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Dynamic binding of Rad9/53BP1 on DNA lesions promotes accurate repair and genome stability

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CONTENTS

Vi è solo un mezzo per far progredir la scienza: dar torto alla scienza già costituita.

(Gaston Bachelard)

Part I

ABSTRACT

All living organisms are constantly exposed to physical and chemical sources that challenge the integrity of the genome. Considering the high number of chemical and physical insults potentially deleterious to which cells are constantly exposed, the maintenance of genome stability is for all living organisms a main challenge during the cellular life cycle. The ability to cope with DNA damage is crucial for cellular proliferation and, in higher eukaryotes, the loss of function of genes responding to DNA damage often results in genetic syndromes and cancer predisposition. Recognition of DNA damaged structures and their accurate repair are two crucial events that involve several factors and multiple specialized pathways. These events are finely orchestrated by the cell cycle checkpoints aimed to sense DNA damage, arrest cellular proliferation and activate the most accurate repair pathway.

At DNA double strand breaks (DBS, the most cytotoxic lesions), homology-directed repair initiates with the 5' strand nucleolitic degradation of the broken end, a process called *resection*. In all the eukaryotes, resection is tightly regulated and, not surprisingly, mutations in resection machinery genes are associated with high genome instability and therefore cancer predisposition. In the past we proposed that the Rad9 checkpoint factor, through the interaction with modified histones physically inhibits the ssDNA accumulation at DSB. Importantly, this function is conserved with the mammalian counterpart 53BP1.

In this thesis, using budding yeast as model system, I have been involved in three projects focusing on the role of Rad9 in DSB repair pathway choice and how the chromatin positioning of this factor is dynamically regulated in response to these lesions.

In a first part, I collaborated in the comprehension of Rad9 genetic and functional interactions with different repair factors during DSB metabolism. In brief, we found that Rad9 positioning around DSB ends are important for tethering of DSB ends, resection start and, most importantly, recruitment of recombination factors. Our findings provided a molecular explanation how Rad9 inhibition facilitates Homologous Recombination (HR), preventing the Non Homologous End Joining repair (NHEJ).

Later, I studied the role of the Slx4-Rtt107 complex in modulating checkpoint signaling and nucleolitic processing during homology-directed repair of DSBs. Using different genetics and biochemical approaches, I described a novel Slx4 function in supporting DSB resection through the inhibition of the formation of a complex between Rad9 and the checkpoint factor Dpb11 (TOPBP1 in mammals). In mammals, biallelic mutations in SLX4 are associated with the Fanconi Anemia, a genetic disorder associated with defects in DNA repair and high cancer risk. Considering this, our results may be important for understanding how Slx4 protects genome stability and favors cellular proliferation in human beings.

In the last part, I have been involved in an international collaboration with Dr. Marcus B. Smolka (Cornell University, Ithaca, NY, USA). Here I studied the role of Dpb11 in coordinating the recruitment of Rad9 during the resection process. We found that a constitutive interaction between Dpb11 and Rad9 severely abrogates ssDNA accumulation in cells responding to DSB lesions, suggesting that this interaction is a crucial point of regulation regarding this process. In human cells, SLX4 shares functional homology with BRCA1, whose interaction with TOPBP1 is mutually exclusive with TOPBP1-53BP1. Our results suggest that TOPBP1, through the coordinated recruitment of pro- and anti-recombination factor, is an essential regulator of DNA repair and genome stability.

STATE OF THE ART

The DNA damage and genome integrity maintenance

In all living organisms, the integrity of the genetic material is constantly threatened by various endogenous and exogenous sources. The aqueous nucleoplasmic environment makes DNA double helix susceptible to spontaneous hydrolysis of the phosphodiester bonds between bases in the DNA backbone. Cellular metabolism often releases free reactive oxidant species that produce highly mutagenic alterations in the DNA double helix such as 8-oxo-dG and others (Lindahl, 1993; Marnett, 2000). Non-enzymatic methylation of DNA bases is also detrimental as alters replication, transcription and chromatin conformation.

Misincorporation of dNTPs or rNTPs during DNA replication is another important source of genome instability as mismatches are inherited by daughter cells after chromosome segregation altering gene function in the progeny (Cerritelli & Crouch, 2016).

Exogenous chemical and physical agents such as Ionizing Radiations (IR) or Ultra Violet rays (UV) severely alter DNA structure, also generating breaks inside the backbone (Lindahl & Wood, 1999). In response to that, cell activate various pathways that modify these substrates and make them accessible to specific repair factors. The enzymatic activity of these factors is *per sé* potentially detrimental as during the repair of DNA lesions instable intermediates, such as ssDNA stretches, are exposed. It is important to note that many of the chemotherapy compounds used to treat solid tumors (cisplatin, mytomicin C, camptothecin, etoposide and others…) cause various type of DNA lesions and their efficacy depends on the inability of many cancer

cells to properly sense and repair DNA damage (Pommier et al., 2010; Samadder et al., 2016; Schärer, 2005).

The DNA double strand break repair pathways

Among the others, DNA double strand breaks (DSBs) are certainly the most cytotoxic lesions and even a single DSB may cause cellular death. In higher eukaryotes, DSBs are a frequent source of translocations and genomic rearrangements, typical hallmarks of the karyotype of cancerous cells.

For this reason, all the cells evolved specialized pathways to quickly and faithfully repair these lesions for a correct maintenance of genome integrity.

Two main distinct DSB repair pathways exist and they are strongly conserved throughout the evolution: Non Homologous End Joining (NHEJ) and Homologous Recombination (HR) (see Figure 1) (Heyer et al., 2010; Symington & Gautier, 2011). These two repair mechanisms are in a dynamic competition depending upon temporary and spatially causes. It is interesting to note that the general mechanisms that control this balancing as well as the factors involved in these processes are highly conserved. This may suggest that a correct combination of NHEJ and HR events is crucial for protection of genome stability and cellular lifespan in all the eukaryotes.

Homologous sequences present on the sister chromatid are likely preferred during HR and therefore, by definition, this error free repair pathway is restricted to S/G2 phases of the cell cycle. Indeed, the use of other templates during HR may lead to loss of heterozygosity, imprecise recombination events and would require longer time to complete the repair process.

By contrast, the fast religation of broken ends makes the NHEJ a quick and easy process to repair DSB lesions and for this reason, higher eukaryotes prefer the NHEJ mode of repair. As very often DSB ends are not an immediate

substrate for a ligation reaction, NHEJ core complex modifies DSB ends introducing short insertions and deletions. The wide presence of non-coding regions as well as repeated sequences allows higher eukaryotes to tolerate point mutations arising by the action of this fast repair process.

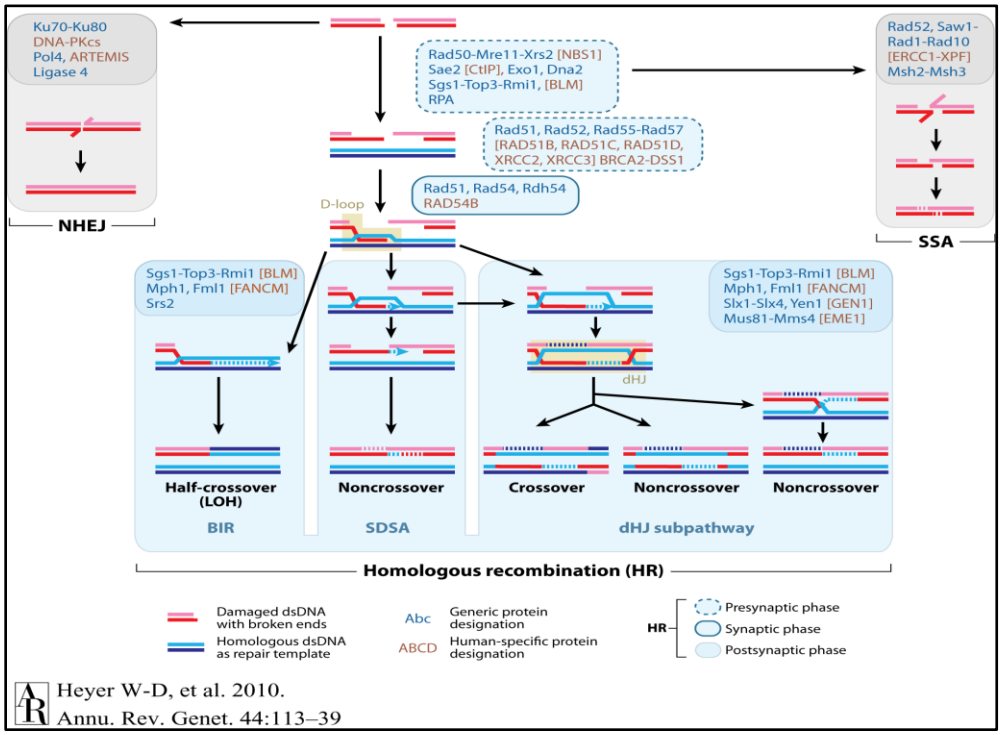

Figure 1: A cartoon showing the various DSB repair pathways and the competition between NHEJ and HR (Heyer et al., 2010).

Non Homologous End Joining (NHEJ)

As discussed in the above paragraph, NHEJ is a major pathway to repair DSB lesions especially in higher eukaryotes and, as its name says, does not utilize any homologous template. NHEJ is often used also in lower eukaryotes like budding yeast, and its employment is favored in the G1 phase of the cell cycle, when HR is prevented (Daley et al., 2005).

Broken ends are recognized and avidly bound by the Ku70-Ku80 heterodimer (Davis et al., 2013; Pierce et al., 2001). This not only favors the NHEJ mode of repair, through the recruitment of DNA ligase IV, Lif1 and Nej1, but also has been proposed to protect DSB ends from extensive degradation and unwanted recombination events (Bernstein et al., 2013; Daley et al., 2005; Davis et al., 2013).

Short processing that happens in the absence of "clean ends" often causes deleterious mutations and loss of genomic information. In mammals, the nuclease ARTEMIS is part of the NHEJ core complex, while in budding yeast different nucleolytic enzymes modify broken ends to make them compatible for the ligation reaction (Daley et al., 2005; Davis et al., 2013).

In mammals, the core complex has the DNA-PKcs (DNA-protein kinase, catalytic subunit) as additional component. DNA-PKcs is a serine/threonine kinase of the family of the phosphatidylinositol 3-kinase-related kinase (the same family of Mec1/ATR and Tel1/ATM kinases). Artificial inhibition of DNA-PKcs prevents NHEJ repair and sensitizes cells to various DSB inducing agents (Ciszewski et al., 2014; Kim et al., 2002). For this reason, specific DNA-PKcs inhibitors have been recently developed in cancer research (Davidson et al., 2013; Ma et al., 2015). Indeed, tumor cells carrying mutations in HR genes (*e.g.* BRCA1/2) would be specifically targeted treating tumors

with DNA-PKcs inhibitor as these cancerous cells rely only on NHEJ pathway for DSB repair.

DNA-PKcs is downregulated in various type of cancers suggesting that its activity is indeed required for DSB repair and tumor suppression (Goodwin & Knudsen, 2014). By contrast, there are conflicting evidences that in other tumors DNA-PKcs, and other NHEJ genes, are upregulated, suggesting the possibility that malignant cells, with the error prone NHEJ repair pathway, acquire an advantage for their growth progression (Beskow et al., 2009).

In vertebrates, NHEJ is also involved in the repair of programmed DSB created during variable (diversity) joining [V (D) J] recombination and class switch recombination (CSR). Downregulation of NHEJ pathway alters the outcome of immune system development and germline mutations in NHEJ core genes predispose to severe pathologies associated with immunodeficiency (Bassing et al., 2002; Malu et al., 2012).

Homologous Recombination (HR)

Besides its well-characterized function in DSB repair, HR is essential also during the meiotic genetic diversification process and for replication restart after fork collapse. In the mitotic cell cycle, HR takes advantage of the homologous sequence present on the sister chromatid to avoid loss of heterozygosity and homeologous events (recombination between sequences with imprecise homology) and therefore is restricted to the S/G2 phases of the cell cycle. The discovery that CDK1, the master cell cycle kinase, targets many of the factors involved in the HR pathway explains at the molecular level why HR is not happening in G1 (Chen et al., 2012; Huertas et al., 2008; Ira et al., 2004; Sartori et al., 2007).

From a molecular point of view, HR is a multistep process involving multiple factors with specialized enzymatic activities. One of the processes taking place in all the HR sub pathways is the search for homology and the pairing between two single stranded DNA sequences. At DSB, ssDNA sequences are exposed after a coordinated process called *DSB resection*. The aim of *resection* is to generate a 3' protruding filaments competent for the homology search and strand invasion processes.

Resection is essential for HR outcome and genome stability maintenance and not surprisingly many of the players involved in DSB *resection* are conserved from yeast to humans (Symington & Gautier, 2011). In yeast, depletion of *resection* genes dramatically increases sensitivity to genotoxic compounds and genomic rearrangements formation. In mammals, germline mutations in same genes predispose to genetic syndromes often associated with high cancer incidence.

Nevertheless, how *resection* is regulated and what is the minimal region for the *strand invasion* process is not yet clear.

DSB resection and recombinogenic filament formation

DSB ends are dangerous substrates prone to formation of rearrangements and fusions between distal regions. Together with the Ku core complex, broken ends are recognized and protected by the heterotrimeric yeast MRX (MRN in mammals) complex formed by Mre11/MRE11- Rad50/RAD50-Xrs2/NBS1 and the accessory protein Sae2 (CtIP in mammals).

As described in the previous paragraph, DSB lesions occurring after replication are channeled into HR by the action of the MRX complex that starts *resection* removing 5' oligonucleotides from broken ends (see Figure 1). The nucleolytic activity of the Mre11 subunit is essential to process 'dirty' ends

and to remove DNA secondary structures that may potentially generate tandem duplication (Deng et al., 2015).

An important point of regulation for Mre11 functioning is the phosphorylation of Sae2/CtIP on conserved residues by the CDK1 kinase (Huertas et al., 2008; Sartori et al., 2007). The phosphorylation of Sae2 is believed to be the molecular event that switches the repair from NHEJ to HR, activating the MRX complex and starting the *resection* (Huertas et al., 2008; Sartori et al., 2007). Sae2 phosphorylation is a 'point of no return' because once the MRX complex is active, the NHEJ is prevented and HR will take place.

After MRX shortly trims DSB ends, other nucleases extend the single stranded region in 5'-3' direction, the opposite polarity respect to that of Mre11 nuclease (Cannavo & Cejka, 2014; Garcia et al., 2011; Llorente & Symington, 2004) (see Figure 2). This bidirectional model for the *resection* initiation has been initially demonstrated during meiotic DSB repair and later proposed also during the mitotic recombination in yeast and humans (Ferrari et al., 2015; Garcia et al., 2011; Shibata et al., 2014).

ssDNA accumulation depends by the coordinated activity of the nucleases Exo1 and Dna2 and the helicase Sgs1 which likely unwinds DNA strands providing the substrate for Dna2 flap endonuclease activity (Mimitou & Symington, 2008; Zhu et al., 2008). These pathways act redundantly, as only the simultaneous depletion of Exo1 and Dna2/Sgs1 results in a complete loss of extensive *resection* (Ferrari et al., 2015; Mimitou & Symington, 2008; Zhu et al., 2008). Moreover, many other enzymes participate in the resection process including kinases, chromatin remodelers and others.

As discussed previously, the aim of DSB processing is to generate the ssDNA stretch essential for the homology search and pairing. Before that, once ssDNA is exposed the filament is immediately stabilized by the single stranded binding protein complex RPA as defects in its loading result in the

formation of deleterious secondary structure and nucleolytic degradation of ssDNA (Chen et al., 2013; Deng et al., 2015). Besides that, ssDNA regions covered by RPA recruit Mec1, the apical kinase of the DNA damage checkpoint (ATR in mammals), and therefore this step is essential to coordinate DSB repair with checkpoint activation (discussed later).

Extensive and uncontrolled *resection* might be a detrimental event in cells responding to DSB lesions. Recent studies in human cell lines proposed novel mechanisms of regulation of *resection*. This year, Tkác and colleagues discovered that HELB, a DNA helicase, is a feedback inhibitor of EXO1 and DNA2 and its inactivation may cause resistance to genotoxic drugs such as PARP inhibitor, a chemical compound that causes DSB during DNA replication (Tkáč et al., 2016). Another group last year described for the first time the Translesion Synthesis Polymerase REV7 as a novel player in the *resection* process (Xu et al., 2015). In brief, as *resection* proceeds, REV7 favors the NHEJ mode of repair synthetizing DNA in the opposite direction of the nucleolytic processing.

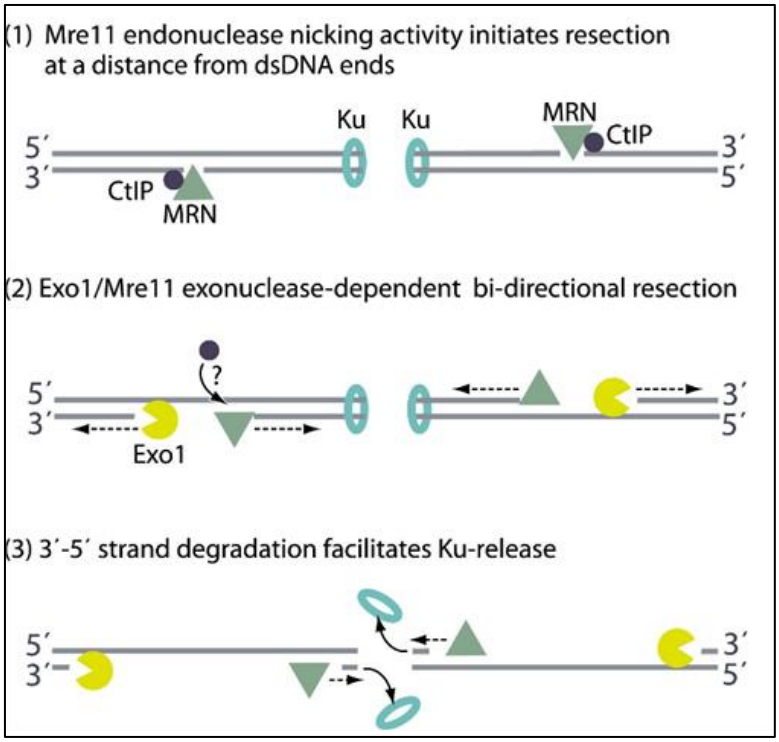

Figure 2: A model for DSB resection initiation in mammalian cells (Chapman et al., 2012).

As *resection* is ongoing, the Rad51 recombinase is exchanged with RPA, covering the ssDNA tail and constituting a recombinogenic competent filament for the strand invasion process. Rad52 is essential for Rad51 loading, HR and strand annealing between homologies in budding yeast (Shinohara et al., 1998; Sugiyama et al., 1998). In vertebrates, Rad52 shares structural homology with RAD52 although its inactivation, surprisingly, does not give strong phenotypes in terms of DNA repair (Yamaguchi-iwai et al., 1998). BRCA2 interacts with RAD51 and RPA and is essential for DNA repair,

recombination and tumor suppression and now is considered as a *bona fide* functional homolog of yeast Rad52, even if does not share any ssDNA strand annealing activity *in vitro* (Liu et al., 2010; Liu & Heyer, 2011).

After Rad51 nucleofilament formation, the broken ends invade the homologous sequence. The pairing of the broken end with the homologous template creates the displacement-loop (D-loop) and the invading 3' terminus serves as substrate for DNA synthesis priming. The capture of second end of the DSB will create a double Holliday Junction (dHJ) structure. These intermediates are processed by several enzymes and may generate different product outcomes. The dissolvasome complex (Sgs1-Top3-Rmi1, BLM-TOPIIIα-RMI1 in mammals) has a prominent role in the repair of dHJ and its activity gives rise only to non-crossover products and normally occurs during DNA replication. Joint molecules, indeed, form frequently during replication restart and Template switching when either one of the parental strands is damaged or modified and thus cannot be copied by replicative polymerases.

A small percentage of joint molecules persist till late S, G2 and mitosis stages and are later resolved by structure specific endonucleases (reviewed in Wild & Matos, 2016) (see Figure 3). Resolution is achieved through the redundant activity of the endonucleases Mus81-Mms4, Yen1 and to a minor extent Rad1-Rad10 (Mazón et al., 2012; Mazón & Symington, 2013) (Figure 3). The activity of resolvases gives rise in the 50 % of the products to crossover outcomes and therefore is considered detrimental during mitotic recombination but essential for genetic diversification during meiotic recombination.

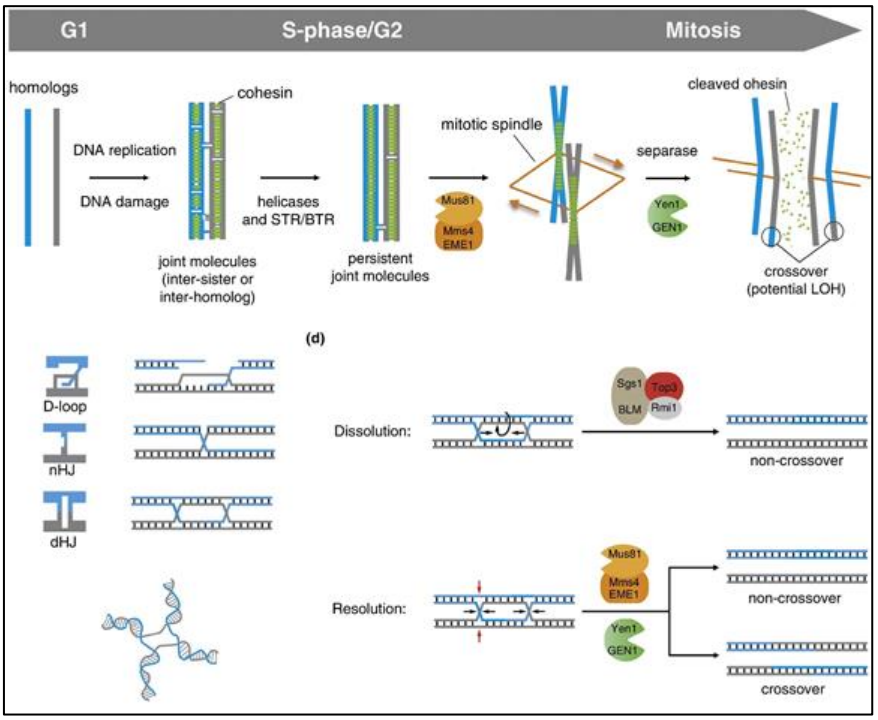

Figure 3: Temporal regulation of joint molecule processing throughout the cell cycle (Wild & Matos, 2016).

Here I listed the two main HR sub-pathway, Synthesis Dependent Strand Annealing and Break Induced Replication, and an additional pathway, Single Strand Annealing, which does not require any strand invasion process (see also Figure 1):

Synthesis Dependent Strand Annealing (SDSA)

Considering that the vast majority of DSB occurring during mitotic recombination do not give rise to crossover products, cells normally repair these lesions through the use of a specialized pathway that does not create double Holliday junctions, the Synthesis Dependent Strand Annealing (SDSA). According to this model, the 3' invading strands, after a limited repair synthesis reaction, are displaced by various factors including Srs2 in yeast. After strand displacement, the newly synthetized complementary strands will anneal completing the repair after limited gap filling and ligation reactions. It is important to note that SDSA gives rise to non-crossover repair products only (Ira et al., 2003).

Break Induced Replication (BIR)

If only one of the broken ends shares homology with the donor template, after the invasion process, a conservative replicative bubble migrates from the invasion site towards the end of the chromosome (McEachern & Haber, 2006) (see Figure 1). BIR results in a loss of heterozygosity (LOH) between sister chromatids and it produces non-reciprocal translocations, when the donor template is used on another chromosome.

Single Strand Annealing (SSA)

SSA is a repair process that does not require any strand invasion process and therefore does not rely on the Rad51 recombinase (see Figure 1). This is the main pathway to repair DSB occurring between direct repeats. After resection exposes the 3'-ssDNA filaments, complementary single stranded repeats anneal facilitated by the Rad52 protein. Non-homologous sequences present between the repeats are removed after their annealing by the action of the

Rad1-Rad10 heterodimer (Flott et al., 2007; Ma et al., 2003; Toth et al., 2010). Its enzymatic activity requires the prior stimulation by Slx4 and the cooperation of the DNA binding protein Saw1 (Li et al., 2008, 2013; Toth et al., 2010). Slx4 functions and regulation will be detailed in dedicated paragraphs of this thesis. Both Slx4 and Saw1 are essential during flap removal as inactivation of these two genes impairs non-homologous tails cleavage, similarly to Rad1 and Rad10 (Flott et al., 2007; Li et al., 2013; Toth et al., 2010). The phosphorylation by Mec1 of a cluster of SQ/TQ residues present in the C-terminal region of Slx4 is required to stimulate the nucleolytic activity of Rad1, without affecting its recruitment to SSA intermediates. However, how this modification alters Slx4 protein functionality is completely obscure (Toth et al., 2010).

Conversely, Saw1 has a prominent role in Rad1-Rad10 recruitment underlying its essential role in the SSA mode of repair (Li et al., 2013). Completion of DSB repair trough SSA requires a final gap filling and ligation reaction.

It is important to underline that SSA is highly mutagenic as it always results in loss of DNA sequence between the homologous sequences causing extensive deletions, translocation between dispersed genomic loci and contraction of tandem repeats.

A detailed list of the factors involved in the homologous recombination processes and their mode of action can be found in the following review (Symington & Gautier, 2011; Symington, 2016).

The DNA Damage Checkpoint

The cell cycle checkpoints allow the cell to integrate stimuli from the exogenous environment with the cellular physiological processes. Genome stability is safeguarded constantly and even a single unrepaired DSB lesion may cause loss of acentric fragments and genetic information during cellular division and ultimately cellular death (Lee et al., 1998; Sandell & Zakian, 1993). The DNA Damage Checkpoint (DDC) controls chromosomal integrity and, in presence of lesions or alterations in the DNA structure, delays or arrests the cell cycle giving more time for the repair.

DDC is organized as a hierarchical signal transduction cascade that is activated locally at the level of DNA lesions and spreads downstream involving and reprogramming almost all the cellular physiological processes.

In budding yeast depletion of genes involved in DDC increases genome instability and sensitize cells to different genotoxic compounds whereas inactivation of the mammalian counterparts either predispose to severe genetic syndromes or to solid tumors (Broustas & Lieberman, 2014). This highlights the importance of this pathway for genome integrity protection and tumor suppression.

Each cell cycle phase has its own checkpoint with the aim of maintaining genome integrity before progressing into the following stage. In G1, checkpoints score genome integrity before DNA replication, intra-S checkpoints monitor replication ongoing and ensure faithful copy of parental genomic material whereas G2/M checkpoints control that DNA lesions are not inherited by daughter cells during mitosis. In this thesis, I will focus describing how cells trigger checkpoint activation in response to DSB lesions during the G2/M phase.

Our current knowledge of DSB-induced G2/M arrest derives from original studies in budding yeast. By using the HO-inducible system where a single

irreparable DSB is formed on the chromosome III of *Saccharomyces cerevisiae* (Lee et al., 1998; Moore, 1996), in the past it was observed that a single DSB is sufficient to trigger a strong DDC activation and a robust G2/M arrest (Pellicioli et al., 1999; Pellicioli et al., 2001). Further studies carried using this genetic system elucidated almost all the components that collaborate in the checkpoint response and were later found to be fully conserved in higher eukaryotes (Harrison & Haber, 2006) (see Figure 4).

Table 1 DNA damage checkpoint proteins

	Budding yeast	**Fission yeast**	**Human**
PIKK	Mec1	Rad3	ATR
PIKK	Tel1	Tel1	ATM
Adaptor	Rad9	Crb2	53BP1, MDC1, BRCA1?
Rfc1 homolog	Rad24	Rad17	Rad17
9-1-1 clamp	Rad17	Rad9	Rad9
	Mec3	Hus1	Hus1
	Ddc1	Rad1	Rad1
MRX complex	Mre11	Mre11	Mre11
	Rad50	Rad50	Rad50
	Xrs2	Nbs1	Nbs1
BRCT domain adaptor?	Dpb11	Rad4/Cut5	TopBP1
Signaling kinase	Rad53	Cds1	Chk2
Signaling kinase	Chk1	Chk1	Chk1
Polo kinase	Cdc5	Plo1	Plk1
Securin	Pds1	Cut2	Securin
Separase	Esp1	Cut1	Separase
APC-targeting subunit	Cdc20	Slp1	$p55^{CDC}$/CDC20

Figure 4: A table showing the main factors involved in DNA Damage Checkpoint signaling and their conservation throughout the evolution (adapted from Harrison & Haber, 2006).

At DSB, the checkpoint is initiated with the recognition of broken ends by specific sensors (Grenon et al., 2001). The MRX complex (see above) not only recruits the nucleases involved in the resection process but also the apical

kinase Tel1 through a direct interaction with the subunit Xrs2 (Nakada & Matsumoto, 2003). Tel1 can phosphorylate the histone H2A on serine 129 in the regions surrounding DSB ends. This modification is strongly conserved with the mammalian histone variant H2AX and specifically marks DNA damage sites spreading for several kilobases around DSB (Iacovoni et al., 2010; Lee et al., 2014; Shroff et al., 2004).

Nevertheless, Tel1 has a prominent function in telomerase recruitment and telomere length homeostasis whereas its exact role during DSB response is still elusive since Tel1 depletion does not significantly sensitize cells to genotoxic treatments (Wellinger & Zakian, 2012).

As resection proceeds and the RPA-covered ssDNA stretch is formed, checkpoint is fully activated (see Figure 5). At the junction between ssDNA and dsDNA, Rad24 in complex with RFC2-5 proteins load the PCNA-like 9-1-1 heterotrimeric complex (Rad17-Ddc1-Mec3), a crucial component of the checkpoint cascade (Majka, Binz, Wold, & Burgers, 2006). 9-1-1 complex triggers DDC activation through the stimulation of the other apical kinase, Mec1.

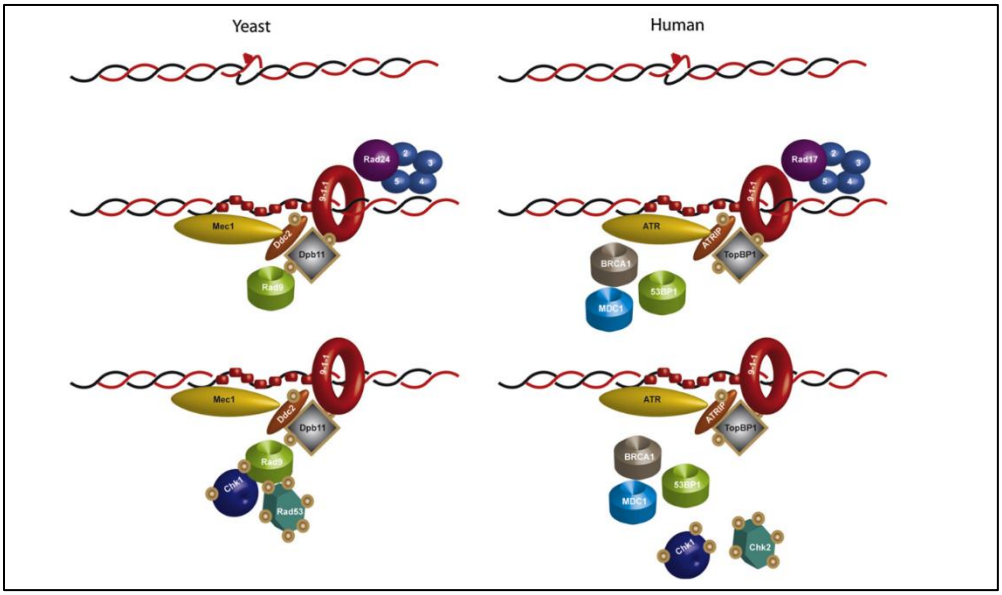

Figure 5: a schematic model for DNA Damage Checkpoint activation in budding yeast and human cells (Novarina et al., 2011).

Mec1/ATR is a member of the PIKK kinases family together with Tel1/ATM and in mammalian cells with DNA-PKcs. It is covalently bound to its binding partner Ddc2/ATRIP which recognizes RPA stretches and mediates the recruitment of Mec1 on resected DSB ends (Zou & Elledge, 2003). The mode of Mec1 kinase activation is a complicated network of positive feedback activation loops which involve different proteins. Once recruited through the Ddc2-RPA interaction, Mec1 interacts with and is stimulated by Ddc1, a subunit of the 9-1-1 complex through the interaction with its unstructured C-terminal domain (Majka et al., 2006). How Ddc1 modifies the kinase activity of Mec1 is still unknown, but Ddc1 depletion completely abrogates Mec1 signaling. More recently, 9-1-1 complex has been also observed to follow

resection acting as a clamp and increasing the processivity of nucleases (Ngo & Lydall, 2015).

Mec1 full activation is latter sustained trough the recruitment of Dpb11/TOPBP1 achieved after the Mec1-depedent phosphorylation on Ddc1 Threonine 602 (Puddu et al., 2008). After Dpb11 binds Ddc1, it interacts with Mec1 in a positive feedback loop to further stimulate its kinase activity (Mordes et al., 2008; Navadgi-patil & Burgers, 2011; Puddu et al., 2008) (see Figure 5).

A recent report discovered that the nuclease/helicase Dna2 also physically stimulates Mec1 during replicative stress through an unstructured C-terminal domain similar to the one shared also by Ddc1 and Dpb11 (Kumar & Burgers, 2013).

In budding yeast, Mec1 has an essential role during DSB response and its depletion strongly sensitize cells to all the genotoxic compounds (Paciotti et al., 2001). Indeed Mec1 was shown to phosphorylate different repair factors but also to promote spindle checkpoint activation and facilitate chromatin mobility and reorganization (Chen et al., 2011; Dion et al., 2012; Kaye et al., 2004). In contrast, in mammalian cells, ATM is the main checkpoint kinase activated only at DSB lesions whereas ATR has a prominent function during the replicative stress response.

Checkpoint cascade follows and ends with the activation of the effector kinases Rad53 and Chk1. For this purpose, cells recruit the checkpoint adaptor Rad9 near DSB to facilitate the following binding of the effector kinases. Rad9 focal recruitment is promoted by the interactions with phosphorylated histone H2A (γH2A), Dpb11 (through the CDK1-dependent phosphorylation on Rad9 Serine 462 and Threonine 474) and the constitutive interaction with methylated Lysine 27 of histone H3 (Giannattasio et al., 2005; Granata et al.,

2010; Hammet et al., 2007; Pfander & Diffley, 2011; Wysocki et al., 2005) (see Figure 6).

Figure 6: map of domains and motives of budding yeast Rad9 protein.

In mammals, 53BP1, the functional orthologue of Rad9, is recruited through the interaction with other complex chromatin modifications, besides its interaction with TOPBP1 and γH2AX (Baldock et al., 2015; Cescutti et al.; 2010). 53BP1 Tudor domain recognizes mono- and dimethylated H4K20 whereas the UDR motif (Ubiquitylated-Dependent Recognition) recognizes ubiquitylated H2AK13 and H2AK15 (Botuyan et al., 2006; Fradet-Turcotte et al., 2013; Huyen et al., 2004) (see Figure 7).

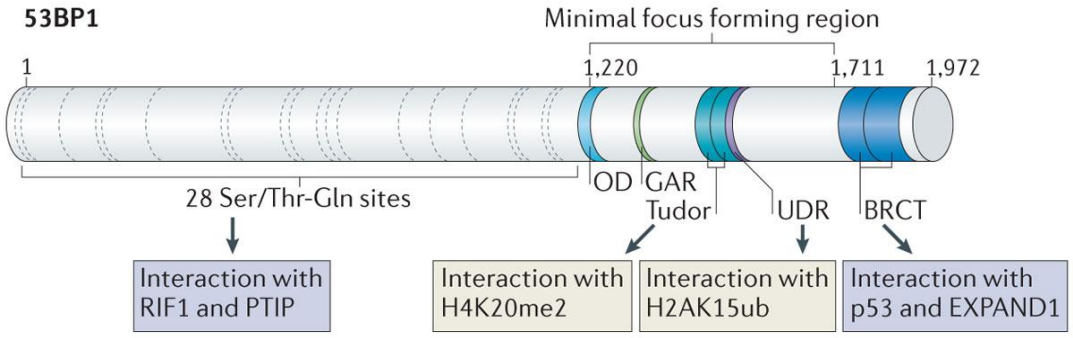

Figure 7: map of domains and motives of 53BP1 protein (Panier & Boulton, 2014).

Moreover, both Rad9 and 53BP1 have a cluster of SQ/TQ motifs on the central and the N-terminal region of the protein respectively (see Figure 6 and 7). Indeed, after DNA damage, Rad9 and 53BP1 are highly phosphorylated by Mec1 and ATM respectively and these modifications are essential for their checkpoint signaling and repair functions (DiTullio et al., 2002; Schwartz et al., 2000; Silverman et al., 2004; Sweeney et al., 2005) . In yeast, Rad53 FHA (Fork-Head Associated) domains recognize Rad9 phosphorylated SQ/TQ residues whereas Chk1 interacts with the CAD (Chk1 Activated Domain) domain on the Rad9 N-terminal part of the protein (Conde et al., 2009; Sun et al., 1998).

Rad53 and Chk1 recruitment achieved through the interaction with Rad9 is essential for their activation. Rad53 in particular, once bound to Rad9, is trans-phosphorylated by Mec1 and, in this partial active state, different Rad53 molecules undergo multiple auto-trans-phosphorylation events that determine the latter separation from Rad9 platform and full kinase activation (Pellicioli & Foiani, 2005). Various Rad53 targets have been identified in the past and many of them are repair factors like Exo1 and Rad9 themselves (Morin et al., 2008; Usui et al., 2009). In addition to that, the Chk1 kinase amplifies checkpoint signaling and impairs cell cycle progression into mitosis inhibiting cohesion degradation and therefore aberrant chromosome segregation (Wang et al., 2001).

As already anticipated above, checkpoint kinases also phosphorylate various repair factors besides controlling cell cycle arrest. Another essential

checkpoint factor that determine DNA damage repair is the adaptor Rad9/53BP1.

In the past, its ability to accumulate near DNA lesions and to form stable oligomers has been proposed to inhibit the resection process as in Rad9-depleted cells, resection speed is 2.5 fold higher compared to wild type cells (Lazzaro et al., 2008). How Rad9 limits the accumulation of ssDNA at DSB is still unclear even though the most probable explanation would be that it physically inhibits the nucleases during the resection process (Ferrari et al., 2015; Lazzaro et al., 2008). In agreement with this, inactivation of Rad53 and Chk1 kinases does not cause the same phenotype as *rad9* mutations, suggesting that Rad9 plays a checkpoint signaling independent function.

This role was latter observed also in mammalian cells where 53BP1 recruits RIF1, RAP80 and PTIP (see Figure 8) at DSB and together they limit RPA and RAD51 foci formation, two clear markers of resected DSB (Feng et al, 2013; Zimmermann et al., 2013). As resection is the molecular switch between HR and NHEJ, 53BP1 is considered the main factor determining the balancing between these two repair pathways (Ramnarain et al., 2014). 53BP1-dependent NHEJ is indeed physiologically essential for the VDJ process during the immune system diversification, as *53bp1*$^{-/-}$ mice show severe adaptive immune response defects (Morales et al., 2003).

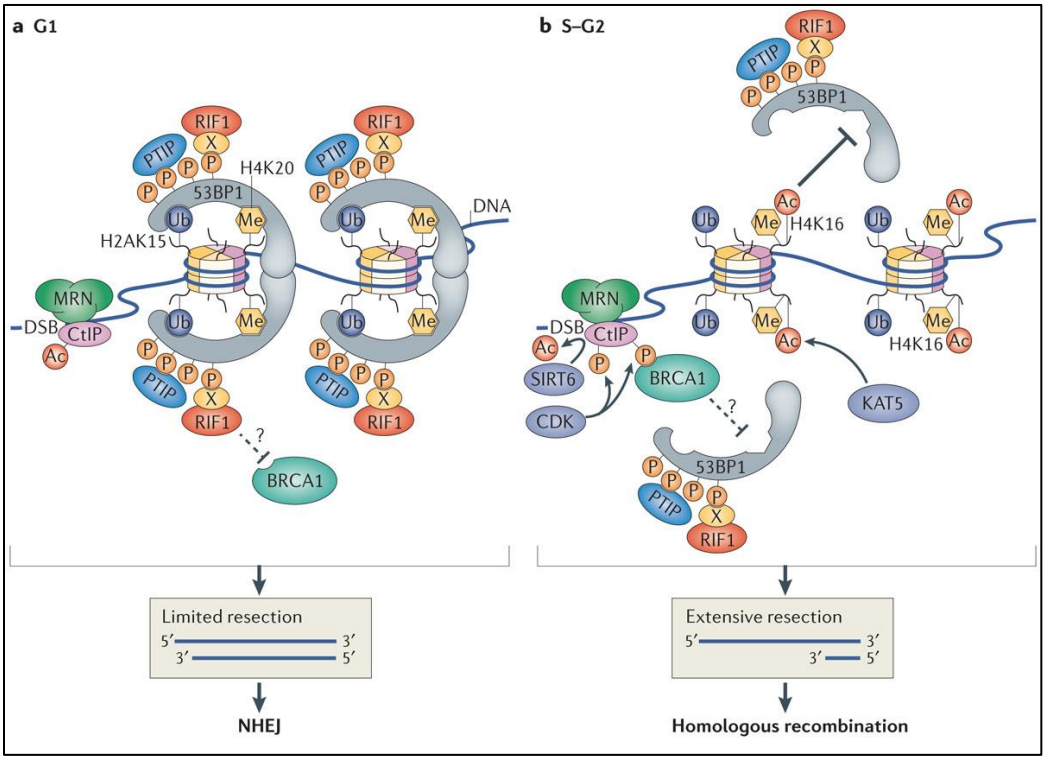

Figure 8: 53BP1 recognition of modified histones influences repair pathway choice (Panier & Boulton, 2014).

If on one end 53BP1 favors the classical NHEJ of DSB, BRCA1 favors the homology-directed repair through the interaction and stimulation with distinct factors (Densham et al., 2016; Yun & Hiom, 2009). Indeed, BRCA1 occupies in a mutually exclusive manner with 53BP1 DNA repair foci and is believed to stimulate resection start preventing NHEJ (Chapman et al., 2012; Feng et al., 2015) (see Figure 9). From a molecular point of view, BRCA1 interacts, among the others, with CtIP and stimulates its activity as resection promoting factor (Cruz-García et al., 2014; Yun & Hiom, 2009). Consistent with a

positive function in the HR repair, BRCA1 mutations predispose to breast cancer and interestingly 53BP1 depletion alleviates cellular proliferation and DNA damage hypersensitivity of *Brca1-null* cells (Bouwman et al., 2010; Bunting et al., 2010; Jaspers et al., 2012). In according with this, early embryonic lethality occurring in *Brca1-null* mice can be surprisingly suppressed by 53BP1 knock-out (Cao et al., 2009).

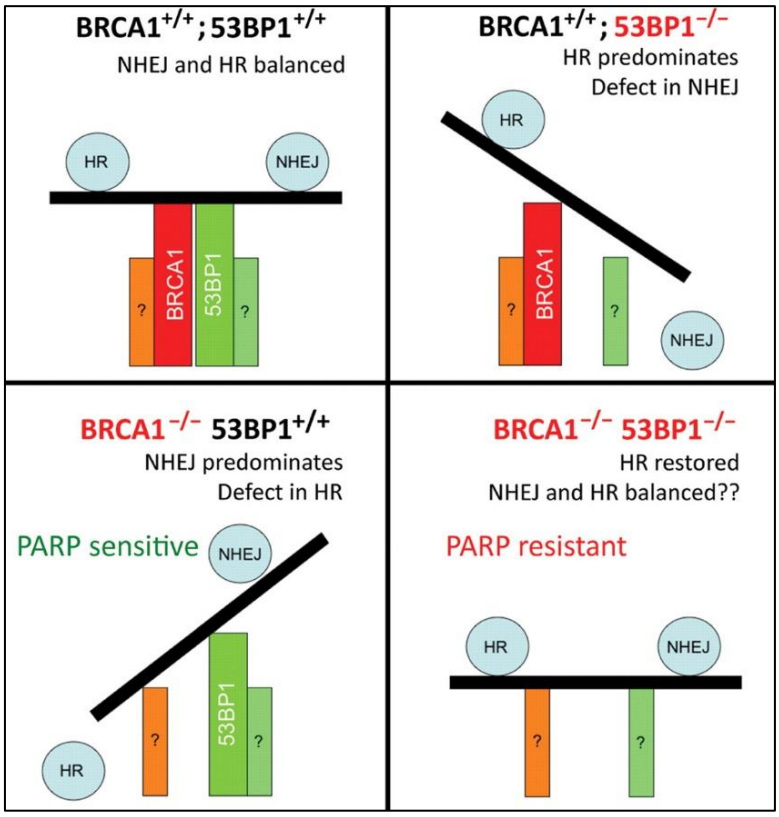

Figure 9: a schematic view of 53BP1 and BRCA1-mediated competition between HR and NHEJ repair pathways (Aly & Ganesan, 2011).

Checkpoint inactivation and cell cycle restart: adaptation and recovery

The main purpose of DNA damage checkpoint is the delay or arrest of the cell cycle in order to have more time to repair DNA lesions. Once lesions are repaired, checkpoint must be switched off to progress with cell cycle and to proliferate. This process of checkpoint inactivation after the damage is repaired, is usually termed *checkpoint recovery* (Bartek & Lukas, 2007).

Early studies reported that a significant yeast population normally has the ability to terminate checkpoint signaling, still in presence of unrepaired DNA lesions through a process called *checkpoint adaptation* (Lee et al., 1998; Moore & Haber, 1996; Sandell & Zakian, 1993). As the deletion of various genes impairs *checkpoint recovery* and/or *adaptation* in yeast, it is now clear that these two are active and genetically controlled processes.

Even though it is widely accepted that checkpoint inactivation requires the prior dephosphorylation and inactivation of the Rad53 kinase, how exactly this happens at the molecular level is not completely clear. During these processes, all the phosphorylation events that sustain checkpoint activation must be removed to interrupt the checkpoint signaling.

If the process of *checkpoint recovery* after repair of DNA lesions is rational, the biological significance of the *checkpoint adaptation* process is still under investigation. In budding yeast, the adaptation process with the progression into the following cell cycle can be considered as a last chance of cellular survival (Sandell & Zakian, 1993).

Even though adaptation was originally proposed to be orchestrated by the Cdc5 (orthologue of the human PLK1) and CKII kinases (Toczyski et al., 1997), over the years many proteins have been shown to collaborate in this process including factors like phosphatases and others involved in DNA

damage repair, chromatin remodeling and autophagy (Clerici et al., 2006; Dotiwala et al., 2013; Eapen et al., 2012; Lee et al., 1998, 2003).

Recently Dr. Smolka's laboratory identified a novel regulator of checkpoint recovery after replication stress in *Saccharomyces cerevisiae*. Authors reported a previously undescribed interaction between Slx4 and Dpb11 checkpoint factor mediated by the CDK1-dependent phosphorylation on Slx4 Serine 486 (Gritenaite et al., 2014; Ohouo et al., 2013). Multisequence alignment later identified the Threonine 1260 on hSLX4 as docking site for the interaction with hTOPBP1 (Gritenaite et al., 2014). In yeast, Slx4-Dpb11 interaction was proposed to compete with Dpb11-Rad9 interaction thus limiting the intensity of checkpoint signaling (see Figure 6). Cells lacking Slx4 show a Rad9-dependent checkpoint hyperactivation, which increase cellular sensitivity to replicative stress agents and impairs the recovery from the checkpoint arrest. Slx4-Dpb11 interaction is positively sustained by the Slx4 binding partner Rtt107 that anchors the complex to γH2A and by Mec1, as deletion of Rtt107 or mutation of a cluster of SQ/TQ sites in Slx4 phenocopies checkpoint hyperactivation reported in *slx4* mutant cells (Ohouo et al., 2013; Ohouo et al., 2010) (see Figure 10).

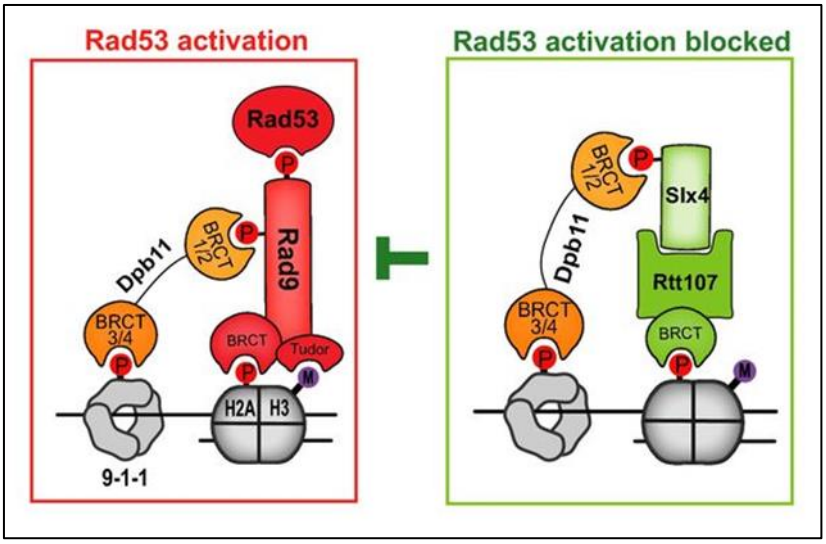

Figure 10: a competition-based mechanism between Slx4 and Rad9 determines Rad53 activation in *Saccharomyces cerevisiae* (Cussiol et al., 2015).

Recently, we observed that the Slx4-Rtt107 complex, through the regulation of Rad9 accumulation near DSB, regulates Rad53 signaling and the process of *checkpoint adaptation* (see the manuscript session) (Cussiol et al., 2016; Dibitetto et al., 2016).

Interestingly, *adaptation* has been observed also in higher eukaryotes supporting an important biological mean for this process. Recently, a study reported that a small population of U2OS cancer cells previously exposed to Ionizing Radiation and arrested in the G2 phase could progress to the following cell cycle with visible γH2AX foci, a clear marker of unrepaired DNA lesions (Syljuåsen et al., 2006). This observation may provide a molecular explanation of how cancer cells escape to the checkpoint arrest

preceding the onset of different genomic abnormalities and loss of genetic information.

Slx4 in genome stability maintenance

Synthetic Lethal of unknown function 4 (Slx4) was identified in a screening from Stephen Brill's group searching for genes synthetic lethal with the Sgs1 helicase in budding yeast (Mullen et al., 2001). Joint molecules and HJ can be repaired either through dissolution or resolution enzymatic reaction. As previously discussed, Sgs1 is the catalytic subunit of the dissolvasome complex (together with Top3 and Rmi1), and *sgs1*Δ cells accumulate several DNA structures especially after exposure to replicative stress compounds such as MMS.

Based on the rational of the screening, Slx4 and the other genes found are essential during joint molecule and HJ resolution, as accumulation of undissolved or unresolved DNA structures impairs cellular proliferation and causes cell death. *SLX* genes are associated in three distinct protein complexes and some of them exhibit strong nuclease activity based on *in vitro* characterization (Bastin-Shanower et al., 2003; Fricke & Brill, 2003).

Slx4 is stably associated with Slx1, a nuclease with a clear 5'-flap endonuclease activity *in vitro* (Fricke & Brill, 2003). Slx4 is essential for Slx1 activity during rDNA replication, where the complex nature of DNA template causes frequent fork collapse recovered by homologous recombination events. A recent study suggested that Slx4 competes with Slx1 homodimerization, a condition that normally inhibits Slx1 endonuclease activity (Yin et al., 2016). Another component of the Slx4-Slx1 complex is Rtt107/Esc4. Rtt107 is a gene originally identified as a Ty1 element mobility regulator with no clear conservation across the evolution (Scholes et al., 2001). Rtt107 is essential for

replication stress recovery and during the deposition of cohesins on the newly replicating sister chromatids (Leung et al., 2011; Ohouo et al., 2010; Roberts et al., 2005; Ullal et al., 2011). Its functions are promoted by various Mec1-dependent phosphorylation events on the C-terminal region in a Slx4 dependent manner, suggesting the possibility that Slx4 regulates Rtt107 localization and/or its association with the chromatin (Roberts et al., 2005).

In addition, Rtt107 seems to facilitate Slx4 localization during replication stress, as deletion of *RTT107* abolishes Slx4-Dpb11 interaction and Slx4 localization at DSBs lesions (Dibitetto et al., 2016; Ohouo et al., 2010).

Slx4 constitutively interacts with the Rad1-Rad10 heterodimer in a protein pool that is different from the one in complex with Slx1 and Rtt107 (Flott et al., 2007). Slx4 association with Rad1-Rad10 is relevant in the context of the SSA mode of repair as already discussed in a previous paragraph but not in the context of replication stress recovery (Flott et al., 2007; Toth et al., 2010).

Many of the Slx4 interactors that were originally found in budding yeast, were later found in a large proteomic study done in human cells (Svendsen et al., 2009). However, across the evolution mammalian SLX4 acquired other interactions that are essential for genome integrity maintenance, as it will be discussed in more details in the following paragraph.

Fanconi Anemia pathway

Fanconi anemia (FA) is a rare heterogeneous genetic disorder characterized by chromosome instability caused by germline biallelic mutations in genes of 20 different complementation groups (A-V) (reviewed in Kottemann & Smogorzewska, 2013; Michl et al., 2016; Walden & Deans, 2014). Patients develop various abnormalities like microcephaly, sarcopenia, immune system deficiency, congenital abnormalities and infertility. Although the clinical picture is usually complex and variegated, all FA patients are characterized by bone marrow failure and susceptibility to acute myeloid leukemia. At the cellular level, cells from FA patients are highly sensitive to agents that cause complex deleterious, interstrand crosslinks lesions (ICLs), such as Mytomicin C, Cisplatin and aldehyde derivatives (see these reviews Kottemann & Smogorzewska, 2013; Michl et al., 2016; Walden & Deans, 2014).

The high incidence and the early onset of cancer in FA patients highlights the importance of Fanconi Anemia pathway in chromosome stability maintenance and tumor suppression. Indeed, whereas mutations in upstream FA genes result in acute myeloid leukemia, heterozygous mutations in genes involved in the HR step of ICL repair predispose also to solid tumors (breast and ovarian cancer are the most frequent).

The precise molecular cause driving bone marrow failure in FA patients is far from being clear and recent studies proposed an interesting correlation between early aging and the global exhaustion of hematopoietic stem cells population in these individuals (Brosh et al., 2016). Other recent works explored a global change in epigenetic marks as an important source of genome instability in FA patients (Belo et al., 2015; Brosh et al., 2016).

Even if it is wide accepted that FA pathway acts primarily in the processing of ICLs lesions, many factors have been reported to exhibit also other functions. FANCD2 and FANCP (SLX4), for example, have been both

observed to mark slowly replicating genomic sites forming microscopy visible foci during late anaphase (Pedersen et al., 2015). Various reports have also described these same proteins to localize at telomeres in ALT cells, where telomere homeostasis is achieved through an alternative Homologous Recombination process (Sarkar et al., 2015; Wan et al., 2013; Wilson et al., 2013). Finally, R-loops-dependent replication inhibition triggers a robust activation of the FA pathway (García-Rubio et al., 2015).

The current model describing the FA pathway functioning in ICL repair consists of various steps each involving different proteins with specialized enzymatic activity (see Figure 11).

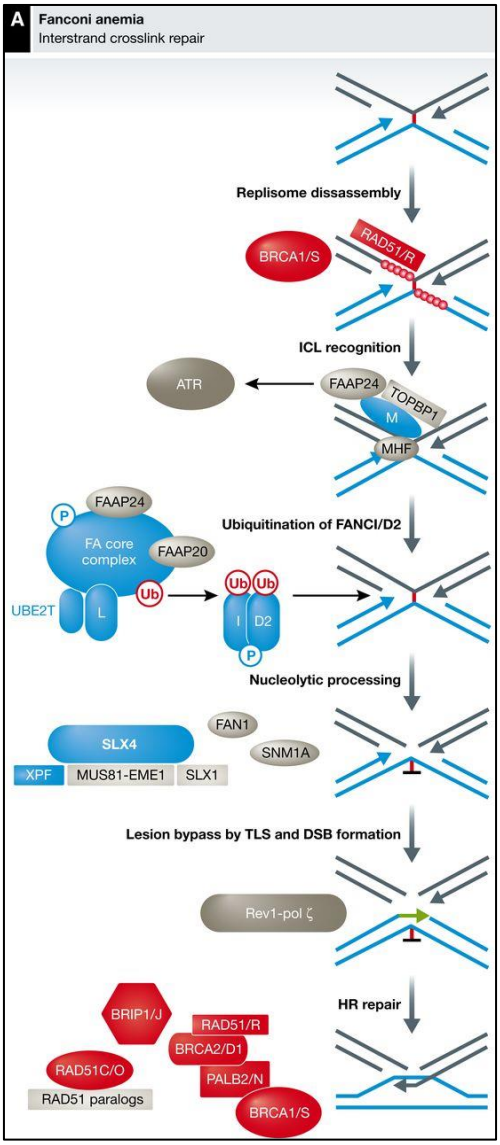

Figure 11: A schematic illustration of the Fanconi Anemia pathway in the repair of ICL lesions (Michl et al., 2016).

Lesion recognition

The main risk for ICL lesions occurs when replication fork encounters the inter-strand block. This modification impairs replicative helicases to unwind double helix allowing progression of replication. Therefore, replication forks, after approaching to the lesion site, pause at a distance of approximately 20 to 40 bp from the ICL, based on *in vitro* studies (Klein et al., 2014). Here replisome is dismantled and specific sensors, FANCM and FAAP24, recognize ICLs. These two factors recruit near ICL lesions the FA core complex and trigger the ATR/CHK1 checkpoint-dependent activation (Collis et al., 2008; Kim et al., 2008).

The FA core complex is composed by 14 different proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM, FANCT, FAAP100, MHF1, MHF2, FAAP20 and FAAP24) (reviewed in Walden & Deans, 2014) and its recruitment on the chromatin triggers downstream FA repair pathway activation. Once recruited, this complex exhibits a strong ubiquitin ligase activity, in particular through the catalytic subunits FANCT and FANCL, the E2 and E3 enzymes respectively (Alpi et al., 2008; Machida et al., 2006). FANCD2-FANCI heterodimer is the first target of the core complex, as monoubiquitinated FANCD2/I foci are widely accepted marker of ICL sites (Smogorzewska et al., 2007; Taniguchi et al., 2002). Ubiquitination of FANCD2 is a crucial event to recruit multiple downstream factors and it is a clear marker of FA pathway activation.

DNA incision

As FA pathway uses an HR-dependent process acting during DNA replication, an incision reaction is required to unhook the parental and the nascent strand, allowing the restoration of a template sequence (see below). Different

nucleases have been reported to be involved in the *incision* or *unhooking step* such as MUS81-EME1, SLX1-SLX4, XPF-ERCC4 and FAN1 (Y. Kim et al., 2013; Klein Douwel et al., 2014; Lachaud et al., 2016), but the latter seems to have a prominent role in the *incision* step (Klein et al., 2014). Even though the precise molecular mode of action is currently unclear, the XPF-ERCC4 activation requires the activation by SLX4/FANCP in a similar fashion to what was observed in yeast during the *Single Strand Annealing* mode of repair (Flott et al., 2007; Toth et al., 2010). A possible explanation would be that SLX4 modifies post-translationally XPF-ERCC4, as recently SLX4 has been observed to exhibit a SUMO E3 ligase activity towards different substrates among which XPF and SLX4 itself (Guervilly et al., 2015; Ouyang et al., 2015). Once unhooked the ICL, a DSB forms on the newly replicating strand whereas on the parental strand remains a short ssDNA gap in front of the lesion.

Lesion bypass

Multiple *Translesion Synthesis Polymerases* (*TLS)* work during lesion bypass introducing multiple mutations at the level of the ICL still present on the parental strand. Although different TLS polymerases have been associated with ICL repair (REV1 and REV3-REV7) both in yeast and in mammals, just recently two groups described a FA-like patient with germline mutations in the TLS polymerase REV7 (also known as MAD2L2) (Bluteau et al., 2016).

Lesion Repair

Given that ICL lesions results in fork stalling during DNA replication, the involvement of HR machinery for the repair is quite expected. Indeed inactivation of NHEJ machinery genes not only have not yet been found in FA patients, but depletion of NHEJ core complex does not result in ICL

sensitivity. Moreover, NHEJ inhibition alleviates genome instability and chromosomal aberrations induced by ICL in several FA patient cells (see below).

By contrast, many genes involved in homology-directed repair work in protection of nucleolytic degradation of stalled fork and nascent strand (Schlacher et al., 2012). Some of these genes (*e.g.* RAD51C/FANCR, BRCA1/FANCS, BRCA2/FANCD1 and PALP2/FANCN) have monoallelic or biallelic mutations with a significant frequency in the FA disease scenario. The proficient activation of FANCD2 by the core complex is normal in these FA patient cells and excludes any role in the apical branch of the FA pathway. The BRCA2-PALB2 complex works loading and stabilizing RAD51 on the ssDNA stretches on the broken end and for this reason, patients with mutations in these genes, develop the most dramatic phenotypes (Howlett et al., 2002; Xia et al., 2007). This not only serves during the latter strand invasion process but also protects replication fork from extensive nucleolytic degradation that would threaten the stability of the genome. A nice example of such a function is in a recent work where a FA-like patient with a negative dominant mutation in the *RAD51* gene that causes nascent strand degradation by DNA2 and WRN is described (Wang et al., 2015). Interestingly, cells from this patient are extremely sensitive to ICLs but still proficient in classical homology-directed repair (*e.g.* during IR repair or in GFP-reporter HR assays), suggesting that RAD51 is a multifunctional element in the FA pathway (Wang et al., 2015).

Further evidences that extensive DNA *resection* may be a detrimental event during ICL repair is given by the restored ICL sensitivity of FANCD2$^{-/-}$ cells upon DNA2 inactivation (Karanja et al., 2014). This suggests that FANCD2 might protect end degradation by CtIP-MRE11 dependent *resection.*

If on one hand extensive *resection* is believed to be a major cause of genomic aberrations, on the other it is necessary to channel broken ends into HR

preventing the NHEJ mode of repair in S phase. In agreement with this, FANCD2 has been recently shown to recruit TIP60, a histone acetylase that modifies lysine 16 of the histone H4 (Renaud et al., 2016). This is known to counteract 53BP1 recognition of histone H4K20me2, one of the main modification that recruits 53BP1 near damaged chromatin (Hsiao & Mizzen, 2008; Tang et al., 2013) (see Figure 8). This event has been proposed to limit 53BP1-RIF1 accumulation, favoring the *resection* process and therefore is of pivotal importance for opposing NHEJ-dependent repair of broken ends. In line with this idea, inactivation of NHEJ either through KU70 downregulation or through the chemical inactivation of DNA-PKcs, partially restores crosslink sensitivity of FANCA and FANCD2 human cells and *Fanca* and *Fancc* MEFs (Adamo et al., 2010; Sivasubramaniam & Patel, 2010).

Moreover FANCD2 was also shown to physically interact with CtIP stimulating its *resection* activity thereby inhibiting the NHEJ process (Unno et al., 2014).

SLX4/FANCP in the FA pathway and in protecting genome integrity

A few years ago, two reports described new patients with all the characteristics of FA patients at the molecular level (chromosomal instability and ICL sensitivity), carrying previously undescribed mutations in the *SLX4* gene. In one case, the individual, EUFA-1354, had a homozygous 1-bp deletion that generates a frameshift and a premature stop codon in the N-terminal region of the protein (Stoepker et al., 2011). In another case, a FA patient (RA-3331 cells) carried two different allelic mutations that result both in frameshifts with premature stop codon at aminoacid 172 and 672 respectively (Y. Kim et al., 2011). These cells are hypersensitive to MMC treatment, as observed also in EUFA-1354 patient, but displayed normal FANCD2 ubiquitination. Moreover, the hypersensitivity to different genotoxic compounds (such as CPT) and its well-characterized function in lower eukaryotes strongly suggested that SLX4 collaborates in the HR repair pathway also in human cells.

FA disease-like phenotype was also observed in mice Btbd12$^{-/-}$, the murine homolog of human SLX4 (Crossan et al., 2011). These mice display abnormalities in the shape of central neural system, alterations in proliferation of hematopoietic progenitor cells and defective spermatogenesis. From a cellular point of view, MEFs isolated from these mice were hypersensitive to MMC and CPT and show a large number of broken and radial chromosomes after treatment with these compounds (Crossan et al., 2011).

Mammalian SLX4 acquired throughout the evolution new interactions with MUS81, MSH2-MSH3 mismatch repair proteins, PLK1 and TRF2 (Svendsen et al., 2009) (see also Figure 12). The interacting regions with XPF-ERCC1 (Rad1-Rad10) and SLX1 (Slx1) are strongly conserved between yeast and human and are located in the N and C terminal region of the protein respectively (Figure 12). In lower eukaryotes, the interactions with Mus81 and

Cdc5 (hPLK1), which in mammalian cells are constitutive, seem apparently not direct but mediated by the formation of a bigger complex with Dpb11 (Gritenaite et al., 2014).

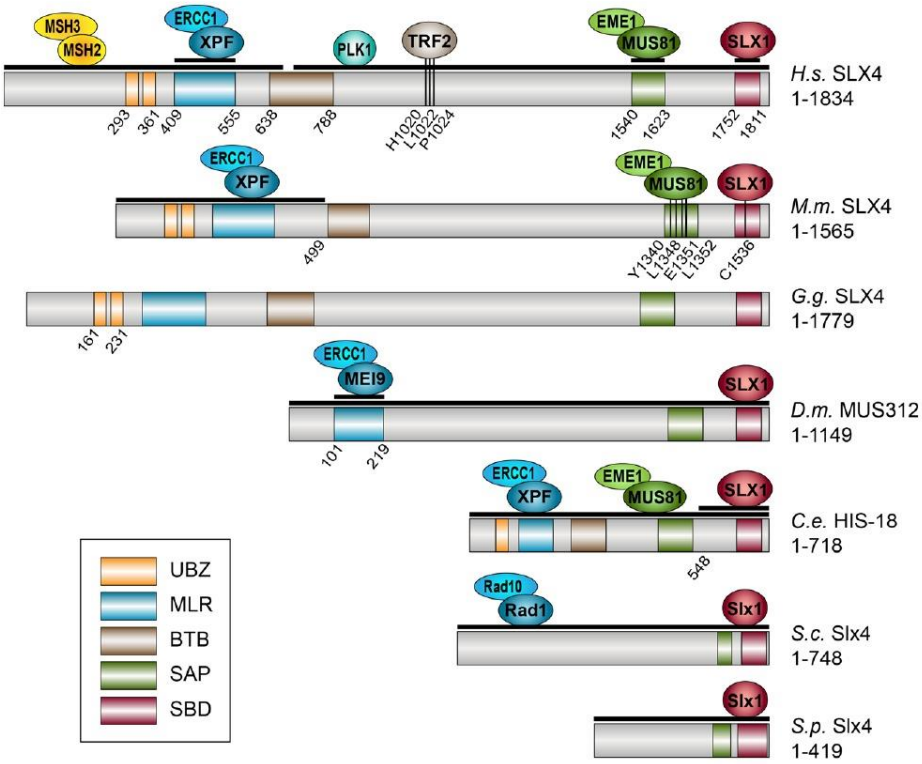

Figure 12: Alignment of Slx4 domains and interactors across the evolution (Y. Kim, 2013).

It is interesting to note that since now XPF is the only nucleolytic enzyme whose inactivation predispose to FA disease even though other nucleases like

MUS81 and SLX1 contribute in homology-directed repair (Fekairi et al., 2009; Muñoz et al., 2009; Svendsen et al., 2009). The fact that XPF is the only gene of the SLX4 complex to be clearly involved in the FA disease can be explained by its prevalent function in the unhooking process (see above) (Klein et al., 2014). For this reason, more efforts are required to uncover novel genes involved in FA pathway.

The association of SLX4 with SLX1 and MUS81 becomes particularly relevant in the context of replication restart or rDNA locus replication. SLX4-mediated cleavage of collapsed forks prevents the formation of HJ-like structures between palindromic sequences in regions highly repeated (Fekairi et al., 2009). Besides that, the SLX4 scaffold cleaves a wide range of cruciform structures deriving from the recombination-dependent replication restart. This is an agreement with a previous report where the highest level of SLX4 mRNA has been found in mice testis and oocytes (Su et al., 2002), likely suggesting a SLX4 essential role during meiotic crossing-over.

How SLX4 is regulated during DNA damage response and eventually how its action is coordinated with other FA components is not known. A recent study reported that SLX4 recruitment to damaged sites depends on the two UBZ domains present in the N-terminal region of the protein suggesting that its localization may depend on the ubiquitin pathway (Lachaud et al., 2014). None of the E3 ubiquitin-ligases BRCA1, RNF8, RNF168 nor ubiquitylated FANCD2 are required to recruit SLX4 at these sites (Lachaud et al., 2014), even though FANCD2 was previously shown to physically interact with SLX4 (Yamamoto et al., 2011).

Over the last years, many groups described SLX4 as an essential platform for telomeric DNA methabolism (Sarkar et al., 2015; Wan et al., 2013; Wilson et al., 2013). Telomeres are highly dynamic elements with repeated sequences where normal processes of replication and recombination often create specific

structures. In particular, SLX4 contribution in these processes is exacerbated in ALT cells, where telomere elongation is achieved through recombination events on the repeated sequences of other chromosomes. Here SLX4 is a scaffold for SLX1, XPF and MUS81 for the cleavage of replication, recombination intermediates and T-loops. As previously discussed, SLX4 physically interacts with TRF2 and this interaction enables SLX4 localization to long telomeres. Loss of SLX4 causes a wide aberrations of telomeric elements such as overlengthening, partial non replication and others (Sarkar et al., 2015; Wan et al., 2013; Wilson et al., 2013). In agreement with these data in humans, we recently found that Slx4 in budding yeast participates in checkpoint silencing regulation at uncapped telomeres, favoring cellular proliferation and avoiding permanent cell cycle arrest (Dibitetto et al., 2016).

As discussed in the previous paragraph, FA patients develop solid tumors earlier and with a high incidence. Over the last years, various groups sequenced large cohorts of patients with breast tumors, with no mutations in the *BRCA1/2* genes, to find a possible correlation between *SLX4* mutations and breast cancer predisposition (Catucci et al., 2012; Shah et al., 2013). Out of almost 2000 patients from different countries, besides the multiple missense mutations, only two truncations in the *SLX4* gene were found and this raised the possibility that *SLX4* may not be a breast cancer suppressor gene as *BRCA1/2* and *PALB2* (Catucci et al., 2012; Shah et al., 2013). A possible hypothesis could be that SLX4 may become essential as a tumor suppressor in those cells with other mutations in checkpoint or DNA repair enzymes, as we recently suggested in yeast Slx4 functioning (Dibitetto et al., 2016).

Aims of the Project

In this thesis, I'm presenting work related to different projects but all characterized to a deeper cohomprension of Rad9/53BP1 functions and regulation.

Aim 1: To characterize the regulation of Rad9 dynamics at DSB lesions through the study of Slx4-Rtt107 complex functioning

At DSB, homology-directed repair is achieved after the creation of ssDNA stretches generated by the resection process. In parallel, ssDNA accumulation triggers the DNA damage checkpoint that in turns avoids the arise of chromosomal aberrations arresting the progression through the cell cycle. Besides that, DDC supports resection making chromatin more accessible for repair enzymes and modifying their affinity for repair intermediates.

Different redundant enzymes participate in the resection process among which the most important are Sae2-MRX complex, Exo1 and Sgs1-Dna2. This process is counteracted by the physical interaction of the checkpoint adaptor Rad9/53BP1 with modified histones and the checkpoint platform Dpb11/TOPBP1. Rad9 is the central transducer of DDC and promotes the full activation of effector kinases Rad53 and Chk1.

Rad9-dependent checkpoint signaling during replicative stress has been recently shown to be counteracted by Slx4, through a competition-based mechanism that inhibits Rad9-Dpb11 interaction. In brief, upon MMS treatment, Professor Smolka and his group proposed that Slx4 and Rtt107 compete with Rad9 for the interaction with γH2A and Dpb11 respectively. Cells lacking a functional Slx4-Rtt107 show a stronger Rad9-Dpb11

interaction and thus a more intense checkpoint signaling. This ultimately impairs checkpoint recovery after DNA damage and cellular proliferation.
Our specific aim was to investigate a positive role of the Slx4-Rtt107 complex in checkpoint regulation and 5'-3' resection at double strand breaks rather than only during replication stress.

Aim 2: To characterize Dpb11/TOPBP1 contribution in the recruitment of the anti-recombination factor Rad9/53BP1

Homologous recombination is an essential pathway to repair DSB and restart replication after fork collapse. Depending on the cell cycle phase, homology-directed repair is counteracted by NHEJ enzymes whose mode of action is the immediate ligation of broken ends. Resection, the 5' strand degradation of DSB ends, is the molecular switch between HR and NHEJ and Rad9/53BP1 is a key regulator of this balancing. Others and we previously proposed both in yeast and in mammals that Rad9/53BP1 accumulation around DSB lesions facilitates the unfaithful NHEJ repair pathway preventing the resection process and thus HR.
Rad9 has been shown to interact with Dpb11 in yeast and humans and this interaction is essential to amplify the DNA Damage Checkpoint signaling. In yeast, Slx4 has been shown to interact with Dpb11, competing with Rad9 and we proposed that this interaction positively supports DSB resection through the inhibition of the Rad9-Dpb11 complex formation. Moreover, in humans, 53BP1-TOPBP1 interaction, which is particularly relevant in the context of the G1 checkpoint, is abrogated upon S-phase entry, suggesting that the inhibition of the TOPBP1-53BP1 interaction might favor homology-directed repair.

This body of evidences suggested a functional model where Dpb11/TOPBP1 is a dynamic platform for the recruitment of pro- and anti-recombination factor with the aim of recruiting and excluding Rad9/53BP1 in a spatially and timely regulated manner.

Our research aim was to investigate what is the contribution of Dpb11/TOPBP1 in the recruitment and stabilization of Rad9/53BP1 at DNA lesions in yeast and humans. Furthermore, this project was aimed to characterize whether an artificial stabilization of Dpb11-Rad9 interaction in yeast and TOPBP1-53BP1 in human cells could inhibit the resection process with a clear implication and interest in genome stability and the cancer research field.

Important Result & Conclusions

1. Functional Interplay between the 53BP1-Ortholog Rad9 and the Mre11 Complex Regulates Resection, End-Tethering and Repair of a Double-Strand Break

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Synopsis of the work and specific contributions:

In all the eukaryotes, Sae2/CtIP is a crucial factor for resection initiation and repair of DNA DSBs. Even though its precise mode of action is still poorly understood, it is now widely accepted that Sae2 primes resection activating the endonucleolytic MRX/MRN complex, determining the repair pathway choice between Homologous Recombination (HR) and Non Homologous end Joining (NHEJ).

In this study, we observed a novel Rad9 checkpoint-independent function in inhibiting resection initiation and repair of DNA breaks, in the absence of a functional Sae2-MRX complex. More in details, we proposed a model where Rad9 protects DSB ends from extensive degradation that occurs prior to mutagenic repair events such as microhomology mediated end joining or single strand annealing (SSA), likely explaining at the molecular level how Rad9 determines DSB repair pathway choice.

In brief, we started our study from the observation that *RAD9* deletion rescues the inability of *sae2*Δ cells in the SSA mode of repair (Fig.1). We also found that *RAD9*-dependent rescue of *sae2*Δ cells completely depends on the Sgs1-

Dna2 pathway but not on the Exo1, suggesting that Rad9 resection-barrier specifically inhibits the Sgs1-Dna2 branch.

To further proof these results, we took advantage of a genetic system where we could carefully measure resection by a quantitative Real Time PCR-based approach. *RAD9* deletion significantly increases resection initiation at DSB ends in *sae2*Δ cells in a Sgs1-dependent manner, reinforcing previous results obtained by viability assays and Southern blotting (Figure 1 and 2 respectively). On this line, we investigated whether *RAD9* deletion might rescue the MRX persistence near broken ends and affect the loading of a pro-recombinogenic factor such as Rad52. Surprisingly, we found that *RAD9* deletion significantly accelerates Mre11 removal and Rad52 foci formation in *sae2*Δ cells, facilitating HR and promoting DSB repair.

Finally, by introducing different Rad9 mutations in *sae2*Δ cells, we aimed to characterize which are the factors that recruit Rad9 near broken ends to inhibit resection initiation.

Finally, we substituted to Alanine different Rad9 residues and we replaced Rad9 mutated alleles to see which are the minimal interactions essential to inhibit DSB repair in *sae2*Δ cells. Substitution of two residues (Serine 462 and Threonine 474) to Alanine that specifically abrogate Rad9-Dpb11 interaction dramatically reduced Rad9 recruitment to DSB by ChIP analysis and thus efficiently rescued SSA repair in *sae2*Δ cells. In contrast, impaired Rad9 interaction with modified histones H3 and γH2A did not or only mildly decreased Rad9 binding to DSB lesions suggesting that the major pathway that recruits Rad9 in *sae2*Δ cells is the Dpb11 checkpoint platform.

As previously discussed, Rad9 resection-inhibition function is highly conserved with its mammalian orthologue 53BP1. Many groups reported that 53BP1 depletion increases RAD51 and RPA foci formation decreasing the

efficiency of NHEJ repair. More recently, a paper reported that 53BP1 downregulation, increasing resection

speed, predispose cells to mutagenic SSA repair events increasing short and extensive genomic deletions. Taking this body of evidence in consideration, we think our report will certainly contribute to better understand how exactly 53BP1 protects genome integrity at the molecular level. In the future, it will certainly be interesting to investigate whether a BLM (human Sgs1 orthologue) mediated resection is essential for MRE11 removal and resection initiation in 53BP1 depleted cells.

In this study, I contributed to generate several mutant strains used throughout the work. I also performed cellular viability assays shown in Figure 1 and 5, together with the Rad9 Chromatin Immunoprecipitation shown in Figure 5. Moreover, I participated to the general discussion of all the obtained results and to the manuscript preparation.

2. Slx4 and Rtt107 control checkpoint signaling and DNA resection at double-strand breaks

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Synopsis of the work and specific contributions:

The Slx4-Rtt107 complex is a crucial component for the maintenance of genome stability as a scaffold for structure specific endonucleases during Holliday junction resolution and cleavage of collapsed forks.

More recently, Slx4 together with Rtt107 has been shown to biochemically compete with Rad9 for its binding partner Dpb11, dampening the Rad53 checkpoint signaling during replication stress.

In this study, we expanded previous findings observing a Slx4 function in inhibiting Rad9-Dpb11 association also at DSBs, controlling both the checkpoint signaling and, more importantly, the Rad9 resection barrier.

As first, we used a system where we could conditionally induce a single and irreparable DSB on Chr III, the JKM139 genetic yeast background. In this system, we observed that *slx4*Δ and *rtt107*Δ cells show a more robust and prolonged Rad53 phosphorylation compared to control cells if assayed by Western blot technique with specific antibodies against the protein. This impaired the resumption of the cell cycle through the adaptation process

scored by a micro-colony formation assay. Importantly, a single Slx4 point mutation, Serine 486 to Alanine, which abrogates Slx4-Dpb11 interaction completely phenocopied *SLX4* deletion in terms of checkpoint signaling and microcolony formation. According to our model, where Slx4-Rtt107 functions at DSB are a functional competition with Rad9, *RAD9* deletion rescued microcolony formation defects of *slx4*Δ, *rtt107*Δ and slx4-*S486A* cells.

This suggested that Slx4 functions at DSBs essentially depend upon Dpb11 interaction. In agreement with this, Slx4 binding analyzed through ChIP analysis depends upon Rtt107 and Dpb11 interaction as in *rtt107*Δ cells or in cells expressing a slx4-*S486A* allele, Slx4 binding is almost abolished near DSB. In these same regions (5 kb from DSB ends) we found by ChIP that Rad9 accumulates more intensively in *slx4*Δ, *rtt107*Δ and slx4-*S486A* cells providing for the first time evidences of an *in vivo* competition between the Slx4-Rtt107 complex and Rad9 for DSB lesions. We expanded these results involving Professor Grant W. Brown (University of Toronto, CA) who performed Rad9 ChIP-Seq analysis all along the chromosome III in control cells and *slx4*Δ.

According to our functional model, Slx4-Rtt107 depleted cells show a significant reduction in ssDNA accumulation at distal but not proximal regions from broken ends, suggesting that Rad9 inhibition is a dynamic process occurring as resection is ongoing. As earlier discussed, *RAD9* deletion completely bypassed these defects, suggesting that the main function of the Slx4-Rtt107 complex is the exclusion of Rad9 from DSB.

In second part of the work, we explored a possible genetic interaction between Slx4 and Sae2, a protein that we previously show to work on resection initiation. Combined deletion of *SAE2* and *SLX4* results in a severe synergistic reduction in ssDNA exposure at proximal and distal regions. This dramatic decrease in resection speed resulted in a severe DSB repair defect measured

by a typical ectopic gene conversion system assayed with viability tests and Southern blotting techniques.

Our molecular data explained the dramatic sensitivity of *sae2*Δ *slx4*Δ, *slx4*Δ *rtt107*Δ and *slx4*Δ slx4-*S486A* to genotoxic stress induced by MMS and Camptothecin. Importantly *RAD9* deletion suppressed the genotoxic sensitivity of these double mutants.

This is my main PhD project, and since the first year, I worked producing various yeast strains that have been used in all the figures. I performed experiments related to checkpoint analysis in Figure 1, Slx4 ChIP analysis in Figure 2, Real Time resection analysis in Figure 3E-D, epistatic analysis with Sae2 in Figure 4, viability assays and Southern blot in Figure 5 and MMS and CPT sensitivity drop test on Figure 6C. Hence, I contributed to the manuscript preparation and revision of the manuscript.

3. Slx4 scaffolding in homologous recombination and checkpoint control: lessons from yeast

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Synopsis of the work and specific contributions:

Over the last two years we intensively collaborated with Dr. Marcus B. Smolka (Cornell University, Ithaca, NY) focusing on the role of Slx4 protein in the regulation of checkpoint signaling and resection during DSB response.
In this paper, we reviewed data from the literature integrating recent updates coming from our laboratories studying a Slx4 novel function in controlling checkpoint signaling and resection at DSBs. Even though this review focuses primarily on recent updates from yeast, we discussed how these findings could be relevant also in the cancer research field.
Here I contributed writing the paragraph concerning Slx4 role in the control of resection and checkpoint at DSB and revising the manuscript draft prior submission.

4. TOPBP1/Dpb11 controls DNA repair through the coordinated recruitment of 53BP1/Rad9

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Synopsis of the work and specific contributions:

Homologous Recombination is essential for repair of DSB lesions, replication restart and cancer suppression. By using different biochemical approaches, we investigated the role of the evolutionary conserved Dpb11/TOPBP1 in the control of Homologous Recombination process. Early studies in budding yeast unraveled a positive function uncovered by Dpb11 in the Mating Type Switching, an HR process used by *Saccharomyces cerevisiae* in the sexual isotype switch. Recently human TOPBP1 has been shown to facilitate RAD51 foci formation at DSB by promoting its PLK1-mediated phosphorylation, an event essential for RAD51 nucleation. Based on our previous studies in yeast, we investigated a different role of Dpb11/TOPBP1 in coordinating the recruitment of anti- and pro-recombination factors at DSB lesions.

We generated a chimeric protein fusing part of the BRCT domains of Dpb11 with the full-length Rad9 protein, the main antagonist of resection and Homologous recombination processes. By ChIP analysis done in yeast, we observed that Dpb11-Rad9 chimera is heavily recruited near DSB lesions and there severely blocks resection at distal regions from DSB ends if analyzed by

Real Time PCR. In agreement with this, Dpb11-Rad9 chimera inhibits formation of RPA and Rad52 foci during replicative stress.

In human cells, adopting a wide proteomic approach, we found that TOPBP1 interacts with and mediates the recruitment of 53BP1 and the pro-recombination factor BRCA1 in a mutually exclusive manner as we previously saw between Rad9 and Slx4 in budding yeast. TOPBP1-BRCA1 interaction is positively regulated by ATR phosphorylation in a similar fashion to what previously observed in the interaction between Dpb11 and Slx4 in yeast.

Finally, similarly to what we did in budding yeast, we fused the full length 53BP1 to a CTR sequence that mediates a constitutive interaction between 53BP1 and TOPBP1. Cells expressing CTR-53BP1 enhanced foci formation of pro-NHEJ factors such as RIF1 and RAP1 during replicative stress and increased the formation of chromosomal aberrations such as metaphase breaks and radial chromosomes. Hence, in agreement with these findings, CTR-53BP1 never colocalized with RPA and RAD51 foci. Together with these results, we proposed a novel TOPBP1 mode of action of how it regulates the balancing between NHEJ and HR repair pathways.

I worked in this project over the last year of my PhD and I mainly contributed with the analysis of Dpb11-Rad9 chimera ChIP and resection analysis in budding yeast that are presented in Figure 1. To this aim, I generated yeast strains, planned and performed experiments. In more details, I obtained that the Dpb11-Rad9 chimera, but not a mutated chimera where the Lysine 544 responsible for the interaction between Dpb11 and Ddc1 has been substituted to Alanine, binds in the early surrounding region of DSB ends. Moreover, resection analysis done with a Real Time PCR-based approach shows that Dpb11-Rad9 chimera severely block ssDNA exposure compared to wild type cells as resection is ongoing far away from broken ends. These experiments

provided evidences that the Dpb11-Rad9 chimera is functional and works inhibiting the resection process, likely masking the dsDNA/ssDNA junction, the accessible entry point for the nucleases.

Besides that, I was also involved in experimental planning, data analysis and manuscript preparation and for this reason my contribution was recognized with a Co-first authorship.

Discussion and Future Perspectives

Considering the various aspects and factors discussed in this thesis, I chose not to present a general discussion. Instead, a detailed discussion with future implications of the presented findings is presented in the dedicated session of each published and submitted manuscript.

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Part II

Published Paper I

Functional Interplay between the 53BP1-Ortholog Rad9 and the Mre11 Complex Regulates Resection, End-Tethering and Repair of a Double-Strand Break

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Functional Interplay between the 53BP1-Ortholog Rad9 and the Mre11 Complex Regulates Resection, End-Tethering and Repair of a Double-Strand Break

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Abstract

The Mre11-Rad50-Xrs2 nuclease complex, together with Sae2, initiates the 5′-to-3′ resection of Double-Strand DNA Breaks (DSBs). Extended 3′ single stranded DNA filaments can be exposed from a DSB through the redundant activities of the Exo1 nuclease and the Dna2 nuclease with the Sgs1 helicase. In the absence of Sae2, Mre11 binding to a DSB is prolonged, the two DNA ends cannot be kept tethered, and the DSB is not efficiently repaired. Here we show that deletion of the yeast 53BP1-ortholog *RAD9* reduces Mre11 binding to a DSB, leading to Rad52 recruitment and efficient DSB end-tethering, through an Sgs1-dependent mechanism. As a consequence, deletion of *RAD9* restores DSB repair either in absence of Sae2 or in presence of a nuclease defective MRX complex. We propose that, in cells lacking Sae2, Rad9/53BP1 contributes to keep Mre11 bound to a persistent DSB, protecting it from extensive DNA end resection, which may lead to potentially deleterious DNA deletions and genome rearrangements.

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Introduction

Similarly to what is seen in higher eukaryotes, in *S. cerevisiae* the ends of a double-strand DNA break (DSB) are recognized and bound by the Mre11-Rad50-Xrs2 (MRX) complex and the Ku70-Ku80 heterodimer, which compete for end binding. Once the MRX complex, together with CDK1-phosphorylated Sae2 (CtIP in human), initiates resection of the DNA ends, Ku70-Ku80 binding and NHEJ (non-homologous end-joining) are prevented [1,2,3,4]. Subsequent 5′–3′ long-range resection can then occur by one of two pathways: the first utilizes the RecQ helicase Sgs1 (BLM in human), in cooperation with the endonuclease Dna2, and the second utilizes the exonuclease Exo1 [5,6,7,8,9].

The regulation of DSB end resection is very important to choose the right pathway to repair a DSB and avoid chromosomal rearrangements [10,11]. Whereas classical NHEJ requires little or no resection, HR (homologous recombination) is characterized by extensive exonucleolytic degradation of one strand. Blocking DNA end resection affects the efficiency and accuracy of how a DSB is repaired. For example, inhibiting resection leads to de novo telomere addition, and eventually loss of a portion of a chromosome [12,13]. On the other end, extensive DNA end resection could lead to accumulation of unstable DNA intermediates and eventually to the highly error-prone microhomology-mediated end joining (MMEJ) and single-strand annealing (SSA) events, which may cause DNA deletions and translocations [14,15,16].

It is now clear that the DNA damage checkpoint response (DDR) plays a central role in regulating DSB end resection. In fact, while resection proceeds, the formation of RPA-coated ssDNA activates the upstream kinase Mec1 (ATR in mammals) and the effector kinase Rad53 (Chk2 in mammals), which in turn phosphorylates and inhibits Exo1 [17]. Interestingly, Exo1 is regulated through a DDR pathway in human cells, too [18,19].

Moreover, studies both in yeast and mammals showed that Exo1 and other DNA end-processing enzymes are inhibited through a physical structural "barrier" formed by Rad9 oligomers (53BP1 in mammals) bound near a DSB [10]. *RAD9* was originally identified as the first checkpoint gene in *S. cerevisiae* and recognized as an "adaptor" protein, linking the upstream kinase Mec1 to the activation of effector kinases Rad53 and Chk1. Rad9 is recruited to chromatin through three different pathways: i) the constitutive interaction with the histone H3 methylated at the K79 residue by Dot1 [20,21,22]; ii) the binding to the histone H2A phosphorylated at the S129 residue by Mec1 [23]; iii) the interaction with Dpb11 [24,25]. In particular, phospho-H2A mediated Rad9 recruitment spreads many kilobases around a

Author Summary

DNA double strand breaks (DSBs) are among the most deleterious types of damage occurring in the genome, as failure to repair these lesions through either non-homologous-end-joining (NHEJ) or homologous recombination (HR) leads to genetic instability. The 5′ strand of a DSB can be nucleolytically degraded by several nucleases and associated factors, including Mre11, CtIP/Sae2, Exo1 and Dna2 together with Bloom helicase/Sgs1, through a finely regulated process called DSB resection. Once resection is initiated, error-prone NHEJ is prevented. Several findings suggest that DSB resection is a double-edged sword, if not finely regulated, since on one hand it is needed for faithful HR, but on the other it may lead to extensive DNA deletions associated with genome instability. Both in mammals and yeast, 53BP1/Rad9 protein binds near the lesion and counteracts the resection process, limiting the formation of ssDNA. By using *S. cerevisiae* as a model organism, here we show that Rad9 oligomers block the removal of hypo-active Mre11 protein from a persistent DSB, thus limiting initiation of resection and the recruitment of the recombination factor Rad52, in the absence of Sae2. Altogether, these findings pinpoint a critical role of 53BP1/Rad9 in balancing HR and NHEJ repair events throughout the cell cycle.

DNA lesion [26]; whereas Dpb11 appears to be more specific at the site of lesion, by binding to a damage-induced phosphorylation in the Ddc1 subunit of the 9-1-1 complex [25,27,28]. All of these three pathways cooperate for efficient checkpoint arrest and cell survival after genotoxic treatments throughout the cell cycle. Moreover, Rad9 contains motifs that are necessary for its oligomerization and DNA damage checkpoint signalling [24,29,30].

Notably, the Rad9-mediated inhibition of DSB resection is a regulatory function conserved throughout evolution. In fact, 53BP1 facilitates NHEJ at the expense of HR, protecting DNA ends from inappropriate 5' resection, in cooperation with the telomere binding protein RIF1 [31,32,33,34,35].

Here, we show that in the absence of Sae2, or in presence of mutations affecting Mre11 nuclease activity, Rad9 dimers and/or oligomers, recruited near a DSB mainly by Dpb11 interaction, inhibit the short-range DNA end processing, thereby preventing Mre11 removal from the lesion and limiting Rad52 recruitment by an Sgs1-dependent mechanism. As a consequence, DSB ends cannot be kept efficiently tethered to each other, and repair through an SSA process is prevented. We propose a novel molecular role of Rad9/53BP1 to protect genome integrity from extensive DNA degradation and rearrangements during DSB repair, also suggesting important implications for malignant transformation in mammalian cells.

Results

Deletion of *RAD9* gene rescues DSB repair defect in *sae2*Δ cells through an Sgs1-Dna2 dependent pathway

It is known that deletion of the *RAD9* gene in yeast leads to faster DSB resection and repair through an SSA process [36,37]. To further understand the role of Rad9 in DSB processing and repair, we decided to combine the deletion of *RAD9* gene with mutations in genes encoding factors either involved in the short-range (*SAE2*), or the long-range (*EXO1*, *SGS1*) DSB resection [38]. We took advantage of the YMV80 background, in which the galactose-induced expression of the HO nuclease causes a single DSB at a specific site on chromosome III. Repair of this DSB occurs mainly through SSA between flanking homologous *leu2* repeats one of which is 25kb from the DSB [39]. We deleted *RAD9*, *EXO1*, *SGS1* and *SAE2* to obtain all viable single, double and triple mutant combinations. Although the *sae2*Δ *sgs1*Δ double mutant is a synthetic lethal combination [40,41], *rad9*Δ interestingly suppresses *sae2*Δ *sgs1*Δ lethality (S1A Fig.). Therefore, it was possible to test the *sae2*Δ *sgs1*Δ *rad9*Δ triple mutant cells. After plating the cells in the presence of galactose to induce one DSB, we found that viability of the *sae2*Δ and *sgs1*Δ single mutant and *sgs1*Δ *exo1*Δ double mutant was severely reduced (Fig. 1A), as expected [6,7,42]. We also found that the deletion of *RAD9* gene effectively rescued the viability of the *sae2*Δ, *sgs1*Δ and *sae2*Δ *exo1*Δ mutant strains following one DSB (Fig. 1A). Interestingly, the viability of the *sae2*Δ *sgs1*Δ *rad9*Δ and *exo1*Δ *sgs1*Δ *rad9*Δ triple mutant cells was very low in the presence of one DSB. Moreover, the HO-induced lethality of the *sae2*Δ *sgs1*Δ *rad9*Δ mutant was not rescued by the expression of the Sgs1-K706A protein variant (S1B Fig.), whose helicase activity is severely reduced [43]. While the failure to repair the DSB in the *exo1*Δ *sgs1*Δ *rad9*Δ triple mutant was expected, since at least one of the Exo1 and Sgs1-dependent pathways is necessary to extensively resect a DSB, the result obtained with the *sae2*Δ *sgs1*Δ *rad9*Δ mutant was surprising. We therefore concluded that an Exo1-independent, Sgs1-dependent pathway is necessary for the viability of *sae2*Δ cells following a DSB in the absence of *RAD9*.

Since Sae2 stimulates the activity of the MRX complex in the first step of the DSB end processing [44], we considered the possibility that *RAD9* deletion may also rescue an Mre11 nuclease defective mutant or the *rad50*Δ mutant, in which the MRX complex is disassembled. Interestingly, we found that *rad9*Δ suppresses the nuclease-defective *mre11*-D56N mutant [45], through an *SGS1*-dependent pathway, while it does not rescue *rad50*Δ mutant, as expected [36] (Fig. 1B). These results suggest that the nuclease activity of the MRX complex is dispensable for the DSB repair in *rad9*Δ cells; however, the MRX complex must be physically present, likely playing an essential structural role. Indeed, *rad50*Δ mutation does not rescue *sae2*Δ cell viability following a DSB (Fig. 1B). Of note, deletion of *RAD9* also suppresses the double mutant *mre11*-D56N *sae2*Δ, further indicating that Mre11 and Sae2 work together in the same pathway (Fig. 1B).

Importantly, the deletion of *RAD9* rescues *sae2*Δ cell viability through an *EXO1*-independent, *SGS1*-dependent pathway also in presence of camptothecin (Fig. 1C), a topoisomerase-aborting agent that causes formation of end-blocked DSBs [46].

To further investigate the findings shown in Fig. 1A at the molecular level, we tested the kinetics of DSB repair by Southern blotting in cells blocked in G2/M cell cycle phase by nocodazole. In agreement with the cell lethality reported in Fig. 1A, we found that the efficiency of the DSB repair is reduced in both the *sae2*Δ and *sgs1*Δ single mutants, as previously described [6,7,42], and it is severely compromised in *sae2*Δ *sgs1*Δ *rad9*Δ (Figs. 2B and 2C). On the contrary, DSB repair is accelerated and very efficient in the *rad9*Δ, *sae2*Δ *rad9*Δ and *sgs1*Δ *rad9*Δ mutants (Figs. 2B and 2C). These results indicate that, in the absence of Rad9, an Sgs1-dependent mechanism is necessary to efficiently repair a DSB in *sae2*Δ cells.

To test if Sgs1 cooperates with Dna2 to repair a DSB in *sae2*Δ *rad9*Δ mutant cells, we took advantage of an auxin-based degradable Dna2 protein variant (Dna2-DEG). This is a common genetic strategy to induce the degradation of a protein by the addition of auxin compound to the cell culture medium [47], and it is particularly useful in the case of an essential gene, such as

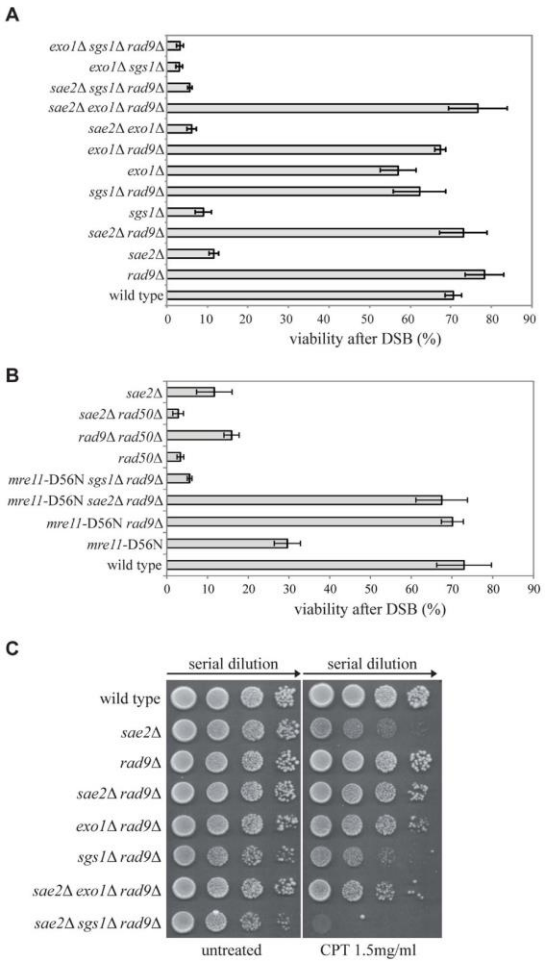

Fig. 1. Deletion of *RAD9* rescues *sae2*Δ and *mre11*-D56N cell viability following DSBs through *SGS1*. (A–B) Viability of the wild type YMV80 strain and the indicated derivatives plated on YEP+gal. In the presence of galactose, one HO-cut is introduced at *leu2* locus (see a scheme in Fig. 2A). For each strain, the number of colonies grown after 3 days at 28˚C in YEP+gal was normalized respect YEP+glu. Plotted values are the mean values ± SD from three independent experiments. (C) Exponentially growing cell cultures of the wild type YMV80 strain and the indicated derivatives were serially diluted (1:10), and each dilution was spotted out into YPD and YPD+camptothecin plates. Plates were incubated 3 days at 28˚C.
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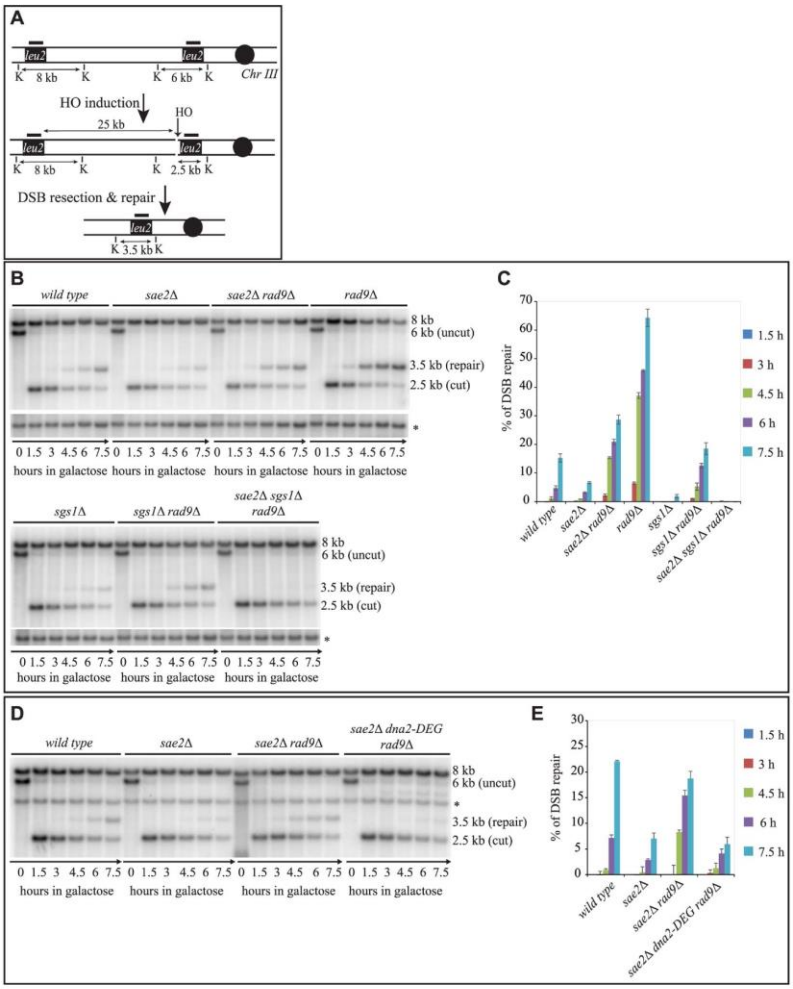

Fig. 2. Deletion of *RAD9* rescues DSB repair defects of *sae2*Δ cells through *SGS1* and *DNA2*. (A) Map of the YMV80 Chr III region, containing the HO-cut site. The indicated vertical bars show KpnI restriction sites. The short thick lines indicate the position where the probe hybridizes. After the HO mediated cleavage, DNA ends are resected. Once the indicated *leu2* cassettes have been exposed as ssDNA, repair through SSA can occur and be monitored by the appearance of an SSA product fragment by Southern blot. (B and D) Exponentially growing YEP+raf cell cultures of the wild type YMV80 strain and the indicated derivatives were synchronized and kept blocked in G2/M phase with nocodazole treatment; galactose was added at

time zero to induce HO-cut. *KpnI*-digested DNA was analysed by Southern blotting with a *LEU2* probe. An *ATG5* (uncut locus on chromosome XVI) probe was also used to normalize the signals. In (D) *LEU2* and *ATG5* probes were added contemporarily to the filter. (C and E) Densitometric analysis of the product band signals of the experiments shown in (B) and (D). The intensity of each band was normalized respect to unprocessed *ATG5* locus (*).

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DNA2. By Southern blotting analysis, we found that the *sae2*Δ *rad9*Δ double mutant cells do not repair a DSB in the absence of Dna2 (Fig. 2D and 2E). Therefore, taking all the data in Fig. 2 together, we concluded that the deletion of *RAD9* rescues *sae2*Δ cells through a DSB resection mechanism mediated by the Sgs1-Dna2 pathway.

In addition, we ruled out the possibility that in the absence of Rad9, the DSB can be repaired more efficiently through a strand invasion-based mechanism (such as a break-induced replication process [48]). In fact, we observed faster DSB repair and high viability when we analysed the *sae2*Δ *rad9*Δ *rad51*Δ triple mutant, in which break-induced replication is impaired, but SSA is not inhibited (S2 Fig.).

Rad9 limits an Sgs1- and Sae2- dependent initial step of DSB processing

A critical step to repair a DSB through SSA is 5′ to 3′ resection of the DSB end. Therefore, based on our results in Figs. 1 and 2, we hypothesized that in *sae2*Δ *sgs1*Δ *rad9*Δ triple mutant DSB resection may be affected, as it was shown in the *sae2*Δ single mutant [6,7,42], while it should be faster in *sae2*Δ *rad9*Δ double mutant. To test the kinetics of DSB processing we used JKM139 background derivatives, where prolonged expression of HO causes an irreparable DSB at *MAT* locus, because of the absence of *HML* and *HMR* homologous cassettes. Therefore, the analysis of the formation of the 3′ single-stranded (ss) DNA is not biased by a repair process [49]. Using Southern blotting of denatured DNA after restriction enzyme digestion [50], we tested the formation of the 3′ ssDNA filament (as depicted in Fig. 3A), after the induction of one DSB in each sister chromatid, in G2/M-blocked cells.

As expected, we found that the formation of a long 3′ ssDNA tail is slightly delayed in the absence of *SAE2*, *EXO1* and *SGS1* genes, and it is severely compromised in the *exo1*Δ *sgs1*Δ double mutant [6,7,51]. Interestingly, we found more extensive 3′ ssDNA in the absence of Rad9 in all the mutants tested, except the *exo1*Δ *sgs1*Δ *rad9*Δ triple mutant (Figs. 3B, 3C and S3). These results support the model that both the Exo1 and the Sgs1-dependent pathways cooperate to resect a DSB, and rule out the hypothesis that additional nuclease(s) may take over to process a DSB in the absence of Rad9. However, we noticed that in the *sae2*Δ *sgs1*Δ *rad9*Δ triple mutant strain the appearance of ssDNA is slightly delayed compared to wild type and *sae2*Δ *rad9*Δ strains (Figs. 3B and 3C). This result may suggest that the initiation of DSB resection is affected in *sae2*Δ *sgs1*Δ *rad9*Δ cells.

To test more precisely DNA processing near a DSB we employed a quantitative PCR-based method [52]. In particular, by this procedure we determined if the *Rsa*I restriction enzyme can cut the DNA at a specific site 150 bp from the HO-cut site, thus indicating whether DSB resection has already passed beyond this site, since, as resection proceeds, the *Rsa*I site becomes single stranded and resistant to digestion, which results in a PCR fragment amplification (see scheme in Fig. 3D). Thus, the rate of PCR fragment amplification, normalized to the efficiency of HO cutting, corresponds to the rate of resection [52]. We also tested with the same procedure another *Rsa*I site 4800 bp from the HO cut site, as a control. Interestingly, we noticed a higher amount of un-resected DNA at 150 bp proximal the DSB site, between 60 and 180 minutes after the cut in nocodazole blocked *sae2*Δ and *sae2*Δ *sgs1*Δ *rad9*Δ triple mutant cells with respect to the wild type and *sae2*Δ *rad9*Δ mutant (Fig. 3E). However, at later time points resection has efficiently passed beyond the *Rsa*I site 4800 bp far from the HO cut site (Fig. 3F), not only in the wild type and *sae2*Δ *rad9*Δ cells, but also in the *sae2*Δ *sgs1*Δ *rad9*Δ triple mutant cells, according to the visualization of the 3′ ssDNA formation by denaturing Southern blotting (Figs. 3B and 3C).

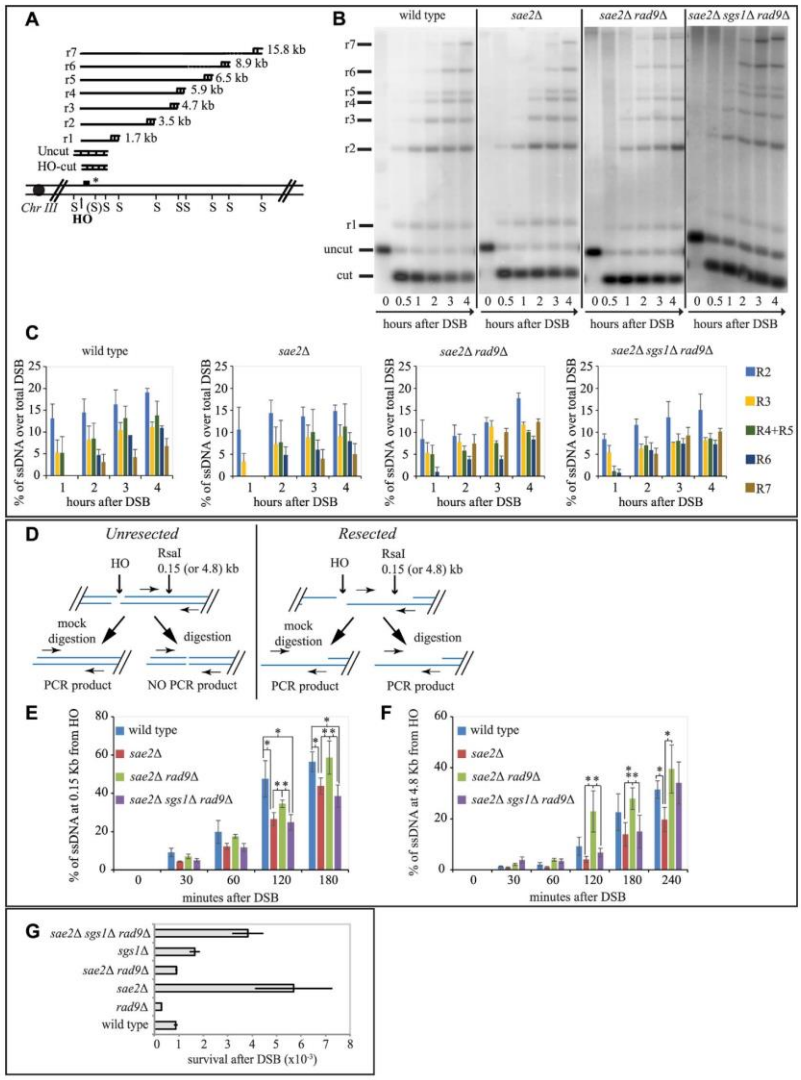
These studies revealed one striking unexpected result: although *sae2*Δ *sgs1*Δ *rad9*Δ triple mutant cells resect a DSB and expose an extended 3′ ssDNA (Figs. 3B, 3E and 3F), they are severely compromised in DSB repair through SSA (Figs. 2B and 2C), suggesting that the long-range resection is not the limiting step to repair a DSB in these cells, rather the defect is different from simply creating enough ssDNA to allow SSA to take place. Therefore, we hypothesize that an Sgs1-dependent mechanism contributes to efficiently initiate DSB processing in the absence of both Rad9 and Sae2, and the kinetics of the initial step of resection would become somehow critical to complete the subsequent steps of the SSA repair.

We then investigated whether the faster DSB end processing that we observed in *sae2*Δ *rad9*Δ cells would be associated with reduced NHEJ events, which are significantly elevated in the *sae2*Δ cells [53]. To this aim, we treated cells of JKM139 strains with nocodazole to block cell cycle in G2/M phase and we added galactose to induce one persistent DSB in each sister chromatid. Cells were kept in nocodazole for 2 hours to avoid potential interference caused by cell cycle transition, before plating in the presence of galactose. In this condition, the continued expression of HO leads to a recurrent cut of the *MAT* locus and precludes precise religation, until the sequence of the HO site is corrupted by deletion/addition of few bases and the ends are joined by imprecise NHEJ [54]. This is a relatively inefficient process in yeast, with a frequency of about $1\text{-}3\times10^{-3}$ in wild type cells [54]. We found that the frequency of imprecise NHEJ events is increased in *sae2*Δ cells, in agreement with previous finding [53], while it is slightly reduced in the absence of Rad9. Interestingly, deletion of *RAD9* reduces NHEJ events to wild type value in *sae2*Δ cells (Fig. 3G).

These results suggest that Rad9 plays a critical role to balance NHEJ and HR events in G2/M phase, likely acting at an early step of DSB processing, leading to increased NHEJ events in the absence of Sae2.

Rad9 limits Mre11 removal from a DSB, affecting Rad52 binding and DSB end-tethering in *sae2*Δ cells

The delay in DSB resection in *sae2*Δ cells has been correlated with a prolonged Mre11 binding at the DSB site [42,55]. More recently, it was also shown that an Sgs1-dependent process can contribute to remove Mre11 from a DSB in *sae2*Δ cells, promoting DSB resection and repair through homologous recombination [56]. Therefore, we decided to investigate Mre11 binding near a DSB by a chromatin immunoprecipitation-after-crosslinking-protocol (ChIP), followed by quantitative PCR (qPCR), with primers specific for the DSB site. Contrary to wild type, *rad9*Δ or *sgs1*Δ cells, we found greater and persistent levels of Mre11 bound near DSB ends in *sae2*Δ cells (Fig. 4A), supporting previous analysis of the Mre11 foci by microscopy [51,56], and by ChIP [55]. Importantly, we found a decrease in fold enrichment of Mre11 binding to the DSB site in *sae2*Δ *rad9*Δ cells, but not in the *sae2*Δ *sgs1*Δ *rad9*Δ triple mutant cells (Fig. 4B). These results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r7
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r1
uncut
cut
0 0.5 1 2 3 4
hours after DSB
C
wild type
sae2Δ
sae2Δ rad9Δ
sae2Δ sgs1Δ rad9Δ
% of ssDNA over total DSB
hours after DSB
R2
R3
R4+R5
R6
R7
D
Unresected
Resected
HO
RsaI
0.15 (or 4.8) kb
mock digestion
digestion
PCR product
NO PCR product
E
% of ssDNA at 0.15 Kb from HO
wild type
sae2Δ
sae2Δ rad9Δ
sae2Δ sgs1Δ rad9Δ
minutes after DSB
F
% of ssDNA at 4.8 Kb from HO
G
survival after DSB (x10⁻³)

Fig. 3. Rad9 limits an Sgs1- and Sae2- dependent initial step of DSB resection. (A) Scheme of the *MAT* locus. The figure shows the positions of the HO-cut site, and the probe used in experiments shown in (B and C) and in S3 and S4 Figs. (B, C) Exponentially growing YEP+raf cell cultures of the wild type JKM139 strain and the indicated derivatives, carrying a unique HO cut site at *MAT* locus and expressing the HO nuclease under GAL1 promoter, were synchronized and kept in G2/M phases by nocodazole treatment. Galactose was added at time 0 to induce HO. SspI-digested genomic DNA, extracted from samples taken at the indicated times, was analysed by Southern blotting to test 3′ filament formation. (C) The mean values ± SEM corresponding to the resection products of two independent experiments were determined by densitometry. (D) Schematic representation of the quantitative PCR method used to monitor HO-induced DSB resection. (E–F) Plots showing the ratio of resected DNA among HO cut DNAs at each time points by qPCR analysis. The mean values from three independent experiments are shown with SEM. Significance was calculated by one-tailed paired Student's *t* test (* for P<0.05; ** for P<0.01; where not indicated, the P value was higher than 0.05) (G) JKM139 derivatives were nocodazole-arrested in G2/M and 2% galactose was added to induce HO cut. After 2 hours of HO induction, cells were plated on YEP+raf and YEP+raf+gal, and incubated at 28°C for three days. Viability results were obtained from the ratio between number of colonies on YEP+raf+gal and YEP+raf. The mean values from three independent experiments are shown with SD.
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suggest that the deletion of *RAD9* gene promotes an Sgs1-dependent process to remove Mre11 from DSB ends in the absence of Sae2, supporting and expanding recent findings [56], and it may explain the high efficiency of SSA repair and viability of the *sae2*Δ *rad9*Δ that we showed in Figs. 1 and 2. Moreover, the prolonged binding of Mre11 near the DSB further supports previous results in Fig. 3, showing that short-range resection in the *sae2*Δ and *sae2*Δ *sgs1*Δ *rad9*Δ triple mutant cells is delayed.

Since it is known that Mre11 persistence at a DSB limits the recruitment of Rad52 [4,57], which is necessary to establish DNA end-tethering and HR pathways [58,59], we investigated by immunofluorescence Rad52 loading onto one DSB in all the mutants described. We found that deletion of *RAD9* totally restores Rad52 binding in *sae2*Δ cells through an Sgs1-dependent mechanism (Fig. 4C). These results correlate with the analysis of Mre11 binding in these mutants (Fig. 4B), and suggest that the limiting step to efficiently complete an SSA process in nocodazole-blocked *sae2*Δ and *sae2*Δ *sgs1*Δ *rad9*Δ cells is not the delay in DSB resection *per se* (Figs. 3B and 3C), but rather the reduced binding of Rad52.

Rad52 is a critical factor to maintain DSB ends tethered to each other, which was suggested to be a relevant event in HR [42,58,59,60,61]. As we showed that the deletion of *RAD9* allows Rad52 binding in *sae2*Δ cells (Fig. 4C), we investigated whether it may also contribute to rescue DSB end-tethering defect in these cells. To this end, we took advantage of a specific yeast background in which the DNA proximal to the irreparable HO break could be visualized by binding of a LacI-GFP (green fluorescent protein) fusion protein to multiple repeats of the LacI repressor binding site, *LacO*. These arrays are integrated at a distance of 50 kb on either side of the HO cleavage site on chromosome VII [58]. Cultures of the original wild type and isogenic *sae2*Δ, *sae2*Δ *rad9*Δ and *sae2*Δ *sgs1*Δ *rad9*Δ derivative strains were arrested in mitosis and kept blocked by nocodazole treatment during break induction by galactose addition. After 2 hours to ensure HO cut formation, we observed two LacI-GFP spots in only 12.5%±2.1% of the wild type cells, and 11.0%±3.1% in *sae2*Δ *rad9*Δ mutant cells, thus indicating their ability to hold the broken DNA ends together. In contrast, 42.3%±3.8% of *sae2*Δ and 42.5%±4.8% of *sae2*Δ *sgs1*Δ *rad9*Δ cells showed two LacI-GFP spots, indicating a failure in DSB end-tethering (Fig. 4D, and see also [42,62]).

Therefore, we conclude that the deletion of *RAD9* rescues both the Rad52 binding and DSB end-tethering in *sae2*Δ cells, contributing to efficiently repair a DSB through an SSA process that requires the resection of 25 kb of DNA between the repeats (Fig. 2A).

Rad9 oligomers limit *sae2*Δ cells viability following a DSB mainly through the interaction with Dpb11

It was previously suggested that Rad9 limits DSB resection acting as a physical barrier toward the actions of nucleases, through a function distinct from its role in DNA damage checkpoint signalling [10]. Therefore, we sought to address if a checkpoint-independent function of Rad9 was involved to limit *sae2*Δ cells viability following one DSB. To this aim, we tested the *chk1*Δ *rad53*-K227A double mutant in the YMV80 background, in which the Rad53 kinase activity is dead and both the two checkpoint-signaling pathways acting downstream Rad9 are abrogated. By plating the cells in the presence of galactose to induce one HO cut, we found that the viability of the *sae2*Δ *chk1*Δ *rad53*-K227A triple mutant cells is reduced, similarly to *sae2*Δ cells (Fig. 5A). This result indicates that signaling through Rad53 and/or Chk1 is not involved into the mechanism by which Rad9 limits SSA repair in *sae2*Δ cells.

In order to further understand how Rad9 inhibits SSA repair in *sae2*Δ cells, we then investigated specific mutations that affect Rad9 binding to a DSB. It is known that Rad9 constitutively binds chromatin through the interaction between its TUDOR domain and the histone H3 methylated at the K79 by Dot1 [20,21,22]. In addition, Rad9 binds chromatin around a DSB site through the interaction of its BRCT domain with the histone H2A phosphorylated at the S129 (γ-H2AX) by upstream kinase Mec1 and Tel1 [23]. Further, Rad9 is recruited near a DNA lesion through the interaction with Dpb11 protein. In particular, Dpb11 binds the CDK1-dependent phosphorylated S462 and T474 Rad9 residues, reinforcing the Rad9 binding to damaged DNA and promoting Rad9 phosphorylation by Mec1 [25].

To test the contribution of the different pathways that mediate Rad9 binding to chromatin, we analysed the viability in the presence of HO-induced DSB of specific mutations that abrogate each of them in the YMV80 background. The deletion of *DOT1* gene eliminates the H3K79 methyl transferase Dot1 protein, and greatly reduces the constitutive binding of Rad9 to chromatin [21,24]. As expected [36], deletion of *DOT1* leads to a faster long-range DSB resection in *sae2*Δ cells (S4A and S4B Figs.). However, by the qPCR-based method, we found that the initial short-range resection is still delayed in these double mutant cells (S4C Fig.), suggesting that the Dot1-dependent resection barrier may have a role only at distal region from the cut site. Indeed, by plating the YMV80 derivative cells in the presence of galactose to induce one DSB, we found that deletion of *DOT1* gene does not rescue *sae2*Δ lethality (Fig. 5A). Further, we deleted *SAE2* gene in a strain that expresses the H2A-S129A histone variant, which is not phosphorylatable by Mec1 and Tel1 kinases and leads to a faster DSB resection [63]. We also deleted *SAE2* gene in a strain that expresses the Rad9-S462A-T474A (hereafter we refer to *rad9*-S462A-T474A as *rad9*-2A) protein variant, which does not interact with Dpb11 [25]. Interestingly, both the failure to phosphorylate the H2A-S129 site and the *rad9*-2A mutation increase the viability of *sae2*Δ cells after one DSB, with the major contribution done by the mutation that abrogates the Rad9-Dpb11 interaction (Fig. 5A). Taking all these genetic results together, we concluded that the recruitment of Rad9 near the

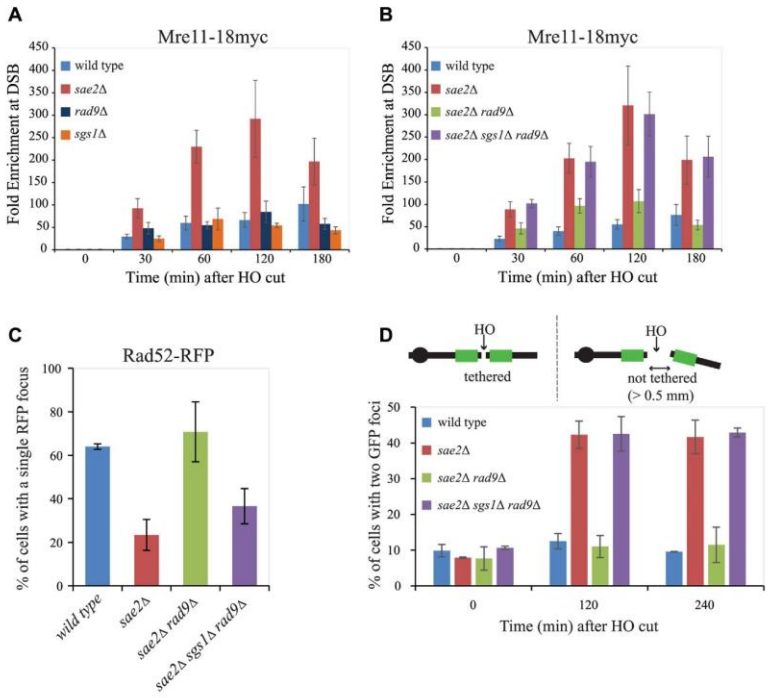

Fig. 4. Rad9 limits Mre11 removal from a DSB, affecting Rad52 binding and DSB ends tethering in *sae2*Δ cells. (A, B) Cells of the wild type JKM139 strain and the indicated derivatives, expressing a Mre11–18Myc fusion protein, were grown in YEP+raf and synchronized in G2/M phases by nocodazole treatment. Galactose was added at time 0 to induce HO. Relative fold enrichment of Mre11–18Myc at 0.1 kb from the HO cleavage site was evaluated after ChIP with anti-Myc antibodies and qPCR analysis. Plotted values are the mean values ± SEM from three independent experiments. (C) Cells of the wild type JKM139 strain and the indicated derivatives, expressing a Rad52-RFP fusion protein, were grown in YEP+raf and synchronized in G2/M phases by nocodazole treatment. Galactose was added at time 0 to induce HO. After 6 hours from DSB, cells were imaged under live cell conditions for Rad52-RFP focus formation. Approximately 100 cells per experiment were analyzed and the percentage of cells displaying a detectable Rad52-RFP focus was quantitated. Error bars reflect ranges from two independent experiments. (D) Cells of the wild type yJK40.6 strain and the indicated derivatives, expressing a LacI-GFP and carrying two *LacO* arrays (green boxes) at 50 kb on either side of one HO cut site on chromosome VII (see a scheme above the graph in Fig. 4D and text for details), were grown in YEP+raf and blocked in G2/M phases by nocodazole treatment. Galactose was added at time 0 to induce HO. Cell samples taken at the indicated times after HO induction were analysed with a fluorescence microscope to determine the percentage of cells in each sample that contained two LacI-GFP foci separated by>0.5 μm. The separation distance between foci was measured for 200 cells/sample.
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DSB site, mediated by its interaction with Dpb11 and partially with γ-H2AX, limits *sae2*Δ cells viability when a DSB must be repaired by SSA.

Consistently with our genetic evidence, we found an increased binding of Rad9 close to an irreparable DSB in *sae2*Δ cells by ChIP analysis (Fig. 5B), which correlates with the increased binding of Mre11 (Figs. 4A and 4B). Of note, the Rad9-2A protein variant does not bind near a break (Fig. 5B), supporting the viability data of the *sae2*Δ *rad9*-2A double mutant cells following one DSB (Fig. 5A). Moreover, Rad9 binding close to the break is only partially dependent on γ-H2AX and not by Dot1 (S5 Fig.), in agreement with cell viability of the *sae2*Δ h2a-S129A and *sae2*Δ *dot1*Δ double mutants (Fig. 5A).

Then we tested if the capability of Rad9 to form oligomers at the DNA damage site [29,30,64] was involved in inhibiting *sae2*Δ cells viability following a DSB. To this aim, we introduced a plasmid vector that expresses either the *rad9*-7xA allele or the *RAD9* gene as a control, by transformation into *rad9*Δ and *sae2*Δ

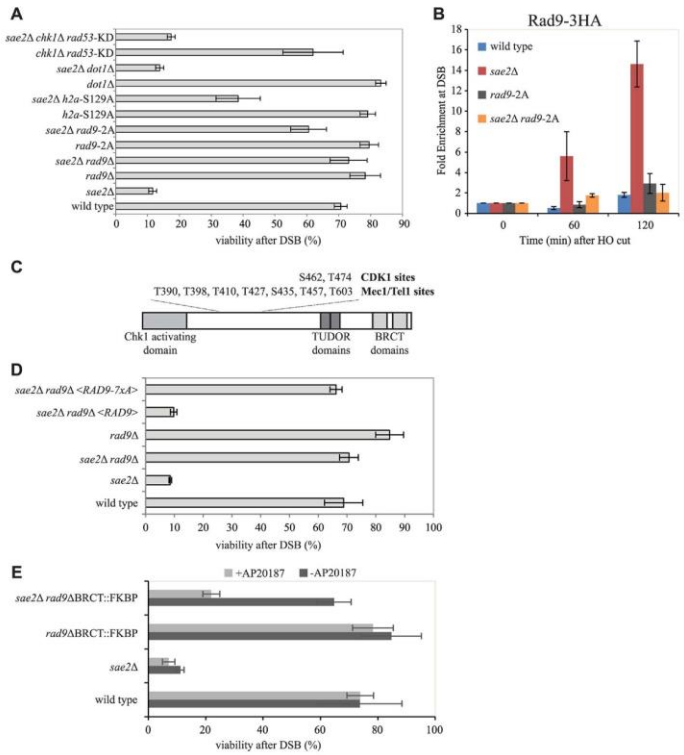

Fig. 5. Rad9 oligomers affect cell viability following a DSB, in the absence of Sae2, mainly through the interaction with Dpb11. (A and D) Viability of the wild type YMV80 strain and the indicated derivatives, plated on YEP+raf+gal. For each strain, the number of colonies grown after 3 days at 28˚C in YEP+raf+gal was normalized respect YEP+raf. Plotted values are the mean values ± SD from three independent experiments. (B) Cells of the wild type JKM139 strain and the indicated derivatives, expressing a Rad9-3HA fusion protein, were grown in YEP+raf and synchronized in G2/M phases by nocodazole treatment. Galactose was added at time 0 to induce HO. Relative fold enrichment of Rad9-3HA at 0.1 kb from the HO cleavage site was evaluated after ChIP with anti-HA antibodies and qPCR analysis. Plotted values are the mean values ± SEM from three independent experiments. (C) Schematic representation of Rad9 functional domains and sites phosphorylated by CDK1, Mec1 and Tel1. (E) Exponentially growing cell cultures of the wild type YMV80 strain and the indicated derivatives were incubated for 2 hours with or without the dimerization-inducing molecule AP20187, before plating in YEP+Raf or YEP+Raf+Gal, with/without AP20187. For each strain, the number of colonies grown after 3 days at 28˚C in YEP+raf+gal was normalized with respect to YEP+raf. Plotted values are the mean values ± SD from three independent experiments. Expression level of Rad9-2A, Rad9-7xA and Rad9-ΔBRCT-FKBP protein variants, described in this Figure, were determined by western blotting in S6 Fig.

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*rad9*Δ YMV80 derivatives. The Rad9-7xA protein variant cannot be phosphorylated at critical sites by upstream Mec1 and Tel1 kinases (see also Fig. 5C), and is unable to oligomerize [29,64]. After plating cells in the presence of galactose to induce one DSB, we found that the expression of the Rad9-7xA protein variant rescues the lethality of *sae2*Δ cells, contrary to the wild type Rad9 (Fig. 5D). This result suggests that the oligomerization of Rad9 molecules is implicated in limiting SSA repair in *sae2*Δ cells. To further support this conclusion, we took advantage of the *rad9*-ΔBRCT-FKBP chimeric allele, which leads to the production of a truncated variant of Rad9 protein, in which the C-terminal BRCT domains are replaced with a FKBP tag [24]. It was shown that the

Rad9-ΔBRCT-FKBP protein variant, which cannot form oligomers due to the absence of the BRCT domains, can dimerize in the presence of the small inducing molecule AP20187, binds chromatin and partially transduces the checkpoint signal (S6B Fig. and see also [24]). Consistent with our hypothesis, we found that the *rad9*-ΔBRCT-FKBP mutation does not rescue *sae2*Δ lethality in the presence of AP20187, while the viability in the *sae2*Δ *rad9*-ΔBRCT-FKBP double mutant cells is almost identical to the wild type value (Fig. 5E), further suggesting that the dimerization/oligomerization of Rad9 affects SSA repair.

Discussion

It is now clear that DSB processing is a finely regulated process, which acts at the crossroad between HR and NHEJ recombination pathways. Indeed, as soon as a DSB is resected, homologous recombination pathways can be used to repair the break in lieu of NHEJ, with important implications for chromosome rearrangements and genome integrity.

Similarly to what seen in higher eukaryotes, three distinct nucleases cooperate to resect a DSB in *S. cerevisiae*. According to a model recently proposed for meiotic DSBs [65], Mre11, activated by Sae2 [44], introduces a nick near a DSB, triggering a bidirectional nucleolytic degradation of the 5′ strand: Exo1 and Dna2-Sgs1 resect the DNA in the 5′-to-3′ direction from the nick, while the Mre11 complex resects the DNA in the 3′-to-5′ direction toward the DSB ends. In G2/M blocked cells, it appears that the Exo1 and Dna2-Sgs1 pathways cannot actively resect a DSB starting from its ends, which are occupied by Ku70-Ku80 complex [1]. Indeed, it was suggested that the Mre11 activity might contribute to the removal of Ku complex, clearing the ends [2,3,11,65,66]. Importantly, in the absence of a functional Sae2, the Mre11-dependent DSB processing is compromised, and Ku-dependent NHEJ events and translocations increased [62]. In addition, Mre11 and Rad52 binding are, respectively, increased and reduced in *sae2*Δ cells (Fig. 4, and see [4,57]), which are severely defective in repairing a DSB through SSA (Fig. 2, and see also [6,42]). Moreover, *sae2*Δ cells cannot keep the DSB ends tethered, which was shown to be relevant for DSB repair (Fig. 4, and see [42,58,60]). Here, we show that the deletion of the *RAD9* gene suppresses all these phenotypes of *sae2*Δ cells. Indeed, we found that deletion of *RAD9* leads to a faster 5′–3′ resection both through the Exo1 and Dna2-Sgs1 pathways, but the Dna2-Sgs1 pathway becomes essential, in the absence of Sae2, to efficiently initiate DSB processing and repair through an SSA process that requires 25 kb DNA resection (Figs. 2 and 3). We also found elevated levels of Mre11 bound near an HO-induced break both in *sae2*Δ and *sae2*Δ *sgs1*Δ *rad9*Δ mutants, accordingly with a defect in Rad52 binding and DNA end-tethering (Fig. 4). The requirement of DSB end-tethering for SSA repair has never been explored before, however it is relevant to underline that Rad52 is important for end-tethering [58], and also our results indicate that a defect in end-tethering is linked with a failure to accomplish SSA repair. Further investigation will be required to fully understand the interplay between SSA and end-tethering. Interestingly, recent findings underlined a role of exonuclease processing of a DSB in maintaining broken chromosome ends in close proximity [61].

Taken all these findings together, we suggest that the prolonged binding of Mre11 near the break site may represent the critical barrier to efficiently initiate DSB resection, load Rad52 and establish end-tethering in the absence of Sae2, and it can be bypassed by a resection-based mechanism mediated by Sgs1-Dna2 in the absence of Rad9.

A similar role to remove Mre11 from a DSB site in *sae2*Δ cells was recently shown for Sgs1, in the absence of Ku70-Ku80 complex [56]. Indeed, deletion of *KU70* suppresses *sae2*Δ cells sensitivity to low doses of CPT and other DSB inducing agents [1,3]. Surprisingly, we did not see a rescue of *sae2*Δ cells lethality by deleting *KU70* after a DSB that can be repaired through an SSA process between two homologous *leu2* repeats 25kb far from each other, although deletion of *RAD9* suppresses the *sae2*Δ *ku70*Δ double mutant (S7 Fig.). One possibility is that Rad9, bound near a DSB site, may limit the Sgs1-Dna2 activity starting from the break ends, leading to prolonged Mre11 binding. This might occur in cooperation with Ku complex, bound to the DSB ends, or rather it might represent a second distinct mechanism to limit DSB ends resection and DNA end-tethering. Alternatively, or in addition, Ku and Rad9 may limit DSB processing in different cell cycle phases. Indeed, the Ku complex acts on a DSB mainly in G1, while Rad9 acts predominantly in G2/M phase [36,67,68].

Genetic and biochemical evidence in Fig. 5 suggest that Rad9 protein dimerization and/or oligomerization, together with Rad9 interactions with Dpb11 and partially with γ-H2AX, are important to limit short-range resection and repair in *sae2*Δ cells. Indeed, Dpb11 is recruited on to the DNA lesion through the interaction with the 9-1-1 complex [28], and both the 9-1-1 complex and Dpb11 are recruited rapidly near a DSB site [69], likely at the ssDNA-dsDNA junction [70]. It is possible that the interactions with γ-H2AX, as well as with the histone H3 methylated at Lys79 by Dot1, become more important to recruit Rad9 in a distal region from the DSB site, contributing to slow down the long-range resection, which is not the limiting step in *sae2*Δ cells. This hypothesis is supported by the fact that DNA damage sensitivity of *fun30*Δ cells, that resect slower a DSB because of their inefficient Rad9 removal from chromatin flanking a DSB [37], is partially rescued in the absence of γ-H2AX or Dot1 [37,63]. Of importance, deletion of *DOT1* gene does not rescue *sae2*Δ cells (Fig. 5A). Notably, although Rad9 binding close to the break is not particularly elevated in wild type cells, it is enriched in *sae2*Δ cells (Fig. 5C). Consistent with our genetic evidence, Rad9 binding close to DNA ends depends on Dpb11, partially on the histone γ-H2AX, but not on the histone H3 methylated at Lys79 by Dot1 (Figs. 5B and S5). Possibly, these data are in agreement with the low amount of modified histones detected in chromatin within 1–2 kb of the break [22,26,71,72,73].

Overall, our genetic and molecular results suggest a model shown in Fig. 6, in which Rad9, in addition to its known role in inhibiting long-range resection, may affect the initial short-range processing of an HO-induced DSB. In fact, Rad9, once recruited close to a DSB end in G2 phase mainly through the interaction with Dpb11, limits the Sgs1 dependent resection starting from DNA ends, whenever Mre11 is blocked near the DNA ends. In the future it will be interesting to investigate whether Rad9 plays a similar role in limiting rapid and coincident resection of dirty radiation-induced DSBs, in cells lacking Sae2 and/or Mre11 [74].

We believe that our findings might have important implications for understanding how the genome stability is preserved, especially in higher eukaryotes, whose genomes are enriched of repeats and SSA events can be particularly frequent. In fact, it becomes clear that too-efficient DSB resection can lead to an excessive initiation of homologous recombination and accumulation of toxic DNA intermediates and rearrangements between repeats [16]. Moreover, DSB resection may lead to highly error-prone alternative ends joining (A-EJ) and MMEJ events [14,16]. In this view, our results in yeast might help to understand recent finding in human cells at the molecular level, showing a role for 53BP1 in protecting from BLM and CtIP-Mre11 dependent A-EJ events and genome rearrangements [75].

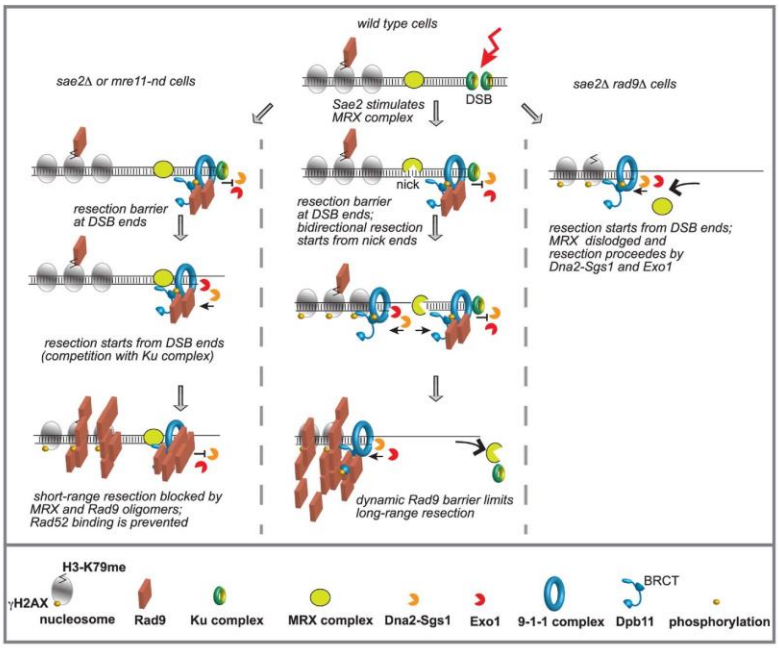

Fig. 6. Model to explain the interplay between Mre11 complex and Rad9 at a DSB in G2/M phase. Ku and Mre11 complexes, together with Rad9, are recruited soon after a DSB formation and limit the action of Exo1 and Dna2-Sgs1 pathways. The order of appearance of the various factors was based on both literature and our results. See details in the text.
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Furthermore, our findings suggest that the functional interplay between 53BP1/Rad9 and Mre11 may also have a physiological relevance to protect from error-prone imprecise NHEJ events in genomic regions containing no repeats. It is also worth mentioning that the inactivation of 53BP1 was shown to potentiate homologous recombination and increase DNA damage tolerance of cancer-prone BRCA1 -/- cells [32,76,77,78], with severe implications for therapeutic treatments.

In conclusion, we show novel insights on the structural barrier induced by Rad9, together with Dpb11 and γ-H2AX, to limit DSB processing and repair. The Sgs1-Dna2 pathway becomes essential to efficiently remove hypo-active Mre11 from a DSB site, in the absence of Sae2 and Rad9, triggering DSB resection and repair. The efficient removal of Mre11 from the DSB site is essential not only to switch to the more processive long-range resection, but also to allow an efficient recruitment of the recombination factor Rad52. This allows the maintenance of DSB end-tethering, which is an important prerequisite to complete repair, especially for those lesions that require extensive resection. These events increase in the absence of Rad9 and might contribute to accumulation of toxic HR events, leading to genome rearrangements and genetic instability.

Materials and Methods

Yeast strains, media and growth conditions

All the strains listed in S1 Table are derivative of JKM139, YMV80 and χJK40.6. To construct strains standard genetic procedures of transformation and tetrad analysis were followed. Deletions and tag fusions were generated by the one-step PCR system [79]. For the indicated experiments, cells were grown in YP medium enriched with 2% glucose (YEP+glu), raffinose 3% (YEP+raf) or raffinose 3% and galactose 2% (YEP+raf+gal). All the synchronization experiments were performed at 28°C.

Measurement of DSB resection at MAT locus

DSB end resection in JKM139 derivative strains was analyzed on alkaline agarose gels using a single-stranded RNA probe as described previously [36,50].

SDS-PAGE and western blot

TCA protein extract was prepared [80] and separated by SDS-PAGE. Western blotting was performed with anti-Rad53 (EL7), anti-HA (12CA5), anti-Rad9 (generously provided by N. F. Lowndes), and anti-actin using standard techniques.

Analysis of SSA repair

Repair of an HO-induced DSB in YMV80 background was analyzed by a Southern blotting procedure described previously [39].

Cell viability assay

YMV80 derivative strains were inoculated in YEP+raf, grown O/N at 28°C. The following day, cells were normalized and plated on YEP+raf and YEP+raf+gal. Plates were incubated at 28°C for three days. Viability results were obtained from the ratio between number of colonies on YEP+raf+gal and YEP+raf. Standard deviation was calculated on three independent experiments.

Non homologous end joining assay

JKM139 derivative strains were inoculated in YEP+raf, grown O/N at 28°C. The following day, after cell cycle block in G2/M by nocodazole, 2% galactose was added to one part of the culture to induce HO cut. After 2 hours of HO induction, cells were normalized and plated on YEP+raf and YEP+raf+gal. Plates were incubated at 28°C for three days. Viability results were obtained from the ratio between number of colonies on YEP+raf+gal and YEP+raf. Standard deviation was calculated on three independent experiments.

ChIP analysis

ChIP analysis was performed as described previously [69]. Input and immunoprecipitated DNA were analysed by quantitative PCR using a Biorad MyIQ2 system or a Biorad CFX connect. The oligonucleotides used are listed in S2Table. Data are presented as fold enrichment at the HO cut site (0.15 or 4.8 kb from the DSB) over that at the *PRE1* locus on chromosome V, then normalized to the corresponding input sample. The obtained fold enrichment values were normalized to the fold enrichment of the t_0 sample. Standard mean error (SEM) was calculated on three independent experiments.

Quantitative analysis of DSB end resection by real time PCR

Quantitative PCR (qPCR) analysis of DSB resection was performed accordingly to [52]. The oligonucleotides used are listed in S2 Table. The DNA was digested with the *Rsa*I restriction enzime (NEB) that cuts inside the amplicons at 0.15 kb and 4.8 kb from the DSB, but not in the *PRE1* control region on chromosome V. qPCR was performed on both digested and undigested templates using StoS Quantitative Master Mix 2X SYBR Green (Genespin) with the Biorad MyIQ2 PCR system. The ssDNA percentage over total DNA was calculated using the following formula: % ssDNA = $\{100/[(1+2^{\Delta Ct})/2]\}/f$, in which ΔCt values are the difference in average cycles between digested template and undigested template of a given time point and f is the HO cut efficiency measured by Southern blot analysis.

DSB end-tethering experiment

Cells of strains derivative from yJK40.6 background were grown in YEP+raf and blocked 3 hours in G2 with nocodazole. 160 μM $CuSO_4$ was added one hour before inducing HO cut with galactose, accordingly to [58]. Samples taken at the indicated time were analysed with a fluorescence microscope. Cells with 2 LacI-GFP foci separated by more than 0.5 μm were considered defective in DSB end-tethering.

Supporting Information

S1 Fig. Deletion of *RAD9* rescues the lethality of the *sae2*Δ cells after a DSB through the helicase activity of Sgs1. (A) Meiotic tetrads from the indicated cross were dissected on YEPD plates that were incubated at 25°C, following by spores genotyping. (B) A plasmid vector expressing either the wild type or *sgs1*-K706A allele of *SGS1* gene was inserted by transformation into the YMV80 derivative *sae2*Δ *sgs1*Δ *rad9*Δ triple mutant. For each YMV80 derivative strain indicated in the Figure, the number of colonies grown after 3 days at 28°C in YEP+gal was normalized respect YEP+glu. Plotted values are the mean values ± SD from three independent experiments.
(TIF)

S2 Fig. Deletion of *RAD9* rescues DSB repair defects of *sae2*Δ cells through a Rad51-independent pathway. (A) Exponentially growing cell cultures of the wild type YMV80 strain and the indicated derivatives were serially diluted (1:10), and each dilution was spotted out into YEP+Raf or YEP+Raf+Gal plates. Plates were incubated 3 days at 28°C. (B) Exponentially growing YEP+raf cell cultures of the wild type YMV80 strain and the indicated derivatives were synchronized and kept blocked in G2/M phase with nocodazole treatment; galactose was added at time zero to induce HO-cut. Genomic DNA, extracted from samples taken at the indicated times, was analyzed for DSB formation and repair, as described in Fig. 2B.
(TIF)

S3 Fig. Rad9 limits an Sgs1- and Exo1- dependent DSB resection. (A) Exponentially growing YEP+raf cell cultures of the wild type JKM139 strain and the indicated derivatives, carrying a unique HO cut site at *MAT* locus and expressing the HO nuclease under GAL1 promoter, were synchronized and kept in G2/M phases by nocodazole treatment. Galactose was added at time 0 to induce HO. Genomic DNA, extracted from samples taken at the indicated times, was analyzed for ssDNA formation, as described in Fig. 3B. (B) Densitometric analysis of the representative experiments shown in (A).
(TIF)

S4 Fig. Analysis of DSB resection in *dot1*Δ derivative strains. (A) Exponentially growing YEP+raf cell cultures of the wild type JKM139 strain and the indicated derivatives, carrying a unique HO cut site at *MAT* locus and expressing the HO nuclease under GAL1 promoter, were synchronized and kept in G2/M phases by nocodazole treatment. Galactose was added at time 0 to induce HO. Genomic DNA, extracted from samples taken at the indicated times, was analyzed for ssDNA formation, as described in Fig. 3B. Wild type and *sae2*Δ blots are the same used in Fig. 3B. (B) Densitometric analysis of the representative experiments shown in (A). (C) Plot showing the ratio of resected DNA among HO cut DNA at each time points by qPCR analysis, measured at 0.15 kb as described in Fig. 3D.
(TIF)

S5 Fig. Analysis of Rad9 binding near a DSB. Cells of the wild type JKM139 strain and the indicated derivatives, expressing a Rad9-3HA fusion protein, were grown in YEP+raf and synchronized in G2/M phases by nocodazole treatment. Galactose was added at time 0 to induce HO. Relative fold enrichment of Rad9-3HA at 0.1 kb from the HO cleavage site was evaluated after

ChIP with anti-HA antibodies and qPCR analysis. Plotted values are the mean values ± SEM from three independent experiments. (TIF)

S6 Fig. Analysis of the expression levels and phosphorylation of various Rad9 protein variants. (A) Cells of the wild type YMV80 strain and the indicated derivatives were grown in YEP+raf. Galactose was added at time 0 to induce HO. Cells have been taken at the indicated times and protein extracts were done. Rad9 and Rad53 were detected by western blotting. (B) Cells of the wild type YMV80 strain and the *rad9*-ΔBRCT-FKBP derivative were grown in YEP+raf. Cell cultures were split in two and one half was treated with AP20187 for 1 hr, before adding galactose to induce HO. Cells have been taken at the indicated times and protein extracts were done. Rad9 and Rad53 were detected by western blotting. (TIF)

S7 Fig. Deletion of *KU70* does not rescue viability of YMV80 derivative *sae2*Δ cells, following a DSB. Viability of the wild type YMV80 strain and the indicated derivatives, plated on YEP+gal. For each strain, the number of colonies grown after 3 days at 28°C in YEP+gal was normalized respect YEP+glu. Plotted values are the mean values ± SD from three independent experiments. (TIF)

S1 Table. List of yeast strains described in this work. (DOCX)

S2 Table. List of the oligonucleotides used for ChIP and DSB resection analyses. (DOCX)

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Author Contributions

Conceived and designed the experiments: MF FL FM JEH AP. Performed the experiments: MF DD GDG CCR VVE MT FM AP. Analyzed the data: MF DD VVE FM AP. Contributed reagents/materials/analysis tools: MF DD GDG CCR VVE FL FM JEH AP. Wrote the paper: FM JEH AP.

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Published Paper II

Slx4 and Rtt107 control checkpoint signaling and DNA resection at double-strand breaks

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Slx4 and Rtt107 control checkpoint signalling and DNA resection at double-strand breaks

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ABSTRACT

The DNA damage checkpoint pathway is activated in response to DNA lesions and replication stress to preserve genome integrity. However, hyperactivation of this surveillance system is detrimental to the cell, because it might prevent cell cycle re-start after repair, which may also lead to senescence. Here we show that the scaffold proteins Slx4 and Rtt107 limit checkpoint signalling at a persistent double-strand DNA break (DSB) and at uncapped telomeres. We found that Slx4 is recruited within a few kilobases of an irreparable DSB, through the interaction with Rtt107 and the multi-BRCT domain scaffold Dpb11. In the absence of Slx4 or Rtt107, Rad9 binding near the irreparable DSB is increased, leading to robust checkpoint signalling and slower nucleolytic degradation of the 5′ strand. Importantly, in *slx4*Δ *sae2*Δ double mutant cells these phenotypes are exacerbated, causing a severe Rad9-dependent defect in DSB repair. Our study sheds new light on the molecular mechanism that coordinates the processing and repair of DSBs with DNA damage checkpoint signalling, preserving genome integrity.

INTRODUCTION

All eukaryotic cells respond to DNA lesions by activating a surveillance network called the DNA damage checkpoint (DDC), which coordinates DNA repair with cell cycle progression (1). Notably, mutations in checkpoint genes lead to genome instability and in higher eukaryotes often give rise to carcinogenesis (2). At double strand DNA breaks (DSBs), the checkpoint is triggered by the formation of long stretches of single-stranded DNA (ssDNA) generated by 5′-3′ nucleolytic degradation (DSB resection) of DNA ends. This action is carried out by multiple conserved factors. In *S. cerevisiae*, CDK1-phosphorylated Sae2 primes the Mre11-Rad50-Xrs2 (MRX) complex to trim DSB ends (short-range resection), which are afterwards extensively processed by the Exo1 and Dna2 nucleases, together with the Bloom helicase Sgs1 (long-range resection). As resection proceeds, the 3′ ssDNA tail is covered by RPA, which then recruits the checkpoint clamp 9–1–1 complex (Rad17, Mec3 and Ddc1 in budding yeast) and the upstream checkpoint kinase Mec1. Proper cooperation of all these factors is critical to establish appropriate DSB resection, repair and checkpoint signalling (3).

A key player in the DDC is Rad9, an ortholog of human 53BP1. Rad9 acts as an adaptor protein, which mediates checkpoint signalling from the sensor kinase Mec1 to the central transducer kinases Rad53 and Chk1 (2,4). Moreover, Rad9 is recruited to DSBs and to uncapped telomeres, limiting the resection of the 5′ strand (5). More recently, we have also shown that increased Rad9 binding close to DSB ends affects the initiation of resection and the balance between non-homologous end joining and homologous recombination events in *sae2*Δ cells (6).

Rad9 recruitment to DSBs is mediated by its interactions with a Mec1-dependent phosphorylation site (S129) in histone H2A (γ-H2AX), and with the multi-BRCT domain protein Dpb11 (TopBP1 in human cells), which is itself recruited to DSBs via interaction with yet another Mec1-dependent phosphorylation site (T602) in the 9–1–1 subunit Ddc1. In addition, the Dot1-dependent methylation of Lysine 79 of histone H3 provides a constitutive docking site for the Rad9 Tudor domain (5,7–11). Up to now, the regulation of Rad9 dissociation from DNA lesions after repair is poorly understood.

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The role of Rad9 in DDC signalling has been recently shown to be counteracted by the action of Slx4, a protein scaffold with established roles in the coordination of structure-specific nucleases (12–15). Upon replication stress caused by the DNA alkylating agent methyl methanesulfonate (MMS), a complex formed by Slx4 with the multi-BRCT domain protein Rtt107 was shown to compete with Rad9 for interaction with Dpb11 and γ-H2AX. As such, cells lacking Slx4 are hypersensitive to MMS treatment due to Rad53 hyper-activation (16). Interestingly, expression of a chimeric protein, in which the BRCT domains 5 and 6 of Rtt107 were fused to BRCT domains 3 and 4 of Dpb11 (MBD, minimal multi-BRCT-domain module), was shown to completely rescue the sensitivity of *slx4*Δ cells to MMS (17). The checkpoint dampening function of Slx4-Rtt107 appears to be distinct from Slx4's role in coordinating DNA repair via regulation of the Rad1 and Slx1 nucleases (16). Disruption of the Slx4-Dpb11 interaction prevents the binding to Mus81 nuclease, leading to the accumulation of unresolved DNA joint molecules (JMs) and RPA foci (indicative of ssDNA gaps), after MMS treatment (18). Therefore, an open question is whether the DDC hyperactivation in *slx4*Δ is primarily due to the defect in dampening checkpoint signalling or the defect in regulation of the JM resolution.

In this study, we investigated the role of the Rtt107-Slx4 complex in the regulation of the DDC in cells responding to an irreparable DSB and to uncapped telomeres. Our results indicate that cooperation between Slx4 and Rtt107 limits Rad9 binding near a DSB, leading to a reduction in DDC signalling and an increase in DNA resection, through a mechanism that does not require the Rad1, Slx1 and Mus81 nucleases. This Slx4-Rtt107 role is critical for the cell to successfully repair DSBs and to survive exposure to MMS and camptothecin (CPT, a topoisomerase-aborting agent), particularly when DSB resection and DDC are already compromised by *sae2*Δ mutation.

MATERIALS AND METHODS

Yeast strains, Media and Growth conditions

All the strains listed in Table S1 are derivative of JKM179 or W303. To generate strains, standard genetic procedures of transformation and tetrad analysis were followed. Deletions and tag fusions were generated by the one-step PCR system (19). All the strains, except the Y603 derivatives, obtained by direct transformation were outcrossed with the parental to clean the background. For the indicated experiments, cells were grown in YP medium enriched with 2% glucose (YEP+glu), 3% raffinose (YEP+raf) or 3% raffinose and 2% galactose (YEP+raf+gal). Unless specified all the experiments were performed at 28°C.

Measurement of DSB resection at MAT locus

DSB end resection in JKM179 derivative strains was analysed by alkaline agarose gels using a single-stranded RNA probe as described previously (5,20), and by quantitative PCR (qPCR) analysis (6,21). The oligonucleotides used in qPCR analysis are listed in Table S2. The DNA was digested with the *Rsa*I restriction enzyme (NEB), which cuts inside the amplicons at 5 kb and 10 kb from the HO-cut site, but not in the *PRE1* control region on chromosome V.

SDS-PAGE and Western blot

TCA protein extracts were prepared as described previously (22), and separated by SDS-PAGE. Western blotting was performed with monoclonal (EL7) or polyclonal (generous gift from C. Santocanale) anti-Rad53 antibodies.

Checkpoint adaptation by micro colony assay

For JKM179 derivative strains, cells were grown O/N in YEP + raf at 28°C. The unbudded cells were micro manipulated on YEP + raf + gal and plates were incubated at 28°C for 24 h. Micro colonies formed by more than 3 cells were scored as 'adapted'. Standard deviation was calculated on three independent experiments. For *cdc13–1* derivative strains, cells were grown O/N in YEP + glu at 23°C and micro manipulated on YEP + glu plates and were incubated at 37°C for 24 h.

ChIP-seq analysis

Cells were grown to log phase in YEP + raffinose and arrested in G2/M with 20 μg/ml nocadozole before addition of galactose to a final concentration of 2%. Cells were sampled immediately (0 h) and at 2, 4 and 6 h after galactose addition. Chromatin immunoprecipitation and sequencing data analysis were performed as previously described (23). Data are presented for chromosome III as a log2 ratio of normalized read counts for each IP:input pair. All sequencing data are deposited in the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra; Study accession SRP062913).

ChIP analysis

ChIP analysis was performed as described previously (6). The oligonucleotides used are listed in Table S2. Data are presented as fold enrichment at the HO cut site (5 kb from DSB) over that at the *PRE1* locus on chromosome V (for Slx4) or *ARO1* locus on chromosome IV (for Rad9), and normalized to the corresponding input sample.

Ectopic recombination assay

We used derivatives of the tGI354 strain (Table S1). The percentage of cell viability of the indicated mutants after HO induction was calculated as a ratio between the number of colonies grown on YEP + raf + gal medium and the number of colonies grown on YEP + raf medium after 2–3 days of incubation at 28°C.

Physical analysis of DSB repair kinetics during ectopic gene conversion was performed with DNA samples isolated at different time points from HO induction. Genomic DNA was digested with *Eco*RI enzyme and separated on a 0.8% agarose gel. Southern blotting was done using a 1000 bp *MATa* probe. To calculate DSB repair values we normalized DNA amount using a DNA probe specific for *IPL1* gene (unprocessed locus).

Drug sensitivity assay

Logarithmically growing cell cultures were serially diluted and spotted on media containing different dosages of MMS or CPT, as indicated. Plates were incubated at 28°C for 3 days.

RESULTS

The Slx4-Rtt107 complex contributes to checkpoint adaptation to one irreparable DSB and to uncapped telomeres

We asked whether the competition between Slx4 and Rad9 for Dpb11 binding might affect the cellular response to DSBs. In particular, we hypothesized that in the absence of Rtt107 or Slx4, the Rad9-dependent checkpoint signalling should be hyper-activated in the presence of one DSB. To address this question, we induced a persistent DSB at the MAT locus by over-expression of HO endonuclease in a JKM139 yeast background (20,24). This genetic system is ideal to correlate the DNA damage checkpoint signalling with the formation of ssDNA. Indeed, in these cells, the DSB induced by HO is extensively 5′-to-3′ resected, and the lack of homology elsewhere in the genome prevents the formation of any recombination intermediate. Thus, G1 unbudded cells were micro-manipulated in galactose containing medium to induce the HO-break. In this condition, the activation of the DNA damage checkpoint blocked cell cycle progression at the G2/M transition for several hours (24). However, wild type cells undergo checkpoint adaptation proceeding through 3–4 divisions after 24 h (25), when we scored the percentage of micro-colonies of 4–8 cells formed (Figure 1A,B). Strikingly, the percentage of cells that underwent adaptation and re-started the cell cycle was severely reduced in *slx4*Δ and *rtt107*Δ cells. A similar result was observed in the *slx4*-S486A mutant (Figure 1B), which specifically affects the Slx4-Dpb11 interaction (16,18), supporting the model that the interaction with Dpb11 is a key event in this regulatory mechanism. Moreover, as an additional indication of the central role of the interaction with Dpb11, the expression of the chimera MBD rescued the checkpoint adaptation defect of *slx4*Δ (Supplementary Figure S1). Importantly, the percentage of cells that underwent adaptation and re-started the cell cycle was not affected in the *slx1*Δ *rad1*Δ *mus81*Δ triple mutant (Figure 1B). These results, obtained after an irreparable DSB, indicate that the Slx4-Rtt107 complex, likely interacting with Dpb11, may have a role in checkpoint adaptation that is distinct from a role in DSB repair and JM resolution, which requires the Slx1, Rad1 or Mus81 nucleases (12–15).

Consistent with the hypothesis that the Rad9 pathway is hyper-activated in the absence of Slx4 and Rtt107, the deletion of the *RAD9* gene completely by-passed the prolonged cell cycle block of *slx4*Δ, *slx4*-S486A and *rtt107*Δ mutants (Figure 1B). The same by-pass was observed in *ddc1*-T602A cells, in which Dpb11 and Rad9 cannot be recruited by the 9–1–1 complex onto chromatin (11,26,27) (Figure 1B).

To monitor DSB-induced checkpoint signalling in the absence of a functional Slx4-Rtt107 pathway, we analysed Rad53 phosphorylation following formation of one irreparable HO-cut. In wild type cells, Rad53 was dephosphorylated 12–15 h after the DSB formation, as expected (28). On the contrary, Rad53 phosphorylation was prolonged and more robust in *slx4*Δ and *rtt107*Δ mutants (Figure 1C), consistent with the checkpoint adaptation defect shown in Figure 1B. The *slx4*-S486A mutant cells also show a robust and prolonged Rad53 phosphorylation, although slightly less than *slx4*Δ cells. Interestingly, Rad53 was only transiently phosphorylated in *ddc1*-T602A and *ddc1*-T602A *slx4*Δ mutants (Figure 1C), consistent with the defect in Rad9 stable association with DNA in *ddc1*-T602A mutant cells (11,18,26,27).

Checkpoint adaptation was previously observed at uncapped telomeres in thermo sensitive *cdc13*–1 mutant cells (29). Therefore we analysed cell cycle block and re-start in *cdc13*–1 derivative strains incubated at the restrictive temperature. Unbudded cells, grown at the permissive temperature, were micro-manipulated on a plate and immediately shifted to 37°C. As expected (29), *cdc13*–1 cells remained blocked at the G2/M transition for several hours, but after 24 h incubation a significant percentage of cells have re-started the cell cycle, producing micro-colonies of 4–8 cells (Figure 1D). Interestingly, *cdc13*–1 *slx4*Δ, *cdc13*–1 *slx4*-S486A and *cdc13*–1 *rtt107*Δ cells did not divide after the shift to 37°C (Figure 1D), suggesting that checkpoint adaptation following telomere uncapping was compromised in the absence of a functional Slx4-Rtt107 pathway, similarly to what we found in the presence of one irreparable DSB (Figure 1B). Importantly, the prolonged cell cycle block was rescued by deleting *RAD9* (Figure 1D). Furthermore, by analysing protein extracts from cells shifted to the restrictive temperature, we found that Rad53 phosphorylation in *cdc13*–1 *slx4*Δ, *cdc13*–1 *slx4*-S486A and *cdc13*–1 *rtt107*Δ mutants occurred earlier than in *cdc13*–1 cells (Figure 1E), indicating that DNA damage checkpoint signalling was hyper-activated in the absence of Slx4 and Rtt107, consistent with the defect in cell cycle re-start by adaptation.

Taking the cellular and molecular results in Figure 1 together, we concluded that the Slx4-Rtt107 pathway plays a regulatory role in dampening the Rad9-dependent checkpoint signalling after one irreparable DSB and after telomere uncapping, leading to checkpoint adaptation and re-start of cell cycle progression after a prolonged G2/M arrest.

The Slx4-Rtt107 complex modulates Rad9 binding to one irreparable DSB

As we and others have recently shown that Rad9 plays a role in preventing DNA resection at a DSB (1,6,30,31), the results in Figure 1 prompted us to test the amount of Rad9 bound near a DSB in the absence of a functional Slx4-Rtt107 pathway. Recruitment of Rad9 oligomers near a DSB is a critical event to elicit a fully active DDC, also affecting DSB processing and repair. Interestingly, Rad9 oligomers are recruited through the interaction with modified histones and Dpb11 soon after DSB formation (6,10).

For high-resolution analysis of Rad9 binding along chromosome III after HO cutting at the *MAT* locus, in wild type and *slx4*Δ JKM139 cells, we used chromatin immunoprecipitation coupled to deep sequencing (ChIP-seq). Interestingly, before the induction of the HO-cut, we found several regions of Rad9 binding along the entire chromosome III,

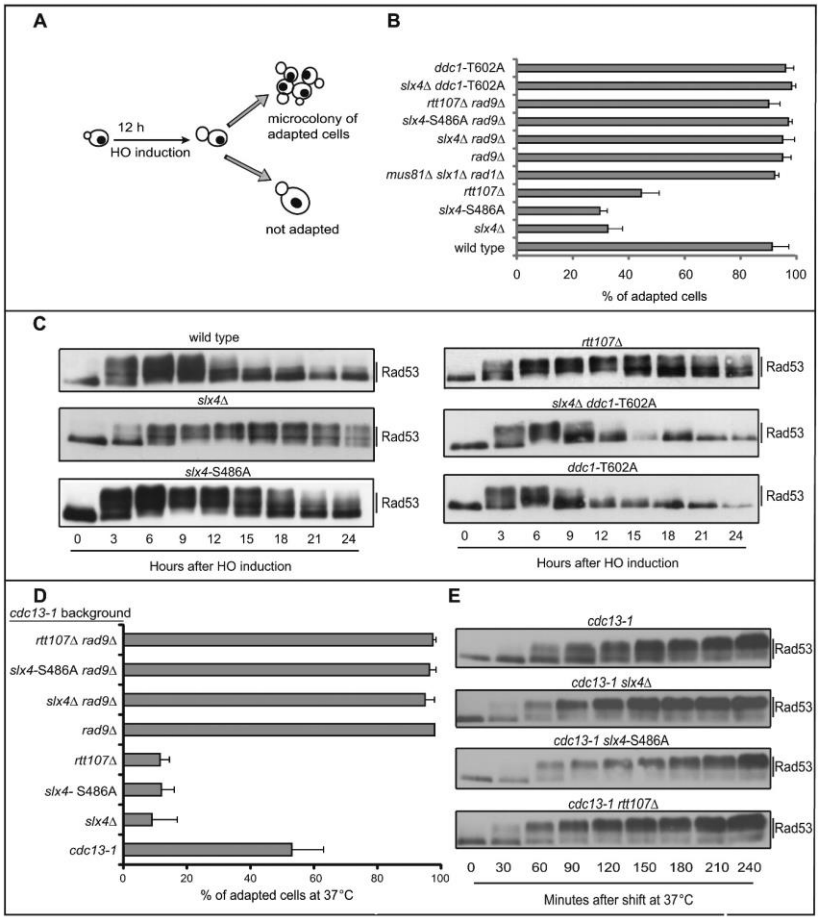

Figure 1. The Slx4-Rtt107 complex is required for checkpoint adaptation to one irreparable DSB and uncapped telomeres. (**A**) Schematic illustration of the HO-cut checkpoint-adaptation assay. (**B**) Graph shows the percentage of adapted cells for each mutant 24 h after plating on galactose containing medium to induce one irreparable HO cut. Values are the mean of three independent experiments ± standard deviation. (**C**) Rad53 phosphorylation analysis by Western Blot in JKM179 derivative strains after HO induction. (**D**) Adaptation assay in *cdc13–1* derivative strains. Graph shows the percentage of adapted cells after 24 h at 37°C. Values are the mean of three independent experiments ± standard deviation. (**E**) Rad53 phosphorylation analysis by Western blot in *cdc13–1* derivative strains after telomere uncapping at 37°C.

both in wild type and *slx4*Δ cells (Supplementary Figure S2). This result is in agreement with previous findings, indicating that Rad9 is recruited to several genome loci through the interaction with the transcription factor Aft1, even in the absence of exogenously induced DNA damage (32). After the induction of the HO cut at *MAT* locus (at ∼0.2 Mb on chromosome III), Rad9 binding increased around the DSB, both in wild type and *slx4*Δ cells (Figure 2A and Supplementary Figure S2), and the binding signal increased and spread along the flanking regions over time. Deletion of *SLX4* resulted in an increase Rad9 binding proximal to the DSB (Figure 2A).

To quantify the difference in Rad9 binding at the irreparable DSB in the presence and absence of Slx4, we performed ChIP followed by quantitative PCR (qPCR) with primers specific for a region 5 kb from the break. We found that deletion either of *SLX4* or *RTT107*, as well as the *slx4*-S486A mutation, led to a significant increase of Rad9 binding 5 kb from the HO cut (Figure 2B). Strikingly, the *ddc1*-T602A mutation, which affects binding to Dpb11 (11,26,27), totally eliminated the increased binding of Rad9 in *slx4*Δ cells (Figure 2C). These results indicate that the Slx4-Rtt107 pathway is critical to limit the accumulation of Rad9, bound to Dpb11 at a persistent DSB, and may provide a molecular explanation for the prolonged checkpoint signalling observed in *slx4*Δ, *rtt107*Δ and *slx4*-S486A cells (Figure 1). Consistent with the proposed model, we also found by ChIP that the Slx4 protein was recruited 5 kb from an HO-induced DSB (Figure 2D). Interestingly, the binding of the Slx4-S486A protein variant was greatly lowered (Figure 2D), according to the effects on Rad9 binding, Rad53 phosphorylation and checkpoint adaptation that we found in *slx4*-S486A cells (Figures 1B,C and 2C). Moreover, deletion of the *DDC1* gene abrogated Slx4 binding near a DSB (Figure 2E), further suggesting that Slx4 binding depends upon the interaction with Dpb11, which in turn is recruited through the 9–1–1 complex (33). We also found that Slx4 binding was severely reduced in *rtt107*Δ (Figure 2E), in agreement with recent findings indicating Rtt107 recruits Slx4 to stressed replication forks (23), and that Rtt107 stabilizes the interaction between Slx4 and Dpb11 (16,34).

Remarkably, Slx4 and Rtt107 were not detectable by ChIP very close to the DSB (35,36), although Dpb11 was recruited soon after the break formation, through the interaction with the 9–1–1 complex (33,37). As Rtt107 interacts with γ-H2AX (36,38), a possible explanation of this discrepancy might be related to the low amount of modified histones close to the break (10,39–42).

The Slx4-Rtt107 complex modulates long-range DSB resection

Rad9 oligomers bound around a DSB represents a physical barrier towards 5′-3′ resection (4). Thus, we hypothesized that DSB resection should be affected in the absence of a functional Slx4-Rtt107 pathway, which leads to increased Rad9 binding (Figure 2). To address this issue, we tested the formation of 3′ ssDNA at one irreparable DSB by Southern blotting of denatured DNA after restriction enzyme digestion (6). The HO-cut was induced in G2/M blocked cells to avoid any possible interference with cell cycle progression. Interestingly, we found that the formation of long 3′ ssDNA tail (specifically the r7 fragment in Figure 3A) was delayed in *slx4*Δ, *slx4*-S486A and *rtt107*Δ mutants, compared to wild type (Figure 3B,C). This may indicate that resection at distal regions from DSB is affected in the absence of a functional Slx4-Rtt107 pathway.

We also analysed DSB resection at different distances from an HO-cut using a more accurate quantitative PCR-based method (Figure 3D) (6,21). Using the same experimental conditions described for the Southern blot (Figure 3B), we found that the percentage of ssDNA accumulated at 5 kb far from the break in *slx4*Δ, *slx4*-S486A and *rtt107*Δ cells was comparable to what found in the wild-type cells (Figure 3E), although Rad9 binding was increased at this site (Figure 2B). Strikingly, a higher amount of unresected DNA was detected 10 kb from the break in *slx4*Δ, *slx4*-S486A and *rtt107*Δ cells (Figure 3F). A possible explanation might be that, as resection was proceeding, the discrepancy between the amount of resected DNA in wild type and *slx4-rtt107* mutant cells increased and became evident only at long distances from the DSB, consistent with what we found by Southern blot (Figure 3B,C).

Importantly, the DSB resection delay observed in *slx4Δ, slx4*-S486A and *rtt107Δ* cells at 10 kb from the break was completely rescued by deleting *RAD9* (Figure 3G), in agreement with the proposed model that the Rad9-dependent DSB resection barrier is higher in *slx4* and *rtt107* mutants.

Taking all the results in Figures 2 and 3 together, we propose that a functional Slx4-Rtt107 pathway contributes to maintaining efficient DSB resection, likely limiting the Rad9 barrier and Rad53 signalling.

DSB resection and DDC inactivation are severely compromised in the absence of both Sae2 and Slx4-Rtt107

Based on the results in Figure 3, we reasoned that deletion of *SLX4* might exacerbate a resection delay in those mutants already defective in DSB processing, particularly short-range resection (3,43). Indeed it is known that double mutants affecting both the short- and long-range resection steps, such as *sae2*Δ *exo1*Δ, show a severe DSB resection defect (6,44–46). To this end, we generated a *sae2*Δ *slx4*Δ double mutant strain and we analysed DSB resection by qPCR, after induction of HO in G2/M blocked cells. In agreement with the hypothesis, the *sae2*Δ *slx4*Δ double mutant cells showed a severe delay in DSB resection (Figure 4A), further supporting our previous conclusion that Slx4 plays a significant role in the long-range DSB resection. Interestingly, we also found that *sae2*Δ *slx4*Δ double mutant cells hyper-activated Rad53 after an irreparable HO-cut, blocking the cell cycle re-start by checkpoint adaptation, even more than the respective single mutants (Figure 4B,C).

We thought that the Slx4-Rtt107 role in the regulation of DDC and DSB processing might contribute to the DSB repair, especially in *sae2*Δ cells. To this end, we took advantage of a genetic system in which interchromosomal recombination between two homologous cassettes on chromosome III and V can occur (47,48). Briefly, in these cells, an HO-induced DSB at an additional *MAT* sequence inserted in chromosome V is repaired by copying the infor-

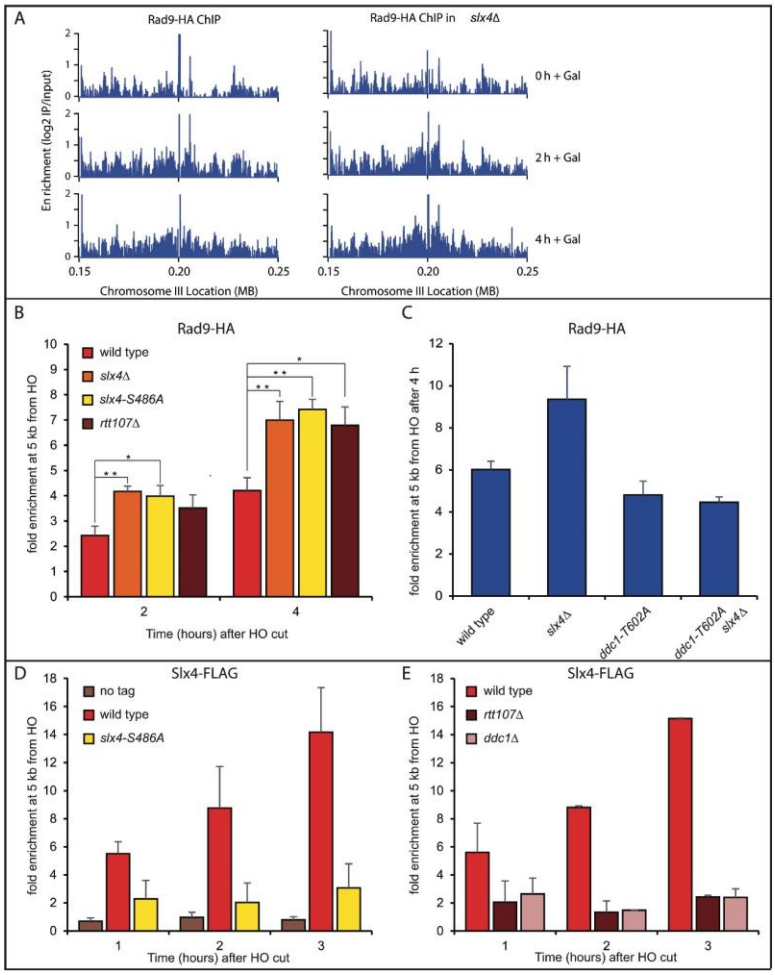

Figure 2. The Slx4-Rtt107 complex modulates Rad9 binding to one irreparable DSB. (**A**) ChIP-seq analysis of Rad9 following induction of a DSB on chromosome III. Rad9 was subjected to chromosome immunoprecipitation at the indicated times after induction of HO endonuclease, in wild type and *slx4*Δ strains. The enrichment scores (the log2 ratio of immunoprecipitate : input) across 100 kb flanking the HO cut site on chromosome III are plotted. (**B**–**E**) ChIP-qPCR analysis showing DSB-induced binding of Rad9, Slx4 or the Slx4-S486A variant at 5 kb from the HO site at the indicated times. All the experiments were performed in nocodazole-blocked cells of the indicated JKM179 derivative strains. In (**B**), (**D**) and (**E**), plotted values are the mean ± SEM of three independent experiments while in (**C**) two experiments were analyzed. Where indicated, significance was determined by single-tailed Student's *t* test (*for $P < 0.05$ and ** for $P < 0.01$).

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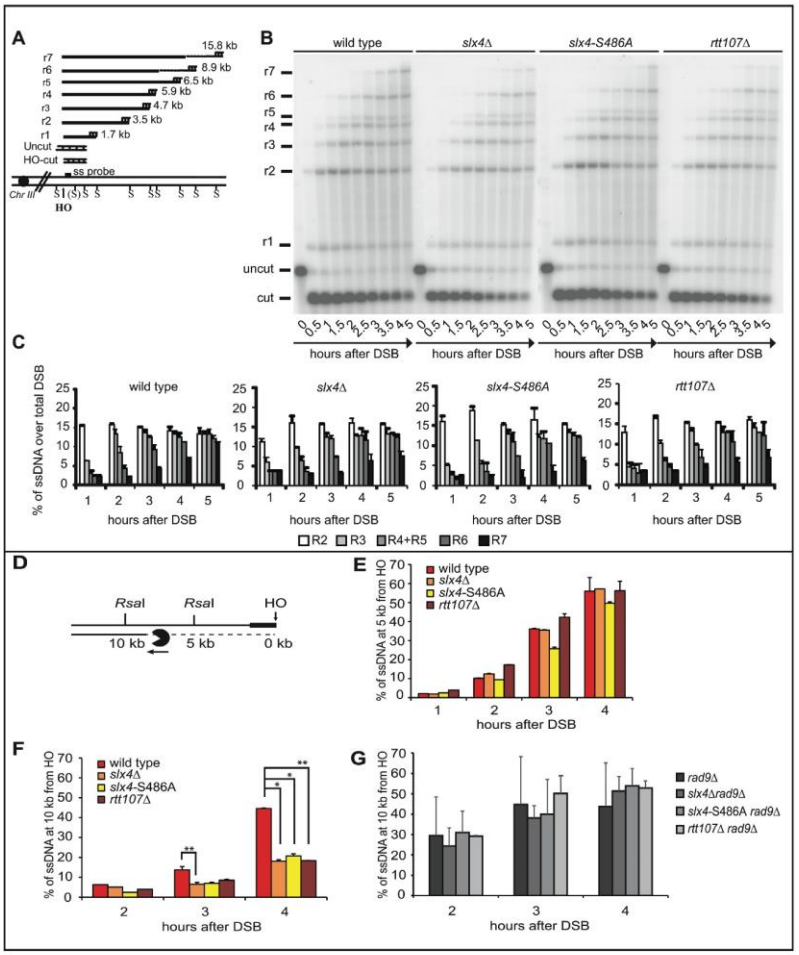

Figure 3. The Slx4-Rtt107 complex modulates long-range DSB resection. (**A**) Schematic illustration of *MAT* locus in JKM179 strains showing positions of HO-cut site, *Ssp*I restriction sites and the RNA probe used. (**B**) ssDNA visualization using RNA probe as described in (**A**) in indicated mutants after inducing HO in nocodazole arrested cells. (**C**) Resection products analysis by densitometry. Plotted values are the mean values ± SEM from two independent experiments performed as in (**B**). (**D**) Schematic illustration of DSB resection at the indicated *Rsa*I sites, 5 kb and 10 kb distal from the irreparable HO cut at the *MAT* locus on chromosome III. (**E**–**G**) DSB resection analysis by qPCR in nocodazole-arrested JKM179 derivative strains. Plotted values are the mean of at least two independent experiments ± SEM. Where indicated, significance was determined by single-tailed Student's *t* test (*for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$).

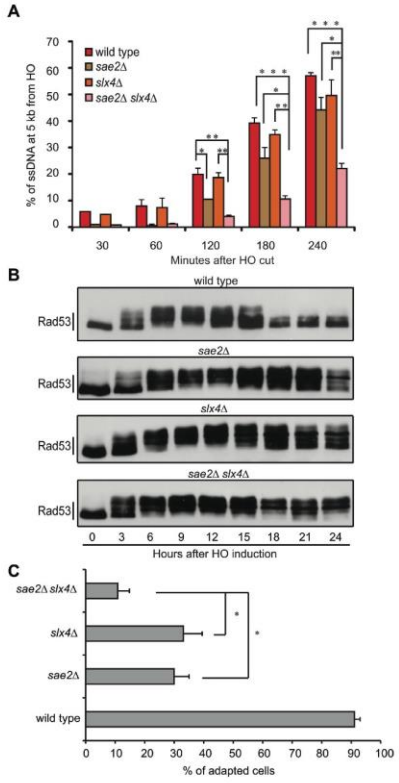

Figure 4. Deletion of *SLX4* exacerbates *sae2*Δ cells phenotypes. (**A**) DSB resection analysis by qPCR in nocodazole-arrested JKM179 derivative strains. Plotted values are the mean of at least two independent experiments ±SEM. Where indicated, significance was determined by single-tailed Student's *t* test (*for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$). (**B**) Rad53 phosphorylation analysis by Western Blot in JKM179 derivative strains after HO induction. (**C**) Graph shows the percentage of adapted cells for each mutant 24 h after plating on galactose containing medium. Values are the mean of three independent experiments ± standard deviation. Where indicated, significance was determined by double-tailed Student's *t* test (* for $P < 0.05$).

mation from the homologous *MATa*-inc locus on chromosome III (Figure 5A), through a gene conversion process that requires 6–8 h and is coupled with DDC activation (47–50). Importantly, Sae2, Rtt107, Rad1 and Mus81 are almost dispensable for DSB repair and cell viability in this assay (47,51,52). By plating the cells in the presence of galactose to induce the HO-cut, we found that the viability of the *slx4*Δ, *slx4*-S486A, *rtt107*Δ and *sae2*Δ single mutants was almost similar to the wild type, while the viability of the *slx4*Δ *sae2*Δ, *slx4*-S486A *sae2*Δ and *rtt107*Δ *sae2*Δ double mutants was severely reduced (Figure 5B). Strikingly, by Southern blotting analysis, we found that the total repair product (as a summary of crossovers and non-crossovers) was reduced in the *slx4*Δ *sae2*Δ double mutant after the HO-cut induction in G2/M blocked cells, although it is not affected in the single mutants (Figure 5C,D). Moreover, Rad53 phosphorylation by western blotting was very robust and prolonged in the *slx4*Δ *sae2*Δ cells during the ectopic recombination assay (Figure 5C), consistent with a persistent DSB. Therefore, our results suggest that the interchromosomal recombination is reduced in *slx4*Δ *sae2*Δ cells mainly as a consequence of their defect in dampening the Rad9-dependent checkpoint and resecting the break.

Supporting the hypothesis that the Rad9 binding near the break, DSB resection and checkpoint signalling are critical events during the interchromosomal recombination in the *slx4*Δ *sae2*Δ, *slx4*-S486A *sae2*Δ and *rtt107*Δ *sae2*Δ cells, the deletion of *RAD9* strikingly rescued the cell lethality in all those double mutants, after DSB induction (Figure 5B,E).

Of importance, *slx4*Δ *sae2*Δ, *slx4*-S486A *sae2*Δ and *rtt107*Δ *sae2*Δ double mutant cells are hypersensitive to both MMS and CPT, even more than the respective single mutant strains (Figure 6), whose sensitivity was already known (16,34,53–55). In particular, *rtt107*Δ cells are reported to be more sensitive to MMS and CPT than *slx4*Δ cells (56,57), therefore in Figure 6C we plated the cells in the presence of lower doses of the drugs, to better show the additive sensitivity of the *rtt107*Δ *sae2*Δ double mutant. Strikingly, the deletion of *RAD9* almost completely rescued the sensitivity of single and double mutants (Figure 6), further suggesting that the hyper-activation of the Rad9-dependent DDC and the slow DNA resection can cause the severe sensitivity to MMS and CPT in these cells.

DISCUSSION

The 53BP1-ortholog Rad9 is crucial for DDC signalling and regulation of DNA end resection in *S. cerevisiae*. Recruitment of Rad9 to DNA lesions is a key aspect of both of these functions, and is mediated by its interaction with modified histones and Dpb11.

Recently, it was shown that the Slx4-Rtt107 complex is in competition with Rad9 for the interaction with Dpb11, contributing to dampen the DDC signalling in the presence of MMS (16). Accordingly, *slx4Δ* cells hyper-activate the Rad9-dependent checkpoint. More recently, it was shown that *slx4*Δ cells accumulate DNA lesions (ssDNA) during stressful replication, and that Slx4-Dpb11 interaction is critical to coordinate the Mus81 nuclease, promoting JM resolution (18). Therefore, these data open a debate on how to discriminate the Slx4 role in checkpoint dampening from its role in DNA replication/recombination. To further understand this issue, here we studied the interplay between the Slx4-Rtt107 complex and Rad9 after the formation of one irreparable HO-cut in the *MAT* locus on chromosome

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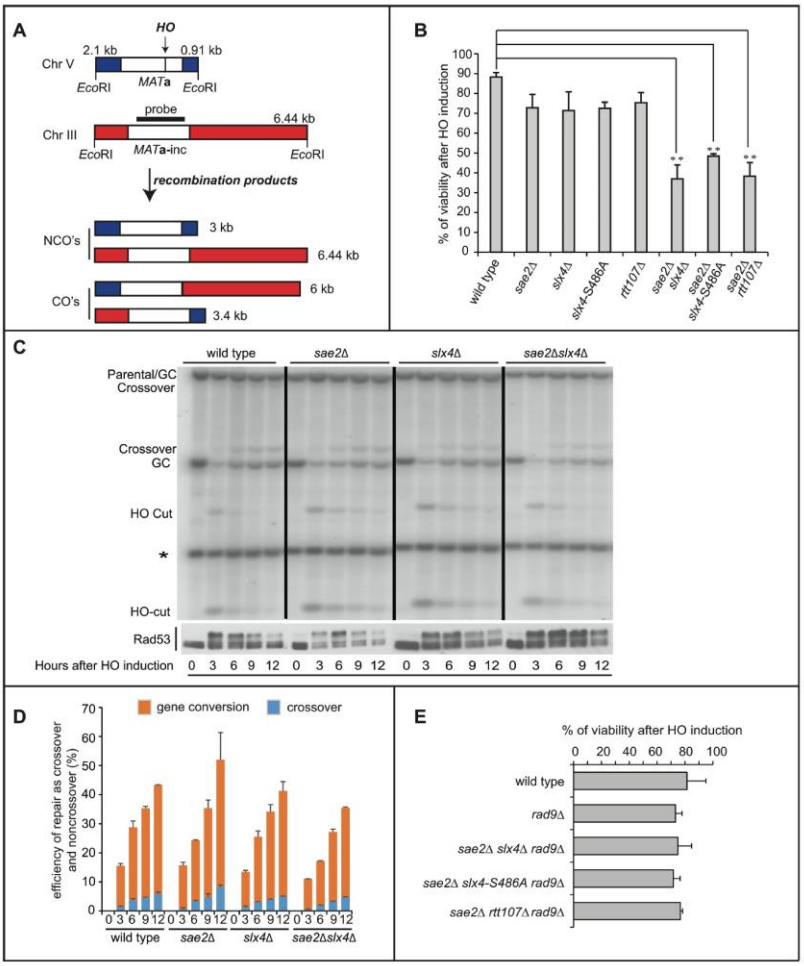

Figure 5. Deletion of *SLX4* affects interchromosomal recombination in *sae2*Δ cells. (**A**) Schematic illustration of *MAT*a-inc locus in Chromosome III and the additional *MAT*a locus in Chromosome V in tGI354 strain, showing positions of HO-cut site, *EcoR1* restriction sites and the probe used to test the interchromosomal recombination. (**B**) Viability of the tGI354 derivatives after the induction of the HO-cut. (**C**) Southern blotting analysis of the interchromosomal recombination using the probe as described in (**A**), in indicated tGI354 derivatives after inducing HO in nocodazole-arrested cells. The intensity of each band was normalized respect to unprocessed *IPL1* locus (*). GC is for Gene Conversion. Western blot analysis shows Rad53 phosphorylation of the same experiment. (**D**) Percentage of crossovers and non-crossovers among all cells in the interchromosomal recombination assay described in (**C**). (**E**) Viability of the tGI354 derivatives after the induction of the HO-cut.

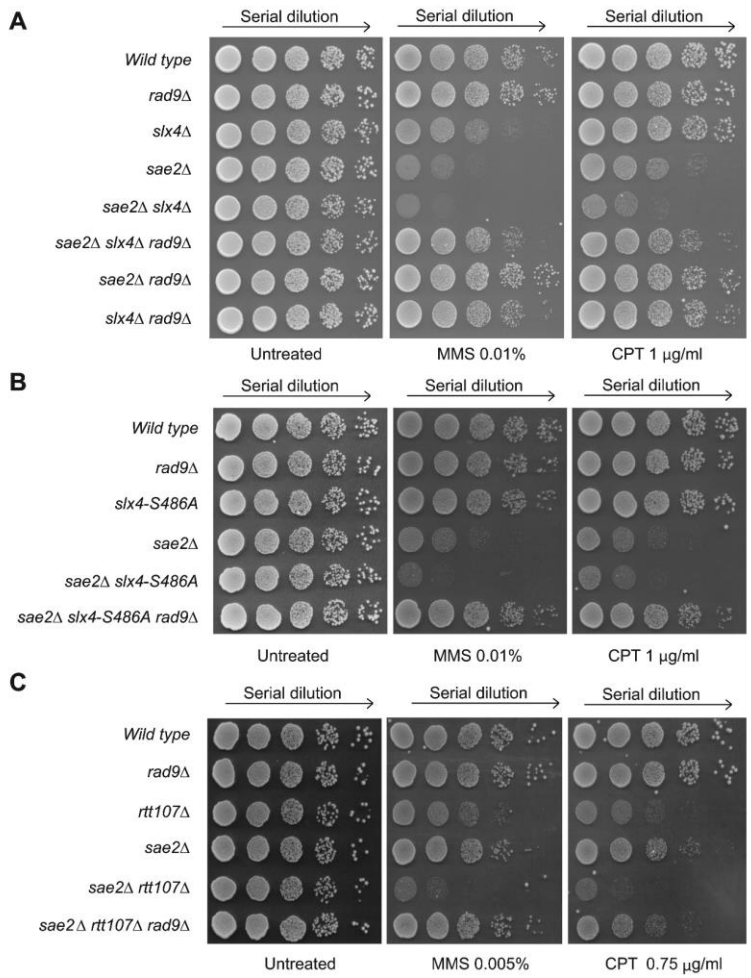

Figure 6. Deletion of *RAD9* rescues the sensitivity to MMS and CPT of *sae2*Δ, *slx4*Δ, *slx4*-S486A and *rtt107*Δ mutant combinations. Exponentially growing cell cultures of the indicated JKM139 derivatives were serially diluted (1:10), and each dilution was spotted out into YPD, YPD + MMS and YPD + CPT plates. Plates were incubated 3 days at 28°C. In panel **C**, we used lower concentration doses of MMS and CPT (see text for details).

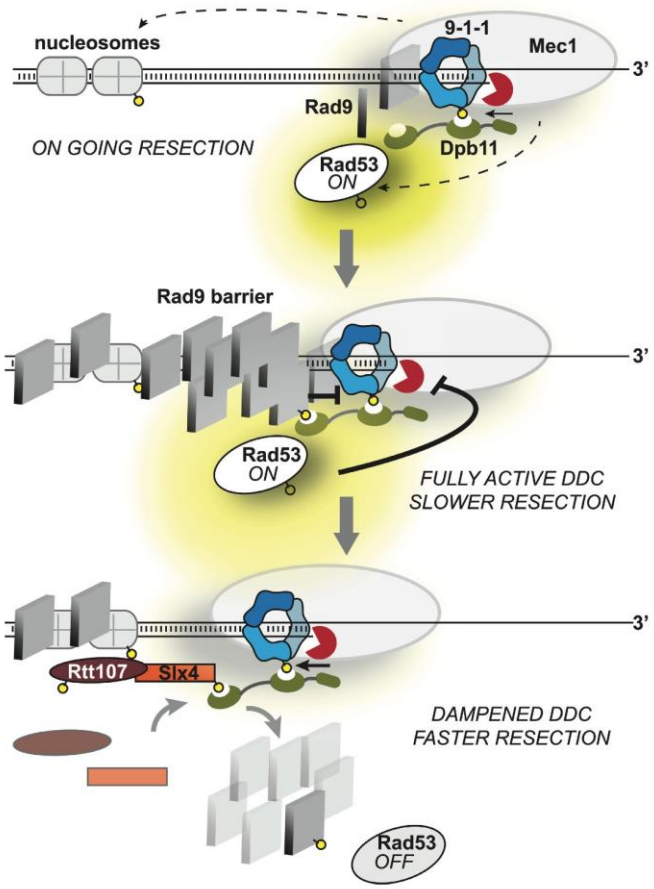

Figure 7. A model for the interplay between Rad9 and the Slx4-Rtt107 complex at a DSB. See text for the details. Yellow circles indicate phosphorylation events.

III, in a strain in which the homologous *HML* and *HMR* sequences were deleted (24). In fact, the Slx4 role in DNA repair and its cooperation with Mus81 and other resolvases is dispensable in this assay, because no recombination intermediate is formed. Therefore, this experimental setup provided us with a defined system to directly investigate the role Slx4 plays independently of these factors.

Strikingly, *slx4*Δ and *rtt107*Δ cells had an increased binding of Rad9 near the DSB, which is dependent on Ddc1 and Dpb11 pathway (Figure 2). As a consequence, the resection of the 5′ strand was slower in *slx4*Δ and *rtt107*Δ cells (Figure 3), Rad53 was hyper-activated and checkpoint adaptation was impaired (Figure 1). Of note, although others showed that Slx4 does not bind close to the DSB (35), we found that Slx4 was recruited within a few Kb from a persistent DSB (Figure 2). Remarkably, the *slx4*-S486A mutation, which prevents Slx4 phosphorylation by CDK1 and its interaction with Dpb11 (16), abolished Slx4 binding to the DSB (Figure 2D), and caused most of the defects found in *slx4*Δ cells (Figures 1–3), suggesting that Dpb11 and CDK1 are important components of this pathway.

Interestingly, we found that *slx4*Δ, *slx4*-S486A and *rtt107*Δ exacerbated the sensitivity of *sae2*Δ cells to HO-breaks, MMS and CPT (Figures 4–6). This additive effect is particularly relevant for the *slx4*Δ and *slx4*-S486A mutations, which per sé do not cause sensitivity to CPT at the dosage tested. Moreover, by using a specific HO-based assay, we found that *slx4*Δ *sae2*Δ cells, but not the single mutants, are defective in interchromosomal recombination (Figure 5C,D). Of note, we recently found that increased binding of Rad9 near a DSB causes all the relevant defects in *sae2*Δ cells: prolonged binding of Mre11, resection delay, reduced recruitment of Rad52 and defect in DSB end-tethering (6). Our data in Figures 4–6 suggest that in the *slx4*Δ *sae2*Δ double mutant the persistent binding of Rad9 limits DSB resection, repair and checkpoint inactivation, even more than the single mutants. In fact, the deletion of *RAD9* rescued the DNA damage sensitivity of cells with dysfunctional Slx4-Rtt107 and Sae2 pathways very well (Figures 5E and 6), strongly suggesting that the defects in dampening the DDC and resecting the DSBs cause cell lethality.

Our results reinforce and expand the notion that Rad9 binding near a DSB is critical for the cell to properly respond and repair DSBs. Indeed, in recent literature there are examples in which the increased Rad9 binding has been associated with a slow DSB resection and a prolonged checkpoint signalling, such as *mec1*-ad and *fun30*Δ cells, which neither recover from, nor adapt to a DSB (58–61). Possibly, the increased Rad9 binding close to DSB ends may affect the balance between NHEJ and HR events, as we showed in *sae2*Δ cells (6). Similar function has been shown for 53BP1 in human cells (62–66).

In conclusion, we show that the Slx4-Rtt107 complex acts as an antagonist of Rad9 binding at DSBs, limiting both the Rad9 checkpoint signalling and DSB resection barrier. Altogether, our findings suggest a working model (Figure 7), in which Dpb11 and Rad9 play a role in the early step of the response to a DSB, activating the DDC. Once extensive resection is on going, the Slx4-Rtt107 complex (likely phosphorylated by Mec1 and CDK1 (16,36,54,67)) competes with Rad9 for Dpb11 binding, dampening DDC and allowing further progression of resection, especially in the presence of nucleosomes. Importantly, the novelties described in our work, after the formation of one persistent DSB, indicate that the role of the Slx4-Rtt107 complex to dampen the DDC is active not only during replication in the presence of MMS (16), but also at DSB lesions. Therefore, it will be important to test in the future whether this mechanism is functional at any types of DNA damage in which the Dpb11-Rad9 axes is engaged. It remains also to be investigated if the Slx4-Rtt107 pathway takes over to counteract Rad9 particularly at persistent DNA lesions. Interestingly, persistent or slowly repaired DSBs re-localize to the nuclear periphery, where they are anchored to the nuclear pore complex (68–70). This phenomenon has been proposed to affect the choice of the repair pathways at persistent DSBs (68). An interesting hypothesis to address is whether the checkpoint dampening and adaptation controlled by the Slx4-Rtt107 pathway occur at the nuclear periphery. This may also correlate with the reduction of DSB repair found in *slx4*Δ *sae2*Δ cells during an ectopic recombination assay (Figure 5C,D), which notably occurs at the nuclear periphery (68).

Considering our data in a wider context, it will be interesting to test if Rad9 may limit ssDNA accumulation during stressful replication in the absence of a functional Dpb11-Slx4-Rtt107 complex (18). In this condition, avoiding the formation of long ssDNA gaps, we can speculate that Rad9 may protect chromosomes from breakages and unscheduled recombination events, preserving genome integrity.

Importantly, mutations in human SLX4 increase sensitivity to DNA damage and are linked with Fanconi Anemia, a genetic disorder associated with high checkpoint marker activation, which could be a cause of bone marrow failure (14,71). Taking that into consideration, in the future it will be relevant to investigate whether SLX4, in addition to its functions in DSB repair, might have a role in controlling DDC and DSB resection in human cells too. Remarkably, we showed that in yeast Slx4 plays an important role in regulating DDC at uncapped telomeres too (Figure 1). An additional open question to address in the future is whether Slx4 might also regulate 53BP1 binding and DDC at eroded telomeres in human cells, where SLX4 localizes to telomeres through TRF2 binding (72,73).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Published Paper III

Slx4 scaffolding in homologous recombination and checkpoint control: lessons from yeast

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REVIEW

Slx4 scaffolding in homologous recombination and checkpoint control: lessons from yeast

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Abstract Homologous recombination-mediated DNA repair is essential for maintaining genome integrity. It is a multi-step process that involves resection of DNA ends, strand invasion, DNA synthesis and/or DNA end ligation, and finally, the processing of recombination intermediates such as Holliday junctions or other joint molecules. Over the last 15 years, it has been established that the Slx4 protein plays key roles in the processing of recombination intermediates, functioning as a scaffold to coordinate the action of structure-specific endonucleases. Recent work in budding yeast has uncovered unexpected roles for Slx4 in the initial step of DNA-end resection and in the modulation of DNA damage checkpoint signaling. Here we review these latest findings and discuss the emerging role of yeast Slx4 as an important coordinator of DNA damage signaling responses and a regulator of multiple steps in homologous recombination-mediated repair.

Keywords Slx4 · Dpb11 · Homologous recombination · Checkpoint · Resection

Introduction

Homologous recombination (HR) is essential for the proper repair of double-strand breaks (DSBs) and several types of replication-associated structures, including collapsed replication forks and ssDNA gaps (Heyer 2015). Not surprisingly, mutations in genes involved in HR are often linked to human diseases, especially cancer predisposition syndromes (Aparicio et al. 2014; Digweed and Sperling 2004; Luo et al. 2000; Moynahan and Jasin 2010). On the other hand, recombination has also the potential to result in genomic instability, such as increased levels of gross chromosomal rearrangements, due to recombination between non-allelic sequences (Carr and Lambert 2013; Kolodner et al. 2002).

HR is a multi-step process that is initiated by 5′ to 3′ exonucleolytic degradation (DNA-end resection) of the DNA lesion, which provides a 3′ ssDNA overhang for the strand exchange protein Rad51 to guide strand invasion and search for homology (for recent reviews please see Cejka (2015), Heyer (2015), Huertas (2010), and Symington (2014)). Successful homology search and subsequent DNA synthesis leads to the formation of recombination intermediates such as Holliday junctions (HJs) that physically link sister chromatids. Disentanglement of these joint DNA molecules (JMs) marks the completion of HR-mediated repair, which needs to occur before chromosomal segregation. This crucial step of JM processing is achieved through either topological dissolution mediated by the Sgs1-Top3-Rmi1 complex (BLM-TOP3α-RMI1-RMI2 in mammals) (Cejka et al. 2012) or via nucleolytic resolution mediated by structure-specific endonucleases such as Slx1-Slx4, Mus81-Mms4, and Yen1 (SLX1-SLX4; MUS81-EME1 and GEN1 in mammals respectively) (Wyatt and West 2014). How cells ensure the proper spatiotemporal coordination of these distinct steps in HR-mediated repair, especially in the context of DNA lesions occurring during DNA replication, remains a major question.

Here, we review the roles of the Slx4 scaffold in the regulation of HR-mediated repair in the model organism *Saccharomyces cerevisiae*. While Slx4 is known to play roles

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in the processing of recombination intermediates by coordinating the action of structure-specific endonucleases, recent studies in budding yeast have revealed additional key roles for Slx4 in other aspects of the DNA damage response and HR repair, including the control of DNA damage checkpoint signaling (DDC) and DNA-end resection, the first step of HR (Fig. 1). Here, we discuss how these emerging roles for yeast Slx4 are now positioning this scaffold protein as a central regulator of multiple steps in HR repair and DNA damage signaling responses.

Slx4 as a scaffold for coordination of structure-specific endonucleases

Slx4 was initially identified in budding yeast in a screen for genes required for cell viability in the absence of the DNA helicase Sgs1 (Mullen et al. 2001). The screen also identified additional "SLX" (synthetic lethal of unknown, "x", function) genes that included the structure-specific nucleases Slx1 and Mus81-Mms4 (Slx3-Slx2). Sgs1 is required for JM processing via dissolution, which in mitotic cells is heavily favored over resolution as the latter can lead to deleterious crossover events (Wu and Hickson 2003). In the absence of Sgs1, the roles of Slx1 and Mus81-Mms4 become critical, especially for the processing of stalled forks generated at hard-to-replicate regions, such as the rDNA locus (Ii et al. 2007; Kaliraman and Brill 2002). Slx4 physically interacts with multiple structure-specific endonucleases and is believed to serve as a platform for the coordination of their action (Flott et al. 2007; Fricke and Brill 2003) (Fig. 2). While Slx4 orthologues are found from yeast to higher eukaryotes (Andersen et al. 2009; Fekairi et al. 2009; Munoz et al. 2009; Saito et al. 2009; Svendsen et al. 2009), they share low amino acid identity with the exception of a conserved C-terminus region consisting of a SAP motif (for SAF-A/B, acinus, and PIAS) that is present in many DNA repair proteins (Aravind and Koonin 2000), and a HtH domain (helix-turn-helix). Strikingly, the ability of Slx4 to physically interact with structure-specific endonucleases is largely conserved from yeast to humans (Fig. 2). Early studies in yeast have revealed that Slx4 physically interacts with Slx1 and with the Rad1-Rad10 complex (ERCC4 (XPF)–ERCC1 in mammals), another structure-specific endonuclease that is not an SLX gene (Flott et al. 2007). Subsequent reports in mammals have identified similar interactions, in addition to an interaction with the MUS81-EME1 nuclease (yeast Mus81-Mms4) (Fekairi et al. 2009; Svendsen et al. 2009).

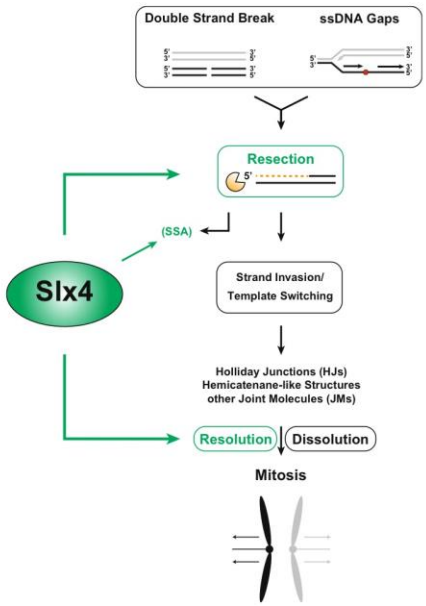

Fig. 1 Simplified depiction of homologous recombination (HR)-mediated repair indicating the steps regulated by Slx4. Repair by HR requires an initial step of resection of 5′ DNA ends, which allows strand invasion and homology search, often leading to the formation of DNA repair intermediates (joint molecules, JMs). These JMs need to be processed via dissolution or resolution pathways before chromosome segregation (see text for more details). Slx4 is important for the regulation of resection and JM resolution. While Slx4 is required for proper single-strand annealing (SSA) repair, it is currently unclear if the SSA defects observed in *slx4Δ* cells are mostly a consequence of improper resection control

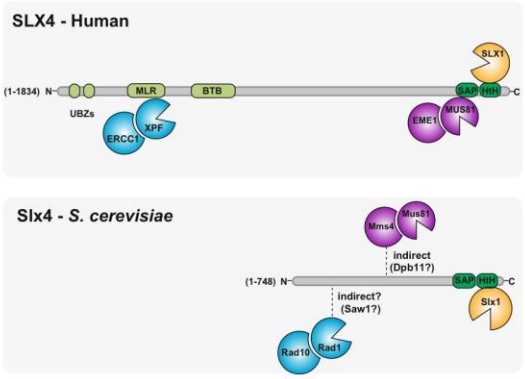

Fig. 2 Domain organization of human and budding yeast Slx4 and modes of interaction with structure-specific endonucleases. *Dashed lines* indicate indirect interaction or lack of evidence for a direct interaction. *UBZ* ubiquitin-binding zinc-finger domain, *BTB* broad-complex, tramtrack, bric-a-brac domain, *MLR* MEI9^{XPF} interaction like region, *SAP* SAF-A/B, acinus and PIAS motif, *HtH* helix-turn-helix motif

Recently, yeast Slx4 was found to also form a complex with Mus81-Mms4 (Gritenaite et al. 2014).

Slx1 is a structure-specific endonuclease that cleaves a wide range of DNA substrates in vitro, but with a preference for 5′-flaps structures in yeast (Coulon et al. 2004; Fricke and Brill 2003; Munoz-Galvan et al. 2012). The interaction of Slx4 with Slx1 is mediated by Slx4's HtH domain (Fig. 2) and a recent structural study in *Candida glabrata* showed that the interaction is responsible for dissociating an inactive Slx1 homodimer to promote its endonuclease activity (Gaur et al. 2015). The importance of Slx1-Slx4, especially in the absence of Sgs1, is likely associated with the fact that replication forks stall with high frequency at the rDNA locus due to the presence of a polar replication fork barrier (RFB) (Brewer and Fangman 1988; Linskens and Huberman 1988). As a consequence, the processing of replication intermediates seems required for proper recovery and restart of these stalled forks (Coulon et al. 2004; Fricke and Brill 2003; Kaliraman and Brill 2002). The role of Slx4 in controlling the action of Slx1 is likely what accounts for the synthetic lethality observed upon deletion of *SLX4* and *SGS1*.

Less is understood about the interaction of Slx4 with Rad1-Rad10. Rad1-Rad10 is required for repair of UV lesions by nucleotide excision repair (NER) and is involved in the repair of DSBs by single-strand annealing (SSA) (Ciccia et al. 2008). It has been proposed that the interaction of Slx4 and Rad1-Rad10 is important for SSA repair, as cells lacking *SLX4* are deficient for SSA, but not for NER (Flott et al. 2007; Li et al. 2008). However, it remains unclear exactly how Slx4 interacts with Rad1-Rad10 and if the interaction plays a role in the regulation of Rad1-Rad10 action. A more recent study suggested a role of the Saw1 scaffold in bridging the Slx4 and Rad1-Rad10 interaction and coordinating their functions (Sarangi et al. 2014). Because human SLX4 binds to XPF-ERCC1 through a long N-terminal region that is absent in yeast Slx4 (Fig. 2), it was proposed that Saw1 may serve the function of the human N-terminal region.

Finally, yeast Slx4 was also shown to indirectly interact with the Mus81-Mms4 nuclease via a phosphorylation mechanism that also involves additional protein scaffolds (Gritenaite et al. 2014). Human SLX4 interacts with MUS81-EME1 through its SAP domain (Fig. 2), and this interaction seems to be crucial for the processing of HJs formed during sister chromatid exchange (Wyatt et al. 2013). However, the functional relevance of the ability of Slx4 to form a complex with Mus81-Mms4 in budding yeast has yet to be established. While it is tempting to extrapolate the identified physical interaction into a role for Slx4 in controlling Mus81-Mms4-mediated resolution, recent findings pointing to roles for Slx4 in the regulation of DNA damage checkpoint signaling and DNA-end resection (see below) reveal a more complex scenario by which Slx4 may be coordinating HR-mediated repair. For more detailed information regarding the roles of Slx4 in the coordination of structure-specific endonucleases, especially in mammals, we refer to recent excellent reviews (Matos and West 2014; Nowotny and Gaur 2016; Rass 2013; Wyatt and West 2014).

Slx4 interactions with multi-BRCT domain proteins point to functions beyond nuclease scaffolding

Recent reports show that Slx4 controls important aspects of the DNA damage response independently of structure-specific endonucleases. Yeast cells lacking *SLX4* are particularly sensitive to methyl methanesulfonate (MMS) (Chang et al. 2002; Fricke and Brill 2003), an alkylating agent that methylates

DNA, leading to replication blocks that are frequently bypassed by replication forks via recombination-mediated template-switching events (Branzei and Foiani 2010). Notably, upon MMS treatment, cells lacking *SLX4* display hyperactivation of the DNA damage checkpoint kinase Rad53 (CHK1/CHK2 in mammals) (Flott and Rouse 2005; Ohouo et al. 2013). However, deletion of any of the nucleases known to interact with Slx4 does not lead to hyperactivation of the checkpoint (Cussiol et al. 2015; Ohouo et al. 2013). Based on these observations, it has been postulated that Slx4 must modulate the DNA damage response independently of structure-specific endonucleases (Ohouo et al. 2013). As discussed later, it is now clear that physical interactions of Slx4 with Rtt107 and Dpb11 (PTIP and TopBP1 in mammals respectively), two scaffolding proteins containing multiple BRCA1 C-terminus (BRCT) domains, are important for the regulation of DNA damage checkpoint signaling and DNA-end resection.

A constitutive interaction with the Rtt107 scaffold

Slx4 was found to interact with the multi-BRCT domain protein Rtt107 ten years ago (Roberts et al. 2006; Zappulla et al. 2006). However, until recently, the role of this interaction has remained obscure. Rtt107 (regulator of Ty1 transposition) was initially identified in screens for mutants with increased Ty transposon mobility or DNA repair defects (Chang et al. 2002; Hanway et al. 2002; Scholes et al. 2001). Structurally, Rtt107 contains six BRCT domains with four tandem BRCT domains at the N-terminus and two at the C-terminus (Bork et al. 1997; Chin et al. 2006; Zappulla et al. 2006) (Fig. 3a). It is believed that only the C-terminal pair of BRCT (5/6) possesses a phospho-binding pocket capable of interacting with phosphorylated proteins (Li et al. 2012). Slx4 constitutively interacts with Rtt107 through its N-terminal BRCT domains and the interaction is not modulated by DNA damage (Roberts et al. 2006). In addition to Rtt107 being the most stably associated Slx4 binding partner, pieces of evidence pointed to the importance of the joint action of the Slx4-Rtt107 complex for the response to replication stress: (i) Cells lacking Rtt107 or Slx4 have similar enhanced sensitivity to MMS and have difficulty recovering from MMS treatment (Roberts et al. 2006); (ii) Rtt107 and Slx4 are heavily phosphorylated by the DNA damage signaling kinase Mec1 (ATR in mammals), especially in response to MMS (Flott and Rouse 2005; Rouse 2004); (iii) Robust phosphorylation of Slx4 by Mec1 requires the presence of Rtt107, and vice versa (Ohouo et al. 2010; Roberts et al. 2006). Of note, Rtt107 is not required for survival in the absence of Sgs1, indicating an Slx1-independent role for the Slx4-Rtt107 complex (Zappulla et al. 2006). It has been proposed that the Slx4-Rtt107 complex is somehow involved in the recovery of stalled forks during replication stress and that Rtt107 works as a scaffold to recruit Slx4 to stalled replication forks (Roberts et al. 2008). This notion was further substantiated by the finding that Rtt107 is recruited to chromatin following replication stress via recognition of γH2A (histone H2A phosphorylated at serine 129) (Balint et al. 2015; Li et al. 2012; Williams et al. 2010), which is generated by the DNA damage signaling kinases Mec1 and Tel1 (ATR and ATM in mammals). Structural and biochemical work have convincingly shown that such recruitment of Rtt107 is mediated by its C-terminal pair of BRCT domains (5/6) (Li et al. 2012; Williams et al. 2010). It is important to note that Rtt107 has Slx4-independent roles as *rtt107Δ slx4Δ* cells are more sensitive to MMS than the single mutants, and *rtt107Δ* cells show enhanced sensitivity to hydroxyurea (HU) while *slx4Δ* cells do not (Roberts et al. 2006). The more central role of Rtt107 is related to its interactions with other genome maintenance factors. In addition to interacting with Slx4, Rtt107 also forms complexes with the Rtt101 ubiquitin ligase complex and the cohesin-like/sumo-ligase Smc5/6 complex (Ohouo et al. 2010; Roberts et al. 2008). These three distinct interactions occur in a mutually exclusive manner (Hang et al. 2015), suggesting a scenario in which Rtt107 functions as a central and multifunctional scaffold for the coordination of DNA damage and replication stress responses.

A phosphorylation-regulated interaction with the Dpb11 scaffold

Mass spectrometry analysis revealed that Slx4 also interacts with Dpb11 (Ohouo et al. 2010), an evolutionarily conserved multi-BRCT domain protein (orthologue of mammalian TopBP1) that plays essential roles in replication initiation and checkpoint activation (Garcia et al. 2005; Mordes et al. 2008; Navadgi-Patil and Burgers 2008; Tanaka et al. 2007; Zegerman and Diffley 2007). While Slx4 directly interacts with Dpb11, Rtt107 was shown to be important to stabilize the interaction (Ohouo et al. 2010). Importantly, the Slx4-Dpb11 interaction is mediated by BRCT domains in Dpb11, mainly BRCT-1/2, and by multiple phosphorylation sites in Slx4 (Fig. 3b) (Ohouo et al. 2010; Ohouo et al. 2013). The interaction is cell cycle regulated and highly dependent on a canonical CDK site on Slx4 (serine 486) (Gritenaite et al. 2014; Ohouo et al. 2013). Furthermore, consistent with the interaction being induced by replication stress or DNA damage, mutation of seven canonical Mec1 sites (7MUT) in Slx4 strongly reduces its interaction with Dpb11 (Ohouo et al. 2010; Ohouo et al. 2013). While yeast cells expressing the *slx4-7MUT* or the *slx4-S486A* alleles are also sensitive to MMS, they are not synthetic lethal with *SGS1* deletion (Ohouo et al. 2010; Ohouo et al. 2013). Together, these findings revealed that a phosphorylation-regulated Rtt107-Slx4-Dpb11 complex plays a key role in the response to replication stress (Fig. 3).

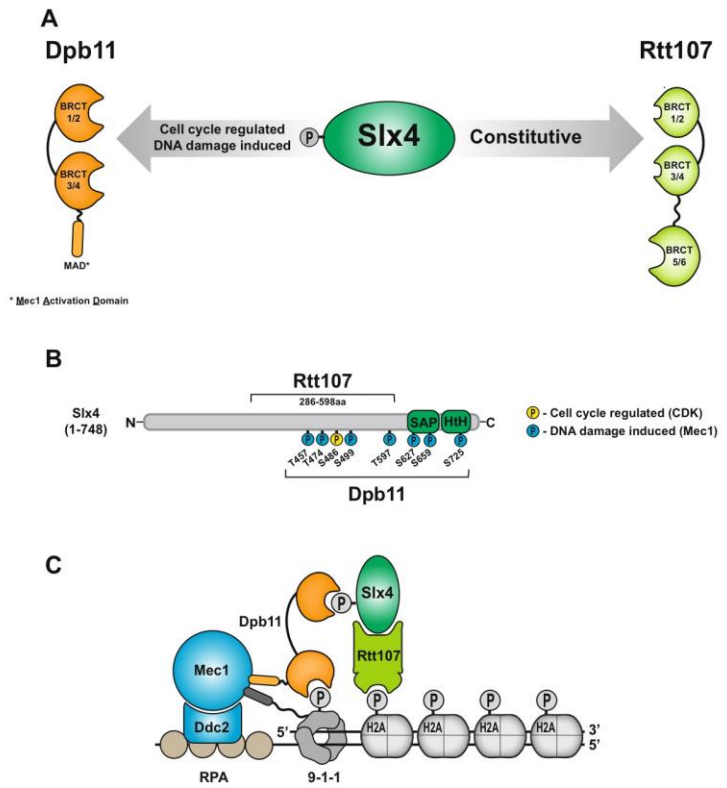

Fig. 3 Modes of interaction of Slx4 with the multi-BRCT domain proteins Dpb11 and Rtt107. **a** While Slx4 interaction with Rtt107 is constitutive and independent of phosphorylation, interaction with Dpb11 requires phosphorylation and is regulated in a cell cycle and DNA damage-dependent manner. **b** Depiction of Slx4 regions and phosphorylation sites important for Rtt107 and Dpb11 binding. **c** Working model for how the Rtt107-Slx4-Dpb11 complex is recruited to DNA lesions. Mec1 phosphorylates the Ddc1 subunit of the 9-1-1 complex (phospho-T602) and histone H2A (phospho-S129) creating docking sites that are recognized by the BRCT domains of Dpb11 (BRCT-3/4) and Rtt107 (BRCT-5/6), respectively

Precise mechanistic understanding of the roles of the Rtt107-Slx4-Dpb11 complex emerged in recent years and points to the control of DNA damage checkpoint signaling and DNA-end resection. Upon DNA damage or replication stress, Dpb11 is recruited to DNA breaks and other replication-induced lesions such as ssDNA gaps via its interaction with Ddc1 (RAD9 in mammals), a subunit of the 9-1-1 checkpoint clamp. Recruitment of Dpb11 to 9-1-1 requires phosphorylation of Ddc1 by Mec1 at threonine 602 that is recognized by BRCT-3/4 of Dpb11 (Fig. 3a). The phospho-dependent Dpb11-Ddc1 interaction is conserved in mammals (TopBP1-RAD9) and in both yeast and mammals, this interaction plays an important role in activation of the DNA damage checkpoint (Delacroix et al. 2007; Navadgi-Patil and Burgers 2009). In budding yeast, both Ddc1 and Dpb11 posses a Mec1 activation domain (MAD), an unstructured region at the C-terminus of these proteins that is required for activation of the Mec1 kinase (Navadgi-Patil and Burgers 2009). In addition to its role in

Mec1 activation, Dpb11 also binds to the checkpoint mediator Rad9 (53BP1 in mammals) in a cell cycle-dependent manner (Pfander and Diffley 2011), which likely helps stabilize Rad9 at DNA lesions. Recruitment of Rad9 by Dpb11 enables Mec1 to extensively phosphorylate Rad9, creating docking phosphosites for the recruitment and activation of the downstream DDC kinase Rad53 (for details on Rad53 activation see Pellicioli and Foiani (2005)). Therefore, Dpb11 is believed to form a complex with Mec1, 9-1-1, Rad9, and Rad53 at sites of lesions, functioning as a pro-checkpoint factor (Fig. 4a). As an intrinsic part of the DNA damage checkpoint response, Rad53 signaling controls a range of functional outputs including cell cycle arrest, inhibition of DNA replication initiation and inhibition of the Exo1 nuclease involved in DNA end resection (for reviews on DNA damage checkpoint, please refer to Branzei and Foiani (2006), Hustedt et al. (2013), and Labib and De Piccoli (2011)). Because the interactions of Dpb11 with Slx4 and Rad9 are largely dependent on BRCT-1/2 of Dpb11, and therefore mutually exclusive, our group proposed a model in which Slx4 binding to Dpb11 counteracts the engagement of Rad9 at DNA lesions, and thereby dampens checkpoint signaling (Fig. 4) (Cussiol et al. 2015; Ohouo et al. 2013). Noteworthy, a recent work provided evidence that the Slx4-Dpb11 interaction is mediated by BRCT-3/4 (Gritenaite et al. 2014), in contrast to our results showing that Slx4 binds preferentially to Dpb11 BRCT-1/2 (Fig. 3c). While we do not exclude the possibility that Slx4 adopts different modes of interaction with Dpb11, as we also observe some level of interaction of BRCT-3/4 with Slx4, more recent experiments demonstrated that mutations in BRCT-1/2 of Dpb11 dramatically reduce its interaction with Slx4, while mutations in BRCT-3/4 have relatively less impact on the interaction (Cussiol et al. 2015). To understand the Slx4-Dpb11 interaction in greater detail, it will be important to study the interaction in vitro using synthetic phosphopeptides that mimic Slx4-phosphorylated surfaces recognized by BRCT domains of Dpb11. Such reconstitution would enable structural and more quantitative analyses. A possible scenario we envision is that the Slx4-Dpb11 interaction undergoes multiple transitions. For example, the interaction may be initiated by recognition of phosphorylated serine 486 in Slx4 via BRCT-1/2, and formation of a transient "bridge" to 9-1-1 that disengages Rad9, as depicted in Figs. 3c and 4a. This "bridging" configuration would allow extensive phosphorylation of Slx4 by Mec1, creating additional phosphosites that are then recognized by BRCT-3/4. It is tempting to speculate that the ability of Slx4 to bind to multiple BRCT domains in Dpb11 may contribute to strengthening the interaction, so Slx4 may more efficiently sequester Dpb11 and prevent it from re-engaging with Rad9. While this transition would free up 9-1-1 to engage with another Dpb11 molecule, the abundance of Dpb11 in the cell is quite low (~200 copies per cell (Ghaemmaghami et al. 2003; Mantiero et al. 2011)), suggesting that it should become limiting.

Recruitment of Slx4 to DNA lesions

How Slx4 is recruited to sites of DNA lesions is a pivotal question for understanding its function as a bona fide scaffold in the control of DNA damage responses. Efficient recruitment of Slx4 to DNA lesions requires interactions with both Rtt107 and Dpb11 (Fig. 3c) (Cussiol et al. 2015; Dibitetto et al. 2016). Recent work demonstrated that recognition of γH2A and phospho-Ddc1^{T602} via BRCT-5/6 of Rtt107 and BRCT-3/4 of Dpb11, respectively, is required for dampening of Rad53 signaling (Cussiol et al. 2015). Based on the available data, Dpb11 seems particularly important for the stabilization of Slx4 precisely at the 5′ recessed end of ssDNA::dsDNA junctions formed at DSBs or ssDNA gaps (we note that the specific

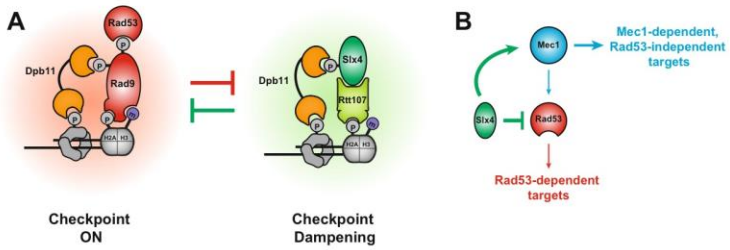

Fig. 4 Slx4 dampens Rad53 signaling by counteracting the Rad9 checkpoint adaptor. **a** Working model for a competition-based mechanism by which Slx4 counteracts the engagement of Rad9 to the 5′ recessed end of a ssDNA::dsDNA junction where the 9-1-1 complex is loaded. "*P*" indicates phosphorylation sites and "*m*" indicates methylation of histone H3K79 (see text for details). **b** Simplified scheme for the role of Slx4 in modulating DNA damage signaling. In addition to uncoupling Mec1 signaling from Rad53 activation, Slx4 contributes to further potentiate Mec1 signaling, likely through the stabilization of Dpb11. As a result, Mec1 partakes on the regulation of processes such as DNA repair without invoking the canonical checkpoint response

recruitment to these junctions may not parallel exactly recruitment to the long track of γH2A). Slx4 is also important to efficiently stabilize Dpb11. A "two-site docking" mechanism was proposed, in which the Rtt107-Slx4-Dpb11 complex recognizes phosphorylated 9-1-1 loaded at these ssDNA::dsDNA junctions, as well as a nearby γH2A-containing nucleosome (Fig. 3c) (Cussiol et al. 2015). Notably, the proposed model predicts that Slx4 can be recruited to any DNA lesion having a ssDNA::dsDNA junction with a 5′ recessed end in which the following events occur: 9-1-1 loading, Mec1 recruitment/activation, and phosphorylation of nearby nucleosomes by Mec1. Consistent with this model, Slx4 is efficiently recruited and phosphorylated upon treatment with MMS, a genotoxin that leads to extensive accumulation of ssDNA gaps behind a moving replication fork and, therefore, multiple ssDNA::dsDNA junctions (Balint et al. 2015; Cussiol et al. 2015; Flott et al. 2007). In addition, resection of double-strand breaks also generates ssDNA::dsDNA junctions that should support Slx4 recruitment. Indeed, ChIP approaches were successfully used to detect recruitment of Slx4 to an HO-inducible DSB (Dibitetto et al. 2016; Leung et al. 2016; Toh et al. 2010). Congruent with these findings, functional studies also reveal important roles for Slx4 in the response to DSBs (Dibitetto et al. 2016).

Slx4 recruitment to DNA lesions counteracts Rad53 signaling

A major step toward understanding the roles for Slx4 that are independent of its nuclease scaffolding functions derived from the observation that cells lacking Slx4 show Rad53 hyperactivation upon MMS treatment (Ohouo et al. 2013). Because Rad53 hyperactivation was not observed in cells lacking the nucleases known to interact with Slx4, we hypothesized that Slx4 is a negative regulator of DDC signaling. Importantly, *slx4Δ* cells did not display enhanced phosphorylation of direct targets of the upstream kinase Mec1, supporting the notion that the increased Rad53 activation in these cells is not due to the higher accumulation of damaged and unrepaired DNA (Ohouo et al. 2013). Compelling genetic evidence of a role for Slx4 in DDC dampening came from the finding that a hypomorphic allele of Rad53 (*rad53-R605A*) is able to rescue the MMS sensitivity of *slx4Δ* cells (Jablonowski et al. 2015; Ohouo et al. 2013). Together, these observations indicate that *slx4Δ* cells are sensitive to MMS due to the inability of properly terminating Rad53 signaling. Because activated Rad53 represses DNA replication and cell cycle progression, it is imperative for the cells to terminate Rad53 signaling in order to resume cell growth. Mechanistically, Slx4 was found to dampen Rad53 signaling by competing with Rad9 for Dpb11 binding (Ohouo et al. 2013) (Fig. 4a). As described above, Slx4 and Rad9 both bind to Dpb11 through a similar mode of interaction that involves BRCT-1/2 of Dpb11 (Ohouo et al. 2013). In addition, the Slx4-Rtt107 complex, as well as Rad9, is recruited to chromatin via a BRCT-mediated interaction with γH2A (Balint et al. 2015; Hammet et al. 2007; Li et al. 2012; Toh et al. 2006; Williams et al. 2010). Therefore, Slx4-Rtt107 and Rad9 are recruited to DNA lesions by interacting with the same docking phosphorylation sites, implying that their recruitment is mutually exclusive. These findings led to the model where Slx4 down-regulates Rad53 signaling via a competition-based mechanism that balances the engagement of the Rad9 adaptor at DNA lesions, a process we named DAMP (Dampens checkpoint Adaptor-Mediated Phospho-signaling) (Ohouo et al. 2013) (Fig. 4a). The checkpoint dampening function of Slx4 is not restricted to MMS-induced replication stress, and a recent report showed that *slx4Δ* cells do not properly down-regulate Rad53 and do not efficiently adapt to one irreparable DSB (Dibitetto et al. 2016). Of importance, these issues were observed in *rtt107Δ* cells, but not in *slx1Δ rad1Δ mus81Δ* cells, which adapt normally to one irreparable DSB, further supporting that the Slx4-Rtt107 complex dampens the checkpoint independently of these nucleases (Dibitetto et al. 2016). In addition, consistent with the competition-based model, the checkpoint adaptation defect of *slx4Δ* cells is correlated with higher recruitment of Rad9 to regions flanking the DSB (Dibitetto et al. 2016). In principle, the competition mechanism does not require dephosphorylation of Rad53 or Rad9, so Slx4-mediated dampening of Rad53 signaling may be considered a phosphatase-independent mechanism for down-regulating the checkpoint. Of note, Slx4-mediated dampening is thought to counteract de novo Rad53 activation, but not de-activate already activated Rad53. This latter function is mostly performed by the Pph3, Ptc2, and Ptc3 phosphatases (Jablonowski et al. 2015; Kim et al. 2011a; O'Neill et al. 2007; Szyjka et al. 2008).

Slx4 promotes DNA-end resection

Why would a bona fide HR factor such as Slx4 control Rad53 signaling? The emerging scenario in yeast is that in addition to arresting the cell cycle and inhibiting DNA replication, Rad53 activity also exerts an inhibitory effect on distinct steps of HR. Therefore, down-regulation of DDC would be critical to promote HR. Recent work revealed that the ability of Slx4 to bind to Dpb11 and counteract Rad9 recruitment is also important to promote DNA-end resection (Dibitetto et al. 2016). This first step in HR is best understood in the context of DSBs and requires the coordinated action of the MRX complex (MRN in mammals) and the Exo1 and Dna2 nucleases (EXO1 and DNA2 in mammals). Resection at a DSB starts through an endonucleolytic nick by the action of the MRX subunit Mre11 (MRE11 in mammals), activated by Sae2 (CtIP in mammals) close to the DSB end (Cannavo and Cejka 2014). This action triggers a short 3′-5′ nucleolytic processing toward DNA ends and allows the more extensive 5′-3′ degradation

mediated by Exo1 and Dna2, in cooperation with the RecQ helicase Sgs1 (Mimitou and Symington 2008; Symington and Gautier 2011; Zhu et al. 2008). Importantly, Rad9 has been shown to block resection (Clerici et al. 2014; Ferrari et al. 2015; Lazzaro et al. 2008), a function that is evolutionary conserved and well documented in the mammalian orthologue 53BP1 (Bunting et al. 2010; Chapman et al. 2013; Di Virgilio et al. 2013; Escribano-Diaz et al. 2013; Zimmermann et al. 2013). In budding yeast, cells lacking *RAD9* resect DSBs faster and more extensively (Chen et al. 2012; Clerici et al. 2014; Lazzaro et al. 2008), and while it remains unknown how Rad9-mediated resection block is achieved, it likely involves multiple mechanisms. One mechanism apparently relies on Rad9-mediated activation of Rad53, which promotes Rad53-dependent phosphorylation and inhibition of Exo1 (Cotta-Ramusino et al. 2005; Jia et al. 2004; Kaochar et al. 2010; Morin et al. 2008; Segurado and Diffley 2008). This Rad53-mediated inhibition of resection is possibly complemented by the ability of Rad9 to oligomerize and form a physical block surrounding the DSB. It is tempting to speculate that the interaction of Rad9 with Dpb11 is important not only to promote Rad53 activation, but also to stabilize Rad9 at the ssDNA::dsDNA junction to efficiently mask the 5′ recessed end, therefore preventing access of Exo1 and Dna2-Sgs1. Congruent with this hypothesis, recent findings have shown that Rad9 reduces the recruitment of Exo1 and Dna2-Sgs1 to DSBs (Bonetti et al. 2015; Ngo and Lydall 2015).

Given the roles of Rad9 in blocking resection, it is logical that the ability of Slx4 to counteract Rad9 recruitment to DNA lesions would help avert the block, therefore promoting resection. Indeed, a recent paper by our groups provided experimental evidence that the Slx4-Rtt107 complex favors resection of DSBs (Dibitetto et al. 2016). Taking advantage of a system for induction of a single irreparable DSB at a specific DNA locus by the HO endonuclease, it is possible to track the amount of ssDNA that is formed once the DSB is resected. Cells lacking *SLX4* or *RTT107* show a significant decrease in ssDNA accumulation at distal regions from a DSB together with a severe defect to dampen Rad53 signaling (Dibitetto et al. 2016). These observations may suggest that Slx4-Rtt107 is particularly important for long-range resection. Consistent with that, Slx4 was not detected close to DSB ends (Toh et al. 2010); however, it is recruited at a few kilobases from the break (Dibitetto et al. 2016; Leung et al. 2016). In agreement with the notion that Slx4-Rtt107 competes with Rad9 for recruitment to DNA lesions (Fig. 4a), decreased resection observed in cells lacking Slx4 or Rttt107 is correlated with increased binding of Rad9 to DSBs (Dibitetto et al. 2016). Moreover, cells expressing the *slx4-S486A* allele phenocopy the resection defects of *slx4Δ* and *rtt107Δ* cells underlining the critical role of the Slx4-Dpb11 interaction. Collectively, these observations corroborate the proposed model for Slx4 recruitment (Fig. 3c) and reveal its role in promoting resection by counteracting Rad9 recruitment and Rad53 signaling (Fig. 4a). While the role of resection in the repair of DSBs by HR is well established, less is known about the importance of resection for the repair of ssDNA gaps by HR. Evidence suggest that expansion of the ssDNA gaps by the action of exonucleases, especially Exo1, would be important to facilitate topological DNA transactions mediated by recombination factors (Giannattasio et al. 2010; Karras et al. 2013; Ma et al. 2013). In agreement with that, Exo1 overexpression can partially rescue the MMS sensitivity of *slx4Δ* cells (unpublished observations).

Checkpoint dampening via Slx4 helps promote Mus81-Mms4 function

In addition to promoting DNA-end resection, the ability of Slx4 to dampen the DDC seems to positively influence other steps in HR. This appears to be the case for Mus81-Mms4-mediated resolution, which is inhibited by DDC signaling. In fission yeast, Cds1 (Rad53 ortholog) binds and phosphorylates Mus81 leading to its dissociation from chromatin, thus preventing the processing of stalled forks (Kai et al. 2005). So far a similar mechanism has not been characterized in budding yeast, but there is circumstantial evidence that DDC signaling through Rad53 inhibits Mus81-Mms4 function indirectly through inhibition of Cdc5 activity (Sanchez et al. 1999; Szakal and Branzei 2013; Zhang et al. 2009). Mus81-Mms4 activity is restricted to late G2/M, before chromosome segregation, when Cdc28 (CDK) and Cdc5 phosphorylate the regulatory subunit Mms4 (Gallo-Fernandez et al. 2012; Matos et al. 2013). This mechanism for temporal restriction is important to prevent premature activation of Mus81 during replication, which could lead to crossover-associated chromosome translocations as well as premature processing of joint molecules (JMs) formed between sister chromatids (Szakal and Branzei 2013). On the other hand, inhibition of Mus81-Mms4 during G2/M would prevent the efficient processing of JMs that escape Sgs1-Top3-Rmi1-mediated dissolution leading to segregation of joint chromosomes and, consequently, chromosome breaks. Consistent with the notion that DDC hypersignaling plays an inhibitory role on Mus81-Mms4 activity, cells lacking the Rad53 phosphatase Pph3 also display MMS sensitivity that seems correlated with the inability to resolve JMs (Jablonowski et al. 2015). These results point to checkpoint dampening as an important mechanism by which Slx4 favors Mus81-Mms4 action, although it remains plausible that this is also achieved via a scaffolding function. Slx4 indirectly interacts with Mus81-Mms4, in a complex that also comprises of Dpb11 and Rtt107 (Gritenaite et al. 2014). Because the SLX4 interaction with MUS81 in human cells has been shown to be important for Holliday junction resolution (Castor et al. 2013), it is tempting to speculate that the interaction of Slx4 with Mus81-Mms4 in budding yeast

is also functionally relevant. However, there are important differences in the architecture of these complexes in yeast compared to humans (Fig. 3), and currently, a separation-of-function mutant that specifically impairs the interaction of Slx4-Dpb11 with Mms4-Mus81 has not been generated. Therefore, the functional relevance of the interaction is yet to be determined. Overall, while yeast Slx4 is likely to play some scaffolding function for Mus81-Mms4 action, lines of evidence point to the control of DDC signaling as being a key mechanism by which Slx4 promotes Mus81-Mms4-mediated resolution.

Slx4 uncouples Mec1 signaling from Rad53 activation

The canonical DDC response involves activation of the apical kinase Mec1 followed by activation of the downstream kinase Rad53, which then mediates several of the established checkpoint outputs (Fig. 4b). As discussed above, Rad53 signaling negatively affects HR, implying that Rad53 needs to be counteracted for pro-HR events such as resection to occur. Consistent with this notion, fork-restart after MMS-induced stalling requires Rad53 deactivation (O'Neill et al. 2007; Szyjka et al. 2008). Interestingly, Mec1 directly

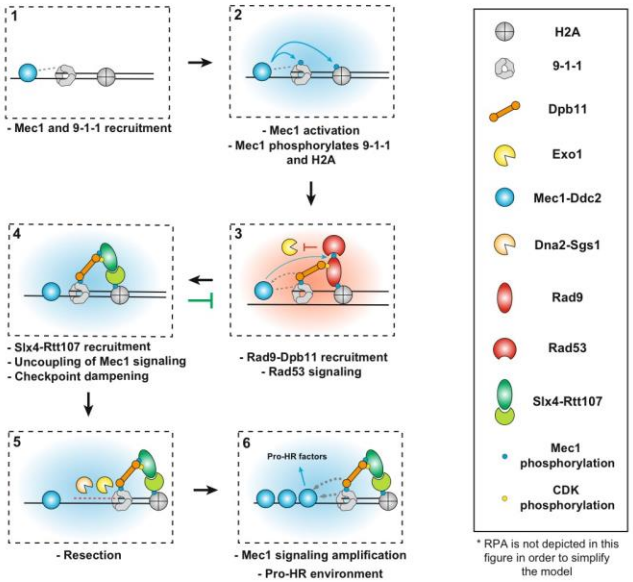

Fig. 5 Spatiotemporal regulation of DNA damage signaling via Slx4. *1* DNA damage leads to the exposure of ssDNA that is rapidly coated by the ssDNA binding protein RPA (not depicted in the cartoon). RPA recruits the Mec1 kinase (via Ddc2 interaction) to DNA lesions, while the 9-1-1 complex is loaded at the 5′ recessed end of the ssDNA::dsDNA junction. The Ddc1 subunit of the 9-1-1 complex interacts with Mec1 through its Mec1 activation domain (MAD), hence stimulating Mec1 kinase activity. *2* Mec1 phosphorylates histone H2A (γ-H2A) and the Ddc1 subunit of the 9-1-1 (phospho-Ddc1) complex. *3* Rad9 and Dpb11 are recruited to the site of lesion via interactions with γ-H2A and phospho-Ddc1, respectively. Once recruited, Dpb11 further stimulates Mec1 kinase activity as it also possesses a MAD. Rad9 becomes extensively phosphorylated by Mec1, which promotes Rad53 recruitment creating the Rad53 activation complex. At this step, resection is not favored due to the inhibition of Exo1 by Rad53 and potentially via Rad9 oligomerization (not depicted in the picture) that could prevent exonucleases from accessing the 5′ recessed end. *4* Build up in the γ-H2A platform surrounding the lesion site helps recruit the Slx4-Rtt107 complex nearby the lesion site, leading to Slx4 phosphorylation by Mec1 (not depicted in the picture), which enhances Slx4 interaction with Dpb11 and competes out Rad9 from the site of lesion. As a result, Mec1 signaling is uncoupled from Rad53 signaling (checkpoint dampening). *5* Resection is favored as Rad53 dampening ceases Exo1 inhibition and Rad9 displacement allows accessibility of nucleases to the recessed 5′ end. *6* Increased ssDNA exposure allows more Mec1 recruitment, while stabilization of Dpb11 and 9-1-1 further stimulates Mec1 signaling that may help regulate subsequent steps in HR-mediated repair

phosphorylates many proteins independently of Rad53 (Bastos de Oliveira et al. 2015; Smolka et al. 2007) and, in contrast to Rad53 signaling, this direct mode of Mec1 signaling apparently exerts multiple pro-HR effects. For example, it has been proposed that Mec1 phosphorylation of Rad51 promotes its ATP hydrolysis and DNA binding activity (Flott et al. 2011). In addition, phosphorylation of the Sae2 endonuclease by Mec1 seems to be important to promote DNA-end resection and repair by HR (Chen et al. 2015; Clerici et al. 2006; Puddu et al. 2015). Finally, Rtt107 phosphorylation by Mec1 has been reported to be important for proper sister chromatid recombination (Ullal et al. 2011). Therefore, the ability of Slx4 to specifically counteract Rad53 activation while maintaining active Mec1 signaling represents a clever mechanism for HR regulation (Fig. 4b). The role of Slx4 in uncoupling Mec1 signaling from Rad53 activation was first noticed using quantitative phosphoproteomic analysis (Ohouo et al. 2013), and more recently, the Brown lab showed that Slx4 in fact contributes to amplify Mec1 signaling, possibly by stabilizing Dpb11 (a Mec1 activator itself) at the DNA lesion (Figs. 3c and 5) (Balint et al. 2015). In addition, Rad9 displacement and Rad53 down-regulation promotes resection, which in turn recruits more Mec1 due to increased ssDNA exposure. Taken together, these findings point to an intricate regulatory mechanism by which Slx4 controls a range of signaling events with the ultimate goal of facilitating HR (see model in Fig. 5).

An integrated model for HR-control via Slx4

In summary, recent reports in budding yeast have revealed unexpected new roles for Slx4 in the control of HR, suggesting a complex mode of action for this scaffold at the intersection of HR and checkpoint signaling (Fig. 6). Importantly, it seems now clear that yeast Slx4 operates in HR control not only by serving as a scaffold for structure-specific endonucleases, but also by interacting with the BRCT-domain scaffolds Dpb11 and Rtt107 to counteract Rad9 function and regulate DDC signaling. This latter role is critical for Slx4 to function as a pro-HR factor promoting DNA-end resection and JM resolution. Figure 6 depicts our current integrated view for how Slx4 functions in the regulation of HR, differentiating the roles that are mediated via physical interaction with structure-specific nucleases and the roles promoted via counteraction of Rad9 and DDC. It remains unclear the precise contribution of the interactions between Slx4 and Mus81-Mms4 and between Slx4 and Rad1-Rad10 for JM resolution and SSA, respectively.

Does mammalian SLX4 also play pro-HR roles independently of its interaction with structure-specific nucleases?

It is currently unclear whether the roles of yeast Slx4 in regulating DNA-end resection and checkpoint signaling are conserved in mammals. In humans, mutations in the *SLX4* gene

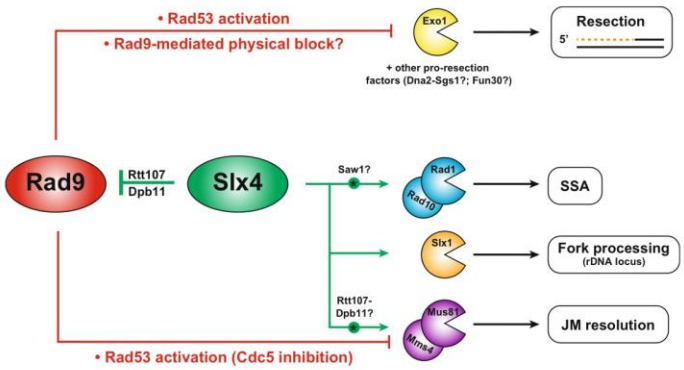

Fig. 6 Integrated view for how Slx4 facilitates HR repair in budding yeast. In addition to functioning as a scaffold for the coordination of structure-specific nucleases, Slx4 plays key roles in the modulation of DNA damage signaling to favor multiple steps in HR-mediated repair. Slx4 positively regulates DNA-end resection by counteracting Rad9 as detailed in Figs. 4 and 5. Moreover, Slx4 dampening of Rad53 signaling favors JM resolution via Mus81-Mms4 by allowing cell cycle progression and timely activation of the cell cycle kinases CDK and Cdc5. *Asterisks* indicate where the role of the physical interaction with Slx4 has not yet been established

are found in Fanconi anemia (FA) patients, an autosomal recessive disease associated with congenital disorders, bone marrow failure and predisposition to cancers (Kim et al. 2011b). The FA pathway plays established roles in protecting chromosome integrity during S-phase, especially when replication forks stall at DNA interstrand cross-links (ICLs). Furthermore, mammalian SLX4 is also important for the repair of other types of lesions induced by camptothecin and PARP inhibitors (Kim 2014). It will be interesting to test whether in mammals, SLX4 functions as a pro-resection factor to initiate HR-mediated repair of distinct DNA lesions. As such, it would be important to explore whether human SLX4 retained across evolution the ability to compete with 53BP1 for binding to TopBP1 (human ortholog of Dpb11) to avert a resection block. Consistent with this hypothesis, human SLX4 was shown to interact with TopBP1 in osteosarcoma tumor cells (Gritenaite et al. 2014) and be targeted by the ATR/ATM kinases (Matsuoka et al. 2007). While there are no evidence pointing to the relevance of ATR/ATM phosphorylation in promoting binding to TopBP1, it is tempting to speculate that, like in yeast, ATR/ATM signaling may somehow facilitate SLX4 recruitment to DNA lesions. However, we do not exclude the possibility that in mammals, other protein scaffolds besides SLX4 are in fact functioning as yeast Slx4 in the coordination of ATR signaling, checkpoint responses, and HR-mediated repair. Coordination of these processes in mammals is likely to be extremely complex, so understanding the exact mechanisms by which this coordination is achieved will be an enormous challenge. Because DDC and HR dysfunctions are closely linked to many human cancers, we anticipate that a better understanding of the mechanisms interfacing DDC signaling and HR control in humans will provide important new directions to understand tumorigenesis and to develop treatment. Interestingly, a recent report pointing to roles for mammalian SLX4 in interfacing with sumoylation-mediated pathways may open new avenues to understand how SLX4 regulates DNA damage responses independently of nuclease scaffolding (Guervilly et al. 2015).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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TOPBP1/Dpb11 controls DNA repair through the coordinated recruitment of 53BP1/Rad9

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TOPBP1/Dpb11 Controls DNA Repair Through the Coordinated Recruitment of 53BP1/Rad9

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Abstract

Genome maintenance and cancer suppression require homologous recombination (HR) DNA repair. In yeast and mammals, the scaffold protein TOPBP1^{Dpb11} has been implicated in HR, although its precise function and mechanism of action remain elusive. Here we show that yeast Dpb11 controls pro- and anti-recombination factors through mutually exclusive interactions. The BRCT domains of Dpb11 mediate opposing roles in the control of DNA end resection by coordinating both stabilization and exclusion of Rad9 from DNA lesions. In human cells, we find that TOPBP1^{Dpb11} engages in interactions with the anti-resection factor 53BP1 and the pro-HR factor BRCA1, suggesting that TOPBP1 also mediates opposing functions in HR control. Consistent with a pro-NHEJ function for TOPBP1, hyper-stabilization of the 53BP1-TOPBP1 interaction enhances the recruitment of 53BP1 and NHEJ factors to nuclear foci in S-phase and induces chromosomal aberrations. Our results support a model in which TOPBP1^{Dpb11} is a regulator of repair pathway choice.

Introduction

The proper repair of double strand breaks (DSBs) that occur during DNA replication is heavily dependent on error-free homologous recombination (HR) (Heyer, 2015; Schwartz and Heyer, 2011). However, DSBs may also be repaired by the direct ligation of DNA ends through non-homologous end joining (NHEJ). Because of the risk of ligating wrong ends and/or deleting DNA sequences, NHEJ is considered an error-prone repair mechanism. During DNA replication, NHEJ repair is particularly deleterious due to the intrinsic increased incidence of breaks, especially one ended DSBs, whose inappropriate joining leads to dicentric chromosomes that initiate break-fusion cycles and complex chromosome rearrangements (Gaillard et al., 2015; Gelot et al., 2015). In fact, NHEJ-mediated mutagenic repair is a major contributor to genomic instabilities and tumorigenesis that arise when the HR machinery is deficient (Deng and Wang, 2003; Prakash et al., 2015). Therefore, the ability of cells to inhibit NHEJ and favor HR during DNA replication is essential for genome integrity.

A critical step in regulating the choice of HR or NHEJ for repair is the control of 5'-to-3' nucleolytic processing of DNA ends (also referred as resection), as the formation of long 3' ssDNA tails naturally promotes HR while preventing NHEJ (for review see (Chapman et al., 2012b; Prakash et al., 2015)). 53BP1 is a scaffolding protein that plays a major role in limiting resection (Bothmer et al., 2010; Bunting et al., 2010). Although the mechanism by which 53BP1 limits resection remains incompletely understood, it involves the 53BP1-dependent recruitment of the additional anti-resection factors such as RIF1 (Callen et al., 2013; Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013; Kumar and Cheok, 2014; Zimmermann et al., 2013). In S-phase, the

tumor suppressor BRCA1 is proposed to play a key pro-HR function by counteracting the recruitment of 53BP1 to DSBs, therefore enabling resection (Bunting et al., 2010). This model is supported by genetic data in mice showing that the loss of 53BP1 suppresses embryonic lethality, genomic rearrangements and tumorigenesis seen in mice lacking functional BRCA1 (Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009; Prakash et al., 2015). DNA end resection is inhibited during S-phase in cells lacking BRCA1, and the increased recruitment of 53BP1 to replication-induced lesions results in mutagenic NHEJ and increased chromosomal aberrations (Bunting et al., 2010; Escribano-Diaz et al., 2013). Collectively, these observations support a model for repair pathway choice in which BRCA1 and 53BP1 compete for the sites of DNA lesions to promote HR or NHEJ. However, there is a fundamental gap of knowledge concerning how BRCA1 counteracts the engagement of 53BP1 in S-phase at the lesions to suppress genomic instability and cancer.

While many aspects of the mammalian DNA repair are conserved in budding yeast, it remains unknown whether key mechanisms of HR-control and DNA repair pathway choice are also conserved. Notably, a clear sequence homolog or a functional analog of BRCA1 has not been identified in fungi. On the other hand, the 53BP1 ortholog Rad9 has been shown to play a conserved role in resection block (Clerici et al., 2014; Ferrari et al., 2015; Lazzaro et al., 2008). Cells lacking *RAD9* resect DSBs faster and more extensively (Chen et al., 2012; Clerici et al., 2014; Lazzaro et al., 2008). Of importance, it was recently proposed that a complex formed by the DNA repair scaffolds Slx4 and Rtt107 is able to counteract the engagement of Rad9 at replication-induced lesions to dampen checkpoint signaling (Ohouo et al., 2013). Given the roles of Rad9 in blocking resection, we predicted that the ability of Slx4-Rtt107 to counteract Rad9 recruitment to DNA lesions would help avert the block, therefore promoting resection. Indeed, recent

work presented experimental evidence that the Slx4-Rtt107 complex favors resection of DSBs (Dibitetto et al., 2016).

TOPBP1 (Dpb11 in yeast) is an essential scaffolding protein that plays evolutionarily conserved roles in the initiation of DNA replication and activation of DNA damage checkpoint signaling (Boos et al., 2011; Navadgi-Patil and Burgers, 2008; Pfander and Diffley, 2011; Puddu et al., 2008; Tanaka et al., 2007; Zegerman and Diffley, 2007). TOPBP1^{Dpb11} is comprised of multiple BRCT (BRCA1 C-terminus) domains (9 in humans and 4 in yeast), which are protein interacting modules that often recognize phosphorylated motifs (Manke et al., 2003; Rodriguez et al., 2003; Yu et al., 2003). TOPBP1^{Dpb11} recognizes phospho-proteins via its BRCT domains and has been shown to assemble multi-subunit complexes required for replication initiation or checkpoint activation (Boos et al., 2011; Pfander and Diffley, 2011; Tak et al., 2006; Zegerman and Diffley, 2007). Although TOPBP1 has been implicated in HR DNA repair (Germann et al., 2011; Liu and Smolka, 2016; Morishima et al., 2007; Moudry et al., 2016), its precise role and mode of action remain largely elusive. Here we show that in budding yeast, Dpb11 plays a decisive role in the control of DNA end resection, the first key step in HR, by mediating a competition between the anti-resection protein Rad9 and the pro-resection scaffolds Slx4-Rtt107 for DNA lesions. In humans, we find that TOPBP1 coordinates the recruitment of 53BP1 via a physical interaction that appears to be mutually exclusive with that of the pro-HR factor BRCA1. Our results support a model in which TOPBP1^{Dpb11} controls the mutually exclusive engagement of antagonistic regulators of DNA repair for the proper control of repair pathway choice and genome integrity maintenance.

Results and Discussion

BRCT domains of Dpb11 mediate mutually antagonistic functions in DNA end resection

In budding yeast, Dpb11 has been shown to recruit Rad9 to the 9-1-1 clamp loaded at DNA lesions to promote activation of the DNA damage checkpoint (Fig. 1A) (Abreu et al., 2013; Granata et al., 2010; Pfander and Diffley, 2011; Wang et al., 2012). Since Rad9 and its human ortholog 53BP1 have both been shown to block DNA end resection, we hypothesized that the role of Dpb11 in mediating the recruitment of Rad9 to DNA breaks plays a decisive role in resection control and HR-mediated DNA repair. To test this, we fused BRCT domains 3/4 of Dpb11 with full-length Rad9 (Fig. 1B), with the expectation that this chimera would hyper-stabilize Rad9 at DNA lesions. Using a system to induce a persistent DSB at the MAT locus through the over-expression of HO endonuclease (Lee et al., 1998; White and Haber, 1990) we found that the Dpb11$^{BRCT3/4}$-Rad9 chimera (hereinafter referred to as 'B3/4-Rad9') is robustly detected at 0.15kb from the break site using ChIP-qPCR (Fig. 1C). Of importance, a point mutation corresponding to K544A in Dpb11, known to disrupt the ability of BRCT-3/4 to recognize phosphorylated 9-1-1, prevents the stabilization of B3/4-Rad9 near the site of DSB (Fig. 1C). Taking advantage of this system, we assessed the effect of Dpb11-mediated Rad9 hyper-stabilization on short-resected DNA ends using an assay to monitor the accumulation of ssDNA flanking an irreparable HO-induced DSB site (Dibitetto et al., 2016; Ferrari et al., 2015). Although we did not observe an impact on resection at 0.15 kb from the break, resection is significantly inhibited at 1.4 kb and severely blocked at 4.8 kb from the break site upon expression of the B3/4-Rad9 chimera (Fig. 1D). The K544A mutation that impairs BRCT-3/4 fully restored resection, arguing for a key role of Dpb11 in bridging Rad9 to the 9-1-1

complex, thus inhibiting long-range resection.

We have recently proposed a model in which Dpb11 also mediates the controlled disengagement of Rad9 from lesions for dampening checkpoint signaling (Fig. 1E) (Cussiol et al., 2015; Ohouo et al., 2013). In this model, the Slx4-Rtt107 scaffolding complex competes with Rad9 for Dpb11 interaction, ultimately preventing Rad9 from stabilizing at DNA lesions. We hypothesized that this competition mechanism is also crucial to control the roles of Rad9 in DNA repair and could provide the molecular basis to understand how 53BP1 recruitment is regulated in mammals. We predicted that a fusion of the Slx4-Rtt107 complex with Dpb11 BRCT-3/4 should be able to antagonize the B3/4-Rad9 chimera and restore resection. We have previously shown that a fusion of Dpb11 BRCT3/4 with Rtt107 BRCT-5/6 (referred as MBD: minimal multi-BRCT domain module) (Fig. 1F) mimics the role of the Dpb11-Slx4-Rtt107 complex in checkpoint dampening (Cussiol et al., 2015). Here we found that expression of MBD prevents hyper-stabilization of the B3/4-Rad9 chimera at DSBs (Fig. 1G) and, strikingly, fully suppresses the resection block induced by B3/4-Rad9 (Fig. 1H). Collectively, the above results are consistent with a model in which Dpb11 plays mutually antagonistic roles in resection by coordinating the stabilization as well as exclusion of Rad9 from DNA lesions (Fig. 1E and I).

Dpb11-mediated recruitment of Rad9 impairs HR-mediated repair in response to replication stress

Slx4 and Rtt107 have been shown to be particularly important in the response to MMS-induced replication stress (Ohouo et al., 2010). We therefore asked whether the B3/4-Rad9 chimera also impairs the control of resection and HR-mediated repair in cells treated with MMS, a DNA alkylating agent that blocks replication fork progression. While MMS treatment resulted in the formation of multiple RPA foci in cells expressing the

mutated BRCT-3/4(K544A)-Rad9 chimera, expression of the chimera B3/4-Rad9 bearing functional BRCT-3/4 prevented most cells from accumulating multiple RPA foci (Fig. 2A), consistent with the less accumulation of ssDNA at replication forks likely due to inhibition of DNA end resection. This defect in RPA foci formation is accompanied by a severe reduction in Rad52 foci (Fig. 2B), pointing to an impairment of HR-mediated repair. These results show that Dpb11-mediated recruitment of Rad9 also plays an important role in coordinating DNA end resection and HR repair in the response to replication blocks. Because the resection block imposed by B3/4-Rad9 at DSB is counteracted by the Slx4-Rtt107-Dpb11-mimicking module MBD as shown in Fig. 1, we speculated that the impaired HR-mediated repair induced by B3/4-Rad9 at replication-induced lesions could also be rescued by the co-expression of MBD. Indeed, MBD was sufficient to rescue B3/4-Rad9-induced MMS sensitivity (Fig. 2C). Expression of B3/4-Rad9 led to hyper-activation of the checkpoint effector kinase Rad53 in cells treated with MMS as evaluated by the mobility shift of Rad53, consistent with the Dpb11-mediated function of Rad9 in promoting checkpoint signaling (Fig. 2D). This aberrant Rad53 hyper-phosphorylation as well as the appearance of a hyper-shifted form of B3/4-Rad9 was suppressed by the co-expression of MBD, which is in agreement with the reduced binding of B3/4-Rad9 nearby an HO-induced DSB upon MBD expression. These data again reinforce the competition-based model in which Dpb11 regulates HR-mediated repair by coordinating the mutually exclusive recruitment of Slx4 and Rad9, and reveal that Dpb11 plays antagonistic roles in HR-mediated repair also in the context of replication stress.

According to our model, the control of Dpb11 interactions with Slx4 or Rad9 is expected to play a key role in the control of DNA end resection. Therefore, the decision to specifically stabilize the Dpb11-Slx4 interaction should be the distinguishing molecular

event that transitions Dpb11's function from blocking resection to favoring resection. Since interactions of Dpb11 with Slx4 and Rad9 are both dependent on CDK (Gritenaite et al., 2014; Ohouo et al., 2013; Pfander and Diffley, 2011; Wang et al., 2012), we reasoned that CDK activity is unlikely to be the discerning molecular event that commands the choice of Slx4 versus Rad9 stabilization at DNA lesions. Previously, we have shown that the Dpb11-Slx4 interaction requires the Mec1 kinase (Ohouo et al., 2010) and here we show that Mec1 is specifically required to enhance the Dpb11-Slx4 interaction but not the Dpb11-Rad9 interaction (Fig. 2E). Therefore, we propose a model in which resection block imposed by the Rad9-Dpb11 complex is counteracted by Mec1 signaling through the formation of a Dpb11-Slx4-Rtt107 complex (Fig. 2F).

Proteomic analysis in human cells reveals TOPBP1 interactions with antagonistic repair factors

Based on our findings in yeast, we speculated that TOPBP1 also plays a role in coordinating the recruitment of antagonistic factors for the proper control of DNA repair in mammals. Previous reports revealed that TOPBP1 indeed interacts with the pro-NHEJ protein 53BP1 and a range of pro-HR factors, including BRCA1-associated proteins (Greenberg et al., 2006; Morishima et al., 2007; Yamane et al., 2002; Yoo et al., 2009). We reasoned that TOPBP1 interactions specifically induced by replication stress should reveal pro-HR functions for TOPBP1. We therefore performed an unbiased mass spectrometry analysis to define the network of TOPBP1 interactions in cells either treated with hydroxyurea (HU) to induce replication stress, or with nocodazole to reveal interactions that are independent of replication stress (Fig. 3A). Next, we measured the changes of the identified interactions by directly comparing cells treated with HU or nocodazole in order to specifically reveal interactions induced by replication stress (Fig. 3B). While most interactions did not display major changes in our comparison (Fig. 3B).

the interaction of human TOPBP1 with a pro-HR factor, in this case BRCA1, is strongly induced by replication stress (Fig. 3B), similar to what we previously observed for yeast Dpb11 (Ohouo et al., 2010),. Of interest, the interaction of TOPBP1 with 53BP1 is reduced under replication stress (Figs. 3B-C) suggesting that the interactions of TOPBP1 with BRCA1 and 53BP1 are mutually exclusive. The finding that both of these interactions require functional BRCT-1/2 and BRCT-5 domains of TOPBP1 (Fig. 3D) further supports this notion (although we note that BRCA1 additionally requires BRCT-7). Because 53BP1 and BRCA1 localize to sites of DNA lesions in a mutually exclusive manner (Chapman et al., 2012a), and have been proposed to compete for DNA lesions to dictate repair pathway choice (Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009; Chapman et al., 2012a), our findings suggest that TOPBP1 could be the mediator of such competition, similar to the role of Dpb11 in coordinating the competition between Rad9 and Slx4 in yeast. Also similar to the yeast model, the ATR kinase plays an important role in promoting the interaction of TOPBP1 with a pro-HR factor, in this case BRCA1, but is not required for enhancing the TOPBP1-53BP1 interaction (Fig. 3E). Overall, these findings are consistent with the hypothesis that yeast Dpb11 and mammalian TOPBP1 have roles in coordinating the action of antagonistic repair factors via a competition-based mechanism (Fig. 3F). The results are suggestive of a key role for ATR^{Mec1} in promoting HR-mediated repair by promoting the $BRCA1\text{-}TOPBP1^{Slx4\text{-}Dpb11}$ interaction upon replication stress. We note that human SLX4 was also identified as a TOPBP1 interactor, consistent with a previous report (Gritenaite et al., 2014). Of note, the interaction of TOPBP1 with SLX4 is not enhanced by replication stress, suggesting a fundamentally distinct mode of interaction compared to the Dpb11-Slx4 interaction in yeast.

Hyper-stabilization of the TOPBP1-53BP1 interaction promotes 53BP1 recruitment

to nuclear foci in S-phase and induces chromosomal aberrations

Based on our findings in yeast, we hypothesized that human TOPBP1 controls the recruitment of 53BP1 to DNA lesions under certain conditions and is important to mediate 53BP1-mediated DNA repair. To test this hypothesis, we engineered a system to stabilize the 53BP1-TOPBP1 interaction. We were unable to generate a similar chimeric protein we generated in yeast, since a fusion of 53BP1 with BRCT domains of TOPBP1 did not express in human cells lines. To circumvent this issue, we fused 53BP1 to a 120 amino acid region from the N-terminal domain of RFC1 (replication factor C subunit 1), which we found to constitutively interact with TOPBP1 (Fig. 3B). Thus, by fusing the N-terminus of RFC1 (hereinafter referred to as Constitutive TOPBP1-interacting Region, CTR) to 53BP1 (Fig. 4A), we reasoned that the interaction of this chimera with TOPBP1 would be stabilized and enhanced during replication stress. Indeed, the CTR-53BP1 chimeric protein displays enhanced interaction with TOPBP1 after hydroxyurea (HU) treatment (Fig. 4B). Strikingly, the CTR-53BP1 chimera forms significantly more nuclear foci compared to 53BP1 alone in cells progressing through S-phase following release from an HU-induced arrest (Figs. 4C-D), suggesting the enhanced recruitment of CTR-53BP1 to replication-induced lesions.

Once recruited to the lesion site, 53BP1 promotes the recruitment of PTIP and RIF1, two proteins believed to function as effectors of NHEJ (Callen et al., 2013; Chapman et al., 2013; Munoz et al., 2007; Zimmermann et al., 2013). To investigate whether the increased recruitment of CTR-53BP1 functionally impacts 53BP1-mediated DNA repair, we first monitored PTIP and RIF1 status. Interestingly, CTR-53BP1 pulled down more PTIP compared to 53BP1 alone, despite the relatively lower expression level of CTR-53BP1 (Fig. 4B). In addition, while we were unable to monitor PTIP foci using available antibodies, we found that CTR-53BP1 induces a significant increase in the number of

RIF1 foci in S-phase cells released from a HU arrest (Figs. 4C-D). Since RIF1 and PTIP recruitment to DNA lesions is believed to require DNA damage-induced phosphorylation of 53BP1 (Callen et al., 2013; Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013; Kumar and Cheok, 2014; Munoz et al., 2007; Zimmermann et al., 2013), our results strongly suggest that the enhanced interaction with TOPBP1 increases the engagement of CTR-53BP1 at sites of lesions, culminating on its phosphorylation and subsequent increased recruitment of RIF1, and likely PTIP.

The foci formed by CTR-53BP1 co-localized with γ-H2AX and RIF1 but not with RPA or RAD51, two markers for HR (Fig. 4E), suggesting that the chimera is engaging in RIF1-mediated DNA repair in a mutually exclusive manner with the HR-machinery. We therefore hypothesized that expression of CTR-53BP1 would induce genomic instability by promoting mutagenic NHEJ repair and/or deregulating HR-mediated repair. Indeed, we observed a significant increase in the number of chromosomal aberrations induced by the expression of CTR-53BP1, but not by expression of ectopic 53BP1, in response to replication stress induced by a combination of PARP inhibitor (AZD2461) and ATR inhibitor (VE821), which we reasoned would induce the formation of collapsed replication forks with concomitant inhibition of the TOPBP1-BRCA1 interaction (Figs. 4F-G).

Collectively, these findings support a model in which TOPBP1 mediates the recruitment of 53BP1 to DNA lesions to promote 53BP1-dependent mutagenic DNA repair and genomic instability (Fig. 4H). We further speculate that the BRCA1-TOPBP1 interaction may play a function analogous to the Slx4-Dpb11 interaction in yeast to prevent TOPBP1-mediated stabilization of 53BP1 at DNA lesions. Of importance, it has been recently reported that depletion of TOPBP1 abrogates RAD51 loading to chromatin and formation of RAD51 foci, but does not impair DNA end resection and RPA loading

(Moudry et al., 2016). Based on our model, we predict that in their experimental setup the absence of TOPBP1 would also impair 53BP1-mediated resection block, thereby allowing productive DNA end processing to occur. In fact, the scenario would be similar to what is observed in cells lacking both BRCA1 and 53BP1, where resection is restored as compared to cells lacking only BRCA1 (Bunting et al., 2010). Therefore, the findings by Moudry et al., are fully consistent with our model that TOPBP1 is important to promote 53BP1 functions in DNA repair. Overall, our findings presented here provide important insights into the mode of action of TOPBP1^{Dpb11} in the control of DNA repair and should have implications to understand how genomic instabilities and cancer arise in individuals with a defective HR-machinery.

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Materials and Methods

Yeast strains, plasmids, media and growth conditions.

Strains generated in this study were derived from S288C or JKM139. All tags were inserted at the C-terminus of the corresponding genes by homologous recombination at the genomic locus and were verified by western blotting. Tagged strains were assayed for sensitivity to MMS to ensure they behaved similarly to the wild-type strain. Standard cloning methods were used to generate the plasmids for this study. B3/4-Rad9 chimera was generated using a stitch PCR protocol. Briefly, we fused the Rad9 promoter (450 base pairs upstream of the start codon) to the Dpb11 BRCT3/4 (292-600aa) and the resulting PCR product was fused to the entire Rad9-3xFLAG sequence (see Fig. 1B for the schematic illustration of the chimeric protein). The final PCR product was subsequently cloned into pRS416 (for ectopic expression) or pFa6a (for integration at the *RAD9* genomic locus). All point mutations were generated by site-directed mutagenesis using the Primestar® Max DNA Polymerase (Takara). All yeast strains and plasmids used in this study are described in Supplementary Tables S1 and S2, respectively. Yeast cells expressing genes with the indicated epitope tags were cultured in YPD or in synthetic complete medium lacking uracil and/or tryptophan when carrying an expression plasmid with *URA3* or *TRP1* (derivatives from pRS416 and pRS414 respectively). Cells were grown to log phase, subjected to MMS treatment as specified in the figures and collected by centrifugation. For the experiments using JKM139 and YMV80 strains, cells were grown in YP medium enriched with 2% glucose (YPD), 3% raffinose (YP raff) or raffinose 3% and galactose 2% (YP raff gal). All the synchronization experiments were performed at 28°C.

Measurement of resection at HO-induced DSB

HO-induced DSB resection was measured in JKM139 background by quantitative PCR analysis as described previously (Ferrari et al., 2015). Cells were arrested in G2/M by nocodazole treatment before HO induction. Genomic DNA was extracted and digested or mock-treated with RsaI restriction enzyme (NEB), which cuts inside the amplicons at 0.15 kb, 1.4 kb and 4.8 kb from the HO-cut site, but not in the *PRE1* control region on chromosome V. PCR values are then normalized by the cut efficiency calculated by southern blot analysis.

ChIP analysis

ChIP analysis for detection of proteins nearby an HO cut site was performed in nocodazole-arrested cells as described previously (Ferrari et al., 2015). The oligonucleotides used are listed in Table S6. Data are presented as fold enrichment at the HO cut site (0.15 kb from DSB) over that at the *PRE1* locus on chromosome V and then normalized by the corresponding input sample.

Co-immunoprecipitation procedure in yeast

Co-immunoprecipitation experiments for yeast lysates were performed as described previously (Cussiol et al., 2015).

Mammalian cell culture and immunoprecipitation procedures

Human U2OS and HEK293T cell lines were grown in DMEM media supplemented with 10% BCS, non-essential amino acid and penicillin/streptomycin (Corning). Microscopy and immunoprecipitation experiments were performed 48 hours post-transfection followed by the according drug treatment. HEK293T Cells were subjected to either 24 hrs of HU (1 mM) or 14hrs of nocodazole (100 ng/mL) post-transfection before

harvesting. For the ATR inhibition experiment, cells were pretreated with 10μM ATR inhibitor (VE821) for 45 min before the additional treatment with 2.5 mM HU for another 30 min in the presence of VE821. Cell pellets were lysed for 30min on ice in modified RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% tergitol, 0.25% sodioum deoxycholate, 5mM EDTA) supplemented with Complete EDTA-free protease inhibitor cocktail (Roche), 5 mM sodium fluoride, 10 mM β-glycerol-phosphate, 1 mM PMSF and 0.4 mM sodium orthovanadate. Protein lysates were cleared by 10 min centrifugation to pellet cell debris and then incubated with anti-TOPBP1 resin, anti-HA or FLAG agarose beads (Sigma-Aldrich) as specified for 4 hrs at 4°C. Immunoprecipitates were then washed three times with the modified RIPA buffer and then eluted using 3 resin volumes of the elution buffer (0.5 μg/mL of FLAG peptide in 100 mM Tris-HCl for anti-FLAG resin; 0.2% tergitol and 100 mM Tris-HCl pH 8.0 and 1% SDS for others).

Immunoblotting analysis

Whole cell lysates and eluents were denatured with 3x SDS sample buffer (composed of bromophenol blue, stacking gel buffer, 50% glycerol, 3% SDS and 60 mM DTT) and resolved on SDS-PAGE gels. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes and probed with desired antibodies.

Mass spectrometry analysis

For mammalian SILAC experiments, HEK293T cells were grown in SILAC DMEM media lacking arginine and lysine (ThermoFisher Scientific 88425) supplemented with 10% dialyzed FBS and penicillin/streptomycin. “Light” DMEM media were supplemented with “light” (normal) arginine and lysine; ‘‘heavy’’ DMEM media were supplemented with “heavy” lysine $^{13}C6$, $^{15}N2$ and “heavy” arginine $^{13}C6$, $^{15}N4$. Cells were treated with 1mM HU for 24hrs or 100ng/ml nocodazole for 14 hrs accordingly before harvesting. TOPBP1

is immunoprecipitated using affinity-purified TOPBP1 antibodies (kindly provided by Dr. Raimundo Freire) or antibodies that recognize the according epitope tags. Immuno-precipitated proteins were then reduced, alkylated, precipitated and digested by trypsin. The peptides were desalted, dried, reconstituted in 80% acenotrile and 1% formic acid and then fractionated by Hydrophilic Interaction Chromatography (HILIC). Fractions were dried, reconstituted in 0.1% trifluoroacetic acid, and analyzed by LC-MS/MS using a Q-Exactive Orbitrap mass spectrometer as described (Ohouo et al., 2010). Database search and quantitation of heavy/light peptide isotope ratios were performed as described (Smolka et al., 2007).

Chemicals

PARP inhibitor (AZD2461) and ATR inhibitor (VE821) were purchased from Selleckchem. Nocodazole was purchased from Calbiochem. Hydroxyurea and MMS were purchased from Acros organics.

Antibodies

Rad53 and yeast epitope tagged proteins were probed using specific antibodies: anti-Rad53 antibody (clone Mab EL7, 1:30 dilution); anti-FLAG (M2 F1804; Sigma, 1:5000 dilution), anti-HA (12CA5; Roche, 1:10000 dilution), ECL HRP-linked secondary antibody (NA931-GE, 1:10000 dilution). The following antibodies were used in the mammalian part: anti-FLAG (M2, F1804; Sigma), anti-HA.11 (MMS-101P; Covance), 53BP1 (NB100-304; Novus Biologicals, RIF1 (sc-55979; Santa Cruz), phospho-KAP-1(S824) (A300-767A-T; Bethyl) phospho-CHK1 (Ser345) (#2341; Cell Signaling), BRCA1(#OP92, MS110; Calbiochem). Previously described TOPBP1 and BRCA1 antibodies were provided by Dr. Raimundo Freire (Danielsen et al., 2009; Kakarougkas et al., 2013). Dr. Kai Ge provided the antibody against PTIP.

Immunofluorescence

U2OS cells grown on glass coverslips, after subjected to the desired drug treatment, were fixed with 3.7% formaldehyde/PBS for 15 min at room temperature. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature, blocked with 5% BSA for 30min at 37°C and incubated with primary antibodies for 1hr at room temperature. This is followed by three washes with PBS and secondary antibody incubation (alexa fluor 568 donkey anti-mouse, 647 donkey anti-goat). Next cells were washed with PBS three times and mounted using vectashield antifade mounting medium with DAPI (H1200; Vector Laboratories).

Microscopy analysis

The images were acquired using a CSU-X spinning disc confocal microscope (Intelligent Imaging Innovations) on an inverted microscope (DMI600B; Leica), with 63×, 1.4 NA objective lens (mammalian cells), 100×, 1.46 NA objective lens (yeast cells) and a charge-coupled device camera (cool-SNAP HQ2, Photometrics) for mammalian cells or electron-multiplying charge-coupled device camera (QuantEM; Photometrics) for yeast cells. SlideBook software (Intelligent Imaging Innovations) was used to obtain Z-stack images. Maximum intensity projections were created in the Slidebook software and exported for analysis in Image J. For mammalian RIF1 and 53BP1 foci analysis, more than 150 transfected cells for each condition were imaged and analyzed per replicate. Cells with more than 10 distinct RIF1 foci or 53BP1 variant foci were scored as foci-positive cells. The percentage of RIF1 foci or 53BP1 variant foci-positive cells was calculated. The arithmetic mean and standard error of the mean (SEM) derived from 3 replicates were used for the plot. A two-tailed Student's t-test with 95% confidence interval was used to determine if the difference between the means of two sets of values

was significant. For yeast Rfa1 and Rad52 foci analysis, cells were grown in SC media until log phase (OD = 0.3) and MMS (0.033%) was added to the cells for 2 hrs at 30°C. Next, cells were washed in sterile water and resuspended in fresh SC media. Live yeast cultures were mounted on an agarose slide pad (1.2% agarose in SC–TRP media). More than 150 cells were scored for each replicate. The percentage of cells with Rad52-mRuby2 or Rfa1-mRuby2 foci was calculated based on the presence of Rad52 foci and the presence of single RPA focus or multiple RPA foci. The graph is plotted using the mean±SEM from three independent experiments.

Metaphase spread preparation

HEK293T cells were co-transfected with 53BP1 or CTR-53BP1 together with H2B-GFP, purchased from Addgene (Addgene plasmid #11680), as a marker for successful transfection when capturing metaphase images. Cells grown in DMEM media were treated with colcemid (150ng/ml) for 1 hr and collected by trypsination followed by centrifugation. Cell pellets were resuspended in hypotonic buffer (0.034 M KCl) for 6min at 37°C and then fixed in fixation buffer (3:1 of methanol and acetic acid) overnight. Fixed cells were then washed, spotted onto microscope slide, and mounted using vectashield antifade mounting medium with DAPI. Metaphase spreads were imaged using the CSU-X spinning disc confocal microscope with 100×, 1.46 NA objective. Chromosomal aberrations were then scored. Each condition is repeated at least two times independently and more than 45 metaphases were analyzed per replicate. The two-tailed Student's t-test was used for statistical analysis.

Figure legends

Figure 1. A competition-based mechanism for the modulation of Rad9 recruitment and DNA end resection via Dpb11 BRCT domains.

(A) A working model for the role of Dpb11 in the recruitment of Rad9 to the 5' recessed end of a DNA lesion.

(B) Schematic illustration of the B3/4-Rad9 ($Dpb11^{BRCT3/4}$-Rad9) chimera.

(C) ChIP-qPCR analysis showing the recruitment of B3/4-Rad9 to an HO-induced DSB site. JKM139 derivative strains expressing the indicated chimera proteins, or an untagged Rad9 control, were arrested with nocodazole and HO endonuclease expression was then induced for the indicated time to trigger an irreparable DSB on chromosome III.

(D) HO-induced DSB resection analysis by qPCR in nocodazole-arrested JKM139 derivative strains expressing indicated Rad9 constructs. For (C) and (D) the graph is plotted using mean ± SEM from at least three independent experiments. P value is determined based on single-tailed Student's t test (*for P<0.05, ** for P<0.01 and *** for P<0.001).

(E) A working model for the role of the Dpb11-Slx4-Rtt107 complex in antagonizing Rad9 recruitment at lesion sites.

(F) Schematic illustration of the MBD (Minimal multi-BRCT-domain module) chimera.

(G) ChIP-qPCR analysis of B3/4-Rad9 or B3/4(K544A)-Rad9 recruitment in cells expressing MBD or not. The graph is plotted using mean ± SEM from at least two independent experiments.

(H) DSB resection analysis by qPCR to determine the effect of MBD expression on resection efficiency in Rad9, B3/4-Rad9 or B3/4(K544A)-Rad9 expressing cells. The

graph is plotted using mean ± SEM from at least two independent experiments.

(I) A working model for the role of the Slx4-Rtt107 complex in counteracting Dpb11-mediated recruitment of Rad9 to promote DNA-end resection.

For the experiments in Fig. 1, B3/4-Rad9 and MBD chimeras were integrated into the *RAD9* and *SLX4* loci, respectively.

Figure 2. Dpb11-mediated hyper-stabilization of Rad9 impairs proper HR repair of replication-induced lesions.

(A) Rfa1-mRuby2 foci were quantified in MMS-treated cells expressing either B3/4-Rad9 chimera or the mutated chimera B3/4(K544A)-Rad9 (fusions were integrated in the *RAD9* locus). Percentage of cells with one Rfa1 focus or multiple Rfa1 foci were plotted as indicated. More than 150 cells were scored per replicate.

(B) Rad52-mRuby2 foci were quantified in cells expressing either B3/4-Rad9 or the mutated chimera B3/4(K544A)-Rad9 (fusions were integrated in the *RAD9* locus). Cells were analyzed after treatment with 0.033% MMS for 2hrs or following recovery of cells for 2 hrs in fresh media after MMS treatment. Cells were evaluated based on the presence or the absence of Rad52-mRuby2 foci. More than 150 cells were scored per replicate.

(C) MMS sensitivity of wild-type cells expressing MBD and/or B3/4-Rad9 from plasmids. Four-fold serial dilutions were spotted on SC-URA-TRP plates and grown for 2–3 days at 30°C.

(D) Immunoblots showing the phosphorylation status of Rad53 and B3/4-Rad9 in cells expressing Rad9 or B3/4-Rad9 or co-expressing B3/4-Rad9 with MBD. The Rad9 fusions were integrated in the *RAD9* locus and MBD was expressed from a plasmid.

(E) Co-IP of Dpb11 with Slx4 or Rad9 in wild-type or *mec1Δ* cells treated with 0.033% MMS for 1 hr.

(F) A model for the role of Dpb11 in resection control via coordination of Slx4 and Rad9.

For (A), (B) and (D), B3/4-Rad9 chimera was integrated into the *RAD9* locus. Graph is plotted using mean ± SEM from at least three independent experiments. *P* value is determined based on single-tailed Student's t test (*for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$).

Figure 3. Proteomic analysis of TOPBP1 interactions modulated by replication stress.

(A) Quantitative mass spectrometry analysis of TOPBP1 interactions in cells arrested with HU or nocodazole. HEK293T cells were grown in "light" and "heavy" SILAC media and treated with 1mM hydroxyurea (HU) for 24 hrs to identify proteins that interact with TOPBP1 in response to replication stress. Two independent experiments were performed, one pulling down endogenous TOPBP1 with an anti-TOPBP1 antibody another puling down over-expressed HA-TOPBP1 with anti-HA resin. Proteins with a TOPBP1-IP / mock-IP ratio above 4 in both experiments were considered specific TOPBP1 interactors. Each dot in dark color represents an identified TOPBP1 interaction. Similar procedures were performed in cells treated with 100 ng/ml nocodazole for 14 hrs to define TOPBP1 interactions in G2/M.

(B) Comparison of the identified TOPBP1 interactions in cells treated with HU and nocodazole. A separate mass spectrometry experiment was performed directly comparing HEK293T cells grown in "light" SILAC media (HU-treated) or "heavy" SILAC media (nocodazole).

(C) Co-IP of TOPBP1 with BRCA1 or 53BP1 in HEK293T cells treated with HU (1mM) or nocodazole (100ng/ml) as described in Fig.3A.

(D) Co-IP experiment determining the contribution of each pair of BRCT domains in TOPBP1 for stabilizing interactions with BRCA1 and 53BP1. HEK293T cells were

transfected with plasmids containing TOPBP1 (wild-type and the following mutants: BRCT-1/2: K154A and K155A; BRCT-5: K704A and W711R; BRCT-7: R1314Q) or empty vector (see plasmid list) and treated with 1mM HU for 24 hrs.

(E) Co-IP of TOPBP1 with BRCA1 or 53BP1 in the presence of ATR, ATM or DNA-PK inhibitors. HEK293T cells transfected with HA-TOPBP1 were pre-treated with ATR, ATM or DNA-PK inhibitors as indicated for 45 min followed by 30 min HU treatment in the presence of inhibitors.

(F) Depiction of an analogous mode of TOPBP1^{Dpb11} interactions with pro-HR (BRCA1 in humans and slx4 in yeast) and anti-resection (53BP1^{Rad9}) factors.

Figure 4. Hyper-stabilization of the 53BP1-TOPBP1 interaction promotes 53BP1 recruitment and genomic instability.

(A) Schematic illustration of the CTR-53BP1 chimera in which a 120 amino acid fragment in the N-terminal domain of RFC1 was fused to full-length 53BP1.

(B) Co-IP experiment pulling-down CTR-53BP1 and probing for TOPBP1 and PTIP in HEK293T cells treated with 1mM HU for 24 hrs. Ectopic 53BP1 and CTR-53BP1 containing an N-terminal FLAG-tag were transiently over-expressed.

(C) Immunofluorescence of U2OS cells transfected with FLAG-53BP1 or FLAG-CTR-53BP1 and treated with 1 mM HU for 24 hrs followed by a 3 hrs release in fresh media. We note that in our experience, this short release period enhances the visualization of replication stress-induced nuclear foci for the indicated proteins. Scale bar, 30μm.

(D) Quantitation of results from the experiment shown in (Fig. 4C) scoring FLAG and RIF1 foci in transfection-positive cells. More than 150 transfected cells were scored per replicate.

(E) Representative confocal images of U2OS cells transiently transfected with FLAG-CTR-53BP1 and co-stained with γ-H2AX; RIF1; RPA and RAD51. Scale bar, 10μm.

(F-G) Analysis of chromosomal abnormalities in metaphases of HEK293T cells treated with 1 μM ATR inhibitor and/or 3 μM PARP inhibitor AZD2461. Metaphase spreads were prepared as described in Material and Methods section. In (F) total aberrations were scored, while in (G) breaks and radial chromosomes per metaphase were scored separately. n>45 metaphases were analyzed in each replicate.

(H) A model for the roles of TOPBP1 in the coordination of DNA repair.

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Figure 1

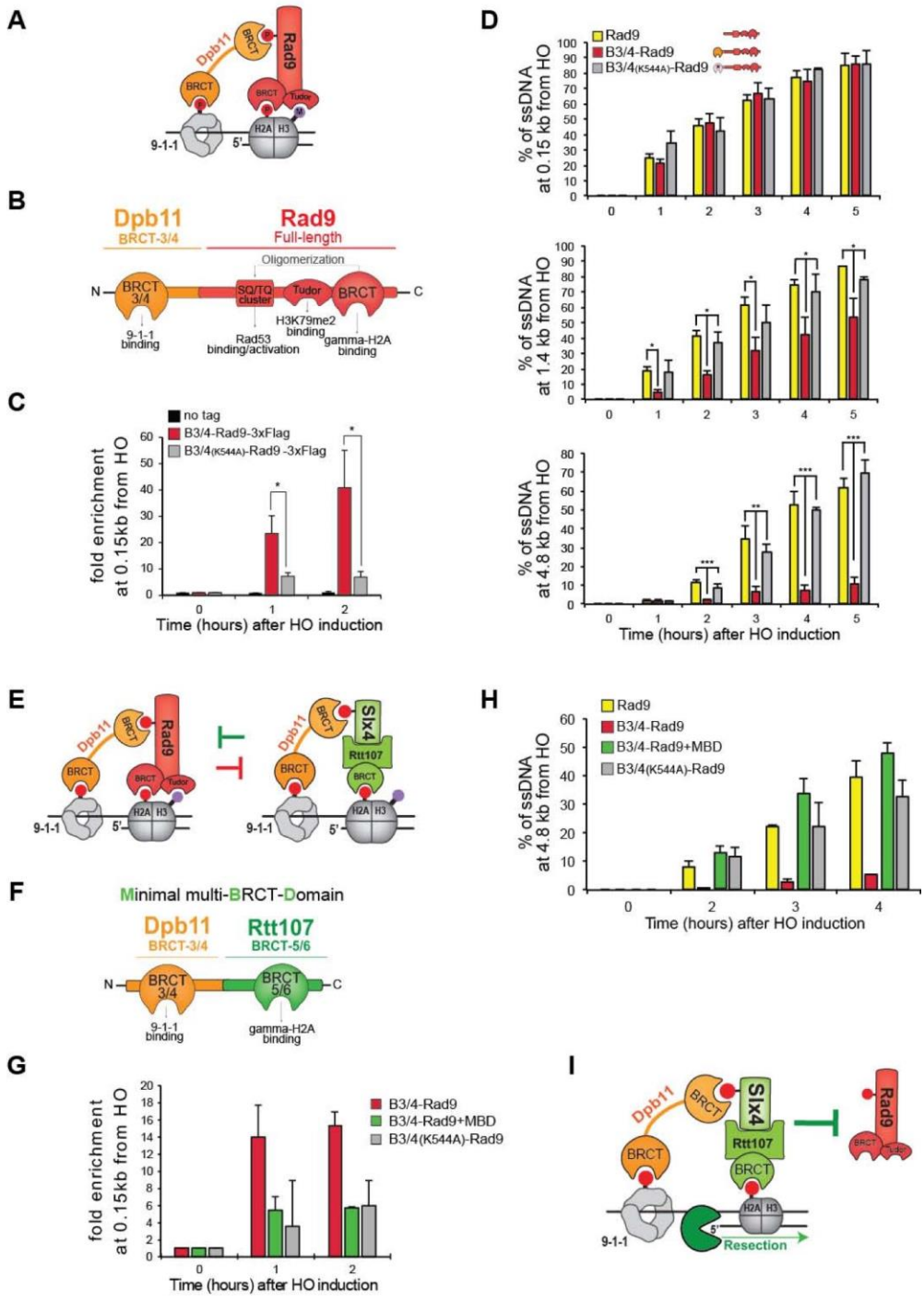

A
Dpb11
BRCT
Rad9
BRCT
BRCT
Tudor
9-1-1
5'
H2A
H3
B
Dpb11
BRCT-3/4
Rad9
Full-length
Oligomerization
N
BRCT 3/4
SQ/TQ cluster
Tudor
BRCT
C
9-1-1 binding
Rad53 binding/activation
H3K79me2 binding
gamma-H2A binding
C
no tag
B3/4-Rad9-3xFlag
B3/4(K544A)-Rad9 -3xFlag
fold enrichment at 0.15kb from HO
Time (hours) after HO induction
D
Rad9
B3/4-Rad9
B3/4(K544A)-Rad9
% of ssDNA at 0.15 kb from HO
% of ssDNA at 1.4 kb from HO
% of ssDNA at 4.8 kb from HO
Time (hours) after HO induction
E
Dpb11
BRCT
Rad9
BRCT
BRCT
Tudor
H2A
H3
9-1-1
5'
Six4
Rtt107
F
Minimal multi-BRCT-Domain
Dpb11
BRCT-3/4
Rtt107
BRCT-5/6
N
BRCT 3/4
BRCT 5/6
C
9-1-1 binding
gamma-H2A binding
G
B3/4-Rad9
B3/4-Rad9+MBD
B3/4(K544A)-Rad9
fold enrichment at 0.15kb from HO
Time (hours) after HO induction
H
Rad9
B3/4-Rad9
B3/4-Rad9+MBD
B3/4(K544A)-Rad9
% of ssDNA at 4.8 kb from HO
Time (hours) after HO induction
I
Dpb11
BRCT
Six4
Rtt107
BRCT
BRCT
Rad9
H2A
H3
9-1-1
5'
Resection

Figure 2

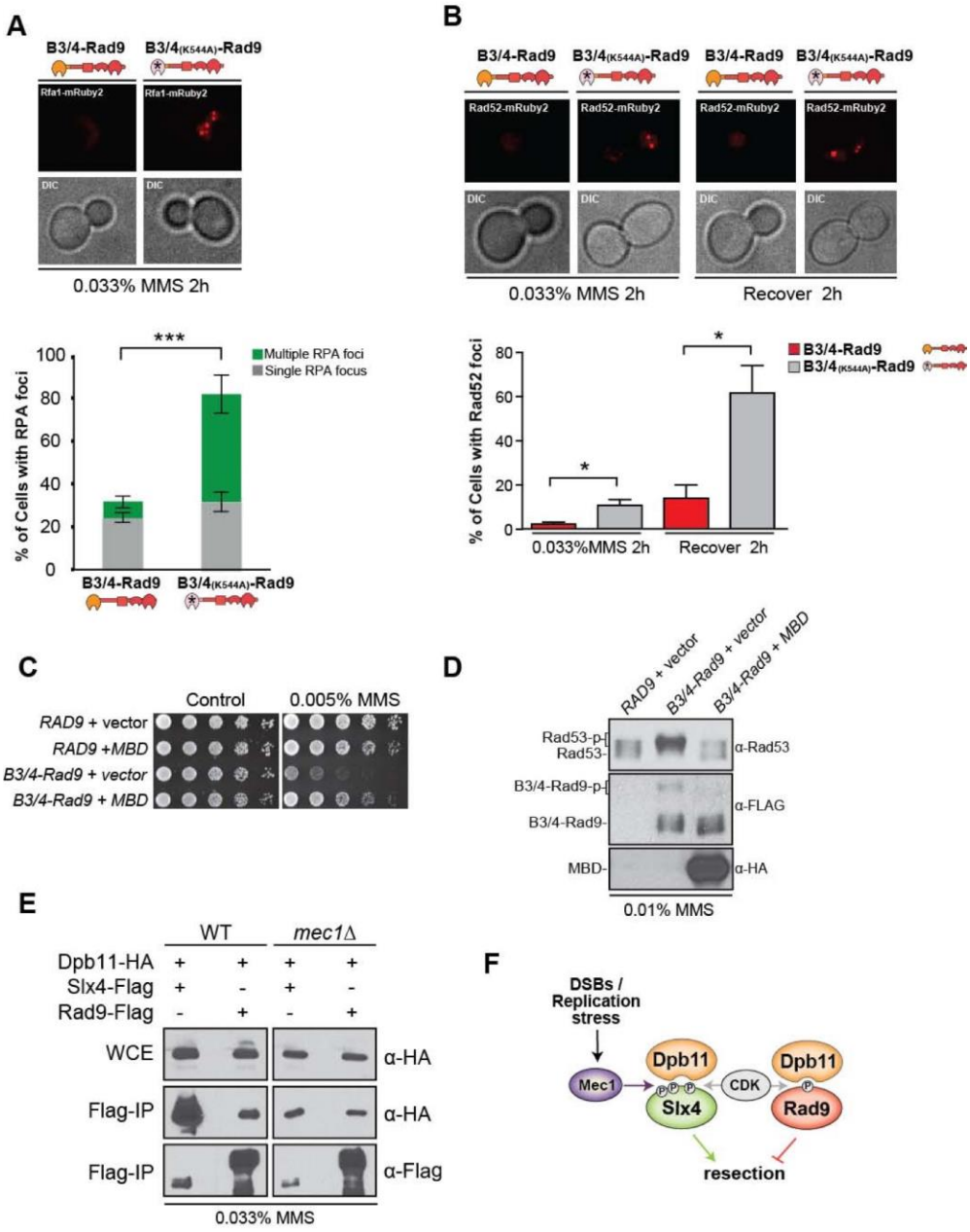

A
B3/4-Rad9
B3/4(K544A)-Rad9
Rfa1-mRuby2
DIC
0.033% MMS 2h
***
Multiple RPA foci
Single RPA focus
% of Cells with RPA foci
B
Rad52-mRuby2
0.033% MMS 2h
Recover 2h
% of Cells with Rad52 foci
C
Control
0.005% MMS
RAD9 + vector
RAD9 +MBD
B3/4-Rad9 + vector
B3/4-Rad9 + MBD
D
RAD9 + vector
B3/4-Rad9 + vector
B3/4-Rad9 + MBD
Rad53-p
Rad53-
α-Rad53
B3/4-Rad9-p
B3/4-Rad9-
α-FLAG
MBD-
α-HA
0.01% MMS
E
WT
mec1Δ
Dpb11-HA
Slx4-Flag
Rad9-Flag
WCE
Flag-IP
α-HA
α-Flag
0.033% MMS
F
DSBs / Replication stress
Mec1
Dpb11
Slx4
CDK
Rad9
resection

Figure 3

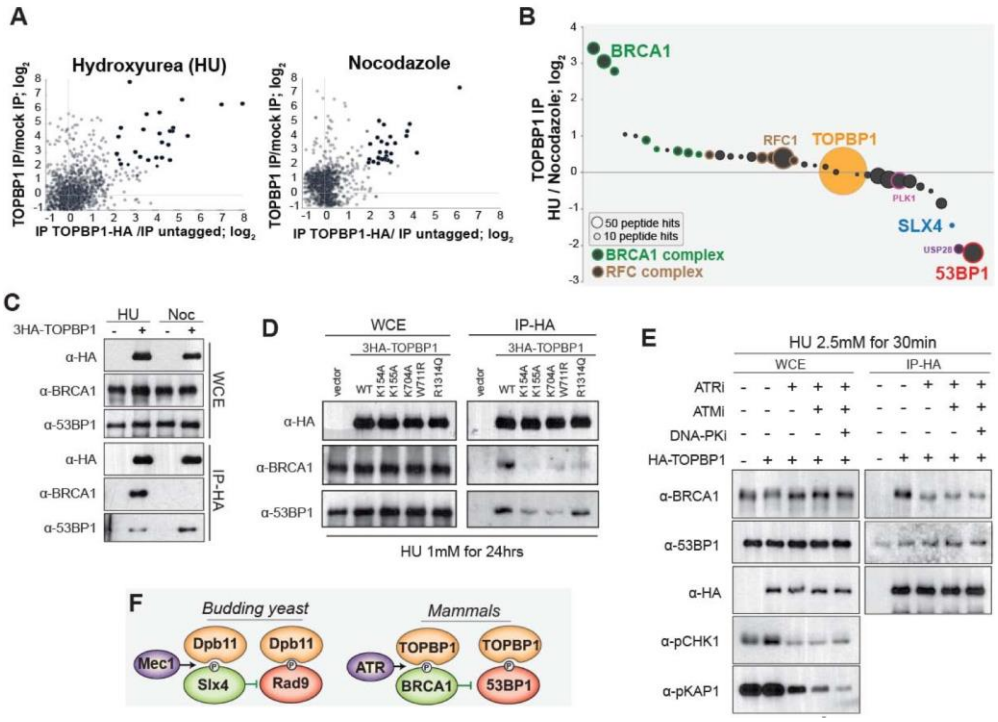
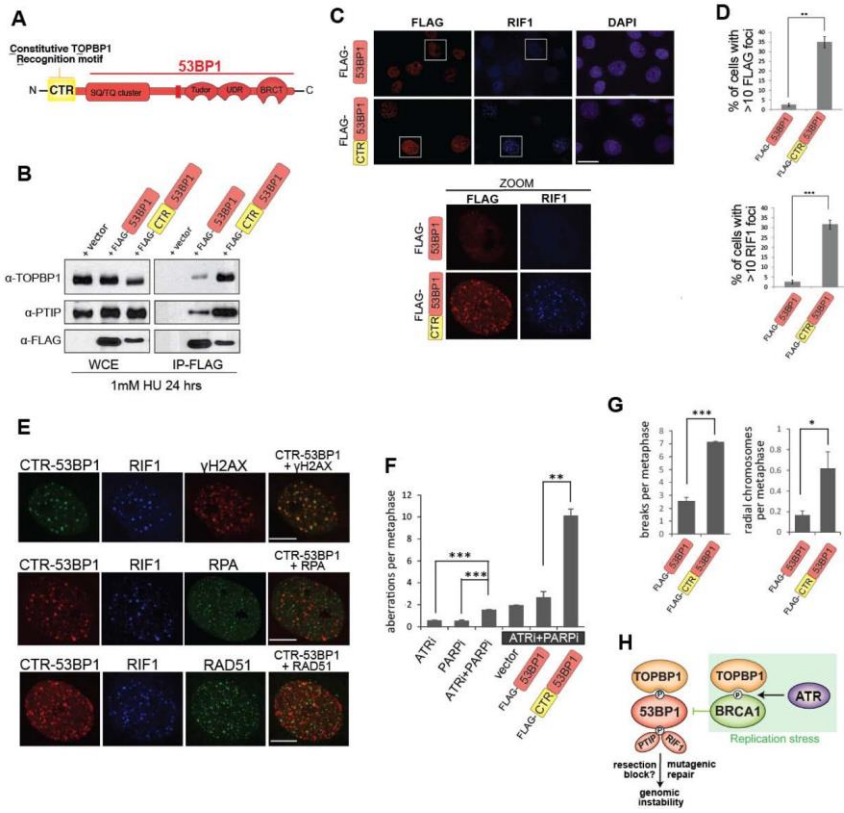

A
Hydroxyurea (HU)
Nocodazole
TOPBP1 IP/mock IP; log2
IP TOPBP1-HA /IP untagged; log2
IP TOPBP1-HA/ IP untagged; log2
B
TOPBP1 IP
HU / Nocodazole; log2
BRCA1
RFC1
TOPBP1
PLK1
SLX4
USP28
53BP1
50 peptide hits
10 peptide hits
BRCA1 complex
RFC complex
C
HU
Noc
3HA-TOPBP1
α-HA
α-BRCA1
α-53BP1
WCE
IP-HA
D
WCE
IP-HA
3HA-TOPBP1
vector
WT
K154A
K155A
K704A
W711R
R1314Q
α-HA
α-BRCA1
α-53BP1
HU 1mM for 24hrs
E
HU 2.5mM for 30min
WCE
IP-HA
ATRi
ATMi
DNA-PKi
HA-TOPBP1
α-BRCA1
α-53BP1
α-HA
α-pCHK1
α-pKAP1
F
Budding yeast
Mammals
Dpb11
Mec1
Slx4
Rad9
TOPBP1
ATR
BRCA1
53BP1

Figure 4


A
Constitutive TOPBP1 Recognition motif
53BP1
N
CTR
SQ/TQ cluster
Tudor
UDR
BRCT
C
B
+ vector
+ FLAG-53BP1
+ FLAG-CTR-53BP1
α-TOPBP1
α-PTIP
α-FLAG
WCE
IP-FLAG
1mM HU 24 hrs
C
FLAG
RIF1
DAPI
FLAG-53BP1
FLAG-CTR-53BP1
ZOOM
D
% of cells with >10 FLAG foci
% of cells with >10 RIF1 foci
E
CTR-53BP1
RIF1
γH2AX
CTR-53BP1 + γH2AX
RPA
CTR-53BP1 + RPA
RAD51
CTR-53BP1 + RAD51
F
aberrations per metaphase
ATRi
PARPi
ATRi+PARPi
vector
ATRi+PARPi
G
breaks per metaphase
radial chromosomes per metaphase
H
TOPBP1
53BP1
BRCA1
ATR
PTIP
Rif1
Replication stress
resection block?
mutagenic repair
genomic instability

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