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Amandine Batté

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THÈSE DE DOCTORAT  
de l'Université Paris-Saclay  
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Structure et Dynamique des Systèmes Vivants

Spécialité de doctorat : Sciences de la Vie et de la Santé

présentée par  
**Amandine BATTÉ**

Impact of nuclear organization and chromatin structure  
on DNA repair and genome stability

Thèse présentée et soutenue à Fontenay-aux-Roses, le 29 juin 2016

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## COMMON ABBREVIATIONS

Å: Ångström	HR: Homologous Recombination
Ac: Acetylation	INM: Inner Nuclear Membrane
ARS: Autonomous Replication Sequence	K: lysine
ATP: Adenosine TriPhosphate	kb: kilobase
BIR: Break Induced Replication	kDa: kiloDalton
bp: base pair	MAT: Mating Type
ChIP: Chromatin ImmunoPrecipitation	me: methylation
CLIP: Chromosome Linkage Inner nuclear membrane Proteins	NAD: Nicotinamide Adenine Dinucleotide
DNA: DesoxyriboNucleic Acid	NAM: NicotinAMide
rDNA: ribosomal DNA	NPC: Nuclear Pore Complex
ssDNA: single-strand DNA	Nup: Nucleoporin
DSB: Double Strand Break	O-AADPR: O-Acetyl ADP Ribose
GC: Gene Conversion	ONM: Outer Nuclear Membrane
GFP: Green Fluorescent Protein	ORC: Origin Recognition Complex
HAT: Histone AcetylTransferase	PCR: Polymerase Chain Reaction
HDAC: Histone DeACetylase	qPCR: quantitative PCR
HM: Homothallic Mating	PIKK: phosphoinositol-3-kinase-related kinase

pre-RC: pre-Replication Complex

PTM: Post-Translational Modifications

RENT: REgulator of Nucleolar silencing  
and Telophase exit

RFB: Replication Fork Barrier

*S. cerevisiae*: *Saccharomyces cerevisiae*

*S. pombe*: *Schizosaccharomyces pombe*

SIR: Silent Information Regulator

SPB: Spindle Pole Body

SUMO: Small Ubiquitin MODifier

RNA: RiboNucleic Acid

mRNA: messenger RNA

rRNA: ribosomal RNA

TPE: Telomere Position Effect

## **PREFACE**

Expression, duplication and transmission of genetic information are the fundamental and essential functions of the cell. However, genome integrity is challenged everyday by endogenous and exogenous factors that can alter DNA structure by creating DNA lesions. These lesions, and double-strand breaks in particular, are sources of mutations or chromosomal rearrangements that can lead to cancer formation or cell death. Many highly conserved repair mechanisms have been developed by the cells to deal with these DNA damages and preserve genome stability.

In the cell, the genetic information is organized in chromatin, a complex structure composed of DNA and proteins that can adopt different levels of compaction. Chromatin is found in a specialized organelle, the nucleus, separated from the rest of the cell by the nuclear envelope. Inside the nucleus, specific interactions between sequences and constraints on DNA result in a non-random organization. Both nuclear organization and chromatin structure participate in the formation of sub-compartments not delimited by a membrane but enriched in particular DNA sequences, proteins and enzymatic activities. They also appear to be identified as regulators of DNA repair. During my thesis, I focused on better understanding this relationship between DNA repair, nuclear organization and chromatin.

I will start by introducing the different mechanisms of DSB repair, before describing the particular organization of the budding yeast nucleus and its consequence on DSB repair. I will then highlight the formation of heterochromatin and depict how more or less compacted chromatin impacts DNA repair. Finally, I will

present the results obtained during my thesis as well as the functional significance and perspectives they imply.

# **INTRODUCTION**

# INTRODUCTION

## 1. The multiple pathways of Double-Strand Break repair

### 1.1. Causes of DSBs

In every single cell, DNA carries the genetic information necessary for the development, functioning and reproduction of all living organisms. All along its lifespan every cell encounter a broad range of damages that threaten the chemical structure of DNA and can result in a break in one or two strands of DNA, a base missing from the backbone of DNA or a chemically changed base. Such DNA damage arises either from endogenous events such as the attack of reactive oxygen species formed as byproducts of metabolic processes or from exogenous factors such as chemical compounds, UV light and ionizing radiation (Hoeijmakers, 2001).

Among these DNA damages, DSBs are generated when the two complementary strands of the DNA double helix are broken simultaneously and can result from ionizing radiation, radiomimetic chemicals, mechanical stress on chromosomes or after a replication fork encounters a single-strand break or another type of DNA lesion. DSBs are also generated deliberately and for a defined biological purpose. In the yeast *Saccharomyces cerevisiae*, mating type switching can occur only after the induction of a DSB at the *MAT* locus by the *HO* endonuclease (Kostriken et al., 1983; Strathern et al., 1982). During meiosis, recombination is a source of genetic diversity and allows the connection and the proper segregation of homologous chromosomes during the first meiotic division (Bishop et al., 1992; Pittman et al., 1998). Meiotic recombination is first initiated at given loci by the induction of DSBs catalyzed by

Spo11 (Keeney et al., 1997). DSBs are also purposely generated at specific loci by Rag1 and Rag2 proteins during V(D)J recombination in developing B- and T-lymphocytes of higher eukaryotes (McBlane et al., 1995). Repair results in the high diversity of immunoglobulin and T-cell receptor proteins. Although these mechanisms are tightly regulated, they can sometimes go awry and it can be devastating for the cell or the organism.

DSBs are considered the most deleterious DNA damages since a single unrepaired DSB is sufficient to induce apoptosis (Rich et al., 2000; van Gent et al., 2001). DSBs can also lead to massive chromosomal rearrangements resulting in duplication or deletion of some genes and are also strong inducers of mutation. All of these events can give rise to the deregulation of genes or the production of proteins that modify cell proliferation that can lead to the development of cancers (van Gent et al., 2001). Eukaryotic cells have therefore developed repair mechanisms conserved from yeast to humans to deal with DSBs, which can be divided in two main pathways: Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR).

## **1.2. DSBs repair mechanisms**

### **1.2.1. Non-Homologous End Joining**

NHEJ corresponds to the direct joining of the two DSB extremities between ligatable 5' phosphates and 3' hydroxyls and it occurs independently of significant sequence homology. Nevertheless the two joined molecules usually utilize short homology of 1-6 bp to direct reannealing of overhanging DNA ends, called microhomology mediated end joining (MMEJ) or alternative nonhomologous end-joining (Alt-NHEJ) (see below). Depending on the source of DSB a wide range of

DNA ends substrates – diverse variety of overhang length, DNA end sequence, and DNA end chemistry – can be generated. If the DSB yielded fully compatible DNA ends with no gaps in the DNA, similar to DSBs produced by restriction enzymes, NHEJ occurs by simple religation. However, DSBs arising from ionizing radiation or multiple single-strand lesions can give rise to damaged termini, such as 3' phosphates or 3' phosphoglycolates, which will prevent ligation. These extremities need to be processed to remove the damaged nucleotides and the gap has to be filled by polymerization before restoring joining (Daley et al., 2005b; Lieber, 2010). Only when NHEJ occurs as simple religation is it considered as a high fidelity repair mechanism. Since limited modifications of DSBs are mandatory when the two ends are not compatible, NHEJ frequently results in small sequence insertions and deletions making it error prone (Heidenreich et al., 2003; Liang et al., 1998). As NHEJ occurs in the absence of sequence homology, NHEJ is preferred when the cells are not replicating their DNA or do not have a homologous donor sequence (G0 and G1 phases). NHEJ is thus utilized before the start of replication or during stationary phase (Lieber, 2010).

Since the aim of NHEJ is to bring together two separate ends in the absence of homologous recombination, NHEJ is accomplished by a series of specific proteins that work together to carry out three basic steps: DNA end-binding and bridging, terminal end processing and ligation.

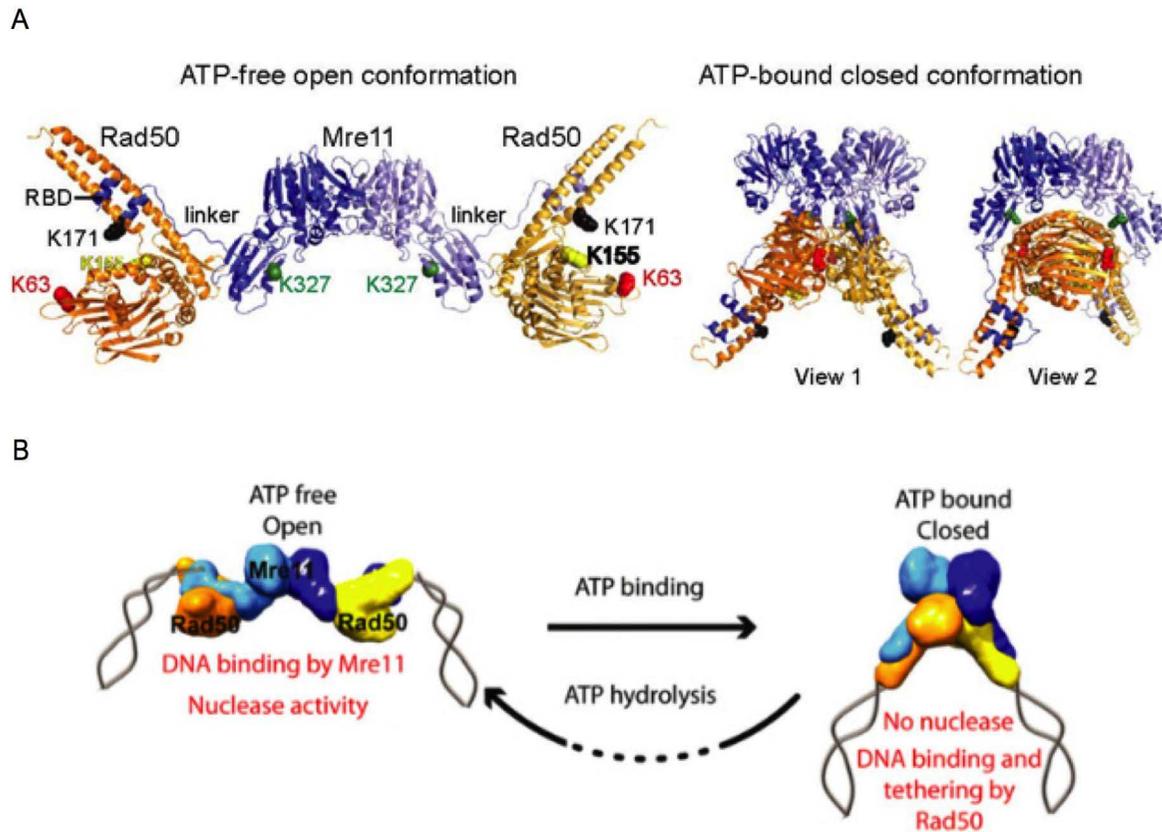
#### *1.2.1.1. DNA end-binding and bridging*

The first step of NHEJ is the binding of the broken ends. Because the two DNA ends can move freely and independently from one another in the nucleus, the

NHEJ machinery must keep them together to mediate the processing and the ligation steps. The recognition and the binding of the Ku protein to the broken DNA extremities initiate NHEJ and the tethering of the two ends requires the Mre11-Rad50-Xrs2 (MRX) complex in yeast and DNA-PKcs in mammals. Ku and MRX are the first proteins to be recruited to the damage sites just after induction (Lisby et al., 2004; Mari et al., 2006). Their recruitment happens at a similar timing but is independent of each other (Kim et al., 2005).

Ku is a heterodimer derived from a duplication of an ancestral gene conserved from bacteria to humans and is composed of yKu70 and yKu80 in yeast (Doherty et al., 2001). The Ku protein forms a ring encircling the DNA that has a high-affinity binding without resorting to sequence-specific binding interactions (Walker et al., 2001). It binds ends in a polarity-dependent manner and slips the DNA through its ring allowing it to slide along the DNA duplex. The C-terminal domain of yKu80 has been characterized to directly bind the DNA Ligase IV Dnl4 (the NHEJ ligase, see below) and favor NHEJ completion (Palmbos et al., 2005; 2008).

Mre11, Rad50, Xrs2 (Mre11, Rad50, Nbs1 in mammals) proteins form a complex called MRX (MRN) and, contrary to the other NHEJ proteins, is also involved in HR (see below). In mammalian cells, the contribution of MRN to NHEJ is debated, but it appears to participate in some NHEJ events (Rass et al., 2009; Xie et al., 2009). However, its role is less preponderant than in yeast and is replaced by a specialized NHEJ protein, DNA-PKcs, recruited by Ku70/Ku80 (Davis et al., 2014). Mre11 and Rad50 are conserved in all kingdoms of life whereas Xrs2/Nsb1 is unique to eukaryotes. Formation of a functional complex requires all three proteins with a stoichiometry of 2:2:1 (Chen et al., 2001; Ghosal and Muniyappa, 2007). Mre11 has both a 3'-5' exonuclease activity and an endonuclease activity which are manganese-



**Figure 1: Representation of the open and closed conformations of the Mre11/Rad50 (MR) complex.**

(A) Structural models of the ATP-free and ATP-bound Mre11-Rad50 complex.

(B) Model of ATP-induced conformational changes in MR and the functions associated with each state. ATP binding to Rad50 leads to a “closed” conformation that promotes DNA end binding and tethering. The MRX complex adopts an “open” conformation upon ATP hydrolysis, unmasking the nuclease sites of Mre11. *Adapted from Deshpande et al., 2014*

dependent and can bind single-stranded DNA as well as double-stranded DNA (Furuse et al., 1998; Trujillo and Sung, 2001). However, function of the MRX complex in NHEJ is independent of its nuclease activities as ends are preserved. Mre11 also forms strong protein-protein interactions with Rad50 and Xrs2. Rad50 belongs to the family of structural maintenance of chromosome (SMC) proteins and is composed of two ATPase domains – where Mre11 binds – separated by a long coiled-coil region. The extremity of this coiled-coil region associates with another molecule of Rad50 to create a Zinc-hook which form a bridge between two DNA molecules that can be separated by up to 1200 Å (Hopfner et al., 2002). Xrs2 is the less characterized component of the complex. It has an intrinsic DNA-binding activity with high affinity for a duplex/single strand junction and is critical for targeting of Mre11 and Rad50 to DNA ends (Trujillo et al., 2003). Moreover, FHA domain of Xrs2 specifically interact with Lif1, the associated partner of Dnl4, and this interaction facilitates the association of MRX with Dnl4 to promote ligation of the DSB ends (Chen et al., 2001; Palmboos et al., 2005; 2008). The MRX complex can adopt two different conformations depending on ATP (Deshpande et al., 2014; Mockel et al., 2012) (Figure 1). When ATP is unbound, the complex adopts an “open” conformation that can engage DNA in a non-end specific manner and the Mre11 nuclease active sites are accessible. However, when the complex binds ATP, the ATPase domains of Rad50 come together and form a “closed” structure which prevents Mre11 nuclease activity and promotes DNA end-binding and tethering.

### 1.2.1.2. Terminal end processing

The next step of NHEJ consists of making the two DSB extremities ready for ligation when the terminal bases are not fully compatible or damaged. It requires the action of polymerases, nucleases and other related proteins.

The yeast DNA Polymerase X-family member, Pol4, has been identified to fill gaps at 3' overhangs (Wilson and Lieber, 1999). Pol4 is a polymerase specific to NHEJ and acts on 3' overhangs of all lengths that necessitate gap filling on both strands. It can be partially complemented by its equivalent proteins Pol  $\lambda$  and Pol  $\mu$  in mammals (Daley et al., 2005a). However, some NHEJ events that necessitate polymerization from a 3' overhang are independent of Pol4 but require Pol3, the large catalytic subunit of the replicative polymerase Pol  $\delta$  (Chan et al., 2008; Daley and Wilson, 2008). DSBs with 5' overhangs are filled in by still unidentified polymerases.

In mammals, Artemis, a 5' and 3' endonuclease, is phosphorylated by DNA-PKcs, which activates its endonuclease activities and can therefore process 5' and 3' overhangs (Ma et al., 2002). On the contrary, its yeast closest ortholog, Pso2, appears to be exclusively associated with hairpin and crosslink repair (Li and Moses, 2003; Yu et al., 2004). In *S. cerevisiae*, Rad27 is a DNA replication protein that has both 5' flap endonuclease and 5' to 3' exonuclease activities. It has been shown that Rad27 plays a role only on a subset of NHEJ events that require processing of 5' flaps (Wu et al., 1999). Another study using a different reporter assay was unable to reproduce the requirement for Rad27 in DSB end processing, suggesting that Rad27 is not involved in this process or is functionally redundant with other nucleases that have yet to be uncovered (Daley and Wilson, 2008). However it has been shown that Rad27 interacts with Pol4 *in vitro* and that they can act in a coordinated way to process a DNA duplex (Tseng and Tomkinson, 2004).

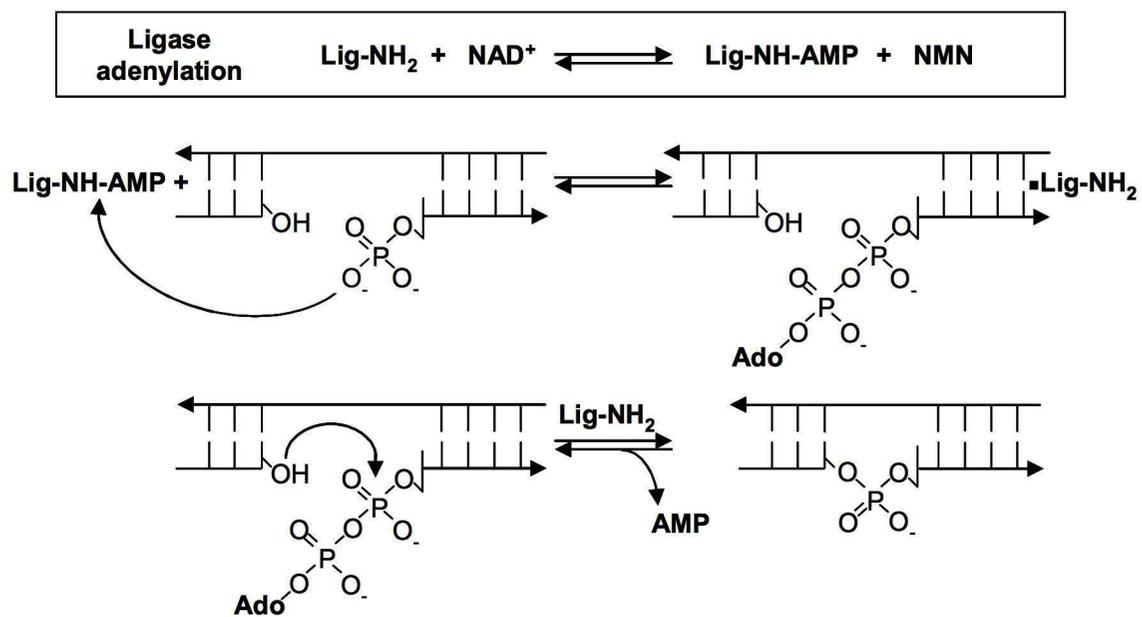
Tdp1 is a general 3' phosphoesterase capable of removing 3'-terminal lesions as well as nucleosides to yield a 3' phosphate. It has been shown to be a component of the NHEJ pathway (Bahmed et al., 2010) and its recruitment at 5' DSBs is restricted by Ku (Liang et al., 2016). Tdp1 seems to compete with other NHEJ factors by regulating the processing of DNA ends by generating a 3' phosphate to temporarily inhibit undesirable filling of 5' overhangs by polymerases prior to rejoining (Bahmed et al., 2010).

In yeast, the end-processing step is still not well defined. Existing data detailed above seems to involve several polymerases and nucleases playing redundant roles that have yet to be determined. This redundancy may explain the different DNA sequences that can be found after repair of the same DSB by NHEJ (Lieber, 2010).

#### *1.2.1.3. Ligation*

The final step of NHEJ is the ligation of one or both strands to restore chromosomal continuity and relies on the DNA Ligase IV complex consisting of Dnl4, Lif1 and Nej1 (Lig4, XRCC4 and XLF in mammals).

Dnl4 is an ATP-dependent DNA ligase strictly required for NHEJ. It is unable to complement the function of the only other known yeast DNA ligase, DNA ligase I (Cdc9), in replication and recombination. Conversely Cdc9 is unable to perform NHEJ (Wilson et al., 1997). Dnl4 is highly conserved from yeast to mammals and harbors a conserved ligase catalytic domain similar to DNA ligase I that has been characterized to encircle the DNA by opening and closing of a non-covalent ring (Pascal et al., 2004). Dnl4 also contains a tandem BRCT domain at its C-terminus that interacts with Lif1 and promotes the stable binding of Dnl4 at a chromosomal DSB



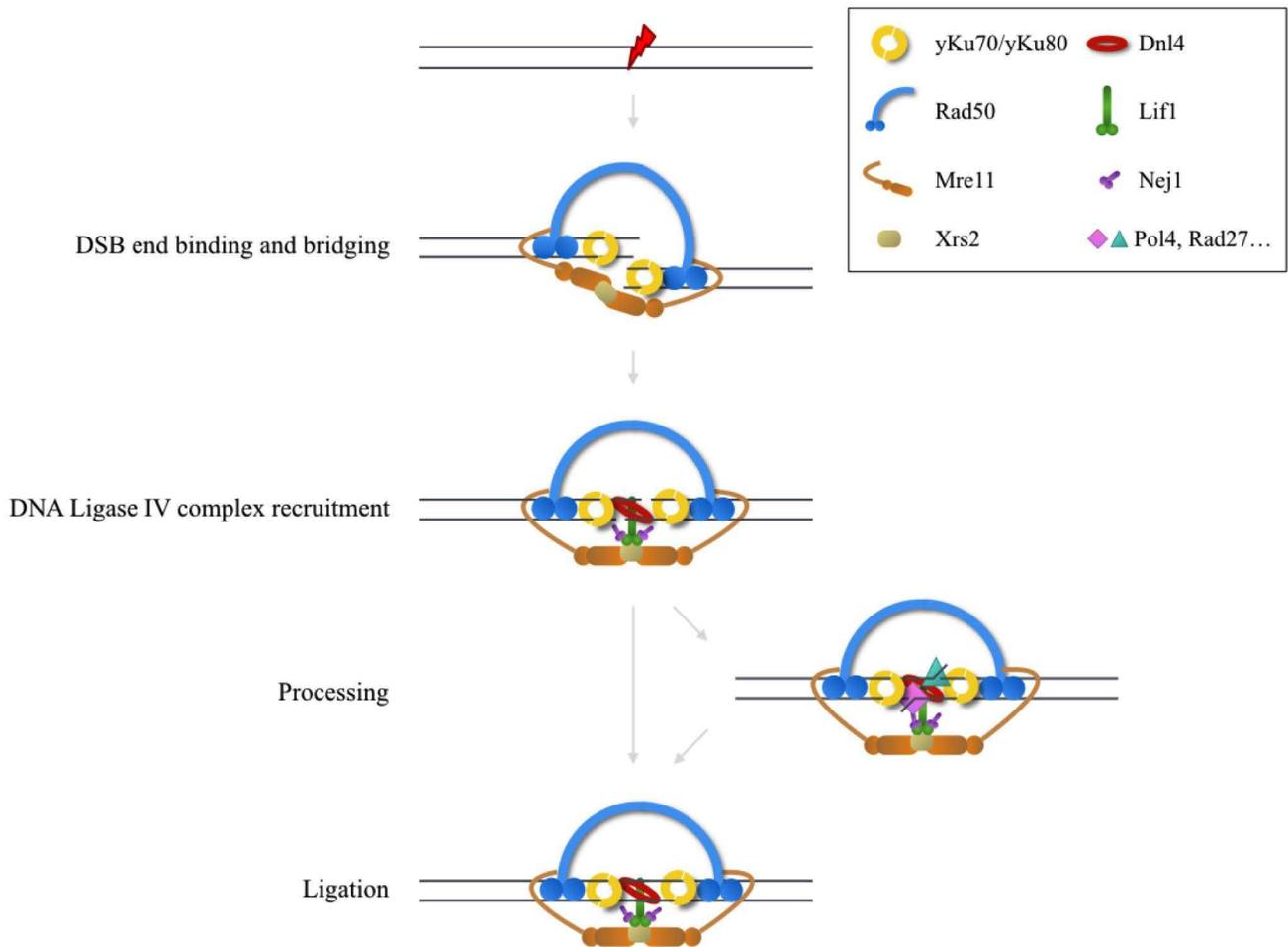
**Figure 2: Reaction mechanism of DNA ligation.**

The first step is the reversible adenylation of ligase with NAD<sup>+</sup> (or ATP) as the adenylyl donor. The second step is the transfer of the AMP to the 5' phosphate end of DNA end. The third step corresponds to the attack of the 5'-phosphate-AMP by a 3'-OH and the release of AMP to close the nick. *Adapted from Miesel et al., 2008*

(Chiruvella et al., 2014; Herrmann et al., 1998). As shown in Figure 2, the enzymatic ligation of DNA is a three steps reaction in which an adenosine 5'-monophosphate (AMP) is first transferred from ATP or NAD<sup>+</sup> to an active-site lysine on the ligase (step 1 = auto-adenylation). The activated AMP is then transferred to a 5' phosphate at a DNA end (step 2) that is attacked by a 3' hydroxyl of a second DNA strand generating a ligated DNA and releasing AMP (step 3) (Ellenberger and Tomkinson, 2008; Miesel et al., 2007).

Lif1 is a Dnl4 associated partner and shows significant sequence divergence from its mammalian homologue, XRCC4, but the structure of the two proteins is very similar (Herrmann et al., 1998; Sibanda et al., 2001). Lif1 is homodimeric with two globular heads and a long coiled-coil region and interacts with Dnl4 by its most conserved segment located in the coiled-coil region (Sibanda et al., 2001). This Dnl4/Lif1 interaction is necessary for the stabilization of Dnl4 (Chiruvella et al., 2014; Herrmann et al., 1998). Thanks to its intrinsic DNA binding activity, Lif1 also plays a role in the targeting of Dnl4 to the DSB site in a Ku-dependent manner (Teo and Jackson, 2000). It has also been suggested by *in vitro* assay that Lif1 could stimulate the catalytic activity of Dnl4 by increasing its auto-adenylation ability (Chiruvella et al., 2014; Teo and Jackson, 2000). The Lif1 globular head also interacts with another partner required for NHEJ, Nej1 (Frank-Vaillant and Marcand, 2001).

Nej1 and its mammalian homologue, XLF, are highly divergent except at their C terminus and harbor the same structure as Lif1/XRCC4, with a shortened coiled-coil region (Andres et al., 2007). It has been shown that the Nej1 C terminus is important for its nuclear localization, its interaction with Lif1 and has a DNA binding activity that is sequence and structure-independent (Mahaney et al., 2014; Sulek et al., 2007). Moreover Nej1 regulates the nuclear localization of Lif1 and Nej1/Lif1



**Figure 3: Schematic representation of the NHEJ reaction.**

After DNA termini binding, Ku and MRX recruit the DNA Ligase IV complex. If DNA ends are ligatable, Dnl4 will perform the ligation. Otherwise the DSB extremities require some processing (polymerization and/or nucleolytic cleavage) before ligation.

complex has a higher affinity to DNA than each single protein (Sulek et al., 2007; Valencia et al., 2001). Altogether it suggests that Nej1 aids in the proper localization of the DNA Ligase IV complex and stabilizes its interaction with DNA. Nej1 is also a major regulator of the NHEJ pathway as its transcription is regulated by the haploid/diploid status in yeast (Frank-Vaillant and Marcand, 2001; Kegel et al., 2001; Valencia et al., 2001).

As mentioned above, after the DSB end-binding and bridging by Ku and MRX, the DNA ligase IV complex is recruited to the DSB site through interactions of Dnl4 and Lif1 to yKu80 and Xrs2 respectively (Palmbos et al., 2008). If the DNA ends are suitable for ligation, DNA Ligase IV can directly perform the reaction. However, if ligation fails, end processing is required and several studies demonstrated that the DNA Ligase IV complex itself mediates the recruitment of processing enzymes. Indeed, an *in vitro* study showed that the interaction between Dnl4 and the BRCT domain of Pol4 stimulates Pol4 polymerization activity and Dnl4-Lif1 DNA joining activity (Tseng and Tomkinson, 2002). Similarly, Dnl4 binds Rad27 and favors its nuclease activity (Tseng and Tomkinson, 2004). *In vivo* recruitment of Rad27 and Pol4 to a DSB depends on Nej1 and Dnl4-Lif1 via additive mechanisms and Nej1 interaction with both Rad27 and Pol4 stimulates their catalytic activities (Yang et al., 2015). The NHEJ reaction thus appears as a dynamic reaction with coordination and iterative testing of ligation and processing to complete DSB repair (Figure 3).

#### *1.2.1.4. Microhomology-Mediated End joining*

In every experimental system studied, when one or more proteins of NHEJ are mutated, the cell can still join DSBs but with a greatly reduced efficiency (Boulton and Jackson, 1996; Wilson et al., 1997) and this alternative end joining process has been referred to as Alt-NHEJ, backup NHEJ or Microhomology-Mediated End Joining (MMEJ). The joints often harbor local deletions with relatively long stretches of microhomology, hence the term MMEJ. However, some joining can also occur independently of the core NHEJ proteins without using any microhomologies. Indeed limited accurate religation exists in the absence of Dnl4 and the efficiency of this NHEJ-independent repair event can be increased by using longer overhangs (> 4bp) or overhangs with a higher GC content (Daley and Wilson, 2005). Considerable confusion exists about the relationship between MMEJ and alternative end joining. Alternative end joining should be defined as any Ku and/or Dnl4-independent end joining process and may encompass many distinct repair mechanisms whereas MMEJ seems to be one of these alternative end-joining processes, the other(s) still needed to be more defined.

MMEJ is a Ku-independent mechanism that uses microhomologies internal to the DSB termini to mediate joining and the repair products generated exhibit deletions that can range from 5 to over 300 base pairs (Boulton and Jackson, 1996; Ma et al., 2003). The mechanism involves end resection, annealing of microhomologies, flap removal, fill-in synthesis and ligation.

End resection corresponds to the nucleolytic processing of the DNA ends to yield 3' single-stranded DNA (ssDNA) overhangs. It is an indispensable step for HR (see below for more details) and is required to expose microhomologies located internally to the DNA termini during MMEJ. Briefly, resection is initiated by the

MRX complex and Sae2/CtIP that promotes the endonucleolytic cleavage of the 5' strand internal to the break by Mre11. The resulting nick is then processed by the 3'-5' nuclease activity of Mre11 in one hand, and the redundant 5'-3' nuclease activities of Exo1 and Dna2 – together with Sgs1/BLM – on the other hand to generate long tracts of ssDNA (Cejka, 2015). Initiation of end resection by the MRX complex and Sae2/CtIP has been identified to be critical for MMEJ in mammals, whereas it is a bit more controversial in yeast (Deng et al., 2014; Lee and Lee, 2007; Ma et al., 2003; Truong et al., 2013). End resection by Exo1 and Sgs1 do not seem to be implicated or only when the microhomologies are located far away one from another and the two redundant pathways are abolished (Deng et al., 2014; Lee and Lee, 2007; Villarreal et al., 2012).

The length of microhomology appears to be a critical feature of MMEJ. Indeed a DSB flanked with 6 or 12 bp microhomology is poorly repaired by MMEJ, whereas this rate increased almost 10-fold for every base pair added between 12 to 17 (Villarreal et al., 2012). Moreover MMEJ efficiency is decreased by mismatched nucleotides in the microhomology. Altogether it indicates that MMEJ is driven by the stability of the annealing between microhomologies. This annealing is prevented by RPA, the main eukaryotic ssDNA-binding protein, as a mutant with lower ssDNA-binding affinity increases MMEJ efficiency (Deng et al., 2014). Rad52 is required for the single-strand annealing (SSA) mechanism occurring between long direct repeats (see below). However Rad52 is not involved in MMEJ when the homology is < 14 nucleotides and is even inhibitory for MMEJ (Deng et al., 2014; Ma et al., 2003; Villarreal et al., 2012).

Following 5' degradation and proper annealing of microhomologies, 3' flaps need to be removed for gap fill-in synthesis and ligation to complete MMEJ. This step

is carried out by an endonuclease complex composed of Rad1 and Rad10 (Lee and Lee, 2007; Ma et al., 2003).

Due to 5' resection at either side of the break, MMEJ needs DNA synthesis to fill these gaps. Pol32, a subunit of the replicative DNA polymerase Pol $\delta$ , and Pol4 are required for DNA synthesis during MMEJ. The involvement of translesion synthesis (TLS) polymerases  $\zeta$  and  $\eta$  in MMEJ is not clear as Rev3 and Rad30 exhibit contradictory effect on MMEJ depending on the assay used (Lee and Lee, 2007; Villarreal et al., 2012).

As NHEJ, MMEJ is completed by the final ligation step where DNA ends are covalently attached to each other. In yeast, MMEJ is independent of Dnl4 (Deng et al., 2014; Villarreal et al., 2012), and the requirement for Cdc9 (Lig1) is still unknown in mediating these events.

### 1.2.2. Homologous Recombination

Homologous recombination corresponds to an exchange of genetic information between a broken recipient and a donor DNA molecule sharing similar or identical sequence. It is an error-free repair mechanism that uses an intact homologous sequence to repair the DNA lesion. It typically involves two DNA molecules that are either the sister chromatids or the homologous chromosomes, so-called allelic recombination. Homologous recombination can also occur between two non-allelic positions on the genome and is, in this case, called ectopic recombination. When given the choice it has been reported that the sister chromatid is the preferred template over a homolog or ectopic recombination (Agmon et al., 2009; Jain et al., 2009; Kadyk and Hartwell, 1992; Wu et al., 1997). Sister chromatid cohesion is

believed to be responsible for this preference (Sjögren and Nasmyth, 2001). It could suggest that HR is restricted to S- and G2 phases of the cell cycle, when the sister chromatid is present for repair. However, HR can also occur in G1 phase between two homologs in diploid cells (Fabre, 1978). Both ploidy and cell cycle phase therefore mainly contribute to the availability of a homologous sequence to perform HR.

HR is a multi-step repair mechanism that requires the sequential recruitment of specific proteins and can be separated into various sub-pathways.

#### *1.2.2.1. Resection*

All pathways of homology-dependent DSB repair initiate by nucleolytic degradation of the 5' strands to yield 3' single-stranded DNA (ssDNA) overhangs and is referred to as 5'-3' resection. In most eukaryotes DNA end resection is a two-step process called short- and long-range resection. The average rate of resection measured by molecular analysis and live-cell imaging was estimated ~ 4kb/h and resection can degrade thousands of nucleotides (Fishman-Lobell et al., 1992; Saad et al., 2014; Zhu et al., 2008).

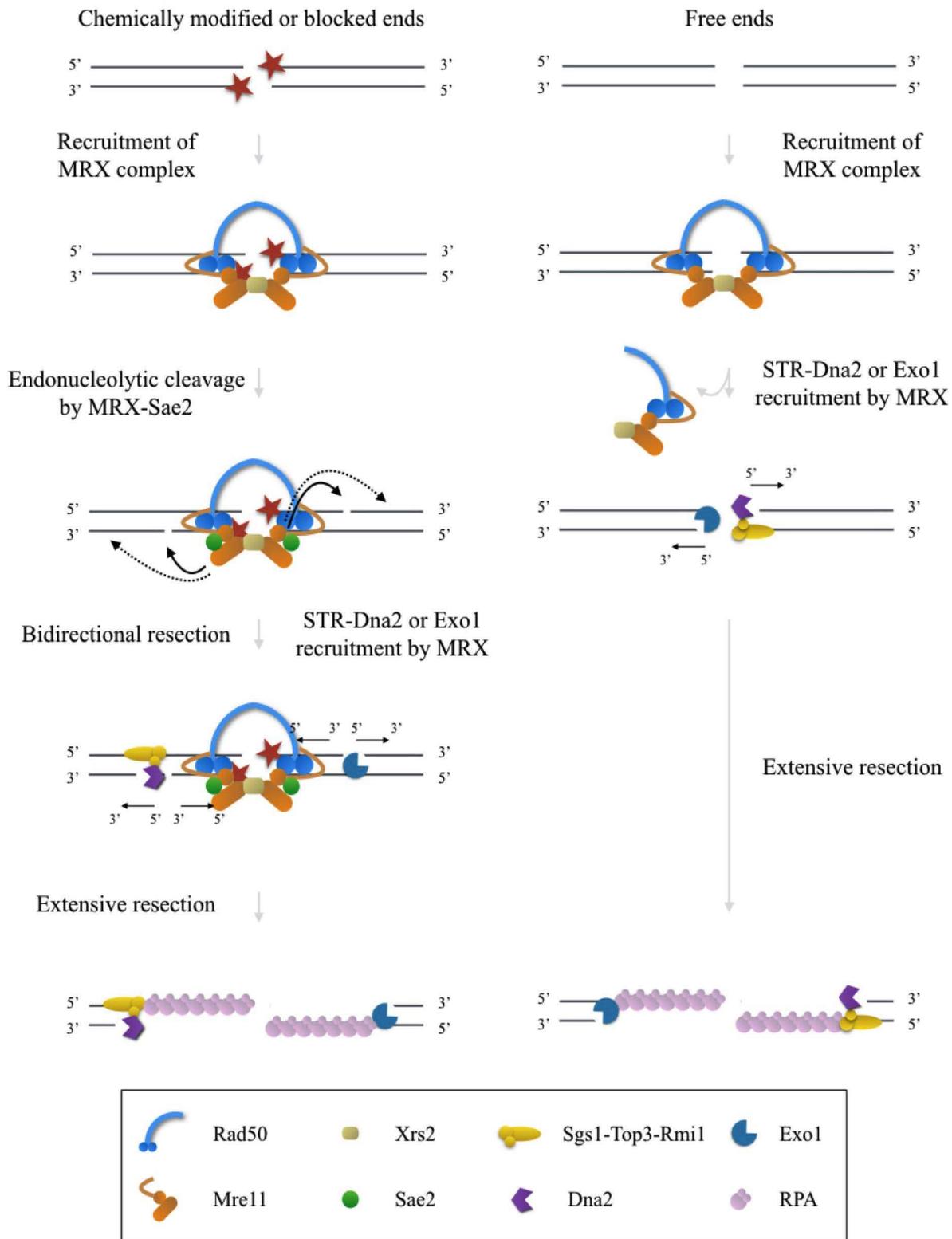
Short-range resection corresponds to a nucleolytic processing limited to the vicinity of DNA ends and is dependent on the MRX/N complex together with Sae2/CtIP. As mentioned above, the MRX complex is one of the first proteins recruited to DSB sites and can bind DNA through both Mre11 and Xrs2 (Furuse et al., 1998; Lisby et al., 2004; Trujillo et al., 2003). It has both catalytic and structural roles in resection. *In vitro* studies demonstrated that Mre11 has a 3' to 5' exonuclease activity and an endonuclease activity that are both manganese-dependent (Cannavo and Cejka, 2014; Paull and Gellert, 1998; Trujillo and Sung, 2001). Conserved

residues within the phosphoesterase motifs, including D56 and H125, are required for endo- and exonuclease activities *in vitro* (Moreau et al., 1999). The 3' to 5' exonuclease activity of Mre11 releases mononucleotide products and has a strong preference for double-stranded DNA (dsDNA) ends (Trujillo and Sung, 2001). Whereas HsRad50 has been shown to increase by 3-4 fold the exonuclease activity of HsMre11 (Paull and Gellert, 1998), ScRad50 does not affect the catalytic activity of ScMre11 (Trujillo and Sung, 2001). However Xrs2 promotes the exonuclease activity of Mre11 alone and Mre11-Rad50 subcomplex (Trujillo et al., 2003). The endonuclease activity of Mre11 is structure-specific and can cleave diverse secondary structures. It gives rise to two major endonucleolytic products depending where the enzyme cuts. The first product results from an incision at hairpin loops whereas the second product comes from a cleavage at the junction between the duplex DNA molecule and the 3'ss DNA extension (Trujillo et al., 2003). Rad50 moderately stimulates the endonuclease activity of Mre11 in the presence of hydrolysable ATP and Xrs2 increases by 2-fold this activity within the Mre11-Rad50 subcomplex (Trujillo and Sung, 2001; Trujillo et al., 2003). MRX has also a structural role in resection by recruiting the long-range resection machinery. Indeed Sgs1 associates with Mre11 upon DNA damage while Mre11 favors Exo1 binding to DNA independently of its nuclease activities (Chiolo et al., 2005; Nicolette et al., 2010).

Sae2 is a poorly conserved protein that shares a limited number of conserved residues with its apparent orthologs, HsCtIP and SpCtp1, restricted to the C-terminus and a homodimerization domain at the N-terminal region, but whose functions are largely conserved (Lengsfeld et al., 2007; Limbo et al., 2007; Sartori et al., 2007). Thanks to *in vitro* assays it has been primarily shown that Sae2 has a ssDNA endonuclease activity that cleaves at ssDNA/dsDNA transitions and is stimulated by

MRX (Lengsfeld et al., 2007). Nevertheless, until now no active site for nuclease activity has been detected and no obvious domains or functional motifs has been identified in Sae2. A recent study found no nuclease activity for Sae2 alone and actually demonstrated that combination of Sae2 and MRX leads to a strong dsDNA endonucleolytic cleavage inherent to Mre11 (Cannavo and Cejka, 2014). Furthermore stimulation of the endonuclease activity of Mre11 by Sae2 is specific to 5'-terminated DNA strand (Cannavo and Cejka, 2014). Accordingly, many genetic studies found similar phenotypes between *sae2*Δ cells and *mre11* nuclease-dead mutants and Mre11 nuclease functions were affected in cells lacking *SAE2* (Lobachev et al., 2002; Rattray et al., 2001). Moreover, it was shown that Sae2 is transiently phosphorylated by the cyclin-dependent kinase Cdc28 during the S/G2 phases and Mec1 and Tel1 upon DNA damage (Baroni et al., 2004; Huertas et al., 2008). This phosphorylation is required for Sae2 functions *in vivo* (Baroni et al., 2004; Huertas et al., 2008). In addition Sae2 phosphorylation regulates its capacity to trigger MRX endonuclease activity (Cannavo and Cejka, 2014), indicating a tight regulation of resection depending on cell cycle and genome integrity .

The polarity of the Mre11 exonuclease described *in vitro* (3'-5') was in disagreement with the polarity of resection observed *in vivo* (5'-3') and the generation of 3' ssDNA tails required for the later repair steps. Insight from meiotic recombination has contributed to explain this paradox. Indeed, DNA molecules of ~10-15 nucleotides and ~20-40 nucleotides in length were found attached to Spo11 *via* their 5' end with a free 3' terminus, suggesting a processing of meiotic DSBs initiated by an endonucleolytic cleavage (Neale et al., 2005). The recent finding that stimulation of Mre11 endonuclease activity by Sae2 leads to a preferential cleavage of 5' strand ~15-25 nucleotides from the DNA end supports the idea that resection



**Figure 4: Model of DNA end resection of free or blocked/modified DNA ends.**

MRX complex is rapidly recruited to DSB site. Sae2 stimulates endonucleolytic cleavage of the ends by the MRX complex and is required to release chemical modifications or proteins blocked at DNA end (*left panel*). This clipping could occur in the vicinity of the DSB to remove a short oligonucleotide (*black arrows*) and/or it could occur several hundred nucleotides from the end (*dashed black arrows*). It is followed by bidirectional exonucleolytic processing by STR-Dna2 or Exo1 that carry out long-range resection and likely by Mre11 that degrade DNA in a 3'-5' direction toward the DSB. At free ends, MRX is also rapidly recruited but the nuclease activity of Mre11 is not required (*right panel*). MRX favors the recruitment of Exo1 or STR-Dna2 to directly initiate resection. Exo1 or STR-Dna2 then extensively resect DNA to produce long stretch of 3' ssDNA overhangs that are coated by RPA.

initiates by Mre11 endonuclease activity, rather than its exonuclease activity (Cannavo and Cejka, 2014). In some cases, DSB processing is initiated by a nick located farther away from the DSB end, distant up to ~300 nucleotides (Garcia et al., 2011). It suggests multiple rounds of MRX-Sae2 endonucleolytic cuts from the DNA ends, or a more distant single endonucleolytic cleavage, followed by bidirectional exonucleolytic processing. This bidirectional resection may use the Mre11 3'-5' exonuclease activity towards the DSB ends in one hand, and create an entry point for the long-range machinery, Exo1 and Sgs1-Dna2, that will digest DNA in the 5'-3' direction away from the DSB in the other hand.

Depending on the nature of the DSB extremities, the requirement for MRX-Sae2 to initiate resection will differ whereas MRN and CtIP are mandatory in human for all types of DSBs (Sartori et al., 2007) (Figure 4). As mentioned above, DSBs generated by restriction endonucleases are “clean” DSBs with 3' hydroxyl and 5' phosphate groups and do not necessitate further processing before ligation or extension by polymerases. In this case Mre11 nuclease activity is largely dispensable for resection (Llorente and Symington, 2004; Moreau et al., 1999). In fact the initiation of resection is delayed in the absence of MRX and Sae2 but, once initiated, the rate of resection is then identical to WT cells (Clerici et al., 2005; Zhu et al., 2008). This delay may be due to a delay in the recruitment of Sgs1 and Exo1, but once recruited these enzymes can directly process free DSB ends. Sometimes, rare DSBs can be terminated by hairpins and need to be clipped by MRX and Sae2 (Lengsfeld et al., 2007; Lobachev et al., 2002). DSBs induced by ionizing radiation give rise to double- and single-strand breaks, base and sugar damage, and DNA-protein crosslinks with complex DNA ends. Mre11 nuclease deficient mutants and *sae2* $\Delta$  cells are less sensitive to IR than *mre11* $\Delta$  strain (Krogh et al., 2005; Mimitou

and Symington, 2010). Nevertheless they are still a lot more sensitive than WT cells, indicating that MRX-Sae2 short-range resection is important for repair of IR-induced DSBs. Moreover DSBs bound by a protein at 5' ends strictly require MRX and Sae2 nuclease activity. Topoisomerases are transiently attached to 5' or 3' DNA ends and abortive reaction can occur either spontaneously or upon drug treatment and can form a DSB; the protein being trapped at the DNA end. It has been well defined for a long time in meiosis where Spo11, a Topo II-like protein, induces a DSB and stays covalently bound to the DNA end and has to be released by MRX and Sae2 (Moreau et al., 1999; Neale et al., 2005). In mitotic cells, MRX and Sae2 participate in Ku and MRX removal from DNA ends to allow resection by Exo1 and Sgs1 (Chen et al., 2015; Mimitou and Symington, 2010). Accordingly endonucleolytic 5' end clipping by Mre11-Sae2 is increased by protein blocks at DNA ends (Cannavo and Cejka, 2014).

Long-range resection machinery is carried-out by two separate pathways depending on the enzymatic activities of Exo1 and Sgs1-Dna2 (Mimitou and Symington, 2008; Zhu et al., 2008) (Figure 4).

Exo1 in yeast (and its human ortholog EXO1), is a member of the Rad2/XPG nuclease family and has been characterized for having a 5'-3' exonuclease activity on dsDNA and a flap endonuclease activity (Tran et al., 2002). Both nuclease activities are carried by the N-terminal region of the proteins, which contain two nuclease domains, the N-nuclease domain and the I-nuclease domain (Tran et al., 2004). Both Exo1 nuclease activities are strongly promoted by MRX and Sae2, in particular when Exo1 concentration is limiting (Cannavo et al., 2013; Nicolette et al., 2010). This stimulation is independent of protein-protein interactions and Mre11 nuclease activity (Nicolette et al., 2010; Shim et al., 2010). The major effect of MRX-Sae2 on Exo1

activity is through an increase in the affinity of Exo1 for DNA (Nicolette et al., 2010). Exo1 preferentially acts on the 5'-terminal strand of a dsDNA with a 3'-ssDNA tail that would be produced by short-range resection (Cannavo et al., 2013). MRX and Sae2 thus likely stimulate Exo1 activity by creating a specific DNA structure that results in a higher-affinity binding site for Exo1. DNA resection by Exo1 is also stimulated by RPA, the ssDNA-binding protein, which prevents nonspecific binding and sequestration of Exo1 to ssDNA that could titrate the enzyme (Cannavo et al., 2013; Chen et al., 2013).

Sgs1 is a 3'-5' DNA helicase belonging to the highly conserved RecQ family. Five RecQ helicases exist in human, but the mechanistically related protein is BLM (Bernstein et al., 2010). DNA helicases unwind dsDNA by travelling on ssDNA and are necessary for all aspects of DNA metabolism. Sgs1 binds preferentially to duplex DNA with 3' overhangs of at least 3-4 nucleotides (Bennett et al., 1999). Sgs1 directly interacts at its N-terminus with Top3, a Type1A topoisomerase that cleaves one DNA strand and relaxes only negatively supercoiled DNA (Bennett et al., 2000; Gangloff et al., 1994). Sgs1 and Top3 form a heteromeric complex with Rmi1, a structure-specific DNA binding protein with a preference for cruciform structures (Mullen et al., 2005). Rmi1 and Top3 can form a stable complex, but the binding of Rmi1-Top3 to Sgs1 is codependent (Chen and Brill, 2007; Mullen et al., 2005). Rmi1 promotes the superhelical relaxation activity of Top3 and its ssDNA binding activity (Chen and Brill, 2007). However Top3 catalytic activity is not required for resection (Niu et al., 2010). Rmi1 more likely plays a role in targeting Top3-Sgs1 to appropriate substrates for resection (Mullen et al., 2005). Sgs1-Top3-Rmi1 (STR complex) does not harbor any nuclease activity by itself. The ssDNA formed by Sgs1 unwinding is degraded by the bifunctional endonuclease/helicase Dna2 (Zhu et al., 2008). Dna2

helicase function is not required for resection (Cejka et al., 2010a; Zhu et al., 2008). Its ssDNA endonuclease activity can cleave both 5'-3' and 3'-5' strands (Bae and Seo, 2000). It acts preferentially on a free ssDNA 5' terminus but then degrades DNA endonucleolytically, resulting in degradation products of 5–10 nucleotides in length (Kao et al., 2004). Sgs1 and Dna2 directly interact, suggesting a model where Sgs1 unwinds DNA by translocating along one strand with a 3'-5' polarity whereas Dna2 degrades unwound DNA by translocating in a 5'-3' direction; both proteins going in the same direction at the end (Cejka et al., 2010a). Sgs1-Dna2 are stimulated by Top3-Rmi1 and the MRX complex via direct complex formation with Sgs1 and this interaction promotes Sgs1 recruitment to a DSB site (Cejka et al., 2010a; Niu et al., 2010). STR-Dna2 also requires RPA that enforces the correct polarity of DNA end resection (Chen et al., 2013). RPA stimulates the helicase activity of Sgs1 in a species-specific manner (Cejka et al., 2010a). It also directs Dna2 nuclease activity to 5'-terminated strand, leading to the generation of 3' ssDNA overhangs, and inhibits 3'-5' degradation by Dna2 by coating the newly formed 3'-tailed DNA (Cejka et al., 2010a). In humans, resection by BLM-DNA2 is similarly promoted by the human RPA, MRN, and Topo III -RMI1-RMI2 proteins (Nimonkar et al., 2011). DNA2 also interacts with another RecQ family helicase, WRN, to promote resection (Sturzenegger et al., 2014).

Exo1 and Sgs1 pathways are non-overlapping in yeast and don't stimulate one another (Mimitou and Symington, 2008). Furthermore activities of Sgs1 and Exo1 at DNA ends are mutually exclusive. Indeed, the helicase-deficient Sgs1-K706A mutant inhibits degradation by Exo1 whereas DNA unwinding by Sgs1 is inhibited by the nuclease-deficient Exo1-D173A mutant (Cannavo et al., 2013). On the contrary, BLM favors EXO1 recruitment to DSB end in mammals even though this structural role in

nonessential (Nimonkar et al., 2011). Extensive resection gives rise to long tracks of 3' ssDNA overhangs that are coated by RPA as soon as they are generated (Figure 4). In the absence of both Exo1 and Sgs1, resection can still occur but is limited to the vicinity of DSB ends and depends on the MRX complex and Sae2 (Zhu et al., 2008).

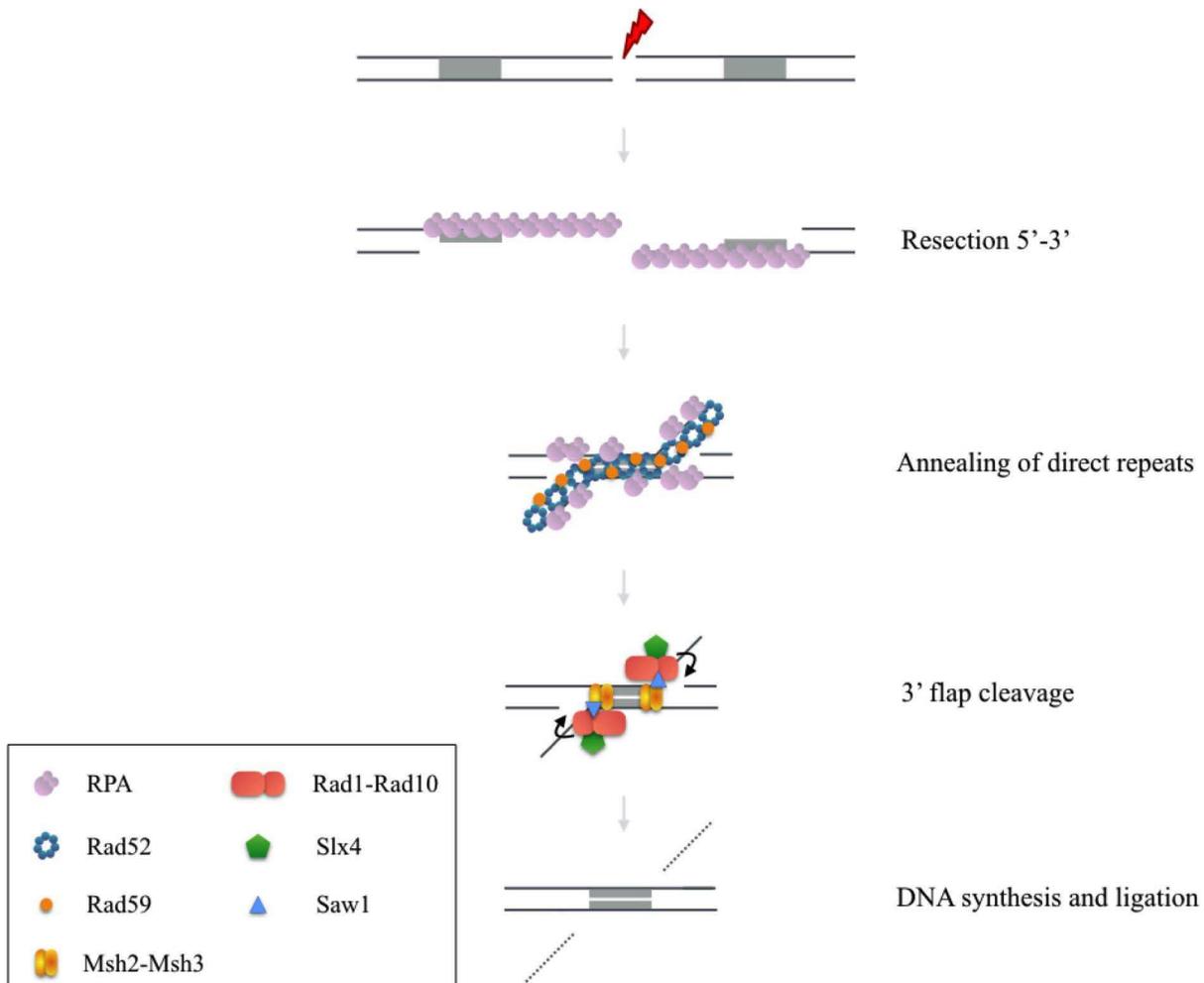
The generation of a 3' ssDNA tail is mandatory for repair by HR and resection has been identified as a crucial step for repair pathway choice between NHEJ and HR (Symington and Gautier, 2011). As already mentioned, 3' ssDNA overhangs are rapidly coated by RPA once formed. RPA is a heterotrimeric complex composed of three subunits coded by essential genes *RFA1*, *RFA2* and *RFA3* (Brill and Stillman, 1991). It is a highly conserved ssDNA binding protein with high affinity for ssDNA without sequence specificity (Alani et al., 1992). In yeast, the binding site size of RPA when bound to ssDNA has been measured and it varies over a wide range from 20-90 nucleotides for one heterodimer (Alani et al., 1992; Sibenaller et al., 1998; Sugiyama et al., 1997). In other species, the reported site size for RPA binding ranges from 20 to 30 nucleotides. One heterotrimeric complex binds ssDNA every 90-100 nucleotides. RPA is the first complex to bind ssDNA and favors the stability of the nucleofilament. It prevents its degradation and the formation of secondary structures within ssDNA such as DNA hairpins due to intra-strand annealing of palindromic sequences (Chen et al., 2013; Deng et al., 2015). Moreover RPA participates in the recruitment of additional repair proteins required for the next steps of HR (Lisby et al., 2004; Sugawara et al., 2003).

#### 1.2.2.2. *Single-Strand Annealing*

Single-Strand Annealing (SSA) is a particular mechanism of HR that relies on annealing but does not necessitate a strand invasion step and is therefore independent of the Rad51 recombinase (Ivanov et al., 1996). It is restricted to repair of DSBs that are flanked by direct repeats (Figure 5). Direct repeats can be as short as ~15-18 bp, although the frequency of SSA is very low (Sugawara et al., 2000; Villarreal et al., 2012). With increasing lengths of homologous sequence, SSA efficiency also increases until it reaches a plateau at approximately 400 bp; SSA being already efficient from 200 bp (Sugawara et al., 2000).

After DSB formation between two direct repeats, 5' DNA ends are degraded to generate 3' ssDNA tails that are coated by RPA. If resection is sufficient to unmask complementary single-stranded regions corresponding to the direct repeats, the two complementary ssDNA then anneals to form a heteroduplex. Annealing is mediated by Rad52, a central protein involved in all recombination pathways (Fishman-Lobell et al., 1992; Mortensen et al., 1996). In human cells, RAD52 is also dedicated to promote the annealing of complementary RPA-covered ssDNA strands whereas other roles in HR are mediated by BRCA2 (Jensen et al., 2010).

Rad52 binds both dsDNA and ssDNA through its N-terminus, with a higher affinity for ssDNA (Mortensen et al., 1996), and interacts with Rad51 at its C-terminus (Milne and Weaver, 1993). It harbors a ring structure when bound to ssDNA (Shinohara et al., 1998). Its strand annealing activity is enhanced by direct interaction with RPA (Shinohara et al., 1998; Sugiyama et al., 1998). Furthermore annealing reaction mediated by Rad52 is also promoted by Rad59, a paralog of Rad52 that can bind ssDNA preferentially over dsDNA and anneals complementary ssDNA only in the absence of RPA (Davis and Symington, 2001; Wu et al., 2006). The requirement



**Figure 5: Model for DSB repair by Single-Strand Annealing (SSA).**

After DSB formation, DNA is resected in both directions by 5' to 3' exonucleases (Exo1 and STR-Dna2). If resection progresses past two direct repeats (*grey boxes*), the complementary ssDNA can anneal through the action of Rad52, promoted by RPA and Rad59. It leaves 3' heterologous flaps that need to be removed by Rad1-Rad10 endonucleases (*black arrows*) with the help of Msh2-Msh3, Saw1 and Slx4 before gap filling and ligation. It results in the deletion of one of the repeats and the intervening sequence.

for Rad59 increases as the repeat length decreases, suggesting that Rad52 becomes more dependent on Rad59 when the direct repeats are short or when short homologies are embedded within extensive nonhomologous regions (Sugawara et al., 2000).

Following annealing of the complementary ssDNA, two noncomplementary 3' ssDNA flaps are formed and need to be removed by Rad1-Rad10 endonucleases with the help of Msh2-Msh3, Saw1 and Slx4. Msh2-Msh3 are thought to stabilize annealed intermediates while Saw1 recruits Rad1-Rad10 to heteroduplexes with 3' flaps by direct interaction with Rad1 and affinity for 3' flaps (Li et al., 2013; Sugawara et al., 1997). Flap clipping by Rad1-Rad10 is stimulated by Slx4 which is phosphorylated upon DNA damage (Toh et al., 2010). Any remaining gaps are then filled in by new DNA synthesis and nicks are ligated. SSA results in the deletion of one of the repeats as well as the sequence in between the direct repeats and is therefore highly mutagenic (Fishman-Lobell et al., 1992) (Figure 5).

#### 1.2.2.3. *Strand invasion*

Besides SSA, HR can be separated in two main pathways namely Gene Conversion (GC) and Break-Induced Replication (BIR). Both pathways rely on the recognition and pairing of 3' ssDNA tail generated by end resection with an intact homologous sequence located on a sister chromatid, an allelic locus or at an ectopic region in the genome (Pâques and Haber, 1999). The formation of this heteroduplex DNA (hDNA) is catalyzed by the Rad51 recombinase (Symington, 2002).

Rad51 is a member of the RecA family of recombinases and is highly conserved in all eukaryotes (Shinohara et al., 1992). Whereas *S. cerevisiae rad51* mutants are viable mitotically, ablation of the *RAD51* gene in vertebrates engenders

mitotic lethality (Symington, 2002). Rad51 is a DNA-dependent ATPase harboring a large ATP binding domain composed of Walker A and B motifs as well as two DNA binding loops L1 and L2. It can bind both dsDNA and ssDNA in presence of ATP, with a higher affinity for ssDNA, to form nucleoprotein filaments that can span thousands of nucleotides (Ogawa et al., 1993; Sheridan et al., 2008; Shinohara et al., 1992; Sung, 1994). Only the filament formed with ssDNA is active for strand invasion. This presynaptic filament is an open, right-handed helix in which each Rad51 protein is bound to three nucleotides. The DNA is significantly stretched between every triplet, which enhances the length of the B-form DNA structure by 50% (Chen et al., 2008). Each helical turn thus contains six protein monomers bound to a total of 18 nucleotides (Chen et al., 2008; Sheridan et al., 2008). ssDNA is bound by the L1 and L2 loop regions of Rad51, which form a short  $\alpha$ -helix and a  $\beta$ -hairpin respectively, while ATP is present at the interface of two Rad51 molecules (Chen et al., 2008).

The formation of the Rad51 filament is a complex reaction. RPA can exert a stimulatory or an inhibitory effect on the assembly of the presynaptic filament depending on the circumstances (Sung, 1997a). Rad51 protein and RPA exclude one another from ssDNA by competing for the same binding sites but RPA also favors presynaptic complex formation by eliminating secondary structure in the 3' ssDNA overhangs (Sugiyama et al., 1997). In any case, displacement of RPA is required to allow Rad51 binding to ssDNA and it necessitates a number of mediator proteins. The main mediator of the presynaptic filament assembly is Rad52 in yeast and BRCA2 in mammals (Jensen et al., 2010). Rad52 interacts directly with both RPA and Rad51 (Milne and Weaver, 1993; Shinohara et al., 1998; Sugiyama et al., 1998). *In vitro* studies have demonstrated that inhibition of RPA on Rad51 activity can be overcome

by the addition of Rad52, which facilitates the loading of Rad51 onto RPA-ssDNA (New et al., 1998; Sung, 1997a). Both RPA-Rad52 and Rad52-Rad51 interactions are required to stimulate the displacement of RPA by Rad51 (Krejci et al., 2002; Sugiyama and Kowalczykowski, 2002). Moreover the rate-limiting step of the displacement reaction is the nucleation of Rad51 protein onto ssDNA. Once nucleation occurs, extensive displacement of RPA occurs by growth of the Rad51 filament along ssDNA. As Rad52-RPA-ssDNA form an intermediate co-complex but Rad52 can not displace RPA by itself, it is proposed that Rad52 favors the nucleation of Rad51 onto ssDNA (Sugiyama and Kowalczykowski, 2002). Similarly it has been shown that *in vivo* recruitment of Rad52 requires RPA, and presence of Rad51 depends on Rad52 after on HO-induced DSB (Lisby et al., 2004; Sugawara et al., 2003) (Figure 6).

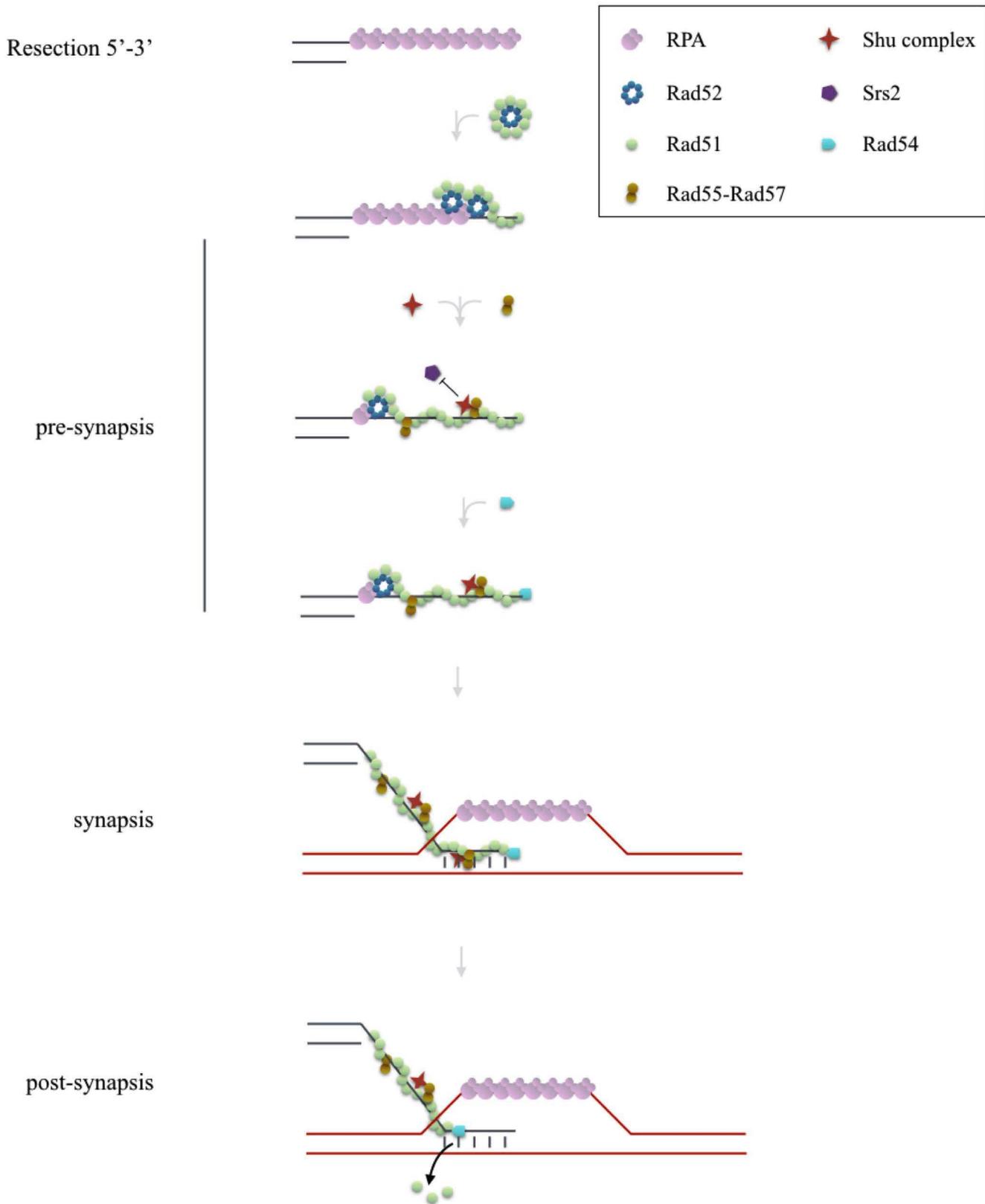
Other mediators involved in presynaptic filament assembly consist of Rad55-Rad57 and the Shu complex that are all paralogs of Rad51. The five human Rad51 paralogs associate into two different complexes, the heterotetrameric BCDX2 (RAD51B-RAD51C-RAD51D-XRCC2) complex and the heterodimeric CX3 (RAD51C-XRCC3) complex (Masson et al., 2001). Rad55 and Rad57 share 20% identity with the catalytic region of Rad51 (Lovett, 1994). Rad55 and Rad57 form a stable heterodimeric complex that interacts with Rad51 through Rad55 (Hays et al., 1995; Johnson and Symington, 1995; Sung, 1997b). Despite the similarity of both Rad55 and Rad57 to Rad51, the Rad55-Rad57 complex has no recombinase activity (Sung, 1997b). However the complex is able to counteract the inhibition of RPA on Rad51-mediated homologous DNA pairing and strand exchange (Sung, 1997b). This result suggests that Rad55-Rad57 promote the formation of the nucleoprotein filament between Rad51 and ssDNA. Furthermore Rad51 recruitment at an HO-induced DSB

is delayed in the absence of Rad55 (Sugawara et al., 2003; Wolner et al., 2003). A Rad51 mutant protein (Rad51-I345T) with stronger affinity for DNA is able to alleviate the recombination defect of *rad55* $\Delta$  and *rad57* $\Delta$  cells, confirming the role for Rad55-Rad57 in the stabilization of the Rad51-ssDNA formation (Fortin and Symington, 2002). It was later demonstrated that Rad55-Rad57 integrates the Rad51-ssDNA filament *in vitro* and stabilizes it (Liu et al., 2011). Rad55-Rad57 have also a role in the regulation of Srs2. Srs2 is a DNA helicase/translocase that disrupts Rad51 nucleoprotein filament via direct interaction with Rad51 triggering ATP hydrolysis and dissociation of Rad51 from ssDNA (Antony et al., 2009; Veaute et al., 2003). Rad55-Rad57 can bind simultaneously to Srs2 and Rad51 in a 1:1:1 ratio even though the heterodimer binds more strongly to Srs2 than Rad51 (Liu et al., 2011). Rad55-Rad57 are thus incorporated onto the ssDNA to stabilize Rad51 filaments while blocking Srs2 translocation.

Similarly, the Shu complex is involved in Rad51 nucleoprotein filament stabilization by antagonizing the effect of Srs2 (Bernstein et al., 2011). The Shu complex is composed of Psy3, Csm2, Shu1 and Shu2. Psy3 and Csm2 have been identified as Rad51 paralogs on a structural point of view and are able to bind ssDNA (Tao et al., 2012). Deletion of *SHU1* results in more Srs2-YFP foci that correlates with a decrease number of cells with Rad51 foci, consistent with a mediator role for the Shu complex through an inhibition of Srs2 (Bernstein et al., 2011). Csm2-Psy3 binds preferentially to forked DNA or 3' ssDNA and is able to stabilize Rad51 binding to ssDNA (Godin et al., 2013; Sasanuma et al., 2013). Furthermore Csm2 interacts with Rad55 and Rad57 and these proteins are part of the same epistasis group (Godin et al., 2013). Csm2 also interacts with Rad51 and Rad52 through Rad55-Rad57 (Gaines et al., 2015; Godin et al., 2013).

Another protein has been identified to mediate the presynaptic filament formation. Rad54, a member of the Swi2/Snf2 family of chromatin remodeling enzymes, indeed favors nucleation of Rad51 on ssDNA and can form a co-complex with Rad51-ssDNA (Mazin et al., 2003; Wolner et al., 2003). However, it is not dependent on its ATPase activity, and the protein is recruited after Rad51, Rad52 and Rad55 (Lisby et al., 2004; Mazin et al., 2003; Wolner et al., 2003). Together these data suggest that a pre-synaptic role of Rad54 is not sufficient to reflect the critical ATPase-dependent function of Rad54 in recombination. The pre-synaptic function may be necessary and important to target Rad54 to the pairing site, where it can engage its ATPase activity and act at the synapsis and post-synapsis steps. Rdh54 is a paralog of Rad54 in yeast and the two proteins share many biochemical features and some overlapping functions even though they have also independent roles in HR, DSB repair and other processes (Mazin et al., 2010; San Filippo et al., 2008).

Rad52, Rad55-Rad57, Rad54 and the Shu complex therefore act together to promote nucleation of Rad51 on ssDNA coated by RPA and to stabilize this nucleoprotein filament, in part by counteracting the effect of Srs2. The presynaptic filament then searches for a distant homologous sequence and subsequently invades the duplex homologous donor sequence to form a D-loop (synapsis). Pairing between Rad51-ssDNA and the donor duplex DNA is facilitated through the specific arrangement of the presynaptic filament. Indeed its stretched B-DNA form favors canonical Watson-Crick hydrogen bonds with complementary triplets in the homologous sequence. This DNA-DNA interaction is crucial for a stable interaction and pairing as Rad51 itself makes few contact with the donor sequence (Chen et al., 2008). Homology search and pairing take ~20-60 min (Hicks et al., 2011; Miné-Hattab and Rothstein, 2012). Rad54 is able to stimulate strand pairing on



**Figure 6: Schematic representation of the Rad51 nucleoprotein filament formation and D-loop formation.**

Rad52 mediates the replacement of RPA by Rad51. Rad55-Rad57 and the Shu complex are integrated in the Rad51 nucleoprotein filament to stabilize it, in part by inhibiting Srs2. Rad54 is then loaded on the presynaptic filament and promotes strand invasion by translocating onto DNA, displacing the nucleosomes and disrupting the donor duplex DNA. After D-loop formation, RPA stabilized the displaced ssDNA strand. Rad54 finally removes Rad51 from the 3' end to facilitate new DNA synthesis.

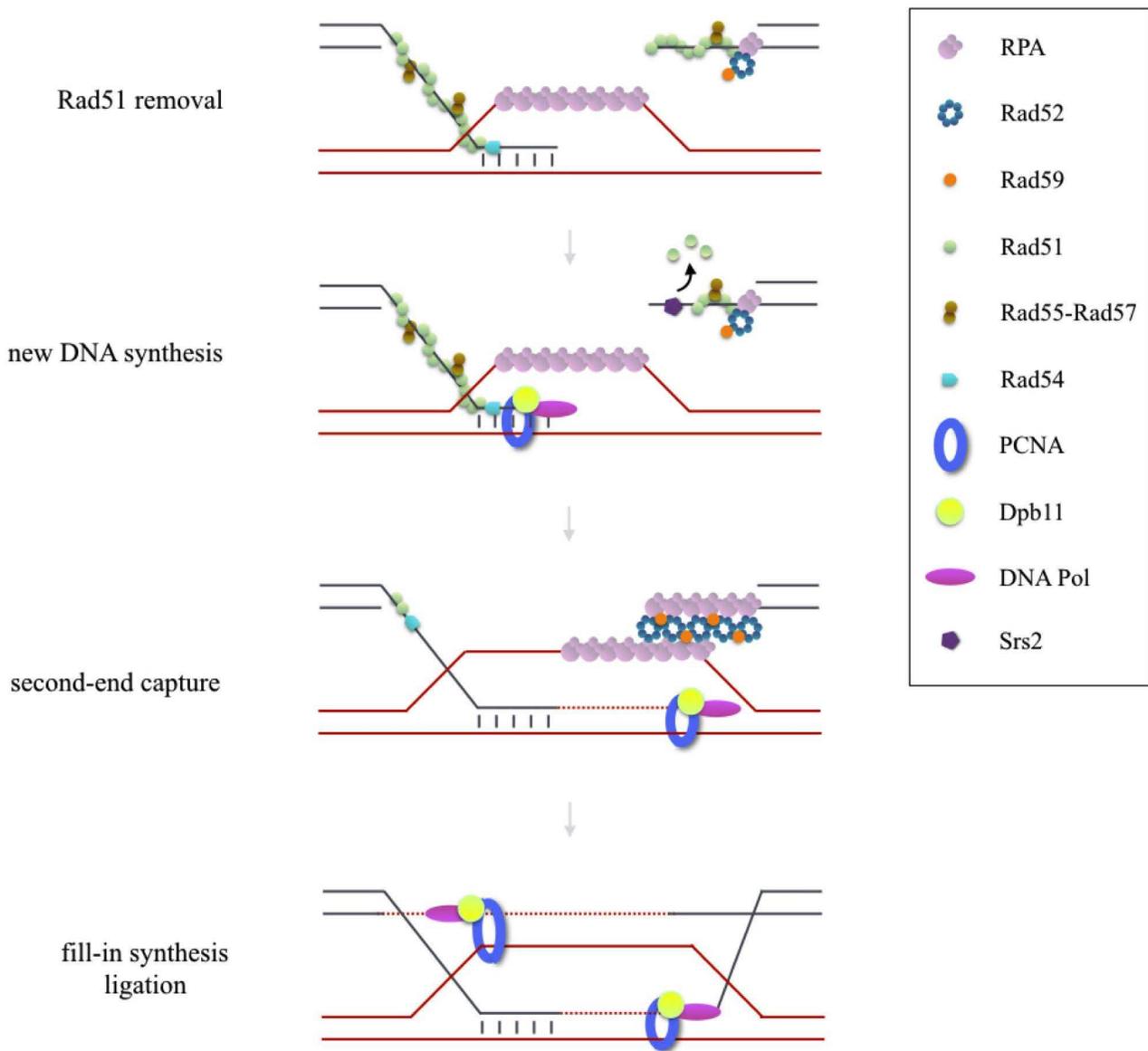
chromatinized substrates and naked DNA, with a higher efficiency in the presence of chromatin (Alexiadis and Kadonaga, 2002). Its dsDNA-dependent ATPase activity, and therefore its chromatin remodeling activity and its ability to induce supercoils into DNA, are promoted by the Rad51-ssDNA nucleoprotein filament (Alexeev et al., 2003; Mazin et al., 2000; Van Komen et al., 2000). Furthermore Rad54 has been detected simultaneously at the DSB site and at the homologous donor (Wolner et al., 2003). Altogether it suggests that Rad54 promotes strand invasion and pairing of the nucleoprotein filament by three non-exclusive mechanisms that likely cooperate. Rad54 would first act as a motor protein translocating on dsDNA and thus facilitating the recognition of the homologous sequence. Its chromatin remodeling activity would then displace the nucleosome and other chromatin bound proteins at the pairing site to allow access to the donor template. Finally the induction of negative supercoils results in transient disruption of base pairing in the dsDNA donor partner allowing joint molecule formation (Figure 6).

After D-loop formation, all steps from new DNA synthesis until restoration of two intact duplex DNAs are referred to as post-synapsis. RPA stimulates strand exchange by sequestering the displaced ssDNA from the donor region that can inhibit the pairing reaction during post-synapsis (Eggleter et al., 2002). Since deletion of *RAD54* delays but does not prevent homologous pairing between recipient and donor sequences, Rad54 function during post-synapsis seems to be more preponderant than during pre-synapsis and synapsis (Sugawara et al., 2003). After heteroduplex formation, Rad51 needs to be removed. However ATP hydrolysis is not sufficient for dissociation of Rad51 from dsDNA (Solinger et al., 2002). Removal of Rad51 from the 3' end actually depends on Rad54 by a species-specific interaction that requires the ATPase activity of Rad54 (Li and Heyer, 2008; Solinger et al., 2002). This step is

necessary to allow access of DNA polymerase to the invading 3'-OH end that is used as a primer to synthesize new DNA (Li and Heyer, 2008; Sugawara et al., 2003). Depending on the requirement of specific factors for 3' invading strand extension, and resulting in different outcomes, HR can be divided in two main alternative pathways: Gene Conversion (GC), that group Double-Strand Break Repair (DSBR) and Synthesis-Dependent Strand Annealing (SDSA) in one hand, and Break-Induced Replication (BIR) in the other hand.

#### *1.2.2.4. Double-Strand Break Repair and Synthesis-Dependent Strand Annealing*

GC corresponds to the nonreciprocal transfer of genetic information in which a short patch of DNA is copied from the homologous template to repair the broken sequence. The yeast mating type interconversion system requires GC and has been used extensively to study the timing of various aspects of this process. After induction of the HO endonuclease, there is a ~40 min delay between the initial detection of pairing and the initiation of new DNA synthesis (Hicks et al., 2011). This delay may be largely explained by the need to assemble a DNA synthesis complex, which differs from the replication complex. Indeed, it does not require the pre-replication (pre-RC) complex (ORC, MCM proteins, Cdc45) and the lagging-strand replication components (primase-Pol $\alpha$ ) (Wang et al., 2004). Nevertheless new DNA synthesis necessitates the leading-strand machinery as the loading of PCNA and Dpb11 at DSB site recruits their associated DNA polymerases Pol $\delta$  and/or Pol $\epsilon$ , Pol $\delta$  and Pol $\epsilon$  playing redundant roles in new DNA synthesis (Germann et al., 2011; Hickson and Mankouri, 2011; Li et al., 2009; Wang et al., 2004). The processivity of Pol $\delta$  and Pol $\epsilon$



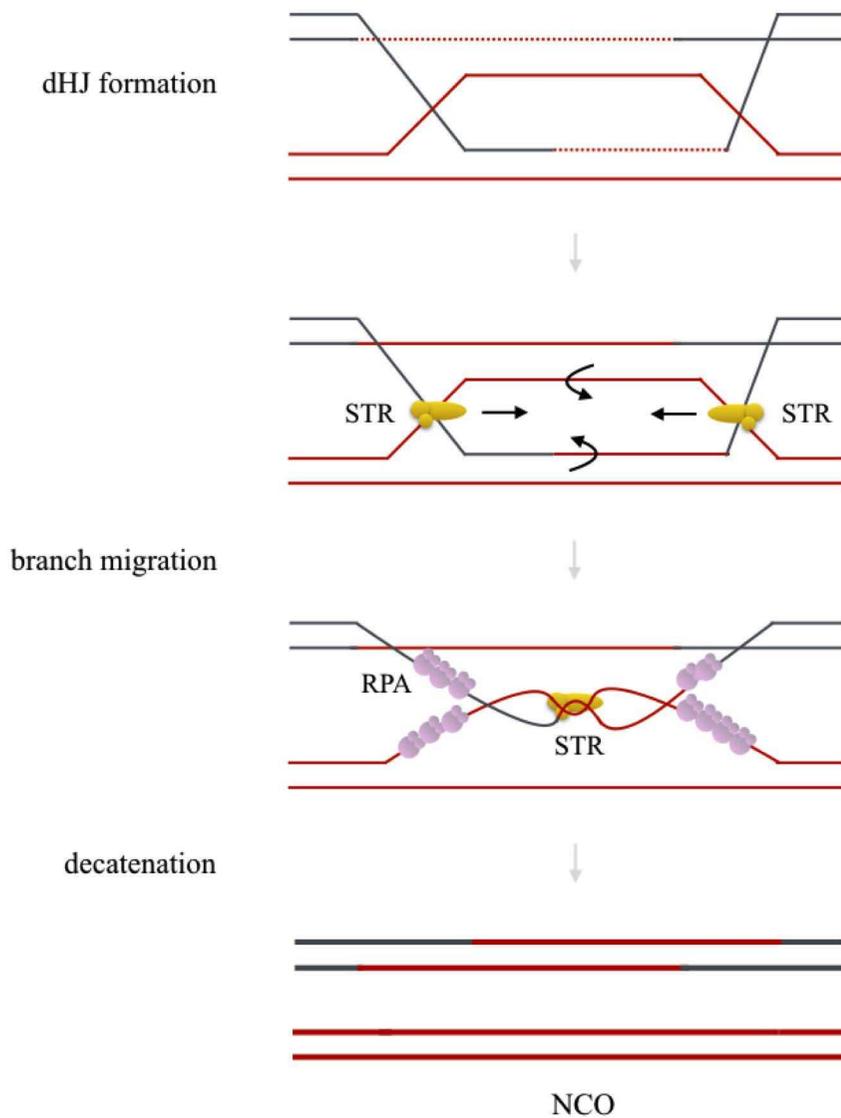
**Figure 7: double Holliday junction formation.**

After Rad51 removal from the 3' end by Rad54, the leading strand replication machinery is loading by PCNA and Dpb11 to initiate DNA synthesis using the invading strand as a primer. The displaced strand from the donor duplex anneals with the 3' ssDNA tail at the other side of the break and primes a second round of leading strand synthesis. After ligation of the newly synthesized DNA to the resected 5' strands, a double Holliday junction intermediate (dHJ) is generated.

during GC is decreased compared to S-phase replication with a high rate of mutation (up to 1400 times) associated with frequent template switches (Hicks et al., 2010; Maloisel et al., 2008). The fact that mating type interconversion does not require many of normal DNA replication complex, in particular the MCM proteins that participate in the initiation and elongation of the replication fork, might lead to a premature dissociation of the nascent DNA strand from the template that might explain this mutation rate, and reinitiation of DNA synthesis might be associated with a switch of DNA polymerases.

After extension of the invading strand, the repair intermediate can have many potential fates leading to either non cross-over (NCO) or cross-over (CO) events. CO between homologous chromosomes can lead to a potential loss of heterozygosity of the segment distal to the CO, involved in tumorigenesis, whereas CO between repeats can result in deletions or duplications (LaRocque et al., 2011; Moynahan and Jasin, 2010). In mitosis, ~20% of GC events are accompanied by CO at an allelic position whereas only ~5% with an ectopic template (Ira et al., 2003). These proportions of GC associated with CO in mitosis are much lower than in meiosis (Pâques and Haber, 1999; Symington, 2002). Two different mechanisms can explain this discrepancy among GC events associated with CO between meiosis and mitosis: DSBR and SDSA.

The DSBR model, also known as the double Holliday junction (dHJ), allows an explanation of the presence of a CO associated with GC during HR (Szostak et al., 1983). Following DSB formation, resection and strand invasion, the D-loop is formed and is extended by new DNA synthesis. The 3' ssDNA at the other side of the break coated by RPA can then anneal to the displaced strand from the donor dsDNA

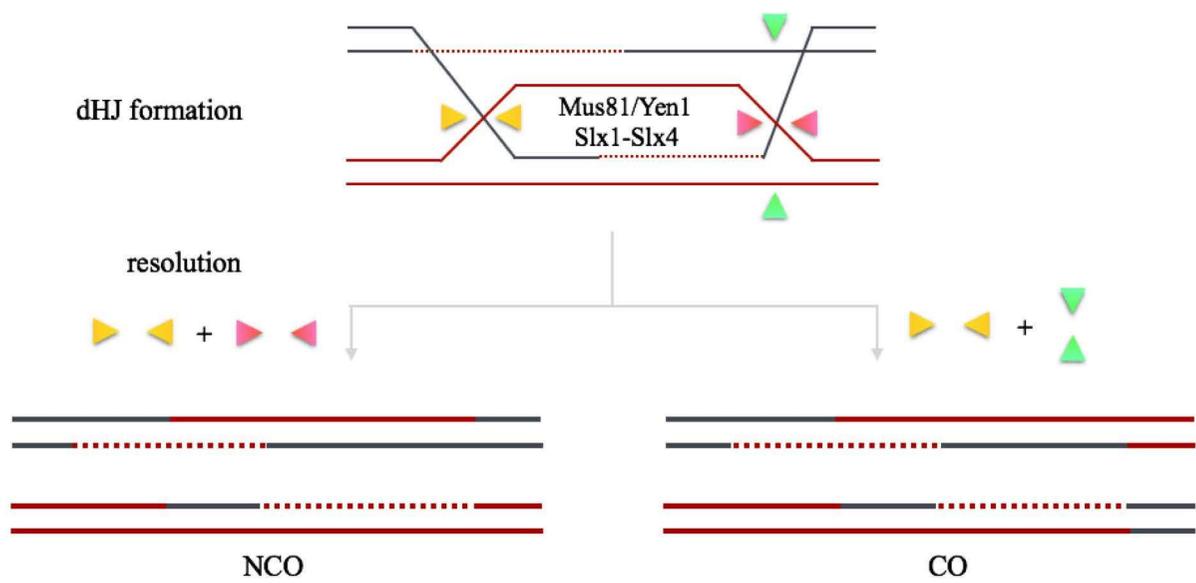


**Figure 8: Dissolution of a dHJ leads to NCO products.**

After dHJ formation, Sgs1 unwinds the DNA while Top3 removes the supercoils. Rmi1 finally stimulates both Sgs1 and Top3 to remove the last linkages between the repaired sequence and the donor template.

generating a second region of heteroduplex DNA. This process called second-end capture occurs through the interaction of RPA with Rad52, whose annealing activity is enhanced by Rad59 (McIlwraith and West, 2008; Nimonkar et al., 2009; Sugiyama et al., 2006; Wu et al., 2008). The second heteroduplex primes a second leading strand synthesis and the ligation of the two newly synthesized DNAs to the resected 5' strands generates a dHJ intermediates. The ligation step is not carried out by Cdc9 or Dnl4, the two yeast ligases, during mating type interconversion suggesting that there is another ligase activity involved yet to be identified (Wang et al., 2004) (Figure 7). Alternatively, two independent strand invasions from both DSB ends, followed by simultaneous DNA synthesis and annealing could also result in a dHJ intermediate. Until now no experimental evidence has demonstrated whether dHJ formation results from a single or a double strand invasion process. Once formed, a dHJ can branch migrate along the DNA axis, a process in which one DNA strand is progressively exchanged for another. Branch migration of dHJ extends or shortens the heteroduplex DNA formed after DNA strand invasion, affecting the length of conversion tracks and thereby the amount of genetic information transferred between the two DNA molecules. To segregate the recombinant complexes, dHJ must be removed, which can occur by dissolution or resolution.

Dissolution results exclusively in NCO and is taken in charge by the STR complex. Indeed, it was demonstrated *in vitro* that the helicase activity of Sgs1 is required to migrate the dHJ toward the center, unwinding the DNA to create ssDNA available for Top3 that removes the supercoils. RPA stabilize the reaction by binding and stabilizing the ssDNA while Rmi1 stimulates DNA decatenation by Sgs1 and Top3 that remove the last linkage between the repaired molecule and the donor sequence (Cejka et al., 2010b; Plank et al., 2006; Wu and Hickson, 2003) (Figure 8).



**Figure 9: Resolution of dHJ.**

Endonucleolytic cleavage of the two inner strands (yellow and red) leads to NCO events while cut of an inner strand and an outer strand (yellow and green) results in CO products.

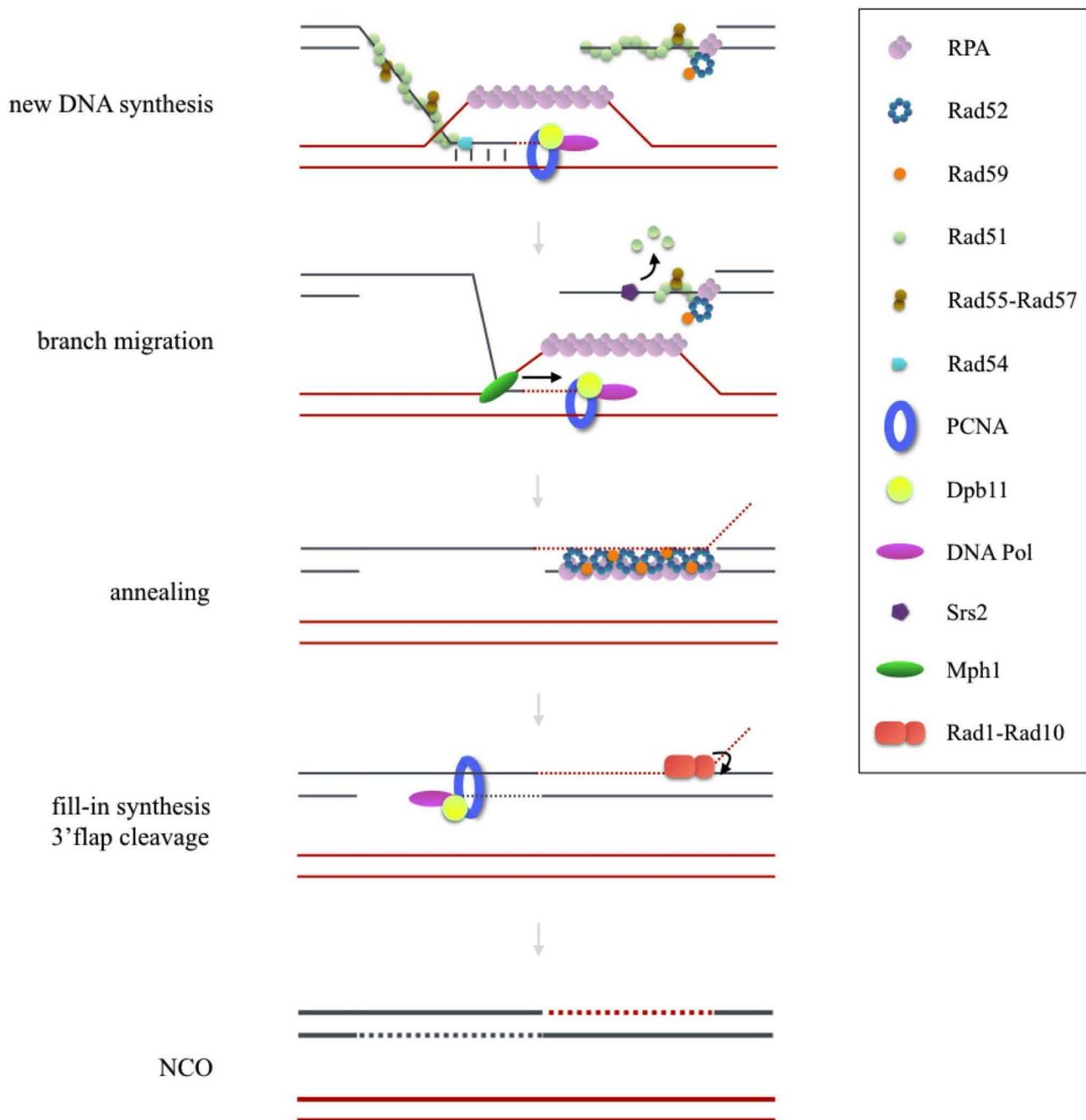
Consistently, deletion of *SGS1* and *TOP3* leads to a higher rate of GC associated with CO and lower resolution of joint molecule (Bzymek et al., 2010; Ira et al., 2003; Mankouri et al., 2011).

Resolution is the alternative way to remove dHJ and occurs through endonucleolytic cleavage. After second-end capture, dHJ may also be extended by branch migration from the center outward which can be mediated by Rad54 through its ATPase activity (Bugreev et al., 2006; Deakynne et al., 2013), and the intermediate is then cut by dHJ resolvase ending up in either NCO or CO events. Cutting the inner strands of both HJs yields NCO products, whereas cleavage of the inner strands of one HJ and the outer strands of the other generates COs (Figure 9). Mus81-Mms4, Yen1 and Slx1-Slx4 are structure-specific endonucleases that can cleave numerous DNA branched structure *in vitro*, including dHJ (Fricke and Brill, 2003; Ip et al., 2008; Kaliraman et al., 2001). *mus81* and *mms4* harbor a synthetic lethality with *sgs1*, indicating that they operate in a parallel pathway (Mullen et al., 2001). Mus81 forms a heterodimer with Mms4 with a preference for nicked dHJ, suggesting an endonucleolytic cleavage prior to ligation (Ehmsen and Heyer, 2008; Mazón and Symington, 2013; Mukherjee et al., 2014). Rad54 participates in the recruitment of Mus81 to its substrate independently of its ATPase activity, and also strongly promotes Mus81 nuclease activity (Matulova et al., 2009). Furthermore while a *yen1Δ* mutant is repair-proficient, *mus81Δ yen1Δ* and *mms4Δ yen1Δ* double mutants shows a stronger sensitivity to DNA damaging agents than *mus81Δ* or *mms4Δ* alone (Agmon et al., 2011; Blanco et al., 2010; Ho et al., 2010). *mus81Δ* and *mms4Δ* mutants also have a decreased number of CO events following a HO- or I-SceI-induced DSB, and *mus81Δ yen1Δ* and *mms4Δ yen1Δ* double mutants present a stronger decrease (Agmon et al., 2011; Ho et al., 2010). Taken together these data suggest that Yen1

acts as a back-up system in absence of Mus81 or on structures that Mus81 finds difficult to process. Slx1 and Slx4 forms a heterodimer that preferentially cleaves 5' branches at the ssDNA/dsDNA junction (Fricke and Brill, 2003). *slx1* and *slx4* are also synthetic lethal with *sgs1* (Mullen et al., 2001). However this lethality is not suppressed in a *rad52* background contrary to *mus81*, suggesting that Mus81-Mms4 and Slx1-Slx4 act on different pathways or DNA structures (Bastin-Shanower et al., 2003; Fricke and Brill, 2003).

It has been proposed that dHJ dissolution by the STR complex is the main pathway to resolve dHJ intermediates even though Mus81-Mms4 can also process these intermediates (Ashton et al., 2011). The DSBR model explains meiotic recombination events and GC events associated with CO. However, it does not explain the low percentage of COs observed during mitotic recombination.

SDSA results exclusively in NCO products and has been identified as the preferred HR repair pathways in eukaryotes (Mitchel et al., 2010). The first steps are similar to DSBR, until D-loop formation and new DNA synthesis (Ferguson and Holloman, 1996; Nassif et al., 1994). However, the D-loop structure formed during SDSA is more dynamic than during DSBR. Indeed, after the beginning of DNA synthesis, the heteroduplex is rapidly dissociated by branch migration of the D-loop, the extended invading strand being therefore only transiently associated with its donor template. D-loop dissociation is taken over by Mph1. Mph1 is a DNA helicase with a 3'-5' polarity and can dissociate Rad51-generated D-loop *in vitro* (Prakash et al., 2009). *mph1* $\Delta$  or *mph1* helicase-dead mutants show a higher rate of CO products and an accumulation of joint molecules, consistent with a role in promoting SDSA (Mazón and Symington, 2013; Mitchel et al., 2013; Prakash et al., 2009). Srs2



**Figure 10: Mechanistic view of SDSA.**

After D-loop formation, the leading strand machinery is loading on the 3' end of the invading strand to synthesize new DNA. Mph1 can dissociate the D-loop by branch migration. Disruption of the Rad5 nucleoprotein filament by Srs2 prevents second end capture. After annealing of the two extremities of the DSB, the 3' flap is cut by Rad1-Rad10 and the gap is filled in by the leading strand machinery. Final ligation ends up by a NCO product.

possibly removes Rad51 from the unpaired 3' DNA tail to prevent second end capture and dHJ formation. Rad54 is also able to branch migrate the D-loop toward the DSB and to dissociate it, but this activity is structure-specific and depends on the length of the invading ssDNA (Bugreev et al., 2006; Wright and Heyer, 2014). Overexpression of wild-type Rad54 reduces GC tract lengths while ATPase-deficient Rad54 increases them, in agreement with a role for Rad54 in SDSA (Kim et al., 2002). After D-loop dissociation, the extended strand can then anneal to the resected 3' strand on the other side of the DSB through RPA-Rad52-Rad59 concerted action. A heterologous flap can be formed after strand annealing if DNA synthesis went further than the region of homology. As in SSA, the flap is removed by Rad1-Rad10 with the helps of Msh2-Msh6, Saw1 and Slx4 (Fishman-Lobell et al., 1992; Li et al., 2013; Sugawara et al., 1997; Toh et al., 2010). It is followed by gap filling through the leading strand machinery using the newly-synthesized strand as a template and a ligation step involving an unidentified ligase (Wang et al., 2004) (Figure 10).

SDSA thus appears as an optimized mechanism to promote high fidelity repair with a limited risk of loss of heterozygosity.

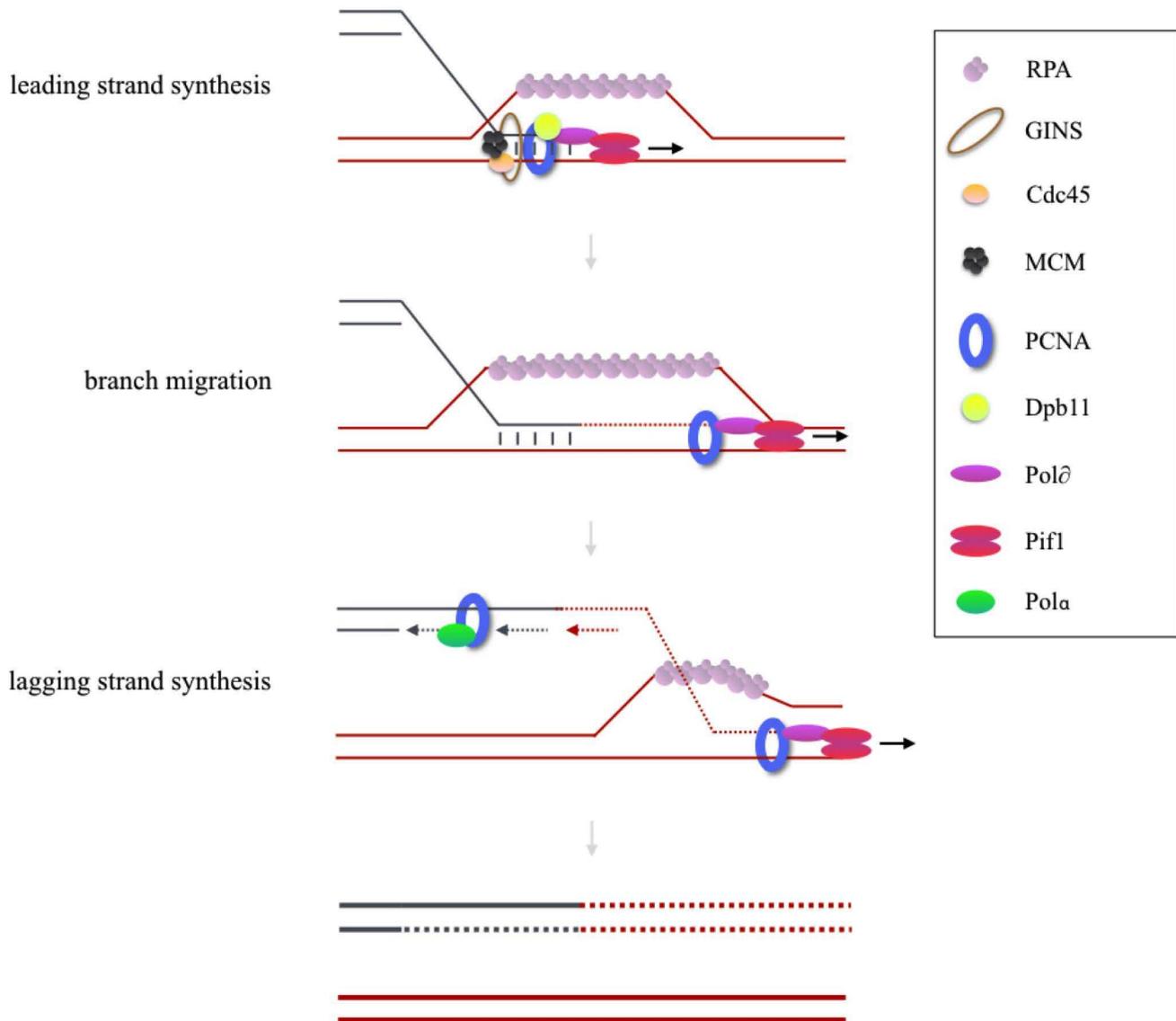
#### *1.2.2.5. Break Induced Replication*

BIR corresponds to a nonreciprocal translocation from the donor template to the broken sequence and is accompanied with extensive loss of heterozygosity. It relies on recombination-dependent DNA replication (Bosco and Haber, 1998; Malkova et al., 1996). It is thought to occur when only one DSB end shares homology with the template or when only one DSB end is available for repair. Indeed, BIR is

outcompeted by GC as it represents <2% of HR events in the case of a DSB with two homologous ends (Malkova et al., 2005).

The initial steps of BIR appear to be identical to those of GC, requiring end resection, homologous pairing and strand invasion, and necessitate all of the same proteins (Davis and Symington, 2004; Jain et al., 2009). However, after strand invasion, a unidirectional replication fork is set up and is capable of copying >100kb of DNA from the sequences distal to the site of homology up to the telomere. BIR efficiency is increased when the DSB is placed closer to the telomere and new DNA synthesis has been identified as the rate-limiting step for BIR, indicating that the formation of the replication fork and the length it needs to copy are crucial for BIR (Donnianni and Symington, 2013; Jain et al., 2009). BIR requires some proteins of the replication machinery (Cdc45, GINS, PCNA), including the three major DNA polymerases responsible for leading and lagging strand synthesis (Pol $\delta$ , Pol $\epsilon$ , Pol $\alpha$ -primase), but not the proteins needed for the assembly of the pre-RC required at replication origins (ORC, Cdc6) (Lydeard et al., 2007; 2010b). In contrast to GC, it specifically requires Pol32, the non-essential subunit of the Pol $\delta$  complex (Lydeard et al., 2007). The Pol $\alpha$ -primase complex and Pol $\delta$  are necessary for the initiation of new BIR replication while Pol $\epsilon$  is only required for processive elongation of the newly synthesized ssDNA (Lydeard et al., 2007). Moreover, Pol $\delta$  is specifically recruited by the Pif1 helicase that stimulates its DNA synthesis activity (Wilson et al., 2013).

Nonetheless the replication fork established during BIR differs from the S-phase replication fork. Indeed, BIR is much more mutagenic than replication and is often associated with template switching, indicative of several rounds of strand invasion, DNA synthesis and dissociation (Deem et al., 2011; Pardo and Aguilera, 2012; Ruiz et al., 2009; Smith et al., 2007). Furthermore replication during BIR



**Figure 11: BIR sets up a replication fork that copy the donor template up to the telomere.**

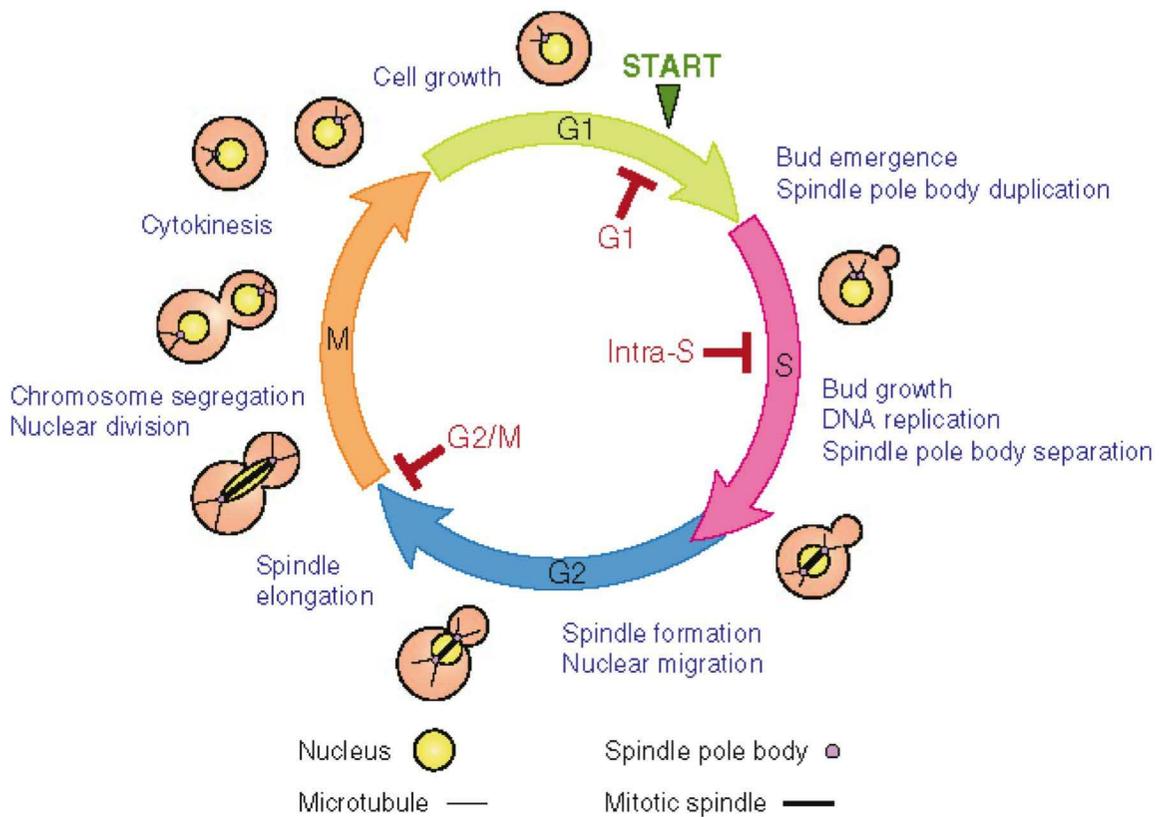
After D-loop formation, almost all the component of the leading strand machinery is recruited. Pif1 promotes DNA synthesis by extending the D-loop. Lagging strand synthesis occurs on the newly repaired strand, resulting in a conservative DNA synthesis.

occurs through a migrating D-loop and the newly synthesized strands segregate with the broken chromosome. It indicates a conservative mode of DNA synthesis in which the lagging-strand synthesis initiates on the nascent ssDNA ejected from the tail end of the D-loop (Donnianni and Symington, 2013; Saini et al., 2013). Although the MCM proteins have first been implicated in the extension of the D-loop (Lydeard et al., 2010b), migration of the D-loop up to the telomere is mainly mediated by Pif1, thus facilitating extensive DNA synthesis by both recruiting Pol $\delta$  and liberating the newly synthesized ssDNA (Saini et al., 2013; Wilson et al., 2013) (Figure 11). The BIR migrating D-loop needs to be stable to process extensive DNA synthesis. Indeed, overexpression of Mph1 has been shown to decrease the frequency of BIR by promoting D-loop dissociation whereas the *mph1* mutant exhibits an increased BIR rate (Luke-Glaser and Luke, 2012; Stafa et al., 2014). Mph1 plays also an important role in BIR template switching (Stafa et al., 2014).

BIR is not the pathway of choice to repair a DSB. In a haploid context, the loss of telomeric proximal sequences can be lethal for the cell if an essential gene was present on it. Depending on the homology and the orientation of the replication fork, the BIR product can result in an acentric or a dicentric chromosome, both of which are also lethal for the cell. The percentage of cells engaging in BIR events compared to GC may thus be underestimated.

### 1.2.3. DSB signaling

To deal effectively with DNA lesions, which is crucial for cellular survival and maintenance of genomic stability, all organisms have developed sophisticated and



**Figure 12: *S. cerevisiae* cell cycle and DNA damage checkpoints.**

The G1 checkpoint arrests the cell before START. The intra-S phase checkpoint slows the rate of replication. The G2/M checkpoint blocks the cells at the metaphase/anaphase transition. *Adapted from Finn et al., 2011*

well-conserved surveillance mechanisms called DNA damage checkpoints (Hartwell and Weinert, 1989). Checkpoints activation leads to cell cycle arrest and recruitment and coordination of DNA repair proteins to give time for the cell to repair. The coordinated cellular response between DNA repair and checkpoints activation is referred to as the DNA damage response (DDR). After repair completion, checkpoints are down-regulated and cells can re-enter the cell cycle in a process called recovery. If the lesion is irreparable, it leads to cellular senescence or apoptosis. Alternatively, the cells may go through adaptation and eventually re-enter the cell cycle in the presence of DNA damage (Lee et al., 1998).

Checkpoints operate at three distinct stages in the cell cycle in response to DNA damage (Finn et al., 2011) (Figure 12). The G1 checkpoint arrests cells at the G1/S transition prior to START (restriction point: control of the cell size and nutrients available) and delays bud emergence, spindle pole body duplication and S phase entry. The intra-S phase checkpoint slows the rate of replication to allow fork repair mechanisms before entry to mitosis. The G2/M checkpoints arrests cells at the transition between metaphase and anaphase, inhibiting cells to progress through mitosis with DNA damage. Checkpoint activation is much more efficient in G2/M than in G1 phase. Indeed, a single DSB is sufficient to trigger checkpoint activation in G2/M while multiple DSBs are required in G1 phase (Gerald et al., 2002; Pellicioli et al., 2001; Zierhut and Diffley, 2008).

The DNA damage checkpoint has been commonly presented as a protein kinase cascade with three main steps. Sensors first detect the lesion and adaptors then communicate the information from sensors to effectors. Effectors are kinases that phosphorylate particular targets to ensure cell cycle arrest and DNA repair.

#### 1.2.3.1. Sensing a DNA double-strand break

The key components of the signaling pathway are several phosphoinositol-3-kinase-related kinase (PIKK) family members. *S. cerevisiae* possesses two PIKK proteins, namely Mec1 and Tel1 (respectively ATR and ATM in humans). PIKK proteins contain a unique kinase domain at their C-terminus flanked by two regions of similarity called FAT and FATC, which may interact and participate in the regulation of the kinase activity (Bosotti et al., 2000).

The first sensor of a DSB is actually the MRX complex (Kim et al., 2005; Lisby et al., 2004). Once bound to the DSB end, the MRX complex recruits Tel1 through a direct interaction between the C-terminal region of Xrs2 and the FATC domain of Tel1 (Nakada et al., 2003; Ogi et al., 2015). MRX also stimulates the kinase activity of Tel1 (Fukunaga et al., 2011). Consistently, Tel1 foci are formed very rapidly at DSB sites and depend on Mre11. Furthermore Tel1 foci form before RPA foci, thus indicating that Tel1-dependent checkpoint activation occurs prior to DNA end processing (Lisby et al., 2004). After its activation, Tel1 phosphorylates Mre11, Xrs2 and Sae2, promoting their functions in DNA repair and checkpoint activation (Baroni et al., 2004; Clerici et al., 2006; Usui et al., 2001). Tel1 also phosphorylates histone H2A on S129 ( $\gamma$ -H2A), an important landmark in the DDR that can spread over ~50 kb domain of chromatin around the DSB (Downs et al., 2000; Shroff et al., 2004).

Mec1 recruitment at DSB site requires the presence of RPA-coated ssDNA 3' overhangs and this recognition depends on the Mec1-interacting protein Ddc2 (ATRIP in mammals) (Zou and Elledge, 2003). Ddc2 forms a complex with Mec1 and recognizes and binds RPA-coated ssDNA (Paciotti et al., 2000; Zou and Elledge, 2003). The interaction between Mec1-Ddc2 and RPA-coated ssDNA is sufficient for

Mec1-Ddc2 recruitment to DNA damage site but is not sufficient to fully activate Mec1. The heterotrimeric ring-shaped complex 9-1-1 composed of Ddc1-Rad17-Mec3 (RAD9-RAD1-HUS1 in humans) is loaded onto the 5' junction between ssDNA and dsDNA by the Rad24-RFC (Rad17-RFC in humans) clamp loader via direct interaction between RPA and Rad24 (Majka et al., 2006a). The 9-1-1 clamp can fully activate the kinase activity of Mec1 either directly via interaction of Ddc1 with Mec1 or indirectly through recruitment of Dpb11 by Ddc1 (Majka et al., 2006b; Navadgi-Patil and Burgers, 2009). Recruitment of Dpb11 requires the phosphorylation of Ddc1 by Mec1, suggesting that RPA-recruited Mec1-Ddc2 may have sufficient residual kinase activity (Navadgi-Patil and Burgers, 2009). Once activated, Mec1 is able to phosphorylates a wide number of targets, including Rfa1, Sae2, the checkpoint adaptators Rad9 and the checkpoint effector Rad53 (Baroni et al., 2004; Brush and Kelly, 2000; Sweeney et al., 2005). As for Tel1, Mec1 also participates in the establishment of  $\gamma$ -H2A (Downs et al., 2000; Shroff et al., 2004).

Both kinases preferentially phosphorylate serines and threonines preceding a glutamine residue on numerous target proteins in response to damage. However Tel1 seems to have a minor role in DSB signaling compared to Mec1. Indeed, *tell1* $\Delta$  cells do not show obvious hypersensitivity to DNA damaging agents and are not defective in checkpoint activation in response to a single DSB (Mantiero et al., 2007). It may be explained by the ability of yeast cells to rapidly convert the DSB ends into ssDNA substrates that preferentially stimulate Mec1 kinase activity, thus masking Tel1 contribution to the checkpoint. Tel1 activation promotes the accumulation of ssDNA at DSB ends through phosphorylation of Sae2 and therefore is critical for the subsequent activation of Mec1 (Baroni et al., 2004; Mantiero et al., 2007). As

generation of ssDNA ultimately leads to Tel1/ATM inactivation, this mechanism ensures an efficient switch from Tel1/ATM to Mec1/ATR (Shiotani and Zou, 2009).

#### *1.2.3.2. Adaptors of the checkpoint response*

Two mediators of the checkpoint reaction exist in yeast, Rad9 and Mrc1 (respectively 53BP1 and Claspin in metazoans). They ensure the transmission of the signal from Tel1 and Mec1 to the protein kinases Rad53 and Chk1.

Mrc1 is a component of the replisome and is specific to the S phase of the cell cycle. It regulates Rad53 activation in response to replication stress (Alcasabas et al., 2001). Mrc1 directly interacts with Rad53 and favors the interaction between Mec1 and Rad53, increasing by 70 fold the ability of Mec1 to activate Rad53 (Chen and Zhou, 2009).

Rad9 protein contains two BRCT domains at its C-terminus and a central Tudor domain, both important for its functions (Usui et al., 2009). Recruitment of Rad9 involves several pathways. In normal conditions, Rad9 is already bound to chromatin through an interaction between its Tudor domain and methylated H3K79 (Grenon et al., 2007). This constitutive Rad9 recruitment to chromatin is thought to facilitate the efficiency of the Rad9-dependent response to DNA damage, which requires additional histone modifications (Huyen et al., 2004). Upon DNA damage, interaction of Rad9 with chromatin is strengthened by the interaction of the Rad9 BRCT domain with  $\gamma$ -H2A (Granata et al., 2010; Hammet et al., 2007). Another pathway for Rad9 recruitment is independent of histone modifications and is particularly important in G2/M checkpoint activation. It requires the phosphorylation of Ddc1 by Mec1, creating a docking site for Dpb11 (Navadgi-Patil and Burgers,

2011). Dpb11 can directly interact via its BRCT domain with two CDK-phosphorylated residues of Rad9 (Pfander and Diffley, 2011). Dpb11 thus functions to recruit Rad9 at DNA damage site through interaction with both Ddc1 and Rad9.

Upon DNA damage, Rad9 is phosphorylated by both Mec1 and Tel1 which creates a binding site for FHA domains of Rad53 (Sweeney et al., 2005; Vialard et al., 1998). Rad9 functions as a signaling scaffold to bring Rad53 in close proximity to Mec1 at sites of damage to facilitate the Mec1-dependent phosphorylation of Rad53 (Sweeney et al., 2005). Rad9 participates also in the activation of Rad53 independently of Mec1 and Tel1. Indeed, it brings Rad53 molecules into close proximity facilitating Rad53 *in trans* autophosphorylation (Gilbert et al., 2001). Rad9 phosphorylation also triggers its oligomerization that is necessary for the maintenance of checkpoint signaling through a feedback loops involving Rad53-dependent phosphorylation of the Rad9 BRCT domain (Usui et al., 2009). Fully activated Rad53 is then released from the hyperphosphorylated Rad9 complex (Gilbert et al., 2001).

#### 1.2.3.3. *Effector kinases and their targets*

Effectors kinases are one of the last steps of the checkpoint cascade, regulating their targets involved in cell cycle control and transcriptional regulation. In yeast, the two main effectors are Rad53 and Chk1, corresponding to CHK2 and CHK1 in vertebrates. In contrast to *S. cerevisiae* where Rad53 is the principal effector kinase, CHK1 is the primary effector of both the DNA damage and replication checkpoints in vertebrates, with CHK2 playing a subsidiary role (Stracker et al., 2009).

Chk1 is a serine/threonine kinase and only regulates the G2/M transition (Liu et al., 2000; Sanchez et al., 1999). Chk1 is phosphorylated by both Mec1 with the

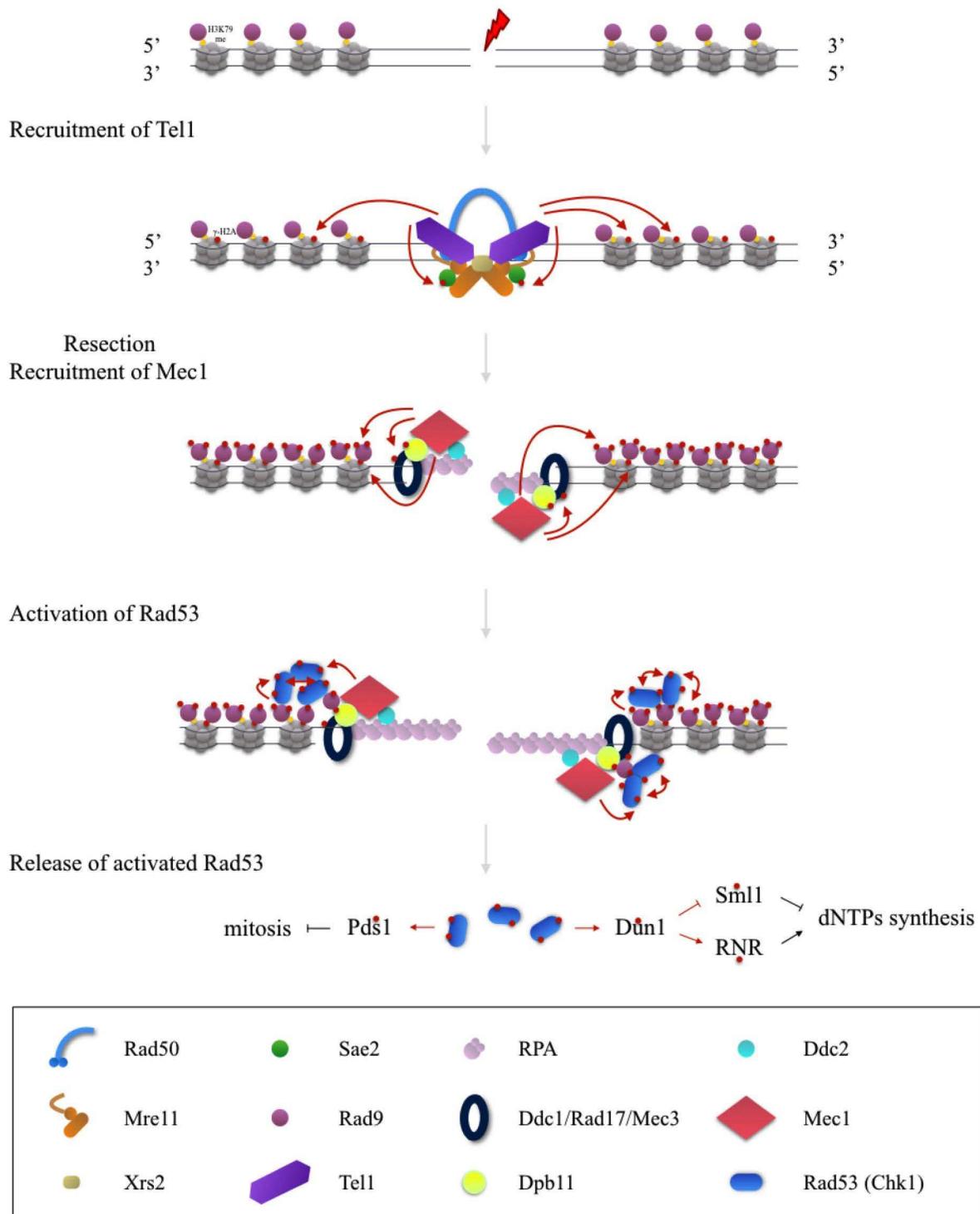
help of Rad9 after DNA damage (Sanchez et al., 1999). Indeed, the N-terminal domain of Rad9 interacts specifically with Chk1 and recruits it to DSB sites (Blankley and Lydall, 2004).

Rad53 is an essential protein composed of a central serine/threonine kinase domain and two FHA domains, one at its N-terminus and the other at its C-terminus (Sweeney et al., 2005). As mentioned above, Rad53 activation requires Mec1, Rad9 and Mrc1 in response to DNA damage and once autophosphorylated, it is released from chromatin.

Mec1 and Tel1 signal through Rad53 primarily and Chk1 that synergistically regulate the expression of more than 600 genes (Jaehnig et al., 2013). The best-identified targets are Pds1 and Dun1.

The main target of Chk1 is Pds1, an inhibitor of anaphase progression whose degradation by the anaphase-promoting complex (APC) through an ubiquitin-mediated pathway is a prerequisite for mitotic progression. Upon DNA damage, Pds1 is phosphorylated by Chk1, which inhibits the ubiquitination reaction (Agarwal et al., 2003; Wang et al., 2001). Rad53 also plays a role in the stabilization of Pds1 by regulating the interaction between Pds1 and Cdc20, the anaphase-specific factor for the APC ubiquitin ligase (Agarwal et al., 2003). Stabilization and accumulation of Pds1 by the combined action of Chk1 and Rad53 prevent its ubiquitination and causes a cell cycle arrest before anaphase, presumably to allow the cell to repair the damaged DNA before sister chromosomes segregate.

Rad53 also regulates the action of Dun1, a kinase paralog of Rad53 with a serine/threonine domain and two FHA domains. Rad53 activates Dun1 by



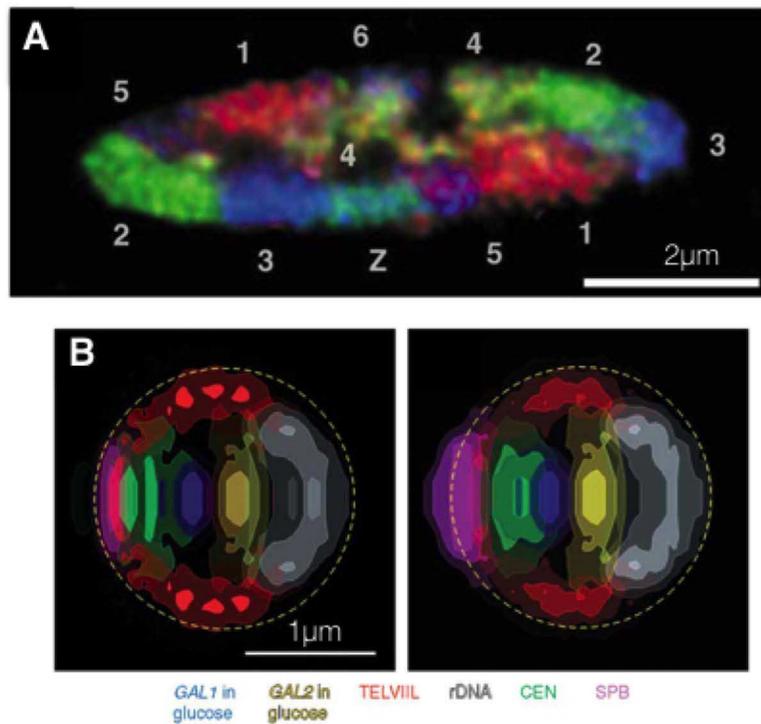
**Figure 13: DNA damage-induced checkpoint activation.**

Following DSB formation, MRX binds to DNA ends and recruits Tel1, which phosphorylates histone H2A and Sae2. After resection, the ssDNA is coated by RPA that recruits Mec1-Ddc2. Mec1 full activation requires Dpb11 and the 9-1-1 clamp. Once activated, Mec1 activates the downstream checkpoint kinase Rad53 by phosphorylating Rad9 and Rad53 itself. Moreover, phosphorylated Rad9 promotes activation of Rad53 by allowing its *in-trans* autophosphorylation. Activated Rad53 is then released from DNA and can regulate its specific targets.

phosphorylating a particular residue in its FHA domain (Bashkirov et al., 2003; Chen et al., 2007). Once phosphorylated, Dun1 regulates deoxyribonucleotides (dNTPs) synthesis by two mechanisms. On the one hand, it activates the transcription of *RNR2*, *RNR3* and *RNR4* coding for ribonucleotide reductase subunits necessary for dNTPs synthesis (Elledge et al., 1993; la Torre Ruiz and Lowndes, 2000). On the other hand, activated Dun1 phosphorylates Sml1, an inhibitor of Rnr1. Sml1 phosphorylation leads to its degradation, thus allowing dNTPs synthesis by Rnr1 (Andreson et al., 2010; Zhao and Rothstein, 2002). Dun1 phosphorylation by Rad53 thus results in an increase of the dNTP pools in the cells. Control of the cellular dNTPs levels may be essential after DNA damage to carry out new DNA synthesis.

Rad53 also phosphorylates Exo1, which negatively regulates Exo1 activity on resection (Morin et al., 2008). Limitation of ssDNA accumulation can be part of a negative feedback loop to prevent extensive resection, regulate Mec1 activation and allow shut down of checkpoint response.

Instead of a simple cascade reaction with sensors, adaptors and effectors, the checkpoint response may be more considered as a complex regulatory network with feedback loops and threshold response (Figure 13). The crosstalk between DNA repair proteins and checkpoint proteins during the DDR ensures and coordinates cell cycle arrest and repair completion.



**Figure 14: Nuclear organization in chromosome or gene territories.**

(A) Visualization of chromosome territories in chicken cell. Mid-plane optical section through a chicken fibroblast nucleus with seven pairs of painted chromosomes. *Adapted from Habermann et al., 2001*

(B) Representation of gene territories in yeast. Combined map showing relative arrangement of five selected gene territories without transformation (*left panel*) or after normalization to the nuclear envelope ellipsoid semi-axes (*right panel*). *Adapted from Berger et al., 2008*

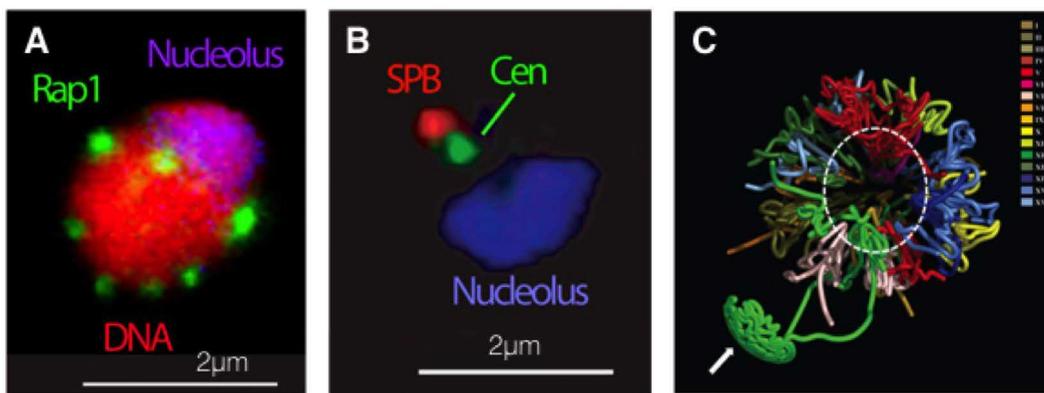
## **2. Nuclear organization of *Saccharomyces cerevisiae***

In budding yeast, like in other eukaryotes, the genetic information contained in DNA molecules is sequestered in the nucleus; a specialized organelle separated from the other cellular components by a double membrane called the nuclear envelope. The nucleus is home to many DNA metabolic steps. It ensures the proper expression, replication, repair and segregation of chromosomes as well as the proper processing and export of mRNA and rRNA.

Chromosomes or genomic loci are not arranged randomly in the nucleus. They occupy preferential positions with respect to each other, mediated by long-range interactions in *trans*, until forming chromosome territories in vertebrates or gene territories in yeast (Berger et al., 2008; Cremer et al., 2006; Habermann et al., 2001) (Figure 14). Chromatin also interacts with several components of the nucleus. This stable interaction creates anchoring sites that physically constrain the movement of the chromatin, thus contributing to the maintenance of chromosome or genomic loci positioning in the interphase nucleus (Avşaroğlu et al., 2014; Chubb et al., 2002; Hediger et al., 2002; Heun et al., 2001). The combination of physical constraints on chromatin movement and specific interactions helps generate nuclear subcompartments that are not delimited by a membrane but enriched for specific DNA sequences, factors, and enzymatic activities.

### **2.1. DNA based compartments located at the nuclear periphery**

In yeast, the anchoring of particular DNA sequences at the nuclear periphery associated with specific proteins appears to be responsible for creating



**Figure 15: Chromosome organization within the yeast interphase nucleus.**

(A) Clustering of yeast telomeres visualized by anti-Rap1 IF (*green*) while the nucleolus is stained with anti- Nop1 (*blue*). Ethidium-bromide staining of nucleic acids (*red*). *Adapted from Taddei and Gasser, 2012.*

(B) Confocal fluorescence images of a centromere detected by FISH (*green*) near SPB (*red*) and the nucleolus (*blue*) detected by anti-Nop1. *Adapted from Bystricky et al., 2005.*

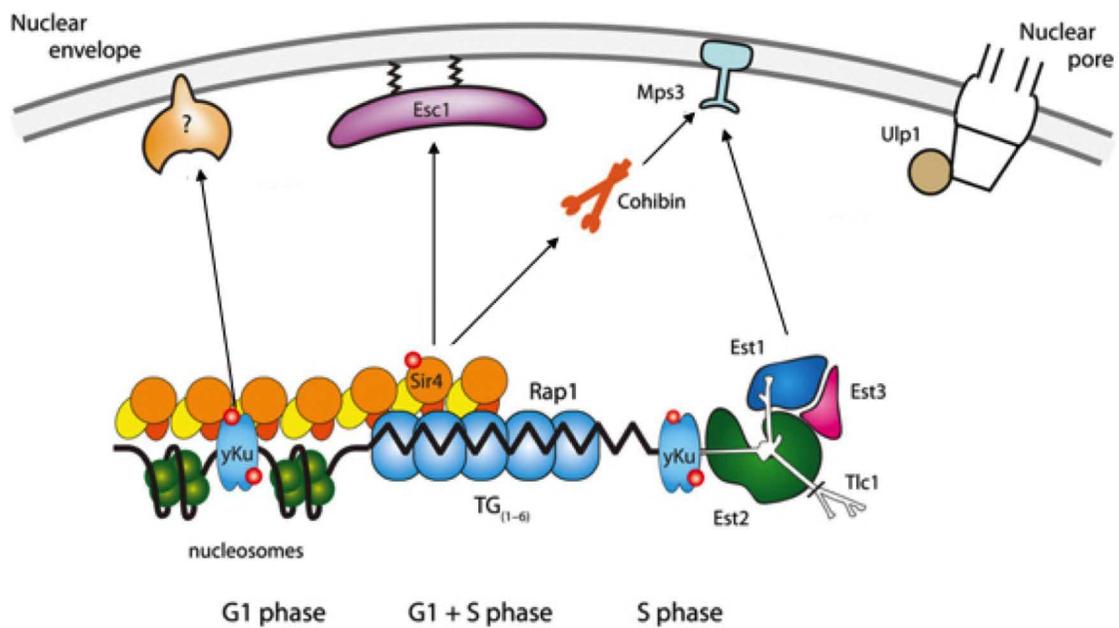
(C) Three dimensional model of the yeast genome. All chromosomes cluster via centromeres at one pole of the nucleus (*dashed oval*), while chromosome 12 extends outward toward the nucleolus, which is occupied by rDNA repeats (*white arrow*). *Adapted from Duan et al., 2010.*

microenvironments that promote or prevent DNA or RNA-based activity

### 2.1.1. Centromeres clustering and telomeres positioning dictate a Rab1-like conformation

The centromere is a unique and specific region present on each chromosome that ensures accurate chromosome segregation. In yeast, centromeres comprise a ~125 bp DNA sequence that contains three conserved DNA sequence elements wrapped around a single nucleosome containing a specific histone H3 variant not found in other nucleosomes (Cse4 in yeast) (Furuyama and Biggins, 2007). Centromeres were shown to cluster in a rosette-like structure around the spindle pole body (SPB; the microtubule organizing center), which is embedded in the nuclear envelope opposite to the nucleolus (Bystricky et al., 2004; Duan et al., 2010; Jin et al., 2000) (Figure 15). Each centromere directs the assembly of the kinetochore, a protein complex of ~70 subunits that binds the plus end of a single microtubule (Joglekar et al., 2009). During interphase, the microtubules maintain the attachment between the centromeres and the SPB (Bystricky et al., 2004; Jin et al., 2000).

Chromosome extremities are composed of telomeres and ~30 kb regions of DNA upstream of telomeres termed subtelomeres. The 32 telomeres of *S. cerevisiae* corresponds to stretches of TG<sub>1-3</sub> repeats with ~250 bp in length. Rap1 binds this repeat on average once every 18 bp (Gilson et al., 1993). The Rap1 C-terminus interacts with the silencing factors Sir3 and Sir4 and the telomerase-repressive factors Rif1 and Rif2 and their binding is mutually antagonistic (Marcand et al., 1997; Moretti et al., 1994; Wotton and Shore, 1997). Telomeres are also bound by the Ku heterodimer (Gravel et al., 1998; Martin et al., 1999). Subtelomeres contain only a

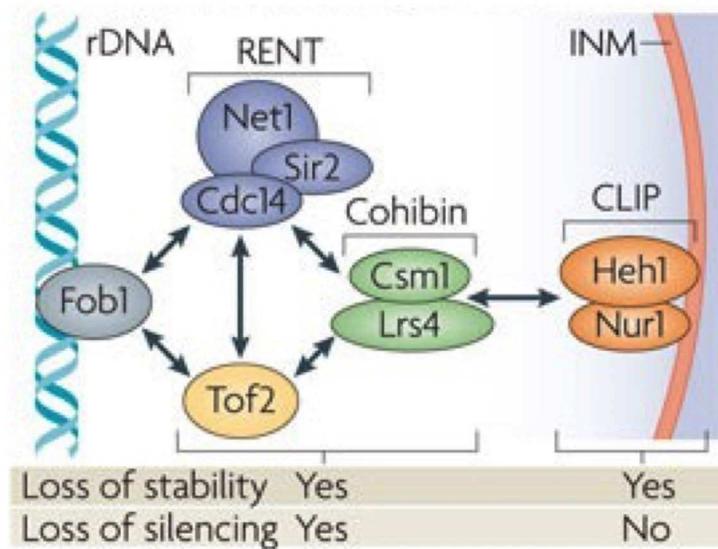


**Figure 16: Telomere anchoring pathways.**

Sir4 binds Esc1, as well as yKu80 and Mps3. yKu80 binds telomerase, which also associates with Mps3 in S phase through Est1. There is an unidentified anchor for yeast Ku in G1 phase that is neither Esc1 nor Mps3 dependent. *Adapted from Taddei and Gasser, 2012*

few genes, all nonessentials, and are constituted of repeated elements. All subtelomeres shared a highly conserved core X sequence while Y' elements are only present on a subset of subtelomeres, their number varying in copy number and location among strains (Wellinger and Zakian, 2012). The telomeres and subtelomeres cluster into three to eight foci and are mainly located near the nuclear periphery (Gotta et al., 1996; Hediger et al., 2002; Palladino et al., 1993; Therizols et al., 2010). The anchoring of telomeres is realized by several protein-protein interactions that can differ according to the cell cycle and the telomere considered (Hediger et al., 2002; Schober et al., 2009; Taddei et al., 2004) (Figure 16). The major telomere-anchoring pathway requires Sir4, which anchors silenced chromatin to the nuclear periphery via direct interaction between its C-terminus and Esc1, an acidic protein associated exclusively with the inner face of the nuclear envelope (Andrulis et al., 2002; Taddei et al., 2004). Sir4 can also tether telomeres through its association with an integral SUN-domain containing protein of the nuclear membrane, Mps3 (Bupp et al., 2007). This interaction is indirect as it requires Lrs4, a component of the cohibin complex (Chan et al., 2011). Mps3 also participates in a Sir-independent anchoring pathway during S phase through its interaction with telomerase, which is recruited at telomeres by Ku (Schober et al., 2009). Ku is also able to anchor telomeres to the nuclear periphery by interacting with nuclear pore proteins or a still unidentified factor of the nuclear membrane (Galy et al., 2000; Taddei et al., 2004; Therizols et al., 2006).

Due to centromere clustering near the SPB and telomere positioning close to the nuclear periphery, arrangement of chromosomes is nonrandom and polarized in interphase yeast nuclei (Figure 15). Carl Rabl first described such folded conformation for anaphase chromosomes in salamander larvae (Rabl, 1885). By



**Figure 17: Protection of rDNA repeats.**

Fob1 recruits the RENT complex, cohibin and Tof2 that promote silencing assembly. rDNA is anchored at the nuclear envelope via interaction between the CLIP complex and cohibin. *Adapted from Mekhail and Moazed, 2010.*

analogy, nuclear organization of chromosomes in interphase yeast has thus been described as a Rabl-like conformation.

### 2.1.2. The nucleolus and the rDNA array on chromosome 12

The nucleolus is the most prominent subnuclear compartment and it is the site of RNA pol I-mediated rDNA transcription and ribosome subunit assembly. This compartment dedicated to ribosome biogenesis has a crescent-shaped structure in yeast and occupies up to one-third of the nuclear volume; abutting the nuclear envelope and lying opposite the SPB (Bystricky et al., 2005; Yang et al., 1989) (Figure 16). In budding yeast, unlike in other yeasts and most other species, the tandem array of rDNA is confined to a single genomic region, on the right arm of chromosome 12 (12R). rRNA is encoded in 100-200 tandem repeats and each repeat unit is 9.1 kb in size and yields a 35S precursor rRNA, transcribed by RNA pol I, and a 5S rRNA, transcribed by RNA pol III (Mekhail et al., 2008; Pasero and Marilley, 1993). rDNA repeats are spatially separated from the bulk of nuclear DNA and provide the foundation for the nucleolus (Duan et al., 2010) (Figure 15).

Because of the number of tandem repeats constituting rDNA, it is critical to maintain its stability to ensure cell growth and survival, otherwise it leads to replicative senescence (Sinclair and Guarente, 1997). Budding yeast has thus evolved two mechanisms to preserve rDNA stability (Figure 17). The first one consists of blocking Fob1, a nucleolar proteins involved in replication fork blocking and recombination, by inducing rDNA silencing through the action of Tof2, the RENT complex (regulator of nucleolar silencing and telophase exit; composed of Net1, Cdc14 and Sir2) and the cohibin (corresponding to Lsr4 and Csm1) (Huang and

Moazed, 2003; Huang et al., 2006; Smith and Boeke, 1997; Straight et al., 1999). The second pathway involves long-range chromatin tethering to the nuclear envelope through the CLIP complex [chromosome linkage inner nuclear membrane (INM) proteins]. The CLIP complex is composed of two integral inner nuclear membrane proteins, Heh1 and Nur1, and physically associates the rDNA-associated cohibin complex to the nuclear envelope (Chan et al., 2011; Mekhail et al., 2008).

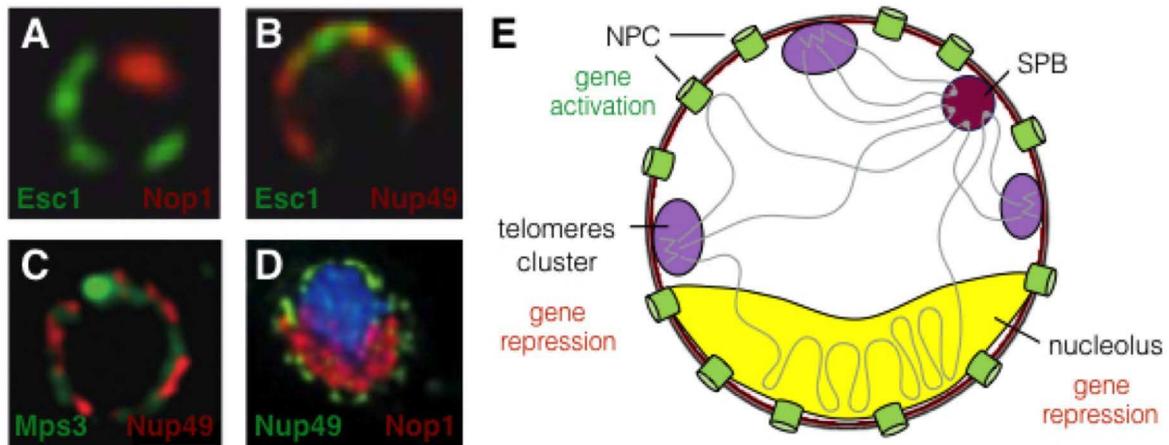
## **2.2. Nuclear envelope and the Nuclear Pore Complex**

The nuclear envelope consists of two lipid bilayers – the inner nuclear membrane (INM) and the outer nuclear membrane (ONM) – that separate chromatin from the cytoplasm and is contiguous to the endoplasmic reticulum. The nuclear envelope is discontinuous as the INM and the ONM fuse in a sharply curved structure called the nuclear pore membrane to form an eyelet within which a Nuclear Pore Complex (NPC) sits (Wente and Rout, 2010). Approximately 200 NPCs span the yeast nuclear envelope (~3,000 to 5,000 NPCs in mammalian cells).

NPCs are large proteinaceous assemblies of ~50 MDa composed of 456 nucleoporins of 30 different types (Alber et al., 2007; D'Angelo and Hetzer, 2008). Each NPC is a doughnut-shaped structure that displays eightfold symmetry around a central channel. The NPC also comprises two rings from which extend flexible protein filaments into both the cytoplasm and the nucleoplasm, termed cytoplasmic filaments and nuclear basket respectively (Alber et al., 2007; Wente and Rout, 2010). The NPC is attached at the nuclear pore membrane thanks to its membrane ring composed of three insoluble proteins (Alber et al., 2007).

NPCs mediate the bidirectional exchange between the cytoplasm and the nucleoplasm. They allow free diffusion of small molecules (less than ~40 kDa in mass or ~5 nm in diameter) while transport of macromolecules across the nuclear envelope depends on either their direct interaction with NPC or their recognition by transport factors that interact with NPC (Cook et al., 2007; Macara, 2001). NPCs play also a role in genome organization by transiently anchoring highly transcribed or inducible genes upon transcriptional activation (Brickner and Walter, 2004; Casolari et al., 2004). In addition to provide a platform for mRNA transcription, NPCs participate in mRNA quality control and export to the cytoplasm (Dieppo and Stutz, 2010).

Beside nucleoporins, many yeast proteins were shown to be associated with the nuclear envelope by microscopy (Huh et al., 2003). Among them, proteins of the INM play a particular role in several nuclear functions and genome organization. Heh1, Nur1 and Mps3 are integral proteins of the INM and anchor rDNA and telomeres to the nuclear envelope as described above (Bupp et al., 2007; Mekhail et al., 2008; Schober et al., 2009). In mammals, the equivalent proteins bridge from the lamina or the INM to chromatin. Mps3 is a shared component of the SPB and the INM and also facilitates the insertion of the SPB in the nuclear membrane by modulating the nuclear envelope composition (Friederichs et al., 2011). Ndc1 is another integral membrane proteins that participates in the insertion of both NPCs and SPB in the nuclear envelope (Chial et al., 1998). Esc1 is a yeast-specific protein that associates tightly to the inner face of the INM. In addition to its role in telomere tethering, it is also involved in the plasticity of shape of the nucleus (Andrulis et al., 2002; Hattier et al., 2007).



**Figure 18: Visualization of distinct domains of the nuclear envelope.**

(A) Localization of Esc1 (*green*) and the nucleolar protein Nop1 (*red*).

(B) Localization of Esc1 (*green*) and the nucleoporin Nup49 (*red*). *Adapted from Taddei et al., 2004.*

(C) Localisation of Mps3 (*green*) and the nucleoporin Nup49 (*red*). The green foci represents the SPB. *Adapted from Horigome et al., 2011.*

(D) Localisation of the nucleoporin Nup49 (*green*), the nucleolar protein Nop1 (*red*) and the nucleoplasm (*blue*). *Adapted from Porter et al., 2005.*

(E) Schematic representation of the yeast nuclear organization. Chromosomes are organized in a Rab1-like conformation with centromeres clustered at the SPB and telomeres tethered at the nuclear periphery in several foci. Telomeres and rDNA anchoring leads to gene repression while chromatin anchoring to NPC is coupled with gene activation.

Thus, the nuclear periphery has an important role in nuclear organization by anchoring chromatin and participating in both gene repression and gene activation. However, it should be seen as a dynamic network with functional crosstalk between its different components. Indeed, Mps3 and Ndc1 interact together and this interaction is important for the distribution of Ndc1 between the NPC and SPB (Chen et al., 2014). Esc1 is necessary for the proper localization of Nup60 while Mlp1 and Mlp2 are required for normal localization of Esc1, indicating a specific interaction between the nuclear basket and Esc1 (Lewis et al., 2007; Niepel et al., 2013). Nevertheless INM proteins, NPC and SPB constitute spatially distinct domains of the NE as they do not overlapped when localized at high resolution (Horigome et al., 2011; Porter et al., 2005; Taddei et al., 2004) (Figure 18). This proximity and interactions between the different perinuclear subcompartments could favor the efficiency and reversibility of gene induction.

### **2.3. Nuclear organization and DSB repair**

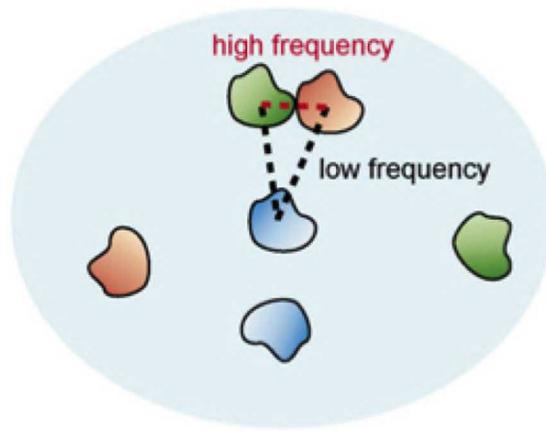
The non-random organization of the genome has an impact on transcription and other processes linked to DNA metabolism are also likely affected by this 3D organization. These past few years, an increased number of studies have started to uncover the importance of nuclear organization on DSB repair.

#### **2.3.1. DSB position influences its repair**

The efficiency of DSB repair is highly dependent on the position of the break in the nucleus and was first unraveled in the 90's for both mitotic and meiotic recombination (Burgess and Kleckner, 1999; Goldman and Lichten, 1996).

Telomeres are less permissive to homologous recombination than subtelomeres (Louis et al., 1994). Indeed intertelomeric recombination occurs at less than 0.3% (Claussin and Chang, 2015; Teixeira et al., 2004). Inhibition of recombination at telomeres is independent of silencing or tethering at the nuclear periphery and involves yKu, Cdc13 and Est1 (Grandin et al., 2001; Marvin et al., 2009b; Stavenhagen and Zakian, 1998; Tong et al., 2011). However, impaired telomere tethering correlates with a decrease in recombination efficiency in subtelomeric regions, indicating that chromatin positioning at the nuclear periphery is important for subtelomeric DSB repair (Chung et al., 2015; Schober et al., 2009; Therizols et al., 2006). This perinuclear anchoring of telomeres strongly promotes DSB repair by BIR (Chung et al., 2015). Furthermore, the distance of a DSB from chromosome ends influences repair efficiency in subtelomeric regions. The ratio of NHEJ to HR decreases progressively as telomeres are approached. This change in ratio is due to an increase frequency of recombination events when the break is positioned closer to chromosome ends, even though the different non-NHEJ pathways are differentially affected (Ricchetti et al., 2003).

Better understanding of this highly ordered chromosomes organization due to the polarized Rab1-like conformation has given new insights on its importance for DSB repair (Agmon et al., 2013; Lee et al., 2016). Indeed, centromeres clustering at the SPB favors inter-chromosomal interactions between centromere proximal sequences within a 20 kb window (Duan et al., 2010). Consequently, recombination between two centromere-proximal cassettes or between a centromere-proximal cassette and a subtelomeric region of a short arm shows a high recombination efficiency (Agmon et al., 2013). Telomeres are grouped according to their chromosomal arm lengths, i.e. subtelomeres of small arms are found in closer



**Figure 19: Spatial proximity influences DSB repair.**

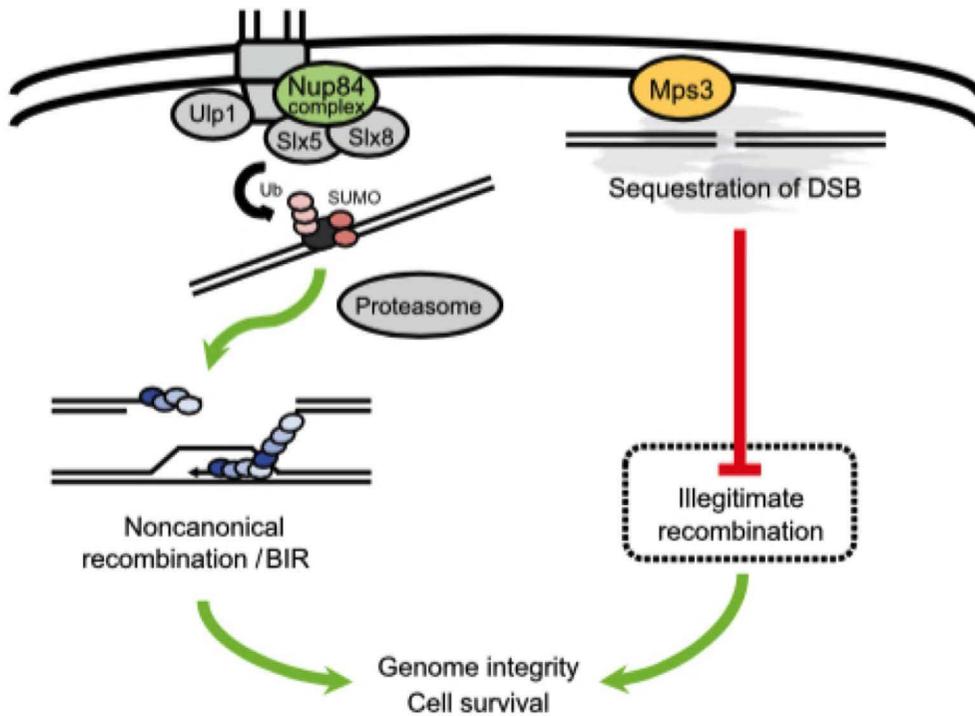
Two close loci recombine more frequently than two more distant ones. *Adapted from Meaburn et al., 2007.*

proximity than subtelomeres on arms of different sizes, and are more likely to interact (Bystricky et al., 2005; Duan et al., 2010; Guidi et al., 2015; Schober et al., 2008; Therizols et al., 2010). This correlates with the decreased efficiency of recombination observed when two homologous cassettes are integrated at subtelomeric regions of different chromosomal arms lengths (Agmon et al., 2013). Thus centromeres tethering at the SPB and telomeres anchoring to the nuclear envelope prevent recombination between differentially localized chromosomal sequences. Accordingly, there exists a strong correlation between contact frequencies of two genomic regions and their ability to recombine. GC efficiency strongly depends on the distance between the DSB site and the homologous donor (Lee et al., 2016). A similar correlation was made in mammals whereby proximally positioned chromosomes or genes undergo translocation events more frequently than distally positioned genomic regions (Meaburn et al., 2007; Parada and Misteli, 2002; Roukos and Misteli, 2014; Roukos et al., 2013).

Altogether, these data suggest that recombination between two loci depends on their spatial proximity and their ability to interact one with another; strongly supporting the idea that homology search is the rate-limiting step in DSB repair (Figure 19). However, until now, this postulate has only been based on correlation between recombination rates and spatial distances and has never been directly proved.

### 2.3.2. Relocalization of a DSB to the nuclear periphery

In yeast, a Rad52 DNA repair focus is able to recruit more than one DSB, suggesting that distinct and dedicated repair centers exist as preferential sites of repair where repair factors concentrate (Lisby et al., 2003). These Rad52 foci, formed either



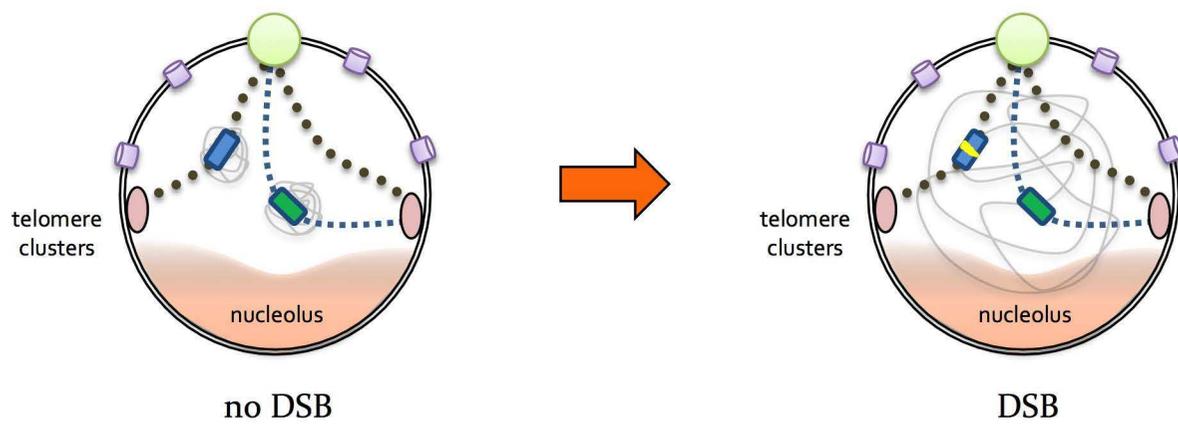
**Figure 20: Model of DSB relocation to the nuclear periphery.**

DSB binding to NPC promotes noncanonical recombination through Slx5/Slx8 and Ulp1 action while tethering by Mps3 prevents recombination. *Adapted from Horigome et al., 2014.*

spontaneously during S phase or induced by DNA damage, are strongly enriched in the nuclear interior, suggesting that HR does not occur at the nuclear periphery (Bystricky et al., 2009).

Nevertheless, certain types of DNA damage are recruited to the nuclear periphery where they bind either the Nup84 complex (located at the NPC central channel) or Mps3 (Horigome et al., 2014; Kalocsay et al., 2009; Khadaroo et al., 2009; Nagai et al., 2008; Oza et al., 2009). The same observation was made in *Drosophila* (Ryu et al., 2015). On the contrary, DSBs generated in mammalian cells do not migrate to the nuclear envelope or the NPC (Lemaître et al., 2014; Soutoglou et al., 2007). Relocation to both Mps3 and nuclear pores requires the deposition of the histone variant Htz1 (H2A.Z) by the chromatin remodeler SWR1 (Horigome et al., 2014).

Persistent or slowly repaired DSBs are recruited to nuclear pores during all phases of the cell cycle through the action of Mec1, Tel1 and Slx5/Slx8 in addition to Htz1 deposition (Horigome et al., 2014; Nagai et al., 2008). Similarly, eroded telomeres that activate the DDR also move to the NPC (Khadaroo et al., 2009). Persistent DSBs interact with the Nup84 complex that recruits the SUMO-dependent ubiquitin ligase Slx5/Slx8 and the SUMO protease Ulp1 (Nagai et al., 2008; Palancade et al., 2007). Sumoylated proteins may accumulate at slowly repaired DSBs that require Slx5/Slx8 ubiquitylation for proteasomal degradation, or alternatively, desumoylation by Ulp1 - even though this last step has not been demonstrated to interact with persistent DSBs so far - to enable appropriate repair using BIR or MMEJ (Horigome et al., 2014). A similar mechanism is proposed in *Drosophila* (Ryu et al., 2015).



**Figure 21: Increased local and global mobility upon DNA damage**

Increase in both local and global chromosome mobility upon DNA damage promotes homology search and the chance of two homologous sequences to collide in the nucleus to increase DSB repair by HR.

Relocation of an unreparable DSB to the INM protein Mps3 is constrained to S and G2 phases and specifically requires the chromatin remodeler INO80 and the deposition of Htz1 (Horigome et al., 2014; Kalocsay et al., 2009). Binding to Mps3 results in repression of recombination, arguing that Mps3 confines persistent DSBs to prevent illegitimate recombination (Horigome et al., 2014; Oza et al., 2009; Schober et al., 2009).

Relocation of a slowly repaired DSB to the nuclear periphery thus appears as a particular mechanism that promotes genome stability by preventing unequal sister chromatid exchange and undesired repair events (Horigome et al., 2014) (Figure 20).

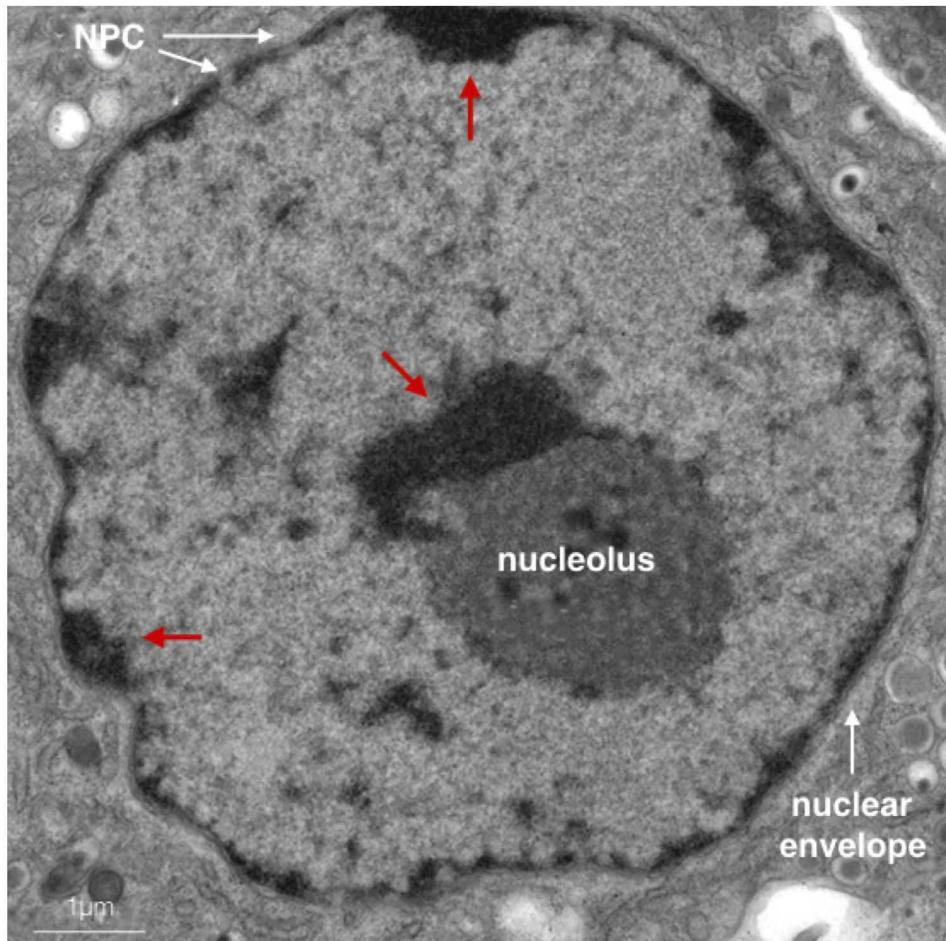
### 2.3.3. Genome mobility upon DNA damage

A broken chromosome is four to five times more mobile than an undamaged locus (Dion et al., 2012; Miné-Hattab and Rothstein, 2012), arguing that DSBs are dynamic and may scan the nucleus to find their appropriate template for repair. The increased mobility of the DSB site initially depends on Sae2 and then the repair factors Rad51 and Rad54 (Dion et al., 2012; Miné-Hattab and Rothstein, 2012). It also requires Mec1 and Rad9 but is independent of Rad53, suggesting that downstream checkpoint functions do not regulate DSB mobility (Dion et al., 2012).

The induction of DNA damage not only affects the movement of the broken locus but also induces a global increase of chromatin mobility (Miné-Hattab and Rothstein, 2012; Seeber et al., 2013) (Figure 21). Global chromatin mobility increase requires a threshold level of damage with the induction of the full checkpoint response through Mec1 and Rad53, which likely act by regulating INO80 (Neumann et al., 2012; Seeber et al., 2013). Upon DNA damage, phosphorylation of Cep3, a

kinetochore component, also favors global chromatin mobility by relieving the centromeric constraint imposed on chromosomes (Strecker et al., 2016). However, Cep3 is phosphorylated by Rad53 and not by Mec1 questioning the role of Cep3 phosphorylation in DSB mobility, although it could account for the Rad53 dependent global genome mobility. This study also questions the fact that increased mobility favors homology search and hence HR as HR efficiency is not affected in *cep3* mutants that cannot be phosphorylated.

In mammals, DSB mobility is also controversial (Dion and Gasser, 2013; Lemaître and Soutoglou, 2015). On the one hand, several findings are similar to the yeast model where damaged chromatin displays a twofold increased mobility compared to unbroken DNA (Dimitrova et al., 2008; Krawczyk et al., 2012; Lottersberger et al., 2015). This enhancement of chromatin mobility requires 53BP1, which limits resection in favor of NHEJ, and the INM proteins SUN1 and SUN2 as well (Dimitrova et al., 2008; Lottersberger et al., 2015). Moreover, it was shown that spatially close DSBs can relocalize in repair centers as in yeast (Aten et al., 2004; Caron et al., 2015; Krawczyk et al., 2007). On the other hand, other studies have demonstrated that chromatin containing DSBs exhibit limited mobility and do not cluster in repair centers (Jakob et al., 2009; Kruhlak et al., 2006; Soutoglou et al., 2007). The constrained motion of DSB ends is partially dependent on Ku80 (Soutoglou et al., 2007). Some of the discrepancies in the movement of DNA damage may be attributed to the fact that different types of DNA damage were induced and that cells activate the ATR/ATM checkpoint response to different degrees, which was shown in yeast to contribute to DSB movement (Seeber et al., 2013). Accordingly, it was shown recently that ATM mediates DSB mobility and relocation of proximal DSBs in a repair center (Becker et al., 2014; Caron et al., 2015).



**Figure 22: Electron radiograph of the mammalian liver nucleus.**

The majority of the nucleus volume is occupied by euchromatin. Heterochromatin is dense and mainly present at the nuclear periphery and next to the nucleolus (*red arrows*). Adapted from Akhtar and Gasser, 2007.

### 3. Chromatin and repair

One key component of nuclear architecture is higher-order chromatin structure. DNA molecules are associated with specific proteins to form a particular structure called chromatin in order to fit in the limited volume of the nucleus. The basic element of chromatin is the nucleosome composed of histone octamers (two H2A/H2B dimers associated with two H3/H4 dimers) wrapped by 147 bp of DNA. The chromatin fiber adopts a wide range of structures *in vivo* corresponding to at least five different levels of compaction (Albert et al., 2012). The heterogeneous distribution of chromatin in the nucleus reveals the existence of two kinds of chromatin defined as euchromatin and heterochromatin (Akhtar and Gasser, 2007; Tooze and Davies, 1967) (Figure 22). Euchromatin is a lightly packed form of chromatin that allows gene expression, contains a high concentration of genes and is replicated at the beginning of S phase. In contrast, heterochromatin is tightly packed and corresponds to the non-transcribed portion of DNA located at the nuclear and nucleolar periphery. It is replicated late during S phase. Heterochromatin can be divided in two types: constitutive and facultative. Constitutive heterochromatin is found in all cells and is associated with repeated sequences while facultative heterochromatin designates genomic regions that have the opportunity to adopt open or compact conformations within temporal and spatial contexts and can differ from one cell to the other. Altogether, chromatin is a dynamic structure that is not regularly compacted and can be transiently modified on both DNA and histones.

Chromatin condensation and its repartition in the nucleus contribute plenty to nuclear organization and one can expect DSB repair to be differentially regulated in euchromatin and heterochromatin.

### **3.1. Repair in euchromatin**

Even though euchromatin is lightly packed, DSB repair requires the action of several chromatin remodelers and histone modifications enzymes to render damaged DNA accessible to repair proteins (Figure 23).

#### 3.1.1. Influence of histone modifications

Post-translational modifications (PMTs) of histones include phosphorylation, acetylation, methylation and ubiquitination. Upon DNA damage, the most characterized histone modification is the phosphorylation of histone H2A mediated by Mec1 and Tel (Downs et al., 2000). Nevertheless other histone modifications occur in regions surrounding a DSB. Indeed, acetylation of H3 and H4 N-tails by Esa1 (part of the NuA4 complex) and Gcn5 HATs are important for DSB repair (Bird et al., 2002; Downs et al., 2004; Tamburini and Tyler, 2005). Histone acetylation confers a negative charge to histone tails leading to chromatin relaxation which favors access of repair proteins to DNA (Murr et al., 2005). All these histone modifications also form a platform mediating the recruitment of repair proteins (Bennett and Peterson, 2015; Downs et al., 2004; Morrison et al., 2004; van Attikum et al., 2007).

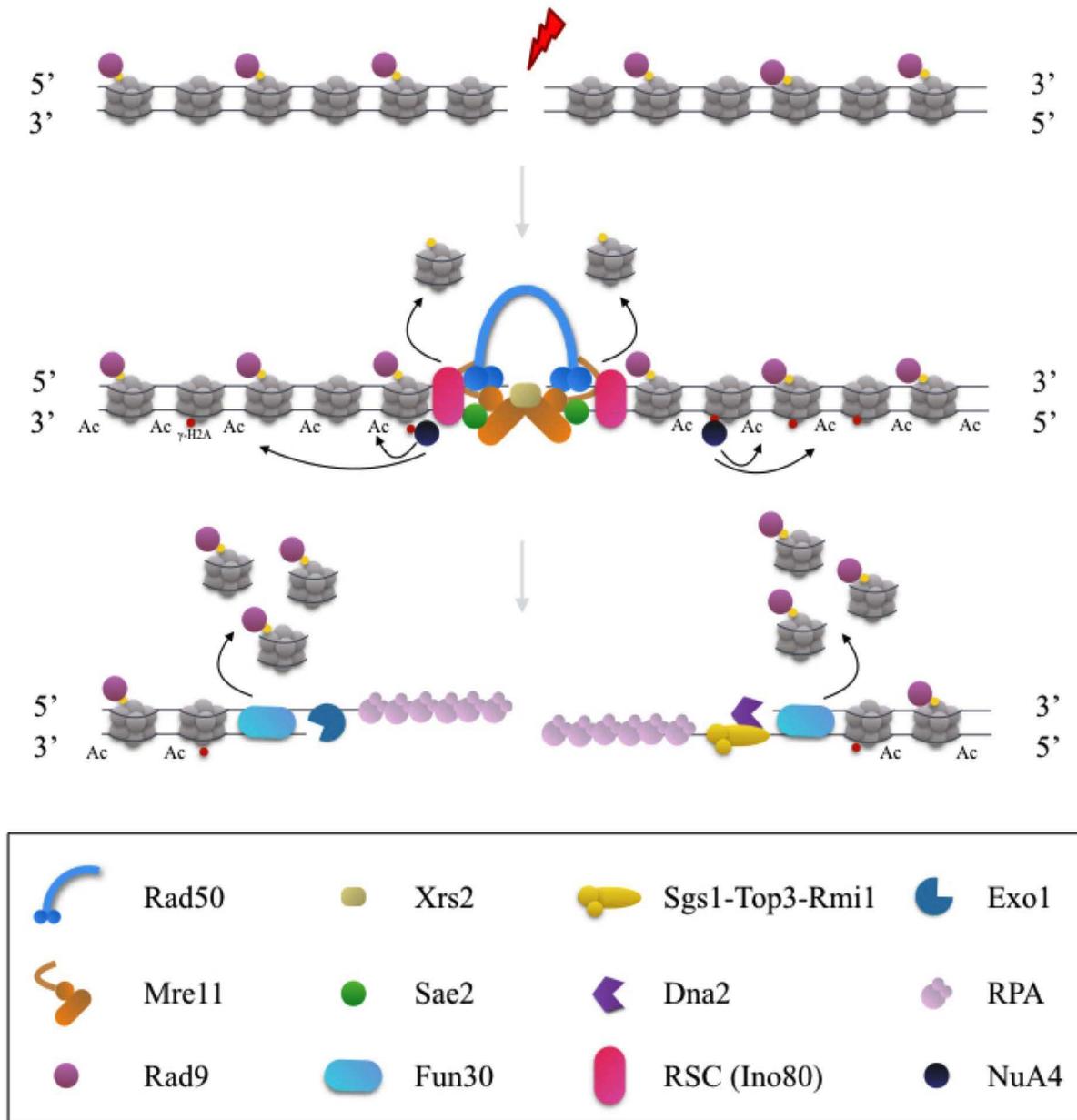
In addition to DNA damage induced histone modification, preexisting chromatin marks can also influence DSB repair. Indeed, trimethylation of H3K36, a histone mark associated with transcriptionally active chromatin, favors the recruitment of the HR machinery in human (Aymard et al., 2014; Carvalho et al., 2014; Pfister et al., 2014). SETD2-dependent H3K36me3 establishment leads to the binding of LEGDF, a chromatin-associated protein (Aymard et al., 2014; Pfister et al.,

2014). Upon DNA damage, LEGDF helps recruiting CtIP, which therefore facilitates resection and Rad51 filament formation (Daugaard et al., 2012). As SETD2 recruitment and H3K36me3 levels do not change upon DSB induction, it suggest that preexisting established H3K36me3 channels DSB repair to HR in active regions (Aymard et al., 2014; Carvalho et al., 2014; Pfister et al., 2014). In both budding and fission yeasts, Set2, the SETD2 homolog, also participate in DSB repair pathway choice. However, Set2/H3K36me is enriched at DSB sites upon DNA damage and favor NHEJ by blocking resection in this case (Jha and Strahl, 2014; Pai et al., 2014). In *S. pombe*, counteracting methylation of H3K36 by Gcn5-dependent acetylation leads to a more open chromatin structure that favors resection and HR (Pai et al., 2014). The differences between mammals and yeasts may be explained by the methylation states of H3K36. Set2 mediates mono-, di- and trimethylation of H3K36 in yeasts (Wagner and Carpenter, 2012). On the contrary, SETD2 only trimethylates H3K36 while dimethylation of H3K36 is mediated by Metnase that is enriched at DSBs and favors NHEJ (Fnu et al., 2011). The reduced resection linked to an increase in NHEJ events in cells deleted for *SET2* in yeasts is thus likely due to the loss of H3K36me2.

Another histone mark was shown to influence DSB repair pathway choice. Indeed, the acetylation/deacetylation state of H3K56 influences repair by sister chromatid recombination at replication-born DSBs (Muñoz-Galván et al., 2013).

### 3.1.2. ATP-dependent chromatin remodelers are recruited to DSB site

ATP-dependent remodelers are large multi-subunit complexes mostly recruited through histone modifications, binding and coupling ATP hydrolysis to



**Figure 23: Chromatin remodeling and histone modifications at a DSB site.**

RSC and INO30 are recruited early to a DSB site and help remove few nucleosomes in the DSB vicinity. NuA4 binds phosphorylated H2A and acetylate H4-tails, leading to chromatin relaxation. Fun30 alleviates the barrier formed by Rad9 bound to chromatin to allow extensive resection by Sgs1-Dna2 and Exo1.

movement of histones or nucleosomes (exchange, eviction and/or repositioning). Amongst the steps occurring at DSBs, resection seems particularly sensitive to the chromatin environment and likely necessitates chromatin remodeling. Indeed, Sgs1-Dna2 activity necessitates a nucleosome-free gap adjacent to the DSB whereas Exo1 activity is completely blocked by nucleosomes *in vitro* (Adkins et al., 2013).

RSC is a SWI/SNF-related remodeling complex composed of 15 subunits with nucleosome sliding activity that is rapidly recruited to a DSB site *via* Mre11 and Ku (Shim et al., 2005). It participates in the repositioning of nucleosomes around the cut that is required for a stronger recruitment of Mre11 and Ku, creating a positive feedback loop (Kent et al., 2007; Shim et al., 2007). It is also required for efficient H2A phosphorylation by facilitating the recruitment of Mec1 and Tel1 (Liang et al., 2007). RSC promotes both NHEJ and HR through different mechanisms (Shim et al., 2005; 2007). By favoring the recruitment of Ku, RSC facilitates the joining of DSB ends (Shim et al., 2005; 2007). Due to its involvement in Mre11 recruitment, RSC is also involved in short-range resection and HR. Indeed mutants of RSC subunits show compromised resection and RPA enrichment (Kent et al., 2007; Liang et al., 2007; Shim et al., 2007), although Rad51 binding to DSB is not markedly affected and it does not impact on strand invasion (Chai et al., 2005; Shim et al., 2007). RSC also facilitates the loading of cohesin at the DSB site, which is particularly important for sister-chromatid recombination (Liang et al., 2007; Oum et al., 2011).

INO80 and SWR1 are two closely Snf2-related chromatin remodeling complexes that share several subunits. They are both recruited to DSB site by binding  $\gamma$ H2A (Morrison et al., 2004; Tsukuda et al., 2005; van Attikum et al., 2007; 2004). Their recruitment is further facilitated by a local increase of histone H4 acetylation by NuA4 (Downs et al., 2004). SWR1, and its human orthologs SCRAP and p400,

replaces H2A with H2A.Z (Htz1 in yeast) in a dimer exchange reaction whereas INO80 mediates the reverse reaction (Luk et al., 2010; Mizuguchi et al., 2004; Papamichos-Chronakis et al., 2011; Ruhl et al., 2006). In *S. cerevisiae*, SWR1-dependent deposition of H2A.Z promotes Exo1-mediated resection while Swr1 does not affect ssDNA formation by itself and favors Ku recruitment (Adkins et al., 2013; van Attikum et al., 2007). In contrast, human H2A.Z appears to inhibit resection by CtIP at DSBs by recruiting the NHEJ proteins while SCRAP interacts with CtIP and facilitates resection (Dong et al., 2014; Xu et al., 2012). Using a different human cell line, other studies demonstrated that p400 promotes HR while they did not find any role for H2A.Z in DSB repair (Courilleau et al., 2012; Taty-Taty et al., 2013). These results indicate that both SRCAP and p400 chromatin remodelers may affect DSB repair in an H2A.Z-independent manner. Differences in the role of H2A.Z deposition between yeast and mammals suggest that it may differentially affect short-range and long-range resection. Indeed, H2A.Z deposition at first seems to prevent short-range resection by recruiting the NHEJ proteins but later favors Exo1-mediated resection in chromatin. INO80 is able to slide and evict nucleosomes around the DSB site that may favor nucleolytic cleavage and explain its slight effect on ssDNA formation (Tsukuda et al., 2005; Udugama et al., 2011; van Attikum et al., 2004; 2007). Mammalian INO80 was also identified as a mediator of 5'-3' resection (Gospodinov et al., 2011). Upon DNA damage, both INO80 and H2A.Z deposition by SWR1 increase DSB mobility in yeast and trigger the relocation of persistent DSBs to the nuclear periphery, indicating that these two remodelers play a major role in the maintenance of genome integrity (Horigome et al., 2014; Kalocsay et al., 2009).

While RSC, SWR1 and INO80 show slight effect on resection, a poorly characterized Snf2-like chromatin remodeler, Fun30 in yeast and SMARCAD1 in mammals, was shown to mainly contribute to 5'-3' resection (Chen et al., 2012). Fun30 promotes extensive resection by both Exo1-dependent and Sgs1-Dna2-dependent pathways through its ATP-dependent remodeling activity (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012). *In vitro*, Fun30 ATP-dependent chromatin remodeling activity is higher in H2A-H2B dimer exchange than in nucleosomes repositioning, even though *in vivo*, Fun30 mediated transcriptional repression is associated with nucleosomes sliding on promoters (Awad et al., 2010; Byeon et al., 2013). Interestingly, Fun30 is less important for resection in the absence of Rad9 or the histone modifications recruiting Rad9, i.e. H3K79me and  $\gamma$ H2A (Chen et al., 2012). As Rad9 blocks resection mainly by inhibiting the binding/persistence of Sgs1 and to a lesser extent of Exo1 at DSB ends (Bonetti et al., 2015; Lazzaro et al., 2008), Fun30 is therefore proposed to promote extensive resection by overcoming the resection barrier formed by Rad9-bound chromatin.

### **3.2. Heterochromatin in *S. cerevisiae***

In budding yeast, cytological observation of chromatin compaction is difficult to observe, if not impossible, and heterochromatin refers to regions of silent chromatin. Moreover, *S. cerevisiae* does not establish the methylation of H3K9, a histone modification typical of constitutive heterochromatin, and lacks heterochromatin protein HP1 that is recruited by H3K9me, which are both conserved from fission yeast to humans (Grewal and Jia, 2007). Sir3 can however be proposed as a functional ortholog of HP1 in *S. cerevisiae* (see below). Yeast silent chromatin is

characterized by deacetylation of H4K16, which contributes to the compaction of the chromatin fiber *in vitro* and *in vivo* (Robinson et al., 2008; Shogren-Knaak et al., 2006). Both heterochromatin and yeast silent chromatin share common features, namely hypoacetylated histones, late replication, RNA pol II transcription repression and are epigenetically transmitted to daughter cells (Bi, 2014).

### 3.2.1. The SIR complex

In *S. cerevisiae*, silent chromatin establishment requires the recruitment of the SIR (silent information regulator) complex. The SIR complex is a heterotrimer composed of Sir2, Sir3 and Sir4 in stoichiometric amounts although Sir2/Sir4 can also exist as a separate complex (Hoppe et al., 2002; Moazed et al., 1997). The SIR complex sits between adjacent nucleosomes with one SIR complex per linker (Martino et al., 2009).

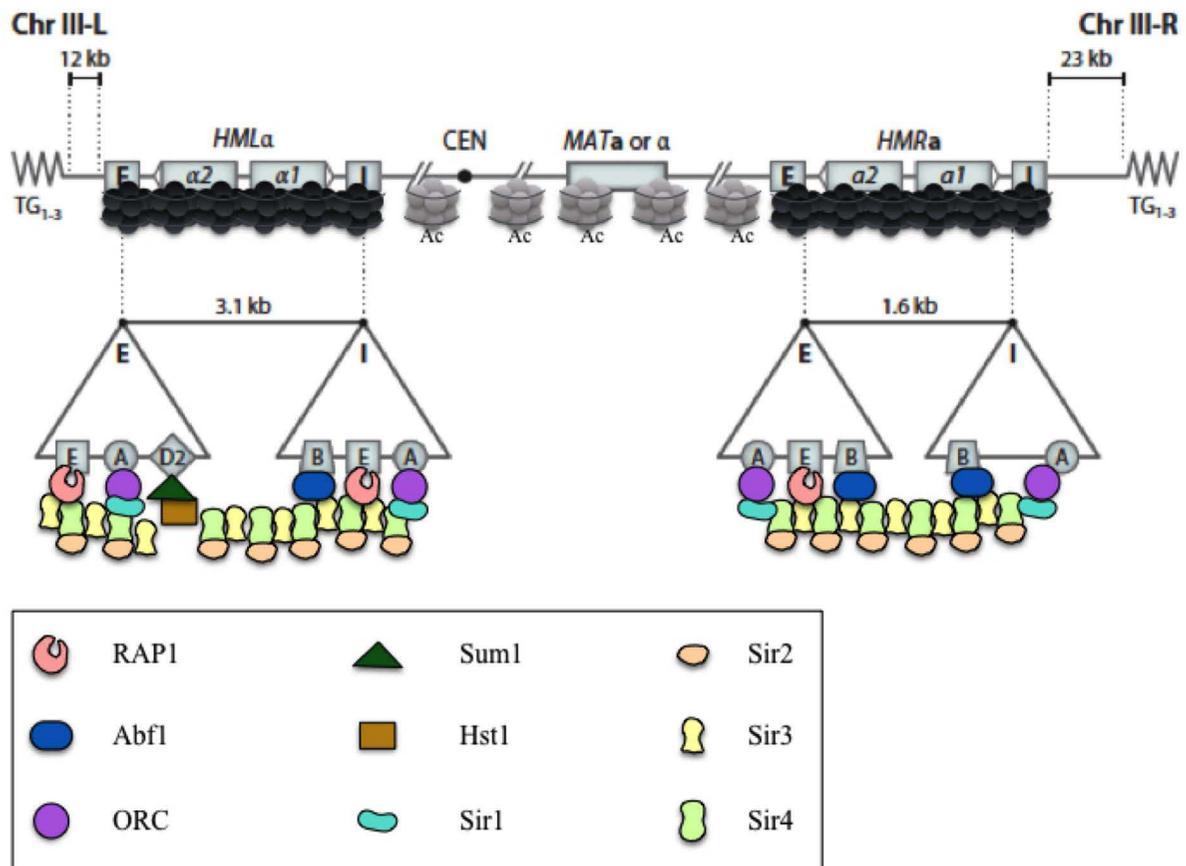
Sir2, a class III NAD-dependent histone deacetylase HDAC, is a founding member of the sirtuin family, which is evolutionary conserved from prokaryotes to man (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000). Sir2 is the enzymatic member of the SIR complex acting at *HM* loci and telomeres and is part of the RENT complex at the rDNA. Sir2, and more generally sirtuins, can deacetylate histones and non-histones substrates in a reaction that couples lysine deacetylation to the hydrolysis of NAD<sup>+</sup> and the subsequent production of nicotinamide (NAM) and O-acetyl-ADP-ribose (O-AADPR). Consequently, NAM inhibits Sir2 enzymatic activity while variations in NAD<sup>+</sup> levels modulate it and are important for rDNA and telomeric silencing (Sandmeier et al., 2002). Supporting a role for a crucial coupling of NAD<sup>+</sup> availability for Sir2 activity, Sir2 physically associates with Thd3, a

GAPDH protein that maintains normal levels of NAD<sup>+</sup> in the nucleus (Ringel et al., 2013). The enzymatic reaction mediated by Sir2 releases considerable free energy that is proposed to serve for conformational changes in proteins and DNA (Rusche et al., 2003; Tanny and Moazed, 2001). In addition, the O-AADPR was shown to induce a change in the conformation of the SIR complex by electron microscopy and to increase both Sir3 and the SIR complex affinity for nucleosomes *in vitro* (Liou et al., 2005; Martino et al., 2009). While Sir2 may act on other substrates, its interaction with Sir4, in addition to promoting its deacetylase activity, also targets it specifically to H4K16Ac (Hsu et al., 2013). H4K16 deacetylation in the presence of NAD<sup>+</sup> and Sir3, results in a decreased accessibility of linker DNA and favors the interaction of Sir3, and to a lesser extent for Sir4, with the N-terminus of histones H3 and H4 (Hecht et al., 1995; Imai et al., 2000; Martino et al., 2009; Oppikofer et al., 2011).

Sir3 has no known enzymatic function but rather plays a structural role in silencing. It harbors a BAH domain on its N-terminus required for its interaction with nucleosomes through unacetylated H4K16 (H4 tail) and unmethylated H3K79 (nucleosome core) (Armache et al., 2011; Onishi et al., 2007). Its central AAA<sup>+</sup> ATPase-like domain no longer possesses an ATPase activity but is required for its interaction with Sir4 and also to bind H3K79 (Ehrentraut et al., 2011). The Sir3 C-terminus is essential for its homodimerization and spreading along nucleosomes (Liaw and Lustig, 2006; Oppikofer et al., 2013b). Sir3 also interacts with the C-terminal domain of Rap1 that participates in the recruitment of the SIR complex (Chen et al., 2011; Moretti et al., 1994). It appears that Sir3 is a limiting factor for silencing (Renauld et al., 1993). Indeed, in normal conditions, Sir3 spreads ~2-3 kb along the subtelomeric regions whereas its overexpression leads to its propagation over ~15-20 kb (Hecht et al., 1996; Radman-Livaja et al., 2011; Ruault et al., 2011;

Strahl-Bolsinger et al., 1997). Furthermore, Sir3 function in silencing can be separated from telomere clustering as a non-acetylatable mutant of Sir3, sir3A2Q, is unable to spread in subtelomeric regions but is efficient for telomere clustering (Ruault et al., 2011). These results indicate that telomere clustering is not a consequence of silencing but can rather favor silencing by concentrating silencing factors.

The Sir4 protein has several fundamental roles in telomeric silent chromatin formation, as it anchors the whole SIR complex to the nuclear envelope through its interactions with Esc1 and Ku, it functions as a scaffold and is required for the nucleation step (Kueng et al., 2013; Oppikofer et al., 2013a). Moreover, it is involved in telomere homeostasis by inhibiting telomere-telomere fusions and by regulating telomere length (Marcand et al., 1997; 2008). Sir4 has also no known enzymatic activity and its preponderant role in silencing is related to its interaction with numerous proteins (Martino et al., 2009). It can bind DNA and chromatin with high affinity but little specificity and can be recruited independently of Sir2 and Sir3, suggesting a role in the initiation of silencing (Johnson et al., 2009; Luo et al., 2002; Martino et al., 2009; Rusche et al., 2002). It binds DNA through its N-terminus, whereby it also interacts with Sir1 and yKu80 that help recruiting the SIR complex to sites of repression (Kueng et al., 2012; Martino et al., 2009; Roy et al., 2004; Triolo and Sternglanz, 1996). The extreme C-terminal coiled-coil domain of Sir4 dimerizes to generate binding sites for Sir3 on its outer face (Chang et al., 2003). This coiled-coil domain also binds Rap1 and yKu70 that serves to recruit Sir4 to telomeres (Mishra and Shore, 1999; Moretti et al., 1994; Tsukamoto et al., 1997). Sir4 also harbors two more central domains on its C-terminus half. The PAD domain binds



**Figure 24: Establishment of silencing at *HML* and *HMR*.**

SIR proteins are recruited to E and I silencers surrounding the HM loci by proteins bound to specific sites (site A = ORC, site B = Abf1, site E = Rap1, site D2 = Sum1). *Adapted from Kueng et al., 2013*

Esc1 and mediates anchorage to the nuclear envelope while the SID domain is the site of interaction with Sir2 (Andrulis et al., 2002; Hsu et al., 2013).

Initiation of silencing begins by the recruitment of the SIR by sequence-specific DNA binding factors (Rap1, Sir1, Ku; see below), which create a nucleation site. Sir2/Sir4 binds H4K16Ac while this histone mark, together with H3K79me, inhibits Sir3 binding to nucleosomes in active chromatin. Sir2 deacetylates histones in the presence of NAD<sup>+</sup> and Sir3, which triggers conformational changes in proteins and DNA and allows Sir3, and to a lesser extent Sir4, to bind to nucleosomes. Through Sir4 and Sir3 homodimerization, the SIR proteins can then spread along nucleosomes, creating an extended domain coated by the SIR complex. It is suggested that SIR proteins spreading is not linear but rather happens through simultaneous loading mediated by long-range interactions (chromatin loop or fold-back structure) between nucleation sites. This spreading is limited by the titration of Sir3 and the combinatorial effect of histone modifications (H3K79me, H2A.Z incorporation) and transcription factors binding (Tbfl, Reb1) that act as boundary elements (Fourel et al., 1999; Martino et al., 2009; Meneghini et al., 2003; Onishi et al., 2007; Renauld et al., 1993).

### 3.2.2. Regions of silent chromatin

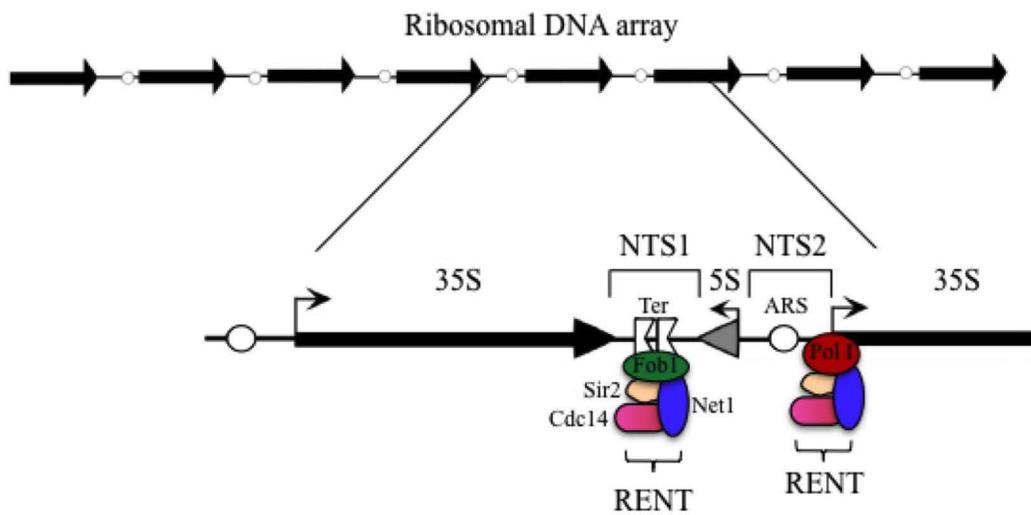
Heterochromatin is found at three distinct domains in yeast, namely the two silent mating type loci *HML* and *HMR*, telomeres and subtelomeres and the rDNA locus. While the whole SIR complex is required for silencing at chromosome ends and *HM* loci, only Sir2 is necessary at the rDNA locus. Depending on the silent domain considered, the proteins required for the recruitment of the SIR proteins are



different. In addition, Fun30 was shown to participate in silencing at all loci by regulating the chromatin structure within or around silent loci (Neves-Costa et al., 2009; Yu et al., 2011).

Existence of silent chromatin was first discovered through the study of factors required for the mating of yeast cells. Haploid *S. cerevisiae* cells exist in two different cell types, a or  $\alpha$ , depending on the gene expressed at the mating type (*MAT*) locus on chromosome III. Nonetheless, a and  $\alpha$  genes are also present at the homothallic mating-type (*HM*) loci, *HMRa* on the right arm and *HML $\alpha$*  on the left arm of chromosome III, which are kept silent. *HM* loci are flanked by *cis*-acting DNA sequences of ~140 bp required for repression called E and I silencers (Abraham et al., 1984; Feldman et al., 1984). They consist of DNA motifs termed A, B, E and D2 that recruit the sequence-specific DNA-binding proteins ORC, Abf1, Rap1 and Sum1 respectively (Brand et al., 1987; Diffley and Stillman, 1988; Irlbacher et al., 2005; Shore and Nasmyth, 1987). Sum1 interacts with Hst1, a paralog of Sir2 (Xie et al., 1999). Rap1 and Abf1 participate in the recruitment of Sir3 and Sir4 while Orc1, a subunit of ORC, binds Sir1 that in turn recruits Sir4 and altogether establish silent chromatin at *HM* (Moretti and Shore, 2001; Triolo and Sternglanz, 1996) (Figure 24).

At telomeres, SIR proteins are recruited by both Ku and Rap1 (Figure 25). As described previously, Rap1 binds the terminal TG<sub>1-3</sub> repeats every 18 bp and interacts with both Sir3 and Sir4 (Gilson et al., 1993; Moretti and Shore, 2001). The Ku complex binds all chromosome ends and interact specifically with Sir4 (Gravel et al., 1998; Roy et al., 2004; Taddei et al., 2004; Tsukamoto et al., 1997). Ku also promotes silencing by overcoming the inhibitory effect of Rif1 and Rif2 that both compete with Sir4 for Rap1 binding (Mishra and Shore, 1999). Telomeric silencing, also called TPE (telomere position effect), is highly variable in strength and shows a strong



**Figure 26: Organization of the rDNA array.**

Model of a ribosomal unit with two transcriptional unit 35S and 5S separated by two intergenic sequences NTS1 and NTS2. NTS1 contains a replication fork barrier (TER sites) bound by Fob1 which recruits the RENT complex. The RENT complex is also recruited at the NTS2 region through Pol I binding.

dependence both on the level of SIR proteins and on boundary complexes that antagonize their binding (Fourel et al., 1999; Renauld et al., 1993). TPE is variable depending on the telomere observed and the variability in TPE strength is attributed to sequence divergence in subtelomeric elements (Kueng et al., 2013). Indeed, the subtelomeric X element harbors binding sites for transcription factors such as Abf1 and ORC that act as protosilencers while others, Reb1 and Tbf1 in particular, act as insulators in Y' elements (Lebrun et al., 2001; Smith et al., 2011). These protosilencers act as a relay of silencing initiated at telomeres to adjacent regions.

As mentioned above, rDNA is composed of ~200 tandem repeats and their silencing through Sir2 is one of the two pathways to maintain their stability. The coding regions of 35S RNA and 5S RNA are separated by two intergenic spacers called NTS1 and NTS2 that contain two tandem Ter sites – also called RFB (replication fork barrier) site – and a single ARS, respectively. Silencing is initiated at both NTS1 and NTS2 and requires the recruitment of the RENT complex composed of Net1, Sir2 and Cdc14 (Huang and Moazed, 2003). At NTS1, the replication terminator protein Fob1 binds to Ter and interacts with Net1 and to a lesser extent Sir2 (Bairwa et al., 2010; Huang and Moazed, 2003). Net1 tethers Sir2 to rDNA and is required for rDNA silencing (Straight et al., 1999). Net1 also recruits the phosphatase Cdc14 and inhibits its activity and therefore exit from mitosis but has no known role in silencing (Shou et al., 1999; Straight et al., 1999). The RENT complex was also shown to be recruited around the Pol I promoter in the NTS2 region and extending into the 35S rRNA coding region independently of Fob1 (Huang and Moazed, 2003; Stegmeier et al., 2004). Both Net1 and Sir2 interacts with Pol I, suggesting that establishment of rDNA silencing at the NTS2 region occurs through

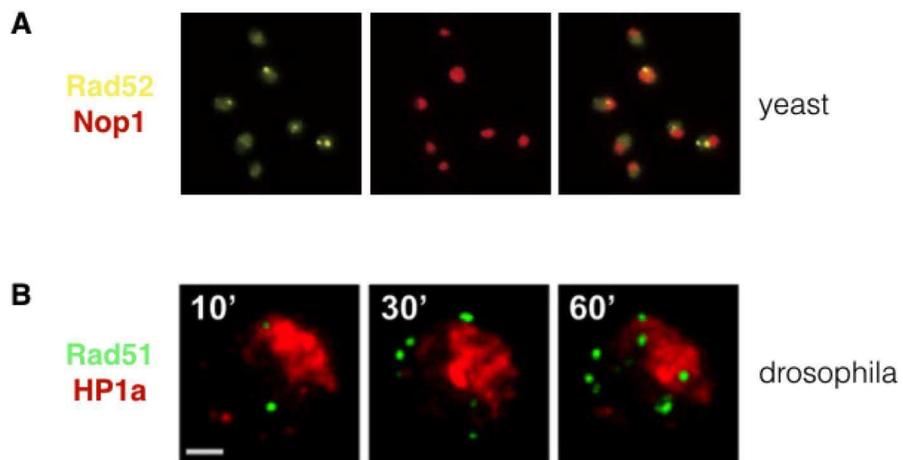
the recruitment of the RENT complex by Pol I (Huang and Moazed, 2003; Shou et al., 2001) (Figure 26).

### **3.3. Repair in heterochromatin**

Heterochromatin ensures genome stability by preventing extensive or illicit recombination between repeated sequences (Gottlieb and Esposito, 1989; Peng and Karpen, 2007; 2009). Furthermore, heterochromatin is thought to constitute a barrier for DNA repair and DSBs in mammals that are formed in heterochromatin are repaired more slowly than in euchromatin (Goodarzi et al., 2008; Lemaître et al., 2014; Lorat et al., 2012). HR seems to be the major pathway to repair a DSB in heterochromatin (Beucher et al., 2009; Geuting et al., 2013; Kakarougkas et al., 2013; Ryu et al., 2015), although it was shown that H3K9me2/3-associated chromatin or chromatin targeted to the repressive nuclear lamina rather favor repair by NHEJ or alt-NHEJ (Aymard et al., 2014; Lemaître et al., 2014). These results indicate that not all heterochromatin domains within the nucleus behave in the same manner but how this is achieved is unclear.

#### **3.3.1. Recruitment of repair proteins in heterochromatin**

It was long thought that DSBs are not formed in heterochromatin because telomeric regions showed very low spontaneous recombination rates and  $\gamma$ H2A formation was prevented in heterochromatic regions both in yeast and mammals (Cowell et al., 2007; Kim et al., 2007; Louis et al., 1994; Marvin et al., 2009b). DSBs can actually be formed in heterochromatin and the initial steps take place in these regions, but the loading of the recombination machinery requires the relocation of the



**Figure 27: Relocalization of a DSB outside of a heterochromatic domain.**

(A) Rad52 foci are excluded from the nucleolus in yeast. *Adapted from Torres-Rosell et al., 2007*

(B) Rad51 foci relocate at the periphery of the heterochromatin domain in drosophila. *Adapted from Chiolo et al., 2011*

DSB outside of the heterochromatic domains in yeast, *Drosophila* and mammals (Chiolo et al., 2011; Jakob et al., 2011; Torres-Rosell et al., 2007) (Figure 27).

In *S. cerevisiae*, Rad52 foci are excluded from the nucleolus whereas Mre11, RPA and Ddc2 foci are visualized in this heterochromatic region, indicating that DSBs in the rDNA are recognized and resected while inside the nucleolus. Recruitment of Rad52 necessitates the relocation of the DSB outside the nucleolus and this requires Smc5/Smc6 and Rad52 sumoylation (Torres-Rosell et al., 2007).

Similarly, in *Drosophila*, resection and ATRIP loading occurs within pericentromeric repeats while RAD51 binding requires the relocation outside of this heterochromatic domain. This relocation involves functional resection, checkpoint activation and the recruitment of Smc5/Smc6 by HP1, which binds the heterochromatic mark H3K9me (Chiolo et al., 2011). Sumoylation by Nse2 and dPIAS, two SUMO ligases, prevents Rad51 recruitment to heterochromatic DSBs in addition to promoting DSB relocalization (Ryu et al., 2015).

In mammals, the repair factor XRCC1 and the ssDNA binding protein RPA are recruited at DSB sites within chromocenters before the movement of DSBs to the periphery of this heterochromatic domain (Jakob et al., 2011). Although ATM is required for DSB repair in heterochromatin, it is not involved in DSB relocation and the mechanism by which DSBs are mobilized is yet to be uncovered (Goodarzi et al., 2008; Jakob et al., 2011). These results suggest that the relocalization outside the heterochromatin domain is not mandatory for repair and may be the consequence of changes in histone modifications and/or the recruitment of particular DSB repair factors.

In all organisms, DSB relocation outside heterochromatin regions is thought to favor genomic stability by confining the DSB away from the repeated sequences, therefore ensuring recombination with the sister chromatid. This relocation is required for the loading of the recombination machinery while the generation of 3' ssDNA overhangs can occur within heterochromatin. Interestingly, DSB targeted to the nuclear lamina are preferentially repaired by NHEJ and alt-NHEJ, which requires little or no resection, and do not move to less repressed chromatin domains (Lemaître et al., 2014). It suggests that the chromatin environment may differentially regulate resection and alter DSB repair outcome.

### 3.3.2. A role for heterochromatin proteins in DSB repair

In mammals, ATM plays an important role in DSB repair within heterochromatin by phosphorylating KAP1 (Geuting et al., 2013; Goodarzi et al., 2008; Ziv et al., 2006). KAP1 is a transcriptional corepressor that interacts with HP1, the histone H3, lysine 9-specific methyltransferase SETDB1 and the HDAC/chromatin remodeler CHD3 to induce heterochromatin formation (Goodarzi et al., 2011; Ryan et al., 1999; Schultz et al., 2002). ATM-dependent KAP1 phosphorylation is further enhanced by 53BP1-mediated MRN accumulation that concentrates active ATM activity and leads to strong and localized KAP1 phosphorylation to repair heterochromatic DSBs (Kakarougkas et al., 2013; Noon et al., 2010). KAP1 phosphorylation reduces KAP1's autoSUMOylation, which impedes the interaction between KAP1 and CHD3. It ultimately leads to the release of CHD3 from heterochromatin and to a local chromatin decompaction (Goodarzi et al., 2011). Chromatin decompaction is further facilitated by the phosphorylation of the ubiquitin

ligase RNF20, which enables physical relocalization of ACF1-SNF2H (ISWI class) chromatin remodeling complex to DSBs in heterochromatin (Klement et al., 2014). ISWI respaces heterochromatic nucleosomes after CHD3 dispersal to facilitate Artemis nuclease-dependent repair by NHEJ in G0/G1 phase and HR during G2 phase (Beucher et al., 2009; Klement et al., 2014).

Despite the necessity for heterochromatin proteins to leave the DSB site, some of them have been determined to have an active role in DSB repair. Indeed, HP1 is rapidly, but transiently, recruited to damaged regions in both euchromatin and heterochromatin within a few minutes after DNA damage (Ayoub et al., 2009; Baldeyron et al., 2011; Luijsterburg et al., 2009; Zarebski et al., 2009). *De novo* accumulation of HP1 depends on the largest subunit of the histone chaperone CAF1 (Baldeyron et al., 2011). Once recruited, HP1 does not affect NHEJ but favors HR by promoting RPA loading and phosphorylation at damaged sites, thus participating indirectly in RAD51 and BRCA1 recruitment (Baldeyron et al., 2011; Lee et al., 2013; Soria and Almouzni, 2013). In addition, HP1 is also recruited at damage sites by chromatin PARylation as part of a single KAP1/HP1/SUV39 complex (Ayrapetov et al., 2014). SUV39-dependent H3K9 trimethylation spreading activates the acetyl transferase Tip60, which promotes the subsequent acetylation and activation of ATM ultimately leading to KAP1/HP1/SUV39 release (Ayrapetov et al., 2014). This rapid, but temporary, formation of repressive chromatin may ensure the coordination between transcription and repair, compact the local chromatin structure, and rewrite the local epigenetic landscape to create a common template for the DSB repair machinery.

In yeast, little – if nothing – is known about the early steps required for DSB repair within heterochromatin and whether specific proteins are required. Although

KAP1 is not conserved, multiple chromatin components are phosphorylated in a checkpoint-dependent manner (Chen et al., 2010; Smolka et al., 2007). It may suggest that checkpoint protein function in DSB repair within heterochromatin may also be conserved and signal for a less compacted chromatin.

### 3.3.3. Effect of the heterochromatin status of the donor locus

In *S. cerevisiae*, the contribution of the heterochromatic donor can be easily assessed by inducing a DSB at *MAT*, which can recombine with one of the two silent *HM* loci. However, it was demonstrated that the heterochromatic context of *HM* does not affect repair at *MAT* (Coic et al., 2011; Tsaponina and Haber, 2014). Indeed, unsilencing of the *HML* and/or *HMR* donors by deleting the adjacent E and I silencer sequences and inserting an homologous sequence to *MAT* gave the same repair outcome than when the *HM* were silent (Coic et al., 2011). Moreover, deletion of *SIR3* does not influence interchromosomal template switch during gene conversion between *MAT* and *HMR*, suggesting that neither the initial dissociation nor the reinvasion of the nascent strand after copying sequences from the interchromosomal donor is altered by loss of the heterochromatic state of *HMR* (Tsaponina and Haber, 2014).

However, an *in vitro* study demonstrated that presence of Sir3 is sufficient to repress nucleosome accessibility and Rad51-dependent joint formation, which corresponds to the strand invasion step during HR (Sinha et al., 2009). This inhibition requires the Sir3 BAH domain and unacetylated H4K16 and is strengthened by Sir2 and Sir4 (Sinha et al., 2009). The SWI/SNF chromatin remodeler directly interacts with Sir3 and is able to evict it from the chromatin, which results in partial restoration

of joint formation (Manning and Peterson, 2014; Sinha et al., 2009). Rad54 is not able to substitute for SWI/SNF for Sir3 removal but addition of Rad54 further enhances joint formation (Sinha et al., 2009). Accordingly, *in vivo* studies showed that SWI/SNF is required for synapsis between the Rad51 filament and the heterochromatic *HML* donor while Rad54 participates in nucleosome remodeling after strand invasion and is necessary for new DNA synthesis (Chai et al., 2005; Hicks et al., 2011; Sugawara et al., 2003). Rad54 also plays a role in DSB repair in euchromatin whereas SWI/SNF is dispensable (Sinha et al., 2009). While loss of the heterochromatic state of *HM* loci does not alter DSB repair outcome, it is likely that additional factors overcome the heterochromatic barrier *in vivo*.

We have seen all along this introduction that DSB repair is a complex reaction involving the sequential recruitment of many proteins. This reaction necessitates a crosstalk between repair proteins but also a crosstalk between repair and checkpoint proteins in order to tightly regulate the DNA damage response and ensure genomic stability. The molecular steps have been extensively studied these past decades and most of them were reconstituted *in vitro* on naked DNA. However, in the nucleus, DSB repair occurs in the context of chromatin, more or less compacted depending on the regions considered. In addition, chromatin is non-randomly organized in the nuclear space, creating subcompartments enriched in specific enzymatic activities and clustering particular sequences together. Thus, it appears that DSB repair is even more complex. We have just started to uncover what additional factors are required and how DSB repair outcome differs depending where the DSB is formed in the nucleus, what the chromatin context is and where the homologous sequence is located in the case of HR.

## 4. PhD project

Yeast chromosomes are arranged in a Rabl-like configuration with their centromeres anchored to the spindle pole body and their telomeres grouped into 3 to 5 foci at the nuclear periphery. Telomeric foci form subnuclear compartments repressive for transcription in which the yeast heterochromatin factors (SIRs - silent information regulators) concentrate.

We designed a genetic system composed of two homologous cassettes that we inserted at different loci in the genome and allow us to create a unique DSB by the expression of the I-SceI endonuclease upon galactose induction. We coupled this system with another one that allows us to change the nuclear organization of telomeres and enables us to modulate heterochromatin on subtelomeric regions to ask how proximity between telomeres and/or their chromatin status impact DSB repair.

# RESULTS

# RESULTS

## 1. Scientific article

### **Nuclear organization and chromatin status modulate homologous recombination efficiency and outcome**

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### **1.1. Summary**

Homologous Recombination (HR) uses an intact homologous sequence to repair a broken chromosome through a conserved mechanism. How different

genomic, chromatin, and subnuclear contexts influence HR efficiency and outcome is still poorly understood. Using a new assay to assess double-strand break induced HR between pairs of genomic loci, we reveal that subtelomeric DSBs show limited Gene Conversion (GC) efficiency but are repaired efficiently through Break Induced Replication despite the presence of homology on both side of the break. Actively grouping telomeres, through *sir3A2Q* mutant overexpression, increases the GC rate between subtelomeres demonstrating that physical distance limits this process. However, spatial proximity is not the only limiting factor as repair efficiency between intrachromosomal and subtelomeric sequences varies up to 50 fold depending on which locus is damaged. Subtelomeric DSBs are poorly repaired with intrachromosomal matrices, but limiting resection notably through *EXO1* deletion improves recombination efficiency through favoring GC. Heterochromatinization of the broken locus upon *SIR3* overexpression also favors recombination in a process counteracted by *EXO1* overexpression. Thus heterochromatin limits DSB resection and avoids genetic information loss upon subtelomeric DSBs.

## 1.2. Introduction

DNA lesions arising either from environmental stress or from endogenous events cause DNA lesions that challenge genomic integrity. The subsequent cellular response is a coordinated sequence that allows DNA damage detection, signaling and repair (Gobbini et al., 2013; Goodarzi and Jeggo, 2013). Among the forms of DNA damage, double strand breaks (DSBs) are the most genotoxic, and improper repair leads to genomic instability or cell death. DSB repair occurs through two different mechanisms: non-homologous end joining (NHEJ) and homologous recombination (HR). HR is usually an error-free mechanism limiting loss of heterozygosity (LOH) to

a small region surrounding the DSB, since two-ended double-strand breaks (DSBs) are generally repaired through gene conversion (GC). However, HR can produce break induced replication events (BIR) when the formation of the initial displacement loop (D-loop) strand invasion intermediate follows the establishment of a processive replication fork and DNA synthesis that continues to the end of the chromosome resulting in a kilobase long tracks of LOH (Llorente et al., 2008). BIR is the predominant repair, when only one DSB end is available for strand invasion, but may aide to restart collapsed replication forks and to elongate telomeres, when telomerase is absent or telomeres are uncapped (Llorente et al., 2008; McEachern and Haber, 2006). While GC and BIR lead to very different HR outcomes, little is known about the relative use of these pathways.

Generating 3' single-strands, required for homology search and strand invasion, promotes DSB repair using HR. The concerted action of MRX/Sae2, Exo1 and Sgs1/Dna2 proteins (Gravel et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008) generate 3' ss overhangs, which are rapidly stabilized by RPA (Alani et al., 1992) and recruit Rad52 epistasis group proteins (Krogh and Symington, 2004). Once coated by Rad51, the broken 3' ssDNA end engages in genome probing, likely through repeated interactions with nearby sequences to find a homologous sequence (Renkawitz et al., 2013). GC then engages following the invasion of the 3' ssDNA ends into a homologous template and the copying of the DNA sequences needed to seal the break. The second 3' end of the break anneals with either the extended and displaced 3' end that initiated strand invasion during synthesis dependent strand annealing (SDSA) or the single-stranded DNA in the D-loop to form a double Holliday junction. In the later case, the D-loop resolves to produce either a non-cross over (NCO) or a cross over (CO) (Heyer et al., 2010).

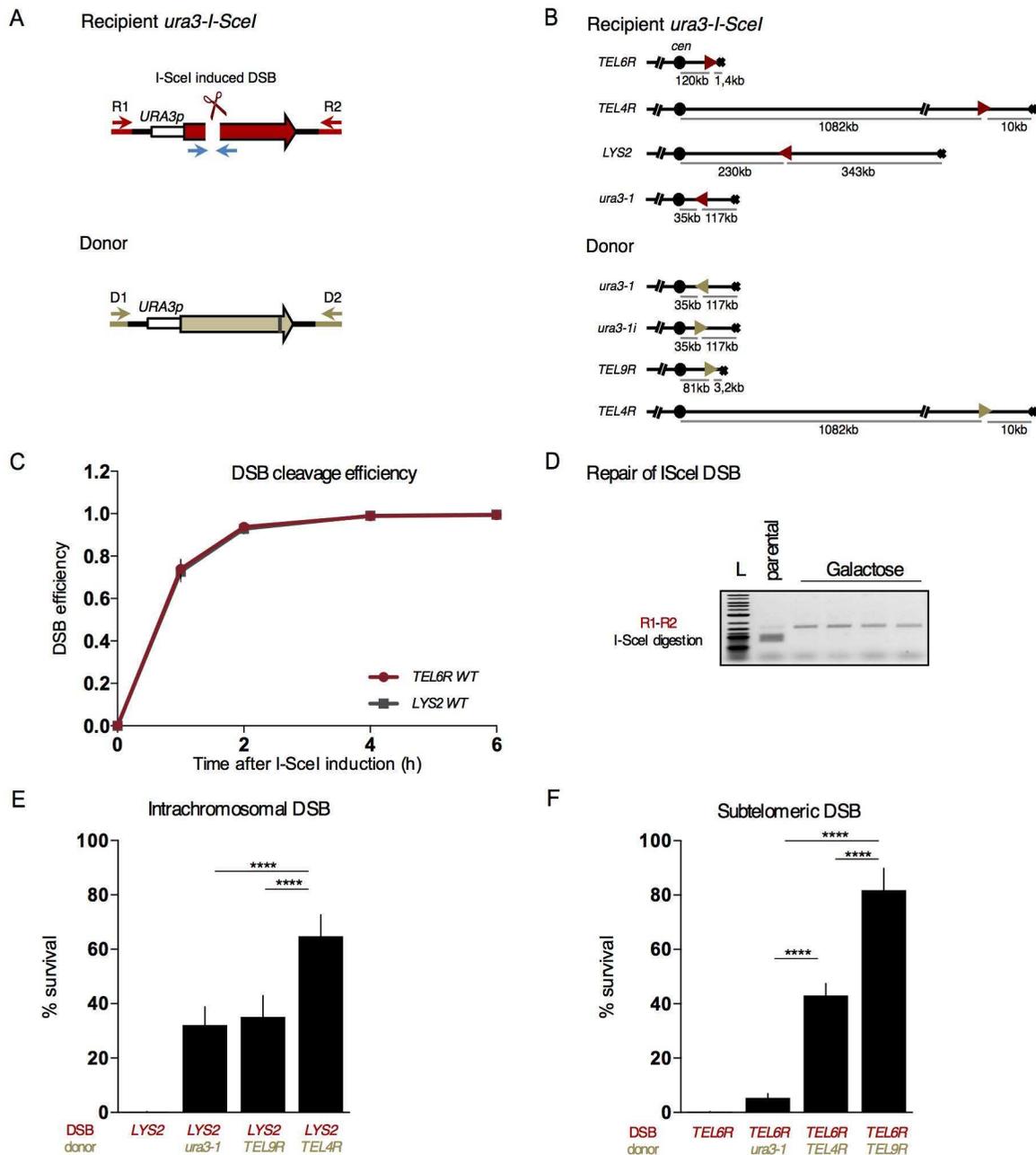
Individual DNA repair molecular mechanisms have been characterized, but genome 3D organization and its packaging into chromatin have emerged as putative regulators of DNA repair pathway choice and repair outcome (Soria et al., 2012). The nuclear organization of *Saccharomyces cerevisiae* is well defined through microscopy and chromosome conformation capture experiments (Taddei and Gasser, 2012). During exponential growth, interphase budding yeast chromosomes assume a Rabl-like conformation with the 16 centromeres held by the spindle pole body (SPB) at one nuclear pole. The 32 telomeres are found at the nuclear periphery to form 3 to 4 foci where the yeast heterochromatin factors (SIRs - silent information regulators) concentrate. These telomere containing foci have no fixed composition, though telomeres of equi-long chromosome arms interact more frequently, presumably due to the geometry imposed by the Rabl conformation (Duan et al., 2010; Guidi et al., 2015; Therizols et al., 2010).

A consequence of this genome organization is that some sequences are more frequently in contact. These timely encounters of homologous sequences may dictate their recombination efficiency. Indeed, studies suggested that the homology search process, which follows the generation of ssDNA and the formation of a Rad51 nucleoproteic filament, is the limiting step for recombination within the *S. cerevisiae* nucleus (Agmon et al., 2013; Burgess and Kleckner, 1999; Lee et al., 2016; Wilson et al., 1994). Recombination efficiency negatively correlates with the spatial distance between a telomeric DSB and its homologous targets (Agmon et al., 2013; Lee et al., 2016). However, how spatial distance between loci controls recombination rates and outcomes has not been directly assessed.

Beside spatial distances, the chromatin state of both donor and acceptor sequences may also regulate recombination steps from early DSB processing into

ssDNA to strand invasion of the homologous sequence, synthesis of new DNA and resolution of repair intermediates. In mammals, DSBs in heterochromatin resolve with slower kinetics and rapidly relocalize to the heterochromatic domain edge (Goodarzi et al., 2008; Jakob et al., 2009). In yeast and *Drosophila*, DSBs formed within heterochromatic domains migrate outside these domains to interact with the recombination machinery to complete repairs (Chiolo et al., 2011; Torres-Rosell et al., 2007). Heterochromatin protects genome integrity by repressing transposition of mobile elements and preventing extensive or illicit recombination between dispersed repetitive DNA elements (Peng and Karpen, 2007; 2008). Despite this repression, recombination does occur at measurable frequencies within heterochromatin (Jaco et al., 2008; Pâques and Haber, 1999). In instances, transpositions into heterochromatin are actually preferred (Zou et al., 1996). However, the molecular mechanisms by which chromatin state and nuclear organization control the detection, processing and repair of DNA lesions remain poorly characterized.

In *S. cerevisiae*, heterochromatin-like domains are localized at the rDNA gene cluster, sub-telomeric regions, and the silent mating type loci, *HML $\alpha$*  and *HMR $\alpha$* . At subtelomeric and silent mating type loci, heterochromatin assembly requires Silent information regulators, Sir2, Sir3 and Sir4. Overexpressing either *SIR3* or *sir3A2Q*, a silencing defective allele, modifies nuclear organization by grouping most telomeres into a single cluster localized into the nuclear interior (Ruault et al., 2011). Since overexpression of *SIR3* or its *sir3A2Q* allele modifies the physical distances between telomeres and heterochromatin spreading in subtelomeric regions, we can determine how chromatin status (euchromatin versus heterochromatin) and nuclear organization controls homologous recombination efficiency and outcome (BIR versus GC). We developed an assay to score DSB-induced recombination events between alleles



**Figure 1: An assay to score recombination efficiency reveals subtelomeric donor sequences efficiently repair intrachromosomal DSB.**

(A) Schematic representation of the two *ura3* alleles used to test recombination efficiencies and outcome: *i*, the recipient *ura3-I-SceI* has a 30bp I-SceI sequence inserted out of frame *ii*, the donor *ura3-1* bears a missense mutation.

(B) The two *ura3* alleles are introduced at chosen loci by PCR gene targeting.

(C) DSB cleavage efficiency measured in donor-less strains by qPCR using primers flanking the DSB site as shown in (A). Error bars represent the standard deviation (s.d.) of at least three independent experiments.

(D) Disappearance of the I-SceI cleavage site in survivors on galactose medium.

(E-F) Survival frequencies observed after induction of a DSB at an intrachromosomal position (E) or at a subtelomeric position (F). Error bars represent the standard deviation of the mean survival (s.d.m.) of at least three independent experiments. Black stars indicate statistical differences (\* =  $p < 0,05$ ; \*\* =  $p < 0,01$ ; \*\*\* =  $p < 0,005$ ; \*\*\*\* =  $p < 0,001$ ).

located throughout the chromosomes. In addition, this assay uniquely allows measurement of the competition between two recombination pathways, namely GC or BIR at chromosomal loci.

## 1.3. Results

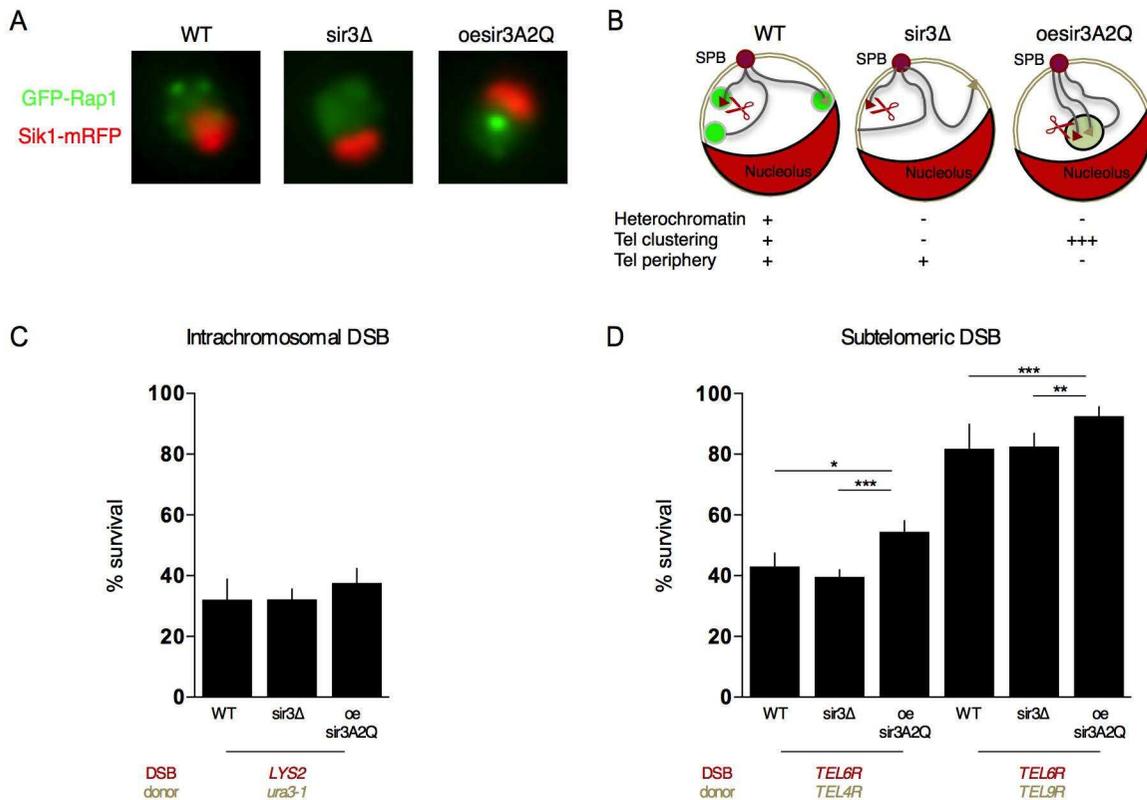
### 1.3.1. An assay to score recombination efficiency

We developed a recombination assay that scores double strand break (DSB) induced repair events between *URA3* alleles inserted at different chromosomal positions (Figure 1A and B). This system includes two types of recombination cassettes: a “recipient cassette” to induce a single I-SceI DSB and a “donor cassette” with an *ura3-1* allele. In the recipient cassette, insertion of 30bp, instead of 18bp (Colleaux et al., 1988), from the I-SceI cleavage site ensured good cleavage efficiency after induction of the I-SceI endonuclease driven by the inducible GAL promoter. Quantitative PCR using primers located on both sides of the DSB revealed DSB occurred in 70% of the cells 1 hour after induction and in over 90% of the cells after 2 hours (Figure 1C). We estimated DSB repair by comparing the ability of cells to form colonies on galactose-containing medium (I-SceI induction) to those on glucose with no I-SceI induction. Elimination of the I-SceI restriction site on the recipient cassette, assessed by in vitro digestion of the PCR amplified cassette, indicates efficient DSB cleavage and subsequent repair through inaccurate NHEJ or recombination based mechanisms. In all the experiments performed, 100% of the cells surviving on galactose containing plates were missing the I-SceI restriction site in the recipient cassette suggesting all cells induced DSB efficiently (Figure 1D). Repair through inaccurate NHEJ rarely occurred, as plating efficiencies of donor-less strains showing

a DSB in the recipient cassettes inserted at the intrachromosomal *LYS2* locus or at the subtelomere of *TEL6R* were less than 0.06% and 0.32% respectively (Figure 1E and 1F). Insertion of a *ura3-1* donor cassette at the *URA3* locus strongly increased cell survival on galactose plates indicating recombination is the most frequent repair pathway when a homologous sequence is present (Figure 1E and 1F).

### 1.3.2. Subtelomeric donor sequence efficiently repair intrachromosomal DSB

Using our assay we determined the recombination efficiency between donors and recipient recombination cassettes inserted at internal or subtelomeric positions in the genome (Figure 1B). We found survival frequencies depended on recombination cassettes position, suggesting that loci at different locations in the genome recombine with different efficiencies. Whereas an intrachromosomal DSB induced at *LYS2* was efficiently repaired with the intrachromosomal *ura3-1* locus, a DSB induced at *TEL6R* was not (Figure 1E and 1F). This observation confirms results obtained in spontaneous recombination assays, which proposed a recombination barrier between subtelomeric and intrachromosomal sequences (Marvin et al., 2009b; 2009a). However, repair of an intrachromosomal DSB at the *LYS2* locus led to similar (*ura3-1* versus *TEL9R* donor) or higher survival frequencies (*ura3-1* versus *TEL4R* donor) when the recombination donor was in a subtelomeric position rather than an intrachromosomic location (Figure 1E). So, subtelomeric cassettes are good recombination donors for intrachromosomal DSBs repair. They remain accessible to intrachromosomal DSBs, despite telomere perinuclear anchoring and no barrier impairs recombination between intrachromosomal and telomeric sequences.



**Figure 2: Reducing spatial distance favors homologous recombination.**

(A) Rap1 foci grouping upon *sir3A2Q* overexpression. The *sir3A2Q* allele is silencing defective and its overexpression leads to telomere clustering at the center of the nucleus. Representative fluorescent images of the telomere-associated protein GFP-Rap1 and of the nucleolus visualized through mRFP-Sik1 in *WT*, *sir3Δ* and *sir3A2Q* overexpressing cells.

(B) Schematic representation of the experimental design showing telomere organization, heterochromatin status and DSB localization.

(C-D) Survival frequencies observed after induction of a DSB in *WT*, in *sir3Δ* and *sir3A2Q* overexpressing cells as in Figure 1(E-F).

### 1.3.3. Reducing spatial distance favors homologous recombination

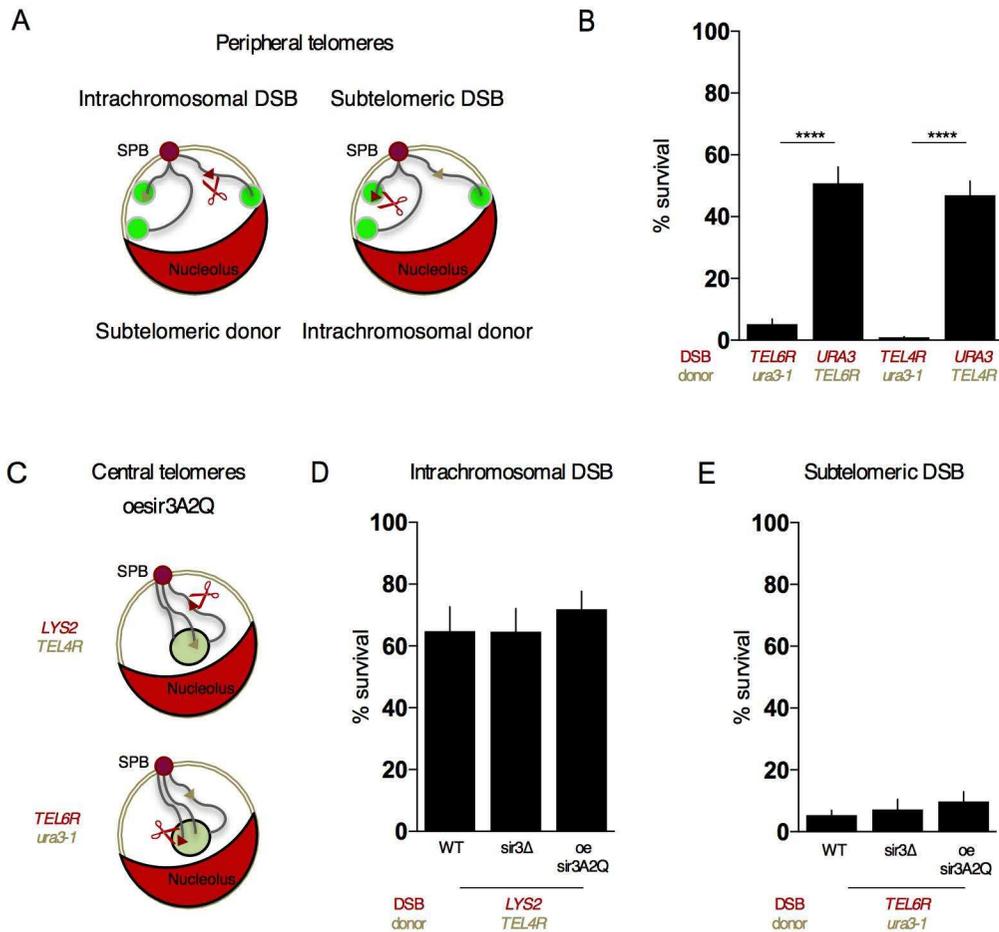
In contrast to the low recombination efficiency observed for subtelomeric DSBs with an internal donor, we found high recombination rates between the *TEL6R* DSB and subtelomeric cassettes inserted at *TEL4R* or *TEL9R* (Figure 1F). The higher recombination rate was observed between *TEL6R* and *TEL9R* subtelomeric loci that are spatially close and do show a higher contact frequency as monitored by HiC (Duan et al., 2010; Guidi et al., 2015; Therizols et al., 2010). The recombination rate between *TEL6R* and the more distant *TEL4R*, which lies at the end of a long chromosome arm, was almost two times lower (Figure 1F). Previous correlations between recombination efficiencies and spatial distances inferred either from the overlap of positions occupied by loci in the nucleus (Agmon et al., 2013) or from HiC contact maps (Lee et al., 2016) suggested that preexisting proximity dictated by nuclear DNA sequence positioning increases recombination efficiency through favoring homology search.

To test this hypothesis directly, we modified inter-loci distances in the nucleus and measured the changes on recombination efficiency. Overexpression of the *sir3A2Q* mutant form of the Sir3 protein promotes telomere clustering within a single focus at the center of the nucleus (Ruault et al., 2011) (Figure 2A, 2B, and S1). So, we assessed recombination efficiencies in cells when the spatial distance between cassettes inserted at subtelomeric loci was reduced following overexpression of *sir3A2Q*. The A2Q mutation was inserted in the endogenous *SIR3* gene with the promoter replaced by a strong GDP promoter for overexpression. As overexpression of *sir3A2Q* also led to loss of subtelomeric silencing (Ruault et al., 2011), we used the *sir3Δ* mutant as a control, since in this mutant, telomeres remain anchored to the nuclear periphery despite being derepressed (Figure S1). To avoid indirect

recombination effects caused by de-repression of the cryptic mating type loci in strains with *SIR3* deletion or *sir3A2Q* overexpression, the *HML* locus was deleted in all strains (Aström et al., 1999; Lee et al., 1999). We observed that recombination efficiency between two intrachromosomal cassettes (*LYS2 ura3-1*) was not significantly affected by Sir3 absence or by *sir3A2Q* protein overexpression (Figure 2C). Deletion of *SIR3* did also not affect recombination levels between *TEL6R* and *TEL4R* or *TEL9R* (Figure 2D). Loss of *SIR3* and subtelomeric silencing had thus no global impact on recombination efficiency. However, reducing the physical distance between subtelomeric cassettes (*TEL6R-TEL4R* and *TEL6R-TEL9R*) upon *sir3A2Q* overexpression significantly increased recombination efficiencies (Figure 2D). These data demonstrate that increased spatial proximity favors recombination between homologous sequences and posits homology search is a limiting factor for recombination efficiency.

#### 1.3.4. Spatial distance is not the only limiting factor for homologous recombination

We next tested whether HR efficiency was the same between reciprocal pairs of donor and acceptor loci as predicted if spatial distance was the only determinant of this process (Figure 3A). Surprisingly, we observed a strong asymmetry in repair efficiency within pairs of intrachromosomal and subtelomeric loci. Indeed DSB induced at the intrachromosomal *URA3* locus was repaired efficiently with sequences inserted in the *TEL6R* or *TEL4R* subtelomeres leading to 54% and 45% survival, but repair of DSBs induced at *TEL6R* or *TEL4R* with the *URA3* locus as a donor only led to 4% and 0.8% survival (Figure 3B). The physical distance between *URA3* and *TEL6R* or *TEL4R* does not change depending on which site the DSB is induced but



**Figure 3: Asymmetry in recombination efficiency between intrachromosomal and telomeric sequences.**

(A) Schematic representation of the assay showing DSB localization and telomere at the nuclear periphery in *WT* cells.

(B) Survival frequencies after DSB induction as in Figure 1(E-F).

(C) Schematic representation of the assay showing DSB localization and telomere at the nuclear center in cells overexpressing *sir3A2Q*.

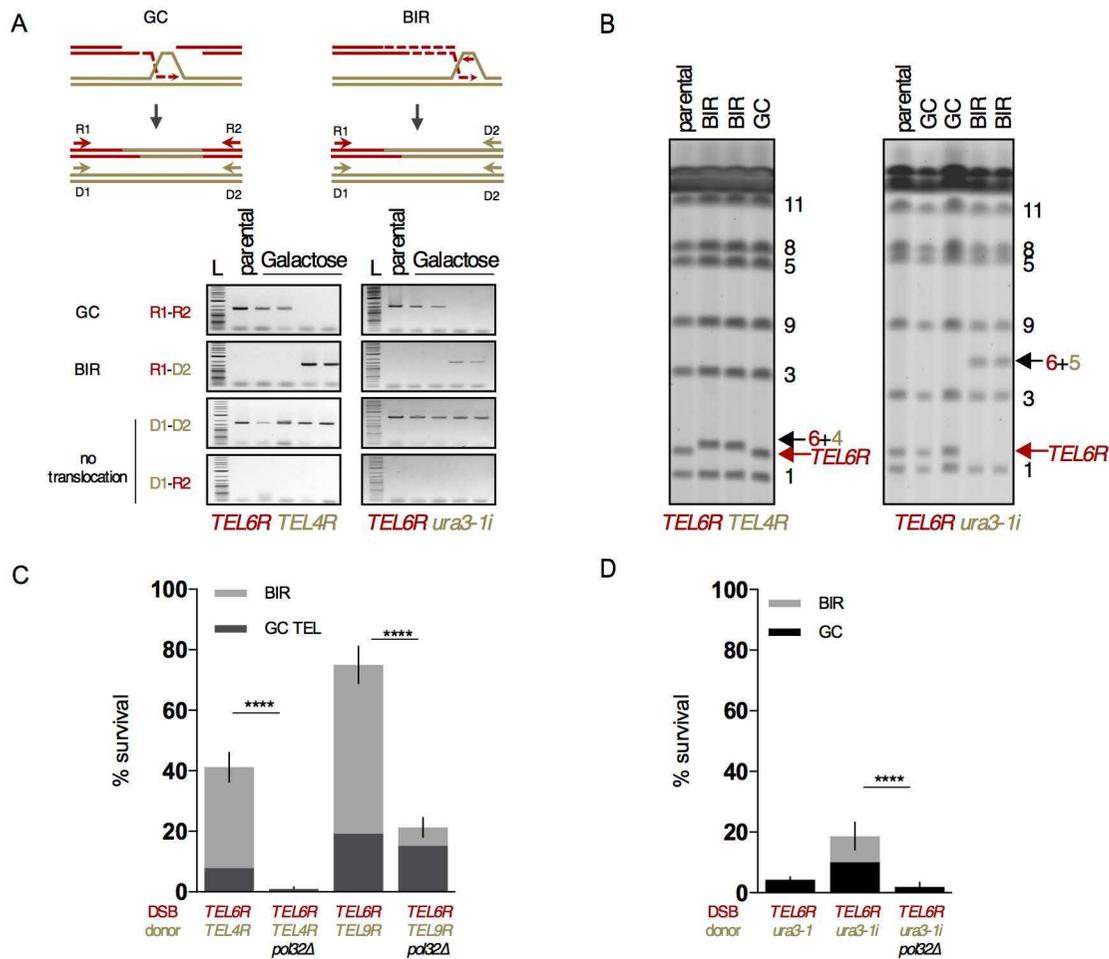
(D-E) Survival frequencies observed after DSB induction at an intrachromosomal position (D) or at a subtelomeric position (E) in *sir3Δ* and *sir3A2Q* overexpressing and *WT* cells as in Figure 1(E-F).

the recombination efficiency varies up to 50 fold. Altogether, these results indicate physical distance between homologous sequences is not the only limiting factor for recombination efficiency but position of the DSB on the chromosome also affects recombination efficiency.

#### 1.3.5. Recombination efficiency between subtelomeric and intrachromosomal loci is independent of telomere perinuclear anchoring

Subtelomeric and intrachromosomal loci differ by their subnuclear position, as telomeres are anchored to the nuclear periphery. We showed DSBs relocate to the nuclear periphery and proposed that change in position favors repair through a still unknown mechanism (Nagai et al., 2008). We hypothesized that relocalization of an intrachromosomal DSB at the nuclear periphery close to telomeric foci would favor its encounter with a subtelomeric donor and explain why a DSB induced at *URA3* is efficiently repaired with subtelomeric donors, while confining a subtelomeric DSB to the nuclear periphery would restrain its ability to find an intrachromosomal donor.

We examined recombination efficiencies between intrachromosomal and subtelomeric sequences upon *sir3A2Q* overexpression to localize telomeres to the nuclear center (Ruault et al., 2011) (Figure S1). Overexpression of *sir3A2Q* or deletion of *SIR3* had no significant effect on repair of an intrachromosomal DSB at the *LYS2* locus using the *TEL4R* subtelomeric donor cassette (Figure 3C). Thus, subtelomeres remain efficient recombination donors for intrachromosomal DSBs, even when located at the nuclear center. Further, a DSB induced at the subtelomere of *TEL6R* was inefficiently repaired with an intrachromosomal cassette displaced at the nuclear center upon *sir3A2Q* overexpression (Figures 3D and S1). Thus this shows that perinuclear anchoring of telomeres is neither favoring the use of subtelomeric



**Figure 4: Subtelomeric DSBs are efficiently repaired through BIR.**

(A) Representative assay to determine the repair pathway used. Schematic of the primers used (top) and representative PCR obtained for the *TEL6R TEL4R* and *TEL6R ura3-1i* strains (bottom).

(B) BIR and GC event are distinguished on resolutive pulsed-field gel electrophoresis. Repair by BIR leads to a shift of the chromosome size as indicated.

(C-D) BIR events are Pol32-dependent. Survival frequencies as in Figure 1(E-F) and proportion of survivors having repaired by GC (dark grey) or BIR (light grey). See Table S1 for statistical analysis of the repair events.

sequences as donors for intrachromosomal DSB nor preventing subtelomeric DSB from being repaired with intrachromosomal donors.

#### 1.3.6. Subtelomeric DSBs are efficiently repaired through BIR mediated non-reciprocal translocations

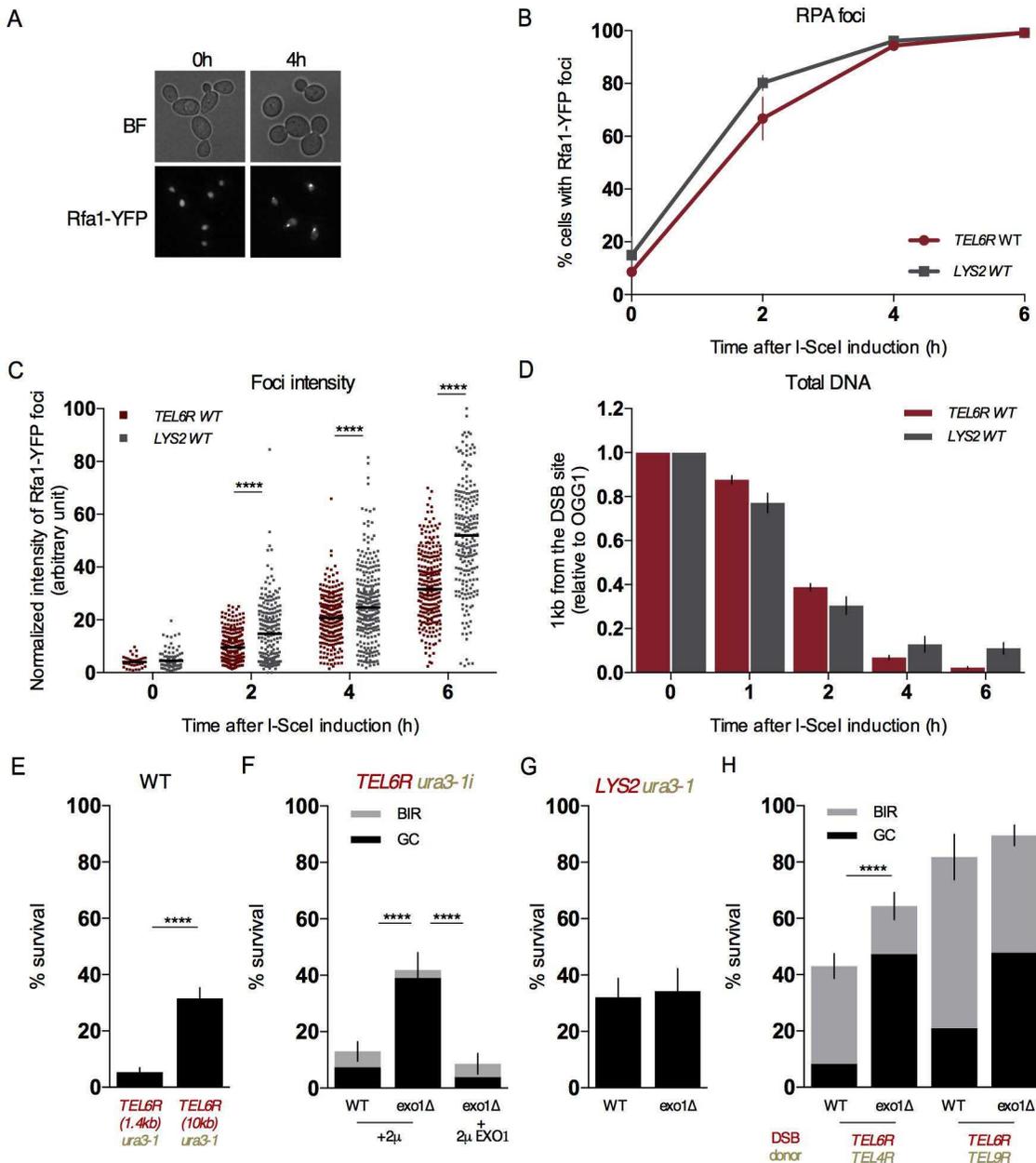
In response to subtelomeric DSBs, two recombination events, GC and BIR, can lead to viable progeny. We investigated whether BIR occurred in our system despite a homologous template with homologies on both sides of the DSB and could account for the observed survival.

To distinguish repair events arising from GC or BIR, we used colony PCR based analysis using primers flanking the recombination cassettes (Figure 4A). BIR events were further confirmed by high-resolution pulsed-field gel electrophoresis (PFGE). As expected, we observed a migration shift of the DSB bearing chromosome corresponding to the size predicted for BIR repair without affecting donor chromosome size. PFGE analysis did not reveal additional gross chromosomal rearrangements (Figure 4B). This molecular analysis revealed that up to 70% of survivors from repair between subtelomeric cassettes exhibited typical BIR NRT (Figure 4C). These NRTs depended on the DNA Pol $\delta$  subunit *pol32*, a factor required for BIR (Deem et al., 2008; Lydeard et al., 2007). BIR events between *TEL6R* and *TEL4R* that required polymerization of 10kb relied entirely on *POL32* (Figure 4C). However, in the absence of *Pol32* recombination between *TEL6R* and *TEL9R* subtelomeres still led to NRTs that accounts for 5% of survival (Figure 4C), which could correspond to gene conversion events, in which D-loop extended over 3.2kb to the end of chromosome 9R (Jain et al., 2009). Together, these results show that BIR is favored at subtelomeric DSBs even when in competition with GC.

If BIR engages at the *TEL6R* DSB using the *ura3-1* locus, oriented toward chromosome V centromere, it should form either acentric or dicentric chromosomes which would account for the low strain viability. To determine whether BIR could occur at the *TEL6R* DSB using intrachromosomal donor, we constructed a strain with an inverted *ura3-1* cassette (Figure 1B; *ura3-1i*). Inversion of the *ura3-1* cassette increased survival rate after inducing a DSB in the *TEL6R* subtelomeric region (*TEL6R ura3-1i*, Figure 4D). This increase stemmed from additional *POL32* dependent BIR repair events with characteristic PCR and pulsed-field profiles (Figure 4A and 4B). Although these events required copying 117kb from the right arm of chromosome V, they occurred in 9% of the cells and accounted for 50% of survivors in this strain. We conclude that subtelomeric DSB shows limited GC efficiency compared to intrachromosomal DSB but are repaired efficiently through BIR accounting for 40 to 80 % of repair events.

### 1.3.7. Loss of the telomeric fragment limits Gene Conversion

We sought to decipher which molecular mechanisms in subtelomeric regions favor BIR. BIR repair may result from the failure to capture the telomeric fragment as a second end (Jain et al., 2009; Malkova et al., 2005). Indeed, both strands of the short telomere proximal fragment disappear shortly after a DSB induction 15kb away from *TEL7L* (Hirano and Sugimoto, 2007). We hypothesized that the high frequency of BIR and a low rate of GC at the *TEL6R* subtelomeric DSB originate from rapid loss by resection of the 1.4kb telomeric fragment generated by I-SceI cleavage. We monitored the appearance of RPA foci following I-SceI DSB induction in donor-less strains to follow single stranded DNA formation and DSB processing. Upon DSB induction at *TEL6R*, the number of cells forming RPA foci was similar to cells with a



**Figure 5: Loss of the telomeric fragment limits gene conversion.**

(A) Representative image of Rfa1-YFP foci in response to an I-SceI-induced DSB at *TEL6R* or at *LYS2* in WT cells.

(B) Quantitation of cells with Rfa1-YFP foci after DSB induction in donor-less strains. Mean of two independent experiments are shown. Error bars indicate SD.

(C) Quantification of Rfa1-YFP foci intensity using Q-foci after induction of I-SceI. Data plotted represent the pool of two independent experiments. Black stars indicate statistical differences using a Mann-Whitney test (\* =  $p < 0,05$ ; \*\* =  $p < 0,01$ ; \*\*\* =  $p < 0,005$ ; \*\*\*\* =  $p < 0,001$ ).

(D) DNA levels measured at 1kb from the I-SceI cut site over time by qPCR in donor-less strains and normalized to DNA levels at the *OGG1* locus. Error bars represent the standard deviation of the mean (s.d.m.) of at least three independent experiments.

(E-H) Survival frequencies and distribution of the repair events of the indicated strains. Error bars represent the standard deviation of the survival mean (s.d.m.) of at least three independent experiments. Black stars indicate statistical differences in survival as in Figure 1(E-F). See Table S1 for statistical analysis of the repair events.

DSB at the intrachromosomal *LYS2* locus indicating that both sites convert to ssDNA with the same kinetics (Figures 5A and 5B). However we observed that the intensity of RPA foci was significantly lower in cells experiencing a subtelomeric DSB indicating fewer single strand (Figure 5C). Monitoring DNA amount at *TEL6R* or *LYS2* I-SceI cleavage sites in donorless strains revealed a faster disappearance of the telomere proximal fragment 1kb away from the I-SceI cleavage site at *TEL6R* compared to the equivalent site at the *LYS2* locus (Figure 5D). As cleavage efficiency was the same at both loci (Figure 1B), the difference in DSB loss in proximal sequences at both loci is likely due to resection. Together these data argue that ssDNA formed by the processing of *TEL6R* telomeric fragment is rapidly lost.

To determine whether this loss was key for DSB repair outcome, we moved the insertion site of the DSB cassette 10kb away from *TEL6R*. Since previous estimates yield a 4kb/hr resection rate (Fishman-Lobell et al., 1992; Mimitou and Symington, 2008; Saad et al., 2014; Zhu et al., 2008), displacing the DSB site 10 kb inward on the chromosome may delay its processing by 2 hours. Inserting the DSB cassette 10kb from *TEL6R* increased repair efficiency 7 fold compared to the 1.4kb *TEL6R* DSB (Figure 5E). This distance-dependent effect of DSB position on repair efficiency suggests that extensive resection of the telomeric proximal fragment limits GC.

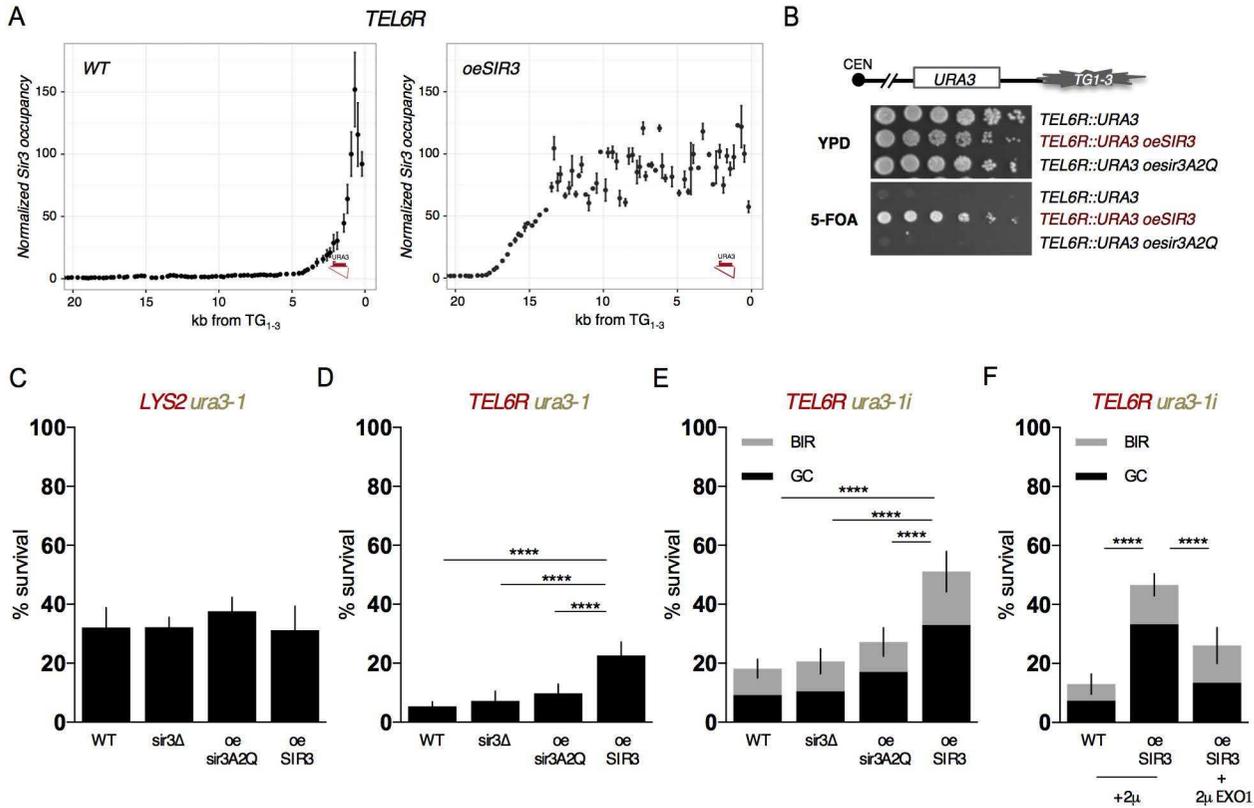
#### 1.3.8. Exo1-mediated resection limits Gene Conversion and favors BIR at subtelomeric DSBs

Next we tested whether altering the resection process affects recombination efficiency and outcome of *TEL6R* subtelomeric DSB. We deleted the *EXO1* gene that codes for a nuclease involved in long-range resection of DSB extremities (Gravel et

al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008) and induced a DSB at 1.4kb from *TEL6R*. Deletion of *EXO1* increased survival almost 3 fold compared to *WT*. This increase reflected a 5-fold increase in GC events, and BIR had an insignificant minimal decrease (Figure 5F, Table S1). Reintroduction of *EXO1* on a high-copy number (2 $\mu$ ) plasmid in *exo1 $\Delta$*  cells restored low levels of GC (Figure 5F). In contrast, limiting ssDNA formation at the *LYS2* intrachromosomal DSB had no effect on GC efficiency as shown previously (Lydeard et al., 2010a; Zhu et al., 2008) (Figure 5G). These results show that Exo1 mediated resection induces lethality at subtelomeric DSB by limiting GC repair. Deletion of *EXO1* in strains where the repair occurs between subtelomeric loci increased GC and significantly decreased BIR events in line with a previous report (Chung et al., 2010) (Figure 5H, Table S1). Loss of the telomeric proximal fragment mediated at least partially by Exo1 dependent resection may be the critical event limiting GC efficiency and favoring BIR repair events in subtelomeric sequences.

### 1.3.9. Heterochromatin spreading at DSB sites counteracts Exo1p induced lethality

Subtelomeric sequences assemble in repressive heterochromatin mediated by the SIR complex in budding yeast. We wondered how this assembly regulates DSB repair of the DSB induced at *TEL6R*. Although we detected low levels of Sir3 binding at the insertion site of the recombination cassette on *TEL6R* by CHIP-Chip, functional assessment of transcriptional repression by FOA assay revealed that the *URA3* gene positioned 1.4kb from *TEL6R* was not silenced (Figure 6A and 6B). Consistently deletion of *SIR3* had no impact on repair of a DSB induced at *TEL6R* compared to a *WT* strain (Figure 6D).



**Figure 6: Heterochromatin spreading at DSB sites counteracts Exo1p induced lethality.**

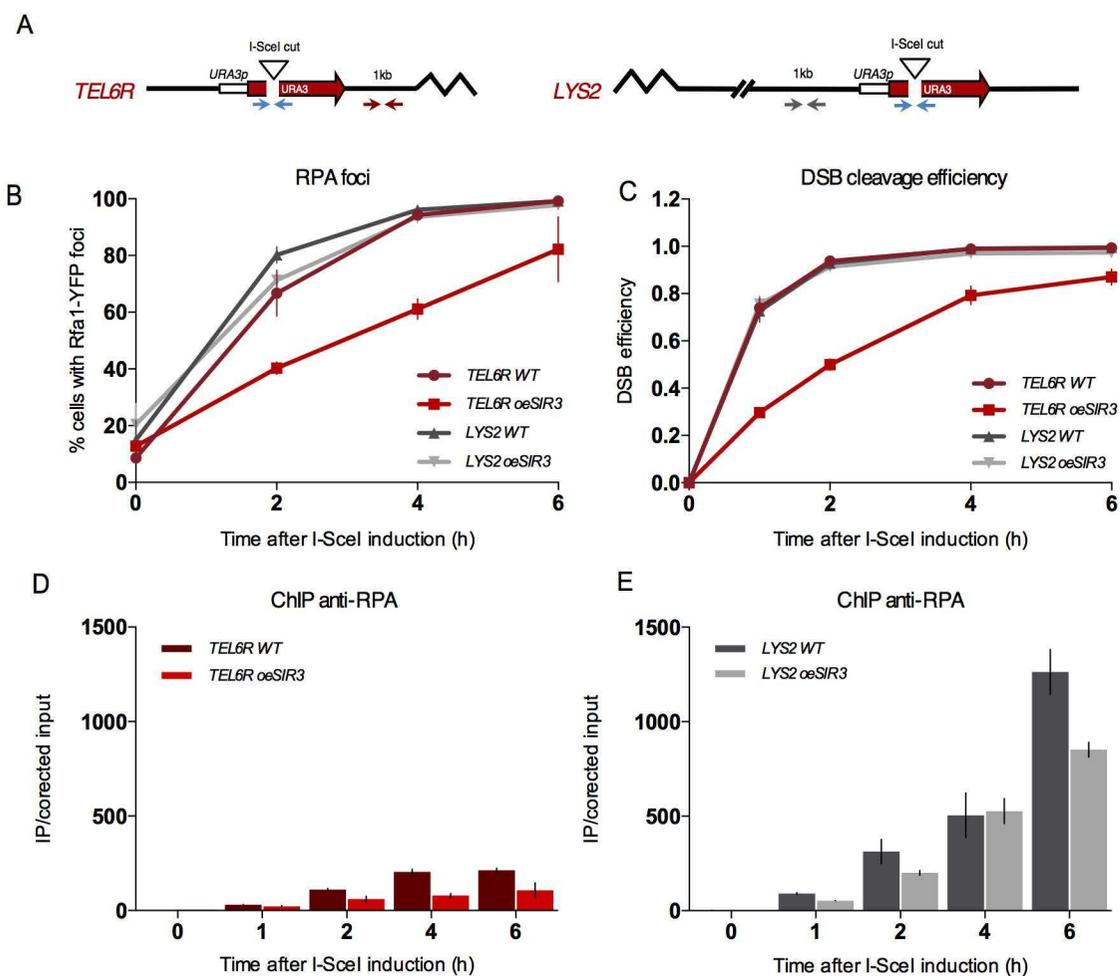
(A) Sir3-binding at *TEL6R* in *WT* cells or in cells overexpressing *SIR3* (*oeSIR3*). Binding is probed by ChIP-chip using antibodies directed against untagged Sir3p. The mean of two independent biological replicates is shown, error bars correspond to the variation between duplicates. The red inserts mark the insertion site of the *URA3* recombination cassette.

(B) Telomeric silencing assay at *TEL6R* in *WT* cells, cells overexpressing *SIR3* (*oeSIR3*) or *sir3A2Q* (*oesir3A2Q*). Increased growth on 5-FOA plates reflects an increase in telomeric silencing.

(C-F) Survival frequencies observed after induction of a DSB in the indicated strains. Survival frequencies for *sir3Δ*, *oesir3A2Q*, and *WT* from Figure 2C and 3D plotted for comparison with strains overexpressing *SIR3*. Error bars represent the standard deviation of the mean survival (s.d.m.) of at least three independent experiments. Black stars indicate statistical differences in survival as in Figure 1(E-F). See Table S1 for statistical analysis of the repair events.

We overexpressed the *WT* form of *SIR3* to determine if heterochromatin altered DSB repair. Increasing *SIR3* expression increased heterochromatin spreading and transcriptional repression in subtelomeric regions (Hecht et al., 1996; Katan-Khaykovich and Struhl, 2005; Ruault et al., 2011; Strahl-Bolsinger et al., 1997). In our strain, overexpressed *SIR3* spread over 15kb on *TEL6R* subtelomeric regions (Figure 6A) and formed a heterochromatic structure repressive for transcription, since cells with a *URA3* gene inserted at the same position as our recombination cassette 1.4 kb away from *TEL6R* grew on FOA plates (Figure 6B).

As Sir3 spreading could alter expression of numerous genes that could indirectly modulate the repair process, we checked the recombination efficiency between two intrachromosomal cassettes (*LYS2* and *ura3-1*) in a *SIR3* overexpressing strain. We observed no change in recombination efficiency between intrachromosomal loci after *SIR3* overexpression compared to *WT* cells (Figure 6C). In contrast, *SIR3* overexpression increased survival and hence GC efficiency, which is the only viable repair event between *TEL6R* and *ura3-1* (Figure 6D). Since *SIR3* overexpression also leads to telomere clustering at the nuclear center (Ruault et al., 2011) (Figure S1), we assessed the effect of telomere clustering on GC rate between *TEL6R* and *ura3-1* in cells overexpressing the separation of function mutant *sir3A2Q*. We found no significant change in GC repair efficiency upon *sir3A2Q* overexpression compared to *WT*, indicating the increase in GC occurs independent of clustering effects (compare *oesir3A2Q* and *oeSIR3* in Figure 6D). In the *TEL6R ura3-1i* strain where both BIR and GC repair produce viable progenies, DSB heterochromatinization favored GC and to a lesser extent BIR (Figure 6E). Since limiting *EXO1* dependent resection favored GC repair at subtelomeric DSB, we predicted that the DSB heterochromatinization regulates resection. In this case, *EXO1* overexpression should



**Figure 7: Heterochromatin at the DSB site impairs resection.**

(A) Schematic of *TEL6R* and *LYS2* loci with primer location indicated for DSB cleavage efficiency (blue) and ChIP measurement (red and grey). The telomeres are represented as wavy lines.

(B) Percentage of cells with Rfa1 foci in response to an I-SceI-induced DSB at *TEL6R* or at *LYS2* in donor-less *WT* and *SIR3* overexpressing cells.

(C) DSB cleavage efficiency at the two loci in *WT* and *SIR3* overexpressing cells measured as in Figure 1D. Error bars represent the standard deviation (s.d.) of at least three independent experiments.

(D-E) ChIP analysis of RPA occupancy at 1kb from the DSB in donor-less *WT* and *SIR3* overexpressing cells after induction of I-SceI cleavage at *TEL6R* (D) or *LYS* (E). The input was corrected for differences in DSB cleavage efficiency (see material and methods for details). Values reflect the ratio of precipitated DNA relative to the corrected input at each region and normalized to OGG1. Error bars represent the standard deviation of the mean (s.d.m.) of two independent experiments.

suppress the effects of *SIR3* overexpression by restoring DSB resection. *EXO1* overexpression counteracted Sir3 mediated heterochromatinization of *TEL6R* DSB and partially restored recombination between *TEL6R* and *ura3-Ii* in strains overexpressing *SIR3* to *WT* levels (Figure 6F), indicating that heterochromatin may modulate resection.

#### 1.3.10. Heterochromatin spreading at DSB sites limits resection

To determine the effect of heterochromatin spreading at the DSB site on resection, we compared DSB processing in *SIR3* overexpressing and *WT* cells. We monitored the appearance of RPA foci following I-SceI DSB induction at *TEL6R* and observed that the number of cells forming RPA foci was strikingly lower in cells overexpressing *SIR3* compared to *WT* cells (Figure 7B). This decrease was specific to cells with a heterochromatic DSB site, as it was not observed when the DSB was induced at the intrachromosomal *LYS2* site in cells overexpressing *SIR3* (Figure 7B). However, we observed a significant delay in DSB cleavage at the heterochromatic DSB site when checking DSB induction efficiency by quantitative PCR (Figure 7A and 7C). This delay could account for a decreased number of cells with RPA foci. To investigate the effect of delayed DSB induction between the two strains, we monitored recruitment of the Rfa1 subunit to DSB by ChIP analysis and normalized immunoprecipitation efficiencies with inputs corrected for differences in DSB cleavage efficiency (Figure 7D and 7E). RPA recruitment was lower at the heterochromatic DSB in the *TEL6R* oe*SIR3* strain than at the euchromatic DSB in the *WT* strain. No significant differences were observed between *SIR3* overexpressing and *WT* cells experiencing a DSB at the control *LYS2* locus. We conclude that increasing

heterochromatin in subtelomeric regions limits resection at subtelomeric DSBs and avoids loss of genetic information.

#### **1.4. Discussion**

Here we show that homologous recombination efficiency is limited not only by the physical distance separating homologous sequences but also by resection and chromatin compaction. We show that the active grouping of two homologous sequences improves recombination efficiency demonstrating directly that the physical distance between two homologous sequences in the nucleus is a limiting factor for homologous recombination rather than indirect correlations (Agmon et al., 2013; Lee et al., 2016; Roukos and Misteli, 2014). However, we also observed that the recombination between two cassettes varied up to 50 fold depending on which one experienced a DSB. A subtelomeric DSB is inefficiently repaired with an intrachromosomal matrix while the converse configuration leads to efficient repair. This shows that the physical distance is not the only rate-limiting factor for subtelomeric DSB.

We show that no barrier impairs contact and recombination between intrachromosomal and subtelomeric sequences as intrachromosomal DSB are efficiently repaired with subtelomeric sequences. As a consequence, the low level of spontaneous recombination previously observed between intrachromosomal and subtelomeric loci (Marvin et al., 2009b) that we recapitulated by inducing a subtelomeric DSB are likely to reflect the propensity of subtelomeric regions to be spontaneously damaged. This fragility may be conserved in humans where subtelomeric regions exhibit high levels of sister chromatid exchanges (Cornforth and Eberle, 2001).

The low HR efficiency between subtelomeric DSB and intrachromosomal loci is not caused by telomere anchoring at the nuclear periphery. Although telomere tethering to the nuclear periphery may be essential for low level of BIR repair of subtelomeric DSB with no ‘homology’ in the genome (Chung et al., 2015; Therizols et al., 2006), we observed that displacing the DSB bearing site at the nuclear center did not decrease BIR nor GC repair of a subtelomeric DSB with an intrachromosomal donor sharing perfect homology (Figure 6E). This held true even when a subtelomeric recombination donor was used (Figure S2). Deletion of *SIR4*, a factor required for telomere anchoring at the nuclear membrane (Hediger et al., 2002; Taddei et al., 2004), did also not affect recombination efficiency and outcome in our system (Figure S3). So, efficient HR in subtelomeric regions did not require the initial perinuclear position of telomeres.

Interestingly, we found that subtelomeric DSBs are efficiently repaired by BIR mediated events that accounts for 50 to 80% of the survivors depending on the configuration tested despite the presence of a two-ended homologous template in *WT* cells.

Favoring BIR in subtelomeres has implication for genome stability and evolution. On the one hand BIR heals damaged ends with telomeric sequences ensuring chromosome stability but on the other end BIR causes loss of heterozygosity and is highly mutagenic (Deem et al., 2008). However BIR induced duplication and exchange in subtelomeres could also be beneficial and serve as a nursery for new genes where diversity evolves faster than in single copy genomic regions.

As BIR preferentially engages when only one end of the DSB shares homology with the donor site, this suggests that the telomeric fragment freed by cleavage of the subtelomeric cassette is not available for GC. One possibility is that

telomere proximal repeated sequences interfere with HR repair by engaging subtelomeric DSB into competing recombination events as shown for multi-genes family or TY elements (Jain et al., 2016). If this was the case, DSB end tethering should drag the centromere proximal side of the DSB away from the URA3 cassette and impair BIR as well (Jain et al., 2016). An alternative hypothesis is that the telomeric proximal fragment is lost upon resection. We show that limiting resection of the telomere proximal fragment by moving the DSB further away from the TG repeats or by deleting *EXO1* increases recombination efficiency. Although in both cases this would limit unmasking of the subtelomeric repeated elements, our molecular analysis showing that telomere proximal sequences rapidly disappear suggest that BIR is favored as the consequence of the loss of the telomere proximal fragment. Interestingly limiting the loss of the telomeric fragment in the *TEL6R-I-SceI ura3-1 exo1Δ* strain increased recombination to levels comparable to those in the *URA3-I-SceI TEL6R* reciprocal strain. These results argue that resection-mediated loss of the telomeric proximal DSB extremity limits repair of subtelomeric DSBs.

The impact of resection on recombination efficiency may not be limited to subtelomeric loci. It was recently shown that resection limits recombinational repair at some intrachromosomal sites (Lee et al., 2016). This could occur following the loss of homology at the 3' end, that we show decreases below 50% 1kb away from the DSB accordingly with previous studies showing that 3' ssDNA overhangs are unstable and may couple to 5' strand resection (Chen et al., 2013; Toledo et al., 2013; Zierhut and Diffley, 2008). Loss of genetic information on the 3' overhang concomitantly with 5' resection could explain how limiting 5' strand resection at an intrachromosomal DSB rescues recombination efficiency. This model also accounts for improved recombination by increasing the size of sequence homology (Lee et al.,

2016). Thus, while the recombination process requires sequences unmasking and formation of a ssDNA recombination filament, resection can also be limiting when too extensive, notably if it causes loss of homologous sequences at the 3' end.

Interestingly, we do not detect any impact of *EXO1* deletion on the recombination at the *LYS2* locus consistent with prior reports (Lydeard et al., 2010a; Zhu et al., 2008) (Figure 5G), while we observe a strong effect at subtelomeric DSBs although the size of the homologous sequences were identical in both cases. So, resection possibly proceeds differently depending on the DSB flanking sequences or chromatin contexts. The nature of the chromatin environment also regulates HR efficiency in *Drosophila* and mammalian cells. Studies suggest that HR is the major pathway for repairing breaks in DAPI dense heterochromatin (Beucher et al., 2009; Geuting et al., 2013; Kakarougkas et al., 2013; Ryu et al., 2015). However, targeting damaged loci to the nuclear lamina delays DDR and impairs HR promoting repair by NHEJ or alternative end joining in human cells (Lemaître et al., 2014). Collectively these results suggest that the chromatin environment regulates HR, although it is unclear how this occurs.

We report here for the first time the impact of heterochromatin spreading at a DSB in yeast. Heterochromatic DSBs show improved recombination correlating with decreased resection of sequences surrounding the DSB. This result supports a regulatory role for chromatin structure on resection.

Although *in vitro* experiments have shown that Sir3 loading on the donor molecule impairs joint molecule formation (Sinha et al., 2009), its impact on the broken molecule has not been addressed. We show here that SIR complex spreading at DSB sites favors HR *in vivo*. This occurs as the consequence of a resection defect alleviated by overexpressing *EXO1*. Because *EXO1* overexpression is enough to

bypass the requirement for a functional MRX complex in the resection of a HO-induced DSB (Lewis et al., 2002; Moreau et al., 2001; Tsubouchi and Ogawa, 2000). Sir3 spreading could also restrain resection mediated by other nucleases. How Sir3 mediated heterochromatin limits resection remains to be explored. It could act as a physical block to slow down the progression of one or several nucleases mediating resection. Alternatively, heterochromatin-specific histone modifications could, directly or indirectly, impair nuclease recruitment or activation.

In conclusion, our work directly demonstrates that spatial distance is limiting recombination efficiency but that chromosome position of the DSB has an even more prominent effect. Altogether our data support a model in which recombination efficiency results from a race between the time needed for a resected DSB to find its recombination partner - determined by the physical distance between the DSB and the donor sequence - and the loss of homologous sequence required to invade the donor sequence - determined by the resection rate. We reveal that not all genomic loci are equal in this race as subtelomeric DSBs are much more sensitive to exonuclease activities possibly due to their proximity to the end of chromosomes and/or to repeated elements that once unmasked by resection could commit the broken chromosome into lethal events. Importantly heterochromatin formation can limit resection and thus the loss of genetic information upon subtelomeric DSB. This connection between heterochromatin and DSB processing and repair yields new insights into how cells maintain genome stability to avoid tumorigenesis.

## 1.5. Experimental Procedures

### Plasmids

Plasmid pAT274 was constructed by inserting *URA3* gene amplified from pRS316 with primers containing restriction sites suitable for cloning and the digested PCR product was ligated into pUG6 *SpeI* and *SacII* sites (from EUROSCARF). pAT275 was made by directed mutagenesis from pAT274 using the Pfu Turbo polymerase from Stratagene with primers inserting the 30bp *I-SceI* recognition sequence. The *ura3-1* mutation was introduced into pAT274 with primers changing the GGA to a GAA codon at position 701 giving the plasmid pAT276. pAT277 was made by introducing the C207T mutation into the pAT276 with primers changing the CCA to a CTA codon leading to the elimination of the *NcoI* restriction site.

To construct the 2 $\mu$  plasmid carrying the *EXO1* gene (pKD232), the *EXO1* gene was amplified from pHL546-*EXO1* (Mantiero et al., 2007) and inserted into the pRS424 digested by *PciI* and *PsiI* by SLIC (Li and Elledge, 2007).

### Yeast strains

All strains used in this study are isogenic to W303 (*MATa RAD5 ADE2 leu2-3,112 his3-11,15 trp1-1 ura3-1*) and are listed in Table S2. Strains were constructed by integrating a 1.6kb *ura3-I-SceI-lox-KanMx-lox* recipient cassette from pAT275 at different loci in the yeast genome. For recombination assays a *ura3-1-lox-KanMx-lox* homologous sequence from pAT277 or pAT276 was inserted as a donor cassette in different regions. Loss of the KanMx marker flanked by loxP sequences was selected on appropriate medium after transformation with pSH62 (*pGAL1-CRE-HIS3* from

EUROSCARF) that was further eliminated by successive restreak on non-selective medium.

The cassettes were inserted at the following coordinates: 268813 (*TELVI-R*, subtelomeric), 259499 (*TELVI-R(10kb)*, subtelomeric), 470815 (+3108 *LYS2*, intrachromosomal), 1522383 (*TELIV-R*, subtelomeric), 436650 (*TELIX-R*, subtelomeric), 116167 (*URA3*, intrachromosomal). All insertions were verified by PCR and all PCR primers are listed in Table S3.

Gene deletions (*sir3*, *ura3*, *exo1*, *pol32*, *dnl4*) and insertions of a strong constitutive promoter (pGPD) were performed by PCR-based gene targeting (Longtine et al., 1998).

For DSB induction or *EXO1* overexpression, cells were transformed with pKD89 (pRS413-pGAL1-I-SceI) or pKD232 (pHL546 ExoI TRP1) and selected on glucose-containing synthetic medium lacking histidine or tryptophane respectively.

For DSB end resection and chromatin immunoprecipitation experiments, the *I-SceI* gene was introduced in the yeast genome by transformation of the cells with Pbp2 (pRS404-pGAL1-I-SceI; gift from S. Marcand) digested by *PmlI* to target it to TRP1.

For the silencing test, a wild type *URA3* gene was amplified from pAT274 and integrated at the same position than the recombination cassettes in subtelomeric regions.

### **Media and growth conditions**

Yeast strains were grown in rich medium (yeast extract–peptone–dextrose, YPD) or synthetic complete (SC) medium lacking the appropriate amino acid at 30°C. Rich or synthetic medium containing 2% lactate, 3% glycerol, 0.05% glucose and lacking the

appropriate amino acids were used to grow the cells overnight prior the induction of I-SceI by plating onto 2% galactose plates or addition of 2% galactose to liquid culture.

### **Silencing test**

For telomeric silencing assay, strains were grown overnight in YPD and then plated in fivefold serial dilutions starting at  $OD_{600nm}=1$  (corresponding to  $10^7$  cells/ml) on YPD medium and synthetic complete (SC) medium containing 0.1% 5-fluoroorotic acid (5-FOA).

### **Recombination efficiency measurement**

Each strain was freshly transformed with pKD89 and a single transformant was grown overnight in 2mL of SLGg lacking histidine to select for the plasmid. The day after each culture was appropriately diluted and plated on SC-HIS plates supplemented with 2% glucose (Glc-HIS) to repress I-SceI expression or 2% galactose (Gal-HIS) to induce I-SceI expression. Colonies were counted after 2-3 days of incubation at 30°C. From Gal-HIS plates 48 isolated recombinants were analysed by PCR as exemplified in Figure 3A and 3B. For each strain at least three independent experiments were performed with the corresponding controls.

### **Pulsed-field gel electrophoresis**

Yeast DNA embedded in agarose plugs was prepared as follows: cells were cultured in rich medium overnight and about  $15 OD_{600}$  of cells were washed twice in 1mL of 50mM EDTA, 10mM Tris pH7.5 and resuspended in 150 $\mu$ L of 50mM EDTA, 10mM Tris pH7.5. The suspension was quickly warmed to 42°C with 0.6 $\mu$ L of Zymolyase

(20mg/mL), mixed with 250 $\mu$ L of prewarmed 1% agarose LMP (Low Melting Point) and distributed into 80 $\mu$ L wells placed into a cool surface. The plugs were extruded and incubated for 24h at 37°C in 1.4mL of 500mM EDTA, 10mM TrisHCl pH7.5 followed by 24h at 55°C in 1.25mL of 500mM EDTA, 10mM Tris pH8, 1% N-Laurylsarcosyl and 0.4mg/mL Proteinase K. Plugs were washed for 1h three times in 1.5mL of 50mM EDTA, 10mM Tris pH7.5. Pulse-field electrophoresis was carried out in a 0.9% agarose gel in 0.5X TBE at 14°C with a CHEF DRII from Bio-Rad for 22h (initial time = 10 s, final time = 25 s). After electrophoresis, the gel was stained with EtBr and photographed.

### **Chromatin immunoprecipitation**

**Rfa1 ChIP:** Yeast cells were grown in 2mL of YPD overnight. Cultures were then diluted in YPLGg and grown to OD<sub>600</sub>=0.3-0.8. I-SceI was induced by addition of galactose to a final concentration of 2%. ChIP was carried out as previously described with minor modifications (Sugawara and Haber, 2006). Samples were incubated with 2 mg of rabbit anti-RPA polyclonal antibody (gift from V. Géli) and 50  $\mu$ L of Magnetic Dynabeads Protein A (Invitrogen) were added to each sample. After washes, elution of the proteins and reversal of crosslinks, samples were treated with RNase A (1mg/mL) followed by purification of the DNA with EconoSpin mini spin columns and homemade buffers (Epoch Life Science). Quantitative PCR reactions were performed using primers located 1kb from the I-SceI cut site and primers flanking the I-SceI restriction site. A control primer pair was used to amplify a region of the *OGGI* locus. Quantitative PCRs were performed using Power SYBR Green Master Mix (Applied Biosystems) with the 7900HT Fast Real-Time PCR System and corresponding software (Applied Biosystems). To correct for differences in DSB

cleavage efficiency, the percentage of uncut DNA was subtracted from Input DNA at each time point.

**Sir3 ChIP:** 3 ml YPD cultures were inoculated and grown in YPD for 10 hours. Those cultures were used to inoculate YP Raffinose (3%, i.e 30g/L) at 0.01 OD<sub>600</sub>. Those overnight cultures were used to inoculate 50ml cultures of YP Raffinose 3% at an OD<sub>600</sub>=0.2. Exponentially growing cultures were allowed to grow for an additional 2 hours, after which Galactose was added to reach a final concentration of 2%. Cells were pelleted 2 hours after galactose addition. A total of 20 OD<sub>600</sub> of cells were fixed in 20 mL with 0.9 % formaldehyde for 15 min at 30°C, quenched with 0.125 M glycine and washed twice in cold TBS 1x pH7.6. Pellets were suspended in 1mL TBS 1X, centrifuged and frozen in liquid nitrogen for -80°C storage. Anti-Sir3 ChIP experiments were done in two independent experiments. All the following steps were done at 4°C unless indicated. Pellets were re-suspended in 500 µL of lysis buffer (0.01% SDS, 1.1% TritonX-100, 1.2 mM EDTA pH8, 16.7 mM Tris pH8, 167 mM NaCl, 0.5 % BSA, 0.02 g.L<sup>-1</sup> tRNA and 2.5 µL of protease inhibitor from SIGMA P1860) and mechanically lysed by three cycles of 30 s with 500 µm zirconium/silica beads (Biospec Products) using a Fastprep instrument (MP Biomedicals). Each bead beating cycle was followed by 5 min incubation on ice. The chromatin was fragmented to a mean size of 500 bp by sonication in the Bioruptor XL (Diagenode) for 14 min at high power with 30s on / 30s off and centrifuged 5 min at 13 000 rpm. 10 µL were kept to be used as Input DNA. Cleared lysate was incubated overnight with 1 µL of polyclonal antibody anti-Sir3 (Agro-bio). 50 µL of magnetic beads protein A (NEB) were added to the mixture and incubated for 4h at 4°C. Magnetic beads were washed sequentially with lysis buffer, twice with RIPA buffer (0.1% SDS, 10mM Tris pH7.6, 1mM EDTA pH8, 0,1% sodium deoxycholate and 1% TritonX-

100), twice with RIPA buffer supplemented with 300 mM NaCl, twice in LiCl buffer (250 mM LiCl, 0.5% NP40, 0.5 % sodium deoxycholate), with TE 0.2% TritonX-100 and with TE. Input were diluted 10x with elution buffer (50mM Tris, 10mM EDTA pH8, 1%SDS) and beads were re-suspended in 100  $\mu$ L elution buffer. A reversal cross-linking was performed by heating samples overnight at 65°C. Proteins were digested with proteinase K in presence of glycogen and the remaining DNA was purified on QIAquick PCR purification columns. Finally, samples were treated with 29  $\mu$ g.mL<sup>-1</sup> RNase A 30 min at 37°C.

### **ChIP-chip preparation and hybridization**

Samples used for ChIP-chip were analysed by qPCR prior to microarray hybridization. For microarray hybridization 4/5 of the immunoprecipitated DNA and the DNA from the input were ethanol precipitated and re-suspended in 10 $\mu$ L of water (Gibco). Purified material was amplified, incorporating amino-allyl-dUTP. The size of the amplified fragments (~500 bp) was assessed by gel electrophoresis. For each sample 1.5  $\mu$ g of amplified DNA was coupled either with Cy5 (immunoprecipitated sample) or Cy3 (input sample) and hybridized on 44k yeast whole genome tiling array (Agilent) as described (Borde et al., 2009).

### **Microarray data acquisition, analysis and visualization**

The microarray was imaged using an Agilent DNA microarray scanner and quantified using GenePix Pro6.1 as described (Borde et al., 2009). Data visualization was done using the R package ggplot2. All scripts are available upon request. Data have been submitted to Gene Expression Omnibus (GEO) under the accession.

## Statistical analyses

To compare survival of two different strains, we used a  $\chi^2$  test. Black stars on graphs indicate statistical differences (\* =  $p < 0,05$ ; \*\* =  $p < 0,01$ ; \*\*\* =  $p < 0,005$ ; \*\*\*\* =  $p < 0,001$ ).

To determine change in repair events, we applied a proportional analysis with a confidence limit of 95%. All p-values are listed in Table S1.

To compare Rfa1 foci intensity, a Mann Whitney test was applied. Black stars on graphs indicate statistical differences (\* =  $p < 0,05$ ; \*\* =  $p < 0,01$ ; \*\*\* =  $p < 0,005$ ; \*\*\*\* =  $p < 0,001$ ).

## Microscopy

Live cell images were acquired using a wide field microscope based on an inverted microscope (Leica DMI-6000B) equipped with Adaptive Focus Control to eliminate Z drift, a 100x/1.4 NA immersion objective with a Prior NanoScanZ Nanopositioning Piezo Z Stage System, a CMOS camera (ORCA-Flash4.0; Hamamatsu) and a solid state light source (SpectraX, Lumencore). The system is piloted by MetaMorph software (Molecular Device).

For GFP-mCherry two-color images, 21 focal steps of  $0.25\mu\text{m}$  were acquired sequentially for GFP and mRFP with an exposure time of 200ms using solid state 475 and 575 nm diodes and appropriate filters (GFP-mRFP filter; excitation: double BP, 450–490/550–590 nm and dichroic double BP 500–550/600–665 nm; Chroma Technology Corp.). Three-dimensional data sets were deconvolved using the blind deconvolution algorithm of AutoQuant (Media Cybernetics, Inc.) with the Point

Spread Function appropriate to our microscope at each emission wavelength. Further processing was done using ImageJ software (National Institutes of Health).

YFP images were acquired at indicated time points before and after induction. 21 focal steps of 0.25 $\mu$ m were acquired with an exposure time of 300ms using a solid state 500 nm diode and a YFP filter (excitation 470-510 nm and dichroic 495 nm; Chroma Technology Corp.) Quantification of foci intensity has been performed as previously described using Q-foci (Ruault et al., 2011). All the images shown are a z projection of z-stack images.

## **1.6. Authors' contributions**

AB, CB and HB generated strains and performed recombination assays and molecular analysis. AB, MR and KD performed microscopy experiments and image analyses. CB performed and analyzed RPA ChIP and PFGE experiments. AH performed and analyzed *SIR3* ChIP on ChIP experiments. AA and CB constructed plasmids. KD, AT and AB designed and interpreted experiments, drafted the figures and wrote the manuscript.

## **1.7. Acknowledgements**

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## 1.8. Supplemental Information

Table S1. Statistics

	p-value (Chi-square)	significance	p-value (proportion test)	significance	p-value (proportion test)	significance
<b>TEL6R TEL4R</b>	survival		GC		BIR	
WT vs oesir3A2Q	0.036	*	0.00067	****	0.87	-
WT vs sir3Δ	0.96	-	0.042	*	0.73	-
oesir3A2Q vs sir3Δ	0.0029	***	1.81x10 <sup>-6</sup>	****	0.61	-
WT vs pol32Δ	1.01x10 <sup>-65</sup>	****	0.014	*	4.44x10 <sup>-15</sup>	****
WT vs exo1Δ	6.24x10 <sup>-7</sup>	****	1.11 x10 <sup>-15</sup>	****	0.00046	****
<b>TEL6R TEL9R</b>	survival		GC		BIR	
WT vs oesir3A2Q	0.008	**	0.0012	***	0.31	-
WT vs sir3Δ	0.99	-	0.1	-	0.15	-
oesir3A2Q vs sir3Δ	0.002	***	2.07x10 <sup>-6</sup>	****	0.014	*
WT vs pol32Δ	8.52x10 <sup>-68</sup>	****	0.027	*	0	****
WT vs exo1Δ	0.21	-	1.21x10 <sup>-6</sup>	****	0.0011	***
<b>TEL6R ura3-1i</b>	survival		GC		BIR	
WT vs oesir3A2Q	0.05	-	0.0059	**	0.61	-
WT vs oeSir3	3.09x10 <sup>-17</sup>	****	3.92x10 <sup>-12</sup>	****	0.0015	***
oesir3A2Q vs oeSir3	4.18x10 <sup>-8</sup>	****	2.57x10 <sup>-5</sup>	****	0.0096	**
WT+2μ vs exo1Δ+2μ	4.22x10 <sup>-5</sup>	****	1.26x10 <sup>-12</sup>	****	0.23	-
WT vs pol32Δ	2.02x10 <sup>-5</sup>	****	0.067	-	0.00038	****
WT vs sir4Δ	0.90	-	0.91	-	0.24	-
exo1Δ+2μ vs exo1Δ+2μExo1	1.31x10 <sup>-9</sup>	****	0	****	0.33	-
WT+2μ vs oeSir3+2μ	8.41x10 <sup>-8</sup>	****	2.09x10 <sup>-10</sup>	****	0.0076	**
oeSir3+2μ vs oeSir3+2μExo1	0.00035	****	1.73x10 <sup>-6</sup>	****	0.83	-
WT+2μ vs exo1Δ+2μExo1	0.73	-	0.28	-	0.81	-
WT+2μ vs oeSir3+2μExo1	0.11	-	0.05	-	0.018	*
<b>TEL6R ura3-1</b>	survival (GC)					
WT vs oesir3A2Q	0.25	-				
WT vs sir3Δ	0.82	-				
oesir3A2Q vs sir3Δ	0.92	-				
WT vs oeSir3	9.35x10 <sup>-10</sup>	****				
oesir3A2Q vs oeSir3	3x10 <sup>-5</sup>	****				
WT(1,4kb) vs WT(10kb)	1.93x10 <sup>-15</sup>	****				
<b>LYS2 TEL4R</b>	survival (GC)					
WT vs oesir3A2Q	0.75	-				
WT vs sir3Δ	0.99	-				
oesir3A2Q vs sir3Δ	0.79	-				
<b>LYS2 ura3-1</b>	survival (GC)					
WT vs oesir3A2Q	0.62	-				
WT vs oeSir3	0.99	-				
WT vs sir3Δ	0.99	-				
WT vs exo1Δ	0.99	-				

WT vs sir4Δ	0.99	-
oesir3A2Q vs sir3Δ	0.68	-
oesir3A2Q vs oeSir3	0.45	-
<b>WT</b>	<b>survival (GC)</b>	
LYS2 ura3-1 vs LYS2 TEL4R	1.11x10 <sup>-20</sup>	****
LYS2 ura3-1 vs LYS2 TEL9R	0.97	-
LYS2 TEL4R vs LYS2 TEL9R	1.48x10 <sup>-17</sup>	****
TEL6R ura3-1 vs TEL6R TEL4R	3.31x10 <sup>-33</sup>	****
TEL6R ura3-1 vs TEL6R TEL9R	1.68x10 <sup>-104</sup>	****
TEL6R TEL4R vs TEL6R TEL9R	2.29x10 <sup>-27</sup>	****
TEL6R ura3-1 vs URA3 TEL6R	1.06x10 <sup>-42</sup>	****
TEL4R ura3-1 vs URA3 TEL4R	1.29x10 <sup>-45</sup>	****

**Supplemental Table 1: Statistical significance between strains for survival, GC and BIR rates.** p-values were calculated with the Chi-square test for survival rate comparison and with the proportion test for repair events rates comparison. Black stars indicate statistical differences (- = non significant, \* = p<0,05; \*\* = p<0,01; \*\*\* = p<0,005; \*\*\*\* = p<0,001).

**Table S2. Yeast Strains**

Strain			source or reference
KD336	(1)	<i>TELVI-R::lox-ura3-ISceI</i>	This study
KD342	(1)	<i>TELVI-R::lox-ura3-ISceI sir3::Nat-GPDp-SIR3</i>	This study
KD343	(1)	<i>TELVI-R::lox-ura3-ISceI sir3::Nat-GPDp-sir3-A2Q</i>	This study
KD557	(1)	<i>TELVI-R::lox-ura3-ISceI sir3Δ::TRP1</i>	This study
KD683	(1)	<i>TELVI-R(10kb)::lox-ura3-ISceI</i>	This study
KD522	(1)	<i>TELVI-R::lox-ura3-ISceI ura3-1::ura3-1-lox-KanMx-lox</i>	This study
KD629	(1)	<i>TELVI-R::lox-ura3-ISceI ura3-1::ura3-1-lox-KanMx-lox sir3::Nat-GPDp-SIR3</i>	This study
KD630	(1)	<i>TELVI-R::lox-ura3-ISceI ura3-1::ura3-1-lox-KanMx-lox sir3::Nat-GPDp-sir3-A2Q</i>	This study
KD792	(1)	<i>TELVI-R::lox-ura3-ISceI ura3-1::ura3-1-lox-KanMx-lox exo1Δ::Nat</i>	This study
KD1087	(1)	<i>TELVI-R::lox-ura3-ISceI ura3-1::ura3-1-lox-KanMx-lox pol32Δ::Nat</i>	This study
KD1051	(1)	<i>TELVI-R::lox-ura3-ISceI ura3-1::ura3-1-lox-KanMx-lox sir3Δ::TRP1</i>	This study
KD1251	(1)	<i>TELVI-R::lox-ura3-ISceI ura3-1::ura3-1-lox-KanMx-lox sir4Δ::TRP1</i>	This study

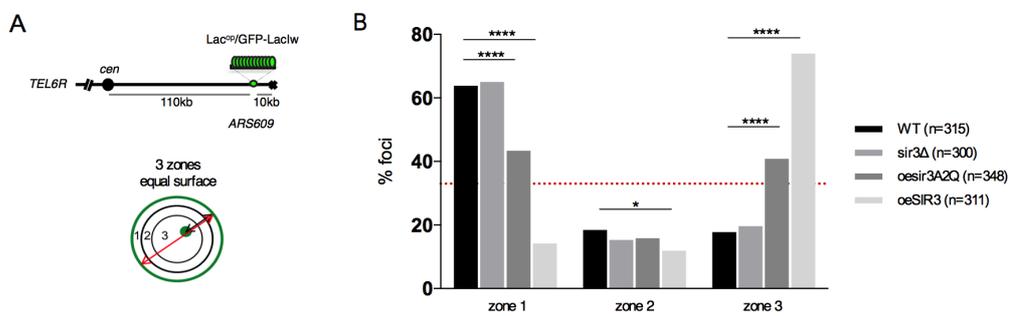
KD420	(1)	<i>TELVI-R::lox-ura3-ISceI TELIV-R::lox-ura3-1,C207T ura3-1Δ::KanMx</i>	This study
KD681	(1)	<i>TELVI-R::lox-ura3-ISceI TELIV-R::lox-ura3-1,C207T ura3-1Δ::KanMx sir3::Nat-GPDp-sir3-A2Q</i>	This study
KD559	(1)	<i>TELVI-R::lox-ura3-ISceI TELIV-R::lox-ura3-1,C207T ura3-1Δ::KanMx sir3Δ::TRP1</i>	This study
KD1085	(1)	<i>TELVI-R::lox-ura3-ISceI TELIV-R::lox-ura3-1,C207T ura3-1Δ::KanMx pol32Δ::Nat</i>	This study
KD1244	(1)	<i>TELVI-R::lox-ura3-ISceI TELIV-R::lox-ura3-1,C207T ura3-1Δ::KanMx exo1Δ::Nat</i>	This study
KD433	(1)	<i>TELVI-R::lox-ura3-ISceI TELIX-R::lox-ura3-1,C207T ura3-1Δ::KanMx</i>	This study
KD435	(1)	<i>TELVI-R::lox-ura3-ISceI TELIX-R::lox-ura3-1,C207T ura3-1Δ::KanMx sir3::Nat-GPDp-sir3-A2Q</i>	This study
KD560	(1)	<i>TELVI-R::lox-ura3-ISceI TELIX-R::lox-ura3-1,C207T ura3-1Δ::KanMx sir3Δ::TRP1</i>	This study
KD1086	(1)	<i>TELVI-R::lox-ura3-ISceI TELIX-R::lox-ura3-1,C207T ura3-1Δ::KanMx pol32Δ::Nat</i>	This study
KD1245	(1)	<i>TELVI-R::lox-ura3-ISceI TELIX-R::lox-ura3-1,C207T ura3-1Δ::KanMx exo1Δ::Nat</i>	This study
KD574	(1)	<i>TELIV-R::lox-ura3-ISceI</i>	This study
KD337	(1)	<i>lys2::lox-ura3-ISceI</i>	This study
KD344	(1)	<i>lys2::lox-ura3-ISceI sir3::Nat-GPDp-SIR3</i>	This study
KD345	(1)	<i>lys2::lox-ura3-ISceI sir3::Nat-GPDp-sir3-A2Q</i>	This study
KD558	(1)	<i>lys2::lox-ura3-ISceI sir3Δ::TRP1</i>	This study
KD1024	(1)	<i>lys2::lox-ura3-ISceI exo1Δ::Nat</i>	This study
KD1248	(1)	<i>lys2::lox-ura3-ISceI sir4Δ::TRP1</i>	This study
KD530	(1)	<i>lys2::lox-ura3-ISceI TELIX-R::lox-KanMx-lox-ura3-1,C207T ura3-1Δ::KanMx</i>	This study
KD713	(1)	<i>lys2::lox-ura3-ISceI TELIV-R::lox-ura3-1,C207T ura3-1Δ::KanMx</i>	This study
KD1017	(1)	<i>lys2::lox-ura3-ISceI TELIV-R::lox-ura3-1,C207T ura3-1Δ::KanMx sir3::Nat-GPDp-sir3-A2Q</i>	This study
KD810	(1)	<i>lys2::lox-ura3-ISceI TELIV-R::lox-ura3-1,C207T ura3-1Δ::KanMx sir3Δ::TRP1</i>	This study
KD935	(1)	<i>ura3-1::lox-KanMx-lox-ura3-ISceI TELVI-R::lox-ura3-1,C207T</i>	This study
KD934	(1)	<i>ura3-1::lox-KanMx-lox-ura3-ISceI TELIV-R::lox-ura3-1,C207T</i>	This study
KD788	(1)	<i>TELVI-R::lox-ura3-ISceI ura3-1Δ::KanMx trp1-1::Gallp-ISceI-TRP1 dnl4Δ::HIS3Mx</i>	This study
KD800	(1)	<i>TELVI-R::lox-ura3-ISceI ura3-1Δ::KanMx trp1-1::Gallp-ISceI-TRP1 dnl4Δ::HIS3Mx sir3::Nat-GPDp-SIR3</i>	This study
KD789	(1)	<i>lys2::lox-ura3-ISceI ura3-1Δ::KanMx trp1-1::Gallp-ISceI-TRP1 dnl4Δ::HIS3Mx</i>	This study
KD802	(1)	<i>lys2::lox-ura3-ISceI ura3-1Δ::KanMx trp1-1::Gallp-ISceI-TRP1 dnl4Δ::HIS3Mx sir3::Nat-GPDp-SIR3</i>	This study

KD565	(1)	<i>TELVI-R::lox-URA3</i>	This study
KD566	(1)	<i>TELVI-R::lox-URA3 sir3::Nat-GPDp-SIR3</i>	This study
KD567	(1)	<i>TELVI-R::lox-URA3 sir3::Nat-GPDp-sir3-A2Q</i>	This study
KD991	(2)	<i>TELVI-R::lox-ura3-ISceI ura3-1Δ::KanMx trp1-1::GalIp-ISceI-TRP1 dnl4Δ::HIS3Mx RFA1-YFP rap1::yEmRFP-RAP1-LEU2</i>	This study
KD992	(2)	<i>TELVI-R::lox-ura3-ISceI ura3-1Δ::KanMx trp1-1::GalIp-ISceI-TRP1 dnl4Δ::HIS3Mx RFA1-YFP sir3::Nat-GPDp-SIR3 rap1::yEmRFP-RAP1-LEU2</i>	This study
KD1005	(2)	<i>lys2::lox-ura3-ISceI ura3-1Δ::KanMx trp1-1::GalIp-ISceI-TRP1 dnl4Δ::HIS3Mx RFA1-YFP rap1::yEmRFP-RAP1-LEU2</i>	This study
KD1007	(2)	<i>lys2::lox-ura3-ISceI ura3-1Δ::KanMx trp1-1::GalIp-ISceI-TRP1 dnl4Δ::HIS3Mx RFA1-YFP sir3::Nat-GPDp-SIR3 rap1::yEmRFP-RAP1-LEU2</i>	This study
AT898		<i>Mat α ade2-1::ADE2 leu2-3,112::Hisp-GFP-LacIw-LEU2 ARS609::LacOp(x256)-TRP1 NUP49::URA3-Nup49-mCherry</i>	This study
KD1254		<i>Mat α ade2-1::ADE2 leu2-3,112::Hisp-GFP-LacIw-LEU2 ARS609::LacOp(x256)-TRP1 NUP49::URA3-Nup49-mCherry sir3::Nat-GPDp-SIR3</i>	This study
KD1255		<i>Mat α ade2-1::ADE2 leu2-3,112::Hisp-GFP-LacIw-LEU2 ARS609::LacOp(x256)-TRP1 NUP49::URA3-Nup49-mCherry sir3::Nat-GPDp-sir3-A2Q</i>	This study
KD1256		<i>Mat α ade2-1::ADE2 leu2-3,112::Hisp-GFP-LacIw-LEU2 ARS609::LacOp(x256)-TRP1 NUP49::URA3-Nup49-mCherry sir3Δ::TRP1</i>	This study
AT340		<i>Mat α ade2-1::ADE2 rap1::GFP-RAP1-LEU2 sik1::SIK1-mRFP-KanMx</i>	Ruault et al., 2011
KD941		<i>Mat α ade2-1::ADE2 rap1::GFP-RAP1-LEU2 sik1::SIK1-mRFP-KanMx sir3::Nat-GPDp-sir3-A2Q</i>	This study
KD942		<i>Mat α ade2-1::ADE2 rap1::GFP-RAP1-LEU2 sik1::SIK1-mRFP-KanMx sir3Δ::TRP1</i>	This study
		All strains are isogenic to W303: <i>ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	Thomas and Rothstein, 1989
	(1)	All those strains are MATa RAD5+ <i>ade2-1::ADE2 rap1::GFP-RAP1-LEU2 hmlΔ::HPH</i>	
	(2)	All those strains are MATa RAD5+ <i>ade2-1::ADE2 hmlΔ::HPH</i>	

Table S3. Primers table

Gene	Primer Name	Primer Sequence
insert_TEL6R_F	am572	GGAGCGATTCTGGACTGTACAAAAAAGAAATAGACTACTTACATCCTcgtagctgcagctgac
insert_TEL6R_R	am573	CATATGGTGGATCTTTTAAACGGAAAAGCGTTTTATTCTCTGACCTTCATccgattcattaatgcagg
TEL6R_F	pr64	GGAATAAATCCTGCCTCAATTGC
TEL6R_R	pr65	CTGAGGTCTCTGGGGATTCC

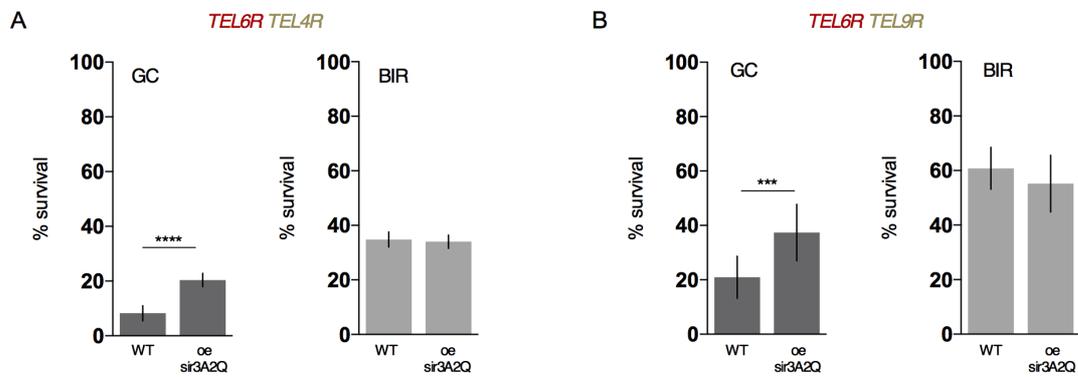
insert_TEL6R(10kb)_F	pr155	GTACGTAATACTTCGGCATTAAATTTGGCCTACCGCTTTGCCACAGTTGAGcgtacgctcaggtcgac
insert_TEL6R(10kb)_R	pr156	GCCTGGATATACAAGGGATCATTITTTTCATGGCTAATACTCCAGTGACCAccgattcattaatgcagg
TEL6R(10kb)_F	pr157	CACGGAATGGAAGCGCCTTG
TEL6R(10kb)_R	pr158	TGCCTAGTCGTCAACCTCCA
insert_LYS2_F	pr339	GGCAGGTATCACCTATGGTACTTGAACGAAAAATTTGCCTCAAATATCgtacgctcaggtcgac
insert_LYS2_R	pr340	CTTCTCATCTGAAAGACCAAATTTGGCTTTTAGATAAAATCGCCTAATACAACccgattcattaatgcagg
LYS2_F	pr62	CGTCAGGGCCAAGGATGAAG
LYS2_R	pr63	GTCAAGAGTAGAAGTGGAGG
insert_TEL4R_F	am671	AAACTGCGCCATAAATTCAGGAGGCACTACCAACCCCTGCCACAGAATAcgtacgctcaggtcgac
insert_TEL4R_R	am672	TCATCTATGTGCTTGGTTCTTGCAACTGTTAAACACTTATACCTAGTAAAccgattcattaatgcagg
TEL4R_F	pr66	CAGAAGCTTGTTGGGCAGG
TEL4R_R	pr67	GAAACATCTTCGATACCCGGG
insert_TEL9R_F	am784	CTCCATTCAACTGCTTGACTTTAAAATGATGCTGGCTCATGAGAAGCCTCcgtagctcaggtcgac
insert_TEL9R_R	am785	GCACAGAGCGGATCGAAAGTATTGGAATAGTAACACCGATATTATTTCTTGccgattcattaatgcagg
TEL9R_F	pr68	GGAGACAAATTCAGCGAGTG
TEL9R_R	pr69	CCTTAAAGGCACAAATCTCCG
insert_URA3i_F	pr77	GTTTTGACCATCAAAGAAGGTTAATGTGGCTGTGGTTTCAGGGTCCATAAccgattcattaatgcagg
insert_URA3i_R	pr99	ACTTGGTTCTGCGGAGGTATTGGATAGTTCCTTTTATAAAGCCATGAAGCcgtagctcaggtcgac
URA3i_F	pr6	AGGCATATTTATGGTGAAGG
KanMX_R	pr111	AGCCGTTTCTGTAATGAAGG
OGG1_F	pr776	CAATGGTGTAGGCCCAAAG
OGG1_R	pr777	ACGATGCCATCCATGTGAAGT
TEL6R_1kb_F	pr752	TGATGAATTACAAGGGAACAATGAG
TEL6R_1kb_R	pr753	CATCAAACAAGTAGGAATGCGAAA
LYS2_1kb_F	pr764	TGATTTACCATTGGGCACAATTT
LYS2_1kb_R	pr765	AATTTCCGCGCAAAGG
IScelcs_F	pr768	GGAGTTAGTTGAAGCATTAGGTCCC
IScelcs_R	pr769	GCGGCTTAACTGTGCCCTC
SIR3_F	pr233	CAGGGGTTAAGAAAGTTGTTTTGTTCTAACAATTGGATTAGCTAAAATGcgtacgctcaggtcgac
SIR3_R	pr234	TCAAATGCAGTCCATATTTTTGAATCTTCATCCATCGAAAAGCGTAATatcgtatgaattcaggtcgac
GPDSIR3_R	pr238	TCATCTGTAATGATAACTTGCCAACCGTCCAAATCTTCAATGTTTTAGCCATCGATGAATTTCTGTGCG
GPDsir3A2Q_R	pr239	TCATCTGTAATGATAACTTGCCAACCGTCCAAATCTTCAATGTTTTCTGCATCGATGAATTTCTGTGCG



**Supplemental figure 1: Localization of *TEL6R*.**

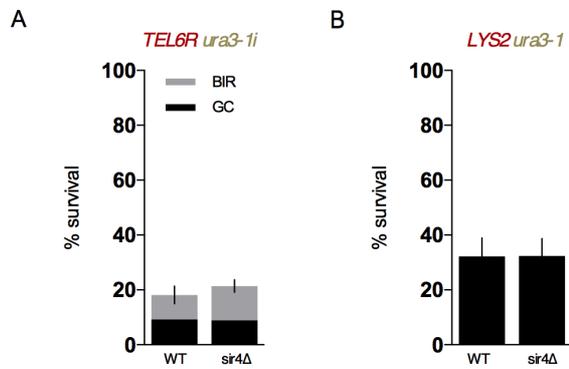
(A) Position of Lac<sup>OP</sup>-tagged *ARS609* on chromosome VI. Position of the GFP-tagged locus was scored relative to the NE (Nup49-mCherry). Ratios of distance from NE and diameter in focal plane are binned into equal surfaces.

(B) Position of *ARS609* in WT, *sir3Δ* cells or cells overexpressing *sir3A2Q* or *SIR3*. Black stars indicate statistical differences compared to WT measured by proportion test (\* = p<0,05; \*\* = p<0,01; \*\*\* = p<0,005; \*\*\*\* = p<0,001).



**Supplemental figure 2 : Perinuclear anchoring is not required for efficient homologous recombination in subtelomeric regions.**

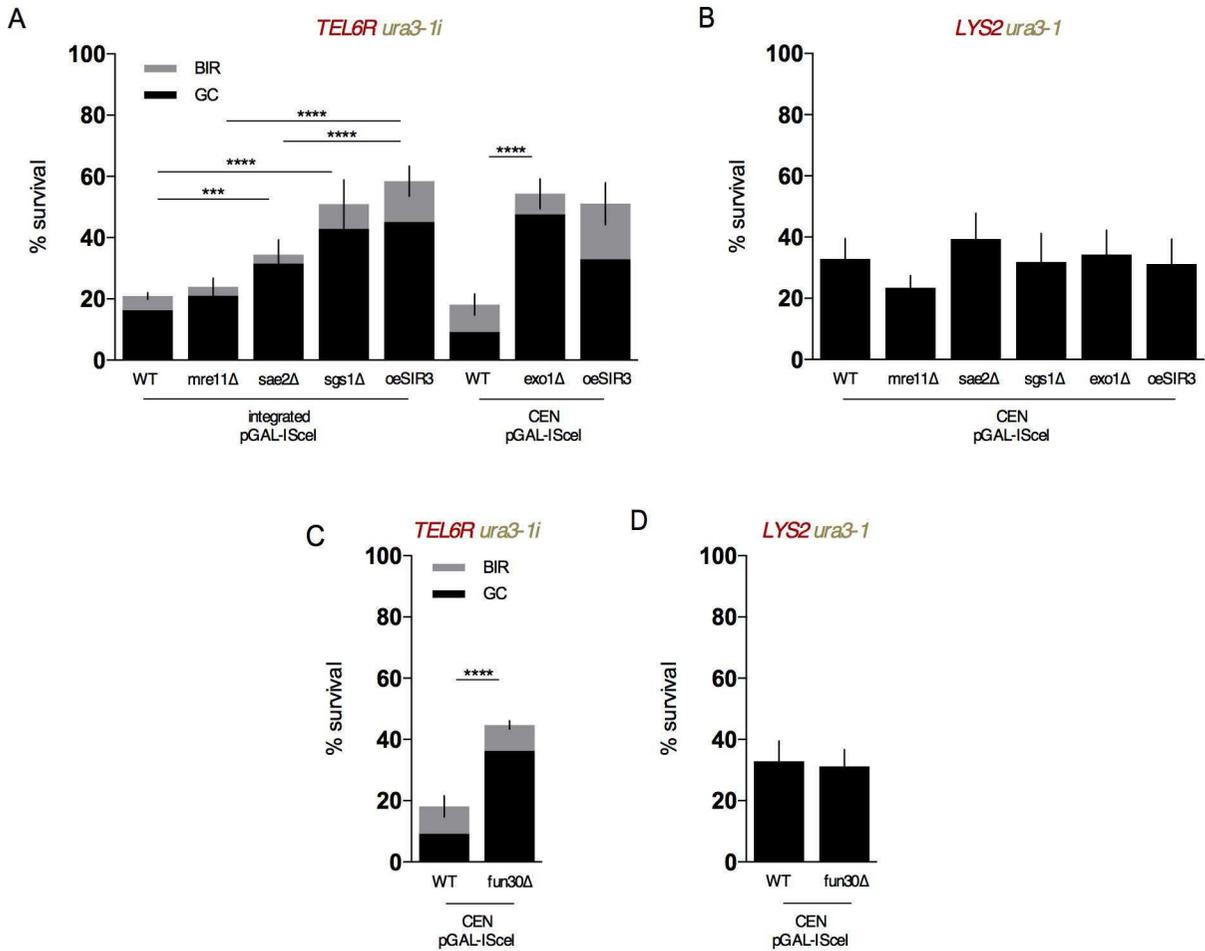
(A-B) Efficiency of repair events (GC and BIR) after induction of a DSB at *TEL6R* with *TEL4R* (A) or *TEL9R* (B) as a donor in WT and in cells overexpressing the sir3A2Q mutant protein. Error bars represent the standard deviation of the mean (s.d.m.) of at least three independent experiments. Black stars indicate statistical differences (\* =  $p < 0,05$ ; \*\* =  $p < 0,01$ ; \*\*\* =  $p < 0,005$ ; \*\*\*\* =  $p < 0,001$ ).



**Supplemental figure 3: Telomere anchoring is not limiting for recombination.**

(A) Survival frequencies observed after induction of a DSB at a subtelomeric position in WT and *sir4Δ* cells.

(B) Survival frequencies observed after induction of a DSB at an intrachromosomal position in WT and *sir4Δ* cells. Error bars represent the standard deviation of the mean (s.d.m.) of at least three independent experiments.



**Figure 28: Short-range and long-range resection differentially affect repair at a subtelomeric DSB.**

(A-D) Survival frequencies and distribution of the repair events of the indicated strains. Error bars represent the standard deviation of the survival mean (s.d.m.) of at least three independent experiments. Black stars indicate statistical differences in survival.

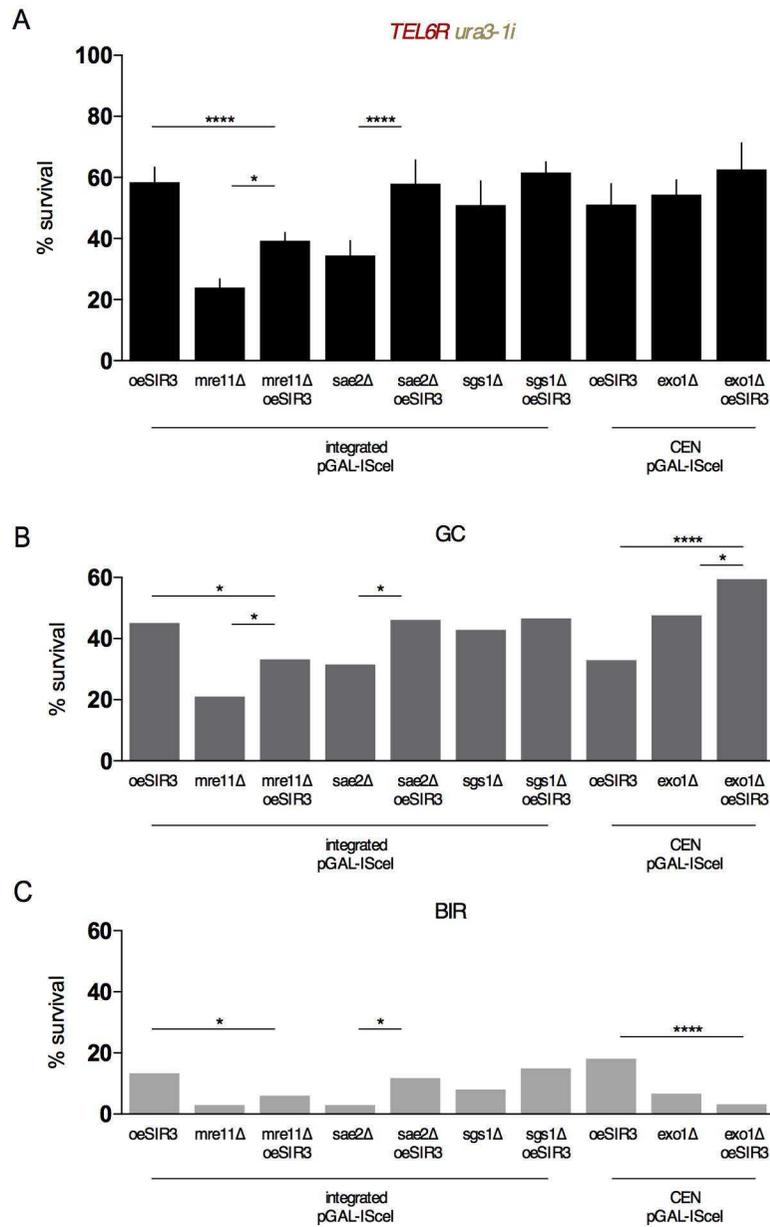
## 2. Complementary results

### 2.1. Presence of heterochromatin at DSB site prevents nucleases activity

To further investigate how resection is regulated in the context of heterochromatin, I started a candidate gene approach, which has been followed up by H el ene Bordelet, another PhD student in the laboratory. While I initiated the experiments with the pGAL-I-SceI on a CEN plasmid, she repeated and expanded some results with pGAL-I-SceI integrated in the genome of *S. cerevisiae* to facilitate the recombination assay. Experiments with the CEN plasmid were performed with synthetic medium whereas those with pGal-I-SceI integrated were run in rich medium. We noticed that depending on the medium, the balance between GC and BIR is altered, BIR being disfavored on rich medium (Figure 28A). We checked that this is not due to the integration of I-SceI or the CEN plasmid. Whether this is linked to a differential transcriptional activation of the *URA3* gene in which the DSB is induced needs to be further investigated.

Following the induction of a DSB, 5'-3' resection is initiated at the broken site by the combined action of Mre11 and Sae2 around the DSB vicinity. Further degradation of the 5' strand is then supported by Exo1 and Sgs1-Dna2 (Mimitou and Symington, 2008; Zhu et al., 2008). We first generated single mutants for each of these nucleases and measured their recombination efficiency at a subtelomeric or intrachromosomal DSB to test whether they can, as seen for *exo1*Δ mutants, mimic the effect of *SIR3* overexpression (Figures 28A and 28B). At *TEL6R*, *mre11*Δ cells recombined with the same efficiency as the WT strain whereas deletion of *SAE2* increased survival by 50%. Nonetheless, this increase in survival was not sufficient to

fully mimic *SIR3* overexpression (Figure 28A). As Sae2 does not present any nuclease activity by itself but rather stimulates the endonuclease activity of Mre11 (Cannavo and Cejka, 2014), we expected a similar phenotype with *sae2Δ* and *mre11Δ* mutants. However, in addition to its role in initiating resection, Mre11 is also important for DSB end tethering and recruitment of Tel1 and the long-range resection machinery (Chiolo et al., 2005; Deshpande et al., 2014; Nakada et al., 2003; Nicolette et al., 2010). These additional functions may explain the difference between *sae2Δ* and *mre11Δ* cells. We are currently constructing the strains with *mre11* nuclease-dead mutants (D56N or H125N) to test this hypothesis. Another possibility to explain this discrepancy is that Sae2, in addition to stimulating Mre11 endonuclease activity, also favors its eviction from DSB ends, facilitating the access of Sgs1 and Exo1 to DSB termini (Cannavo and Cejka, 2014; Chen et al., 2015). Deletion of *SGS1* or *EXO1* led to a 2.3- and 2.8-fold increase in survival compared to WT respectively, mimicking survival observed upon *SIR3* overexpression (Figure 28A). Accordingly, deletion of *FUN30*, a chromatin remodeler that facilitates both Exo1- and Sgs1-Dna2-mediated resection (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012), also increased recombination by almost 2.3-fold (Figure 28C). On the other hand, and in agreement with previous studies, limiting resection at *LYS2* did not significantly affect GC rates (Lydeard et al., 2010a; Mimitou and Symington, 2008) (Figures 28B and 28D). Altogether, these data suggest that the presence of heterochromatin at a DSB site preferentially impedes resection mediated by the long-range resection machinery. Nonetheless, *SIR3* overexpression is likely to have an additional effect at the DSB site because it does not perfectly mimic the recombination outcome of long-range resection mutants. Indeed we observed an increase in BIR events that is not obtained after *EXO1* deletion (Figure 28A).



**Figure 29: Heterochromatinization at the DSB site prevents nucleases activity.**

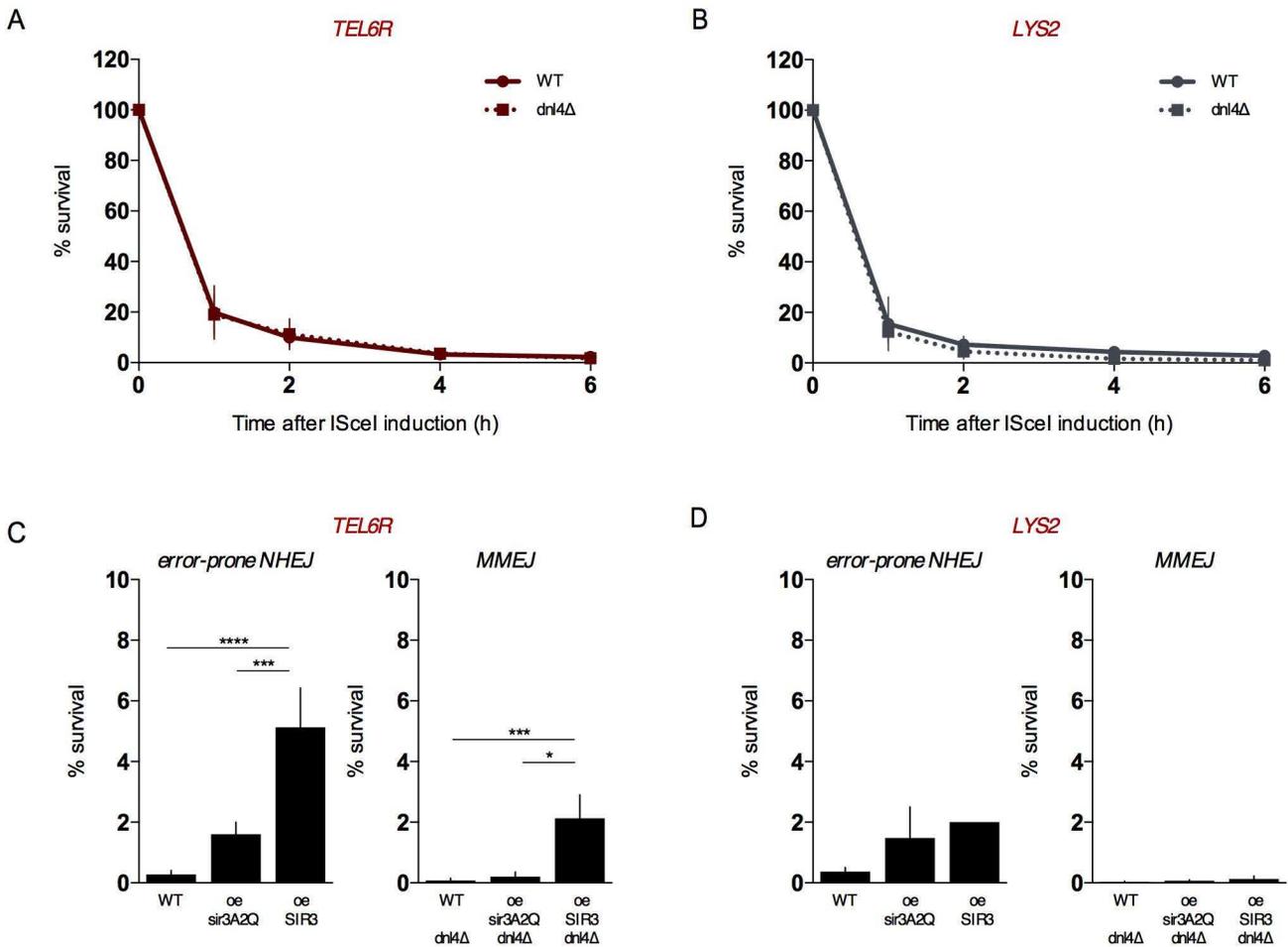
(A) Survival frequencies of the indicated strains in WT cells or cells overexpressing Sir3. Error bars represent the standard deviation of the survival mean (s.d.m.) of at least three independent experiments. Black stars indicate statistical differences in survival.

(B-C) Distribution of GC (B) and BIR events (C) in strains in Figure 29A.

To determine which nucleases are inhibited by the presence of heterochromatin, we deleted each of them in strains in which *SIR3* was overexpressed (Figure 29). Absence of Mre11 counteracted *SIR3* overexpression (Figure 29A), leading to a decrease in both GC and BIR events (Figures 29B and 29C). On the contrary, deletion of *SAE2* did not further affect recombination due to the heterochromatinization of the DSB site (Figure 29), suggesting that Sae2 is blocked by the overexpression of Sir3. Because *sae2* $\Delta$  cells and *mre11* nuclease-dead mutants show similar phenotypes in numerous survival assays (Lobachev et al., 2002; Rattray et al., 2001), we hypothesize that the requirement for Mre11 to repair a heterochromatic DSB is independent of its nuclease activity. Testing directly the nuclease-dead mutants will give us further insight on this last point. Deletion of *EXO1* or *SGS1* did not impact on the survival of *SIR3* overexpressing cells (Figure 29A), suggesting that presence of heterochromatin at DSB site prevents both long-range resection pathways. However, deletion of *EXO1* in the presence of heterochromatin enhanced GC rates compared to both *exo1* $\Delta$  and *SIR3* overexpression, while the distribution of repair events was not affected in *sgs1* $\Delta$  cells overexpressing *SIR3* (Figure 29B and 29C). These results will have to be confirmed using integrated pGal-I-SceI for *exo1* $\Delta$  cells. Altogether, these results show that heterochromatin likely impairs resection by blocking Sae2 and Sgs1 and partially Exo1. Further experiments will be required to determine whether heterochromatin blocks the recruitment or the nuclease activity of the different enzymes.

## **2.2. Heterochromatin favors error-prone NHEJ and MMEJ**

Resection is a key step committing DSB repair toward HR instead of NHEJ. Because heterochromatinization of the DSB site impairs resection, we hypothesized



**Figure 30: Heterochromatin favors error-prone NHEJ and MMEJ.**

(A-B) Survival test after galactose induction of ISceI endonuclease at a subtelomeric (A) or intrachromosomal (B) site of WT or dn14Δ strains. Time points were taken at 0h, 2h, 4h and 6h. Error bars represent the standard deviation between at least three independent experiments.

(C-D) Survival frequencies of the indicated strains after DSB induction at a subtelomeric locus (C) or an intrachromosomal locus (D). Error bars represent the standard deviation of the survival mean (s.d.m.) of at least three independent experiments. Black stars indicate statistical differences in survival.

that it could promote NHEJ. To test this hypothesis, we measured NHEJ by plating donorless strains on glucose after I-SceI induction in galactose to repress I-SceI induction and allow repair by NHEJ (Figures 30A and 30B). However, we observed no differences in survival at both *TEL6R* and *LYS2* between WT and *dnl4Δ* strains, indicating that NHEJ does not occur. We supposed that this is caused by the stability of the I-SceI protein that may still be present in cells to induce DSB on glucose plates despite transcriptional repression. This contrasts with the HO endonuclease that has a half-life of 10 minutes, allowing to assess NHEJ by plating cells on glucose following pulses of galactose induction (Haber, 2012; Kaplun et al., 2003; 2006). We are currently constructing strains bearing a HO cutting site inserted at *TEL6R* and *LYS2* to assess the effect of heterochromatin on NHEJ. Alternatively, we also intend to develop an inducible degron tagged version of I-SceI.

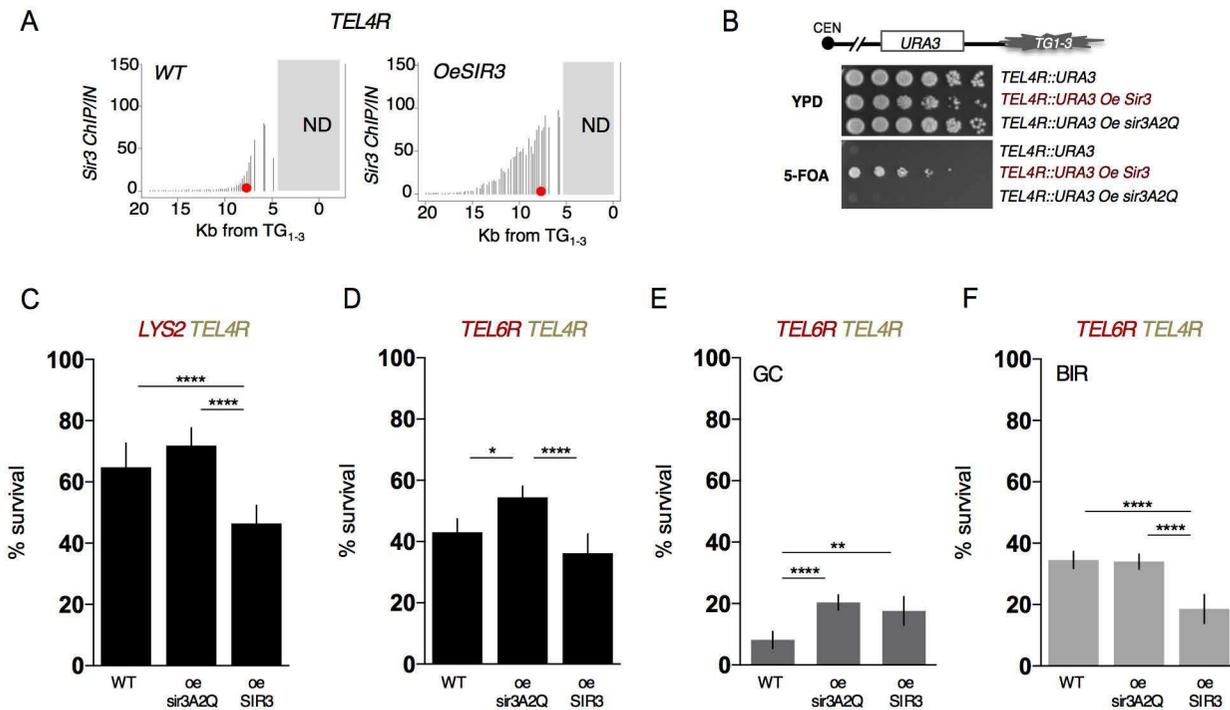
Nonetheless, we were able to monitor error-prone NHEJ and MMEJ. Error-prone NHEJ occurs when ligation of the two DSB ends results in the insertion of a mismatched nucleotide. MMEJ is a Ku- and Dnl4-independent end-joining pathway arising between microhomologies and ends up by the deletion of the sequence between the two microhomologies. Both inaccurate NHEJ and MMEJ lead to the mutation or the deletion of the I-SceI recognition sequence and thus prevent the constitutive cutting on galactose plates (Figures 30C and 30D). We first measured error-prone NHEJ after plating WT donorless cells on galactose medium. We observed a slight, although not statistically significant, increase in error-prone NHEJ upon *sir3A2Q* overexpression at *TEL6R* (Figure 30C, left panel). Similarly, we noticed the same nonsignificant increase in error-prone NHEJ at the intrachromosomal *LYS2* locus when *SIR3* or *sir3A2Q* were overexpressed (Figure 30D, left panel). These results suggest that the higher concentration of the protein

may slightly favor inaccurate NHEJ. Alternatively, it may come from a difference in cell cycle that can impact on NHEJ. In particular, cells in which *SIR3* is overexpressed present a growth defect, although it does not seem to be the case for *sir3A2Q* overexpression. However, heterochromatin spreading upon *SIR3* overexpression at a subtelomeric DSB significantly enhanced survival by 3.2-fold compared to *sir3A2Q* overexpression (Figure 30C, left panel). We then measured MMEJ by deleting *DNL4* in our strains and plating them on galactose plates. Similarly to unfaithful NHEJ, MMEJ was also affected by the presence of heterochromatin at the DSB site (Figure 30C, right panel), while *SIR3* overexpression did not change Dnl4-independent NHEJ events at *LYS2* (Figure 30D, right panel). Together, these results indicate that heterochromatinization of the DSB site promotes error-prone NHEJ and MMEJ and is reminiscent of what was observed at the nuclear lamina in mammals (Lemaître et al., 2014). As we observed that *SIR3* overexpression at a subtelomeric DSB impairs resection, and the long-range machinery in particular, it suggests that slower resection favor inaccurate NHEJ and MMEJ.

## **2.3. Heterochromatinization of the donor locus impairs HR**

### **2.3.1. A heterochromatic recombination donor impairs both GC and BIR**

Previous studies demonstrated that the loss of the heterochromatic state of *HM* does not affect recombination at *MAT* (Coic et al., 2011; Tsaponina and Haber, 2014). However, *MAT* switching is a particular recombination process that has been selected by evolution and does not necessarily reflect all the recombination events taking place in the cell. Taking advantage of our system, we overexpressed Sir3 in strains where the recombination donor was located at the subtelomeric region of the right arm of



**Figure 31: Heterochromatin spreading at donor site impairs GC and BIR.**

(A) Sir3-binding measured by ChIP-chip of anti-GFP Sir3 IPs at *TEL4R* in WT cells or in cells overexpressing *SIR3* (*oeSIR3*). The red dot marks the insertion site of the recombination cassette on the chromosome.

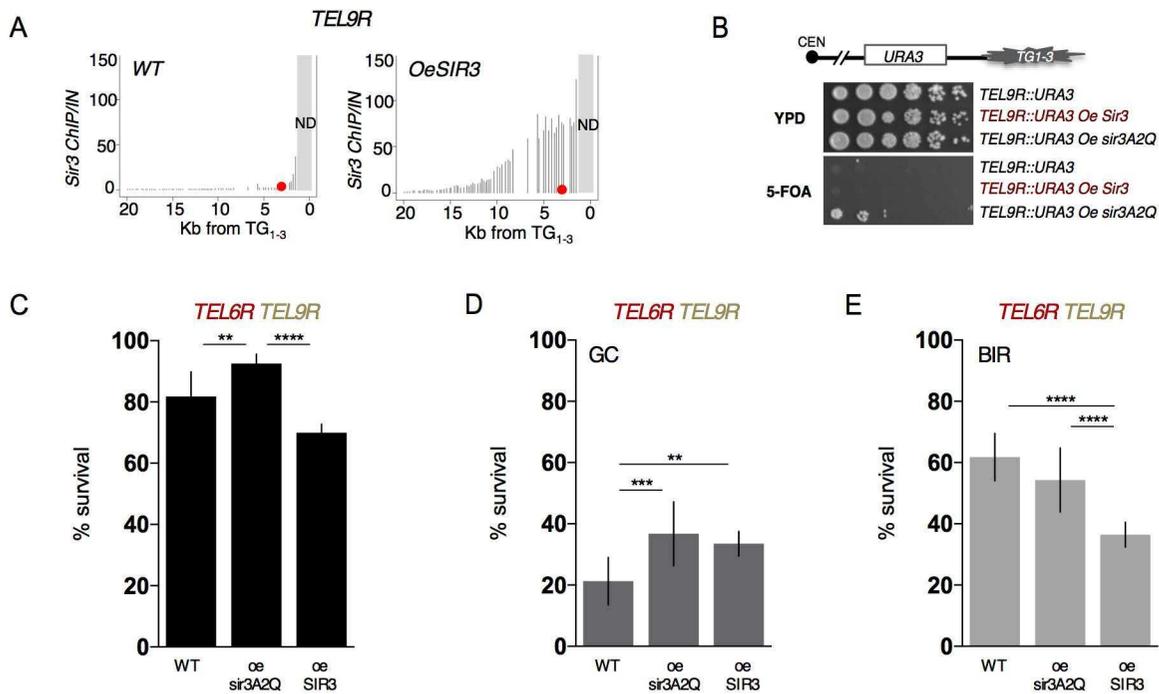
(B) Telomeric silencing assay at *TEL4R* in WT cells, cells overexpressing *SIR3* (*oeSIR3*) or *sir3A2Q* (*oesir3A2Q*). Increased growth on 5-FOA plates reflects an increase in telomeric silencing.

(C-D) Survival frequencies observed after induction of a DSB in the indicated strains. Error bars represent the standard deviation of the mean survival (s.d.m.) of at least three independent experiments. Black stars indicate statistical differences in survival.

(E-F) Distribution of GC (E) and BIR events (F) in strains in Figure 31D.

chromosome 4 (*TEL4R*), which leads to a strong heterochromatin spreading able to induce silencing of a *URA3* gene inserted at this locus (Figures 31A and 31B). To assess the effect of the heterochromatinization of the donor template on HR, we first induced a DSB at the intrachromosomal locus *LYS2* and monitored the recombination efficiency with *TEL4R* in strains overexpressing or not the Sir3 protein (Figure 31C). To assess effects of the change in telomere organization that occurs concomitantly with Sir3 spreading in subtelomeric regions, we used as a control strains overexpressing *sir3A2Q*. We observed that heterochromatin spreading on the recombination donor site upon *SIR3* overexpression decreased survival compared to WT cells or cells overexpressing *sir3A2Q*. As survival rate directly mirrors GC rate in these strains, this indicates that the presence of heterochromatin at the donor locus impairs GC.

To gain insight into the impact of a heterochromatic donor on BIR, we looked at the recombination between two subtelomeric regions (Figure 31D). We noticed that overexpression of *SIR3* resulted in a decrease of survival compared to WT cells or cells overexpressing *sir3A2Q*. This decrease is mainly due to a decrease in BIR events compared to *sir3A2Q* overexpression while GC rate was not affected (Figures 31D, 31E and 31F). However, in this strain, both the DSB site and the recombination donor are heterochromatic when *SIR3* is overexpressed. We previously showed that heterochromatin at the DSB site strongly promotes GC and moderately favors BIR. We thus expected an increase in GC events, however it did not change. In addition, we noticed a decrease in GC rate in the strain in which only the recombination donor is heterochromatic (Figure 31C). Taken together, these results suggest that the absence of difference in GC rate actually comes from a balance between



**Figure 32: Heterochromatinization of the donor site at *TEL9R* also prevents HR.**

(A) Sir3-binding measured by ChIP-chip of anti-GFP Sir3 IPs at *TEL9R* in WT cells or in cells overexpressing *SIR3* (*oeSIR3*). The red dot marks the insertion site of the recombination cassette on the chromosome.

(B) Telomeric silencing assay at *TEL9R* in WT cells, cells overexpressing *SIR3* (*oeSIR3*) or *sir3A2Q* (*oesir3A2Q*). Increased growth on 5-FOA plates reflects an increase in telomeric silencing.

(C) Survival frequencies observed after induction of a DSB in the indicated strains.

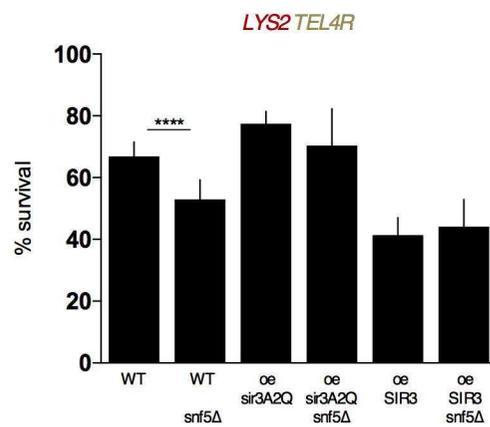
(D-E) Distribution of GC (D) and BIR events (E) in strains in Figure 32C. Error bars represent the standard deviation of the mean survival (s.d.m.) of at least three independent experiments. Black stars indicate statistical differences in survival.

heterochromatinization of the recipient and the donor loci whereas heterochromatin at the donor strongly prevents BIR.

These results are not locus-specific as heterochromatinization of a recombination donor located at *TEL9R* lead to the same outcome (Figure 32). Presence of heterochromatin at the donor locus therefore impedes both GC and BIR, presumably by affecting a common step of the two sub-pathways, namely strand invasion or initiation of new DNA synthesis.

### 2.3.2. Snf5 is not sufficient to overcome the heterochromatic barrier

*In vitro* experiments showed that the presence of Sir3 on nucleosomes blocks joint formation (Sinha et al., 2009). The chromatin remodeler SWI/SNF interacts with Sir3 and this interaction is required for the eviction of Sir3 from chromatin, which facilitates joint formation (Manning and Peterson, 2014; Sinha et al., 2009). Furthermore, deletion of the *SNF5* subunit prevented synapsis between *MAT* and the heterochromatic *HML* donor locus *in vivo* (Chai et al., 2005). Thus, we suspected that SWI/SNF was required to support strand invasion when the recombination donor is heterochromatic. Absence of *Snf5* decreased recombination in WT strain whereas it did not change survival when *sir3A2Q* is overexpressed (Figure 33). There is no heterochromatin spreading in strains overexpressing *sir3A2Q* (Ruault et al., 2011) while Sir3 is present in WT strain at the donor locus even though not enough to induce silencing (Figure 31A and 31B). In agreement with *in vitro* and *in vivo* studies, these results suggest that *Snf5* is required to evict Sir3 and favor GC completion. Surprisingly, deletion of *SNF5* did not further affect GC rate in the strain overexpressing *SIR3* (Figure 33). This result indicates that the strong heterochromatin formation on the recombination donor upon *SIR3* overexpression may block the



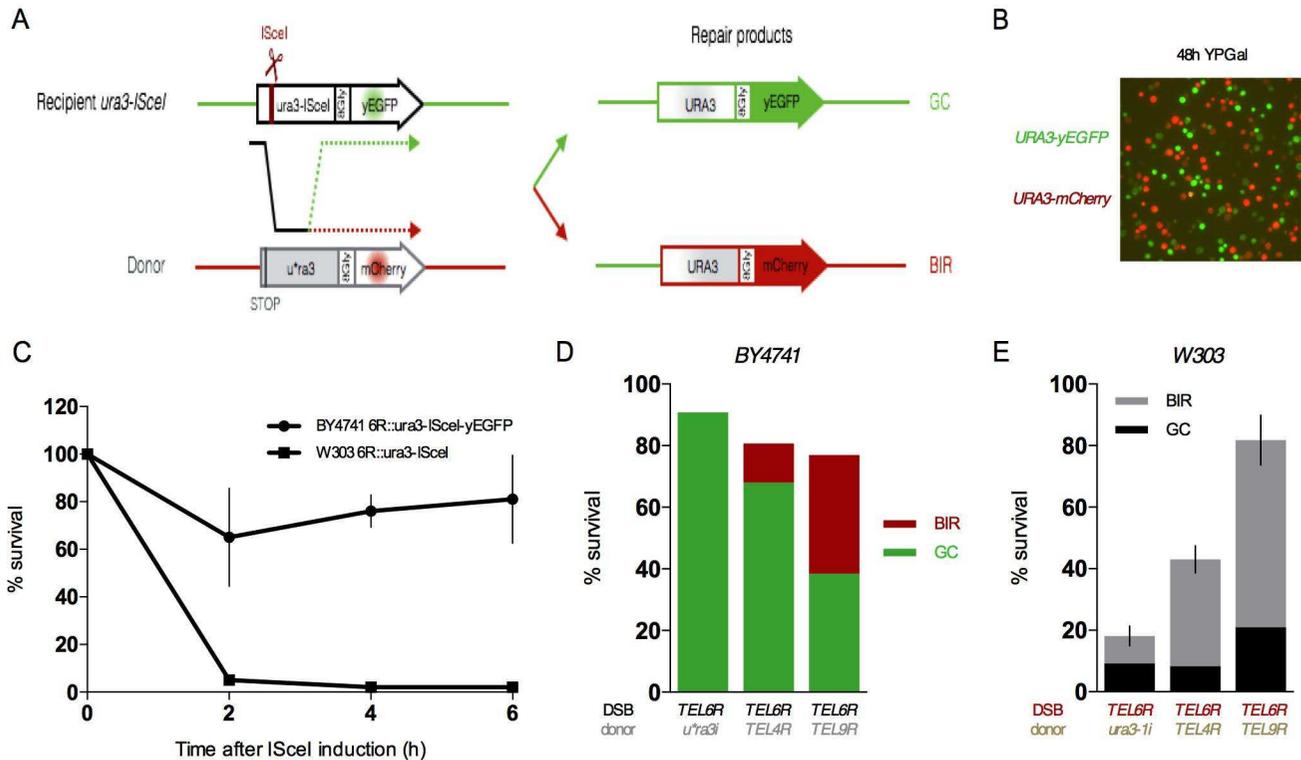
**Figure 33: *Snf5* is not sufficient to overcome the heterochromatic barrier.**

Survival frequencies observed after induction of a DSB in the indicated strains deleted or not for *SNF5*. Error bars represent the standard deviation of the mean survival (s.d.m.) of at least three independent experiments. Black stars indicate statistical differences in survival.

action of Snf5 and may account for the lower survival rate in this strain. Nevertheless, these results support the hypothesis that heterochromatinization of the donor locus may inhibit strand invasion, although we cannot exclude a more general impact of Snf5 in recombination for the moment. To test this last possibility, we have to delete *SNF5* in strains with an intrachromosomal donor that will not be affected by variation of Sir3 levels.

## **2.4. Identification of new actors implicated in GC/BIR regulation**

All the factors involved in recombination identified so far have been mainly characterized based on their requirement for repair in euchromatin and when only one HR sub-pathway can be used at a time. Thanks to our new recombination assay, we can determine which mechanisms take place when GC and BIR are in competition and assess the effect of the presence of heterochromatin on DSB repair. We started to modify this system to perform a genome wide genetic screen in order to uncover new regulatory pathways of GC and BIR repair in different chromatin contexts. To screen for the different repair events, we tagged the recipient *ura3-I-SceI* cassette with *yEGFP* while we inserted a STOP codon in the *URA3* gene tagged with *mCherry* to constitute the donor cassette (Figure 34A). Strains containing the two cassettes were not able to grow in absence of uracil. However, after DSB induction and recombination cells a functional *URA3* gene is restored and cells grew in absence of uracil. If repair occurred through GC, cells will express *yEGFP* whereas they will produce *mCherry* if repaired by BIR. The recombination pathway preferentially used by the cells can thus be monitored either by FACS or by microscopy through the expression of the *yEGFP* and *mCherry* fluorescent proteins (Figure 34B).



**Figure 34: Assay to identify new actors of GC/BIR regulation.**

(A) Schematic representation of the two *ura3* alleles used to test recombination efficiencies and outcome. The recipient cassette and the donor cassette were tagged with yEGFP and mCherry respectively with a 8Gly linker. Repair by GC results in yEGFP expression while repair by BIR produces mCherry.

(B) Representative image of recombinant cells after 48h on YPGal plates.

(C) Survival test after galactose induction of IScel endonuclease in BY4741 and W303 backgrounds. Time points were taken at 0h, 2h, 4h and 6h. Error bars represent the standard deviation between two independent experiments.

(D-E) Survival frequencies and distribution of the repair events observed after induction of a DSB in the indicated strains in BY4741 (D) or W303 (E) genetic backgrounds. Error bars represent the standard deviation of the mean survival (s.d.m.) of at least three independent experiments.

To identify the actors involved in GC/BIR regulation, we will cross this test strain with the deletion library through the Synthetic Genetic Array (SGA) protocol (Tong et al., 2001). To assess whether the GC/BIR regulation differs when the DSB site and/or the donor locus are bound by high level of Sir3, we will couple the SGA protocol with the Selective Ploidy Ablation (SPA) protocol (Reid et al., 2011), which will allow to overexpress *SIR3* on a plasmid in all the deletion strains containing our recombination system.

However, the deletion library is available in the BY4741 genetic background while our recombination assay was set up in the W303 background. We thus introduced the fluorescent recombination system described above in BY4741 and tested the recombination efficiency and outcome. In donorless strains, we observed a higher survival in BY4741 than in W303, which directly correlates with the DSB cleavage efficiency (Figure 34C). In addition, recombination in BY4741 with the fluorescent cassettes was higher than in W303 with the non-fluorescent cassettes (Figures 34D and 34E). These results suggest that the I-SceI endonuclease is not well expressed in BY4741 and we are currently trying to improve the induction to perform the screen in the best conditions. Nevertheless, this system should allow us to detect differences between GC and BIR levels.

**DISCUSSION  
&  
PERSPECTIVES**

## **DISCUSSION & PERSPECTIVES**

During my doctoral work, I was interested in better understanding how non-random nuclear organization affects DSB repair and what molecular steps are modulated by the presence of heterochromatin either at the DSB site or at the donor template. We directly demonstrated the importance of spatial proximity on HR by reducing the physical distance between two homologous sequences. We also shed light on how heterochromatin regulates homologous recombination. We showed that it acts as a barrier for the resection of heterochromatic DSBs and for synapsis and/or post-synapsis steps when the recombination donor is heterochromatic.

Some points are already discussed above in the scientific article and will not be discussed further in this part.

### **1. Impact of DSB position on HR sub-pathways**

#### **1.1. Spatial proximity is more restricting for GC than BIR**

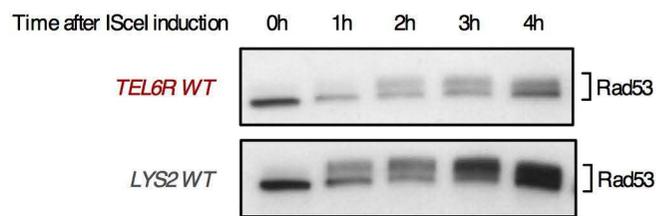
One likely hypothesis to explain the limiting role of spatial proximity is that it favors the encounter of homologous sequences rendering strand invasion more likely to occur. However, we showed that this increase in recombination due to homologous sequences colocalization causes an increase in GC events while BIR was not affected (Figure S2). As homology search and strand invasion are steps common to both GC and BIR pathways, the fact that BIR efficiency is not increased upon sequences grouping suggests that an additional factor influence the balance GC/BIR. The factor

could be the initiation of DNA synthesis that is delayed several hours for BIR compared with GC (Jain et al., 2009; Malkova et al., 2005).

Nevertheless, physical distance is likely important for both GC and BIR as two naturally close subtelomeres show a stronger use of both pathways than two distant ones. Together our data suggest that DSB repair must take place in a limited time window that is restricted both by the preexisting distance between homologous sequences and the resection rate of DSB ends (Lee et al., 2016). BIR is the pathway of choice for one-ended DSBs whereas two-ended DSBs are primarily repaired by GC. Accordingly, we demonstrated that the loss of the telomeric proximal fragment was limiting for GC while it favored BIR. Together, it indicates that spatial proximity may be more important for GC than BIR because it likely favors encounter between homologous sequences and completion of DSB repair before extensive resection leads to the loss of one of the two DSB ends.

## **1.2. Differences in resection between subtelomeres and internal loci**

The asymmetry of recombination efficiencies between subtelomeric and intrachromosomal loci depending on which experiences the DSB is actually due to a difference in resection with the faster disappearance of the telomere proximal fragment at the subtelomere. The simplest explanation may be that this loss only depends on the distance of the DSB from the chromosome end. Nonetheless, we cannot rule out that an active mechanism may be at play in subtelomeric regions to accelerate resection.



**Figure 35: Checkpoints are less activated in case of a subtelomeric DSB.**

Western blots of protein extracts after IScelI induction with anti-Rad53 antibodies. Upper band represents Rad53 phosphorylation.

H3K79me3 is apparently absent at subtelomeres in normal conditions (A. Hocher and A. Taddei, personal communication). Rad9 is recruited to DSB site through  $\gamma$ -H2A binding but its natural binding to chromatin through H3K79me3 is thought to promote the efficiency of the Rad9-dependent response to DNA damage (Grenon et al., 2007; Huyen et al., 2004). Absence of H3K79 methylation at subtelomeres may thus imply a lower recruitment of Rad9, which in turn may result in the lower activation of Rad53. Indeed, we observed that Rad53 phosphorylation was less important following a subtelomeric DSB compared to an intrachromosomal DSB (Figure 35). This difference may affect resection through two distinct ways. On the one hand, Rad9 inhibits extensive resection, in particular by preventing the binding of Sgs1 (Bonetti et al., 2015; Lazzaro et al., 2008). On the other hand, activated Rad53 phosphorylates Exo1 to negatively regulate its activity (Morin et al., 2008). Altogether both Rad9 lower recruitment and Rad53 lower phosphorylation at subtelomeres may cause faster resection.

To test this hypothesis, it would be interesting to see whether the deletion of *RAD9* or the H3K79 methyltransferase *DOT1* differentially affects recombination at subtelomeres and at an internal region. We could also directly measure the recruitment of Rad9 by ChIP around the DSB site at subtelomeric and intrachromosomal loci.

## **2. Heterochromatin is a barrier to HR**

Although HR is thought to be the main repair pathway for a DSB in heterochromatin (Beucher et al., 2009; Geuting et al., 2013; Kakarougkas et al., 2013;

Ryu et al., 2015), the need to relocate the DSB to more permissive environment to load the recombination machinery defined heterochromatin as a barrier to HR (Chiolo et al., 2011; Jakob et al., 2011; Ryu et al., 2015; Torres-Rosell et al., 2007). Accordingly, we demonstrated that the presence of heterochromatin impairs several molecular steps during HR.

## **2.1. Resection is prevented in heterochromatin**

The difference in resection between a subtelomeric and an intrachromosomal DSB led us to investigate whether the chromatin state would play a role in the regulation of resection. We showed that heterochromatin spreading decreases resection by blocking Sae2, Exo1 and Sgs1. However, Mre1 seems to be necessary for DSB repair in heterochromatin. Mre11, as part of the MRX complex, is one of the main actors of the DDR and plays multiple roles in the detection, signaling and repair of a DSB. We are constructing the *mre11-D56N* and *mre11-H125N* mutants defective in resection in our system to precisely assess the impact of heterochromatin on the nuclease activities of Mre11. As *sae2Δ* cells present the same phenotypes as *mre11* nuclease-dead mutants in many genetic assays (Lobachev et al., 2002; Rattray et al., 2001), we can expect the nuclease activities of Mre11 to be similarly affected by the presence of heterochromatin than the deletion of *SAE2*. It would suggest that another function of Mre11 is required to repair a heterochromatic DSB.

The processive resection machinery appears to be more severely impacted by the presence of heterochromatin than the short-range resection enzymes. How Sgs1-Dna2 and Exo1 are affected by heterochromatin has yet to be uncovered. It would be interesting to decipher whether their recruitment or their activity is impaired by

heterochromatin. To test their recruitment, we could first measure their binding to DNA around the DSB site by ChIP analysis in euchromatin and in heterochromatin. *In vitro* assays described different requirements to allow resection by Sgs1-Dna2 and Exo1 in presence of nucleosomes (Adkins et al., 2013). Sgs1-Dna2 requires a nucleosome free-region while Exo1 is blocked by a single nucleosome. Testing the different mediators would give us more information on the mechanisms set up in heterochromatin and how resection is affected by the presence of heterochromatin.

Exo1-mediated resection is blocked *in vitro* by the presence of a single nucleosome containing an H2A-H2B dimer. Efficient processing requires the incorporation of the histone variant H2A.Z (Htz1 in yeast) by the chromatin remodeler Swr1 (Adkins et al., 2013). H2A.Z is enriched in euchromatic regions but absent at the mating type loci and telomeres and protects euchromatin from the spreading of Sir-dependent silencing (Meneghini et al., 2003). In mammalian cells, H2A.Z is excluded from constitutive heterochromatin but its monoubiquitinated form is associated with facultative heterochromatin (Sarcinella et al., 2007). H2A.Z is incorporated early after DSB induction by Swr1 in euchromatin (Kalocsay et al., 2009; Papamichos-Chronakis et al., 2006). Whether this histone variant can be incorporated in heterochromatin after a DSB is not known and may explain the inhibition of Exo1.

As for Sgs1-Dna2 pathway, nucleosomes located adjacent to a DSB inhibit Sgs1 helicase activity. Efficient resection necessitates a nucleosome-free region of more than 50 bp next to the DSB site to allow unwinding of DNA by Sgs1, which can then traverse a nucleosome, and allow subsequent degradation by Dna2 (Adkins et al., 2013). This finding suggests that Sgs1-Dna2-mediated resection requires chromatin remodeling events to generate a short nucleosome-free region. However, once

initiated, extensive processing by the Sgs1-Dna2 machinery may not require additional remodeling. RSC and INO80 remodeling enzymes are able to slide and evict a single nucleosome and appear as potential candidates to generate nucleosome-free region around the DSB (Chen et al., 2012; Kent et al., 2007; Shim et al., 2007; Udugama et al., 2011; van Attikum et al., 2004; 2007). Their recruitment or their remodeling activities may thus be affected by the presence of heterochromatin and may account for the impairment of Sgs1-Dna2-mediated resection.

In the DSB vicinity, RSC and INO80 play redundant roles with Fun30, the chromatin remodeler with the strongest impact on resection that participates in both Exo1- and Sg1-dependent resection (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012). We showed that its deletion gave the same recombination efficiency as *exo1Δ* and *sgs1Δ* single mutants and *SIR3* overexpression. Interestingly, Fun30 is naturally present in heterochromatic regions where it promotes gene silencing (Byeon et al., 2013; Neves-Costa et al., 2009). Fun30 thus appears as a potential regulator of the two long-range resection pathways in heterochromatin even though their mechanism of action differs.

Analyzing the effect of the deletion of *RSC*, *INO80*, *SWR1* or *FUN30* on recombination efficiency at a heterochromatic DSB would allow to determine which one(s) are required for resection. To determine whether they directly regulate resection in heterochromatin, we could target these different chromatin remodelers to the DSB site through LexA-fusion proteins that would bind LEXA sites inserted next to the break and measure ssDNA formation.

## 2.2. Heterochromatin impairs late repair steps

### 2.2.1. Blocking of strand invasion or new DNA synthesis

Resection is not the only molecular step of HR to be impaired by the presence of heterochromatin. Indeed the presence of heterochromatin at the recombination donor also affects recombination. It was previously shown that the loss of the heterochromatic state of *HM* does not affect *MAT* switching (Coic et al., 2011; Tsaponina and Haber, 2014). In contrast, we observed that recombination efficiency is reduced by the heterochromatinization of the donor locus. *MAT* switching is a very efficient process with almost 100% success that necessitate only few remodeling at the heterochromatic *HM* loci (Hicks et al., 2011). We can consider that yeast cells have evolved a natural and tightly regulated recombination event at *MAT* that may not fully reflect recombination at other loci and may account for the discrepancy between the different studies.

The fact that both GC and BIR are impaired using a heterochromatic donor suggests that a step common to the two sub-pathways is impacted. The presence of heterochromatin may prevent homologous pairing or the strand invasion step. Opening the DNA could also be more difficult in heterochromatin as well as the priming of DNA synthesis. To determine whether strand invasion is impaired, we could directly measure Rad51 interaction with the donor sequence by ChIP experiments in euchromatin and heterochromatin. To assess the effect on initiation of repair DNA synthesis, we could use a primer extension assay corresponding to a PCR method that amplifies a unique product formed only after a small extent of new DNA synthesis has occurred.

### 2.2.2. Chromatin remodeling required at a heterochromatic donor

To further understand how heterochromatin modulates recombination using a heterochromatic donor template, we started to look whether some chromatin remodelers were particularly required during this process. Although SWI/SNF was identified to facilitate strand invasion in heterochromatin both *in vitro* and *in vivo* (Chai et al., 2005; Sinha et al., 2009), we found no effect of the deletion of *SNF5* on GC rate at a silenced donor. However, *Snf5* was important for recombination in a WT strain in which *Sir3* is slightly present on a subtelomeric region, but not enough to induce silencing. The result suggests that the presence of heterochromatin at the recombination donor site prevents *Snf5* action. A more recent study failed to reproduce the key role for SWI/SNF in heterochromatic recombinational repair by deleting *SWI2*, which specifically interacts with *Sir3* (Manning and Peterson, 2014). To assess more clearly the role of SWI/SNF when the donor is heterochromatic and to eliminate a possible unique phenotype of *snf5Δ* on recombination, we could construct the strains with *swi2Δ* and the *swi2-Δ10R* mutant, which both specifically abolish the interaction between SWI/SNF and *Sir3* (Manning and Peterson, 2014).

For now we cannot rule out that SWI/SNF can indeed evict *Sir3* from the heterochromatin to allow strand invasion. Nonetheless, other proteins involved in stabilizing the joint molecule and extending it also seem to be inhibited by heterochromatin. In particular, *Ino80*, which is responsible for the displacement of nucleosomes from chromatin surrounding the initial joint necessary for efficient strand invasion (Tsukuda et al., 2009), could be required to counteract heterochromatin at the donor site.

Another candidate is *Rad54*, which is not strictly required for strand invasion but helps to convert the transient interaction between the invading strand and its

homologous donor into a stable joint molecule (Sinha and Peterson, 2008). In addition, SWI/SNF primary action enhances Rad54-dependent formation of a strand invasion product (Sinha et al., 2009). Rad54 is also required before DNA polymerisation machinery recruitment to remodel nucleosomes within the donor locus and allow subsequent new DNA synthesis (Chai et al., 2005; Hicks et al., 2011; Sugawara et al., 2003; Wolner and Peterson, 2005).

Testing the deletion of *SWI2*, *RAD54* and *INO80* on recombination would give new insight on their ability to act on heterochromatin substrate. However, to easily test their impact on both GC and BIR at the same time, it would be interesting to construct a strain in which only the donor locus is heterochromatinized but with BIR events leading to viable products. To do so, we could move the DSB site to the *CANI* locus on chromosome V located 33kb away from the telomere. This region does not contain any essential gene on the telomere proximal side and is not covered by Sir3 when it is overexpressed (A. Hocher and A. Taddei, personal communication). We could then assay Rad51 enrichment and primer extension at the donor locus of these different mutants to directly assess their role in strand invasion and new DNA synthesis and decipher which one(s) are modulated by the presence of heterochromatin.

### **2.3. Could heterochromatin be repressive for histone mark establishment required for subsequent DNA repair?**

Interestingly, SWR1 and INO80 chromatin remodeling complexes are recruited to DSB site by binding  $\gamma$ -H2A and this recruitment is further enhanced by H3 acetylation dependent of the Gcn5 catalytic subunit of the SAGA complex and H4

acetylation mediated by the Esa1 subunit of the NuA4 complex (Bennett and Peterson, 2015; Downs et al., 2004). In addition, the incorporation of H2A.Z by SWR1 is directly stimulated by acetylation of H2A or H4 N-terminal tails by NuA4 *in vitro* (Altaf et al., 2010). The SWI/SNF complex is also recruited to a DSB site by NuA4 and Gcn5, which promotes phosphorylation of H2A, suggesting an early role of SWI/SNF in DSB repair (Bennett and Peterson, 2015). Although the RSC complex has not yet been identified to be recruited to DSB site through NuA4 and Gcn5 but rather by Mre11 and Ku (Shim et al., 2007), it was shown to be recruited by these two HAT to coding sequences where it stimulates transcriptional elongation by histone eviction together with SWI/SNF (Ginsburg et al., 2009).

NuA4 and Gcn5 histone acetyltransferases thus appear to be important regulators of the recruitment of different chromatin remodeling complexes. NuA4 is recruited to a DSB site through binding to  $\gamma$ -H2A but also recognizes methylated H3K4 and H3K36 which enhances NuA4 activity (Downs et al., 2004; Ginsburg et al., 2014; Su et al., 2016). NuA4 binding to methylated H3 promotes nucleosomal binding and H3 acetylation by Gcn5 (Ginsburg et al., 2014). Upon DNA damage, methylation of both H3K4 and H3K36 by Set1 and Set2, respectively, are enriched around the DSB site in euchromatin (Faucher and Wellinger, 2010; Jha and Strahl, 2014).

We can hypothesize that these chromatin marks may be less enriched around the DSB site in heterochromatin, which may then impact histone acetylation, chromatin remodeler recruitment and accessibility to other DSB repair proteins, in particular those involved in resection. By using histone mutants, Esa1-degron protein and deletions of *GCN5*, *SET1* and *SET2*, we could decipher whether some of the

chromatin modifications are required to allow DSB repair in heterochromatin and impact all the downstream response.

### **3. Impaired resection in heterochromatin favors end-joining events**

We were not able to measure classical NHEJ in our system. Nevertheless, the initiation of 5'-3' resection of DNA ends is a critical determinant of repair pathway choice, which directs cells to HR and prevents NHEJ (Symington and Gautier, 2011). As the presence of heterochromatin at the DSB site prevents resection, we can thus hypothesize that repressive chromatin would favor NHEJ events, accordingly to what was seen in mammalian cells (Aymard et al., 2014; Lemaître et al., 2014).

We also observed that the presence of heterochromatin increased error-prone NHEJ principally due to an increase in MMEJ events. In yeast, stimulation of Mre11 endonuclease activity by Sae2 has been implicated in favoring MMEJ by exposing microhomologies at DNA ends for annealing (Deng et al., 2014; Lee and Lee, 2007; Ma et al., 2003). A similar role was identified for CtIP in mammals (Zhang and Jasin, 2010). Loss of extensive resection was proposed to stabilize the partially resected 3' overhangs to provide increased opportunity for MMEJ to occur between microhomologies proximal to the DSB site (Deng et al., 2014). Furthermore, RPA accumulation is delayed and less robust at the nuclear lamina in mammalian cells, indicating a semi-functional resection pathway which promotes MMEJ (Lemaître et al., 2014). Accordingly, we observed that resection is delayed in heterochromatin but can still occur. In particular, we showed that the presence of heterochromatin at the

DSB site affects more the long-range machinery than the short-range resection. The presence of heterochromatin at a DSB site thus likely allows the initiation of resection required to reveal microhomologies but inhibits the long-range resection machinery. This situation may stabilize the annealing between microhomologies and account for the increase in MMEJ events.

These models presented here are highly speculative and need to be tested experimentally. Nevertheless, they open a broad new range of perspectives to understand how cells evolved repair pathways according to the chromatin state and promise interesting discoveries in the future. Elucidating the mechanisms that protect genome stability is an essential step towards understanding and fighting devastating diseases like cancer. Uncovering novel chromatin-regulated repair pathways are fundamental to a better understanding of the maintenance of genome integrity and may allow us to define new therapeutic targets.

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# **ANNEX**

## **Annexe 1: Résumé substantiel de la thèse en français**

Chaque jour, nos cellules subissent de très nombreuses lésions au niveau de leur ADN. Elles résultent aussi bien de stress environnementaux (UV, produits chimiques..) que d'évènements endogènes liés à la vie et à la division cellulaire (métabolisme, réplication..). Parmi ces dommages à l'ADN, les cassures double-brin sont les plus délétères pour l'intégrité des cellules. En effet, une cassure double-brin non réparée est létale pour la cellule. De plus, une mauvaise réparation de cette lésion peut entraîner des réarrangements chromosomiques pouvant modifier l'expression de certains gènes qui peuvent être à l'origine du développement de certains cancers ou de maladies génétiques. Des mutations dans de nombreuses protéines régulant les systèmes de réparation sont aussi associées au développement de cancers (Helleday, 2010). Pour préserver l'intégrité de leur génome, les cellules eucaryotes ont développé des mécanismes de réparation des cassures double-brin qui sont conservés de la levure à l'homme (Sonoda et al., 2006). La réparation par recombinaison homologue utilise une séquence homologue intacte présente ailleurs dans le génome et peut se diviser en deux sous-voies de réparation. La conversion génique (GC) transfère l'information génétique d'une molécule à son homologue, tandis que le Break Induced Replication (BIR) établit une fourche de réplication qui peut procéder jusqu'à la fin du chromosome (Symington et al., 2014).

Les étapes moléculaires de la réparation des cassures double-brin ont été largement étudiées ces dernières décennies et pour la plupart reconstituées *in vitro* sur de l'ADN nu. Or, dans le noyau, la réparation intervient dans le contexte de la chromatine, plus ou moins compactée suivant les régions où elle a lieu. De plus, le génome est organisé de façon non aléatoire dans le noyau, créant ainsi des sous-

compartiments enrichis en certaines activités enzymatiques et plaçant certaines séquences à proximité les unes des autres.

Mon projet de thèse visait à mieux définir l'impact de l'organisation du noyau et de l'hétérochromatine sur la réparation des cassures double-brin en utilisant la levure *S. cerevisiae* comme modèle. Pour cela, j'ai utilisé un système qui permet de changer l'organisation nucléaire des télomères et de moduler l'état chromatinien des séquences subtélomériques (Ruault et al., 2011). Chez la levure, les chromosomes sont organisés selon une configuration Rab1 où les centromères sont ancrés au niveau du Spindle Pole Body (l'équivalent du centrosome des mammifères) et les télomères sont regroupés en trois à huit foyers à la périphérie du noyau dans lesquels sont concentrés les facteurs hétérochromatiques SIR (Silent Information Regulators) (Taddei et al., 2004). Cette organisation peut être modifiée grâce à la surexpression de la protéine Sir3 ou de la protéine mutante sir3A2Q qui conduit au groupement de la majorité des télomères en un « hypercluster » localisé au centre du noyau. Alors que ce groupement a lieu en absence d'hétérochromatine lors de la surexpression de sir3A2Q, la surexpression de Sir3, quant à elle, aboutit à la propagation de l'hétérochromatine le long des régions subtélomériques. Ce système a été couplé à un système génétique permettant de mesurer l'efficacité de la réparation d'une cassure double brin par recombinaison entre deux cassettes homologues insérées à différents sites dans le génome.

Des études ultérieures avait observé une forte corrélation entre la propension de deux séquences à interagir l'une avec l'autre et leur efficacité de recombinaison, suggérant que la recherche d'homologie est une étape limitante pour la recombinaison homologue (Agmon et al., 2013; Lee et al., 2016). Au delà de cette corrélation, nous avons montré que la réduction de la distance entre deux séquences homologues par le

groupement des télomères via la surexpression de sir3A2Q accroît le taux de recombinaison entre deux séquences subtélomériques. Nous avons ainsi donc pu directement démontré le rôle clé de la proximité spatiale et donc de la recherche d'homologie dans le succès de la réparation par recombinaison.

Nous avons également pu observé que deux séquences équidistantes l'une de l'autre ne recombinaient pas avec la même efficacité suivant où était induite la cassure double-brin. En effet, une région subtélomérique est un bon donneur pour une cassure intrachromosomique alors qu'une cassure subtélomérique recombine mal avec une séquence intrachromosomique. Cela souligne que la proximité spatiale n'est pas le seul facteur limitant pour la recombinaison. Nous avons pu démontré que l'ancrage des télomères à la périphérie du noyau n'est pas le facteur limitant dans ce cas.

Grâce au système que nous avons développé, nous pouvons mesurer la compétition entre la conversion génique et le BIR. Nous avons pu observé que les cassures subtélomériques sont principalement réparées par BIR. La proportion plus élevée de BIR par rapport à la conversion génique aux subtélomères est due à une disparition plus rapide du fragment télomère proximal qu'à un site intrachromosomal.

Les subtélomères sont des régions hétérochromatiques régulées par la présence du complexe SIR. La surexpression de Sir3 a pour effet de propager fortement l'hétérochromatine le long des régions subtélomériques et nous nous en sommes servis pour déterminer si le statut chromatinien pouvait réguler la recombinaison. Dans le cas où seul le site de cassure est hétérochromatique, nous avons constaté que la surexpression de Sir3 augmente la fréquence de recombinaison. La présence d'hétérochromatine au niveau du site de cassure limite la résection, ce qui permet probablement une disparition plus lente des extrémités, qui resteraient

disponibles plus longtemps pour réaliser la recherche d'homologie et achever la réparation. Nous avons pu démontrer que l'hétérochromatinisation du site de cassure bloque les différentes nucléases responsables de la résection, et plus particulièrement la résection à longue distance médiée par Exo1 et Sgs1-Dna2.

Enfin, nous avons observé que la présence d'hétérochromatine au niveau du site donneur diminue l'efficacité de recombinaison à la fois par conversion génique et par BIR. Cela suggère qu'elle doit moduler une étape commune aux deux voies de réparation, à savoir l'invasion de brin ou l'initiation de la synthèse d'ADN.

Ce travail de thèse a ainsi permis de démontrer directement l'importance de la proximité spatiale entre les deux séquences homologues sur la recombinaison. Nous avons également mis en évidence comment l'hétérochromatine régule la recombinaison homologue. Nous avons montré qu'elle représente une barrière pour la résection d'une cassure hétérochromatique ainsi que pour les étapes plus tardives de la recombinaison quand la séquence donneuse est hétérochromatique. Ces données ouvrent de nouvelles perspectives pour comprendre comment les cellules ont développé les voies de réparation selon l'état de la chromatine et promettent des découvertes intéressantes dans l'avenir. La compréhension des voies de réparation de l'ADN est d'une importance cruciale pour la compréhension du développement cancéreux. Une meilleure compréhension de la réparation de l'ADN dans les différents contextes chromatinien s'avère donc primordiale pour une amélioration des diagnostics et pourrait permettre de définir de nouvelles cibles thérapeutiques.

**Titre :** Impact de l'organisation du noyau et de la structure de la chromatine sur la réparation de l'ADN et la stabilité du génome

**Mots-clés :** cassure double-brin, recombinaison, chromatine, organisation nucléaire

**Résumé :** L'organisation non-aléatoire du noyau des cellules eucaryotes et la compaction de l'ADN en chromatine plus ou moins dense peuvent influencer de nombreuses fonctions liées au métabolisme de l'ADN, y compris la stabilité du génome. Les cassures double-brin sont les dommages à l'ADN les plus néfastes pour la cellule. Pour préserver l'intégrité de leur génome, les cellules eucaryotes ont développé des mécanismes de réparation des cassures double-brin qui sont conservés de la levure à l'homme. Parmi ceux-ci, la recombinaison homologue utilise une séquence homologue intacte présente ailleurs dans le génome et peut se diviser en deux sous-voies de réparation. La conversion génique transfère l'information génétique d'une molécule à son homologue, tandis que le Break Induced Replication (BIR) établit une fourche de réplication qui peut procéder jusqu'à la fin du chromosome.

Mon travail de thèse s'est attaché à caractériser la contribution du statut chromatinien et de l'organisation tridimensionnelle du génome à la réparation des cassures double-brin. L'organisation du noyau de la levure *S. cerevisiae* ainsi que la propagation de l'hétérochromatine au niveau des régions subtélomériques peuvent être modifiées via la surexpression des protéines Sir3 et sir3A2Q. Nous avons montré que le groupement des télomères accroît la conversion génique entre deux séquences subtélomériques, soulignant le rôle clé de la proximité spatiale et de la recherche d'homologie. Nous avons également constaté que la présence d'hétérochromatine au niveau du site de cassure limite la résection, ce qui permet une disparition plus lente des extrémités, qui resteraient disponibles plus longtemps pour réaliser la recherche d'homologie et achever la réparation. Enfin, nous avons observé que la présence d'hétérochromatine au site donneur diminue l'efficacité de recombinaison et qu'elle doit moduler une étape commune aux deux voies de réparation, à savoir l'invasion de brin. Ces travaux nous ont permis de décrire de nouvelles voies de régulation de la réparation de l'ADN.

**Title :** Impact of nuclear organization and chromatin structure on DNA repair and genome stability

**Keywords :** double-strand break, recombination, chromatin, nuclear organization

**Abstract :** The non-random organization of the eukaryotic cell nucleus and the folding of genome in chromatin more or less condensed can influence many functions related to DNA metabolism, including genome stability. Double-strand breaks (DSBs) are the most deleterious DNA damages for the cells. To preserve genome integrity, eukaryotic cells thus developed DSB repair mechanisms conserved from yeast to human, among which homologous recombination (HR) that uses an intact homologous sequence to repair a broken chromosome. HR can be separated in two sub-pathways: Gene Conversion (GC) transfers genetic information from one molecule to its homologous and Break Induced Replication (BIR) establishes a replication fork than can proceed until the chromosome end.

My doctorate work was focused on the contribution of the chromatin context and 3D genome organization on DSB repair. In *S. cerevisiae*, nuclear organization and heterochromatin spreading at subtelomeres can be modified through the overexpression of the Sir3 or sir3A2Q mutant proteins. We demonstrated that reducing the physical distance between homologous sequences increased GC rates, reinforcing the notion that homology search is a limiting step for recombination. We also showed that heterochromatinization of DSB site fine-tunes DSB resection, limiting the loss of the DSB ends required to perform homology search and complete HR. Finally, we noticed that the presence of heterochromatin at the donor locus decreased both GC and BIR efficiencies, probably by affecting strand invasion. This work highlights new regulatory pathways of DNA repair.

