, whereas in cells expressing GFP-lacI, this colocalization is as low as 10% (Supplemental Fig. S1B,C).

To determine whether tethering of the lacO/I-SceI locus to the nuclear lamina has an effect on the accessibility of the I-SceI endonuclease, we performed ligation-mediated PCR (LM-PCR) in cells expressing GFP-lacI or GFP-lacI-ΔEMD. We found that the cutting efficiency is equivalent in both environments (Supplemental Fig. S1D), demonstrating that the I-SceI endonuclease is able to recognize its target sequence and cleave its substrate regardless of its nuclear localization.

DSBs activate the DDR, which allows recognition of breaks and the activation of checkpoints. Consequently, cell cycle progression is paused, which allows time for the cell to repair the lesions before dividing (Misteli and Soutoglou 2009). DDR involves a megabase-wide spreading of a phosphorylated form of the histone variant H2AX (γ-H2AX) around them (Rogakou et al. 1998; Misteli and Soutoglou 2009).

To assess the impact of repositioning the lacO/I-SceI locus at the nuclear lamina compartment on DDR efficiency, we compared the kinetics of induction of γ-H2AX at the I-SceI break in cells expressing GFP-lacI or GFP-lacI-ΔEMD by immuno-FISH. Although repositioning of the lacO/I-SceI break at the nuclear lamina did not affect the maximal percentage of γ-H2AX, cells expressing GFP-lacI showed the highest percentage of γ-H2AX colocalization with the lacO/I-sceI locus 14 h after Dox addition, whereas GFP-lacI-ΔEMD cells only achieved the same level 24 h after Dox was added (Fig. 1A, B). This observation was further confirmed by chromatin immunoprecipitation (ChIP) experiments (Fig. 1C). We also investigated the recruitment of another DDR factor, 53BP1, which has been implicated in the choice of the DSB repair pathway (Bunting et al. 2010; Panier and Boulton 2014). Similarly to γ-H2AX, the recruitment of 53BP1 was also delayed and showed a maximal accumulation at 24 h after I-SceI expression in GFP-lacI-ΔEMD cells compared with 20 h in GFP-lacI cells (Fig. 1D,E). A similar difference was observed in a lacO/I-SceI system integrated in the I-Hela111 cell line (Supplemental Fig. S2A,B), suggesting that the effect is not tissue-specific but rather is a general mechanism. Taken together, these results reveal a general delay in DDR in lesions occurring in chromatin associated with the nuclear lamina and suggest that this compartment is a repressive microenvironment for DDR.

To rule out the possibility that this defect was due to the expression of the ΔEMD in the context of the GFP-lacI-ΔEMD fusion protein, we performed an immuno-FISH experiment in the presence of IPTG. Under these conditions, the GFP-lacI-ΔEMD fusion protein is expressed but does not bind to the lacO array, and the array is not relocalized at the nuclear lamina, which was confirmed by the markedly reduced colocalization of the array and laminB (Supplemental Fig. S3A–C). As shown in Supplemental Figure S3B and quantified in Supplemental Figure S3D, there was no difference in the degree of γ-H2AX at the I-SceI break in cells expressing either GFP-lacI or GFP-lacI-ΔEMD in the presence of IPTG and 14 h after Dox where there was the maximal difference in DDR between the two compartments (Fig. 1B), confirming that the decreased phosphorylation of H2AX is a consequence of a lesion induced at the nuclear lamina.

In light of the above observations, we investigated whether the delay in DDR at the I-SceI lesion at the nuclear membrane impacts on its repair. To evaluate the effect of the I-SceI break repositioning at the inner nuclear membrane on NHEJ, we compared the degree of colocalization of Ku80 (Britton et al. 2013) with the lacO/I-SceI array by immuno-FISH and the recruitment of XRCC4 by ChIP in cells expressing GFP-lacI and GFP-lacI-ΔEMD, two main proteins of the NHEJ pathway (Lieber 2010). We observed no difference in the recruitment of KU80 in I-U2OS19 (Fig. 2A; Supplemental Fig. S4A) and I-Hela111 (Supplemental

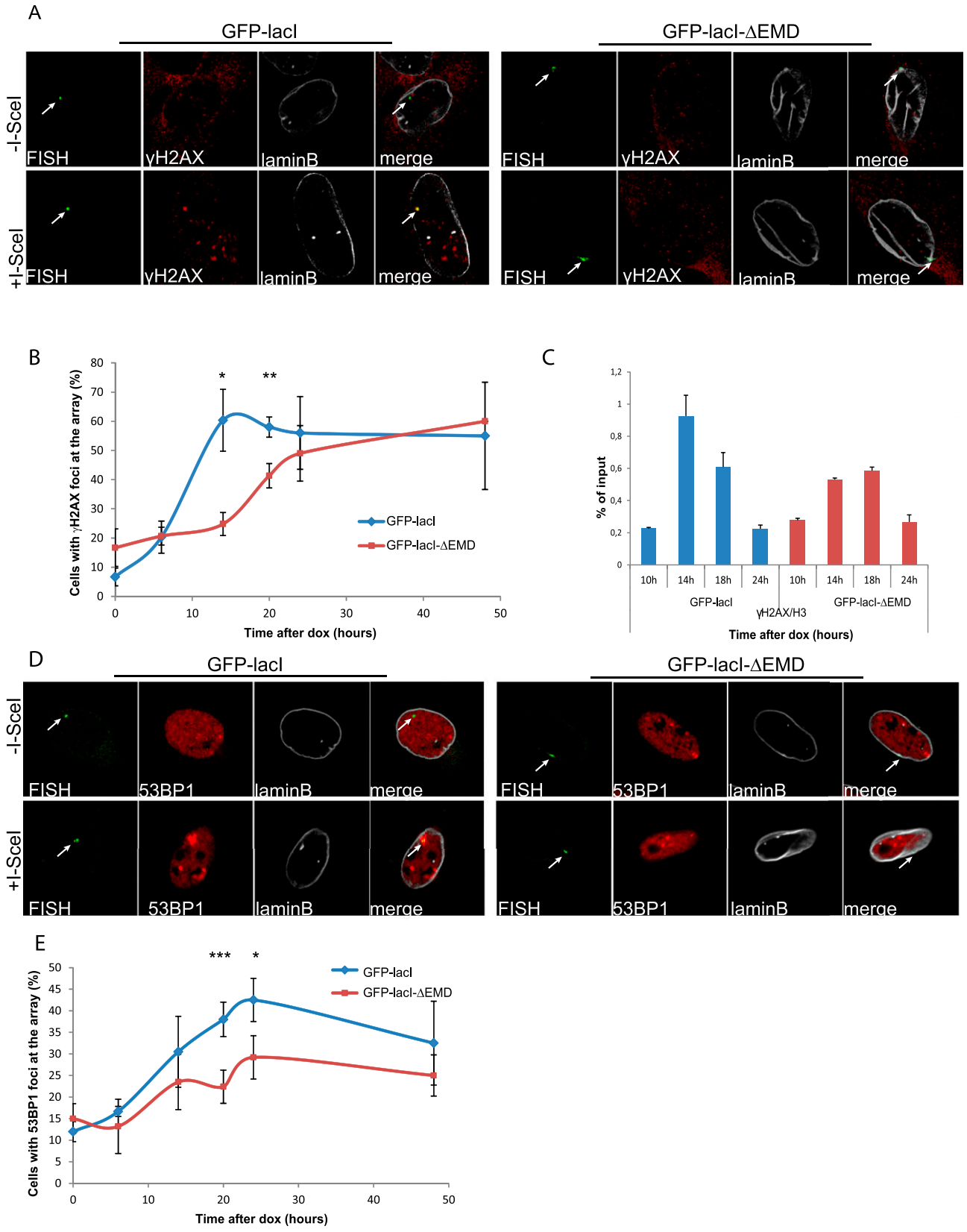

Figure 1. The DDR is delayed at the nuclear lamina. (*A*) Immuno-FISH single-Z confocal images of the lacO array (green), γ-H2AX (red), and laminB (gray) in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD and treated or not with Dox for 14 h. (*B*) Time course of the percentage of colocalization of the lacO array with γ-H2AX. (*C*) γ-H2AX ChIP at the indicated time points after Dox addition in cells expressing GFP-lacI or GFP-lacI-ΔEMD. Values were normalized to input DNA and H3 ChIP and are representative of three independent experiments. (*D*) Immuno-FISH single-Z confocal images of the lacO array (green), 53BP1 (red), and laminB (gray) in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD and treated or not with Dox for 20 h. (*E*) 53BP1 after Dox addition in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD. Values represent mean ± SD of three independent experiments with $n > 50$ cells. For statistical analysis, a *t*-test was performed. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$. In all figures, the arrow depicts the position of the lacO array.

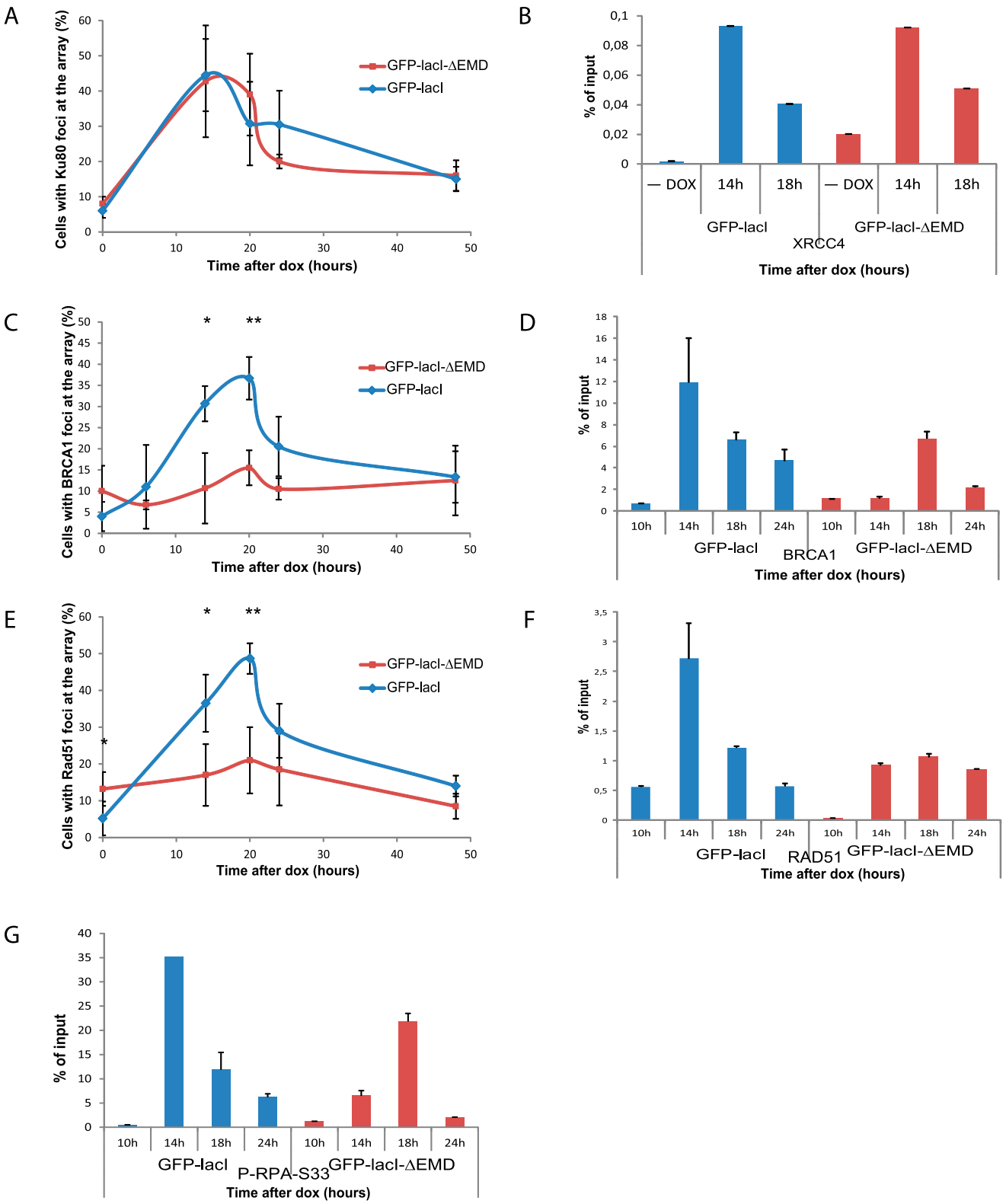

Figure 2. Recruitment of HR factors is impaired at the nuclear lamina. (*A*) Time course of the percentage of colocalization of the lacO array with Ku80 after Dox addition in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD. Values represent mean ± SD of three independent experiments with $n > 50$ cells. ChIP for XRRC4 (*B*), BRCA1 (*D*), RAD51 (*F*), or P-RPAS33 (*G*) at the indicated times upon Dox addition in I-Hela111 cells (XRCC4) or I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD is shown. Values were normalized to input DNA and are representative of three independent experiments. The percentage of colocalization of the lacO array with BRCA1 (*C*) and Rad51 (*E*) at the indicated times after Dox addition in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD is shown. Values represent mean ± SD of three independent experiments with $n > 50$ cells. For statistical analysis, a *t*-test was performed. (*) $P < 0.05$; (**) $P < 0.01$.

Fig. S5A–D) cells or XRCC4 at I-Hela111 (Fig. 2B) at the I-SceI break induced at the nuclear lamina compared with the nuclear interior, suggesting that NHEJ can occur efficiently in both compartments. Interestingly, the recruitment of NHEJ factors was not delayed, which is indicative of an uncoupling of DDR and repair by NHEJ.

HR is mainly active during the S phase of the cell cycle and uses the homologous sister chromatid as a template for error-free repair (San Filippo et al. 2008). Contrary to what was observed for NHEJ proteins, the recruitment of HR factors such as BRCA1, Rad51 (Fig. 2C–F; Supplemental Figs. S4B,C, S5B,C,E,F), and Rad54 (Supplemental Fig. S6A) at the broken lacO residing at the inner nuclear membrane was markedly decreased. Interestingly, the phosphorylation of RPA was delayed and less robust but not entirely abolished, suggesting a semifunctional resection pathway (Fig. 2G) and a more dramatic effect specific to late HR factors. To verify that this difference was not due to an impaired cell cycle progression in the cells expressing GFP-lacI-ΔEMD, we compared the cell cycle profiles of the two cell lines by flow cytometry and observed no difference (Supplemental Fig. S6B). Our results suggest that the nuclear lamina is a repressive environment for HR.

In the mammalian nucleus, chromatin is organized into structural domains by association with distinct nuclear compartments (Parada and Misteli 2002; Bickmore 2013). To gain insight into the cause of the DDR delay and HR repression promoted by the nuclear lamina environment, we considered the possibility that the repressive chromatin structure associated with the nuclear lamina (Padeken and Heun 2014) is involved in this phenomenon (Goodarzi and Jeggo 2012; Lemaître and Soutoglou 2014).

To test this hypothesis, we treated cells with an inhibitor of histone deacetylases, trichostatin A (TSA). This treatment resulted in an increase in histone acetylation (Supplemental Fig. S7A) and loss of heterochromatin in the nucleus, including perinuclear heterochromatin, leading to a homogenous chromatin state, as visualized by electron microscopy (Supplemental Fig. S7B–D). TSA treatment did not perturb the repositioning of the lacO/I-SceI locus at the inner nuclear membrane (Supplemental Fig. S7E,F). Interestingly, TSA treatment rescued the defect in γ-H2AX and recruitment of BRCA1 and RAD51 observed after the lacO locus relocalization at the inner nuclear membrane, pointing to an inhibitory role of chromatin compaction in DDR and HR (Fig. 3A–C; Supplemental Figs. S8, S9A,B). Our results are in line with previous studies that showed that reduced gene expression around the nuclear periphery after repositioning of the lacO array depends on the activity of histone deacetylases (Finlan et al. 2008).

To further confirm that the perinuclear heterochromatin in contact with the nuclear membrane is responsible for delayed DDR and repressed HR, we induced decondensation of the lacO/I-SceI chromatin by direct tethering of the chromatin remodeler BRG1. To this end, we expressed cherry-lacI-BRG1 in cells expressing GFP-lacI or GFP-lacI-ΔEMD (Supplemental Fig. S10A). As shown in Supplemental Figure S10B and quantified in Supplemental Figure S10C, tethering of BRG1 at the lacO array resulted in local chromatin decondensation, as visualized by an increased size of the array.

Similar to what we observed after global chromatin decondensation, local chromatin opening by BRG1 rescued the defect in γ-H2AX and the recruitment of BRCA1 and RAD51 upon lacO repositioning at the lamina (Fig. 3D–G; Supplemental Fig. S11A,B). Altogether, these results strongly suggest that the decreased recruitment of HR factors at the nuclear lamina is due to the highly compacted state of the surrounding chromatin.

To further examine whether the localization of a DSB within a nuclear compartment in relation to the state of the chromatin that surrounds the compartment can influence the DNA repair pathway choice, we assessed DSB repair at the nuclear pores, which are subcompartments of the nuclear periphery that represent a permissive environment for gene expression and other DNA-dependent nuclear transactions (Taddei et al. 2006; Ptak et al. 2014). To position the lacO/I-SceI locus at the nuclear pore compartment, we expressed GFP-lacI fused to the nucleoporin Pom121 (Supplemental Fig. S12A). We found that repositioning of the lacO array to the nuclear pores did not affect DDR, as visualized by H2AX phosphorylation and 53BP1 recruitment (Fig. 4A–C; Supplemental Fig. S12B). Furthermore, the recruitment of HR factors was similar in cells expressing GFP-lacI and GFP-lacI-Pom121 (Fig. 4D,E; Supplemental Fig. S12C,D). These observations suggest that in contrast to the nuclear lamina, nuclear pores represent a permissive microenvironment for DDR and DSB repair by HR. Therefore, although the nuclear lamina and nuclear pores are in very close proximity in the nuclear periphery, the difference in chromatin compaction associated with the two compartments regulates the choice of the repair pathway that will be prevalent in lesions occurring in each compartment.

It was previously shown that breaks inflicted at pericentric heterochromatin in *Drosophila* migrate at the periphery of the heterochromatin domain for HR repair in order to avoid recombination between repetitive sequences (Chiolo et al. 2011). Given that tethering of the lacO/I-SceI locus at the nuclear membrane using the GFP-lacI-ΔEMD might limit its potential mobility toward activating environments for DDR and repair, such as the nucleoplasm or the nuclear pores, we asked whether the lacO/I-SceI locus acquires mobility after break induction in the presence of IPTG when the lacI is not bound to the lacO array and cannot constrain its movement (Supplemental Fig. S13A). Surprisingly, we did not detect any migration of I-SceI breaks away from the compartment (Supplemental Fig. S13B).

To further investigate whether breaks occurring at the lamina migrate away from the lamina compartment toward the adjacent pores or the interior of the nucleus, we used an experimental system previously developed to visualize chromatin domains associated with laminB in single cells (Kind et al. 2013). This system uses DNA adenine methylation as a tag to visualize and track LADs

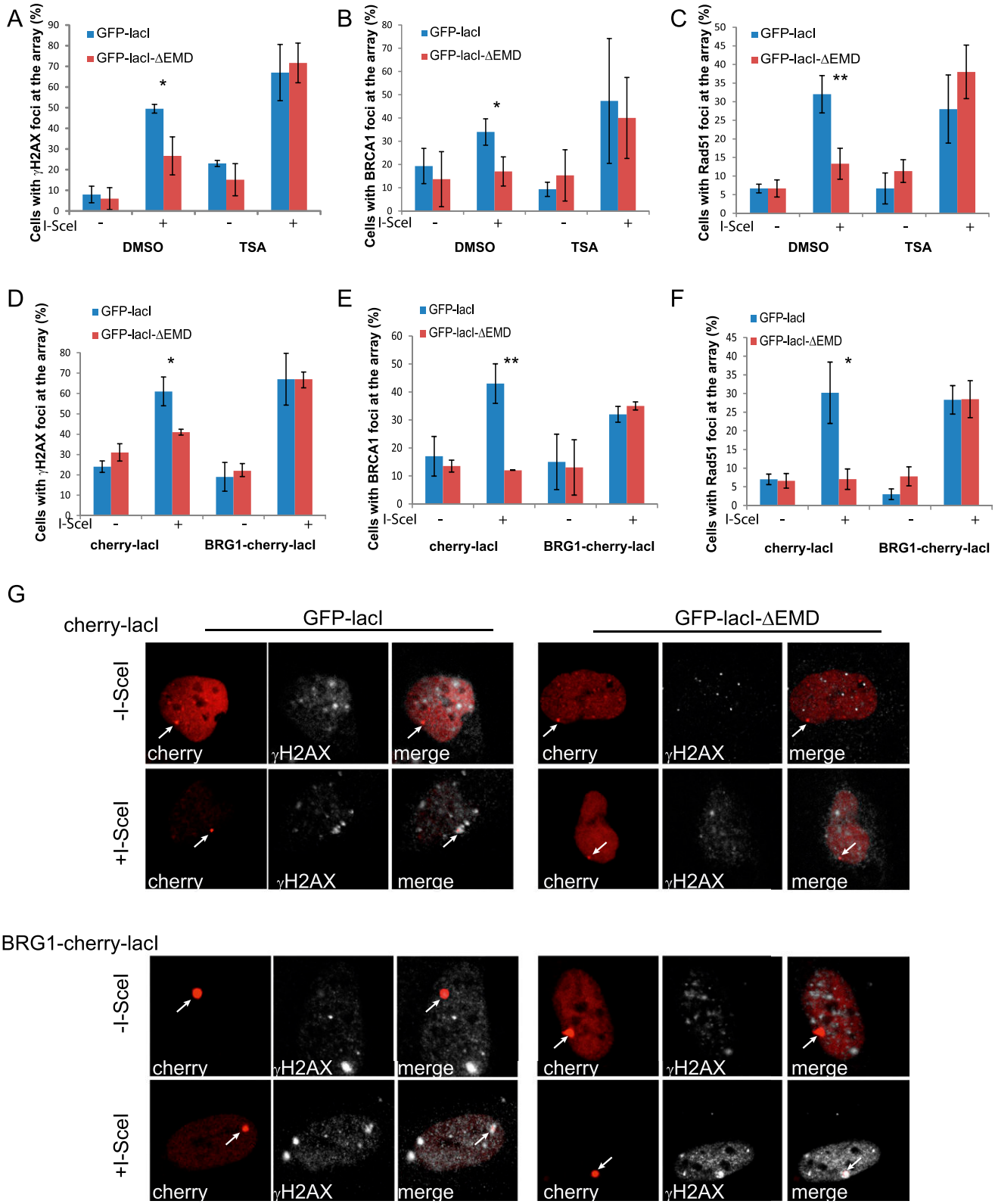

Figure 3. Chromatin decompaction restores DDR and the recruitment of HR factors at the nuclear lamina. Colocalization of the lacO array with γ-H2AX (*A*), BRCA1 (*B*), or RAD51 (*C*) in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD and pretreated for 4 h with DMSO or TSA in the absence or presence of Dox for 14 h or 20 h is shown. The percentage of colocalization of the lacO array with γ-H2AX (*D*), BRCA1 (*E*), or RAD51 (*F*) in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD and cherry-LacI or BRG1-cherry-lacI and treated or not with Dox for 14 h or 20 h is shown. (*G*) Immunofluorescence single-Z confocal images of γ-H2AX (gray) in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD transfected with cherry-lacI or BRG1-cherry-lacI (red) and treated or not with Dox for 14 h. For statistical analysis, a *t*-test was performed. (*) $P < 0.05$; (**) $P < 0.01$.

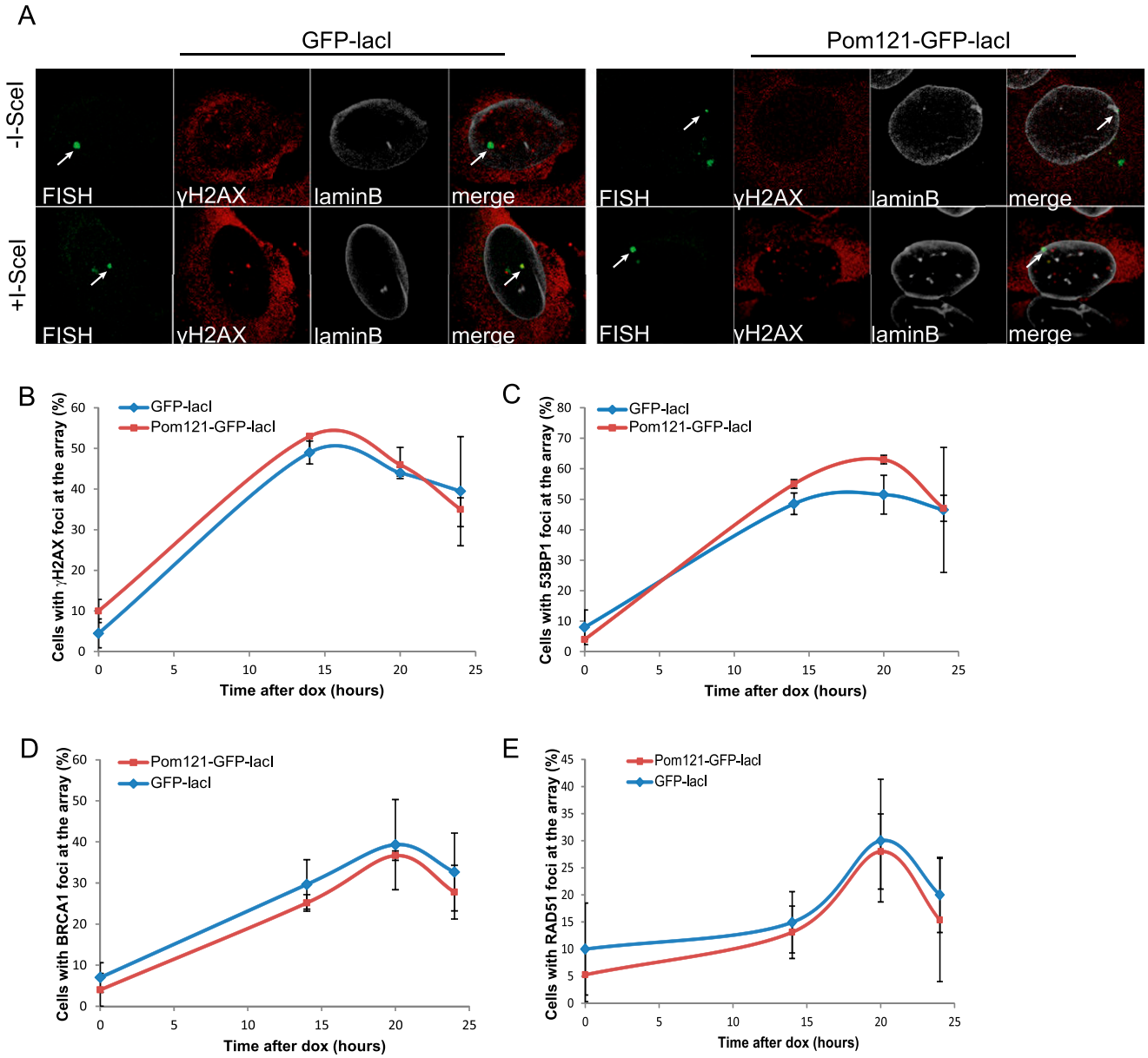

Figure 4. DDR and HR are not affected by tethering at the nuclear pores. (*A*) Immuno-FISH single-Z confocal images of the lacO array (green), γ-H2AX (red), and laminB (gray) in I-U2OS19 cells expressing GFP-lacI or Pom121-GFP-lacI and treated or not with Dox for 14 h. Time course of the percentage of colocalization of the lacO array with γ-H2AX (*B*), 53BP1 (*C*), BRCA1 (*D*), or RAD51 (*E*) in I-U2OS19 cells expressing GFP-lacI or Pom121-GFP-lacI cells after Dox addition is shown. Values represent mean ± SD of three independent experiments with $n > 50$ cells.

using a truncated version of the DpnI enzyme fused to GFP (m6a-Tracer), which recognizes methylated LADs in cells expressing LaminB-Dam (Kind et al. 2013). To probe the behavior of LADs in the presence of DNA damage, we followed the m6a-Tracer localization using live-cell imaging (Supplemental Fig. S13C) or confocal (Fig. 5A,B) or superresolution (Fig. 5C) microscopy. The infliction of DNA damage in the LADs was verified by γ-H2AX (Fig. 5A; Supplemental Fig. S13D). Interestingly, the partition of the LADs between the nuclear membrane and the nucleoplasm did not notably change before and after global DNA damage (Fig. 5A–C; Supplemental Fig. S13C), suggesting that DNA lesions do not lead to massive rearrangements of LADs within the nucleus.

In yeast, persistent DSBs migrate from their internal nuclear positions to the nuclear periphery, where they associate with nuclear pores (Therizols et al. 2006; Nagai et al. 2008; Oza et al. 2009). To more precisely assess the spatial proximity of LADs with laminB and nucleoporin of the nuclear basket TPR before and after DNA damage, we used two-color dSTORM superresolution microscopy (Folling et al. 2008). As expected, we observed juxtaposition and a certain degree of colocalization of LADs with LaminB but not with TPR

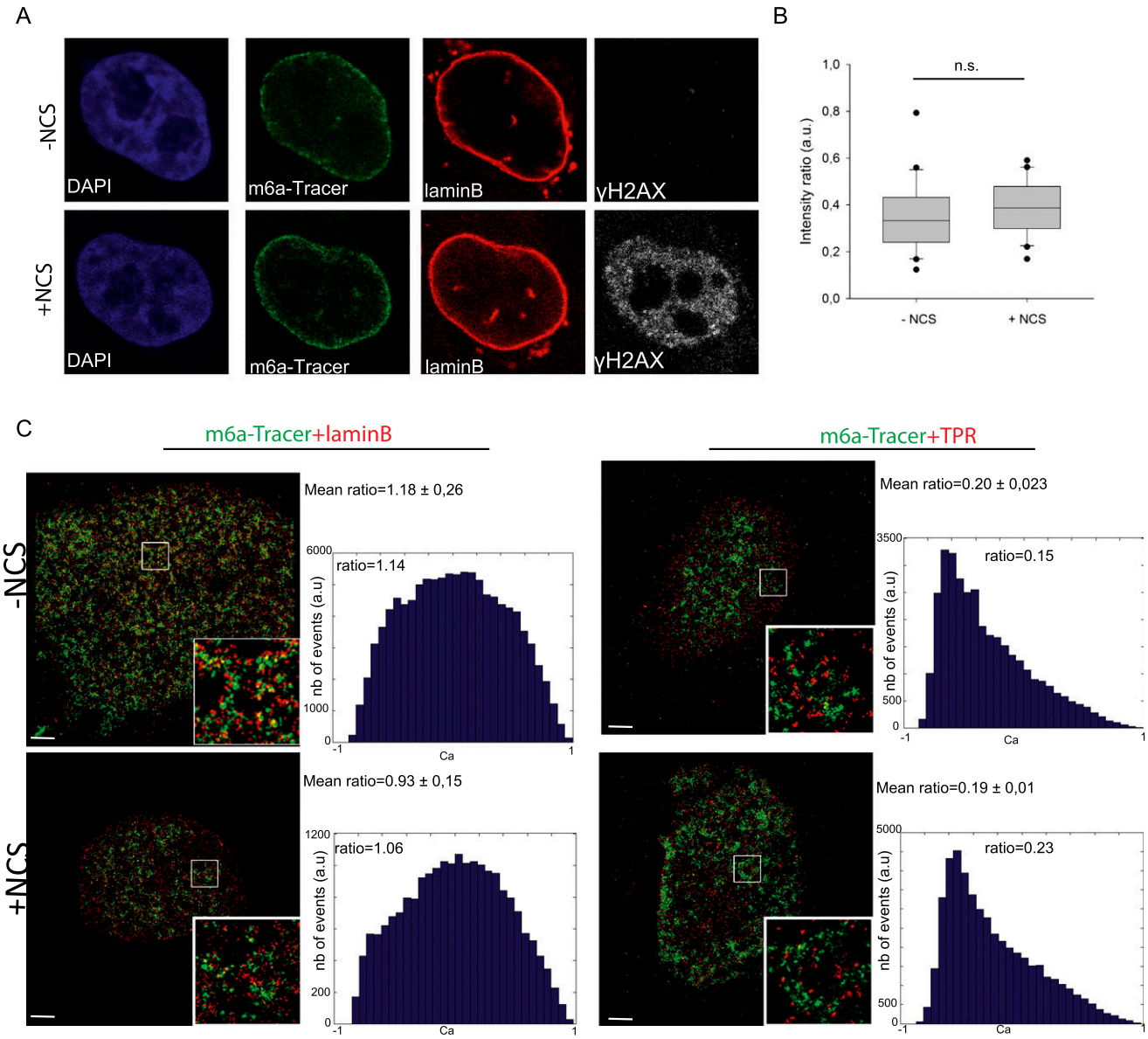

Figure 5. DSBs at the nuclear lamina are positionally stable. (*A*) Immunofluorescence of HT1080 cells expressing Dam-LaminB1 and m6A-Tracer 2 h after treatment (or not) with 50 ng/mL neocarzinostatin (NCS) for 15 min. (*B*) Box plot of GFP intensity ratios of the signal in the nucleoplasm versus the signal at the nuclear envelope in a HT1080-derived clonal cell line expressing a Dam-LaminB1 and the m6A-Tracer. The number of cells analyzed per condition was 20. For statistical analysis, χ^2 tests were performed. (n.s.) Nonsignificant. (*C*) dSTORM microscopy images of LADs (green) and laminB (*left* panel; red) or TPR (*right* panel; red) in the absence (*top* panel) or presence (*bottom* panel) of DNA damage (100 ng/mL NCS for 15 min and released for 2 h) in HT1080 cells expressing Dam-LaminB1 and m6A-Tracer. Images were taken from the bottom of the cells to allow better resolution of nuclear pores. Corresponding colocalization and the ratio of positive over negative colocalization events are displayed at the *right*. The mean ratios for all nuclei analyzed ($n \geq 8$) are displayed *above*.

(Fig. 5A). Interestingly, DNA damage did not induce changes in the proximity of LADs toward both compartments, which further pointed to the positional stability of LADs upon DNA damage (Fig. 5A). Taken together, these results suggest that contrary to what has been shown in yeast, breaks occurring on chromosomes that associate with the nuclear membrane do not travel and seek an environment permissive to HR repair, such as the nuclear pores.

To further investigate the contribution of NHEJ and HR in repairing the I-SceI breaks at the lamina or the nuclear interior, we assessed the degree of persistent breaks in GFP-lacI or GFP-lacI-ΔEMD cells depleted of XRCC4 and RAD51 (knockdown efficiencies verified in Supplemental Fig. S14A). Interestingly, in control cells, breaks were efficiently repaired in both nuclear compartments, which was exemplified by the decrease in γ-H2AX signal at the lacO array 24 h after break

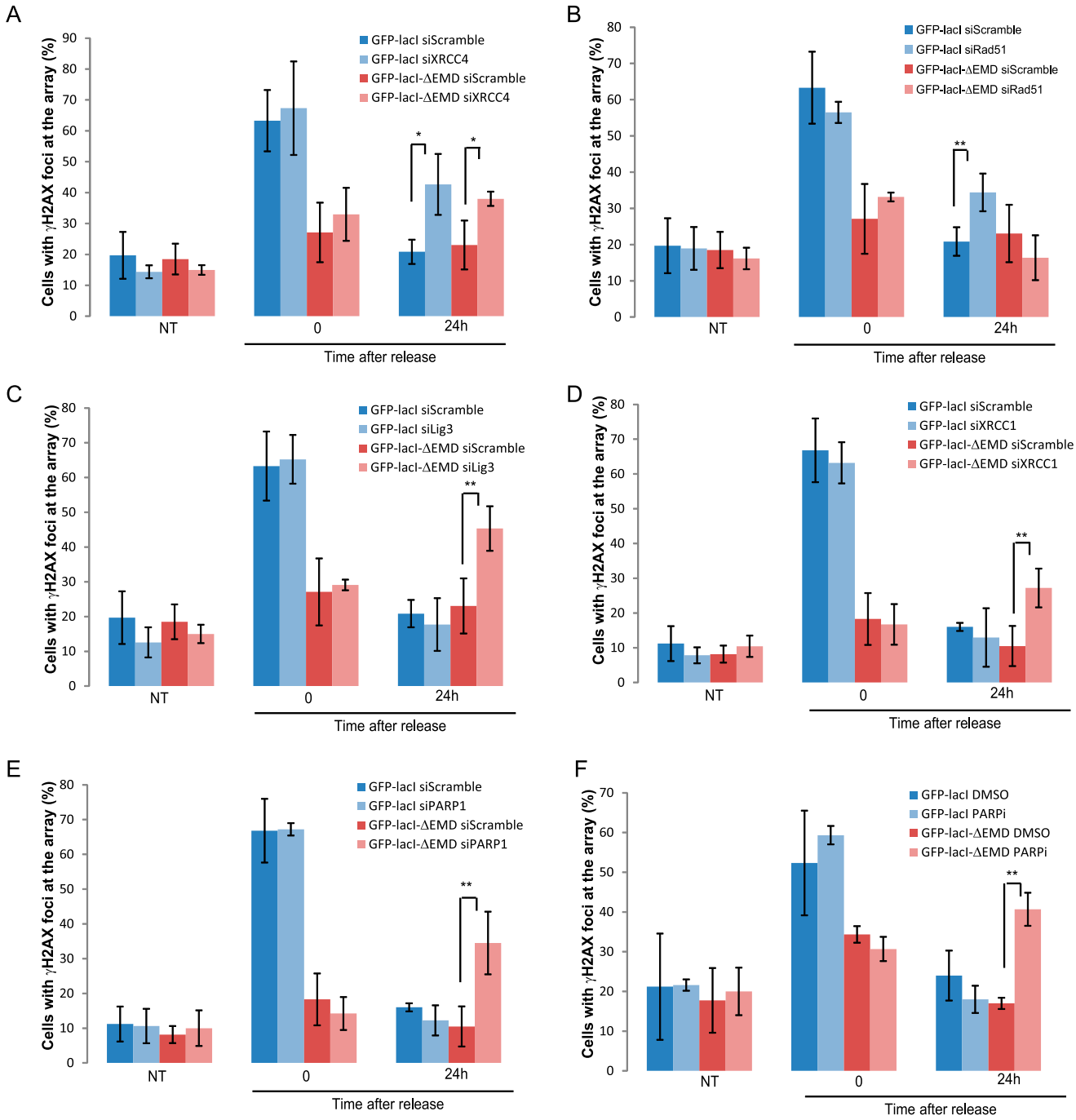

Figure 6. DSBs at the nuclear lamina are repaired by NHEJ or A-EJ. The percentage of colocalization of the lacO array with γ-H2AX in untreated cells (NT) or after 14 h of Dox (time point 0) and subsequent release for 24 h in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD and transfected with XRCC4 (*A*), RAD51 (*B*), ligase 3 (*C*), XRCC1 (*D*), or PARP1-specific siRNAs (*E*) is shown. (*F*) The percentage of colocalization of the lacO array with γ-H2AX upon Dox treatment or release in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD and treated with DMSO or a PARP inhibitor (PARPi, during the entire course of the experiment) is shown. Values represent mean ± SD of three independent experiments with $n > 50$ cells. For statistical analysis, a *t*-test was performed. (*) $P < 0.05$; (**) $P < 0.01$.

induction by a short pulse of Dox (Fig. 6A–E). Although depletion of XRCC4 led to persistent damage in both compartments (Fig. 6A), depletion of RAD51 did not affect the repair of breaks at the lamina (Fig. 6B). These results suggest that lesions at LADs do not depend on HR for their repair.

To test whether repositioning of the lacO/I-SceI break at the nuclear membrane affects the kinetics of repair, we performed LM-PCR in GFP-lacI and GFP-lacI-ΔEMD cells after a short pulse of Dox followed by release for 36 h. We found that breaks at both nuclear locations were efficiently repaired based on the marked decrease in PCR

signal (Supplemental Fig. S14B). These results strongly suggest that efficient DNA repair takes place at the lamina-associated I-SceI breaks even in the absence of functional HR.

Since resection is not abolished at lacO/I-SceI breaks when associated with the nuclear lamina, we sought to determine the fate of the lesions whereby resection has occurred but complete DNA repair by HR cannot occur. To answer this question, we assessed the contribution of the A-EJ pathway in the repair of breaks at the periphery. To this end, we quantified persistent γ-H2AX at the lacO/I-SceI locus 24 h after break induction in GFP-lacI and GFP-lacI-ΔEMD cells where ligase 3, XRCC1, or PARP1 had been depleted (knockdown efficiencies verified in Supplemental Fig. S14A,C) or PARP was inhibited. Interestingly, inhibition of the A-EJ pathway resulted in a repair delay for only breaks that were associated with the nuclear membrane (Fig. 6C–F; Supplemental Fig. S14D). These findings indicate that NHEJ and A-EJ, but not HR, are the most prevalent pathways of DNA repair for lesions occurring at nuclear membrane-associated chromatin and reveal for the first time that A-EJ takes place as a main pathway and not as a backup pathway activated solely in instances where there is a DNA repair factor deficiency (Frit et al. 2014).

Taken together, we showed that breaks occurring in chromatin that surrounds the nuclear membrane do not migrate to other regions of the nucleus, not even to other domains within the nuclear periphery, but rather are repaired within the lamina, where the break occurred by NHEJ and A-EJ.

Discussion

To preserve genomic integrity, different DNA repair pathways have evolved, and multiple layers of regulation like the cell cycle, specific proteins, or chromatin structure exist to ensure the tight balance between these pathways (Kass and Jasin 2010). Here, we propose another layer of regulation of DNA repair pathway choice imposed by nuclear compartmentalization. We show that the nuclear lamina restricts HR and allows NHEJ and A-EJ. These observations are in agreement with data in yeast showing that distinct nuclear compartments of the nuclear periphery like the nuclear pore or the inner nuclear membrane favor different repair outcomes (Nagai et al. 2008; Khadaroo et al. 2009; Oza et al. 2009; Horigome et al. 2014). Similar to what we observed, it was shown that binding of DSBs to Nup84 in yeast facilitates recombination through SUMO protease Ulp1 and the SUMO-dependent ubiquitin ligase Slx5/Slx8 (Nagai et al. 2008) using BIR and microhomology-mediated recombination. On the contrary, binding to the inner nuclear membrane protein Mps3 has two different outcomes: In the case of telomere tethering, it inhibits recombination by sequestering the DSBs from nonspecific interactions with chromatin (Oza et al. 2009; Schober et al. 2009), while in the case of persistent DSBs, it triggers repair by the classical HR pathway (Horigome et al. 2014).

We also found that the chromatin structure at the inner nuclear lamina is mainly responsible for inhibiting HR. This is in keeping with recent studies, which found that HR is activated at DSBs located within actively transcribed genes that reside in euchromatin (Aymard et al. 2014; Pfister et al. 2014). Given that the lacO locus is promoterless and not transcribed, our results indicate that HR is not regulated solely by the transcriptional status. Instead, the exact nature of the chromatin environment and chromatin accessibility appear to be major determinants of HR regulation (Jha and Strahl 2014; Pai et al. 2014). Indeed, other studies have shown that HR is a main pathway in repairing breaks within heterochromatin (Beucher et al. 2009; Geuting et al. 2013; Kakarougkas et al. 2013). However, our data point to the fact that not all heterochromatin domains within the nucleus behave in the same manner and that the specific type of heterochromatin at the nuclear lamina has distinct functions.

In most of the above studies, chromatin structure and histone modifications affect the very first step of the HR pathway that is DNA end resection. Aymard et al. (2014) show that H3K36me3 is essential for the recruitment of CtIP through LEDGF. On the other hand, H3K36me3 in yeast induces chromatin compaction and inhibits resection, as visualized by increased RPA foci when the methyltransferase responsible for this modification is absent (Pai et al. 2014). Here we observed that phosphorylation of RPA at S33 is delayed and not mounted properly at lesions occurring in chromatin associated with the inner nuclear membrane. We also show that BRCA1 recruitment is dramatically affected. Since BRCA1 is acting with CtIP to activate long-term resection (Chen et al. 2008), it is possible that DNA ends are not appropriately resected to create a proper template for recombination, and the short resection channels lesions to A-EJ as was proposed earlier (Zhang and Jasin 2011; Deng et al. 2014). The fact that resection at the lamina is not as dramatically affected as late steps of HR might also suggest that nuclear position dictates the DNA repair pathway choice by regulating only the recruitment of late HR proteins to DSBs.

The use of A-EJ, which is considered a highly mutagenic pathway, instead of the error-free HR pathway might seem dangerous for the maintenance of genomic stability. However, LADs are relatively gene-poor, have a repressive chromatin signature, and are demarcated by repetitive and AT-rich sequences (Meuleman et al. 2013). The inhibition of HR may represent a means to avoid genomic instability provoked by recombination between repetitive sequences, which is a mechanism that has been proposed for the repair of DSBs that form in heterochromatic regions in *Drosophila* (Chiolo et al. 2011). Moreover, activation of A-EJ that is an error-prone pathway might have less impact given that the most of the genes that reside in LADs are not transcribed (Meuleman et al. 2013).

In *Drosophila*, breaks induced in the heterochromatic domain rapidly relocate outside of the domain, where HR is completed (Chiolo et al. 2011). A similar DSB relocation

was observed in mouse cells upon break induction by linear ion tracks in chromocenters (Jakob et al. 2011). On the contrary, we show that breaks occurring in chromatin associated with the inner nuclear lamina are positionally stable, suggesting that different heterochromatic compartments use different strategies to avoid recombination. One of the possible hypotheses to explain such a difference is a different chromatin composition or a difference in the regulation of chromatin mobility. Indeed, in yeast, DSBs were shown to have increased mobility (Dion and Gasser 2013). This mobility is facilitated by chromatin decompaction via chromatin remodelers (Neumann et al. 2012) and HR factors (Dion et al. 2012) and in turn allows the homology search step of HR (Mine-Hattab and Rothstein 2012). In mammalian cells, however, DSB mobility is limited and actively restricted by the NHEJ complex Ku70/Ku80 (Soutoglou et al. 2007; Roukos et al. 2013). In *Drosophila* cells, the relocation of DSBs outside of the heterochromatic domain is accompanied by decondensation of the domain (Chiolo et al. 2011), suggesting a mechanism similar to the one responsible for DSB mobility in yeast. At the nuclear lamina, however, this mechanism does not seem to be active, suggesting that an additional mechanism could repress DSB movement at the nuclear lamina. This hypothesis is in accordance with the observation that chromatin mobility is decreased for genomic loci associated with the nuclear lamina or the nucleoli (Chubb et al. 2002). Furthermore, laminA has recently been identified as a factor inhibiting DSB movement in mammalian cells (Mahen et al. 2013), further pointing to an active inhibition of DSB mobility at the nuclear lamina.

Another difference between our results and the results obtained in the heterochromatic compartment of *Drosophila* cells is the activation of DDR. In *Drosophila* cells, the activation of DDR was faster in heterochromatin compared with euchromatin (Chiolo et al. 2011). On the contrary, our results show a slower DDR activation at the nuclear lamina compared with the nuclear interior. Given the implication of the early steps of DDR in the initiation of resection by the ATM and MRN complexes, and the fact that resection facilitates DSB movement in yeast, one can hypothesize that the delayed DDR at the nuclear lamina inhibits DSB mobility.

Overall, our findings indicate that spatial positioning of a DSB is a new parameter to consider in the study of DSB repair, which has significant implications for our understanding of how the organization of repair in the highly compartmentalized nucleus contributes to maintaining genome stability and avoiding tumorigenesis.

Materials and methods

Cell lines, infections, transfections

I-U2OS19 GFP-lacI and GFP-lacI-ΔEMD cells were generated by infecting the U2OS19ptight13 cell line (Lemaître et al. 2012) with GFP-lacI (Soutoglou and Misteli 2008) and GFP-lacI-ΔEMD (Reddy et al. 2008) plasmids and after FACS sorting. Briefly, BOSC cells were transfected using FuGENE6 (Promega) according to the manufacturer's protocol with GFP-lacI or GFP-lacI-ΔEMD constructs and an amphotropic vector. Cell supernatants were harvested 48 h later and transferred to U2OS19ptight13 cells. Twenty-four hours after infection, cells were FACS-sorted for GFP-positive signal and cultured in the presence of 800 μg/mL G418 and 2 mM IPTG (inhibitor of the lacI/lacO interaction). Cells were plated in the absence of IPTG for 24 h prior to starting an experiment. To induce I-SceI expression, Dox was added to the cells at a concentration of 1 μg/mL. In Supplemental Figure S3, 2 mM IPTG was maintained during the whole experiment, and in Supplemental Figure S7, A and B, cells were plated in the absence of IPTG for 24 h and treated with Dox for 12 h. IPTG was then added for 2 h, while Dox was maintained until the end of the experiment.

Hela111 cells were obtained by transfection of lacO-I-SceI-hygro plasmid and subsequent clonal selection using 300 μg/mL hygromycin. I-HeLa111 cells were generated by transfection of Hela111 cells with pWHE320-HA-I-SceI and pWHE146-Tet activator plasmids and selection using 1 mg/mL G418. I-Hela111 GFP-lacI or GFP-lacI-ΔEMD cells were generated by infection of I-Hela111 cells with GFP-lacI and GFP-lacI-ΔEMD plasmids and FACS sorting for GFP-positive cells.

I-U2OS19 Pom121-GFP-lacI cells were obtained after infection of I-U2OS19 cells with Pom121-GFP-lacI and selection of GFP-positive cells using FACs sorting.

I-U2OS19 GFP-lacI and GFP-lacI-ΔEMD were transfected with cherry-lacI or BRG1-cherry-lacI by using FuGENE6 reagent according to the manufacturer's protocol. The cells were first plated in the absence of IPTG for 24 h and then transfected and treated with Dox 4 h after transfection.

I-U2OS19 GFP-lacI and GFP-lacI-ΔEMD cells were transfected with siRNA scramble (OnTarget Plus nontargeting pool siRNA; Dharmacon, D-001810-10-20), XRCC4 (Dharmacon, M-004494-02), Rad51 (Dharmacon, L-003530-00) or Lig3 (Dharmacon, L-009227-00) using oligofectamine reagent (Invitrogen) according to the manufacturer's protocol. Knockdown efficiency was analysed by Western blot or RT-qPCR. RNA was extracted using the RNeasy minikit (Qiagen) according to the manufacturer's protocol. RT-qPCRs were then processed as in (Pankotai et al. 2012). Proteins were extracted in RIPA buffer and analyzed by Western blot.

PARP inhibitor treatment

I-U2OS19 GFP-lacI and GFP-lacI-ΔEMD were plated in the absence of IPTG for 24 h and treated with PARPi (ABT-888, sc-202901A) at a 10 μM concentration or by DMSO.

TSA treatment

Cells were plated in the absence of IPTG for 24 h and subsequently treated with TSA at 0.5 μM or DMSO for control for 4 h. Dox was added after 4 h of treatment for the indicated time, while DMSO or TSA was maintained during the whole experiment.

Neocarzinostatin (NCS) treatment

Cells were plated in the presence of Shield for 20 h, treated for 15 min with 100 ng/mL NCS (N9162-100UG, Sigma), and fixed 2 h after treatment.

Cell cycle analysis

Cells were fixed in 70% EtOH overnight at −20°C and stained with 25 μg/mL propidium iodide. The acquisition was performed on a FACSCalibur. Results were analysed using FlowJo software.

LM-PCR

Cells were plated in the absence of IPTG for 24 h and subsequently treated with Dox for 14 h. DNA was then extracted with the DNeasy blood and tissue kit (Qiagen). Assymetric adaptator (S21, Phos-GCATCACTACGATGTAGGATG; and Lup, CATCCTACATCGTAGTGATGCTTAT) was annealed in TE for 5 min at 95°C and then allowed to reach room temperature slowly. One-hundred picomoles of assymetric adaptator was added to 1 μg of DNA extracted from cells. Ligation was performed using T4 DNA ligase overnight at 16°C. PCR was performed using Pfu enzyme (Agilent) with an annealing temperature of 58°C. The PCR primers used were LM-I-SceI (CAT CCTACATCGTAGTGATGC) and lacR (TTAATTAATCAAAC CTTCCTCT). The PCR product was then run on a 2% agarose gel.

Immunofluorescence, immuno-FISH, and microscopy

Cells were cultured on coverslips and fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.5% Triton for 10 min, blocked in 1% BSA for 30 min, and incubated with primary antibody for 1 h (see the antibodies table in the Supplemental Material) and secondary antibodies for 45 min. Coverslips were incubated with DAPI and mounted on slides in Prolong Gold (Molecular Probes).

For Rad51 and Ku80 immunofluorescence or immuno-FISH, cells were pre-extracted in CSK buffer (10 mM Hepes at pH 7, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.7% Triton X-100) containing 0.3 mg/mL RNase A prior to fixation (Britton et al. 2013).

For immuno-FISH, the same protocol was used, but after incubation with secondary antibodies, they were submitted to post-fixation in 4% formaldehyde for 20 min. Cells were washed for 5 min in 2× SSC and 45 min in 2× SSC with a increasing temperature from room temperature to 72°C. After one wash in 70% ethanol and two washes in absolute ethanol, coverslips were dried for 5 min at room temperature. They were subsequently incubated with 0.1 N NaOH for 10 min and washed in 2× SSC for 5 min. Coverslips were washed again in 70% ethanol and twice with absolute ethanol. After drying, cells were hybridized with DNA probe (see immuno-FISH probe preparation below) for 30 sec at 85°C and incubated overnight at 37°C.

The immuno-FISH probe was prepared by nick translation from the lacO-I-SceI plasmid that was used to create the I-Hela111 cell line. DNA probe (0.3 μg) was mixed with 9 μg of ssDNA and 3 μg of CotI human DNA (Roche) and precipitated with 2.5× vol of ethanol and 1/10 vol of 2.5 M sodium acetate for 30 min at −80°C. After 20 min of centrifugation, the supernatant was discarded, and the pellet was washed with 70% ethanol and centrifuged again for 5 min. The supernatant was discarded, and the pellet was dried. The pellet was resuspended in 20 μL of hybridization solution (50% formamide, 4× SSC, 10% dextran sulfate) per coverslip by vortexing for 1 h. The probe was denaturated for 5 min at 90°C and preannealed for at least 15 min at 37°C before hybridization with cells.

The day after hybridization, immuno-FISH was revealed. Coverslips were washed twice for 20 min at 42°C in 2× SSC and then incubated with secondary antibody and fluorescein anti-biotin (Vector Laboratories, SP-3040) at 1:100 dilution for 45 min. Coverslips were washed, incubated with DAPI, and mounted in Prolong Gold reagent (Molecular Probes).

Slides were observed, and colocalization counting was done in epifluorescence microscopy. Pictures were taken with confocal microscopy. For experiments with Pom121-GFP-lacI constructs, cells were always costained with laminB to evaluate relocalization of the lacO array at the nuclear pores. For experiments with BRG1-cherry-lacI or cherry-lacI transfections, colocalization was counted using confocal microscopy.

Time-lapse microscopy

Three-dimensional stacks were captured every 10 min for a total of 320 min upon NCS addition using the Leica DM6000 microscope with Leica CSU22 spinning disc and Andor Ixon 897 camera. Twenty different cells were imaged for each condition (±NCS).

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Materials and methods

Cloning and plasmids

Construct	Origin
GFP-lacI-ΔEMD	(Reddy et al. 2008)
GFP-lacI	(Reddy et al. 2008)
cherry-lacI	(Soutoglou and Misteli 2008)
BRG1- cherry-lacI	gift from Tom Misteli
Pom121 GFP lacI	(see below)
pEXPR-EF1α-Pom121A-Venus	gift from Naoko Imamoto
pWHE320-HA-I-SceI	(Lemaitre et al. 2012)
pWHE146-Tet activator	(Lemaitre et al. 2012)

pCXPA-POM121A-EGFP-LacI was assembled with the universal expression system (manuscript in preparation) in a single cloning reaction with 5 fragments and using type IIS restriction enzymes

Super-resolution imaging and analysis

Immunofluorescence was performed as described above. Postfixation in 4% formaldehyde for 20 min was performed prior to imaging. The super-resolution microscopy experiments were performed on a Leica SR GSD system that consists of: Leica DMI6000 B inverted microscope with HCX PL APO 100x/1.47 Oil CORR TIRF PIFOC objective and 1.6x magnification lens for resulting pixel size of 100 nm; Andor

iXon3 DU-897U-CS0-#BV EMCCD camera with field of view of 18x18 μm in GSDIM mode; continuous wave fibre lasers (MPBC Inc., 488 nm 300 mW, 532 nm 1000 mW, 642 nm 500 mW); a diode laser 405 nm 30 mW; supressed motion (SuMo) sample stage with reduced drift.

For the super-resolution imaging the samples were mounted in PBS buffer that contained 10 mM of cysteamine (Sigma) and that was adjusted to pH 7.5 with 25 mM of HEPES. MEA was dissolved at 1M in PBS and was stored at -20°C. The final dilution was done prior to imaging.

For imaging of Alexa-488 we used the 488 nm laser as excitation source, filter cube with excitation filter DBP 405/10 488/10, dichroic mirror LP 496 and emission filter BP 555/100. For Alexa-647 — 642 nm laser, DBP 405/10 642/10, LP 649 and BP 710/100, respectively. The two colour channels were imaged sequentially: first Alexa-647, then Alexa-488. The excitations were performed at 100% power of corresponding lasers; the acquisitions started after beginning of observation of single-fluorophore events ("blinking") that corresponded to 1-2 min of excitation for Alexa-488 and 1-5 s for Alexa-647. The time of exposition of a frame was 50 ms at 488 nm and 10 ms at 647 nm. After few minutes of acquisition, as number of blinking evens dropped, the sample started to be illuminated additionally by 405 nm laser with gradual increase of its intensity in order to keep a constant rate of single-molecular returns into the ground state. The acquisition stopped after complete bleaching of the fluorophore. Duration of acquisitions was typically 10-20 min for Alexa-488 and 7-10 min for Alexa-647.

The localization and fitting of single-molecular events were performed in Leica LAS AF 3.2.0.9652 software with "center of mass" fitting method. Close events on consecutive frames, most likely originating from the same fluorophore, were merged using a corresponding option in the software. Maximal number of events to merge was set to 10, radius – to 50 nm. The obtained event lists, containing for each event: frame ID, coordinates x y, fitted number of photons, standard deviations σ_x σ_y for fitted 2D-Gaussians, were exported in an .ascii file and analysed further using a custom software written in Matlab. Super-resolution images, were calclulated with grey value of a pixel as quantity of localizations detected in the pixel area.

In order to reduce chromatic aberrations, the microscope was calibrated with multi-colour fluorescent beads (Tetraspeck, d=200 nm). The same area of a coverslip with beads was excited by 488 nm and 642 nm laser light; obtained pair of images appeared shifted on 20-60 nm for each bead, depending on lateral position of the bead in the field of view. The values of the offset were fitted to the x and y position on the image by a 2-order polynom. The obtained fit was subtracted from coordinates of each event of the red channel, resulting in residual chromatic offset less than 25 nm through all the field of view.

In order to reduce a drift of the sample, each single-color acquisition was divided onto two successive parts with equal number of events. From each part, a super-resolution image was reconstructed. The shift between the two images was calculated with subpixel precision by cross-correlation using a Matlab function(Manuel Guizar-Sicairos 2008) . The obtained value was fitted linearly into full range of frames and was subtracted from each single-molecular localization. The red channel events were shifted towards the final frame of the red colour acquisition,

the green channel ones – towards the first frame of the green colour acquisition, in order to reduce an additional offset between two colours, produced by drift and sequential imaging.

We were not able to reliably calculate shifts between smaller datasets due to not enough quantity of localizations for reconstruction more than two resembling images. So with this approach only a constant component of drift may be reduced, that is yet the most significant on our system.

We performed coordinate-based colocalization analysis of single-molecule localization data of two species. For each single-molecular event A*i* we calculated a colocalization value C_{Ai} that adapts values from -1 (for anti-correlated distributions) through 0 (for non-correlated) to +1 (for perfectly correlated distributions)(Malkusch et al. 2012). For the calculation of C_{Ai} we took into account all the localizations of both colours around A*i* within radii from 2 nm to 500 nm with step of 2 nm.

A histogram of distribution of C_A showing overall colocalization level was calculated for each double-colour image. We also calculated a global colocalization value for each image by division the sum of all positive values C_{Ai} by the sum of all negative values.

Quantification of the distribution of m6A-Tracer intensity

The quantification of distribution of m6a-Tracer was done using a macro on ImageJ, available upon request. Ratio of intensity of m6a-Tracer in the nucleoplasm over the intensity at the nuclear enveloped was then calculated.

Antibodies

Antibodies	Company	Reference	Application
laminB	Santa Cruz	SC-6216	ImmunoFISH, IF
γH2AX	Abcam	Ab22551	ImmunoFISH, IF
53BP1	Novus	NB100-304	ImmunoFISH, IF
Brca1	Calbiochem	OP92+OP93	ImmunoFISH, IF
Rad51	Calbiochem	PC130	ImmunoFISH, ChIP
Rad54	Abcam	Ab11055	ImmunoFISH
Ku80	Santa Cruz	SC-56136	ImmunoFISH
γH2AX	Abcam	Ab2893	ChIP
RPA	Novus	NB600-565	ChIP
P-RPA	Bethyl	A-300 245A	ChIP
BRCA1	Santa Cruz	SC-642	ChIP
XRCC4	Abcam	Ab145	ChIP, WB
Tubulin	Sigma	DM1A	WB
GFP	IGBMC		IF prior to GSDIM
TPR	Abcam	Ab84516	IF prior to GSDIM

Electron spectroscopic imaging (ESI) of chromatin structure and variation. Human U2OS osteosarcoma cells were treated with either vehicle (0.1% DMSO) or with 500 nM trichostatin A (TSA) for 4 h before being fixed in 4% paraformaldehyde (EMS) for 10 min at room temperature (RT) prior to being permeabilized in PBS containing 0.5% Triton X-100 for 5 min. Cells were then "post fixed" in 1% glutaraldehyde

(EMS) for 5 min at RT to maintain chromatin structure during resin embedding. The cells were then dehydrated in an ethanol series and embedded in Quetol 651 (EMS) before being processed, sectioned and imaged by ESI as previously described (Dellaire G 2004)using a Tecnai 20 transmission electron microscope (FEI) equipped with an energy-filtering spectrometer (Gatan). Energy-filtered electron micrographs of nitrogen (N) and phosphorus (P) were collected, and non-chromosomal protein was segmented by subtracting the N from the P ESI micrograph, which was then false colored in cyan and combined in a composite image with the P ESI micrograph false colored in yellow in Photoshop CS6 (Adobe) to highlight chromatin. The composite elemental maps of N-P (cyan) and P (yellow) were then analyzed for thickness of nuclear-lamina-associated chromatin using Image J v1.48k software (NIH). Pixel measurements (50 measurements taken from 10 cells) were converted into microns (μm) and then averaged per cell, and the data was represented as mean chromatin thickness± SEM (where N=10). Statistical significance between cell lines was generated using the Student's *t* test in Excel (Microsoft). The mean coefficient of variation (CV) in chromatin density was calculated for chromatin within the nucleus of vehicle and TSA treated U2OS cells (N=5), using phosphorus-enriched 155 keV electron micrographs as previously described(Dellaire et al. 2009). Briefly, the mean and SD pixel intensities were first determined from 5 X 10 pixel-wide line scans per cell using Image J. Then for each cell the CV was determined by dividing the mean pixel intensity by the SD, after which the CVs were averaged for vehicle or TSA treated cells and represented as a percentage ± SEM.

Measurement of the size of the lacO array

The lacO array sizes at different conditions were measured on paraformaldehyde fixed samples. The images were taken by Leica DM6000 microscope with Leica CSU22 spinning disc and Andor Ixon 897 camera. For every condition at least 20 individual cells were imaged and analyzed. The Z planes were taken every 0.3 mm. For 3D reconstruction and quantification of volumes the Imaris software (Bitplane) was used.

ChromatinIP

The ChIP analysis was done following the Dynabeads ChIP protocol from Abcam(Pankotai et al. 2012) with a few modifications. Briefly, one 150-mm dish with cells that were 70% confluent was used for each time point. The cells were cross-linked for 30 min in 0.75% (v/v) paraformaldehyde and then sonicated in 1% (v/v) SDS-containing sonication buffer (50 mM HEPES, pH 8, 140 mM NaCl, 1 mM EDTA, 1% (v/v) TritonX-100, 1% (v/v) SDS and a protease inhibitor cocktail (Roche)). Thirty milligrams of chromatin were diluted in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (v/v), 0.1% sodium deoxycholate (w/v) and 0.1% SDS (v/v)) and were used in each immunoprecipitation by adding 4 μg of antibody and 50 μl Dynabeads M-280 (Invitrogen). The beads were washed for 5 min with low-salt buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 1% (v/v) Triton X-100 and 0.1% (v/v) SDS), then 5 min with high-salt buffer (20 mM Tris-HCl, pH 8, 500 mM NaCl, 2 mM EDTA, 1% (v/v) Triton X-100 and 0.1% (v/v) SDS) and for 5 min with LiCl buffer (10 mM Tris-HCl, pH 8, 250 mM LiCl, 1 mM EDTA, 1% (v/v) NP-40 and 1% (w/v) sodium deoxycholate) and two times for 5 min with TE

buffer. The elution was done twice at 65 °C for 15 min. Cross-links were reversed by incubation at 65 °C for 6 h. The DNA was purified after proteinase K and RNaseA treatment by using phenol-chloroform extraction and was resuspended in 50 µl of TE buffer.

The signal in each experiment was calculated using the formula (immunoprecipitated sample–IgG control)/input, and each value represents a relative DNA concentration that is based on the standard curve of the input.

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Supplementary figure legends

Figure S1- Experimental system

(A) Schematic representation of the experimental system: the lac repressor (GFP-lacI) binds to the lac operator (lacO), which allows the relocalization of the lacO array to the nuclear lamina when fused to ΔEMD. The addition of Dox allows the expression of ISceI and induction of a DSB at the I-SceI restriction site, which is located next to the lacO repeats. **(B)** Quantification of the colocalization of the lacO array with lamin B in absence or presence of dox for 14h. Values represent means ± SD from three independent experiments (number of cells analyzed per experiment ≥ 50). **(C)** 3D Z-stacks confocal microscopy images of the lacO array (green) and lamin B (gray) in I-U2OS19 GFP-lacI cells (upper panel) or GFP-lacI-ΔEMD cells (lower panel) **(D)** LM-PCR in GFP-lacI and GFP-lacI- ΔEMD cells 14h after Dox addition. Products are shown (upper panel) and quantified (below) after 26 (left) or 28 (right) PCR cycles.

Figure S2- DDR is delayed at the nuclear lamina

(A) Quantification of the colocalization of the lacO array with γH2AX at the indicated times after dox addition in I-HeLa111 infected with GFP-lacI or GFP-lacI-ΔEMD. Values represent means ± SD of three independent experiments (number of cells analyzed per experiment ≥ 50). (B) Immuno-FISH single-Z confocal images of the lacO array (green) and γH2AX (red) in I-HeLa111 cells expressing GFP-lacI or GFP-lacI-ΔEMD and treated or not with Dox for 14h. For statistical analysis, t test was performed. P value are represented as follows : *<0.05, **<0.01

Figure S3- The expression of GFP-lacI-ΔEMD does not impair DDR activation

(A) Immuno-FISH single-Z confocal images of the lacO array (green) and lamin B (red) in I-U2OS19 GFP-lacI cells (left) or GFP-lacI-ΔEMD cells (right) in presence of 2mM IPTG and in the absence (upper panel) or presence (lower panel) of Dox for 14h. **(B)** Single-Z confocal microscopy images of the lacO array (green) and γH2AX (red) in I-U2OS19 GFP-lacI cells (left) or GFP-lacI-ΔEMD cells (right) in presence of 2mM IPTG and in the absence (upper panel) or presence (lower panel) of Dox for 14h, after immuno-FISH. **(C)** Percentage of the colocalization of the lacO array with lamin B in presence of 2mM IPTG, and in absence or presence of doxycycline for 14h. **(D)** Quantification of the colocalization of the lacO array with γH2AX in presence of 2mM IPTG, and in absence or presence of Dox for 14h. Values represent means ± SD of three independent experiments (number of cells analyzed per experiment ≥ 50).

Figure S4-Recruitment of DSB repair factors at the nuclear lamina in I-U2OS19

Immuno-FISH single-Z confocal images of the lacO array (green), laminB (gray) and (A) Ku80 (red), (B) BRCA1 (red), (C) Rad51 (red) in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD and treated or not with Dox for 14 or 20h

Figure S5-Recruitment of DSB repair factors at the nuclear lamina in I-HeLa111

Immuno-FISH single-Z confocal images of the lacO array (green), laminB (gray) and (A) Ku80 (red), (B) BRCA1 (red), (C) Rad51 (red) in I-HeLa111 cells expressing GFP-lacI or GFP-lacI-ΔEMD and treated or not with Dox for 14 or 20h **(D-F)** Quantification of the colocalization of the lacO array with **(D)** Ku80, **(E)** BRCA1, **(F)** RAD51, at the indicated times after dox addition in I-HeLa111 infected with GFP-lacI or GFP-lacI-ΔEMD. Values represent means ± SD of three independent experiments (number of cells analyzed per experiment ≥ 50). For statistical analysis, t test was performed. P value are represented as follows : *<0.05, **<0.01

Figure S6-Recruitment of Rad54 at the nuclear lamina in I-U2OS19

(A) Quantification of the colocalization of the lacO array with Rad54 at the indicated times upon Dox addition in I-U2OS19 GFP-lacI or GFP-lacI-ΔEMD cells. Values represent means ± SD of three independent experiments (number of cells analyzed per experiment ≥ 50). For statistical analysis, t test was performed. P value are

represented as follows : *<0.05 **(B)** Cell cycle profiles of I-U2OS19 GFP-lacI and I-U2OS19 GFP-lacI-ΔEMD cells.

Figure S7- TSA treatment induces chromatin decompaction

(A) Immunofluorescence images depicting H4acetylation (red) in I-U2OS19 GFP-lacI cells treated with DMSO or TSA. Nuclear stain, DAPI (blue) **(B-C)** Human U2OS osteosarcoma cells were treated with **(B)** vehicle (0.1% DMSO) or **(C)** with 500 nM trichostatin A (TSA) for 4 h before fixation and processing for electron spectrocopic imaging (ESI). In each row a low magnification phosphorus-enriched (155 KeV) electron micrograph is shown at the left, a line-scan of phosphorus intensity across the cell nucleus (between the white arrows) is shown in the middle panel, and on the far right a high magnification ESI electron micrograph is shown of the region outlined by a white dashed box in the low magnification micrograph. The coefficient of variation (CV) is also shown for the phosphorus intensity across the nuclei of vehicle and TSA treated cells (n=5; ± SEM); which represents the degree of variability in chromatin density as a percentage, where a lower percentage indicates a more homogenous chromatin density. The ESI micrographs have been false coloured such that chromatin appears yellow and non-chromosomal protein (e.g. nucleopores, marked by white astericks) appears cyan. The thickness of the nuclear lamina associate chromatin is demarcated by white arrow heads, N = nucleoli, and the scale bars = 1 micron. **(D)** The mean thickness of condensed chromatin associated with the nuclear lamina for cells treated with vehicle or with 500 nM TSA and depicted as a bar graph. Error bars = SEM, N=10. *p < 0.001
(E) Immuno-FISH single-Z confocal images of the lacO array (green) and lamin B (red) in I-U2OS19 GFP-lacI cells (left) or GFP-lacI-ΔEMD cells (right) in presence of DMSO (uper panel) or TSA (lower panel). **(F)** Quantification of the colocalization of the lacO array with lamin B in absence or presence of dox for 14h in cells treated with DMSO or TSA for 4h. Values represent means ± SD of three independent experiments (number of cells analyzed per experiment ≥ 50).

Figure S8- H2AX phosphorylation at the nuclear lamina is rescued upon TSA treatment

(A) Immuno-FISH single-Z confocal images of the lacO array (green) and γH2AX (red) in I-U2OS19 GFP-lacI cells (left) or GFP-lacI-ΔEMD cells (right) in presence of DMSO (uper panel) or TSA (lower panel).

Figure S9- HR factors recruitment at the nuclear lamina is rescued upon TSA treatment

Immuno-FISH single-Z confocal images of the lacO array (green) and (A) BRCA1 (red) or (B) RAD51 in I-U2OS19 GFP-lacI cells (left) or GFP-lacI-ΔEMD cells (right) in presence of DMSO (uper panel) or TSA (lower panel).

Figure S10- BRG1 tethering induces chromatin decondensation

(A) Schematic representation of the experimental system. The lac repressor (GFP lacI/cherry-lacI) binding to the lac operator (lacO) allows the relocalization of the

lacO array at the nuclear lamina when fused to ΔEMD. The expression of BRG1-cherry-lacI allows local decondensation of the lacO/I-SceI locus. The addition of Dox allows the expression of I-SceI and induction of a DSB at the I-SceI restriction site, next to the lacO repeats. **(B)** Images of 3D reconstruction of nuclei (blue) and the lacO array (red). **(C)** Quantification of the volume of the lacO array, normalized to the volume of the nucleus in GFP-lacI and GFP-lacI-ΔEMD cells expressing cherry–lacI or BRG1-cherry-lacI.

Figure S11- BRG1 tethering rescues HR factors recruitment at the nuclear lamina

Immunofluorescence single-Z confocal images of (A) BRCA1 (gray) or (B) RAD51 (gray) in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD, transfected with cherry-lacI or BRG1-cherry-lacI (red) and treated or not with Dox for 20h.

Figure S12- Recruitment of DDR and HR factors are not impaired by tethering at the nuclear pores

(A) Schematic representation of the experimental system (left panel) for relocalization of the lacO locus to nuclear pores. Expression of Pom121-GFP-lacI allows the repositioning of the lacO locus to the nuclear pores. Immuno-FISH single-Z confocal image (right upper panel) of the lacO array colocalizing with lamin B in Pom121-GFP-lacI expressing cells. D-Storm picture of Pom121-GFP-lacI (green) and nucleoporin TPR (red) showing colocalization of the lacO array with the nucleoporin TPR (right lower panel). Immuno-FISH single-Z confocal images of the lacO array (green), laminB (gray) and (B) 53BP1 (red), (C) BRCA1 (red), (D) RAD51 (red) in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD and treated or not with Dox for 20h.

Figure S13- Positional stability of LADs upon DNA damage

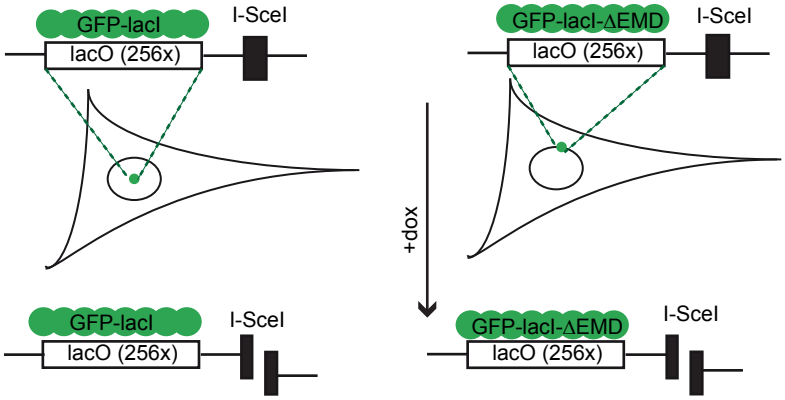
(A) Schematic representation of the experimental system. IPTG addition for 2h in GFP-lacI-ΔEMD after lacO repositioning to the periphery and DSB induction (with 14h dox treatment) allows the dissociation of the lacI from the lacO and a potential movement away from the nuclear lamina. **(B)** Percentage of colocalization of the lacO array with lamin B in absence or presence of dox (14h) in GFP-lacI- ΔEMD treated (for 2h) or not with IPTG. Values represent means ± SD of three independent experiments (number of cells analyzed per experiment ≥ 50). **(C)** Time lapse microscopy on HT1080 cells expressing Dam-laminB1 and m6a-Tracer (green) upon addition (or not) of 50ng/mL NCS for 15min. **(D)** D-STORM pictures of LADs colocalization with γH2AX (red) in HT1080 cells expressing Dam-laminB1 and m6a-Tracer (green) upon addition (or not) of 50ng/mL NCS for 15 min and release for 2 h.

Figure S14- Validation of silencing of ligase 3, XRCC4, RAD51, PARP1, XRCC1 by siRNA

(A) Western blot for tubulin, XRCC4, Rad51, PARP1, XRCC1 in I-U2OS19 GFP-lacI or GFP-lacI-ΔEMD treated with corresponding siRNAs. **(B)** LM-PCR in GFP-lacI and GFP-lacI-ΔEMD cells non-treated, 14h after Dox addition or 36h after a 14h Dox

pulse. Products are shown (upper panel) and quantified (below) after 28 PCR cycles. The intensity of the products depicted is normalized to the products of the non-treated samples. (C) Quantitative RT-PCR analysis of ligase *3* expression levels in I-U2OS19 GFP-lacI or GFP-lacI-ΔEMD cells treated with siRNA that targets a scramble sequence (purple lines) and ligase 3 sequences (blue lines). (D) Percent colocalization of the lacO array with γH2AX in untreated cells (NT) or after 14h of Dox (time point 0) and subsequent release for 24h in I-U2OS19 cells expressing GFP-lacI or GFP-lacI-ΔEMD and transfected with different ligase3-specific siRNAs (siLig3-6 or siLig3-7).

A



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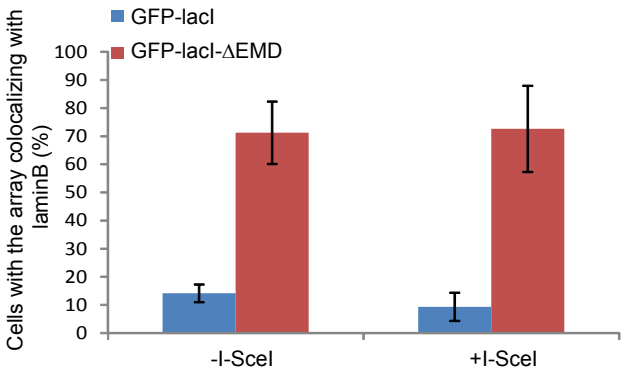

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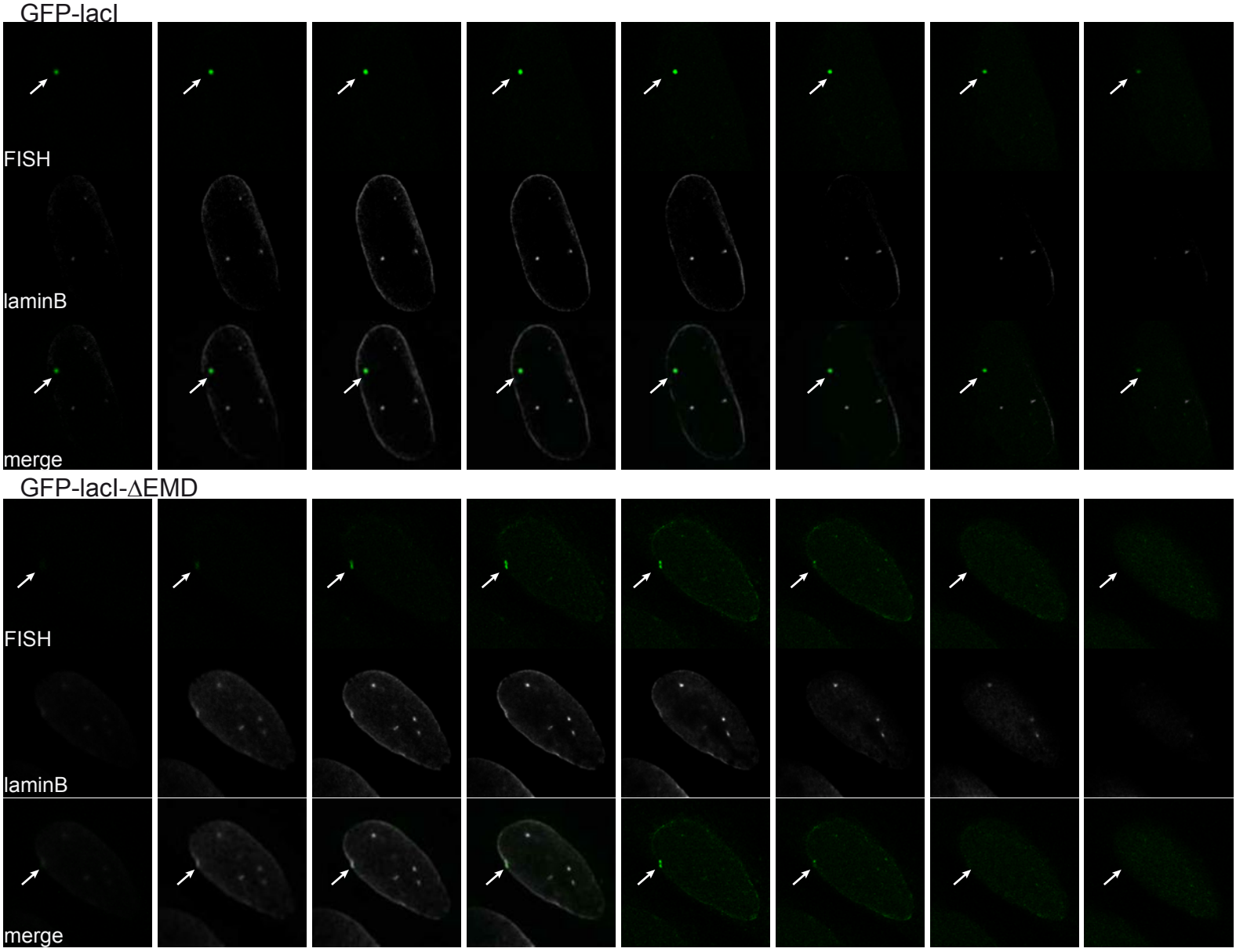

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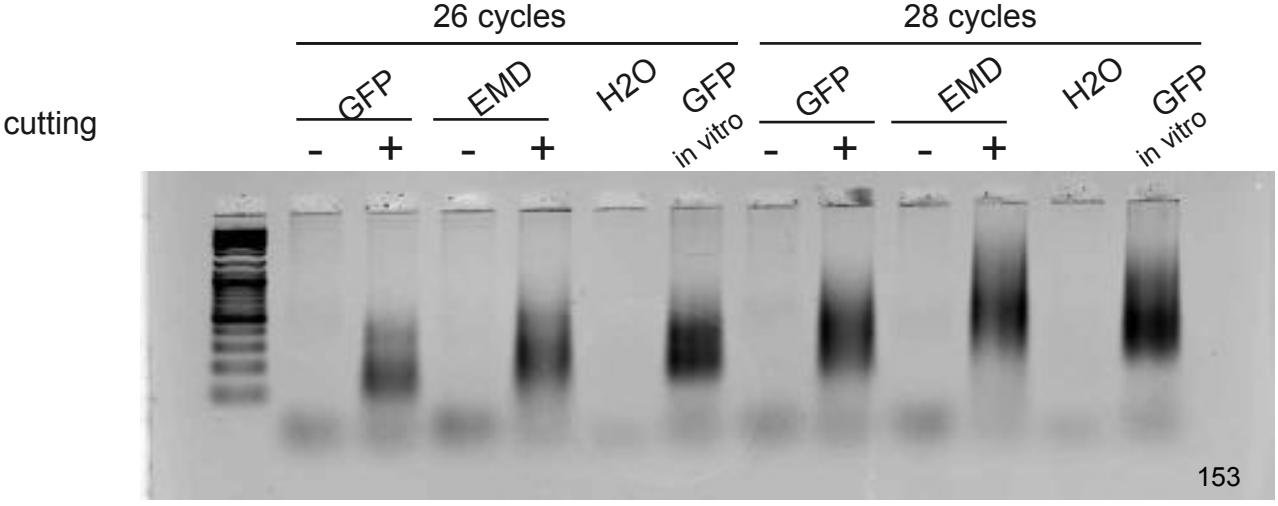
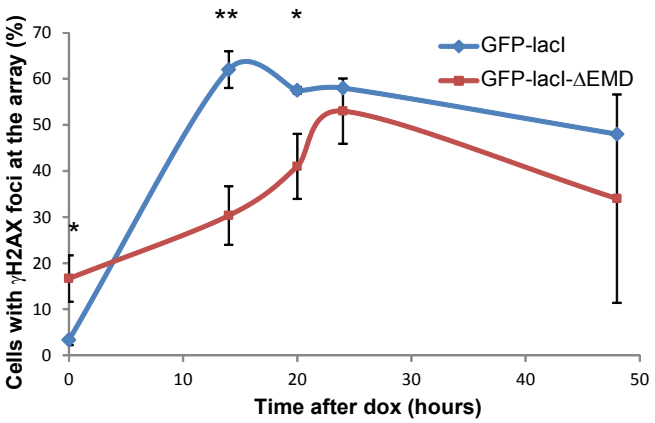


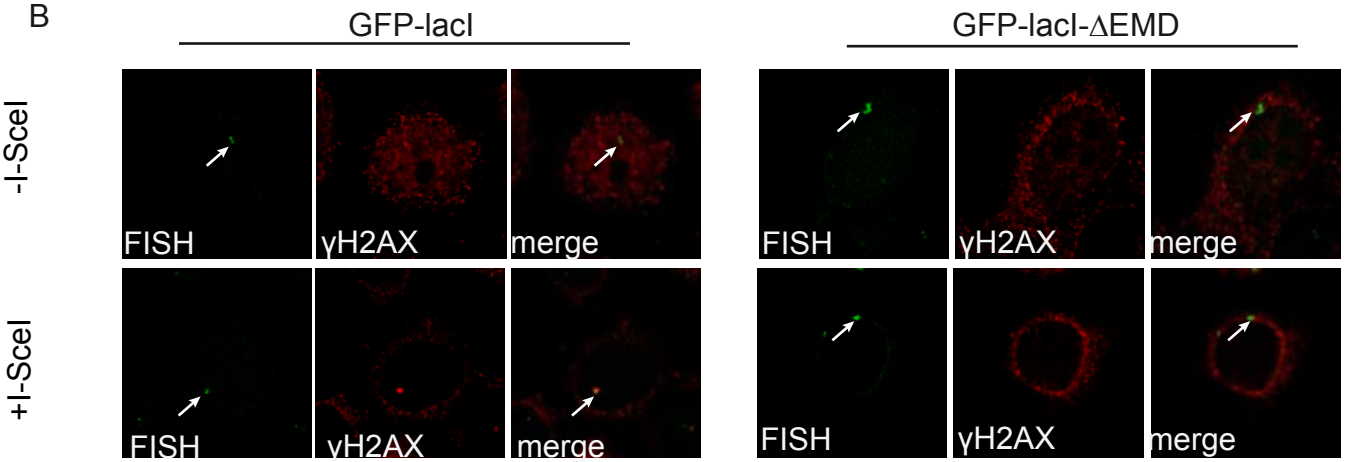
average intensity-background	1.7	**15.8**	-0.1	**22.3**	0	28.2	2.4	**28.6**	1.1	**32.5**	0	36.2

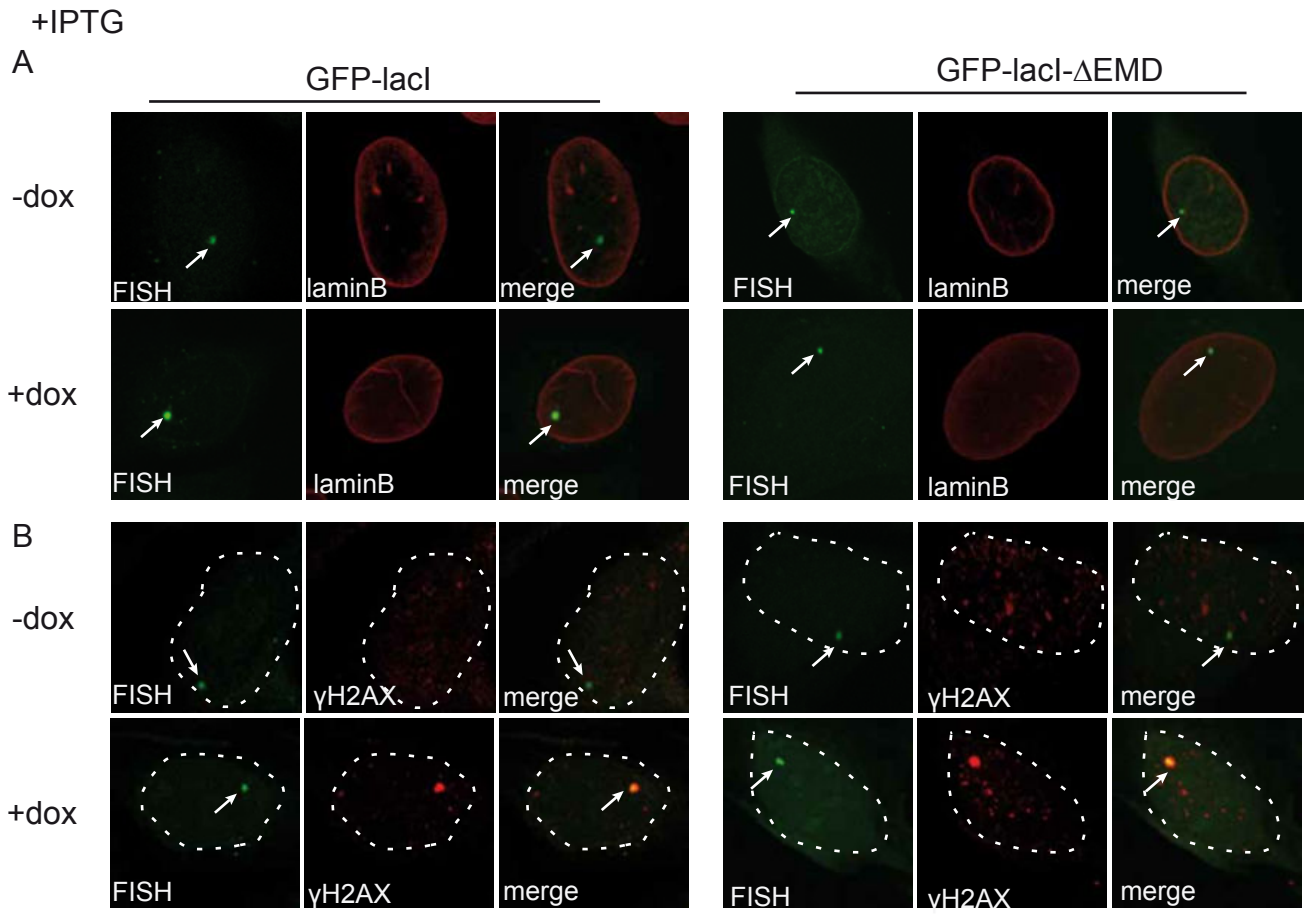
A

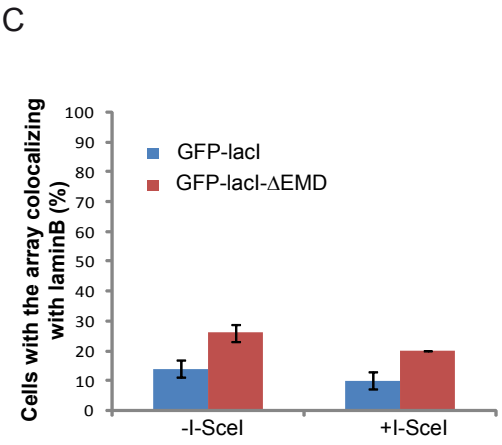
I-HeLa 111

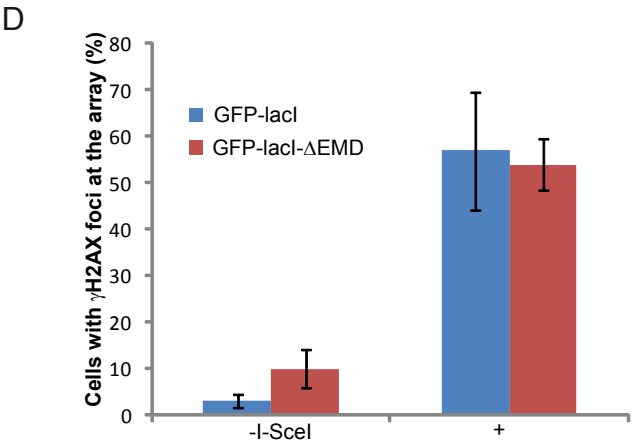

Cells with γH2AX foci at the array (%)
Time after dox (hours)
GFP-lacI
GFP-lacI-ΔEMD
**
*
*
0
10
20
30
40
50
60
70

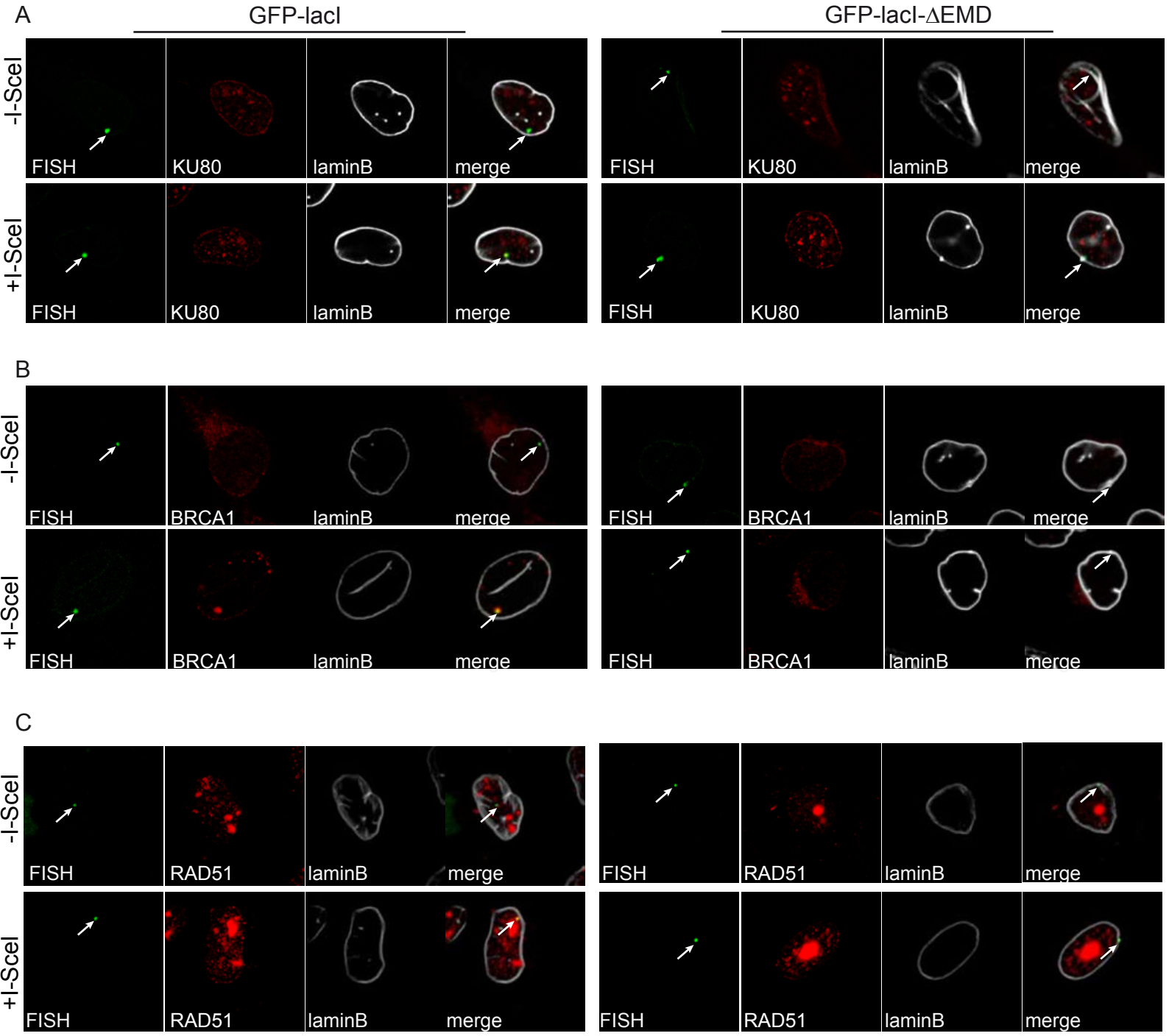
B

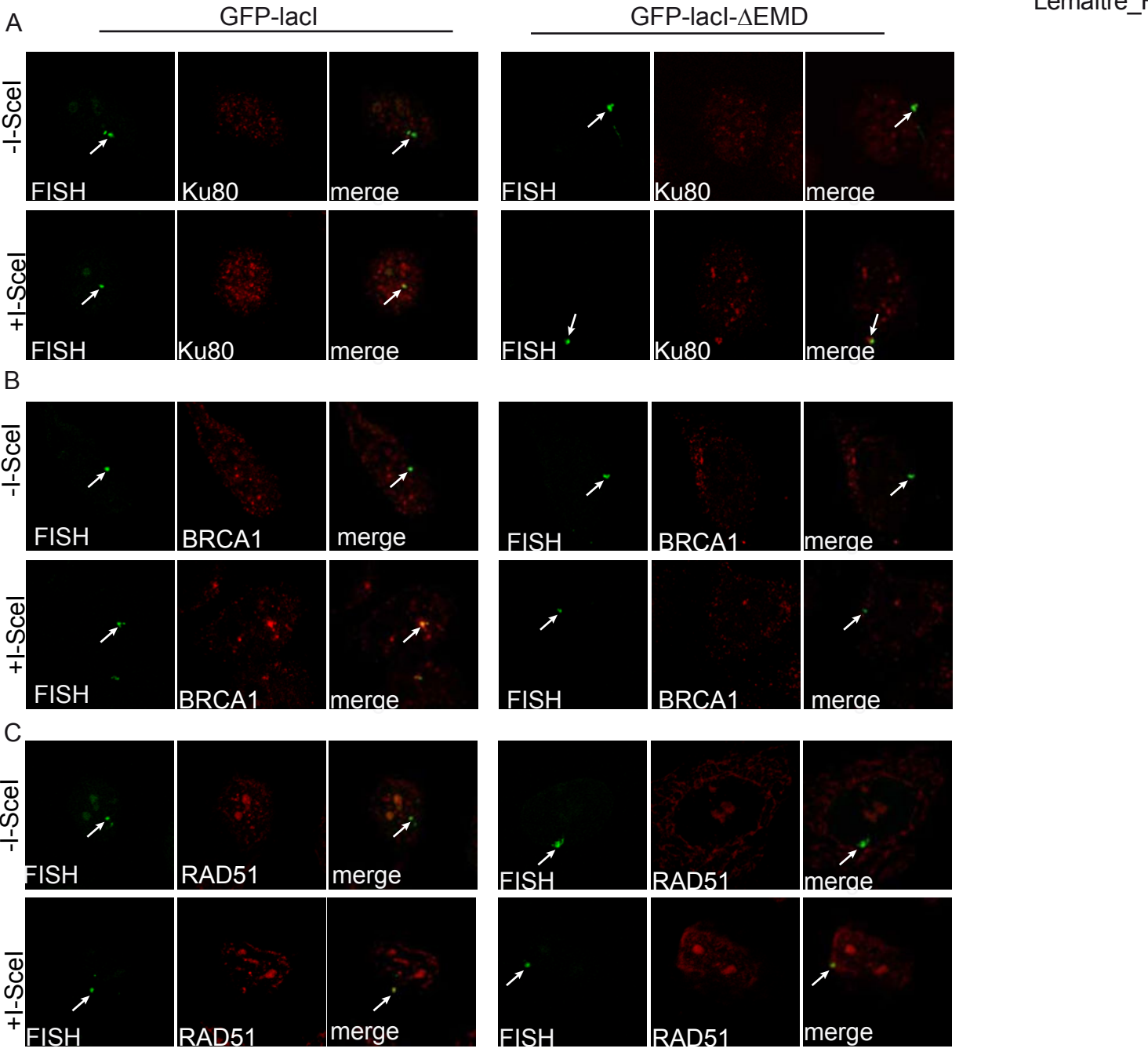

GFP-lacI
GFP-lacI-ΔEMD
-I-SceI
+I-SceI
FISH
γH2AX
merge

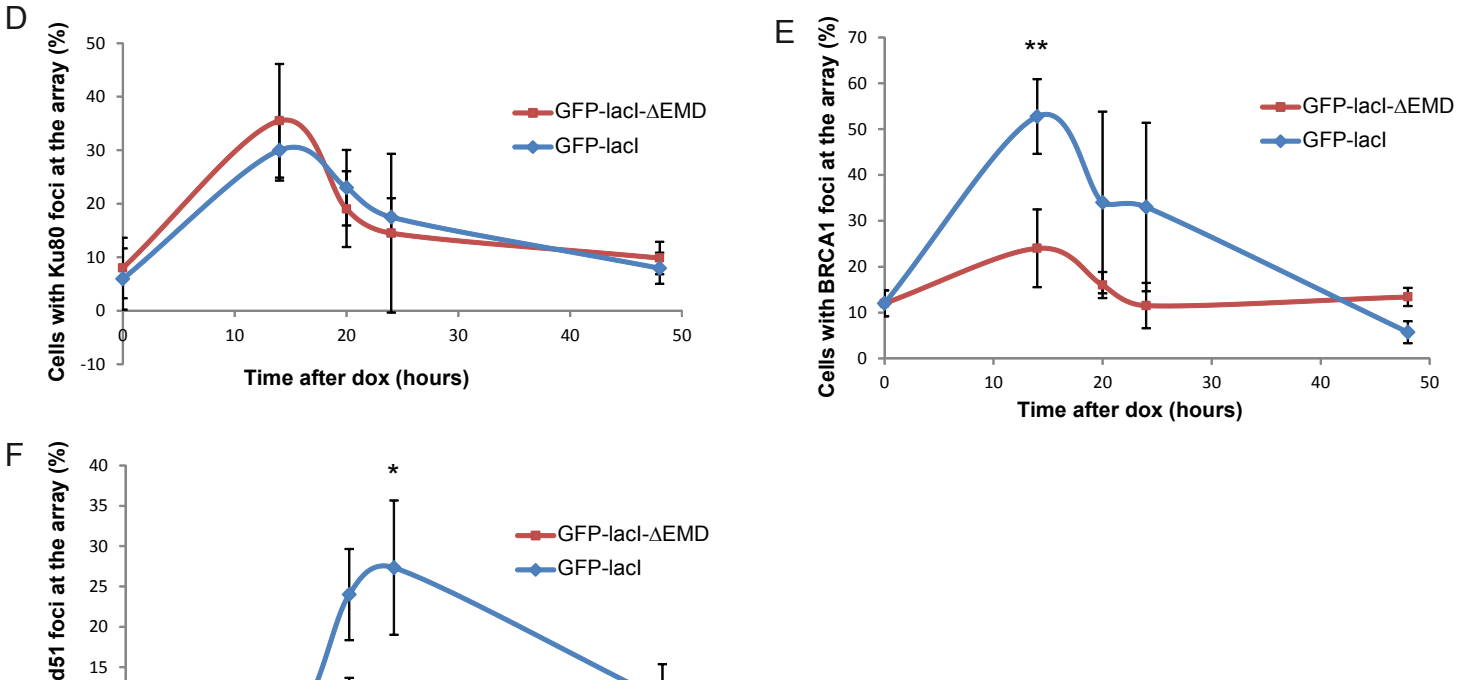

+IPTG
A
GFP-lacI
GFP-lacI-ΔEMD
-dox
+dox
FISH
laminB
merge
B
γH2AX


C
Cells with the array colocalizing with laminB (%)
GFP-lacI
GFP-lacI-ΔEMD
-I-SceI
+I-SceI

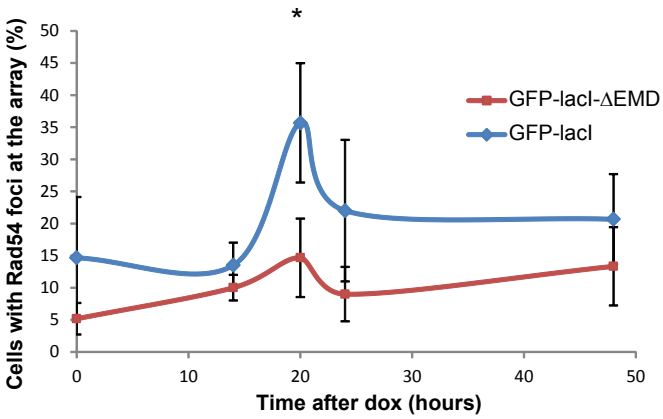

D
Cells with γH2AX foci at the array (%)
GFP-lacI
GFP-lacI-ΔEMD
-I-SceI
+


A
GFP-lacI
GFP-lacI-ΔEMD
-I-SceI
+I-SceI
FISH
KU80
laminB
merge
B
BRCA1
C
RAD51

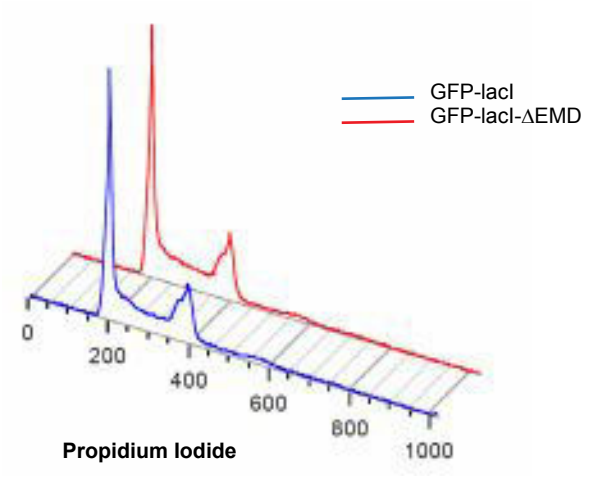

A
GFP-lacI
GFP-lacI-ΔEMD
-I-SceI
+I-SceI
FISH
Ku80
merge
B
BRCA1
C
RAD51

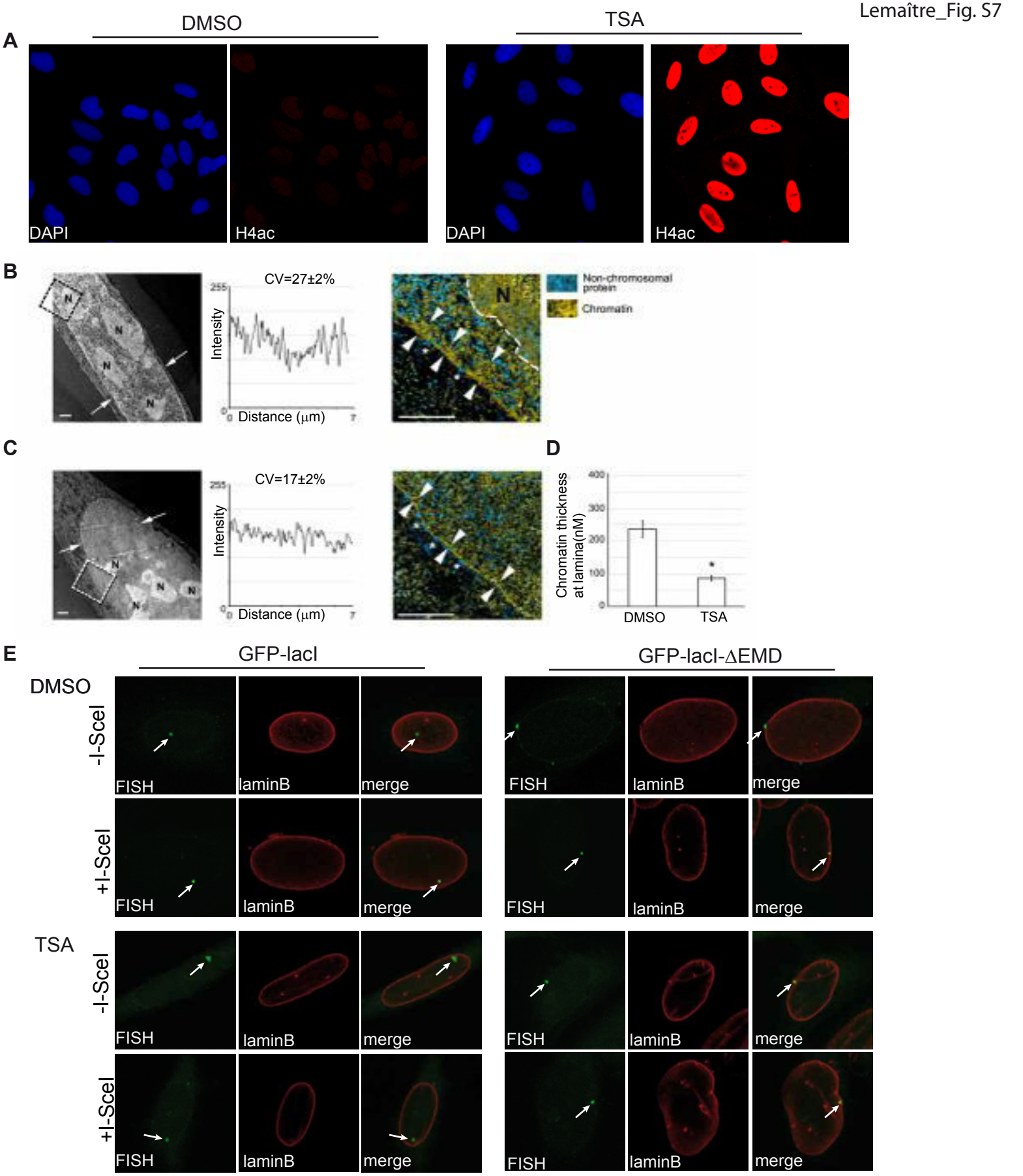

D
Cells with Ku80 foci at the array (%)
Time after dox (hours)
GFP-lacI-ΔEMD
GFP-lacI
E
**
Cells with BRCA1 foci at the array (%)
Time after dox (hours)
F
*
Cells with Rad51 foci at the array (%)
Time after dox (hours)

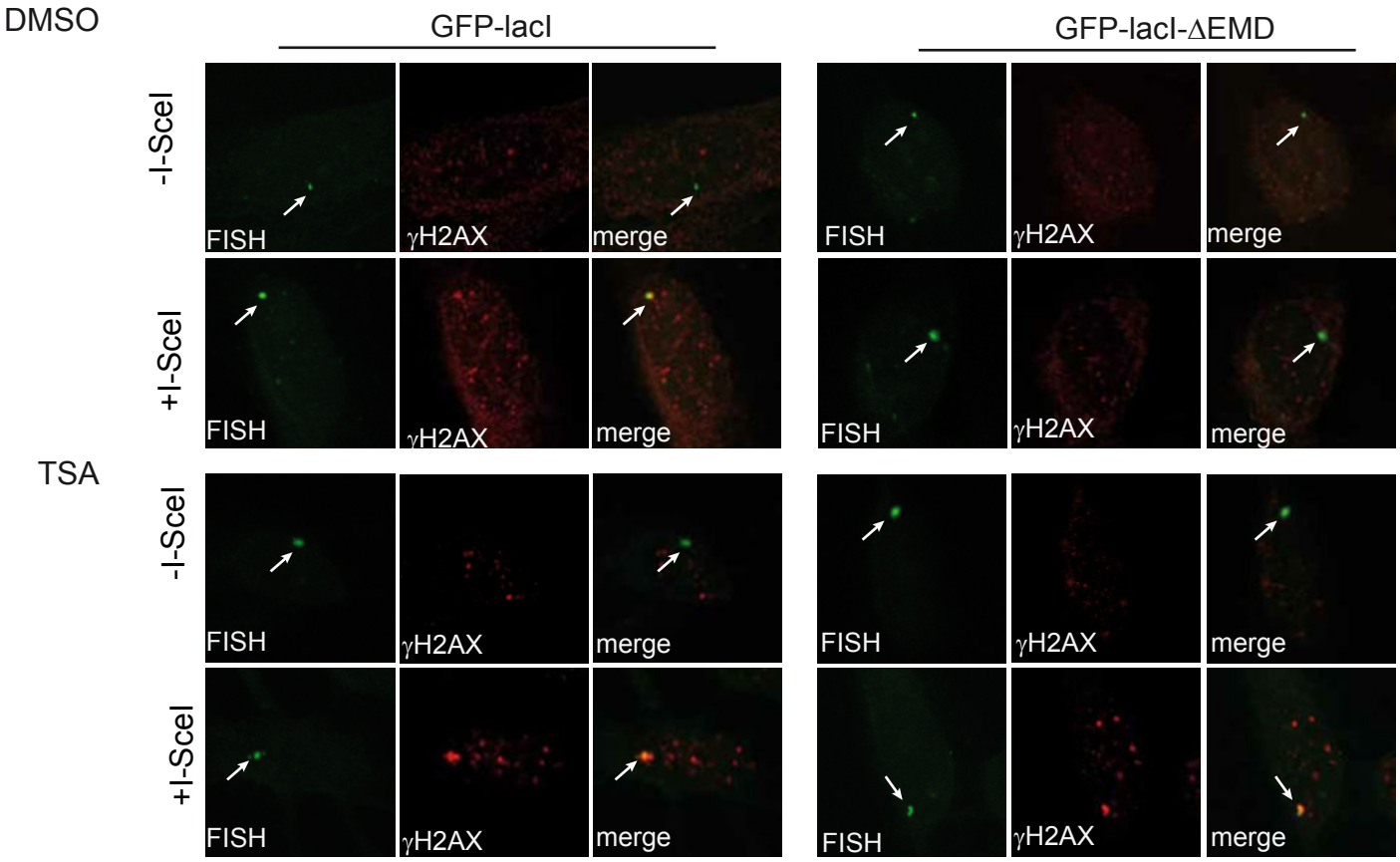
A

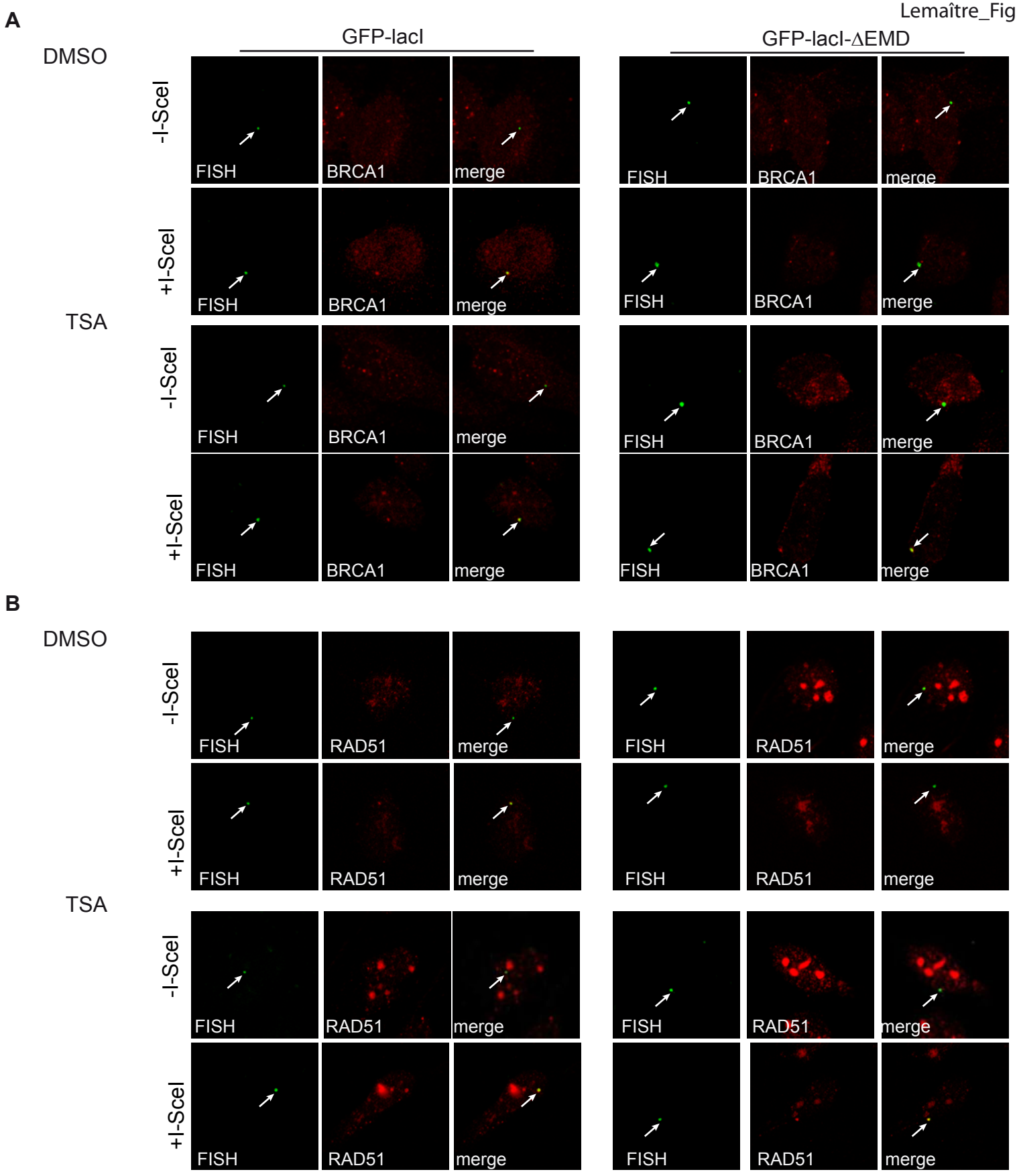

*
Cells with Rad54 foci at the array (%)
50
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
Time after dox (hours)
GFP-lacI-ΔEMD
GFP-lacI

B

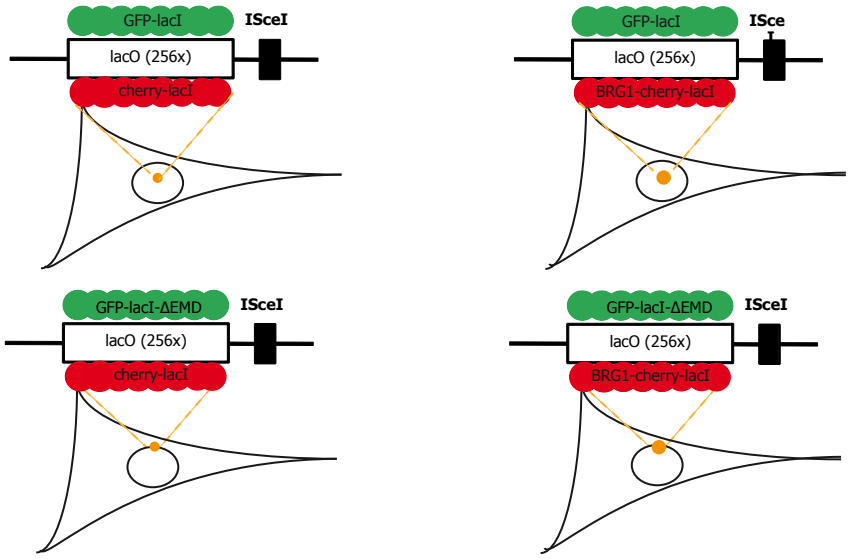

GFP-lacI
GFP-lacI-ΔEMD
0
200
400
600
800
1000
Propidium Iodide


A
DMSO
TSA
DAPI
H4ac
DAPI
H4ac
B
CV=27±2%
Intensity
Distance (μm)
Non-chromosomal protein
Chromatin
C
CV=17±2%
Intensity
Distance (μm)
D
Chromatin thickness at lamina(nM)
DMSO
TSA
E
GFP-lacI
GFP-lacI-ΔEMD
DMSO
TSA
-I-SceI
+I-SceI
FISH
laminB
merge
F
GFP-lacI-ΔEMD
GFP-lacI
Cells with the array colocalizing with laminB (%)
-I-SceI
+I-SceI
-I-SceI
+I-SceI
DMSO
TSA

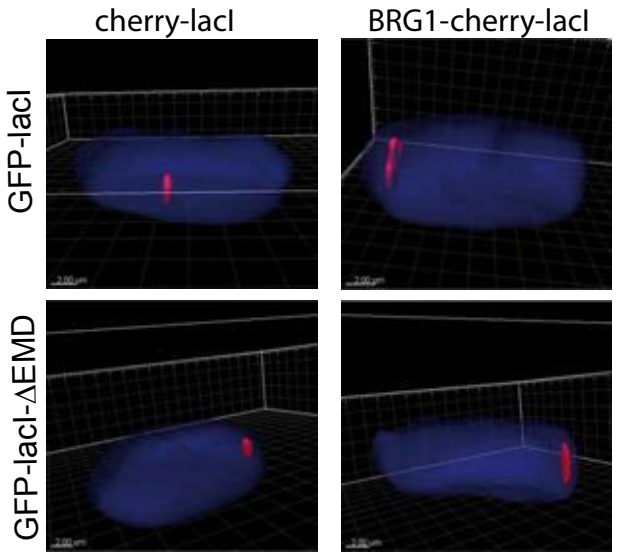

DMSO
TSA
GFP-lacI
GFP-lacI-ΔEMD
-I-SceI
+I-SceI
FISH
γH2AX
merge


A
GFP-lacI
GFP-lacI-ΔEMD
DMSO
TSA
-I-SceI
+I-SceI
FISH
BRCA1
merge
B
RAD51

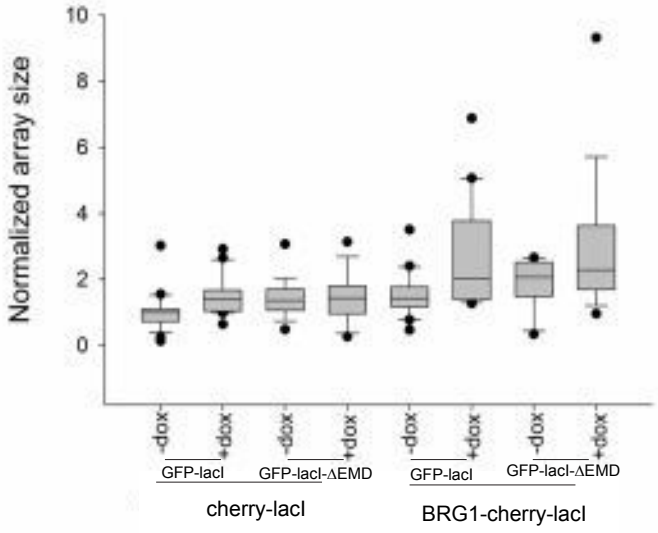
A

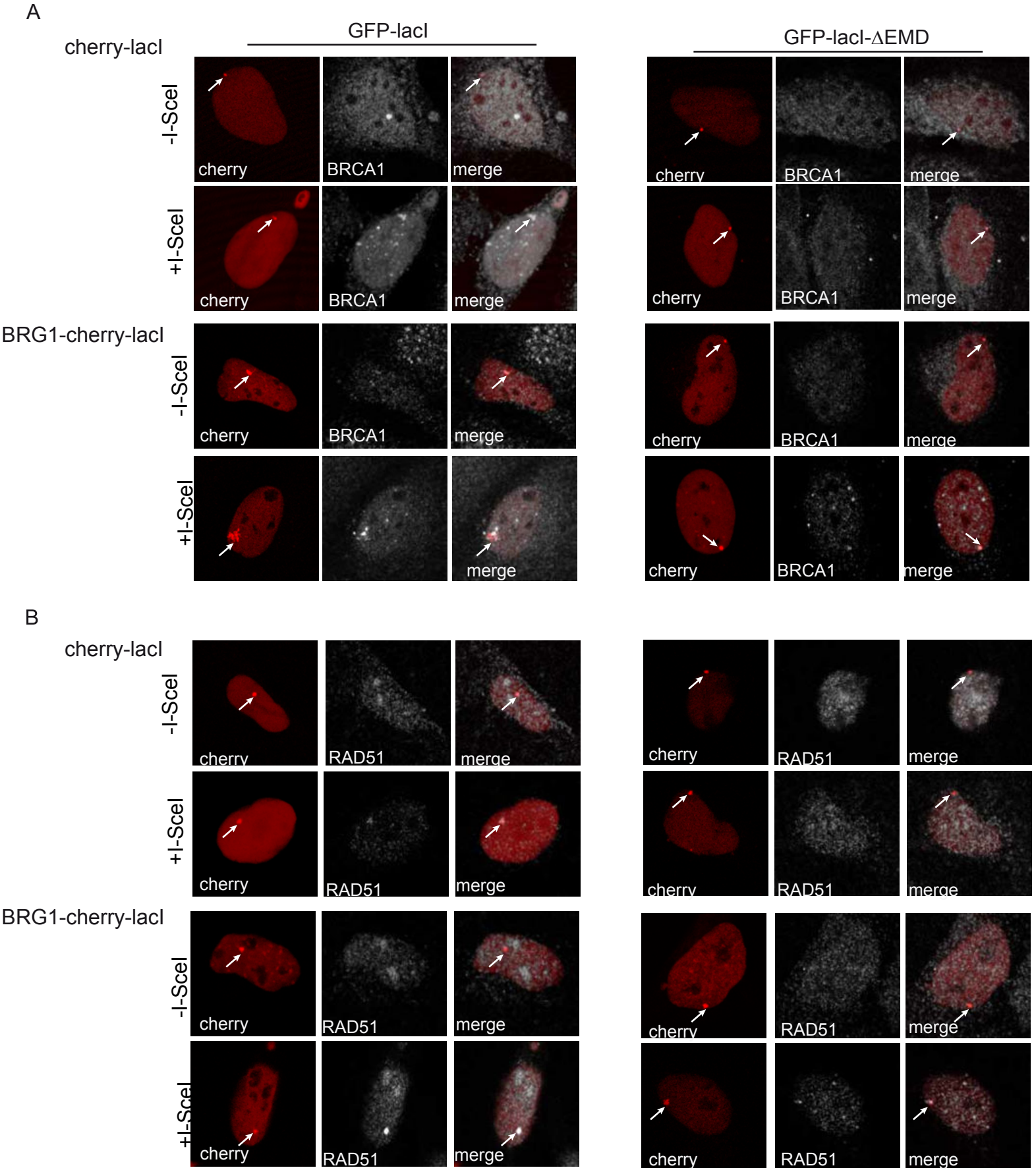

GFP-lacI
ISceI
lacO (256x)
cherry-lacI
GFP-lacI
ISce
lacO (256x)
BRG1-cherry-lacI
GFP-lacI-ΔEMD
ISceI
lacO (256x)
cherry-lacI
GFP-lacI-ΔEMD
ISceI
lacO (256x)
BRG1-cherry-lacI

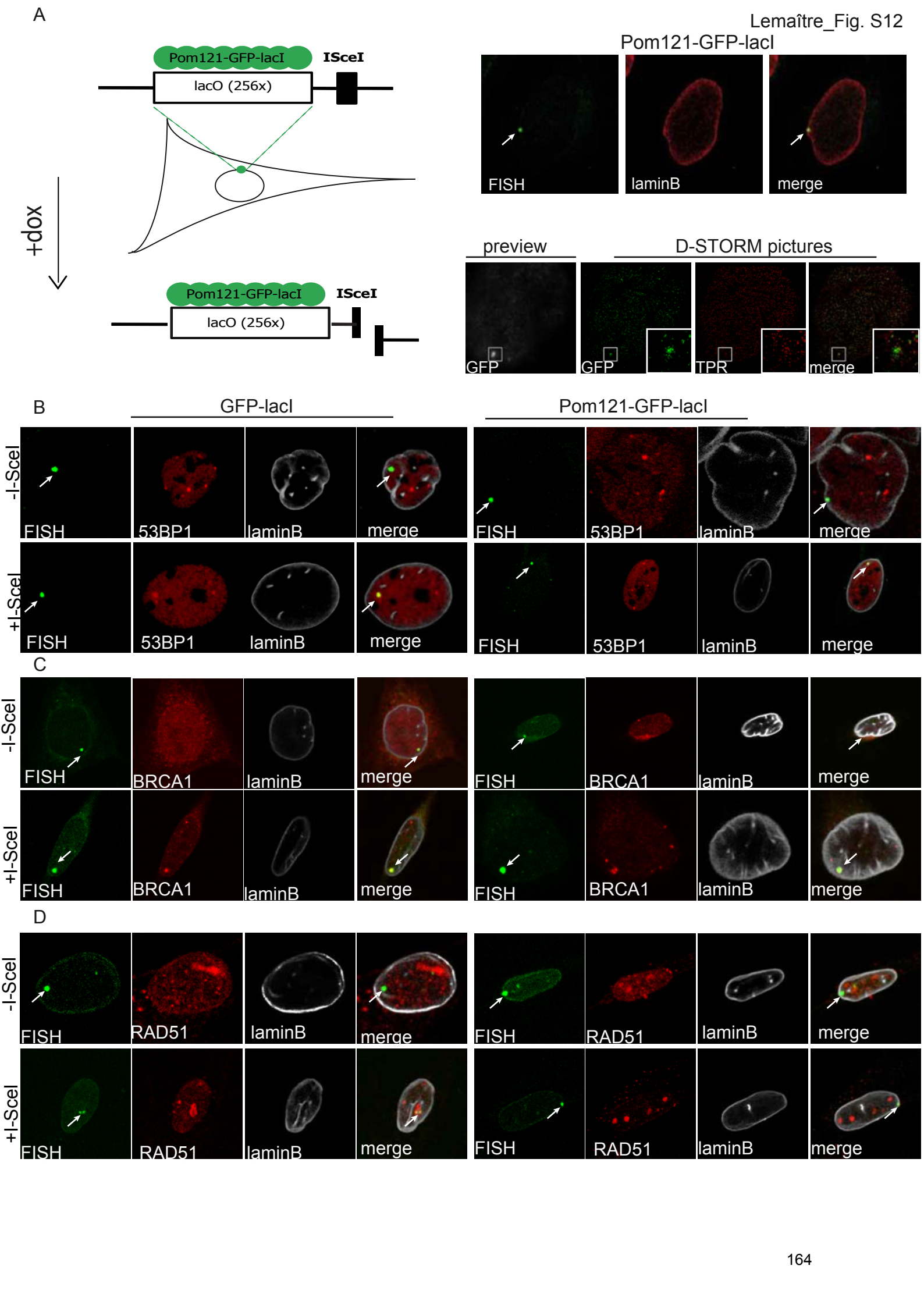
B

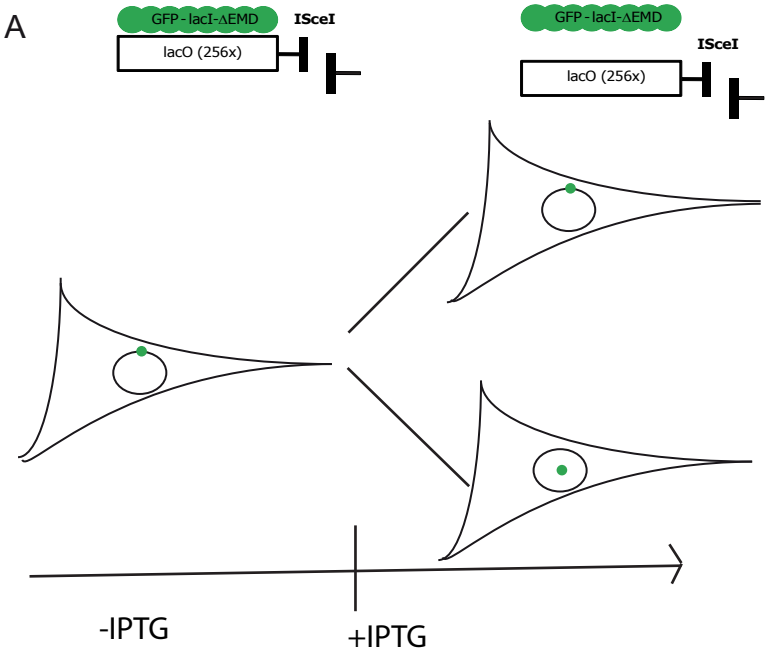

cherry-lacI
BRG1-cherry-lacI
GFP-lacI
GFP-lacI-ΔEMD

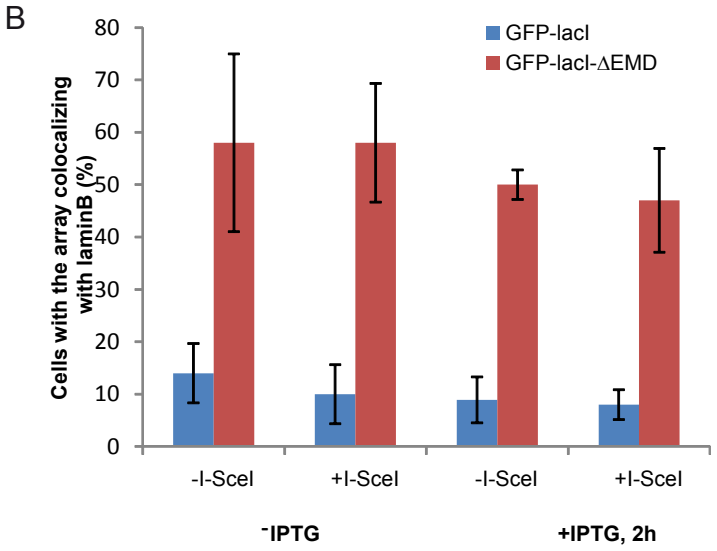
C

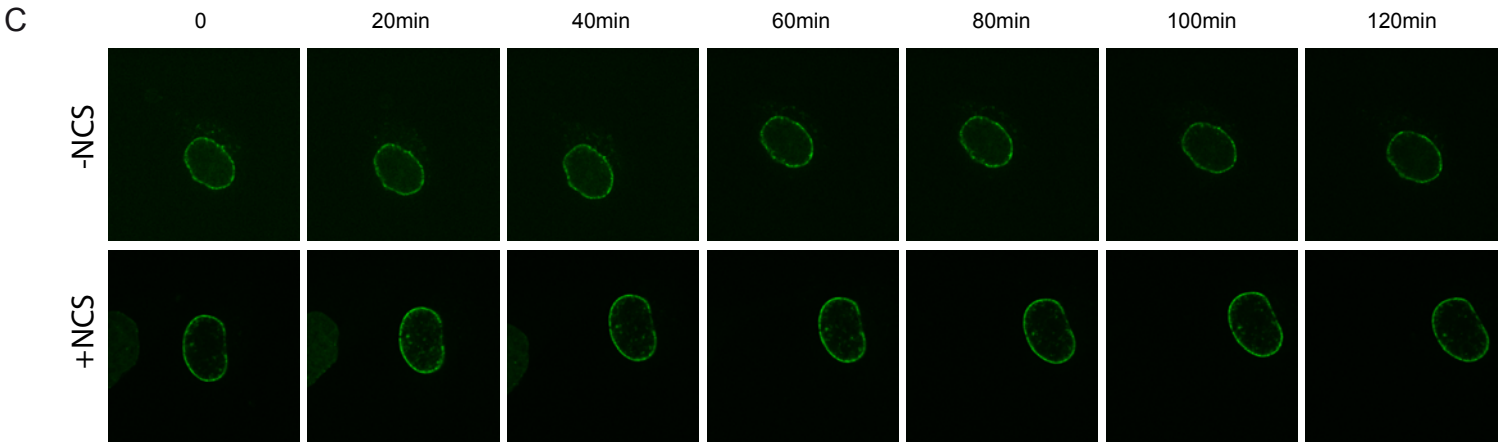

Normalized array size
10
8
6
4
2
0
-dox
+dox
GFP-lacI
GFP-lacI-ΔEMD
cherry-lacI
BRG1-cherry-lacI

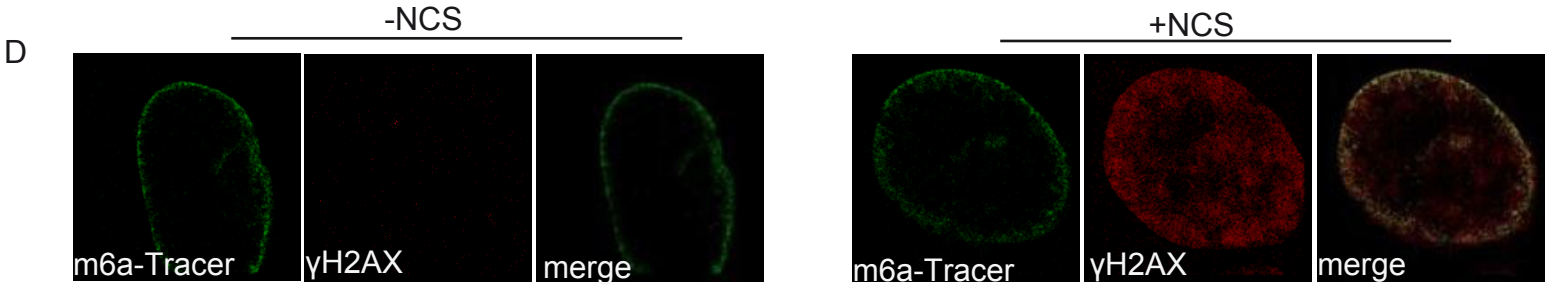

A
cherry-lacI
GFP-lacI
GFP-lacI-ΔEMD
-I-SceI
+I-SceI
BRG1-cherry-lacI
cherry
BRCA1
merge
B
RAD51


A
Pom121-GFP-lacI
ISceI
lacO (256x)
+dox
Pom121-GFP-lacI
ISceI
lacO (256x)
Pom121-GFP-lacI
FISH
laminB
merge
preview
D-STORM pictures
GFP
GFP
TPR
merge
B
GFP-lacI
Pom121-GFP-lacI
-I-SceI
+I-SceI
FISH
53BP1
laminB
merge
C
BRCA1
D
RAD51

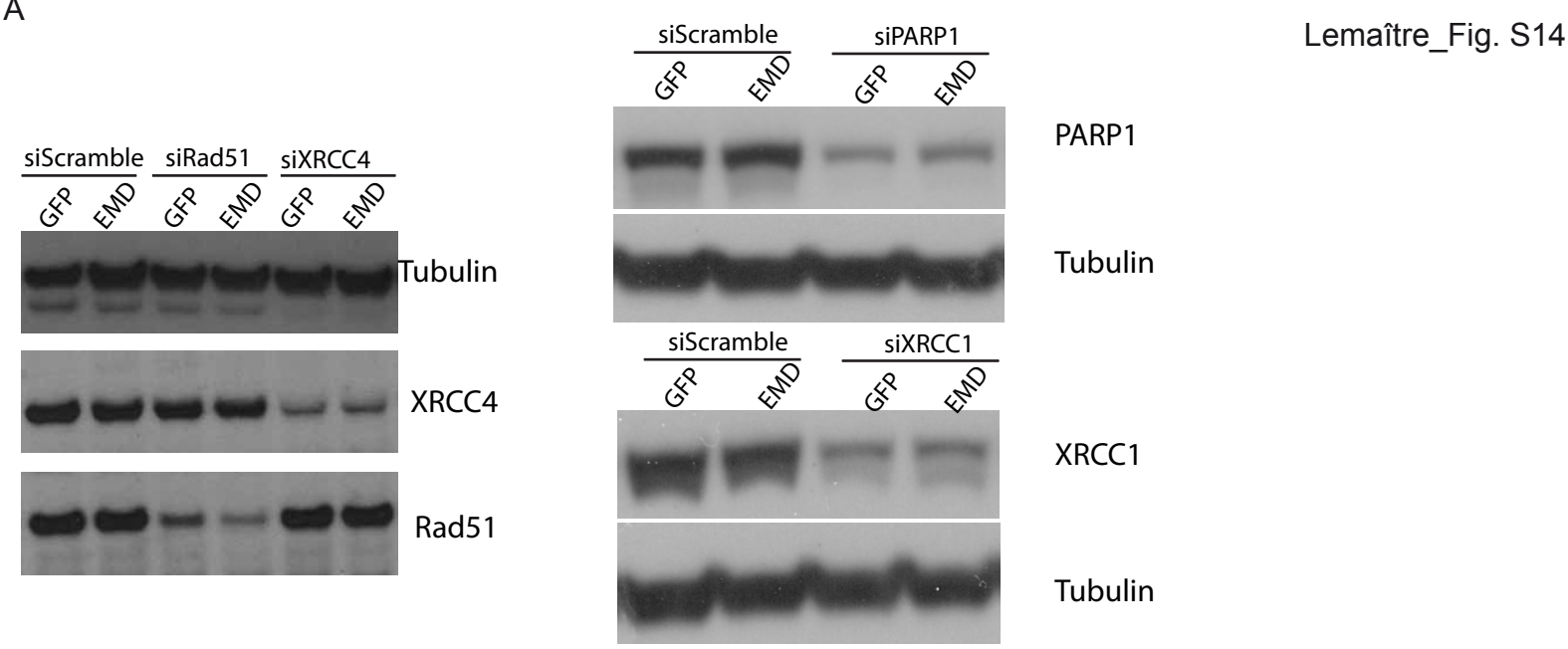

A
GFP-lacI-ΔEMD
ISceI
lacO (256x)
GFP-lacI-ΔEMD
ISceI
lacO (256x)
-IPTG
+IPTG


B
Cells with the array colocalizing with laminB (%)
GFP-lacI
GFP-lacI-ΔEMD
-I-SceI
+I-SceI
-I-SceI
+I-SceI
-IPTG
+IPTG, 2h


C
0
20min
40min
60min
80min
100min
120min
-NCS
+NCS


D
-NCS
+NCS
m6a-Tracer
γH2AX
merge
m6a-Tracer
γH2AX
merge

A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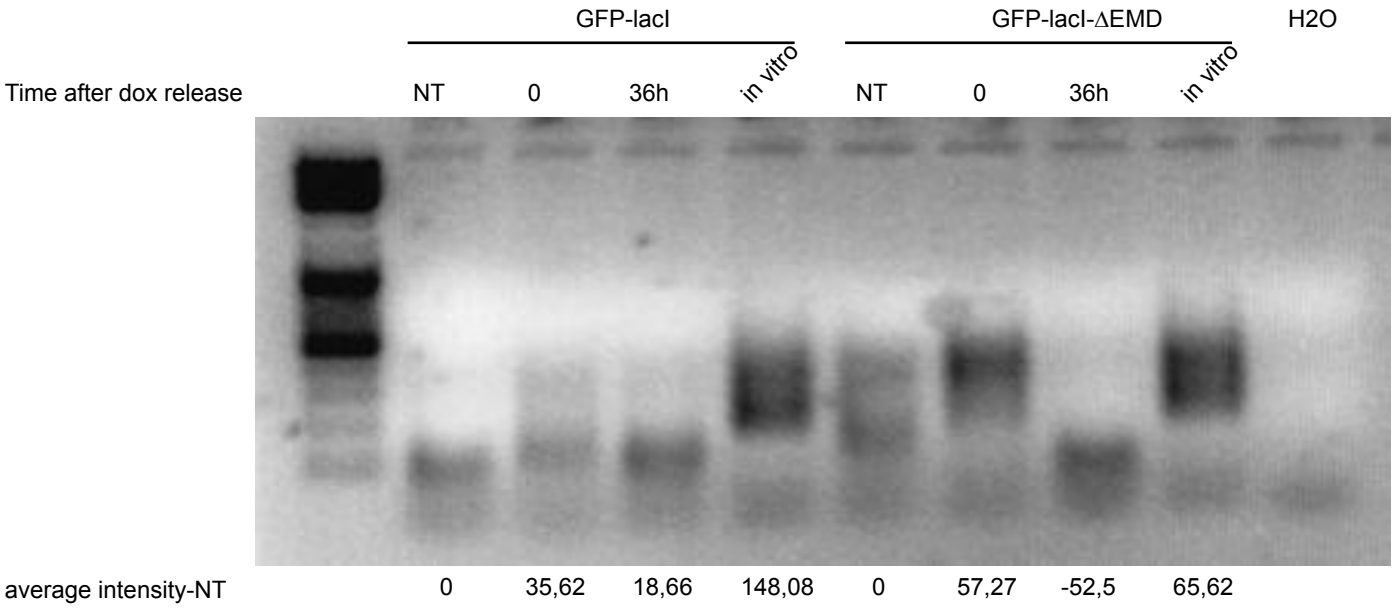
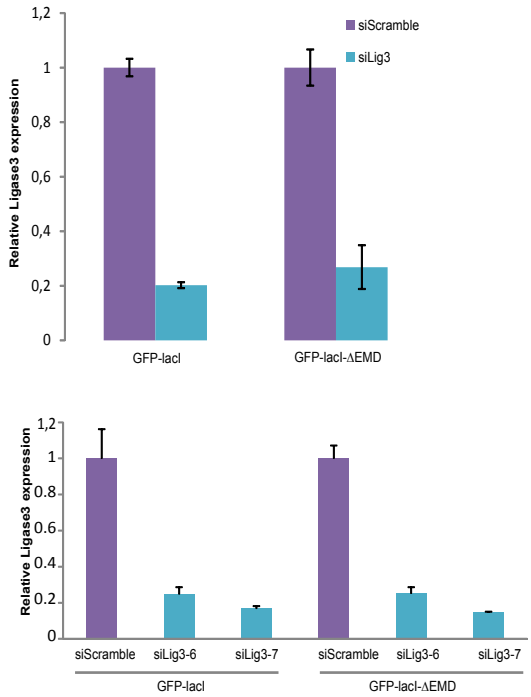
siRad51
siXRCC4
GFP
EMD
Tubulin
XRCC4
Rad51
siScramble
siPARP1
PARP1
Tubulin
siScramble
siXRCC1
XRCC1
Tubulin

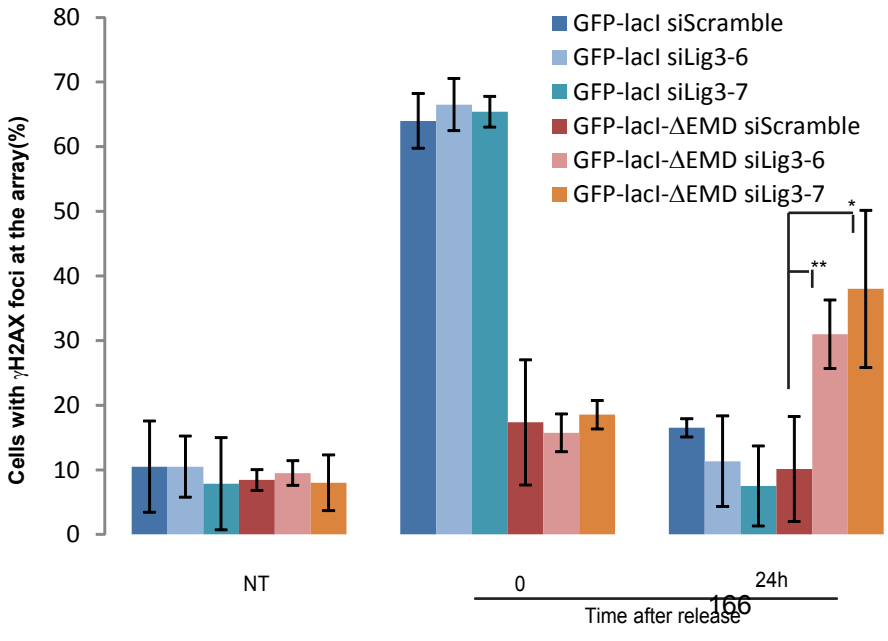
B


GFP-lacI
GFP-lacI-ΔEMD
H2O
Time after dox release
NT
0
36h
in vitro
average intensity-NT
0
35,62
18,66
148,08
0
57,27
-52,5
65,62

C


siScramble
siLig3
Relative Ligase3 expression
GFP-lacI
GFP-lacI-ΔEMD
siScramble
siLig3-6
siLig3-7
GFP-lacI
GFP-lacI-ΔEMD

D


Cells with γH2AX foci at the array(%)
GFP-lacI siScramble
GFP-lacI siLig3-6
GFP-lacI siLig3-7
GFP-lacI-ΔEMD siScramble
GFP-lacI-ΔEMD siLig3-6
GFP-lacI-ΔEMD siLig3-7
NT
0
24h
Time after release

2. Discussion and perspectives

My results demonstrate that DSB positioning at the nuclear lamina -in contrast to positioning at the nuclear pores or at the nuclear interior- delays DDR, inhibits HR but activates NHEJ and alt-EJ (summary in figure 36). In addition to showing the influence of gene positioning on DNA repair, they also raise several intriguing questions, whose answers would contribute to a better understanding of the mechanisms of DSB repair. In the following discussion, I'm addressing some of these questions, in light of the recent literature. In particular, I will discuss the potential mechanisms responsible for the inhibition of homologous recombination at the nuclear lamina, the interplay between DDR and repair pathways, the interplay between alt-EJ, HR and NHEJ, the role of alt-EJ in genomic instability and finally the extent, impact and regulation of DSB movement in mammals and DNA repair compartmentalization.

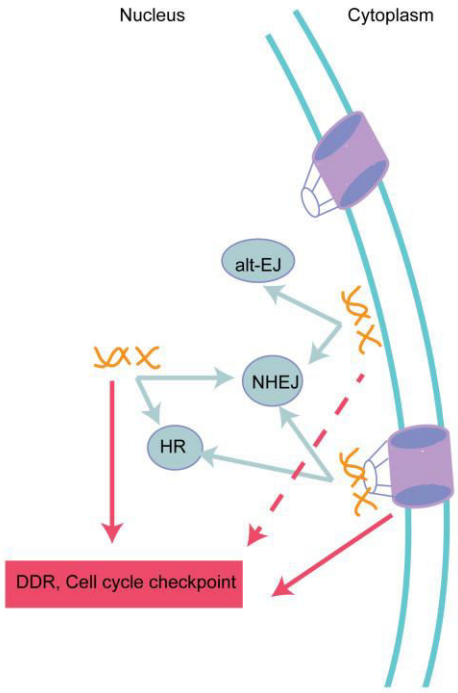


Figure 36- Nuclear position dictates DNA repair pathway choice

DSBs positioned at the inner nucleus or associated with nuclear pores (in purple) are repaired either by NHEJ or HR. They robustly activate DDR. On the contrary, DSBs positioned at the nuclear lamina are repaired by NHEJ or alt-EJ. The DDR activation is delayed. Consequences of this delay on cell cycle checkpoints activation remain to be investigated.

a) Mechanisms inhibiting homologous recombination at the nuclear lamina

Our results show that the state of chromatin at the nuclear lamina inhibits HR. However the exact mechanism underlying this inhibition remains unclear. Aymard

et al. showed that active transcription is necessary for resection, the first step of HR (Aymard et al., 2014) and one could hypothesize that the low transcription activity at the nuclear lamina might render this compartment unable to resect DSBs. However, we showed by ChIP that RPA is phosphorylated at breaks induced at the nuclear lamina whereas later factors of HR are not recruited. This result suggests that resection is active, at least partially. Furthermore the alt-EJ pathway that is active at the nuclear lamina is initiated by resection, also suggesting that resection is not dramatically affected.

Resection is the common initial step of HR and alt-EJ. However alt-EJ is usually associated with short-range resection whereas HR is associated with longer-range resection and the extent of resection might modulate the choice between these two pathways. Whereas the initiation of resection is mediated by the concerted action of MRN and CtIP, longer range resection involves additional nucleases such as Exo1 and DNA2 (Nimonkar et al., 2011). One of the possible hypothesis to explain the inhibition of HR at the nuclear lamina could be that long-range resection is inactive and it would be therefore interesting to study resection more in detail. To directly assess the extent of resection we could analyze the loading of RPA by ChIP using primers located at various distances from the break site or use a recently described method that allows the measurement of ssDNA at resected breaks by qPCR (Zhou et al., 2014). Another possibility would be to perform immuno-FISH allowing the visualization of RPA loading around the lacO array on chromatin fibers. We could also assess whether tethering of nucleases at the lacO locus would rescue HR at the nuclear lamina by fusing them to the lac repressor.

Another possible hypothesis is that the condensed state of chromatin at the nuclear lamina does not allow recruitment or proper polymerization of Rad51 filaments and it would therefore be interesting to assess Rad51 loading more in detail. We could for example test whether tethering of Rad51 at the lacO array could rescue HR or analyze recruitment of BRCA2.

Another intriguing hypothesis is that the delay in DDR constitutes a signal for alt-EJ to take place. Indeed one could imagine that the delay with which the breaks at the

lamina are sensed by the DDR machinery leads to a delay in recruitment of HR factors and cells sense these breaks as persistent breaks and channel them to alt-EJ pathway. This appealing hypothesis would however be difficult to test. Tethering of early DDR factors is sufficient to induce DDR activation even in absence of DSB (Soutoglou and Misteli, 2008). We could therefore induce DDR activation at the nuclear lamina and see whether this DDR activation is enough to rescue repair by HR. Consistantly with this hypothesis, HR efficiency is decreased upon ATM inhibitor treatment (Serrano et al., 2013).

b) Interplay between the DDR and the repair pathways

DDR is considered as a mechanism allowing the coordination between cell cycle progression and DNA repair. This function implies a crosstalk between DDR and the DNA repair pathways. However, we observe that NHEJ factors recruitment at the nuclear lamina has a similar kinetics than in the inner nucleus, although DDR is delayed. This result questions the interplay between DDR and NHEJ. Indeed, both the Ku complex and MRN are able to directly bind DNA ends, suggesting that they might act independently. Additionally, the role of MRN in HR suggests that the two complexes could compete for DSB binding. Therefore, how the cell cycle checkpoints are activated when breaks are channeled to the NHEJ pathway might not be via the classical DDR pathway. A possible hypothesis is that NHEJ is considered as a fast repair pathway and that once Ku is bound to the DNA the repair is almost immediate and therefore the checkpoints might not be activated in the absence of persistent breaks. Another possible hypothesis is that the role of DNAPK in DDR activation might be underestimated.

To fully assess the consequences of a delayed DDR on NHEJ we should study NHEJ efficiency using repair substrates tethered at the nuclear lamina. Indeed, we cannot rule out the possibility that although Ku recruitment is not affected, later steps of NHEJ could be impaired. Indeed, ATMi inhibition has been shown to decrease NHEJ efficiency (Rass et al., 2009).

c) Alt-EJ, an alternative for homologous recombination?

The alt-EJ pathway was identified in cells with genetic deficiencies for NHEJ factors (Boulton and Jackson, 1996; Kabotyanski et al., 1998; Liang and Jasin, 1996). It was therefore considered as a backup pathway for NHEJ. However, our results show that alt-EJ can be active in cells having intact NHEJ pathway, depending on the position where the break occurs. Furthermore, our data suggest that alt-EJ might repair breaks that would normally be repaired by HR if they were located in the inner nucleus. To further investigate whether alt-EJ constitutes an alternative to HR at the nuclear lamina it would be interesting to study cells in different cell cycle phases. Indeed, HR is mainly active during the S/G2 phases of the cell cycle, therefore if alt-EJ is an alternative to HR it should also occur preferentially in S/G2 cells.

Interestingly, a recent paper suggested that coating of ssDNA by RPA promotes HR and inhibits alt-EJ (Deng et al., 2014). RPA could therefore be a master regulator in the competition between alt-EJ and HR at resected breaks. Our results show that RPA phosphorylation at the nuclear lamina is not abolished but delayed and slightly reduced. This difference could be one of the mechanisms leading to alt-EJ use at the nuclear lamina.

d) Alt-EJ and genomic instability

Cells lacking some of the NHEJ factors can mediate rejoining of broken extrachromosomal DNA fragments (Kabotyanski et al., 1998; Liang and Jasin, 1996) or form junctions during V(D)J recombination (Blackwell et al., 1989; Bogue et al., 1997, 1998; Malynn et al., 1988) thanks to the use of the alt-EJ pathway. Analyses of the junctions formed by alt-EJ allowed the description of some of their features, including large deletions, microhomologies, and occasional insertions of large DNA fragments (Deriano and Roth, 2013). Furthermore, NHEJ-deficient mice that are also deficient for p53 develop pro-B cell lymphomas resulting from chromosomal translocations catalyzed by alt-EJ (Zhang et al., 2010). Cultured cells deficient for NHEJ factors also bear increased translocations (Boboila et al., 2010; Simsek and Jasin, 2010; Yan et al., 2007). Additionally, analyses of chromosomal translocations junctions in human tumors also revealed some of the features of alt-EJ mediated

junctions, further pointing to a role for alt-EJ in translocations formation and genomic instability (Stephens et al., 2009; Zhang and Rowley, 2006).

Therefore, the use of alt-EJ for the repair of DSBs at the nuclear lamina, whereas NHEJ is functional might seem dangerous for the maintenance of genomic stability. Furthermore, inhibition of HR, which is considered as the more error-free pathway could also seem hazardous to the cells. Several hypotheses to understand why cells use alt-EJ instead of HR at the nuclear lamina can be proposed. Indeed, HR was proposed to be deleterious in repetitive sequences, for example in the heterochromatic compartment of drosophila cells (Chiolo et al., 2011; Guirouilh-Barbat et al., 2014). Comparably to this compartment, nuclear lamina harbors numerous repetitive sequences (Guelen et al., 2008) in which the use of HR could also be deleterious. Additionally sequences at the nuclear lamina are late-replicating (Hiratani et al., 2010), which reduces the amount of time the sister chromatid is present. So far, no mechanism restricting the use of HR to situations where the sister chromatid is present was described. Totally avoiding the use of HR at the nuclear lamina might be a way to avoid its use in absence of sister chromatid, where it would lead to major genomic rearrangement. Furthermore, genes positioned at the nuclear lamina are mostly silent (Guelen et al., 2008), therefore, deletions induced by the alt-EJ pathway might be less deleterious in these regions than the major rearrangement that could be induced by HR.

e) DSB (im)mobility and DNA repair compartmentalization in mammalian cells

In yeast, DSB repair is compartmentalized: indeed, several DSBs can migrate to be repaired in specialized repair centres (Lisby et al., 2003). Further evidence for spatially restricted DSB repair in yeast comes from the observation that persistent breaks migrate from their internal nuclear position to the nuclear periphery, where they associate either with nuclear pores or with the nuclear envelope. Depending on their anchoring site, these DSBs are repaired with distinct mechanisms (Horigome et al., 2014). The immobility of DSBs in mammalian cells suggested that this DNA repair compartmentalization might not be conserved in mammalian cells, since all breaks must be repaired efficiently in various chromatin environments. However,

our results show that DNA repair compartmentalization is in fact conserved in mammalian cells. I discussed these aspects in a review submitted to the journal of molecular biology and the most recent version of it is reprinted in the following pages.

DSB (im)mobility and DNA repair compartmentalization in mammalian cells

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Abstract

Chromosomal translocations are considered as causal in approximately 20% of cancers. Therefore, understanding their mechanisms of formation is crucial in the prevention of carcinogenesis. The first step of translocation formation is the concomitant occurrence of double-strand DNA breaks (DSBs) in two different chromosomes. DSBs can be repaired by different repair mechanisms, including error-free homologous recombination (HR), potentially error-prone non-homologous end joining (NHEJ), and the highly mutagenic alternative end joining (alt-EJ) pathways. Regulation of the DNA repair pathway choice is crucial to avoid genomic instability. In yeast, DSBs are mobile and can scan the entire nucleus to be repaired in specialized DNA repair centers or if they are persistent, to associate with the nuclear pores or the nuclear envelope where they can be repaired by specialized repair pathways. However, in mammalian cells it is quite the reverse. DSB mobility is limited, therefore raising the question of whether the position at which a DSB occurs, influences its repair. Here, we review the recent literature addressing this question. We first present the reports describing the extent of DSB mobility (or stability) in mammalian cells. In a second part we discuss the consequences of non-random gene positioning on chromosomal translocations formation. In the third part, we discuss the mobility of heterochromatic DSBs in light of our recent data on DSB repair at the nuclear lamina, and finally we show that DSB repair compartmentalization at the nuclear periphery is conserved from yeast to mammals, further pointing to a role for gene positioning in the outcome of DSB repair. When regarded as a whole, the different studies reviewed here demonstrate the importance of nuclear architecture on DSB repair and reveal gene positioning as an important parameter in the study of tumorigenesis.

Nuclear architecture is a key regulator of DNA transactions such as transcription, replication or repair[1]. Nuclear architecture comprises high-ordered chromatin structure, nuclear compartmentalization, non-random spatial genome organization and their interconnection[2]. Eukaryotic nuclei encompass various nuclear compartments, defined by a specific subset of proteins and an associated function. Best-studied nuclear compartments include the nuclear lamina, nucleoli, cajal bodies, PML bodies, and nuclear speckles[3–6]. One of the mechanisms governing genome organization is the association with nuclear compartments[7], which participates in the regulation of DNA metabolism. For example, artificial tethering of certain genes to the nuclear lamina can lead to transcriptional repression[8–11] and gene positioning can be correlated to replication timing[12]. Additionally, components of the transcription machinery have a non-homogeneous distribution in the nucleoplasm[13,14], suggesting that genes should have the potential to move towards or away from this transcription machinery to be regulated. In line with this hypothesis, hundreds of genes are relocated to and from the nuclear periphery during early stages of development and their position is correlated with their expression levels, with repressed genes located at the nuclear periphery and active genes located in the interior of the nucleus[15]. On the other hand, chromatin mobility is constrained by its physical properties and its compaction, and similar limited mobility with a diffusion coefficient ranging from 10^{-4} to 10^{-3} $\mu m^2/s$ was observed in all organisms from bacteria to mammals, regardless of the size of the nucleus[16,17]. This observation raises the question whether chromatin mobility has a significant impact in mammalian cells since a typical 1 μm locus movement represents only 1/10th of the mammalian

nucleus, whereas it represents the entire nucleus in yeast[18]. Of particular interest, chromatin mobility upon DSB induction has been shown to increase in yeast in order to promote repair whereas DSB mobility in mammals is debated[19]. Here, we review the recent literature explaining the extent, impact, and regulation of DSB mobility in mammalian cells. We further analyze the involvement of DSB movement in the formation of translocations and the prevention of recombination in heterochromatin. Finally, we discuss our recent data demonstrating compartmentalization of homologous recombination (HR) and non-homologous end joining (NHEJ) in mammalian cells.

DNA repair centers and (im)mobility of DSBs in mammalian cells

The existence of different nuclear compartments raises the question of whether there are specialized centers for DNA repair in the nucleus and whether DSBs move towards these centers. In *S. cerevisiae*, several DSBs exert increased mobility (see[19] for review) and cluster to form repair centers[20] (figure 1, left panel). Yet, in mammalian cells, DSBs movement and the existence of repair centers are debated. Although the formation of repair centers was observed upon exposure of cells to α particles[21] and tracking of repair foci marked by the repair factor 53BP1 showed higher mobility upon ionizing irradiation[22], DSBs induced by ultrasoft x-rays in human fibroblasts did not show major movement[23]. Furthermore, DSBs induction by laser micro irradiation or γ-irradiation triggers local chromatin decompaction as visualized by extension of chromatin domain but does not display large-scale mobility[24] and the induction of multiple damaged sites didn't lead to the formation of repair clusters as proposed in yeast[24] (figure 1). More precise studies of DSB mobility were permitted

by the development of GFP-lac repressor (LacI) fusion proteins that binds to lac operator (lacO) arrays integrated in the genome of the studied organism[25]. This method allows for the precise tracking of the tagged locus and analysis of its movement over time. The adaptation of this experimental system in mammalian cells demonstrated that DSBs induced by the I-SceI endonuclease are positionally stable[26]. Simultaneous tagging of the two broken chromosome-ends showed very limited end separation and no significant increase in mobility upon induction of DSBs[26,27]. Additionally, when multiple DSBs were induced simultaneously they didn't migrate to common repair centers, even when initially separated by less than 400 nm[26]. Interestingly, DSB mobility and end-separation was increased upon knock-down of Ku80[26], a factor from the NHEJ repair pathway, suggesting an active inhibition of DSB motion in mammalian cells.

A first hypothesis to explain the apparent discrepancy between results obtained in yeast and mammals is the difference in the size of the nucleus. Indeed, analysis of local motion of chromatin showed similar results regardless of the organism or the size of its nucleus[16,17]. Therefore, an increased movement of 1 μm that represents the exploration of the full nucleus in yeast cells is considered as limited motion in mammalian cells and might have different functional implications[18]. Another persuasive hypothesis is that DSB motion is actively inhibited in mammalian cells, as increased DSB mobility was correlated with a higher translocation rate[27]. A first mechanism underlying DSB mobility restriction in mammalian cells might come from the predominant use of NHEJ. Indeed, NHEJ is the major repair pathway that can be used in any phase of the cell cycle in mammalian cells. Yet, HR is restricted to

the S/G2 phases of the cell cycle and uses the homologous sister chromatid as a template for repair. In yeast, the major repair pathway is HR and recombination can happen between sites on different chromosomes[28]. The increased DSB mobility observed in yeast was proposed to facilitate the homology search[29], which is considered as the major rate-limiting step of HR[30] and repair by recombination correlates positively with DSB mobility[31]. Conversely, HR was proposed to be required for the enhanced mobility of DSBs[32]. Therefore, enhanced DSB mobility seems associated with and regulated by HR, which is likely when using templates other than the sister chromatid, as supported by the fact that cohesion, which helps maintaining sister chromatids together[33], restricts DSB movement[34]. In mammalian cells, this situation is very rare and could therefore explain the observed difference. Additionally, NHEJ might directly inhibit DSB movement, as demonstrated by the fact that Ku80 depletion leads to increased mobility[26]. Furthermore, lamin A, which is absent in yeast cells, has recently been identified as an additional factor inhibiting DSB movement in mammalian cells[35], providing further evidence for active inhibition of DSB mobility in mammalian cells.

DSB movement in chromosomal translocations

Translocation formation involves simultaneous occurrence of DSBs in different chromosomal locations[36], physical contact between these DSBs and illegitimate joining of ends[37]. Tumor analysis showed that translocations are often formed by the same translocation partners, suggesting that DSBs pairing is not random. Two main models have been put forward to explain the formation of frequent tumorigenic translocations: the "breakage-first" and "contact-first" hypotheses[38]. The breakage-

first hypothesis postulates that DSBs occur first and then explore the nuclear space to find their translocation partners. In that case, translocations between distant loci could eventually occur. The contact-first hypothesis, however, postulates that translocations occur between loci that were in spatial proximity before the breaks occur. The non-random chromosomal positioning within the nucleus and the immobility of DSBs argue in favor of the contact-first hypothesis and correlations between tissue-specific chromosome locations and tissue-specific translocations have been observed[39]. Genome-wide mapping studies further established the correlation between loci proximity and translocation rate[40,41]. However, some exceptions to this model have been observed. Indeed, Myc-Igh translocations were shown to occur at the same frequency regardless of the position of the Igh locus in the genome[42]. On the other hand, MYC and IGH genes are thought to be transcribed in the same transcription factory, which may contribute to the formation of translocations by retaining these translocations partners together[43]. It would therefore be interesting to see if the IGH loci artificially introduced at various locations of the genome are still maintained in the same transcription factory as MYC, and therefore still maintained in a spatial proximity during transcription. Recently, the observation of translocation formation in living cells[27] allowed the two models to be combined. Indeed, although DSBs were generally positionally stable, the ones that ultimately led to chromosomal translocations showed slightly increased mobility. In accordance with the contact-first hypothesis, the authors demonstrated that more than 80% of translocations occurred when the loci were initially separated by less than 2.5 μm. Nonetheless, a minority of translocations could form between loci separated by more than 5 μm,

therefore suggesting that different mechanisms could give rise to translocations and that the two models might both apply. Therefore, active restriction of DSB mobility in mammalian cells might constitute a protective mechanism against the formation of chromosomal translocations.

Movement of heterochromatic DSBs

The various range of chromatin compaction challenges DNA repair that has to be equally efficient in all chromatin contexts. In particular, the highly compact heterochromatin was proposed to constitute a barrier for DDR and DSB repair[44,45]. Recently, DNA repair in heterochromatin compartments has been associated with DSB movement. Indeed, breaks induced in the heterochromatic domain of drosophila cells rapidly accumulate the early markers of DDR and activate the early steps of HR repair within the domain. However, this step is followed by expansion of the heterochromatic domain and relocalization of breaks outside of the domain, where HR is completed[46]. The authors propose that this relocalization is occurring to avoid recombination between repetitive sequences that are often found in heterochromatin[46]. A similar DSB relocation was observed in mouse cells upon break induction by linear ion tracks in the heterochromatic compartments known as chromocenters[47] (figure 1). Whereas HR is the major pathway used for DSB repair in drosophila[48], in mouse cells both HR and NHEJ are major repair pathways. Therefore, it would be interesting to know which mechanism repairs the relocated breaks in mouse cells and whether this relocation is cell cycle dependent and would happen only to breaks ultimately repaired by HR during the S/G2 phase of the cell cycle. In agreement with this, relocation of heterochromatic DSBs in drosophila

depends on the HR factors[46], as proposed for DSBs mobility in yeast[32]. Furthermore, it would also be in agreement with the inhibitory role of the NHEJ protein KU80 in DSB mobility[26].

Relocation of heterochromatic DSBs was accompanied by expansion of the domain[46,47], suggesting that chromatin decompaction is at the origin of the observed DSBs movement as suggested in yeast[31]. The relocation of DSBs at the periphery of chromocenters in mouse cells is reminiscent of the fact that replication of major satellites in mice cells happens at the periphery of the chromocenters[49,50], therefore raising the question whether a common mechanism underlies the two phenomenon especially since common proteins are involved in the two processes.

These studies raise the question whether DSBs induced in all heterochromatic compartments are subjected to relocation outside of the compartment and are repaired by HR. To address this question, we developed an experimental system allowing us to specifically induce a DSB at the nuclear lamina, at the nuclear pores or in the inner nucleus and compared their fate. We showed that breaks specifically induced at the nuclear lamina, which is also considered as a heterochromatic compartment, in human cells do not relocate outside of the compartment[51]. It would thus be interesting to induce breaks in other heterochromatic compartments in human cells (e.g. in the heterochromatin located around the nucleolus) to understand whether heterochromatic breaks relocation is conserved in humans.

Interestingly, we observed that HR is inhibited at the nuclear lamina[51], suggesting that indeed an evolutionary pressure exists to avoid recombination in repetitive

sequences. Yet, the ways HR is inhibited within heterochromatin compartments seem to be variable, acting through increased mobility and relocation of the breaks or direct inhibition of HR[51]. A hypothesis to explain why these different heterochromatic compartments behave differently might come from the fact that the chromatin composition itself might differ between them. Indeed, the classification of chromatin types into two classes (euchromatin and heterochromatin) is an oversimplification and they can be divided in different subtypes, suggesting that there could be several classes of heterochromatin. In drosophila for example, 5 chromatin types were identified based on their combination of chromatin-associated proteins and histone marks[52]. In mammalian cells, the situation seems more complicated and 51 chromatin types were identified in human lymphocytes[53]. For example, while chromocenters in mouse cells are associated with the tri-methylation of the histone H3 on lysine 9 (H3K9me3), heterochromatin at the nuclear lamina is associated with the dimethylation of H3K9 (H3K9me2). Additionally, chromatin of the chromocenters is associated with the histone variant CENPA, which is supposed to form more flexible nucleosomes than the canonical H3[54]. Therefore, these differences in nucleosome composition or histone modifications might explain the various ways are used to avoid recombination between repetitive sequences. Additionally, the association of loci with the nucleolus or the nuclear envelope was shown to limit their mobility[55], eventually explaining the absence of DSB relocation at the nuclear lamina and the use of a different mechanism in this compartment.

Compartmentalization of DNA repair at the nuclear periphery

Another type of DSB movement observed in yeast is the accumulation of persistent DSBs at the nuclear periphery. These breaks can be anchored to the two compartments of the nuclear periphery, the nuclear pores via interaction with the Nup84 complex and the inner nuclear membrane (INM) via interaction with the Mps3 protein[56–59]. A recent study demonstrated that the association with the two different compartments leads to different repair outcomes[60]. Indeed, breaks associated with the nuclear pores are repaired by the BIR or microhomology-mediated error-prone mechanisms, whereas breaks associated with the inner nuclear membrane are repaired by the error-free HR pathway[60] (figure 1, left panel). Due to limited DSB mobility, persistent breaks in mammalian cells do not migrate to the nuclear periphery. However, we recently showed that DNA repair pathway spatial segregation is conserved across species. Indeed, breaks induced at the nuclear pores or at the inner nucleus were repaired by both NHEJ and HR pathways, whereas breaks induced at the nuclear lamina were unable to recruit HR factors and were instead repaired by NHEJ or by the error-prone mechanism - alternative end-joining (alt-EJ) (figure 2). The exact mechanism by which HR is repressed at the nuclear lamina remains to be characterized but the highly compacted state of chromatin at the nuclear lamina is one of the factors involved in this inhibition and global as well as local induction of chromatin decompaction was sufficient to rescue HR at the nuclear lamina. We therefore demonstrated that gene positioning determines DNA repair pathway choice in mammals[51].

Moreover, we showed that the resection initiation can still occur at the nuclear lamina but the later steps of HR are not completed[51]. It's likely that NHEJ cannot occur once resection has happened and that is probably the reason why alt-EJ takes place. In that case, alt-EJ would only repair breaks that were supposed to be repaired by HR. It would therefore be interesting to test whether alt-EJ is only activated in the S/G2 phase of the cell cycle or whether it can occur at any phase of the cell cycle. Therefore, our study demonstrates the use of alt-EJ under physiological conditions, when the NHEJ pathway is fully functional[51]. However, alt-EJ is considered as a highly mutagenic repair pathway; hence its regular use should lead to important genomic instability and high variability of the sequences associated with the nuclear lamina. In line with this hypothesis, lamina-associated domains (LADs) have a highly conserved size and genomic position but their overall sequence conservation is low[61]. Moreover, the alt-EJ pathway has been involved in the formation of chromosomal translocations[62–64], therefore it would be interesting to study whether loci positioned at the nuclear periphery show an increased translocation rate and consequently might be a new parameter to take in account in the study of tumorigenesis. On the other hand, additional mechanisms might eventually inhibit translocation formation at the nuclear lamina for example through the inhibition of DSB movement by the association with the nuclear envelope[55].

Conclusion

Recent literature shows that, unlike what has been shown in yeast, DSB movement in mammalian cells is actively inhibited and this limited mobility prevents translocation formation. However, DSB mobility can also be increased in some instances, for

example during repair of heterochromatic DSBs in mice chromocenters or during the formation of long-distance translocations. The mechanisms regulating DSB mobility in mammals include active inhibition of movement by the NHEJ protein KU80. Nevertheless, the signals leading to increased mobility of heterochromatic DSBs or during the formation of translocations remain unknown. On the other hand, our recent work demonstrates that similarly to yeast, DSB repair is compartmentalized and the choice of the repair pathway to be used can be regulated by the location of the break. Therefore, studying how nuclear positioning is determined during development may be essential in the understanding of tumorigenesis.

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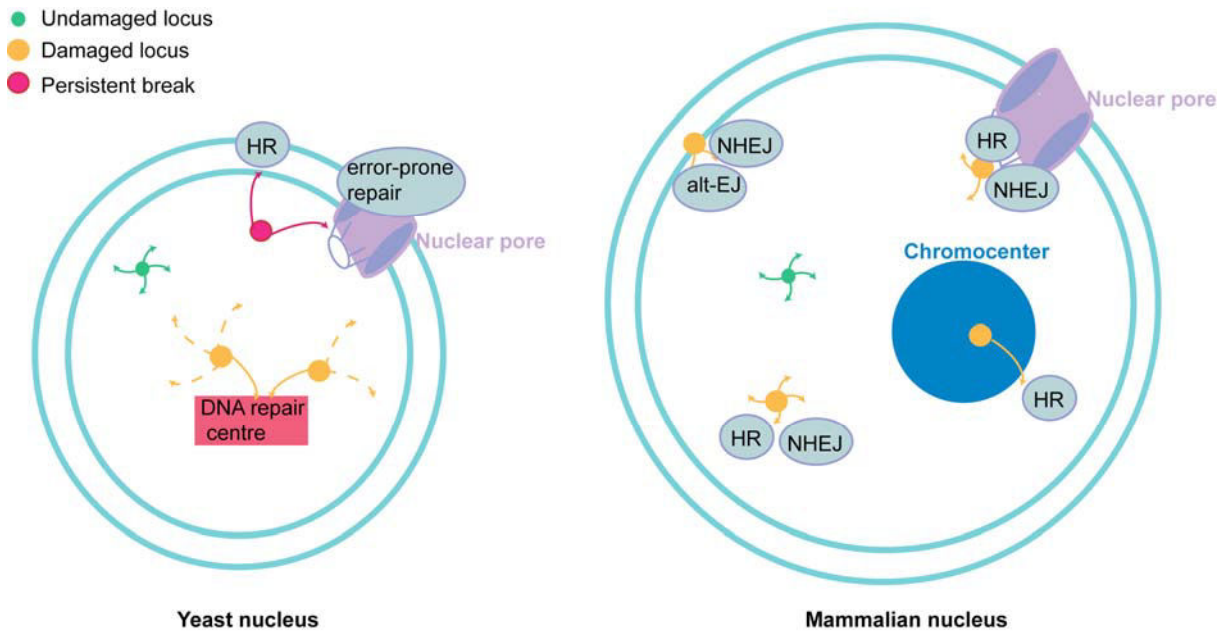

Figure 1- DSB mobility in yeast and mammalian cells
In yeast (left), DSBs (in yellow) exert higher mobility (represented as arrows) than an undamaged locus (in green). Several DSBs can meet in a single DNA repair centre. Persistent DSBs (in pink) can associate with the nuclear membrane where they are repaired by HR or with the nuclear pore where they are repaired by error-prone mechanisms.

In mammalian cells (right), undamaged loci (in green) exert the same mobility than yeast loci. The mobility is not increased upon DSB (in yellow) and breaks are repaired individually by HR or NHEJ. Breaks occuring in heterochromatic compartments such as chromocentres (dark blue) can move outside of the compartment where they might be repaired by HR. Breaks induced at the nuclear lamina do not move towards the inside of the nucleus and are repaired in situ by NHEJ or alt-EJ. Breaks induced at the nuclear pores are repaired by HR or NHEJ.

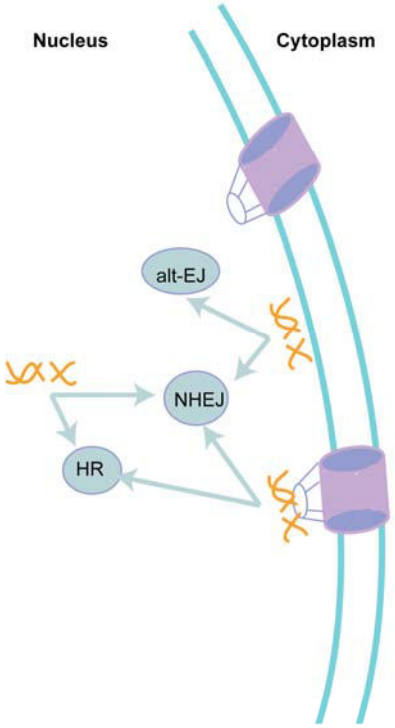

Figure 2- Nuclear position dictates DNA repair pathway choice
DSBs positioned at the inner nucleus or associated with nuclear pores (in purple) are repaired either by NHEJ or HR. On the contrary, DSBs positioned at the nuclear lamina (in blue) are repaired by NHEJ or alt-EJ.

DNA plasticity is crucial to allow genome evolution, diversity during meiosis and for the establishment of the immune repertoire. DNA lesions are, on the other hand, deleterious to the cells, because they cause mutations and rearrangements that can trigger cancer or various other pathologies (see introduction).

DNA repair mechanisms control the balance between the beneficial and deleterious outcomes, by allowing the sensing of the lesions and their repair with a certain degree of fidelity or damage tolerance.

My work emphasizes the importance of nuclear organization on the regulation of DSB sensing and DSB repair. More precisely, I studied the role of the nuclear periphery, which is comprised of the nuclear pores and the nuclear lamina, in these processes. I showed that the nuclear lamina is inhibiting DDR and DNA repair by HR whereas nuclear pores are proficient in both pathways. Moreover, my work demonstrated that the nuclear basket nucleoporin Nup153 is essential for proper DDR mounting and is regulating the choice between HR and NHEJ.

Although Nup153 is mainly localized at the nuclear periphery, my work demonstrates a more general role of this protein in repair of DSBs. Given that Nup153 is involved in the retention of the sumo protease SENP2 at the nuclear envelope, one possible hypothesis for the mechanism of action of Nup153 is the regulation of SUMOylation spatial organization and turnover within the nucleus. Since several DNA repair proteins have been reported to get SUMOylated after DNA damage, the nuclear levels of SUMO could indeed have important implications on DSB repair pathway choice.

The second part of my PhD work showed that DSB repair is compartmentalized within the mammalian nucleus. Indeed, the spatial localization of a DSB influences the choice of the repair pathway to be used. While DSBs induced at the nuclear pores or at the inner nucleus are repaired by NHEJ or HR, DSBs induced at the nuclear lamina are repaired by NHEJ or alt-EJ. Although the localization of a break does not

seem to impair the efficiency of its repair, the use of different repair pathways might influence the fidelity of the repair and have major consequences on genome integrity.

Taken together, these results further demonstrate that spatial organization of proteins and chromatin within the nucleus is a key parameter in the regulation of DSB repair in mammalian cells, as it was also proposed in yeast. The increased DSBs mobility in yeast allows them to scan the entire nucleus to be repaired in specialized repair centers, therefore demonstrating the compartmentalization of DSB repair in yeast. In mammalian cells however, DSBs are not able to roam the entire nucleus and their initial position determines the environment in which they will be repaired. My results demonstrate that these different environments lead to the use of different repair pathways and therefore emphasize the importance of gene positioning in the outcome of DSB repair (for more details see part II.2.e of the results section).

Further studies will shed light on the regulatory role of nuclear organization in DNA repair. On one hand, it will be interesting to investigate whether other nucleoporins exert similar functions as NUP153. Similarly, one could test the role of the different inner nuclear membrane proteins or factors that are sequestered in other compartments such as Cajal bodies or PML bodies in DDR and DNA repair, addressing the question of whether sequestration of proteins within specific nuclear domains contributes to the organization of DSB repair within the mammalian nucleus. On the other hand, it will be exciting to assess DSB repair in other nuclear compartments. In particular, it would be interesting to study DSB repair in the nucleolus. Indeed, some of the lamina associated domains overlap with nucleolus-associated domains (van Koningsbruggen et al., 2010), therefore suggesting that the organization of the two domains could be comparable. It would therefore be appealing to know whether alt-EJ is also active in the nucleolus and what would be the consequences of the use of this error-prone pathway for the repair of ribosomal DNA.

Regarding the use of alt-EJ for the repair of lamina associated domains, another interesting issue is which are the LADs that are repaired by alt-EJ or by NHEJ. Using the knowledge from the laminB-DamID approach regarding constitutive and

facultative LADs in certain cell types (see Introduction), one could target specifically constitutive or facultative LADs by using the CRISPR/Cas9 or TALEN systems and compare DDR and repair factors recruitment in these domains. These results will address whether different lamina associated sequences are more prone to a specific repair pathway.

Finally, an interesting question that my work opens for investigation is whether other DNA repair pathways are compartmentalized within the nucleus. A possible way to study ssDNA repair for example is to induce a single strand break by the nickase Cas9 in the lacO array tethered in different nuclear positions.

The investigation of these different questions in combination with my results and work of others will allow us to better understand the organization of DNA repair within the nucleus, therefore deepening our knowledge on how nuclear structure contributes to the maintenance of genome stability and suppression of tumorigenesis.

Résumé en français

Architecture nucléaire et réparation de l'ADN: réparation des cassures double brins de l'ADN en périphérie du noyau

L'ADN peut être endommagé par des facteurs environnementaux ou intrinsèques au fonctionnement des cellules. Ces facteurs induisent différents types de lésions comme par exemple, les cassures simple brins, les modifications de bases, ou encore les cassures double brins (CDBs) (Garinis et al., 2008; Jackson and Bartek, 2009; Lindahl, 1993). Les CDBs sont particulièrement dangereuses pour les cellules et une réparation inefficace ou non précise de ces cassures peut entraîner des mutations ou des translocations qui peuvent être à l'origine de cancers (Lengauer et al., 1998; Roukos and Misteli, 2014). Afin d'éviter l'instabilité génétique que peuvent induire les CDBs, les cellules ont développé deux principaux mécanismes de réparation: la ligature d'extrémités non homologues (NHEJ pour *non homologous end joining*) et la recombinaison homologue (HR pour *homologous recombination*). La NHEJ est le principal mécanisme de réparation des CDBs pendant la phase G1 du cycle cellulaire et consiste en une simple ligature des deux extrémités endommagées. Ce mécanisme peut parfois entraîner des erreurs de réparation. La HR au contraire est un mécanisme de réparation précis, qui n'induit pas d'erreurs. Il a lieu à la fin de la phase S ou pendant la phase G2 du cycle cellulaire et utilise la chromatide soeur comme modèle pour la réparation de la chromatide homologue (Chapman et al., 2012). Les différents mécanismes de recombinaison homologue sont présentés dans la figure 1.

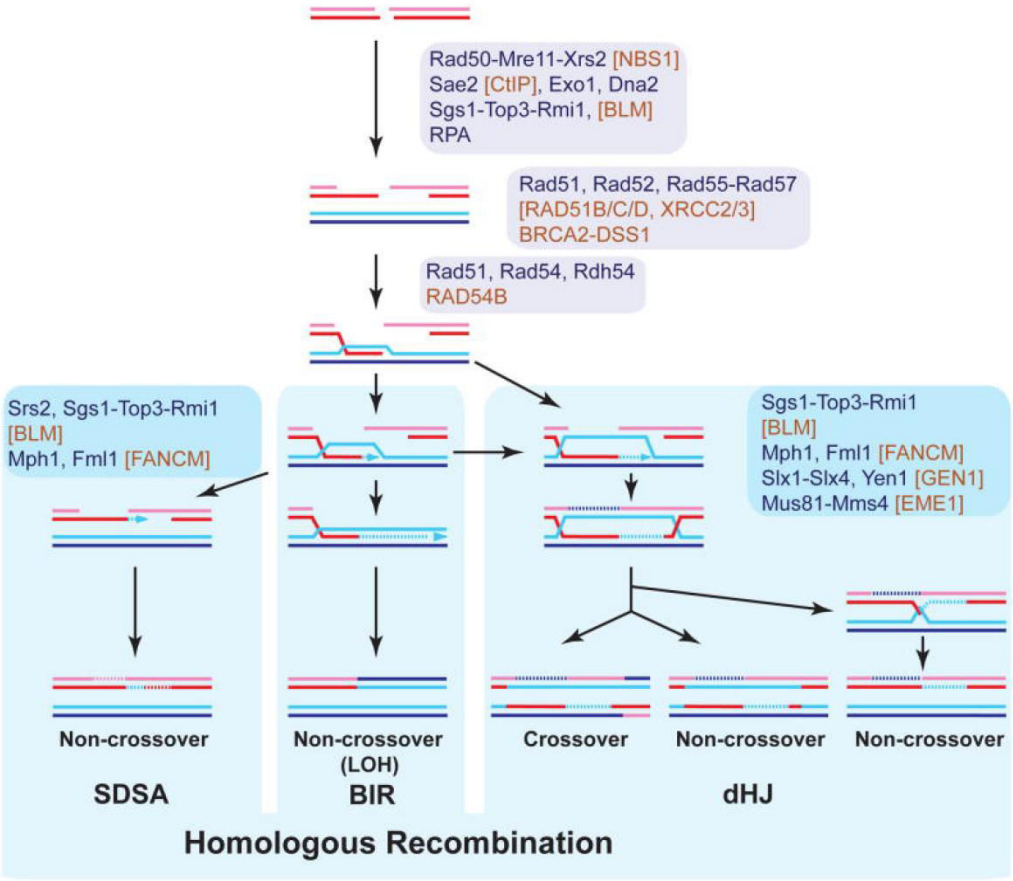

Figure 1- Mécanismes de recombinaison homologue (adapted from Heyer, 2010)

La recombinaison homologue est initiée par une étape de résection qui permet l'apparition d'extremités simple brin. Ces extremités peuvent alors envahir la séquence homologue, formant une structure en D-loop. Selon le mécanisme employé pour la résolution de cette structure, la RH est divisée en trois sous-mécanismes : SDSA, BIR et dHJ.

Lorsque la NHEJ n'est pas fonctionnelle, un mécanisme de réparation alternatif (alt-EJ pour *alternative end joining*) peut également prendre en charge les CDBs. Cependant ce mécanisme induit de nombreuses erreurs et un fort taux de translocations chromosomiques. Ce mécanisme n'a cependant jamais été décrit dans des conditions physiologiques lorsque la NHEJ est intacte et son implication dans l'initiation de cancers reste donc débattue (Frit et al., 2014). Les mécanismes de la NHEJ et du alt-EJ sont représentés dans la figure 2.

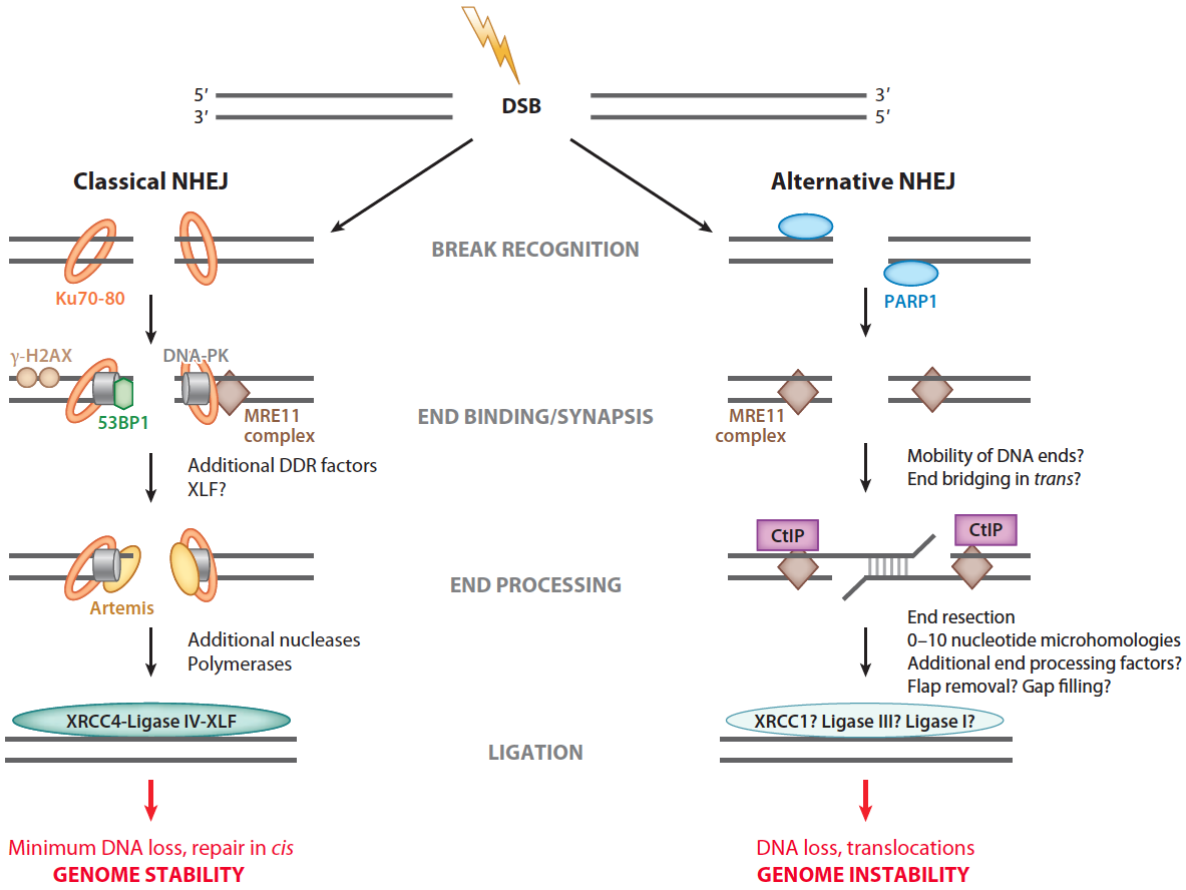

Figure 2- Mécanismes du alt-EJ et de la NHEJ (Deriano and Roth, 2013)

Lors de la NHEJ (à gauche), la CDB est reconnue par le complexe Ku. Les deux extrémités sont maintenues à proximité l'une de l'autre, notamment grâce au complexe DNAPK. Après une éventuelle modification des extrémités, elles sont ligaturées grâce à la ligase IV.

Le mécanisme d'Alt-EJ (à droite) comporte des étapes comparables à la NHEJ. La cassure est reconnue par PARP1 puis une étape de résection est assurée par CtIP et MRN. L'étape finale de ligation est assurée par les ligases III et I. A cause de l'étape de résection, et à l'inverse du NHEJ, alt-EJ est source d'instabilité génomique.

En parallèle de la réparation elle-même, les cellules activent une voie de signalisation nommée réponse aux dommages de l'ADN (DDR for *DNA damage response*). La DDR active les points de contrôle du cycle cellulaire qui permettent l'arrêt du cycle cellulaire le temps que les cassures soient réparées ou qui provoquent l'apoptose ou la senescence des cellules si la réparation est impossible(Misteli and Soutoglou, 2009).

Pour être efficace, la réparation de l'ADN se doit d'être correctement coordonnée dans le temps et dans l'espace. Par exemple, le choix du mécanisme de

réparation doit être strictement régulé. Ainsi par exemple, la HR est un mécanisme de réparation précis et doit être utilisé préférentiellement pendant les phases S/G2 du cylce cellulaire. Par contre, en dehors de ces phases, en l'absence de chromatide homologue, la recombinaison peut être extrêmement dangereuse et entraîner d'importants réarrangements génomiques, éventuellement à l'origine de cancers.

La réparation de l'ADN, comme tous les mécanismes dépendant de l'ADN a lieu dans le noyau des cellules, qui chez les eukaryotes contient plusieurs compartiments associés à différentes fonctions. Ces compartiments sont définis par un ensemble de protéines spécifiques et remplissent des fonctions distinctes. La périphérie nucléaire par exemple joue un rôle important dans la régulation de la transcription (Mekhail and Moazed, 2010). L'organisation du noyau a été démontrée comme étant un facteur important dans la régulation de la réparation des CDBs chez la levure. En effet, lorsque plusieurs CDBs sont induites simultanément elles migrent pour être réparées dans un centre de réparation commun (Lisby et al., 2003). De plus, les CDBs persistantes migrent depuis l'intérieur du noyau jusqu'à la périphérie nucléaire pour être réparées. Cette relocalisation dépend de différents composants des pores nucléaires(Nagai et al., 2008; Oza et al., 2009; Therizols et al., 2006). Dans les cellules de mammifère en revanche, chaque CDB est réparée individuellement et les centres de réparation ne semblent pas exister. De plus, les CDBs ne migrent pas à la périphérie nucléaire et leur mouvement semble très limité (Soutoglou et al., 2007). L'influence de l'organisation du noyau sur la réparation de l'ADN chez les mammifères restait donc à étudier. Par exemple, la question de savoir si la réparation de l'ADN est aussi efficace quel que soit la localisation de la CDB dans le noyau restait à élucider.

Mon projet de thèse consistait en l'étude du rôle de la lamina nucléaire et des pores nucléaires, les deux compartiments qui constituent la périphérie nucléaire, dans la réparation des CDBs.

Dans un premier temps, j'ai étudié le rôle des nucléoporines, les protéines qui forment les pores nucléaires. J'ai démontré que la nucléoporine 153 (Nup153) est nécessaire à la survie des cellules lorsqu'elles sont soumises à des CDBs. Nup153 est impliquée dans le choix entre les mécanismes de HR et NHEJ pour la réparation des

CDBs. Elle agit en favorisant le recrutement de la protéine de DDR 53BP1, qui elle-même inhibe la HR (Lemaître et al., 2012). Ces résultats ont été publiés dans le journal Oncogene (Lemaître et al., 2012). Afin de comprendre si d'autres nucléoporines ont un rôle similaire, nous avons réalisé un criblage par siARN contre les 30 nucléoporines de mamifères connues. Nous n'avons identifié que deux nucléoporines ayant un rôle similaire à Nup153, ce qui suggère que les effets observés ne sont pas dus à une fonction générale du pore nucléaire mais bien à Nup153 elle-même. Un des paramètres impliqués dans le recrutement de 53BP1 aux sites de cassure est la modification post-traductionnelle SUMO1. En effet, en présence de CDBs, 53BP1 est sumoylé et en absence de la ligase de sumo PIAS4, le recrutement de 53BP1 est inhibé. Or, Nup153 interagit avec la protéase de sumo SENP2. Nous avons donc émis l'hypothèse que le rôle de Nup153 dans la régulation du choix du mécanisme de réparation pourrait être médié par SENP2. Nous avons en effet pu démontrer qu'une déplétion de SENP2 entraîne une diminution de l'efficacité de HR, au contraire des résultats obtenus lors d'une déplétion de Nup153 et une déplétion simultanée de SENP2 et Nup153 induit une efficacité de HR comparable à une situation sans déplétion. Un modèle permettant d'expliquer ce résultat pourrait être que SENP2 empêche le recrutement de 53BP1, ce qui entraîne donc une stimulation de HR. Nup153 permettrait la régulation de l'activité de SENP2 en le séquestrant en périphérie nucléaire. En l'absence de Nup153, SENP2 diffuse dans le nucléoplasme et le recrutement de 53BP1 est inhibé, ce qui conduit à une augmentation de HR (données non publiées). Ces résultats ont mis en évidence un nouveau mécanisme de régulation du choix de la voie de réparation des CDBs et donc de maintenance de la stabilité génomique.

Dans un second temps, j'ai étudié l'influence de la position d'une CDB dans le noyau et particulièrement en périphérie nucléaire sur sa réparation. Dans ce but, j'ai créé un système cellulaire permettant l'induction d'une CDB à un site génomique donné et de suivre son évolution en temps réel (figure 3).

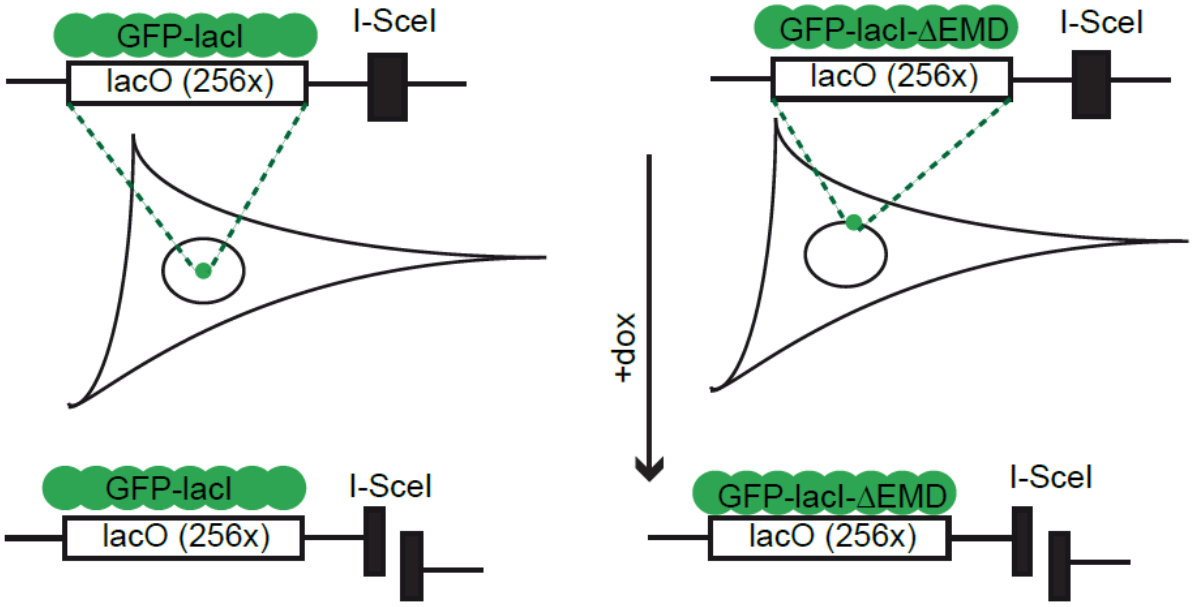

Figure 3- Système cellulaire permettant l'induction d'une CDB au sein de la lamina

Un site de restriction I-SceI, entouré de séquences répétées lacO est intégré de manière stable dans le génome. La protéine lac repressor (lacR) se lie à la sequence lacO. La fusion de lacR à la GFP permet la visualisation de la séquence au sein du noyau. La fusion de la protéine ΔEMD –une protéine de l'enveloppe nucléaire- au lacR permet la relocalisation de la séquence lacO à la lamina nucléaire. L'utilisation d'un système inductible permet l'expression de l'enzyme de restriction I-SceI suite à l'ajout de doxycycline (dox) et conduit à l'induction d'une CDB.

J'ai induit une CDB unique à un site genomique ancré à la membrane nucléaire interne, au pore nucléaire ou à l'intérieur du noyau. La DDR était retardée à la membrane nucléaire en comparaison avec l'intérieur du noyau ou les pores nucléaires. Par ailleurs, les CDBs induites à la membrane nucléaire n'étaient pas réparées par la HR alors que l'efficacité de NHEJ était la même, quel que soit la position de la CDB. Une hypothèse possible pour expliquer cette différence est la présence de chromatine très condensée en périphérie nucléaire. Pour tester cette hypothèse, j'ai induit une décondensation globale ou locale de la chromatine et ai mesuré l'efficacité de la DDR et de la HR. La décondensation de la chromatine était en effet suffisante pour obtenir une efficacité de DDR et HR comparable quel que soit la position de la CDB, ce qui démontre que la présence d'hétorochromatine associée à la membrane nucléaire est en effet responsable du retard de DDR et de la diminution d'efficacité de la HR observée. Contrairement à ce qui a été observé chez la drosophile (Chiolo et al., 2011) ou la levure (Miné-Hattab and Rothstein, 2012), j'ai

montré par imagerie en temps réel et microscopie super resolutive que les CDBs associées à la membrane nucléaire ne migrent pas vers un environnement favorable à la HR, comme par exemple l'intérieur du noyau ou les pores nucléaires, mais sont au contraire réparés *in situ* par alt-EJ. Ces résultats sont résumés dans la figure 4.

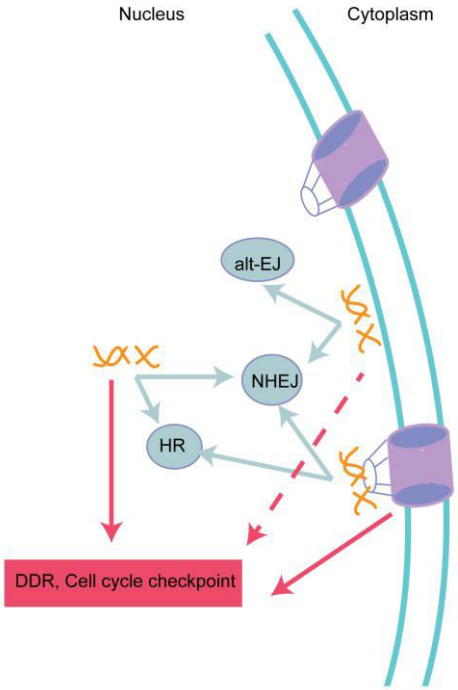

Figure 4- La position d'une CDB determine le mécanisme par lequel elle sera réparée

Les CDBs positionnées à l'interieur du noyau ou associées aux pores nucléaires (en violet) sont réparées soit par NHEJ soit par HR. La DDR est alors robustement activée et le cycle cellulaire est arrêté en attendant la réparation.A l'inverse, les CDBs positionnées à la lamina nucléaire sont réparées par alt-EJ and NHEJ. L'activation de la DDR est retardée. Les consequences de ce retard sur l'arrêt du cycle cellulaire sont inconnues à ce jour.

Mon travail apporte donc la première observation d'alt-EJ dans des conditions physiologiques, en présence d'une NHEJ fonctionnelle. Mes résultats démontrent donc que la position nucléaire d'une CDB peut déterminer le choix de la voie de réparation utilisée et indiquent que la position des gènes dans le noyau est un nouveau paramètre à prendre en compte dans l'étude de la réparation des CDBs. Mon travail a donc d'importantes implications dans la compréhension du rôle de l'organisation du noyau sur le maintien de la stabilité du génome afin d'éviter la formation de tumeurs. Ces résultats ont été publiés dans le journal Genes and Development (Lemaître et al., 2014).

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Charlène LEMAITRE

Nuclear architecture and DNA repair

Résumé

L'ADN peut être endommagé par des facteurs environnementaux ou intrinsèques au fonctionnement des cellules. Ces facteurs induisent différents types de lésions dont les cassures double brins (CDBs). Les CDBs sont particulièrement dangereuses pour les cellules et une réparation inefficace ou non précise de ces cassures peut entraîner des mutations ou des translocations qui peuvent être à l'origine de cancer. Afin d'éviter l'instabilité génétique que peuvent induire les CDBs, les cellules ont développé deux principaux mécanismes de réparation: la ligature d'extrémités non homologues (NHEJ pour *non homologous end joining*) et la recombinaison homologue (HR pour *homologous recombination*). L'utilisation de l'un ou de l'autre de ces mécanismes est finement régulée et une dérégulation de cet équilibre induit une importante instabilité génomique.

Tous ces mécanismes ont lieu dans le noyau des cellules qui, chez les mammifères est fortement hétérogène, comportant différents compartiments et des régions où la chromatine est plus ou moins compacte. Cette hétérogénéité implique que la réparation de l'ADN doit pouvoir être efficace dans différents contextes nucléaires. Au cours de ma thèse, j'ai étudié l'influence de l'architecture nucléaire sur le choix des mécanismes de réparation des CDBs. J'ai montré d'une part que la protéine appartenant au pore nucléaire Nup153 influence l'équilibre entre HR et NHEJ et d'autre part que la position d'une CDB influe sur le choix du mécanisme de réparation.

Mes résultats démontrent que l'organisation des gènes dans le noyau est un nouveau paramètre à prendre en compte dans l'étude des mécanismes de réparation de l'ADN et de tumorigénèse.

Mots clés : réparation de l'ADN, architecture nucléaire, position des gènes, pores nucléaires, enveloppe nucléaire

Résumé en anglais

DNA is constantly assaulted by various damaging agents, leading to different types of lesions including double-strand breaks (DSBs). DSBs are the most harmful lesions to the cells and their inaccurate or inefficient repair can trigger genomic instability and tumorigenesis. To cope with DSBs, cells evolved several repair pathways, including non-homologous end joining (NHEJ) and homologous recombination (HR). A fine regulation of the balance between these two pathways is necessary to avoid genomic instability.

All of these mechanisms happen in the nucleus, which is highly heterogeneous in mammalian cells. Indeed, it encompasses several compartments and regions of various chromatin compaction levels. My PhD project focused on the influence of nuclear architecture on DNA repair pathway choice. I demonstrated on one hand that the nuclear pore protein Nup153 influences the balance between HR and NHEJ and on the other hand that the position of a DSB influences the choice of the repair pathway that will be used.

My results demonstrate that gene positioning is a new important parameter in the study of DNA repair and tumorigenesis.

Keywords: DNA repair, nuclear architecture, gene positioning, nuclear pores, nuclear envelope