UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze Biologiche e Molecolari

XXVIII Ciclo

Role of Polo kinase Cdc5 and Slx4-Rtt107 complex in checkpoint signaling during DNA damage in *S. cerevisiae*

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PhD Thesis

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Academic year: 2014-15

SSD: BIO/11

Thesis performed at Department of Biosciences,
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To Aai Baba and Nupura...

Part I

Abstract

The integrity of genomic DNA is continuously jeopardized through of environmental stresses such as UV light, ionizing radiations and various chemicals in addition to cellular byproducts such as reactive oxygen species. Furthermore, structural or chemical hindrances also affect the basic cellular processes (replication, transcription and translation) compromising genome stability. All the eukaryotic cells have thus evolved mechanisms to detect such genomic lesions and activate a surveillance mechanisms termed as checkpoint activation to arrest cell cycle, which in term provide time to repair the lesion using a suitable pathway to maintain genome stability. The resumption of cell cycle after the repair is also an important and finely regulated mechanisms. Indeed, resumption of cell cycle in case of faulty/un-repaired damage compromises genome integrity and may lead to cancer.

In this thesis, I studied the role of Polo-kinase Cdc5 and DNA repair scaffold complex-Slx-Rtt107, specifically in response to one of the most deleterious lesion, DNA double strand break (DSB) in budding yeast *Saccharomyces cerevisiae.* The human counterpart Polo-like kinase 1 is overexpressed in many cancers, while Slx4/FANCP is one of the proteins involved in Fanconi anemia repair pathway.

In first part, we characterized the role of phosphorylation of Threonine 238 in the activation loop of the Cdc5 kinase domain in unperturbed cell cycle and in response to repairable and unrepairable DSB. Using alanine/ aspartic acid mutagenesis and genetic approaches we delineated the requirement of T238 phosphorylation of Cdc5. Interestingly, we discovered that absence of T238 phosphorylation of Cdc5, even though doesn't affect the normal cell cycle, affects kinase activity and leads to defect in checkpoint adaptation and recovery after one DSB. Importantly, we also found that *cdc5*-T238A cells

also have altered genome stability, assessed by using multiple genetic approaches.

In second part, we characterized the role of Slx4-Rtt107 complex in modulating the level of checkpoint signalling and initial processing of DSB. Indeed in the absence of functional Slx4-Rtt107 complex, we found slower processing of DSB and hyper-activated checkpoint signalling which is due to increased binding of checkpoint adaptor protein Rad9 at the lesion. Importantly, this hyper-activated checkpoint has consequent effect on cell cycle resumption and proliferation in response to various DNA damaging agents.

State of the art

The DNA damage and genome integrity maintenance:

The integrity of DNA molecules which form the basis of all the living organisms is continuously challenged by endogenous or exogenous agents. In aqueous environment the phosphodiester bond in sugar-base of deoxyribonucleotides is more susceptible for hydrolysis than in ribonucleotides, leading to depurination and formation of abasic site. Also presence of variety of free oxidants such as hydroxyl ions or peroxynitrite due to intracellular metabolism leads to formation of oxidized DNA bases, of which 8-oxo-dG, thymine glycol, 5-hydroxy uracil, uracil glycol are highly mutagenic (Lindahl 1993; Marnett 2000). Another endogenous DNA damage is due to nonenzymatic DNA methylation by *S*-adenosylmethionine which leads to formation of 7-methylguanin and 3-methyladenine. The later one not only alters coding specificity but is a major cytotoxic lesion as it blocks replication. Also the endogenous errors of replication such as misincorporation of dNTPs or incorporation of rNTPs are sources of DNA damages. In addition to these, peroxidation of polyunsaturated fatty acids present in phospholipids produces different aldehyde products of which 4-hydroxynonenal (HNE) and malondialdehyde (MDA) are highly mutagenic due to their ability to form exocyclic products (De Bont & van Larebeke 2004).

Importantly, the chemical and physical exogenous agents also pose a serious threat to genome stability. The ionizing radiations (IR) and ultra violet rays of the sunlight are responsible for hazardous alterations of DNA structure. UV light mainly leads to formation of bulky dipyrimidine photoproducts such as cyclobutane pyrimidine dimers and 6-4 photoproducts. Besides these, near UV light also causes covalent changes in oxidized DNA bases. Instead ionizing radiations mainly cause doubles strand breaks (DSBs) or single strand

breaks (SSBs) (Lindahl & Wood 1999). Among other environmental sources, it is important to mention the cigarette smoke, which induces aromatic DNA adducts and oxidative changes (Phillips et al. 1988; Kiyosawa et al. 1990). Also the chemotherapeutic agents cause different types of DNA lesions: such as alkylating agents (methyl methanesulphonate, temozolomide), interstrand crosslinking agents (cisplatin, psoralen, nitrogen mustard, mitomycin C), topoisomerase inhibitors (camptothecin and etoposide) (Schärer 2005; Pommier et al. 2010) and other drugs which impair replication progression (Hydroxyurea depleting dNTP pool and aphidicolin inhibiting DNA polymerase).

The DNA repair pathways:

All the living organisms have thus evolved mechanisms to detect different types of DNA lesions, recruit appropriate repair machinery and repair them to maintain genome integrity. Different pathways have been discovered which repair specific type of lesion. In some cases, the chemical base alteration can be repaired by direct damage reversal systems. Excision repair system are divided into Base Excision Repair, Nucleotide Excision Repair and Mismatch Repair. In all of these processes, one or more nucleotides are removed from the lesion forming gap which is filled in by DNA synthesis. More details can be found in exciting reviews (Lindahl & Wood 1999; Krokan et al. 2000; Memisoglu & Samson 2000; Marti et al. 2002). Here, I will focus on specific DNA lesion: DNA Double Strand Break (DSB) and its repair pathways.

The DNA double strand break repair pathways:

The DNA double strand break is one of the most deleterious lesion, which if unrepaired or repaired inappropriately will lead to cell death or genome instability. There are two major pathways through which eukaryotic cells repair these lesions: non homologous end joining (NHEJ) and homologous recombination (HR). The use of either of the pathway is dependent of the phase of cell cycle, the nature of DNA ends and importantly the availability of homologous sequence. There is also a newly emerging pathway which utilizes limited or short homology to anneal the ends hence termed as microhomology mediated end joining (MMEJ).

NHEJ:

As the name says, this is the major pathway in higher eukaryotic cells to repair DSB which does not require homologous sequences. Even though, NHEJ can occur throughout the cell cycle, it is the major pathway utilized to repair DSB in G1 phase. The DSB ends are detected by Ku70-Ku80 heterodimer protein complex. Binding of Ku complex prevents degradation of the ends and is required for the recruitment of DNA ligase IV (encoded by Dnl4/Lig4) and the accessory proteins Lif1, Nej1. The budding yeast *S. cerevisiae* does not have the end processing nucleases in its NHEJ machinery (Artemis, PALF in vertebrates), so NHEJ in yeast relies on the availability of compatible DNA ends (with 3'-hydroxyl and 5'-phosphate groups). In absence of 'clean' ends, processing is required which can result in mutagenic insertions or deletions at the site. In fact, NHEJ is responsible for chromosome rearrangements like translocations and DSB telomere fusions (Myung et al. 2001). In vertebrates, NHEJ has a physiological role in repairing DSBs created during variable (diversity) joining [V(D)J] recombination and class switch recombination (CSR), thus in absence of functional NHEJ machinery

patients are radiation sensitive and severely immunodeficient (Daley et al. 2005; Lieber 2010; Frit et al. 2014; Pannunzio et al. 2014).

Homologous Recombination (HR):

Homologous recombination is essential for chromosomal pairing and exchange during meiosis and repair of DNA lesions during mitosis. More specifically, HR is available as an option for repairing lesion at S/G2 phase where homologous sister chromatid is available after replication in haploid mitotic cells. The important steps during HR mediated repair of DSB are:

Presynaptic stage involving initial processing of DSB which forms 3' ssDNA (termed as resection), coating of ssDNA with strand-exchange protein Rad51 to form nucleofilament, **synaptic stage** involving strand invasion and D-loop formation mediated by Rad51 bound ssDNA to search for homology and finally **post-synaptic** stage which involves DNA synthesis to complete the repair using suitable routes. There is also Rad51 independent repair choice (termed as single strand annealing), which uses initial processing of DSB (resection) but does not utilize Rad51 mediated 'synapsis', hence termed as Rad51 independent DSB repair which is different from NHEJ described before.

DSB resection and Rad51 loading:

The ends of the DSB are recognized by Ku70-80 heterodimer complex which competes with Mre11-Rad50-Xrs2 (MRX) complex for binding. As described before, in absence of 'clean' DSB ends or in S/G2 phase MRX (MRN complex in humans) complex in cooperation with Sae2 (CtIP), can remove 5' oligonucleotides resulting in limited end processing. The nucleolytic activity of MRX complex is absolutely essential to process 'dirty' DNA ends produced by IR or to remove hairpin structures produced by

specific drugs (Lobachev et al. 2002), but is not essential to process 'clean' DSB ends generated by activity of endonucleases such as HO or *I-Sce*I (Llorente & Symington 2004; Clerici et al. 2005) . In S/G2 phase Cdk1 phosphorylated Sae2 initiates DSB processing with MRX complex (Ira et al. 2004; Sartori et al. 2007; Huertas et al. 2008; Cannavo & Cejka 2014). Once DSB processing is initiated, the NHEJ in prohibited and the DSB repair is directed to HR (Clerici et al. 2008; Mimitou & Symington 2010; Shim et al. 2010). After this initial processing the extensive 5'-3' nucleolytic degradation occurs due to the activity of Exo1 exonuclease and Cdk1 phosphorylated Dna2 endonuclease together with Sgs1 helicase (Mimitou & Symington 2008; Mimitou & Symington 2010; Cejka et al. 2010; Chen et al. 2011). This process of generation of long 3'ssDNA tails is termed as DSB resection which is bidirectional with Exo1 and Dna2 nucleases act in redundant fashion (Garcia et al. 2011), and it is stimulated by the 9-1-1 complex (Ngo et al. 2014). As soon as ssDNA is formed, it immediately gets coated with ssDNA binding protein, RPA (Replication protein A) which not only prevents its degradation, but also plays important role in checkpoint signalling as discussed later (Zou & Elledge 2003; Wang & Haber 2004; Dubrana et al. 2007). The DSB resection is also regulated by other factors, such as the chromatin modellers RSC complex (Shim et al. 2007), Fun30/SMARCAD1 (Chen et al. 2012; Costelloe et al. 2012; Eapen et al. 2012) and Ino80 (Van Attikum et al. 2004; van Attikum et al. 2007; Morrison et al. 2007).

This initial processing of DSB is prerequisite for repair through either of routes discussed below. Once 3'ssDNA coated with RPA is generated, the central player of HR, Rad51 recombinase is loaded on it through another key recombination protein Rad52 (New et al. 1998; Shinohara & Ogawa 1998; Miyazaki et al. 2004). The resulting Rad51-ssDNA nucleofilament is right handed B-helix with ssDNA stretched to one and half of its length to facilitate

the fast and efficient homology search (Sung et al. 2003; Klapstein et al. 2004; Chen et al. 2008) (Refer to **A** part of Figure 1).

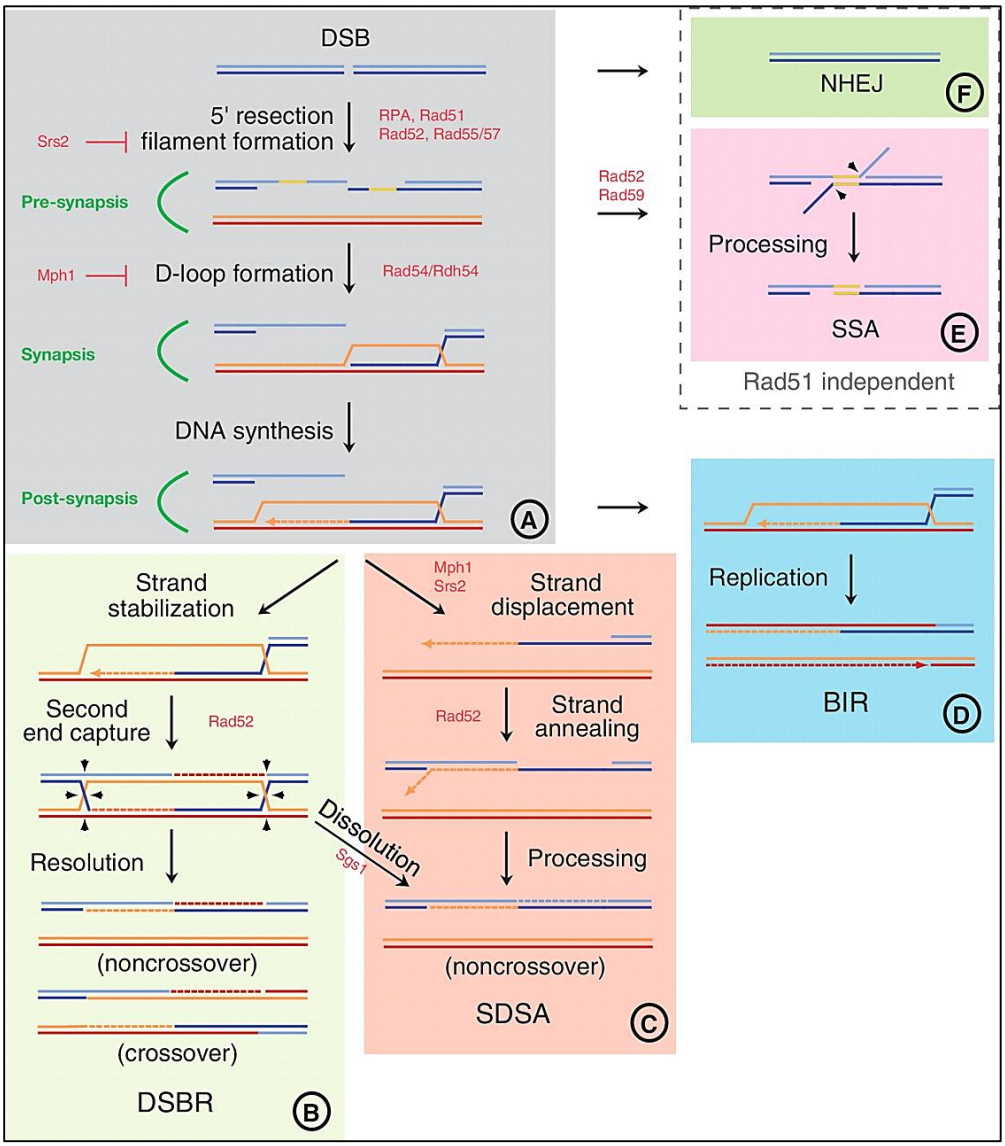


Figure 1. Models for the repair of DNA double-strand breaks. DNA DSBs are resected to generate 3' ssDNA ends followed by formation of Rad51 filaments that invade into homologous template to form D-loop structures. (A) After priming DNA synthesis, three pathways can be invoked. The DSBR pathway (B), the SDSA pathway (C), and the third pathway of BIR (D). Rad51-independent recombinational repair pathways are also depicted: Single Strand Annealing (E), and NHEJ (F).
Adapted from (Krejci et al. 2012)

DSB Repair model:

The DSB repair model is the most well accepted model to account for association of crossing over with gene conversion during HR which was proposed in early studies (Orr-Weaver et al. 1981; Szostak et al. 1983). In this model, after initial DSB resection, one of two Rad51 nucleofilaments invades homologous dsDNA to form a displacement-loop (D-loop), and the 3' end is used to prime DNA synthesis. The displaced strand of dsDNA anneals with other side of the break to initiate second round of DNA synthesis which in turn creates double Holliday Junction (dHJ) after ligation of newly synthesized strands. Such dHJs are formed in mitotic as well as in meiotic cells (Schwacha & Kleckner 1995; Allers & Lichten 2001; Hunter & Kleckner 2001). Furthermore DNA lesions occurring during S phase are also bypassed through template switching mechanism which forms DNA joint molecules (Branzei et al. 2008). The DNA joint molecules are mainly processed by dissolution pathway comprising of STR complex (Sgs1-Top3-Rmi1 in yeast and BLM-Top3-Rmi1-2 in human cells) in S phase, which primarily results in non-crossover products. The persistent joint molecules are later on resolved by the activity of Resolution complex consisting of Mus81-Mms4 (MUS81-EME1 in human cells), which results in formation of crossover and non-crossover products. Yen1/GEN1 is an additional resolvase having functionally overlapping role to Mus81-Mms4, but acting later in time and space (Blanco et al. 2010; Blanco et al. 2014; Eissler et al. 2014). (Refer to **B** part of Figure 1).

Synthesis Dependent Strand Annealing:

As mitotic recombination involved lower incidences of crossing overs, DSBR model was slightly modified to Synthesis dependent strand annealing and migrating D-loop models (Nassif et al. 1994; Ferguson & Holloman 1996; Pâques & Haber 1999). These models propose that one or both 3' tails of DSB

invade the homologous duplex (-es) and after limited DNA synthesis are displaced by the helicases. The newly synthesized complementary strands anneal and after gap filling and ligation it produces exclusively non crossover products. (Refer to **C** in Figure 1)

Break Induced Replication:

This model of DSB repair propose that the single 3' end of invades the homologous dsDNA and initiated replication till the end of the homologous chromosome (Kraus et al. 2001; Llorente et al. 2008). As it results in extensive loss of heterozygosity (LOH), this pathway is usually suppressed when DSBs have two homologous ends to utilize more conservative HR pathways. (Refer to **D** of Figure 1). BIR can occur by multiple rounds of strand invasion, DNA synthesis and dissociation and thus it is proposed to be highly mutagenic and contributory to genome rearrangements.

Single Strand Annealing:

This is Rad51 independent DSB repair mechanism which occurs to repair DSB formed between direct repeats (Pâques & Haber 1999). It efficiently repairs DSB formed between repeats of >200bp, but the frequency drops significantly for repeats of <50bps (Sugawara et al. 2000). After sufficient resection of DSB, 3' ssDNA tails anneal when the complementary repeats are exposed. Then non-homologous 3' tails are removed by the activity of flap nucleases followed by gap filling and ligation (Fishman-Lobell & Haber 1992). (Refer to **E** of Figure 1). The recent pathway of repairing DSB is referred to as microhomology mediated end joining (MMEJ), which involves limited end resection and annealing between short direct repeats (5-25nt) flanking a DSB (Villarreal et al. 2012; Deng et al. 2014). MMEJ and

SSA mechanisms are always mutagenic as they result in deletions altering genome integrity.

The detailed account of DSB repair thorough homologous recombination is available in various extensive reviews (Krejci et al. 2012; Symington & Gautier 2011; Symington et al. 2014).

The Cell Cycle Checkpoints:

The maintenance of genome integrity and its faithful transmission to the progeny is the essential goal of cell cycle progression. Thus all the eukaryotic cells have evolved a surveillance mechanism to detect any kind of impediments in genome integrity as well as in cell morphogenesis. These mechanisms, which are termed as Cell Cycle Checkpoints, serve to halt the cell cycle progression at the required stage and permit appropriate repair through desired pathway. Different types of checkpoints have been found in yeast as well as in higher eukaryotes which serve for different purposes, such as checkpoint to monitor budding in coordination with nuclear event is termed as Morphogenesis checkpoint (Lew 2003). Also correct chromosome segregation is assured through Spindle Assembly Checkpoint which monitors the appropriate anchoring of chromosome kinetochores with microtubule spindle apparatus (Lew & Burke 2003; Lara-Gonzalez et al. 2012). Similar mechanisms exist which monitor the integrity of genomic DNA at each phase of cell cycle, which are known as G1/S checkpoint (analysing integrity before replication), S-phase checkpoint (assuring integrity and faithful replication) and G2/M checkpoint (to avoid faulty chromosome segregation before cell division). Here, I will briefly summarize the important events of G2/M DNA damage checkpoint in response to double strand breaks.

The DNA damage checkpoint:

By using HO endonuclease system in budding yeast it has been demonstrated that, a single DSB is enough to trigger strong checkpoint activation and block cell cycle at G2/M phase (Sandell & Zakian 1993; Moore & Haber 1996). Furthermore it has significantly helped to understand the molecular choreography of DSB response in terms of damage sensors, subsequent DSB processing, signal transduction and signal amplification by effector kinases. As listed below, the checkpoint machinery is well conserved from yeast to human beings, thus making budding yeast as an ideal system to study genetic and biomolecular details of DSB response.

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

Table 1: DNA damage checkpoint proteins: Adapted from (Harrison & Haber 2006)

Choreography of checkpoint activation in response to DSB:

The first sensor of DSB is the MRX complex, which is also responsible for recruitment of upstream PIKK kinase Tel1 (ATM in humans) (Nakada et

al. 2003). Once recruited on to the lesion, Tel1 can phosphorylate the H2A to create a region of γ- H2AX. As described before, Cdk1 phosphorylated Sae2, stimulates nuclease activity of MRX complex thus initiating the processing of the DSB and further promoting removal of MRX complex and Tel1 from the lesion and limiting their signalling potential. After initial processing, the 5' to 3' DNA resection is carried out by exonuclease Exo1 and endonuclease Dna2 with helicase Sgs1. At the junction of ssDNA/dsDNA, Rad24 in complex with Rfc2-5 is responsible for loading of 9-1-1 complex (Rad17-Mec3-Ddc1 in yeast) (Kondo et al. 2001; Majka, Binz, et al. 2006), which has been recently found also to regulate DNA resection (Ngo et al. 2014). The ssDNA generated by this process is immediately coated with ssDNA binding protein RPA which is prerequisite for the recruitment of the important PIKK of yeast: Mec1 (ATR in humans). Many studies have now enlightened the variety of mechanisms through which Mec1 is activated which depends on nature of DNA damage, cell cycle phase and influence of different checkpoint factors. Starting with Mec1 binding partner Ddc2 which is essential for its activation (Paciotti et al. 2001; Zou & Elledge 2003), Mec1 activation has been found to be regulated through other checkpoint factors such as TopBP1/Dpb11 (D. a Mordes et al. 2008; Navadgi-Patil & Burgers 2008; D. a. Mordes et al. 2008; Puddu et al. 2008), the 9-1-1 clamps (Majka, Niedziela-Majka, et al. 2006), and Dna2 endonuclease (Kumar & Burgers 2013). Once activated, Mec1 is the main PIKK kinase responsible for checkpoint activation in budding yeast in response to DSB by activating the transducer kinases Rad53 (Chk2 in humans) and Chk1 in Rad9 dependent manner. The key adaptor protein, Rad9, is recruited to DSB by three different mechanisms: through interaction with γ-H2AX, through interaction with Mec1 phosphorylated Ddc1 which recruits Dpb11 at the damage site and through interaction with methylated histones (Giannattasio et al. 2005; Lazzaro et al. 2008; Pfander & Diffley 2011). These

multiple routes ensure flexibility and robustness of checkpoint activation required for efficient signalling cascade. Finally, the oligomerization and trans-autophosphorylation of Rad53 and Chk1 also has explicit functions for their complete activation (Pellicioli et al. 1999). It is essential to note that the key cell cycle regulator, Cdc28 (Cdk1 in humans), is also one of the most important regulator of checkpoint signalling and DSB processing by phosphorylating several checkpoint factors such as Sae2, Dna2 endonuclease and adaptor protein Rad9 (Ira et al. 2004; Chen et al. 2011; Pfander & Diffley 2011; Granata et al. 2010). Figure 2 depicts the checkpoint signalling cascade in response to DSB.

G2/M Cell Cycle arrest:

Conversely, it is important to mention that, the activity of cell cycle regulators Cdc28 and polo kinase Cdc5 is restrained upon checkpoint activation which is essential for G2/M cell cycle arrest. In addition Chk1 mediated phosphorylation of Pds1 renders it resistant for APC (Anaphase Promoting Complex) mediated ubiquitination and degradation, thus preventing cohesin cleavage and chromosome segregation (Tinker-Kulberg & Morgan 1999; Wang et al. 2001). At the same time, Rad53/Chk2 acts directly on APC subunit Cdc20, thereby inhibiting the complex, ensuring the checkpoint mediated cell cycle block (Agarwal et al. 2003). Recent studies have further added molecular details, describing the role of DNA damage checkpoint in preventing spindle elongation by maintaining Cdh1 in active state (Crasta et al. 2008; Zhang et al. 2009). Active Cdh1, thus limits accumulation of kinesins required for spindle elongation. This mechanism acts in parallel to Pds1 stabilization and APC regulation by checkpoint kinases Rad53 & Chk2. The Spindle Assembly Checkpoint (SAC) proteins (Mad and

Bub proteins) also sense DSBs and contribute in extending Mec1-Rad53 mediated checkpoint arrest (Kim & Burke 2008; Dotiwala et al. 2010).

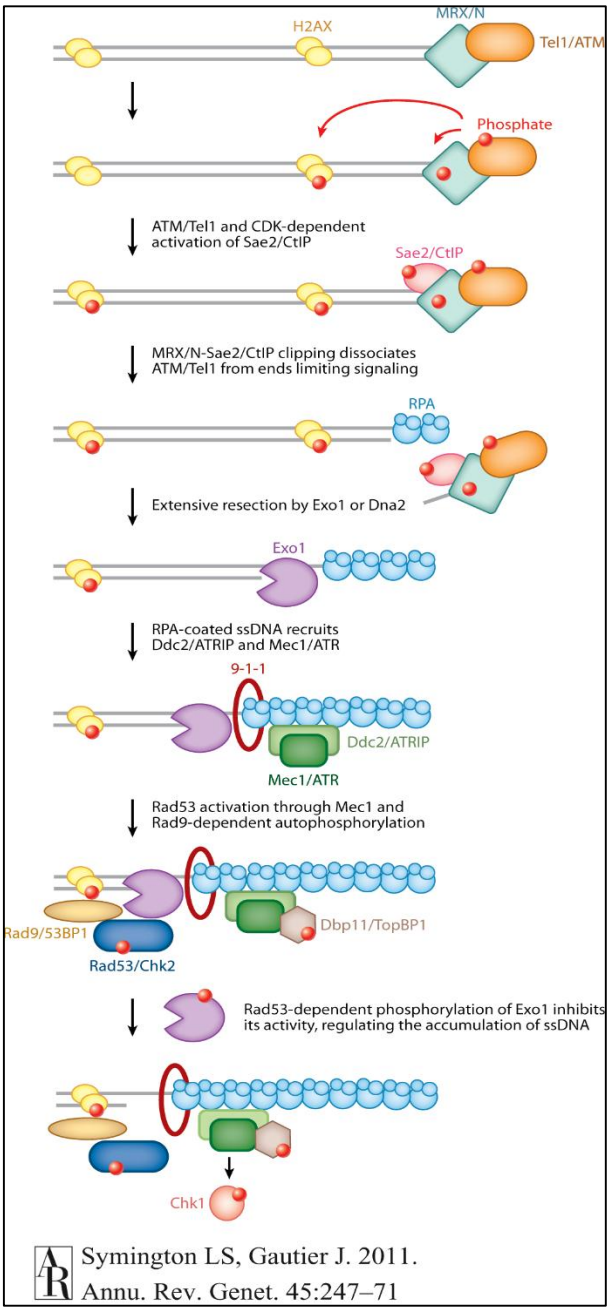


Figure 2: Schematic representation of DSB processing and checkpoint activation, adapted from (Symington & Gautier 2011)

Checkpoint inactivation by Adaptation and recovery:

The purpose of checkpoint mediated cell cycle arrest is to allow repair of DNA lesion through the suitable pathway. As is it essential to inactivate checkpoint to resume the cell cycle, two genetically distinct mechanisms have been found to be responsible for this inactivation. First mechanism, in which checkpoint is inactivated after faithful repair of the DNA lesion is termed as **checkpoint recovery**. A distinct pathway has also been found to be responsible for inactivating checkpoint even in the absence of repair of the lesion, which is termed as **checkpoint adaptation**.

As soon as repair is accomplished, through a feedback mechanism the checkpoint is turned off and cell cycle is resumed. This recovery relies on important kinases, such as Plk1, which was found to be responsible for phosphorylation mediated inactivation of variety of targets. Of these Cdk1 inhibitor Wee1(Van Vugt et al. 2004), and some important checkpoint factors such as Claspin (Yoo et al. 2004), Chk2, 53BP1 (van Vugt & Yaffe 2010) and GTSE1 (Liu et al. 2010) are among the key regulators of checkpoint recovery. Importantly, Aurora-A mediated phosphorylation of Plk1 in its activation domain appears to be the key commencing event in this process of checkpoint recovery (Macůrek et al. 2008; Seki et al. 2008). Along with these, dephosphorylation of γ-H2AX by multiple phosphatases also has important consideration. Even though human Plk1 has been found to have role in checkpoint recovery, Cdc5, the only Plk in budding yeast, seems not to have the same role (Vaze et al. 2002), instead it has been found to regulate checkpoint adaptation, as better described below.

Using specific genetic systems in budding yeast, it was found that cells take the last chance of survival by progressing to next cell cycle even in the presence of irreparable DNA double strand break or telomere dysfunction. Even if this decision allows inactivation of checkpoint and cell cycle

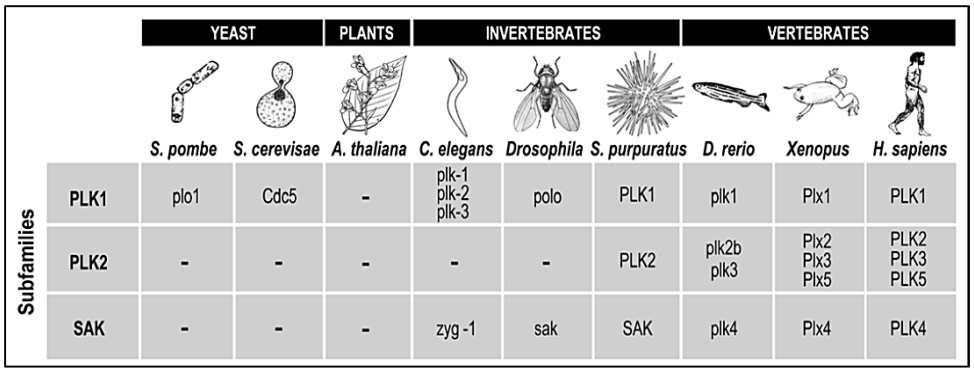
progression, usually it precedes with different forms of genome instability (Galgoczy & Toczyski 2001). Initially, this process was found to be regulated by polo kinase Cdc5 and Casein kinase II (Toczyski et al. 1997). In due course multiple factors were discovered to have role in checkpoint adaptation such as several repair factors including Ku complex, Sae2, helicases like Sgs1, Srs2, Replication protein A, Rad51 recombinase, chromatin re-modellers such as Fun30, Tid1, phosphatases Ptc2-Ptc3 and autophagy factors such as GARP and Vps51 [reviewed in (Harrison & Haber 2006; Pellicioli 2010; Bartek & Lukas 2007; Ciccia & Elledge 2010; Serrano & D'Amours 2014)]. In current scenario, multiple hypothesis have been put forth for reasoning the adaptation defect in these mutants, correlating the extent of DNA damage and level of checkpoint activation with the amount of ssDNA produced after DSB processing in addition to role of cytoplasmic effectors of checkpoint such as spindle assembly checkpoint and spindle elongation. Still the precise mechanism through which cells undergo checkpoint adaptation compromising genome stability remains elusive.

Importantly, it has been also discovered that, checkpoint adaptation exists in higher eukaryotes in response to various genotoxic stresses. Indeed, Plk1 and its orthologues have been found to be required during the process of checkpoint adaptation in response to ionizing radiation or in presence of replication stress in human cancer cells and *Xenopus* egg extracts respectively (Yoo et al. 2004; Syljuasen et al. 2006). It is important to highlight that, Aurora kinases and Plk1 are frequently up-regulated in many cancers which display hallmark of genomic instability.

Now, I would briefly introduce the two important factors which I studied during my thesis: The budding yeast Polo kinase- Cdc5 and Slx4-Rtt107 complex which have been previously known to play important role in DNA damage response and repair.

Polo kinases: Conserved Structure and Functions

Polo kinases (Plks) are a well-conserved subfamily of Ser/Thr protein kinases in eukaryotes. The first Plk was discovered in *D. melanogaster*, and thus termed as polo kinase. Later, several Plks have been isolated from budding yeast to mammalian organisms. Plks play essential role in mitotic transition and cellular proliferation (Glover et al. 1998; Nigg 1998). The lower eukaryotes, *S. cerevisiae* and *S. pombe* have just one Plk, named Cdc5 and Plo1, respectively. In higher eukaryotes, there are at least four Plks members (designated as Plk1-4). Please refer Figure 3.



Subfamilies	YEAST		PLANTS	INVERTEBRATES			VERTEBRATES		
	S. pombe	*S. cerevisae*	*A. thaliana*	*C. elegans*	*Drosophila*	*S. purpuratus*	*D. rerio*	*Xenopus*	*H. sapiens*
PLK1	plo1	Cdc5	-	plk-1 plk-2 plk-3	polo	PLK1	plk1	Plx1	PLK1
PLK2	-	-	-	-	-	PLK2	plk2b plk3	Plx2 Plx3 Plx5	PLK2 PLK3 PLK5
SAK	-	-	-	zyg -1	sak	SAK	plk4	Plx4	PLK4

Figure 3: Polo like kinases in different taxon, adapted from (de Cárcer et al. 2011).

The founding member Plk1 and Cdc5 are the master regulators of cell division with well-established crucial roles in mitosis and cytokinesis [reviewed in (Petronczki et al. 2008; Takaki et al. 2008; Archambault & David M Glover 2009; de Cárcer et al. 2011)]. Importantly, Plk1 is an essential gene and also *CDC5* null mutants are lethal in yeast (Llamazares et al. 1991; Kitada et al. 1993). Each member of the subfamily has been well characterized in terms of localization, substrate specificity and functionality (Barr et al. 2004; Archambault & David M Glover 2009). In this thesis, Plk1 and its budding yeast homolog Cdc5 will be discussed in more details than other members of subfamily.

Plk's possess N-terminal kinase domain and two highly conserved polo-boxes (PB1 and PB2), also referred to as Polo Box Domain or PBD, which forms a non-catalytic C-terminal domain implicated in protein-protein interaction (Hudson et al. 2001; Seong et al. 2002). The comparison between amino acid sequence of human Plk1 and yeast homologs (Cdc5 and Plo1) has revealed that they exhibit 49% identity (almost 70% similarity) in the N-terminal kinase domain and 33-46% identity (53-61% similarity) in the two polo-boxes (Lee et al. 2005). Remarkably, overexpression of human Plk1 and Plk3 completely rescued the lethality associated with the temperature sensitive phenotype of *cdc5* mutant cells. This lead to designate Plk1 and Cdc5 and functional homologs of each other (Lee & Erikson 1997; Ouyang et al. 1997).

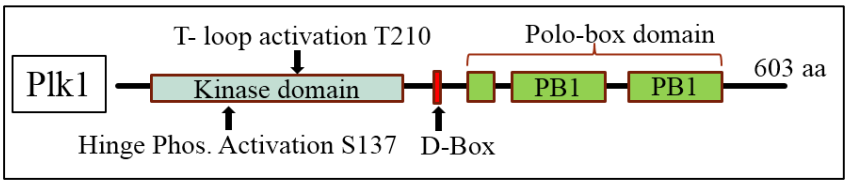


Figure 4: Polo kinase 1: protein structure, adapted from (Archambault et al. 2015)

Subcellular localization and Polo-box domains:

In late S phase or early G2 phase Plk1 localizes to centrosomes and kinetochores, and it remains there till metaphase/anaphase transition. In anaphase it relocalizes to midzone and later to midbody (Golsteyn et al. 1994; Lee et al. 1995; K. S. Lee et al. 1998; Seong et al. 2002). Similar to Plk1, in *S. cerevisiae* Cdc5 localizes to Spindle Pole Bodies (SPBs) from early G1 phase until the end of mitosis (Shirayama et al. 1998; Song et al. 2000). Later on, Cdc5 localization is also observed near septin ring filament, which persists until late mitosis (Sakchaisri et al. 2004). The fundamental purpose of this dynamic localization is to finely choreograph the multiple mitotic transition

and cytokinesis steps: i) localizing at SPB for spindle positioning and orientation (Snead et al. 2007); ii) sister chromatid cohesion through phosphorylation of Scc1 and functions at kinetochores (Alexandru et al. 2001; Hornig & Uhlmann 2004); iii) in meiosis for cohesion of chromosome arms and resolution of cross overs (Lee & Amon 2003b; Clyne et al. 2003); iv) activation of upstream regulators of the Mitotic Exit Network (MEN) and Cdc fourteen early anaphase release (FEAR) pathway to promote mitotic exit (Bardin & Amon 2001; Simanis 2003; Meitinger et al. 2012). These important mitotic and meiotic functions of Cdc5 are well described in reviews (Lee & Amon 2003a; Lee et al. 2005).

Furthermore, subcellular localization of Cdc5 is also regulated through multiple mechanisms. First, it is found to be regulated through the activity of TOR pathway. Indeed, in absence of functional TOR pathway, or in presence of TOR inhibitor Rapamycin, Cdc5 localization was affected at SPB but not in the nucleus (Nakashima et al. 2008). Secondly, recent studies reported that Cdc5 is nuclearized after DNA damage and proposed that it might relocalize to SPB to promote checkpoint adaptation by inactivating MEN inhibitor Bfa1 (Valerio-Santiago et al. 2013). In addition, the detailed localization analysis of Cdc5 in different steps of mitosis also identified Bfa1 as a key cytoplasmic target of Cdc5 for regulating mitotic exit (Botchkarev Jr et al. 2014).

The apparent substantial differences between localizations observed in yeast and mammals may reflect the difference of spatial and temporal regulation of mitosis and cytokinetic machineries in unicellular versus multicellular organisms.

All of above mentioned Plk localizations are mediated through the PBD. A single mutation (W414F) in polo box 1 of murine Plk1 completely disrupted the localization and mitotic functions of the protein, without

affecting kinase activity and stability of the protein (K. S. Lee et al. 1998), thus highlighting the importance of the PBDs.

Delineating the exact details of PBD and interactions with Plk substrates, two pioneering studies shed light on molecular mechanisms how this interaction happens. The PBD of Plk1 binds optimally to phospho-peptide sequence of Ser-pThr/pSer-Pro/X with a critical requirement of Ser at pThr-1 position and loose selectivity for Pro at pThr + 1 position (Elia, Rellos, et al. 2003). Their phospho-proteomic library screening also revealed that Plk1 bind to peptides phosphorylated by Cyclin-dependent kinases (CDKs) or Pro-directed kinases (Elia, Cantley, et al. 2003).

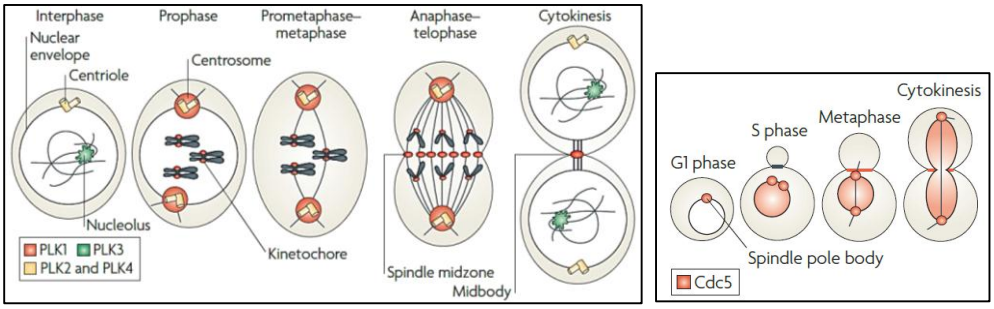


Figure 5: Cell cycle dependent localization of Plks and Cdc5, adapted from (Archambault & David M Glover 2009)

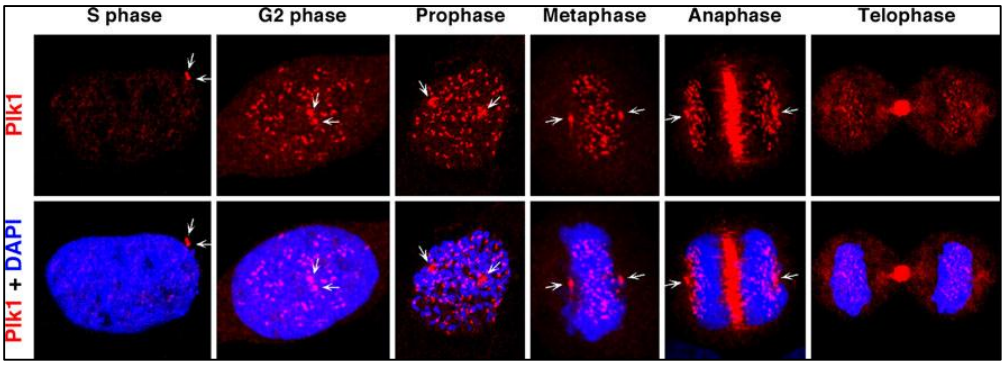


Figure 6: Cell cycle dependent localization of Plk1 adapted from (Park et al. 2010)

Moreover, studies have reported a detailed analysis of different Plks and their specific roles in target specificities. It has been found that PBD of Plk1 confers it the interaction with its substrates and also facilitates its subcellular localization. The PBDs of other Plks (i.e. Plk2-Plk4), even though have higher degree of identity of PBD of Plk1, have limited overlap for interaction with Plk1 substrates; rather they convey a significant deal for substrate specificity keeping the functional redundancy within the Plk-family to minimum (van de Weerdt et al. 2008; Park et al. 2010).

Considering the more conserved similarities between the human Plk1 and budding yeast Cdc5 with respect to the subcellular localization and roles in mitotic transition, it makes Cdc5 as an ideal candidate to study the intricate mechanisms of Plks functionality, not only in normal cell cycle but also in response to DNA damage.

Regulation of Plk1/ Cdc5 activity:

Polo kinases are regulated in both time and space. Cell cycle dependant temporal regulation is achieved through transcriptional control, phosphorylation and proteolysis. The spatial regulation of Plk1/Cdc5 is mediated by its interaction with specific substrates and sub-cellular localization.

PLK1 transcription peaks in G2/M phase and is regulated by forkhead associated transcription factors (Alvarez et al. 2001). Interestingly, both in human and yeast, Plk1 and Cdc5 interacts with forkhead associated transcription factors to reinforce the transcription of itself (Darieva et al. 2006; Fu et al. 2008). Transcription of *PLK1* is repressed in G1 through cell cycle dependent elements (CDE); moreover tumor suppressor protein p53 and its target p21 are negative regulators of *PLK1* transcription (Martin & Strebhardt 2006).

Similarly to the RNA transcripts, the level of Plk1 and Cdc5 protein peaks in late G2 and M phase. The Cdc5 activity peaks when Cdc28-Clbs becomes active and decreases when activity of Anaphase Promoting Complex (APC) rises (Charles et al. 1998). Like Plk1, Cdc5 is degraded through APC/Cdh1 dependent proteolysis in G1 phase (Charles et al. 1998; Shirayama et al. 1998). Moreover, Cdc5 positively regulates the activity of APC/Cdh1 to destroy mitotic cyclins and down regulate Cdc28 activity (Charles et al. 1998; Shirayama et al. 1998), in a feedback regulatory network.

In addition, Plk1 and Cdc5 proteins are regulated through phosphorylation (Kitada et al. 1993; Lee et al. 1995), which is also important for allosteric interaction of kinase domain and PBD.

Activation by phosphorylation:

Plks are activated through the phosphorylation of two critical Threonine residues in their activation segment, which is also termed as T-loop. A sequence alignment of the T-loop of the most relevant Plks from different organisms is reported in Figure below.

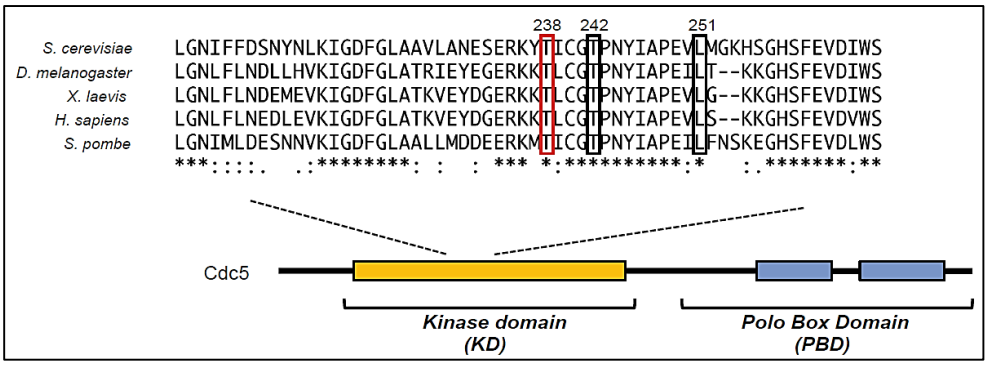


Figure 7: Multiple sequence alignment of activation loop of Polo kinases.

Previous studies have shown the activation and regulation of Plk1 by Aurora kinases in cooperation with Bora cofactor, which phosphorylate the T210 in the T-loop. Interestingly, in budding yeast the corresponding site

T238 of Cdc5 is phosphorylated by an unknown kinase, and it is shown to be dispensable for Cdc5 activation (Mortensen et al. 2005). Remarkably, the phosphorylation of T242 in the T-loop, which is mediated by Cdc28/Cdk1, is indispensable for Cdc5 activity and cell viability (Mortensen et al. 2005). Thus even though Plk1 and Cdc5 are activated by central mitotic kinases Aurora and Cdk1, there are subtle differences in model systems.

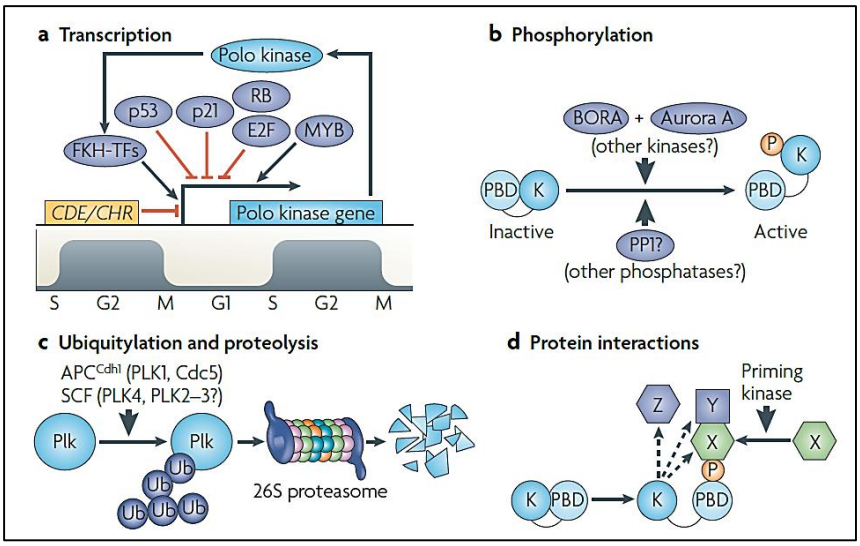


Figure 8: Four levels of regulation shared by polo-kinases**,** adapted from (Archambault & David M Glover 2009)**.**

a | The transcription of Polo kinases is regulated by the cell cycle and peaks in G2 phase **b** | Plks are activated by phosphorylation in their T-loop (or activation loop). This activates the kinase domain (K in the figure) and relieves an intramolecular inhibitory interaction with the Polo-box domain (PBD). In human cells, PLK1 is activated at mitotic entry by Aurora A kinase and its adaptor BORA, which phosphorylate PLK1 in its T-loop. Other kinases and phosphatases probably regulate the T-loop phosphorylation of other Plks. **c** | Different Plks are targeted for degradation by different ubiquitin ligases. Polyubiquitylation is recognized by the 26S proteasome, which destroys the Plk. PLK1 and Cdc5 are targets of the Cdc20 homologue 1 (Cdh1)-activated anaphase promoting complex (APC^{Cdh1}). **d** | Plks engage in protein interactions through the binding of their PBD to targets previously primed by phosphorylation. This increases the kinase domain activity and positions Plks favourably for phosphorylation of either the same target or another proximal target.

Once T210 in Plk1 is phosphorylated by Aurora kinase A in cooperation with Bora cofactor (Seki et al. 2008; Macůrek et al. 2008), it inhibits the interaction of kinase domain with the polo-box domain leading to protein activation. The phospho-mimetic mutation T210D has been shown to

reduce the interaction between KD and PBD, thereby increasing the kinase activity and also increasing the PBD interactions allosterically (Jang et al. 2002; Xu et al. 2013).

Polo kinases in DNA damage response:

Detailed regulation of Plk1 during DNA damage response is reported in many reviews (Dai et al. 2003; Bartek & Lukas 2007; Archambault & David M. Glover 2009; Lens et al. 2010; Bahassi 2011; Hyun et al. 2014; Wang et al. 2014; Archambault et al. 2015). Here I will briefly summarize key aspects.

In yeast, Cdc5 was found to regulate the process of checkpoint adaptation after prolonged G2/M cell cycle block due to telomere dysfunction and persistent DSB (Toczyski et al. 1997). In particular, the miss-sense mutation *cdc5*-L251W (also called *cdc5*-ad) was shown to strongly prevent Rad53 inactivation and cell cycle re-start after unrepairable DSB (Pellicioli et al. 2001), whereas the same mutation did not affect the checkpoint switching off when the damage was repaired (Vaze et al. 2002). It is worth to mention that CKII was also found as a fundamental regulator of checkpoint adaptation, in the same genetic screening in which Cdc5 was identified (Toczyski et al. 1997).

In response to DNA damage, Cdc5 activity is restrained through Mec1 and Rad53 dependent phosphorylation, blocking the mitotic transition (Cheng et al. 1998; Sanchez et al. 1999; Zhang et al. 2009). Interestingly, more recent finding indicate that the Cdc5 protein is nuclearized after telomere erosion in *cdc13-1* cells (Valerio-Santiago et al. 2013). Furthermore, during checkpoint adaptation Cdc5 activity promotes metaphase to anaphase transition by inactivating Cdh1 to allow spindle elongation (Zhang et al. 2009), and by inactivating Bfa1 and Mad2 to allow mitotic exit (Dotiwala et al. 2007; Dotiwala et al. 2010; Valerio-Santiago et al. 2013).

On the contrary, many reports suggests that Cdc5 acts directly on Rad53 to dephosphorylate it and promote checkpoint adaptation (Donnianni et al. 2010; Lopez-Mosqueda et al. 2010; Schleker et al. 2010; Vidanes et al. 2010). In our previous work, we had demonstrated that overproduction of Cdc5 not only prevented the Mec1 dependent Rad53 hyper-phosphorylation, but also affected checkpoint signaling at multiple steps after single irreparable DSB. Importantly, higher levels of Cdc5 altered phosphorylation of several Mec1 targets without affecting their recruitment at the lesion and also slowed down the DSB resection (Donnianni et al. 2010). Moreover, in our study Sae2 was reported to be the target of Cdc5 by Y2H interaction, and overproduction of Cdc5 had a significant effect on accumulation of Sae2 at DSB (Donnianni et al. 2010). This interaction was also found to be conserved in human cells in a recent study in which Plk3 was shown to phosphorylate CtIP, regulating DSB processing and repair (Barton et al. 2014).

In human cells, recent studies have shown that during DNA damage ATM/ATR directly phosphorylate Bora at T501 which promotes its degradation, thus Plk1 activation is blocked and cell cycle is arrested at G2/M phase (Smits et al. 2000; Qin et al. 2013). In addition, the transducer kinases Chk1 and Chk2 also inhibit Cdc25 phosphatase, resulting in inactivation of Cdk1 as it prevent dephosphorylation of inhibitory phosphorylation of Cdk1 at Y14 and Y15. All together, the entry into mitosis is prevented until the DNA damage is repaired (Donzelli & Draetta 2003). When DNA damage is repaired, activated Plk1 restores the activity of Cdc25 phosphatase and importantly it promote phosphorylation mediated degradation of checkpoint adaptor protein Claspin, dissociating it from ATR thus terminating checkpoint signalling (Mamely et al. 2006). The phosphorylation at T210 of human Plk1 by Aurora A in cooperation with cofactor Bora is not only essential for early activation of the protein but also absolutely important for checkpoint recovery

after DNA damage and cell cycle restart (Tsvetkov & Stern 2005; Macůrek et al. 2008; Seki et al. 2008; Archambault & Carmena 2012).

Polo kinase 1/Cdc5 and the recombination process:

Plk1 regulates homologous recombination process through phosphorylation of key factors. For instance, Plk1 in cooperation with CKII has been recently shown to phosphorylate Rad51 to regulate its interaction with member of MRN complex, Nbs1, facilitating homologous recombination (Yata et al. 2012). Moreover, as discussed before, Plk3 and Cdc5 phosphorylate the DSB resection factors CtIP and Sae2, respectively in human and yeast cells (Barton et al. 2014). Importantly, the activity of primary JM resolution complex Mus81-Mms4 is regulated by Cdc5 through the phosphorylation of regulatory subunit of the complex Mms4. The nuclease activity of the Mus81-Mms4 complex is extremely important to process JMs in situation of replication stress due to DNA alkylating agent methyl methanesulphonate, Topoisomerase inhibitor Camptothecin and interstrand crosslinking agents such as cisplatin and mitomycin C (Ciccia et al. 2008; Gallo-Fernández et al. 2012; Wyatt et al. 2013; Sarbajna et al. 2014). Recently, it was shown that Plk1/Cdc5 mediated phosphorylation of Mus81-Mms4 leads to their interaction with Slx4-Dpb11 complex, which functions for resolution of DNA joint molecules in yeast as well as in human cells (Gritenaite et al. 2014). Therefore, it is expected that the activity of Plk1/Cdc5 may be a key regulator to protect genome from unwanted recombination events.

Polo kinase and cancer:

The phenomenon of checkpoint adaptation in presence of persistent DNA damage in yeast (Toczyski et al. 1997; S. E. Lee et al. 1998) was later on further explored in details for its impact on genome stability. It was found that checkpoint adaptation precedes with different forms of genomic instabilities such as chromosome loss, translocations and break induced replication. Importantly, the adaptation defective *cdc5*-ad mutant cells have significantly reduced rates of these anomalies (Galgoczy & Toczyski 2001).

In human cells, several studies reported elevated Plk1 mRNA and protein levels in variety of cancers (Yuan et al. 1997) and it was designated as marker of proliferative cells (also reviewed in (Eckerdt et al. 2005; Takai et al. 2005)). So a question was raised if Plk1 overexpression in tumor is a cause or consequence. An answer to this question came from a study demonstrating that enforced expression of murine Plk1 in NIH3T3 cells confers a transformed phenotype as shown by ability of these cells to form foci on soft agar and more importantly to develop tumors in nude mice (Smith et al. 1997). Later this model was strengthen by a study in which depletion of Plk1 in U2OS osteosarcoma cells completely abrogated their ability to form colonies (Van Vugt et al. 2004). Along with these, recent studies have highlighted an intricate regulatory mechanisms between tumor suppressor protein p53 and Plk1. Plk1 transcription is inhibited in G1 phase in normal cell cycle due to concerted activity of p53 and p21 regulating at the level of transcription. But in cancerous cells, Plk1 has been found to directly inhibit p53 activity thereby reinforcing its expression also at the level of transcription (reviewed in (Martin & Strebhardt 2006)). Very recently, Plk1 has been found to directly up-regulate the activity of telomerase enzyme. Plk1 overexpression and its association with the catalytic subunit of telomerase complex not only

increased its activity but also prevented ubiquitin mediated degradation (Huang et al. 2015).

Remarkably, Plk1 has been also found to regulate checkpoint adaptation in human cells after treatment of ionizing radiations (Syljuåsen et al. 2006), strongly supporting the idea that checkpoint adaptation is a cancer prone event.

Not surprisingly, **Plk1 is a target of anticancer therapy**. Multiple approaches are in trials to inhibit Plk1 activity. Currently, targeting Plk1 relies on two mechanisms: developing drugs against N-terminal kinase domain and second developing drugs against Polo box domains as it mediates Plk1 interaction with its substrates. Variety of drugs are under clinical trials at different phases. For example, GSK461364 is imidazotriazine, ATP-competitive inhibitor, exhibits more than 1,000-fold higher potency toward Plk1 than the majority of 48 other protein kinases tested. Volasertib (BI 6727) is dihydropteridinone derivative which targets Plk1 with selectivity and efficiency [reviewed in (Reindl et al. 2008; Degenhardt & Lampkin 2010; Strebhardt 2010; Medema et al. 2011)].

The Slx4-Rtt107 complex:

Discovery and important functions:

Six *SLX* genes were isolated in a synthetic lethal screening in yeast cells lacking RecQ helicase Sgs1 by Stephen Brill and his group (Mullen et al. 2001). These six genes were found to associate in three protein complexes with nuclease activities to specific DNA structures and intermediates from 5' or 3' flaps till single or double Holliday junctions. As they were identified essential in the absence of RecQ helicase Sgs1 (which by itself is guardian of genome stability), recent studies were focused on all the three complexes addressing their role in DNA damage, repair and genome stability maintenance.

RTT107 (Regulator of Ty1 Transposition 107, also known as *ESC4*) was discovered in a genetic screening for increased Ty transposon mobility in budding yeast (Scholes et al. 2001). It was also found to have synthetic genetic interactions with genes involved in DNA replication and repair *SGS1* and *RRM3* (Tong et al. 2001; Tong et al. 2004). Moreover, it was suggested to have role in replication fork processivity and S-phase progression when cells lacking *RTT107* were found to have increased sensitivity to DNA alkylating agent methyl methanesulphonate (Chang et al. 2002). Consequently, Rtt107 was found to be phosphorylated by Mec1 in response to DNA damage and this phosphorylation was required for recovery from replication stress (Rouse 2004).

Slx4 has been found to interact with multiple proteins and exist in two protein complex pools: first Slx4 bound with Slx1 which forms a 5' endonuclease complex (Fricke & Brill 2003; Coulon et al. 2004); second Slx4 bound with Rad1-Rad10 along with Saw1 which forms 3' endonuclease complex (Flott et al. 2007). More recently, Slx4 has been shown to interact with DNA replication protein Dpb11 and this interaction is independent of

Slx1 and Rad1-Rad10, which is discussed in more details in subsequent sections.

Slx1and Slx4 were subsequently found to be the catalytic and regulatory subunits of 5' endonuclease complex (Fricke & Brill 2003; Coulon et al. 2004). Interestingly, Slx4 is regulated through phosphorylation by Mec1 in response to DNA damage (Flott & Rouse 2005). Furthermore, in addition to 5' endonuclease activity, human Slx1-Slx4 complex has also been shown to possess robust Holliday junction resolution activity (Fekairi et al. 2009). Slx1-Slx4 is required for S-phase dependent recombination at rDNA loci in budding and fission yeast, but deletion of *SLX1* doesn't have any increased DNA damage sensitivity. Instead, the deletion of *SLX4* has been found to increase the sensitivity to variety of DNA damaging agents such as Camptothecin and Methyl methanesulphonate.

Role in DNA damage and repair:

Replication stress:

As deletion of either *SLX4* or *RTT107* were found to increase the sensitivity to replication stress conditions in MMS response, studies were initiated to find the link between two proteins as they were the targets of Mec1 during DNA damage. It was also found that Slx4 and Rtt107 interact with each other in DNA damage response and were hypothecated to have role in checkpoint response and replication restart after alkylation damage (Roberts et al. 2006). Furthermore, Rtt107 was found to be directly recruited to chromatin in Rtt101 and acetyl transferase Rtt109 dependent manner and specifically recognizes γH2AX through its C-terminal BRCT domains ($BRCT_5$-$BRCT_6$) (Roberts et al. 2008; Li et al. 2012). Cells lacking either *SLX4* or *RTT107* failed to recover after MMS induced replication stress, as analyzed by unrepaired chromosomes by pulse field gel electrophoresis (Flott

& Rouse 2005). In the absence of *SLX4*, cells accumulate DNA alterations and unreplicated gaps due to low replication fidelity (Flott et al. 2007). Epistatic analysis of *SLX4*, *SLX1* and *RAD1* showed that neither *SLX1* nor *RAD1* are involved in DNA damage sensitivity to replication stress in cells lacking *SLX4*, even though mutually exclusive physical interaction was investigated between them (Flott et al. 2007).

Slx4-Rtt107 and DSB response:

Cells lacking *SLX4* were found to be defective in SSA pathway of DSB repair. Furthermore, it was found that Mec1 mediated phosphorylation of Slx4 is required for cleavage of 3' non homologous (NH) tails, although it is dispensable for Slx4 recruitment to the 3' NH tail. Indeed in absence of Mec1 phosphorylation, *SLX4* mutant displayed reduced SSA efficiency. Interestingly,Slx4 is dephosphorylated after the completion of SSA repair (Flott et al. 2007; W.-L. Toh et al. 2010). Further studies in yeast emphasized the role of structure specific nuclease complex Slx4-Rad1-Rad10, in promoting mating type switch and DSB repair through gene conversion, when 3' NH tails are formed (Lyndaker et al. 2008). Altogether these observations found a distinct set of gene products involving recombination, mismatch repair and nucleotide excision repair. Recent studies further added a key player in this recombination pathway, *SAW1* (Single strand annealing – weakened 1), which was identified in a specialized microarray based screening (Li et al. 2008). The same research group further explored the order and hierarchy of assembly of Rad1-Rad10/Saw1/Slx4 complex and Msh2/Msh3 complex to 3' NH tails. It was suggested that Saw1 is essential for recruitment of Rad1-Rad10, but not Slx4, at the 3' tailed recombination intermediate. However, Slx4 is essential for the cleavage of 3' tail by stimulating the activity of Rad1-Rad10 (Li et al. 2013). Similarly to Slx4, Rtt107 is a Mec1 target and it was

assessed in response to DSB. It was found that Rtt107 was enriched to 7 fold 0.5-10kb either side of DSB and it was dependent on its Mec1 mediated phosphorylation and interaction with Smc5-Smc6 (Ullal et al. 2011). Furthermore in absence of *RTT107,* the spontaneous sister chromatid exchanges were reduced.

Apart from these specific DNA damage responses and repair mechanisms, human Slx4 has been found to be involved in multiple DNA damage responses. The BTBD12 (human orthologue of budding yeast Slx4) was shown to form multi-protein complex, involving XPF-ERCC1, MUS81-EME1 and SLX1 endonucleases. It was also shown to be associated with MSH2/MSH3 mismatch repair complex, telomere binding complex (TRF2-TER-F2IP) and protein kinase Plk1 (Svendsen et al. 2009). The same study, also highlighted the importance of SLX4 in human cells as in its absence increased the sensitivity to mitomycin C and camptothecin with reduced DSB repair efficiency. At the same time, it was found that human SLX4-SLX1 complex also has Holliday- resolution activity in addition to 3' flap endonuclease activity observed in yeast, and depletion of SLX1-SLX4 results in 53BP1 foci accumulation and increased H2AX phosphorylation and sensitivity to MMS (Fekairi et al. 2009). In summary SLX4 was found to be a scaffold recruiting and delivering structure specific nucleases to maintain genome stability (Muñoz et al. 2009). Later on a detailed characterization showed that SLX4 in human cells is recruited to telomeres through its interaction with TRF2 (component of shelterin comlex) and has role in telomere homeostasis (Wilson et al. 2013). These functions are reviewed in more details (Ciccia & Elledge 2010; Kim 2014).

Post- translational modifications of Slx4 and Rtt107:

Phosphorylations:

Slx4 is phosphorylated in response to DNA damage at all cell cycle stages, but not in unperturbed cell cycle (Flott & Rouse 2005). Slx4 has 18 S/T-Q motifs, which could be phosphorylated by the upstream checkpoint kinases. Of these, the Mec1 and Tel1 dependent phosphorylation of Slx4 (at T72, 113, 319 and S289, 329, 355) has been shown to be required for DSB repair through SSA pathway (Flott et al. 2007). Apart from these, in recent studies using proteomics approach seven other residues (T457, 474, 597 and S499, 627, 569, 725) are found to be phosphorylated in response to MMS induced replication stress by Mec1, which are essential for its interaction with Dpb11 (Ohouo et al. 2010). More importantly, the phosphorylated Serine 486 of Slx4 was found to share similarities to the Cdk1 phosphorylated Sld3 interacting with Dpb11. Indeed, this crucial Proline directed phosphorylation in Slx4 was identified as Cdk1 dependent (Ohouo et al. 2013), regulating Slx4-Dpb11 interaction in DNA damage response, which was also found to be conserved in human SLX4 (Gritenaite et al. 2014).

SUMOylation:

Human SLX4 is found to be component of SUMO E3 ligase complex, and its SUMO Interacting Motifs (SIM) are important for its own SUMOylation along with SUMOylation of XPF (Guervilly et al. 2015; Ouyang et al. 2015). The SIMs of SLX4 are dispensable for ICL repair pathway, but are essential for CPT induced DNA damage response; furthermore they are the mediators and enhancers of interaction between SLX4 and DNA damage sensor proteins such as RPA, MRN complex and TRF2 (Ouyang et al. 2015). Apart from SUMOylation, SLX4 has been found to be PARylated, a modification which increases its binding to DNA damage sites (González-prieto et al. 2015).

Recent study has added much more details of Rtt107 function in budding yeast. It highlighted that Rtt107 interacts separately with its interacting partners: SUMO E3 complex, ubiquitin E3 complex, and Slx4 in mutually exclusive manner. And each Rtt107 involving complex affects unique protein modification pathway to regulate SUMOylation and ubiquitination to regulate replication progression and interaction with Slx4 to regulate checkpoint signalling (Hang et al. 2015).

Regulation of checkpoint signalling:

In response to replication stress, Mec1 phosphorylated Slx4 was found to interact with Dpb11, a replication factor which is also involved in DNA damage checkpoint activation (Ohouo et al. 2010). In particular, Dpb11 binds to checkpoint adaptor protein Rad9 and positively regulate checkpoint signalling (Granata et al. 2010; Pfander & Diffley 2011). Recently, it was discovered that Cdk1 phosphorylated Slx4-S486 interacts with Dpb11, thus counteracting Rad9 binding and signalling to the checkpoint transducer Rad53. At the same time Rtt107, in complex with Slx4, counteracts Rad9 recruitment to γH2AX, thus reinforcing the check on checkpoint hyper-activation at the level of chromatin. Consequently, in cells lacking *SLX4* or *RTT107*, robust Rad53 activation was observed in response to MMS induced replication stress (Ohouo et al. 2013). This Slx4-Dpb11 interaction, apart from modulating the checkpoint signalling, has also found to be important for processing of joint DNA molecule arising due to MMS induced replication stress (Gritenaite et al. 2014; Princz et al. 2014). The Slx4-Dpb11 interaction has also been found to be conserved in human cells, where mutagenizing T1260 of human SLX4 to non-phosphorylable amino acid reduced its interaction with TopBP1 (Gritenaite et al. 2014).

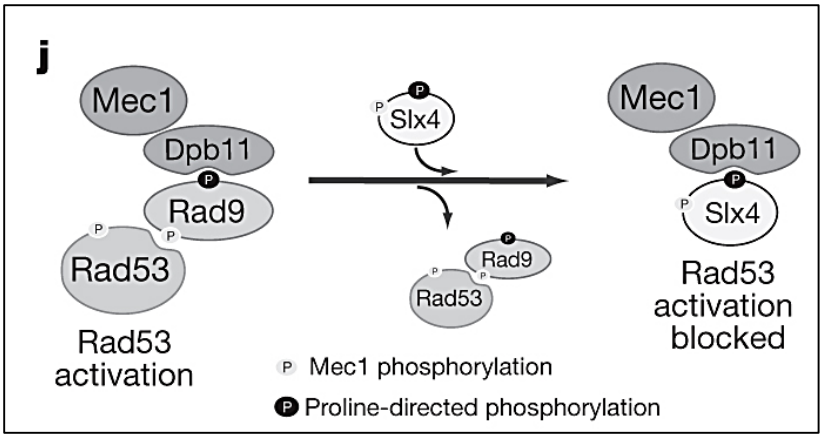


Figure 9: Slx4 binding to Dpb11 counteracts the Dpb11–Rad9 interaction and Rad53 activation (Ohouo et al. 2013).

Interestingly, in human cells SLX4/MUS81/EME1 complex has also been found to interact with *vpr* proteins of lentiviruses (such as HIV-1), which have role in G2/M cell cycle arrest. Furthermore, studies with Vpr proteins from Simian Immunodeficiency Virus have shown that SLX4 is the target of lentiviruses not only to induce G2/M cell cycle arrest, but also for dis-regulating Fanconi Anemia repair pathway (Berger et al. 2015). The molecular mechanism for this *vpr* induced G2/M arrest and role of SLX4 are yet to be explored, but it primarily indicates conserved function in different type of DNA damage and cell cycle regulation.

Fanconi anemia:

Fanconi anemia is a rare genetic disease with cancer predisposition, bone marrow failure, increased hypersensitivity to DNA damaging agents and genome instability, which was identified by Swedish pediatrician Guido Fanconi in 1927 (Walden & Deans 2014). Currently, sixteen FANC genes (FANCA- FANCQ) have been found to be associated with the mutations in patients. These gene products work together in a pathway to repair DNA interstrand crosslinks arising from exposure to chemicals such as mitomycin C, diepoxybutane, cisplatin and potentially aldehydes. Figure below shows the FA repair pathway involving the roles of sixteen currently know gene products at various steps.

FA repair pathway:

Interstrand crosslinks need to be repaired as they covalently link both the strands of DNA double helix inhibiting transcription and replication. The anchor complex recognizes the ICL and when activated it recruits the core complex (FANC -A, -B, -C, -E, -G, -L and FAAP20, 100). The core complex is responsible for the monoubiquitination of FANCI-FANCD1 (ID2) heterodimer which signals nuclease complex involving FANCP (SLX4) and FANCQ (XPF) and downstream repair factors (FANC-J, -N, -D1, -O).

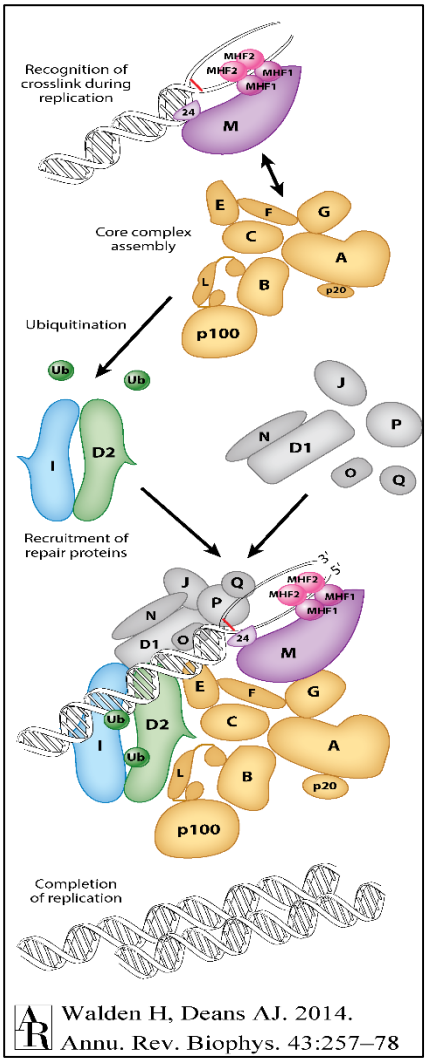


Figure 10:
Schematic of the complexes in the Fanconi anemia pathway.
The FANCI/FANCD2 (ID2) complex is depicted in blue/green. The core complex is shown in gold, and the anchor complex shown in pink/purple. Downstream repair factors are in gray. Each complex is thought to exist separately but converge at sites of DNA interstrand crosslinks (*red lines*). Abbreviation: Ub, ubiquitin.

Along with XPR-ERCC1 complex, SLX4 has been found to be essential for interstrand cross link repair (ICL) (Fekairi et al. 2009; Muñoz et al. 2009; Svendsen et al. 2009; Svendsen & Harper 2010; Kim et al. 2013; Hodskinson et al. 2014). Confirming the initial observations suggesting possible role of SLX4 in ICL repair pathway (Fekairi et al. 2009), mutations in SLX4 have been discovered as a causative of Fanconi Anemia, (Stoepker et al. 2011). SLX4 was involved in FA repair pathway with FANCP as designation. Consistent with these studies, mouse with *Slx4*$^{-/-}$ phenocopies Fanconi anemia with multiple cancers (Crossan et al. 2011).

Recent studies have found various mutations in human *SLX4*, with a direct impact on the Fanconi Anemia pathway. For example, a missense mutation was discovered in SLX4, which abrogated its nuclear localization and thus interaction with Mus81-EME1 and XPF-ERCC1 (Schuster et al. 2013). Additionally, a deletion of two UBZ domains was observed in some patients of FA and it was characterized that one of these UBZ domain is essential for SLX4 recruitment at ICL induced site, whereas other UBZ domain is essential for Holliday junction resolution activity *in vivo* (Lachaud et al. 2014).

Aims of the projects

In this thesis, I'm presenting work related to two factors which were studied with distinct objective as follows:

Aim 1: To characterize the role of Threonine 238 phosphorylation of Cdc5 in response to DNA damage and genome stability.

In response to DNA damage, all eukaryotic organisms activate a surveillance mechanism, called DNA damage checkpoint (DDC), to arrest cell cycle progression and facilitate DNA repair. Several factors are physically recruited to the damaged sites, and specific kinases phosphorylate multiple targets leading to checkpoint activation, repair and subsequent checkpoint inactivation. Interestingly, two different processes have been involved in checkpoint switch off in the presence of repairable or irreparable DNA damage. A process called recovery leads to checkpoint inactivation once the DNA lesion has been repaired, allowing cells to resume cell division after a checkpoint-induced cell cycle delay. A different process, called adaptation, leads to checkpoint switch off also in the presence of persistent DNA damage. The polo-like kinase Cdc5 (Orthologue of Human Plk1) was the first factor involved in checkpoint adaptation in yeast and mammals.

In this project we aimed to characterize the requirement of phosphorylation of T238 in activation loop of Cdc5 in response to DNA double strand break (DSB). Previous work in the lab had shown that absence of T238 phosphorylation (*cdc5*-T238A) reduces the kinase activity of the protein. Importantly, we were interested in investigating the role of Cdc5 in checkpoint adaptation and recovery processes after induction of DSB. Furthermore, we also aimed to assess the effect on genome stability in *cdc5*-

T238A cells using multiple genetic approaches in unperturbed conditions as well as in DNA damage induction.

Aim 2: To characterize the role of Slx4-Rtt107 complex in checkpoint signalling and initial processing after DNA double strand break.

After induction of DNA double strand breaks (DSBs), the checkpoint is triggered due to single-stranded DNA (ssDNA) generated by 5′-3′ nucleolytic degradation (DSB resection) of DNA ends. 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 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.

A key player in the DDC is Rad9, an orthologue of human 53BP1, which acts as an adaptor protein, mediating checkpoint signalling from the sensor kinase Mec1 to the central transducer kinases Rad53 and Chk1. Moreover, Rad9 is recruited to DSBs and to uncapped telomeres, limiting the resection of the 5′ strand.

The role of Rad9 in DDC signalling was recently shown to be counteracted by the action of Slx4, a protein scaffold with established roles in the coordination of structure-specific nucleases. Upon replication stress caused by the DNA alkylating agent methyl methanesulfonate (MMS), Slx4 in complex with Rtt107 (a multi- BRCT domain protein) was shown to compete with Rad9 for interaction with Dpb11 and γ-H2AX. Indeed cells

lacking, either component of the complex had hyper-activated Rad53 dependent checkpoint and increase sensitivity to MMS.

Thus we were interested to investigate if Slx4-Rtt107 complex has the similar checkpoint dampening function by counteracting Rad9 at DNA DSB as it was shown in replication stress. Furthermore we aimed to characterize the role of this complex in checkpoint signalling and 5' to 3' resection at DSB and consequent effect on DSB repair.

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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PLoS Genet. 2015 Jan 8; 11(1):e1004928. doi: 10.1371/journal.pgen.1004928.

Synopsis of the work and specific contribution:

At DNA double strand break, Cdk1 phosphorylated Sae2 along with MRX (Mre11-Rad50-Xrs2) complex primes the end resection which in turn inhibits non homologous end joining and commits the repair through homologous recombination pathways.

In this study, we demonstrated checkpoint independent role of Rad9 (orthologue of 53BP1), regulating DSB repair through single strand annealing pathway in absence of Sae2 or functional MRX complex.

In brief, we found that deletion of *RAD9,* restores the DSB repair defect in terms of viability of *sae2Δ* cells and nuclease defective *mre11*-D56N mutant through a Sgs1 dependent mechanism (Fig. 1). We confirmed our observations by Southern blot analysis that *RAD9* deletion restores the repair through SSA in *sae2Δ* cells, which requires Sgs1 helicase and Dna2 endonuclease (Fig. 2). Interestingly, Exo1 is not involved in the mechanism, suggesting that the Rad9 barrier is specific for the Sgs1-Dna2 resection pathway. Consequently, we found that *sae2Δ sgs1Δ rad9Δ* cells are completely defective in SSA repair, even though the DSB is processed through

long range resection by the Exo1 pathway (Fig. 3). Further exploring the effect of *RAD9* deletion, we found that Rad9 inhibits short range resection in absence of Sae2, but not much the long range resection (at 4.8 kb from DSB) (Fig. 3 E, F). Indeed, deletion of *RAD9* allows the initial step of resection through Sgs1. Understanding the importance short range resection and freeing the DSB ends, we discovered that Rad9 is responsible for Mre11 persistence as observed by ChIP analysis near the DSB ends. We speculated that this persistent Mre11 binding near the DSB may render them less accessible for Rad52 loading and thus limiting SSA repair in *sae2Δ* cells. We thus analysed Rad52 recruitment in case of *sae2Δ* cells in which we observed less percentage of cells with Rad52-RFP focus. Furthermore, we found that deletion of *RAD9* rescues the defect of DSB end-tethering in *sae2Δ* cells and restores the single strand annealing repair (Fig. 4).

It is known that Rad9 is recruited at lesion by 3 different pathways: i) interaction with Dot1 methylated H3K79, ii) interaction with γH2AX through its BRCT domain and iii) interaction with Dpb11 through Cdk1 phosphorylated residues of Rad9. We found that failure to recruit Rad9 through phosphorylated histones only partially rescued the repair defect of the *sae2Δ* cells, whereas the major contribution was due to the inhibition of Rad9 recruitment through its interaction with Dpb11 and its oligomerization (Fig. 5).

In summary, in this work we highlighted novel role of Rad9 to limit repair of a DSB through SSA, in absence of initial DSB processing factors. Considering that SSA events are associated with large DNA deletions, our finding elucidate a novel role of Rad9 in protecting genome integrity.

In this study, I participated to the general discussion of all the experiments, although my experimental contribution was minimal. In particular, I analysed DSB resection by qPCR in the absence of the histone

methyl transferase, Dot1. I found that deletion of *DOT1* lead to a faster long range resection in *sae2Δ* cells, but it does not rescue the short range resection defect. In fact, I found less resected DNA at 150bp away from DSB in *saeΔ dot1Δ* cells (Supplementary figure S4, pg. no. 96 of thesis). This result was important because it provided a molecular explanation of the SSA defect and lethality of the *saeΔ dot1Δ* cells (Fig. 5A), which was critical during the revision process of the work to rule out any specific contribution of Rad9 bound to methylated histone to limit SSA. Along with this, I also assisted in other experiments and generating mutant strains.

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

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Nucleic Acids Res. 2015 Oct 20. doi: 10.1093/nar/gkv1080

Synopsis of the work and specific contribution:

Slx4-Rtt107 complex is involved in DNA repair, where it functions as a scaffold for a variety of structure specific nucleases, such as Rad1, Slx1 and Mus81. Recently, it was also shown to dampen the Rad53 dependent checkpoint signalling upon replication stress by counteracting Rad9 through interaction with Dpb11.

In this study, we uncovered novel role of Slx4-Rtt107 complex in regulating not only checkpoint signalling but also DNA resection at double strand breaks.

We started our observations with cells lacking functional Slx4-Rtt107 complex in response to one irreparable DSB or telomere dysfunctioning (*cdc13*-1 background which leads to telomere uncapping at non permissive temperatures). In both cases, we observed by western blotting hyper-phosphorylation of Rad53, a marker of active DNA damage checkpoint signalling. The persistent checkpoint activation inhibited cell cycle restart

after damage and, as a consequence, *slx4Δ* and *rtt107Δ* cells were defective in micro-colony formation (Fig. 1). We observed similar phenotypes in *slx4*-S486A mutant which lacks Cdk1 phosphorylation required for interaction with Dpb11 thus counteracting Rad9. As predicted by our working model, deletion of *RAD9* rescued the checkpoint adaptation defect and cell cycle block in all these mutants. Indeed, these results prompted us to analyse Rad9 binding at DSB. Using Chromatin Immunoprecipitation and deep sequencing (ChIP-Seq-Fig. 2A) and Chromatin Immunoprecipitation followed by qPCR (ChIP-Fig. 2B), we found significant increase in Rad9 binding at 5kb from DSB in *slx4Δ, rtt107Δ* and *slx4*-S486A cells. Interestingly, this was dependent on Dpb11 recruitment at the lesion through Ddc1-T602 phosphorylation (Fig. 2C). Consistent with our hypothesis, this Rad9 binding at 5kb from DSB is counteracted by Slx4 binding at the same site observed by ChIP analysis of Slx4 after DSB. Interestingly, Slx4-S486A recruitment is reduced at the lesion, consistent with an increased Rad9 interaction with Dpb11 and checkpoint hyperactivation in *slx4*-S486A cells. Importantly we also found that Slx4 recruitment at 5kb from the DSB is absolutely dependent on Rtt107 and Ddc1, which is responsible for recruitment of Dpb11 (Fig. 2D, E).

Further exploring the physiological role of Slx4-Rtt107 complex in counteracting Rad9 "barrier", we found a significant effect on long range resection. In fact, cells lacking functional Slx4-Rtt107 complex have reduced ssDNA as a result of increased Rad9 binding (Fig. 3). Consistently, deletion of *RAD9* rescued the DNA resection defect in these cells.

In a second part of the work, we combined *SLX4* deletion with *SAE2* deletion, which is known to cause defect in short range resection (see also Ferrari et al., 2015)). Interestingly, we found severe DNA resection defect in *slx4Δ sae2Δ* cells (Fig. 4), as a result of additive defects in both the long and

short range DSB processing. Furthermore, we observed robust and persistent checkpoint signalling, and strong failure in checkpoint adaptation in the double mutant cells (Fig. 4). Underscoring this accumulative resection defect and hyperactive checkpoint, we found significant defect in repairing DSB by interchromosomal recombination assay, which in term reduced viability in *slx4Δ sae2Δ* cells (Fig. 5). Our molecular data were also supported with the viability of the cells lacking functional Slx4-Rtt107 complex in response to genotoxic stress induced by methyl methanesulfonate and camptothecin. Indeed, deletion of *SAE2* exacerbated the sensitivity of *slx4* or *rtt107* mutant cells, whereas deletion of *RAD9* suppressed the drugs sensitivity of all those single and double mutants (Fig. 6).

In summary, our data highlights the novel role of Slx4-Rtt107 complex in counteracting Rad9 barrier in distal regions of a persistent DSB, not only to dampen the checkpoint signalling but also to promote long range resection.
I started working on this project since beginning, and observed checkpoint adaptation and micro-colony formation defect in *slx4Δ* and *slx4*-S486A cells after unrepairable DSB which was suppressed by deletion of *RAD9* (Fig. 1B). These observations were also recorded in response to telomere uncapping by my colleagues. During further studies, I performed experiments to analyse resection in *slx4Δ, rtt107Δ* and *slx4*-S486A by Southern blot (Fig. 3A-C) and by qPCR, where we showed the resection defect is suppressed by deletion of *RAD9* (Fig. 3G). I also worked on the preliminary experiments to set up the Southern blot analysis of ectopic recombination assay presented in Fig. 5. Finally, I assessed the sensitivity assays to MMS and CPT shown in Fig. 6A and 6B.

Apart from experiments, I generated a number of reagents and strains, and also contributed to the experimental planning, data analysis and

manuscript preparation. Importantly, my contribution to the final manuscript was recognized with a Co-first authorship.

3. A distinct role of T238 phosphorylation of polo kinase Cdc5 in response to DNA damage and genome integrity in *S. cerevisiae*

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Manuscript in preparation

Synopsis of the work and specific contribution:

The essential mitotic regulator polo kinase- Cdc5 is has been found to regulate the process of checkpoint adaptation after telomere dysfunction and unrepairable DNA DSB due to the discovery of a missense mutation *cdc5-ad* (L251W), which had persistent checkpoint signalling. Intriguingly, Cdc5 and its human orthologue Plk1, are regulated through multiple post translational phosphorylations not only in normal cell cycle but also in response to DNA damage.

In this study, we characterized the requirement of a well conserved phosphorylation at Threonine 238 of Cdc5, located in its activation loop of kinase domain.

In brief, we started with mutagenesis of conserved T238 of Cdc5 to non-phosphorylable amino acid alanine, and phospho-mimetic amino acid aspartic acid. As reported previously, we found that T238 phosphorylation is not essential for activity of protein and cell viability and it rescued the growth defect of *thermo-sensitive* allele *cdc5*-1 at restrictive temperatures. Interestingly, by *in vitro* kinase assay we found that absence of this

phosphorylation reduces the kinase activity of the protein even though the cell cycle dependent regulation is unaltered (Fig. 1, S1). Furthermore, we investigated the effect of this hypomorphic allele on maintenance of genome stability in undamaged conditions using specific genetic backgrounds. I found that *cdc5*-T238A cells have 50% reduction in spontaneous mitotic recombination rate and threefold increase in chromosome loss rate (Fig. 2). These findings suggest that the T238 phosphorylation, although non-essential for cell viability in unperturbed cell cycle, is important for genome integrity.

Later on, we tested the effect of absence of T238 phosphorylation in response to unrepairable DSB. Interestingly *cdc5*-T238A cells were found to be defective in checkpoint adaptation by micro-colony assay, while cells with phospho-mimetic mutation *cdc5*-T238D were proficient in micro-colony formation. We also found that checkpoint signalling in terms of Rad53 phosphorylation is prolonged in *cdc5*-T238A cells (Fig. 3A, B). By immunofluorescence analysis we discovered that *cdc5*-T238A cells remain blocked at G2/M phase with undivided nuclei and have defect in spindle elongation (Fig. 3C, D). Notably, by genetic analysis we found that deletion of genes for checkpoint adaptor protein Rad9, spindle elongation restrictor Cdh1, and mitotic exit inhibitor Mad2 rescued the checkpoint adaptation defect of *cdc5*-T238A cells. Of these Cdh1 and Mad2 are the known targets of Cdc5, thus we speculated that, in *cdc5*-T238A cells, the reduced kinase activity of cdc5-T238A protein might be insufficient to inactivate the mitotic targets of Cdc5 to promote checkpoint adaptation (Fig. 3E). Recent studies had shown that Cdc5 is nuclearized in response to DNA damage, and postulated that during checkpoint adaptation it should relocalize at spindle pole bodies to promote spindle elongation and mitotic exit. Thus, we visualized Cdc5-eGFP localization by fluorescence in wild type and indicated mutants after induction of unrepairable DSB. We found that Cdc5-T238A

protein was localized in the nucleus after DNA damage, but there was a strong delay in its re-localization at SPBs and consequently a defect in SPB separation in mother and bud, which could be the reason of checkpoint adaptation defect (Fig. 4A, B).

After analysing the effect of unrepairable DSB, we checked the response of *cdc5*-T238A cells to repairable DSB. Using interchromosomal recombination assay, we found no defect in cell viability but there was a partial delay in repair of DSB analysed by Southern blot (Fig. 5B, C, and D). Importantly, in the same experiments, we found a strong delay in checkpoint inactivation and cell cycle restart observed by micro-colony formation under microscope (Fig. 5E, F). These results highlighted a previously underestimated role of Cdc5 in checkpoint recovery in budding yeast. We also observed similar effect on checkpoint recovery and repair kinetics in another pathway in which DSB is repaired through extensive resection mediated single strand annealing (Supp. Fig. S3).

Finally, we investigated the sensitivity of *cdc5*-T238A cells to variety of genotoxic stresses such as alkylating agent MMS and topoisomerase I inhibitor CPT and found increased sensitivity at higher doses. Importantly, we found a significant defect in Mms4 phosphorylation by western blotting in response to MMS, which is required for the activity of Mus81-Mms4 mediated resolution of DNA joint molecules. Consequently, when combined with *SGS1* deletion (component of dissolution pathway acting in S phase for joint molecule processing) we observed increased hypersensitivity of *cdc5*-T238A cells to very mild doses of MMS (Fig. 6A-D).

In summary, we characterized functional role of T238 phosphorylation of Cdc5 in regulating its kinase activity and maintaining genome stability in normal cell cycle. Importantly, T238 phosphorylation becomes crucial in response to DNA damage, and its absence prevents checkpoint adaptation in

response to one irreparable DSB. The T238 phosphorylation of Cdc5 seems also affecting the DSB repair kinetics, delaying cell cycle restart and checkpoint recovery.

My specific contributions to this study regard the characterization of *cdc5*-T238A mutation in maintenance of genome stability using multiple genetic approaches (Fig. 2). Moreover, I studied checkpoint adaptation defect in *cdc5*-T238A cells by checkpoint analysis and immunofluorescence microscopy (Fig. 3 & 4). Furthermore, I explored the role of Cdc5-T238 phosphorylation in checkpoint recovery analysis (Fig. 5) and in regulating activity of Mus81-Mms4 mediated resolution pathway (Fig. 6).

I also investigated the migration of Cdc5 protein by 2 dimensional gel electrophoresis followed by western blotting, in order to understand the effect of absence of T238 phosphorylation on regulating Cdc5 phosphorylation status. However, this approached did not provided substantial advances and I did not report it in this Thesis.

Furthermore, I was involved in planning the experiments, data analysis and manuscript writing. Indeed, a preliminary draft of a manuscript is presented in this Thesis.

Discussion & Future Perspectives:

Considering the studies of various factors presented in this thesis, a general discussion is not presented in this section. Instead, a detailed discussion focussed on respective factor/s studied is presented in the published papers and in manuscript in preparation.

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Acknowledgments

I wish to sincerely thank to Prof. Achille Pellicioli for whole heartedly welcoming me in his lab, supervising my PhD work, providing opportunity to learn variety of techniques and staying along with me since the first moment I joined. A special thanks to Prof. Federica Marini not only for scientific discussions but also for helping in all the required official and beaurocratic work. I would also like to express my sincere gratitude to Prof. Paolo Plevani (also my thesis committee member), Prof. Marco Muzi-Falconi and Dr. Federico Lazzaro for their continuous support. I would like to remember Prof. Gianni Deho (Ex-Director of Phd school) for his help during the initial days of my PhD. I would also like to remember Prof. Giordano Liberi (External thesis committee member) for the annual evaluations of my work and helpful suggestions. I would like to thank Dr. G. Colombo for teaching interesting techniques.

I would like to specially thank my friends and colleagues Matteo and Diego for cordiality and sincere friendship. Off course, it is worthy to mention Sara and Chiara with whom, I worked for a short duration but had wonderful memories. Never the less, I would like to mention Dr. Giacomo Buscemi, Simona and Shyam for providing a joyful company. I would also like to thanks all the members of Lab1 and Lab15 for sincere friendship, healthy discussions and numerous happy moments. I would also like to remember lab technicians Pino and Ciro, and departmental secretaries Margherita and Silvana for their assistance.

I also thank all the friends with whom I shared wonderful memories throughout my PhD. Importantly, I would like to mention Santosh, Sri Harsha, Ranjith, Nitin, Atul staying in Milan and Chetan, Samrudhi, Adwait, Pranav, Priyanka staying in France, who made life easier away from home. Last but not least, I wish to thank my family for the support they provided during all these years.

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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PLoS Genet. 2015 Jan 8; 11(1):e1004928. doi: 10.1371/journal.pgen.1004928.

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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.

Citation: Ferrari M, Dibitetto D, De Gregorio G, Eapen VV, Rawal CC, et al. (2015) Functional Interplay between the 53BP1-Ortholog Rad9 and the Mre11 Complex Regulates Resection, End-Tethering and Repair of a Double-Strand Break. PLoS Genet 11(1): e1004928. doi:10.1371/journal.pgen.1004928

Editor: John L. Nitiss, University of Illinois College of Pharmacy, United States of America

Received June 27, 2014; **Accepted** November 30, 2014; **Published** January 8, 2015

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by grants from AIRC (IG Grant n.10343) and CARIPLO (2013-0790) to AP, and NHI grants GM20056 and GM61766 to JEH. MF was supported by a fellowship from Adriano Buzzati-Traverso Foundation; VVE was supported by an HHIM international predoctoral fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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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

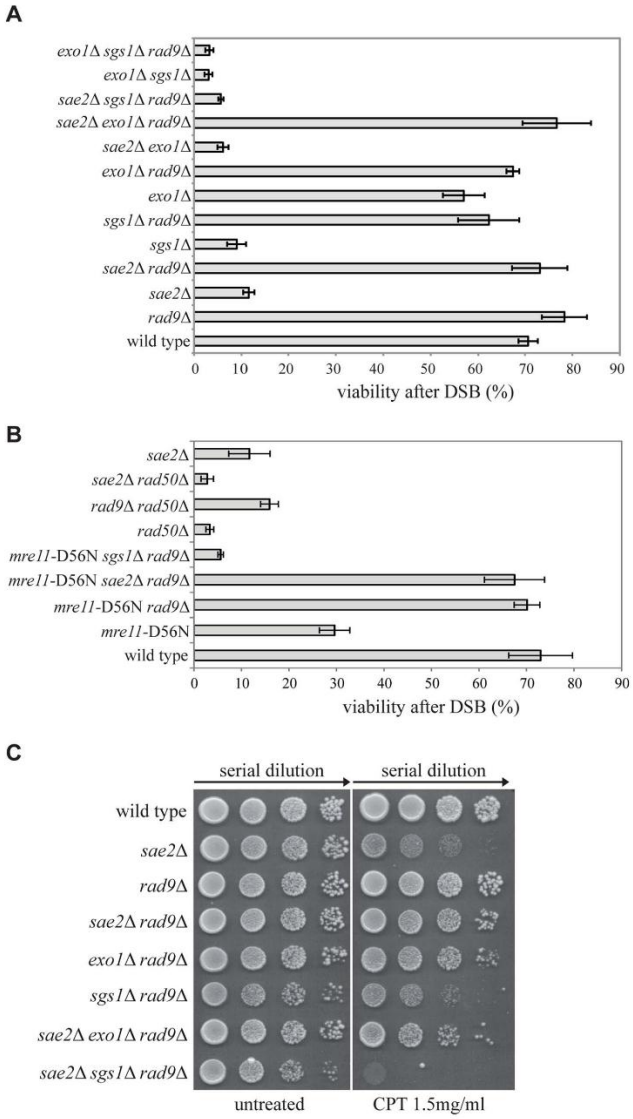


Fig. 1. Deletion of *RAD9* rescues *sae2*Δ and *mre11*-D56N cell viability following DSBs through *SGS1*. (A–B) Viability of the wild type YMV80 strain and the indicated derivatives plated on YEP+gal. In the presence of galactose, one HO-cut is introduced at *leu2* locus (see a scheme in Fig. 2A). For 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.
doi:10.1371/journal.pgen.1004928.g001

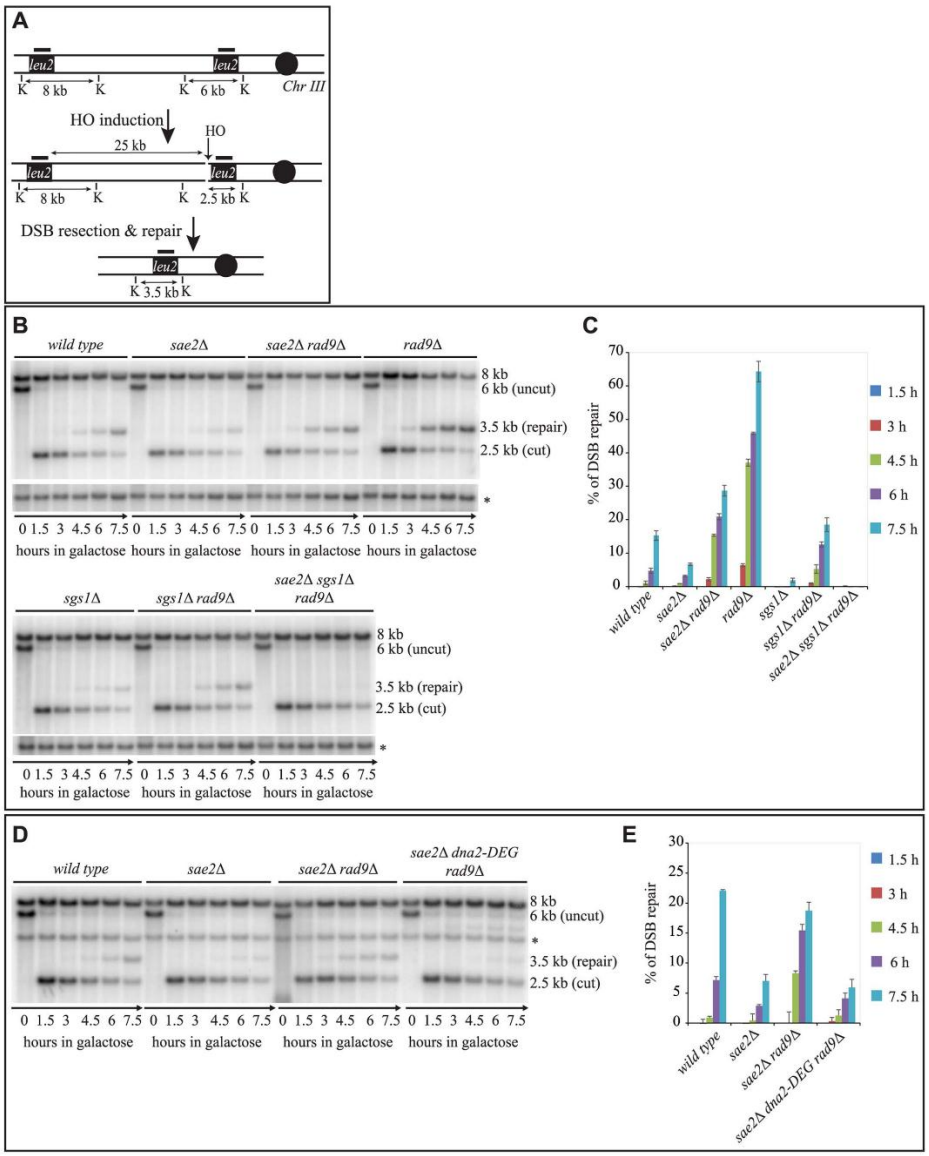


Fig. 2. Deletion of *RAD9* rescues DSB repair defects of *sae2Δ* cells through *SGS1* and *DNA2*. (A) Map of the YMV80 Chr III region, containing the HO-cut site. The indicated vertical bars show KpnI restriction sites. The short thick lines indicate the position where the probe hybridizes. After the 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 (*).

doi:10.1371/journal.pgen.1004928.g002

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).

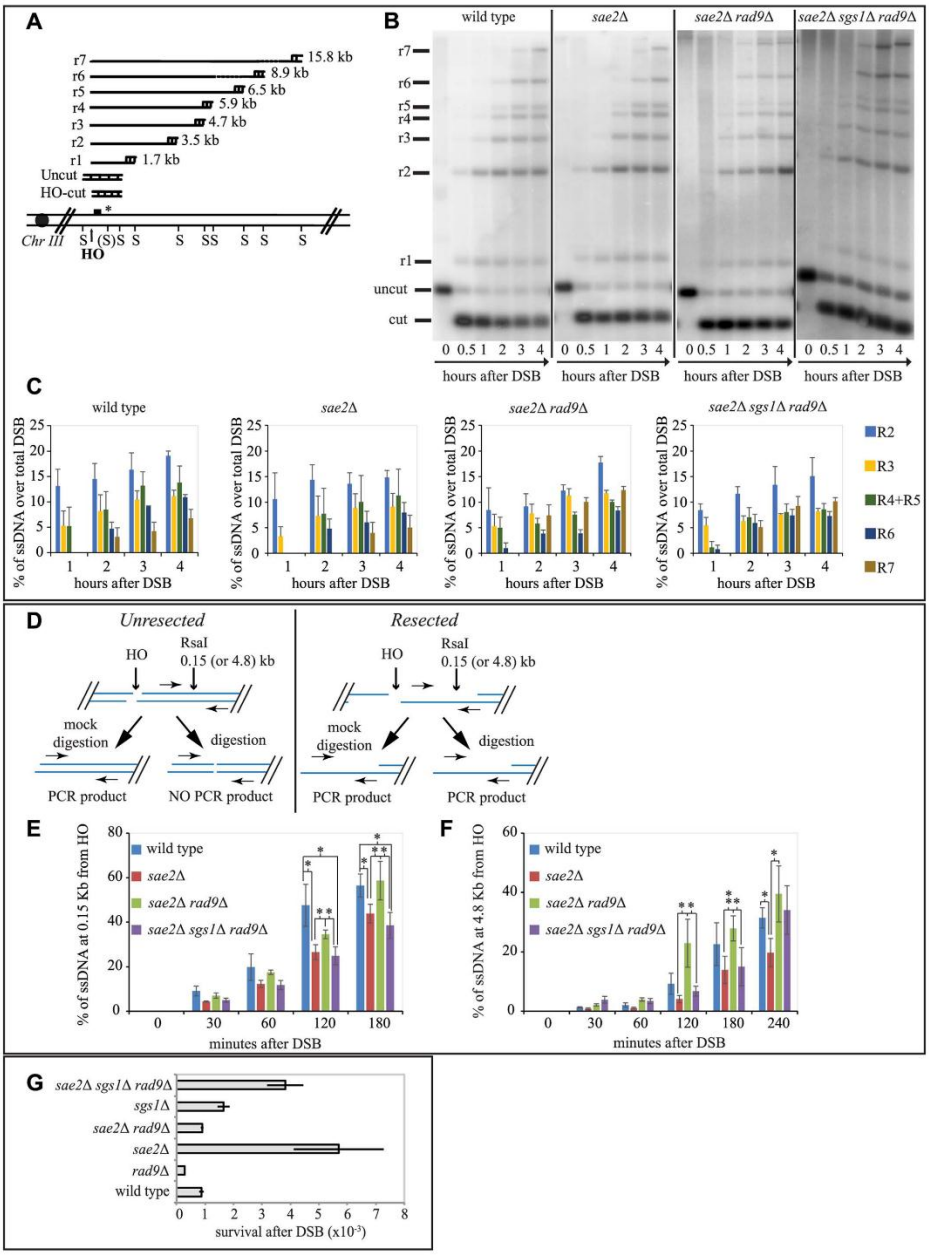
These studies revealed one striking unexpected result: although *sae2*Δ *sgs1*Δ *rad9*Δ triple mutant cells resect a DSB and expose an extended 3′ ssDNA (Figs. 3B, 3E and 3F), they are severely compromised in DSB repair through SSA (Figs. 2B and 2C), suggesting that the long-range resection is not the 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


A
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uncut
cut
hours after DSB
C
% 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
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.
doi:10.1371/journal.pgen.1004928.g003

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 *sae2Δ rad9Δ* 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 *sae2Δ* and *sae2Δ sgs1Δ rad9Δ* 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 *sae2Δ* 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 *sae2Δ* and *sae2Δ sgs1Δ rad9Δ* 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 *sae2Δ* 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 *sae2Δ*, *sae2Δ rad9Δ* and *sae2Δ sgs1Δ rad9Δ* 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 *sae2Δ rad9Δ* mutant cells, thus indicating their ability to hold the broken DNA ends together. In contrast, 42.3%±3.8% of *sae2Δ* and 42.5%±4.8% of *sae2Δ sgs1Δ rad9Δ* 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 *sae2Δ* 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 *sae2Δ* 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 *sae2Δ* cells viability following one DSB. To this aim, we tested the *chk1Δ 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 *sae2Δ chk1Δ rad53*-K227A triple mutant cells is reduced, similarly to *sae2Δ* 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 *sae2Δ* cells.

In order to further understand how Rad9 inhibits SSA repair in *sae2Δ* 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 *sae2Δ* 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 *sae2Δ* 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 *sae2Δ* 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

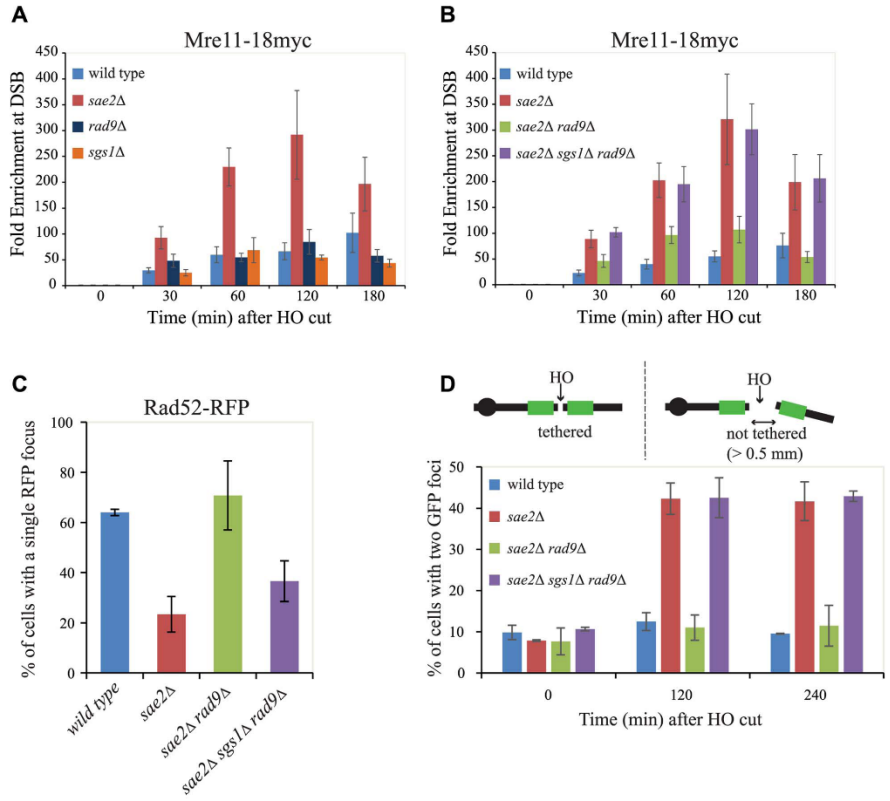

Fig. 4. Rad9 limits Mre11 removal from a DSB, affecting Rad52 binding and DSB ends tethering in *sae2*Δ cells. (A, B) Cells of the wild type JKM139 strain and the indicated derivatives, expressing a Mre11–18Myc fusion protein, were grown in YEP+raf and synchronized in G2/M phases by nocodazole treatment. 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.
doi:10.1371/journal.pgen.1004928.g004

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

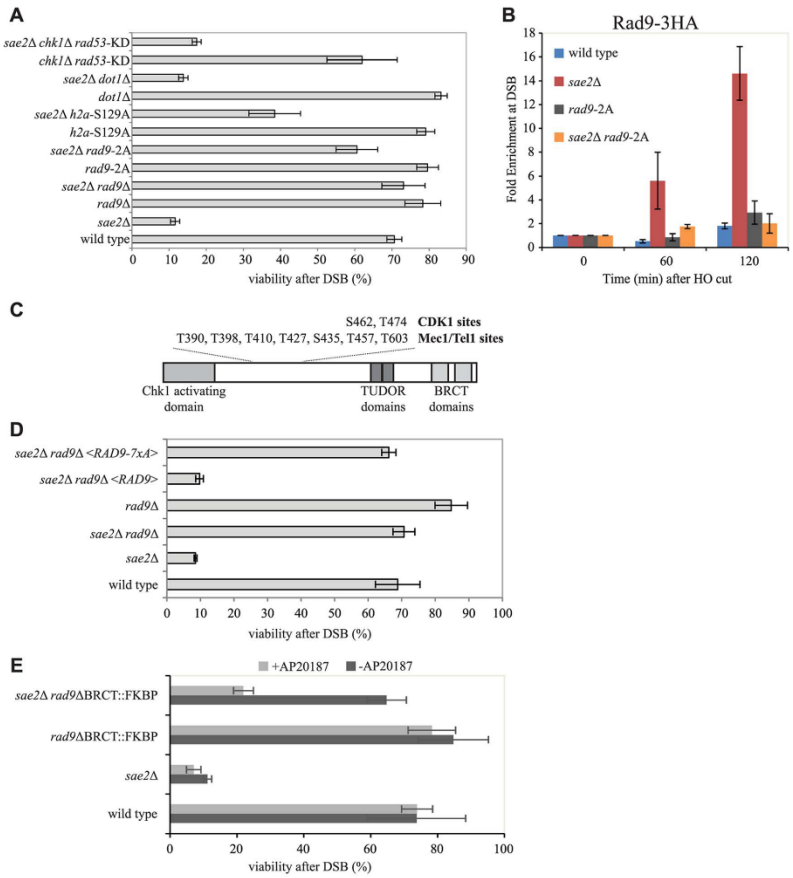

Fig. 5. Rad9 oligomers affect cell viability following a DSB, in the absence of Sae2, mainly through the interaction with Dpb11. (A and D) Viability of the wild type YMV80 strain and the indicated derivatives, plated on YEP+raf+gal. For each strain, the number of colonies grown after 3 days at 28˚C in 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.

doi:10.1371/journal.pgen.1004928.g005

rad9Δ 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 *sae2Δ* 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 *sae2Δ* 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].

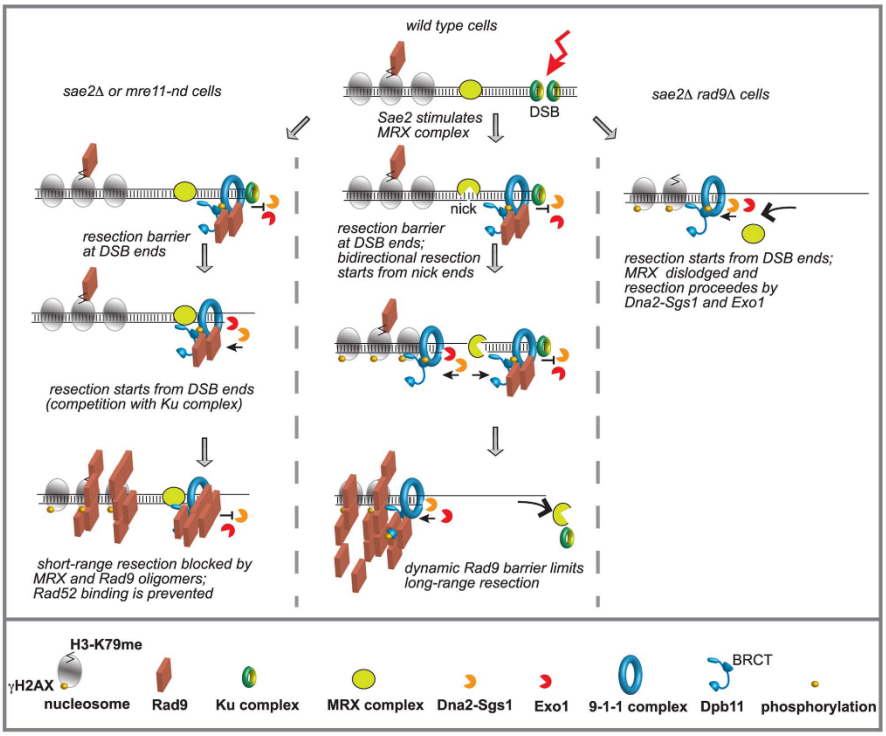

Fig. 6. Model to explain the interplay between Mre11 complex and Rad9 at a DSB in G2/M phase. Ku and Mre11 complexes, together with Rad9, are recruited soon after a DSB formation and limit the action of Exo1 and Dna2-Sgs1 pathways. The order of appearance of the various factors was based on both literature and our results. See details in the text.
doi:10.1371/journal.pgen.1004928.g006

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 yJK40.6. 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)

Acknowledgments

We thank Sarah Sertic and Luca Lorenzi for technical advices on qPCR set up. We also thank all the members of our laboratories for helpful discussions. Noel Lowndes generously provided Rad9 antibodies. We thank Fondazione IRCCS-Istituto Nazionale Tumori (Milano) for technical support.

ARIAD (www.ariad. com/regulationkits) provided the FKBP regulated homodimerization kit.

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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Ferrari et al, Supplementary Figure 4

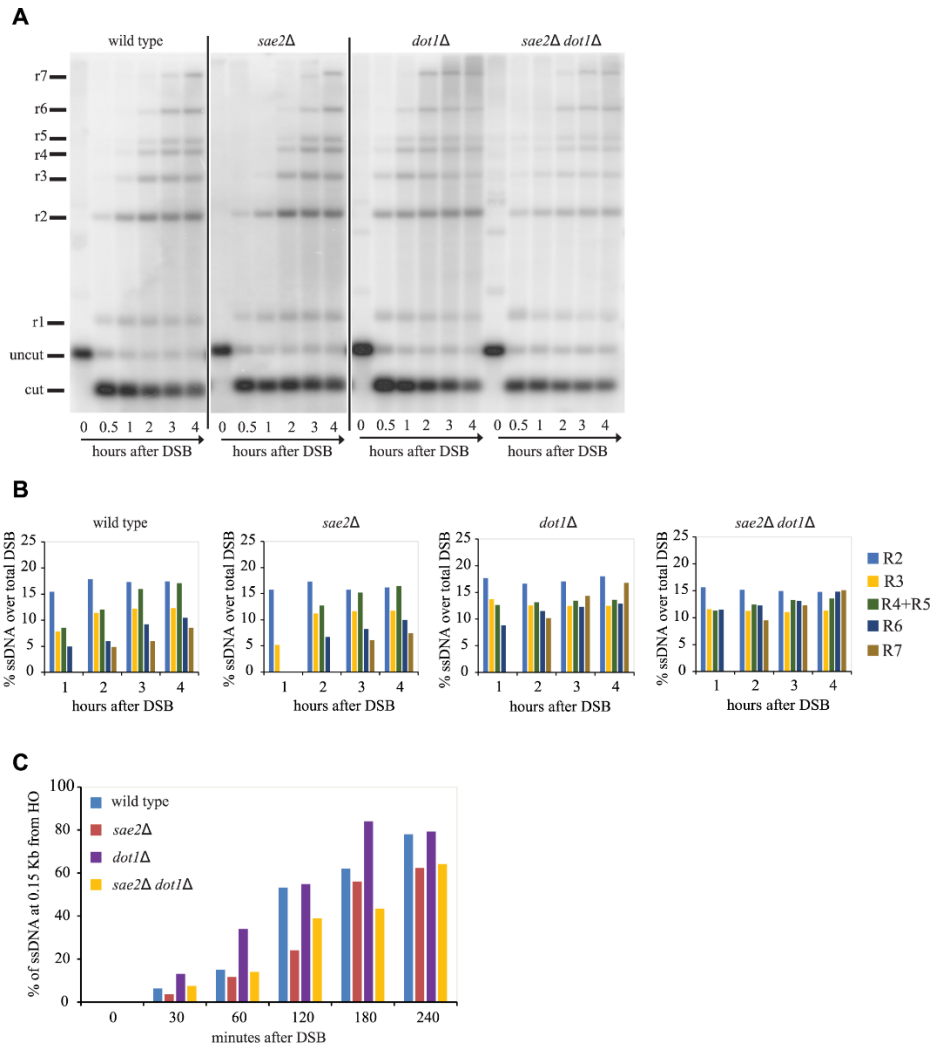

Fig. S4: Analysis of DSB resection in *dot1Δ* derivative strains. (A)Exponentially growing YEP + raf cell cultures of the wild type JKM139 strain and the indicated derivatives, carrying a unique HO cut site at *MAT* locus and expressing the HO nuclease under *GAL1* promoter, were synchronized and kept 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 *sae2Δ* 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.

Published paper II

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

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Nucleic Acids Res. 2015 Oct 20. doi: 10.1093/nar/gkv1080

Nucleic Acids Research Advance Access published October 20, 2015

Nucleic Acids Research, 2015 **1**
doi: 10.1093/nar/gkv1080

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Received August 06, 2015; Revised September 21, 2015; Accepted October 06, 2015

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 *MAT*a 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,

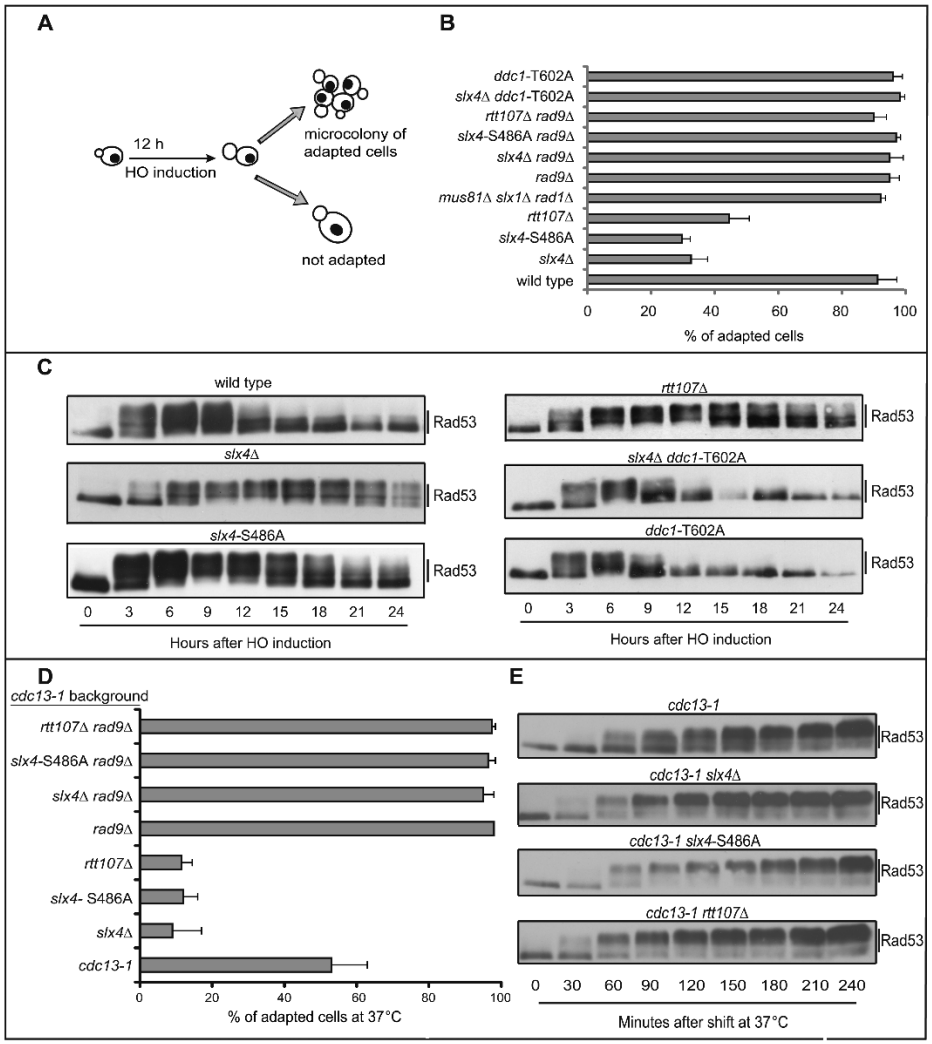

Figure 1. The Slx4-Rtt107 complex is required for checkpoint adaptation to one irreparable DSB and uncapped telomeres. (**A**) Schematic illustration of the HO-cut checkpoint-adaptation assay. (**B**) Graph shows the percentage of adapted cells for each mutant 24 h after plating on galactose containing 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-

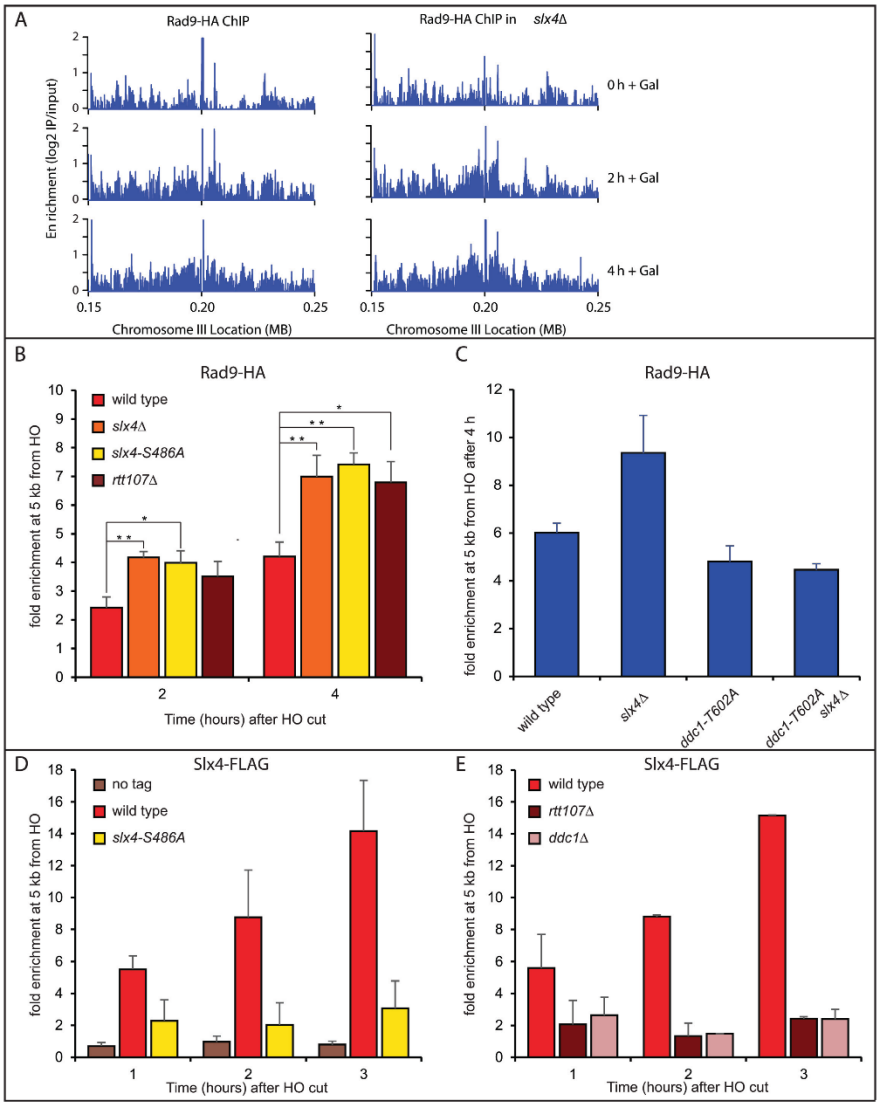

Figure 2. The Slx4-Rtt107 complex modulates Rad9 binding to one irreparable DSB. (**A**) ChIP-seq analysis of Rad9 following induction of a DSB on chromosome III. Rad9 was subjected to chromosome immunoprecipitation at the indicated times after induction of HO endonuclease, in wild type and *slx4*Δ strains. 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 $P < 0.01$).

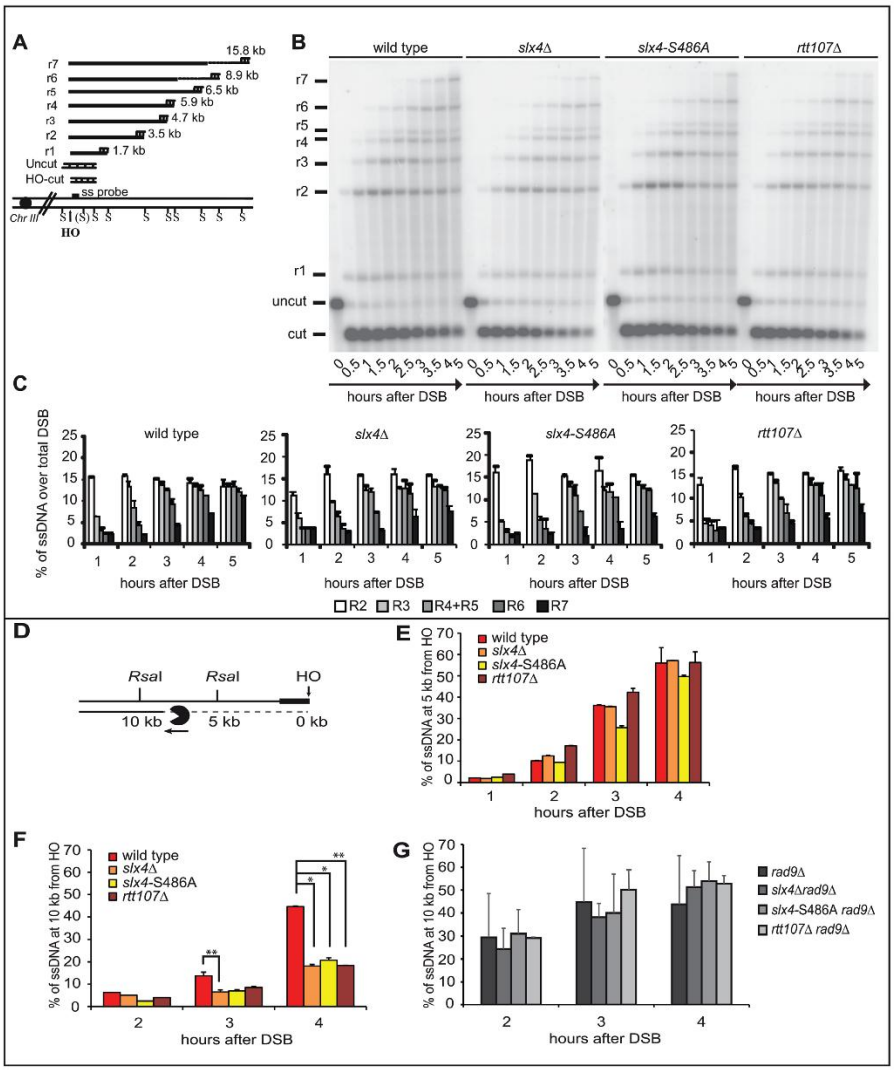

Figure 3. The Slx4-Rtt107 complex modulates long-range DSB resection. (**A**) Schematic illustration of *MAT* locus in JKM179 strains showing positions of HO-cut site, *Ssp*I restriction sites and the RNA probe used. (**B**) ssDNA visualization using RNA probe as described in (**A**) in indicated mutants 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 $P < 0.001$).

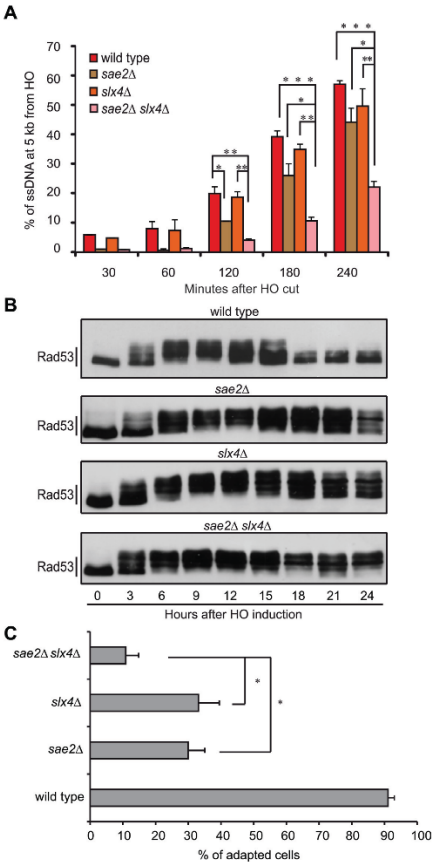

Figure 4. Deletion of *SLX4* exacerbates *sae2*Δ cells phenotypes. (**A**) DSB resection analysis by qPCR in nocodazole-arrested JKM179 derivative strains. Plotted values are the mean of at least two independent experiments ±SEM. Where indicated, significance was determined by single-tailed Student's *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

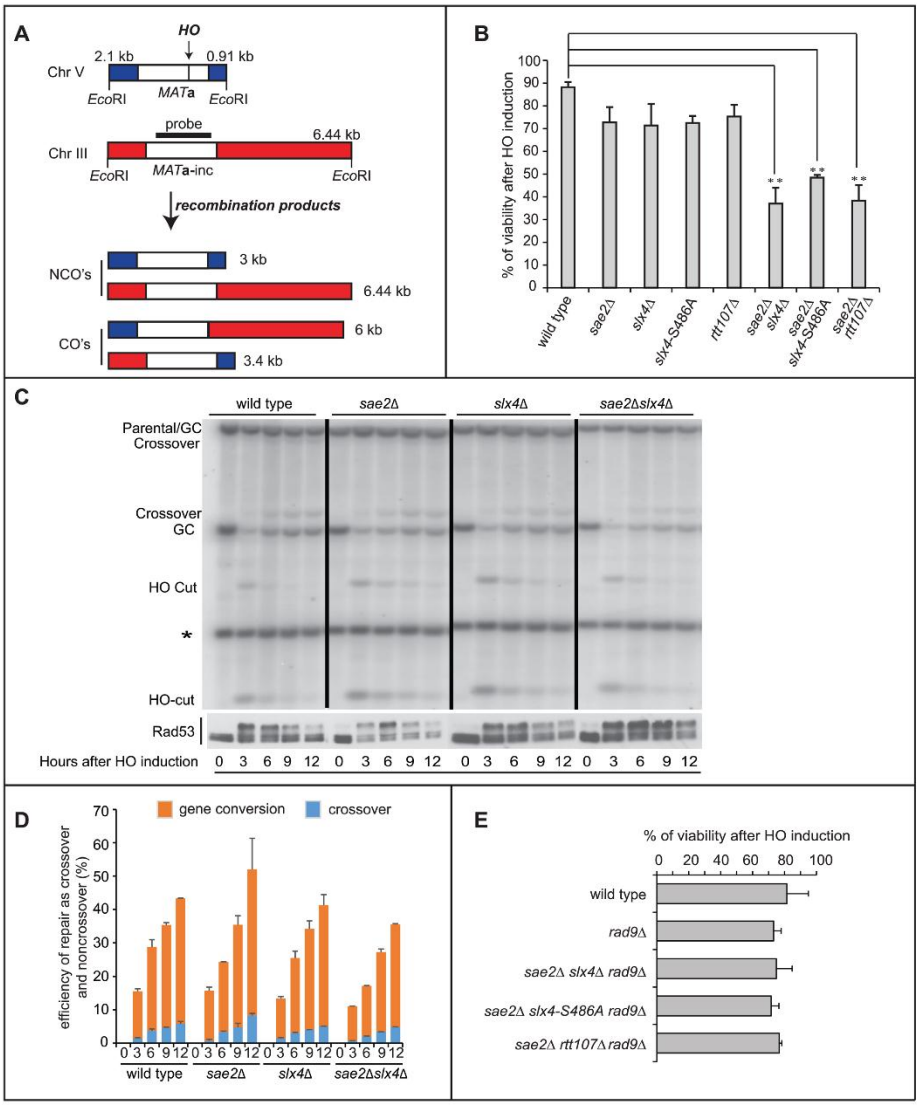

Figure 5. Deletion of *SLX4* affects interchromosomal recombination in *sae2*Δ cells. (**A**) Schematic illustration of *MAT*a-inc locus in Chromosome III and the additional *MAT*a locus in Chromosome V in tGI354 strain, showing positions of HO-cut site, *EcoR1* restriction sites and the probe used to 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.

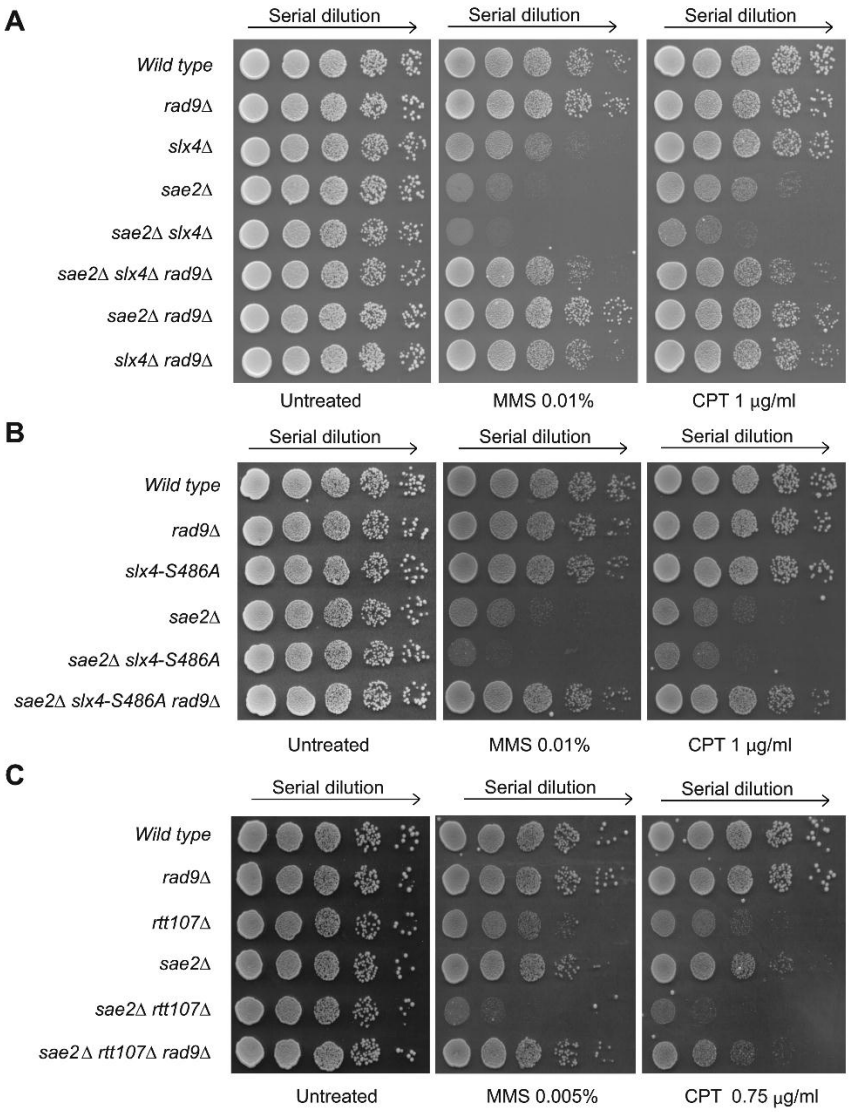

Figure 6. Deletion of *RAD9* rescues the sensitivity to MMS and CPT of *sae2*Δ, *slx4*Δ, *slx4*-S486A and *rtt107*Δ mutant combinations. Exponentially growing cell cultures of the indicated JKM139 derivatives were serially diluted (1:10), and each dilution was spotted out into YPD, YPD + MMS and YPD + 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).

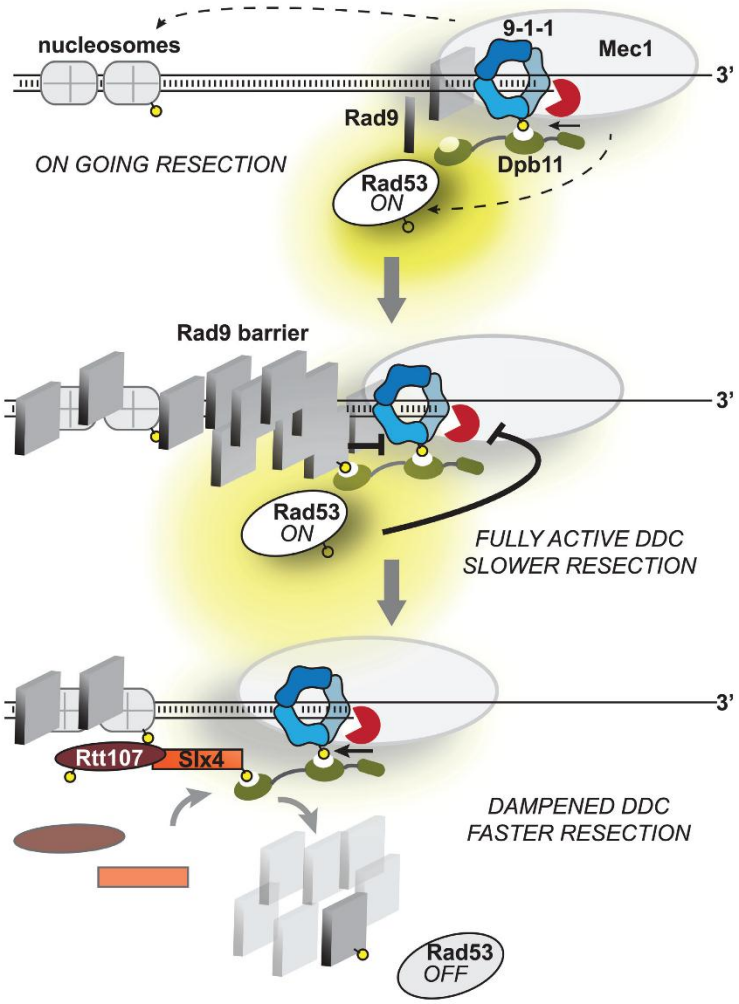

Figure 7. A model for the interplay between Rad9 and the Slx4-Rtt107 complex at a DSB. See text for the details. Yellow circles indicate phosphorylation events.

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.

ACKNOWLEDGEMENT

We thank all the members of our laboratories and M. Neale for helpful discussions. J. Haber generously provided the JKM139 strain, and G. Ira the tGI354 strain.

FUNDING

Associazione Italiana per la Ricerca sul Cancro [AIRC_IG Grant 15488 to A.P.]; CARIPLO [2013-0790 to A.P.]; Canadian Institutes of Health Research and the Canadian Cancer Society Research Institute [to G.W.B.]; and a fellowship from Fondazione VERONESI [to M.F]. Funding for open

access charge: Associazione Italiana per la Ricerca sul Cancro (AIRC) [IG Grant 15488 to A.P.].
Conflict of interest statement. None declared.

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Part III

A distinct role of T238 phosphorylation of polo kinase Cdc5 in response to DNA damage and genome integrity in *S. cerevisiae*

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Abstract

In response to DNA damage all eukaryotic cells activate a surveillance mechanism called DNA damage checkpoint (DDC), arresting cell cycle progression to allow repair. Mechanisms have evolved to switch-off DDC and restart cell cycle when the damage is repaired. However, cells can resume cell cycle even if the damage is unrepaired, compromising genome integrity. In all the eukaryotes, Polo-like kinases (Plks) are key regulators of DDC inactivation and cell cycle re-start. Plks are regulated by multiple mechanisms, and the phosphorylation of well conserved threonine residues in the T-loop of the kinase domain is priority for their activation.

Here, using amino acid substitution and variety of genetic approaches, we delineate the importance of the phosphorylation of Threonine 238 in the activation loop of Cdc5, the only Plk in *S. cerevisiae*. Although this phosphorylation is not required for cells proliferation in unperturbed conditions, the T238A mutation reduces the kinase activity of Cdc5, affecting genome stability. Furthermore, absence of Thr238 phosphorylation hinders DDC inactivation and cell cycle re-start after one irreparable and persistent double strand DNA break. We also found that *cdc5*-T238A cells do not activate the Mus81-Mms4 complex very well, showing sensitivity to DNA damage arising in S phase.

Our data indicate a prominent role of the phosphorylation at Thr238 in the T-loop to trigger Cdc5 activation, in cells responding to DNA damage. Moreover, our results highlight the necessity of T238 phosphorylation of Cdc5 to safeguard the genome stability, even in unperturbed cell cycle.

Key words: Polo kinase/Cdc5, DNA damage, checkpoint adaptation, genetic interaction

1. Introduction

Polo kinases (Plks) are highly conserved mitotic regulators from yeast to mammals. Their number varies from just a single member in budding and fission yeast (Cdc5 & Plo1 respectively) to five members in mammals (Plk1-5), attributing to the variety of functions. However, in all the eukaryotes they perform essential role in mitotic transition and cytokinesis (Archambault & Glover 2009). Interestingly, balance of Plk1 level is very critical for normal cell cycle and genome stability as its overexpression is associated with various cancers; whereas Plk1 depletion has also been found to induce aneuploidy (Eckerdt et al. 2005; Takai et al. 2005; de Cárcer et al. 2011). PLKs were also implicated in response to DNA damage to inactivate the DNA damage checkpoint (DDC) (Bahassi 2011; Hyun et al. 2014). More specifically, PLKs were involved in DDC inactivation and cell cycle restart either when DNA damage is completely repaired, thorough a process called checkpoint recovery, or when the DNA lesions are refractory to be repaired, through a process called checkpoint adaptation. Although checkpoint adaptation is a controversial phenomenon in higher eukaryotes and human cells, it has been associated to tumor development. Indeed, studies in yeast have reported that checkpoint adaptation precedes different types of genome instabilities (Galgoczy & Toczyski 2001).

In budding yeast, induction of single DNA double strand break (DSB) at *MAT* locus through expression of HO endonuclease, has led to important advancement in understanding key regulators of the checkpoint adaptation and recovery processes (White & Haber 1990; Lee et al. 1998; Harrison & Haber 2006).

After irreparable DSB, cell cycle progression is arrested due to upstream kinase Mec1/Tel1and the effector kinases Chk1 and Rad53, which prevent chromosomes separation and mitotic exit (Sanchez et al. 1999; Tinker-

Kulberg & Morgan 1999; Wang et al. 2001; Fenghua Hu et al. 2001). Moreover, Mad-Bub proteins, components of the spindle assembly checkpoint (SAC), also contribute to maintain the cell cycle block after one irreparable DSB (Dotiwala et al. 2010). Several factors have been implicated in checkpoint adaptation in yeast: i) recombination factors (Sae2, Tid1, Sgs1, RPA, Ku complex, Mre11/Rad50, Rad51); ii) chromatin remodelers (Fun30, Ino80); iii) cell cycle and checkpoint kinases (Cdc5, CKII, Mec1); iv) phosphatases (Ptc2-Ptc3) (Toczyski et al. 1997; Lee et al. 1998; Lee et al. 2001; Lee et al. 2003; Leroy et al. 2003; Clerici et al. 2006; Papamichos-Chronakis et al. 2006; Eapen et al. 2012; Costelloe et al. 2012; Clerici et al. 2014; Ghospurkar et al. 2015).

The missense mutation *cdc5*-L251W (also called *cdc5*-ad) identified Cdc5 as the key factor in regulating checkpoint adaptation after telomere dysfunction and irreparable DSB (Toczyski et al. 1997). In *cdc5*-ad cells, even one irreparable DSB is sufficient to trigger a robust and persistent activation of Rad53 (Pellicioli et al. 2001). Importantly, the same mutation does not affect the checkpoint switching off during checkpoint recovery, after the repair of a DSB (Vaze et al. 2002). In human cells, depletion of Plk1 has been found to affect both checkpoint adaptation and recovery and it also lead to cancer cell death (van Vugt & Medema 2004; Van Vugt et al. 2004). Specifically, Plk1 has been found to regulate checkpoint adaptation in response to replication stress and ionizing radiations (Yoo et al. 2004; Syljuasen et al. 2006). Notably, checkpoint adaptation occurs as the final survival attempt, but it also increases the risk of development of cells with chromosomal instability (Galgoczy & Toczyski 2001; Syljuåsen 2007).

It is now clear that in yeast and human cells, Cdc5 and Plk1 act directly on the checkpoint transducer kinases Rad53 and Chk2, inactivating them and promoting checkpoint adaptation (Donnianni et al. 2010; Lopez-Mosqueda et

al. 2010; Schleker et al. 2010; Vidanes et al. 2010; Yoo et al. 2004; van Vugt et al. 2010; Liu et al. 2010). Moreover, the overproduction of Cdc5 affected checkpoint signalling at multiple steps, suggesting that Cdc5 regulate multiple targets during the process (Donnianni et al. 2010). Interestingly, it was shown that Cdc5 phosphorylates Sae2, affecting its binding to a DSB (Donnianni et al. 2010). More recently, a similar mechanism was shown for the human counterparts, Plk3 and CtIP, respectively (Barton et al. 2014). In addition, Plk1 and Cdc5 regulate a number of factors involved in other mechanisms of the DNA damage response. One of the major targets of Plk1 and Cdc5 is the Mus81-Eme1 (Mus81-Mms4 in yeast) complex, whose activity is required for processing homologous recombination intermediates, which accounts for crossover outcomes in mitosis as well as in meiosis (Matos et al. 2011; Matos et al. 2013; Szakal & Branzei 2013).

In response to DNA damage in yeast, checkpoint activation restrains Cdc5 activity and recently it was shown that the protein is nuclearized. (Cheng et al. 1998; Sanchez et al. 1999; Zhang et al. 2009; Valerio-Santiago et al. 2013). Interestingly during checkpoint adaptation, Cdc5 activity is re-activated to inactivate critical mitotic regulators, such as Cdh1 and Bfa1, promoting spindle elongation and mitotic exit (Crasta et al. 2008; Zhang et al. 2009; Valerio-Santiago et al. 2013).

Considering their central role in many aspects of the DNA damage response and cell cycle progression, Plks are finely regulated by different mechanisms (Barr et al. 2004; Archambault & Glover 2009; Clémenson & Marsolier-Kergoat 2009; Bahassi 2011; Archambault & Carmena 2012; Archambault et al. 2015b). All the Plks are regulated through phosphorylation of Threonine residues in the T-loop of the kinase domain. Human Plk1 is phosphorylated at T210 in its activation loop by Aurora A and Aurora B kinases (Macůrek et al. 2008; Seki et al. 2008). The phosphorylation at T210

of human Plk1 by Aurora A with co-factor Bora is essential for checkpoint recovery and for early activation of the protein at centrosomes (Macůrek et al. 2008; Seki et al. 2008; Tsvetkov & Stern 2005; Bruinsma et al. 2015). In budding yeast, the T238 residue in the Cdc5 T-loop, which corresponds to T210 of Plk1, has also been found to be phosphorylated, but was shown to be dispensable for cell viability in unperturbed conditions (Mortensen et al. 2005). Indeed, it was shown that the Cdc5 activity is primed by the Cdk1 (Cdc28)-dependent phosphorylation of T242 in the T-loop of the kinase domain (Mortensen et al. 2005), highlighting differential regulation between mammalian and yeast systems.

In this study, we show that phosphorylation of the T238 residue of Cdc5 reduces the kinase activity of the protein. We also show that *cdc5*-T238A cells have reduced rate of mitotic recombination and increased rate of chromosome loss, indicating altered genome stability in unperturbed cell cycle. Importantly, *cdc5*-T238A mutation affects both the checkpoint adaptation to one irreparable DSB and, marginally, the checkpoint recovery from a persistent DSB. Moreover, *cdc5*-T238A cells cannot activate properly the Mus81-Mms4 complex, resulting in slight sensitivity to DNA damage inducing agents.

In summary, we found that the phosphorylation of T238 site in the T-loop of Cdc5 is important to fully activate Cdc5 in cells responding to DNA damage, thus preserving genome stability. Our results partially reconcile the regulation of Cdc5 in yeast with that described for Plk1 in mammals, in cells responding to DNA damage.

2. Materials and methods:

Yeast strains and media:

All the strains listed in Supplementary Table 1 are derivative of JKM139, YMV80, tGI354 or W303. 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 (Longtine et al. 1998). Mutant alleles of *CDC5* were obtained by site specific mutagenesis of pRS306 plasmid containing wild type *CDC5* with its endogenous promoter and C-terminal –HA tag. *Bcl*I-digested pRS306 plasmid was integrated into the *CDC5* locus and after pop-out by treatment with 5-FOA, the integration of the *cdc5*-T238A and other alleles was confirmed by sequencing. Except, complementation analysis of *cdc5*-1, shown in Figure 1B, all the experiments were performed with *CDC5* mutations integrated at its endogenous locus.

Strains used for chromosome loss assay were generated by transforming *SnaBI* digested CFV/D8B-tg into *RAD5* derivative of W303 background. Stable Ura^+ transformants due to BIR induced extra-chromosome fragment were confirmed by pulse field gel electrophoresis as described previously (Davis & Symington 2004).

All the strains used in this work are haploid; moreover, *mec1*Δ strain also has the *sml1*Δ mutation, to keep cells viable (Zhao et al. 1998).

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.

2.1 Western blot analysis

The TCA protein extraction and the Western blot procedures have been previously described (Muzi Falconi et al. 1993). Rad53 and -3HA tagged proteins were analyzed using Mab.EL7, and 12CA5 monoclonal antibodies, respectively.

2.1 Cell synchrony and flow cytometry

Cells were pre-synchronized in G1 with α-factor (2 µg/ml) and then released in fresh medium. Cells were arrested in G1 and G2/M with α -factor (10 µg/ml) or nocodazole (20 µg/ml), respectively. DNA content was analyzed by FACS Calibur (Bekton-Dickinson) and Cell-Quest software (Bekton-Dickinson).

2.2 Immunofluorescence analysis:

Samples were collected at indicated time points and fixed either in 100% ethanol or K-Phos.-formaldehyde with magnesium chloride buffer. Spheroplasting was done with 1mg/ml of zymoliase. Monoclonal anti-alpha tubulin antibody was used to visualize tubulin and nuclei were stained with DAPI. Images were captured using Leica BG DMR fluorescence microscope and analyzed with LAS AF suite.

2.3 In vitro kinase assay:

Cdc5-3HA kinase activity was measured in 12CA5 immuno-precipitates from nocodazole arrested cells and washed sequentially in LLB, high-salt QA (20 mM Tris-HCl, pH 7.6, 250 mM KCl, 1 mM MgCl2, 1 mM DTT), and 5KB (50 mM Hepes-NaOH, pH 7.4, 200 mM KAc, 10 mM MgCl2, 5 mM MnCl2, 1 mM DTT). Kinase assays (30 µl) were performed in 50 mM

Hepes-NaOH, pH 7.4, 60 mM KAc, 10 mM MgCl2, 5 mM MnCl2, 50 mM ATP, plus 5 mg casein and 2.5 µCi [32P]ATP (Charles et al. 1998).

2.4 Checkpoint adaptation and recovery analysis by micro-colony assay:

JKM139, tGI354 or YMV80 derived strains were grown overnight in YP + raf media and unbudded cells (G1 phase) were micro-manipulated on YEP + raf + gal plates. Percentage of checkpoint adaptation was scored after 24 and 48hrs of incubation in JKM139 derived strains whereas checkpoint recovery was monitored in tGI354/ YMV80 derived strains by following cell cycle progression at indicated time points as described previously (Lee et al. 1998; Vaze et al. 2002)

2.5 Southern blot analysis

DSB repair in YMV80 derivative strains and tGI354 derivative strains were analyzed on agarose gels with DNA probes annealing at *LEU2* and *MAT a* loci respectively (Vaze et al. 2002; Ira et al. 2003). Furthermore, loading was normalized in YMV80 derived southern blots using probe specific for unprocessed locus *ATG5* and in tGI354 derived southern blots using probe specific for unprocessed locus *IPL1* (Ferrari et al. 2015).

2.6 Chromosome loss assay:

Strains with chromosome III fragment (110kb) were grown overnight in SC-uracil liquid medium. The following day, cells were washed with sterile water and plated on SC+Ade (6µg/ml) to enhance red pigmentation. After incubation of 3-4 days, at least 10,000 colonies were screened per strain for exact half red/white sectoring which indicates chromosome loss at first cell division in non-selective medium (Spencer et al. 1990). The data represents 3 independent experiments.

2.7 Cell viability assay

YMV80 and tGI354 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.

2.8 Spot test for DNA damage sensitivities:

Log phase cultures were normalized to 10^7cells/ml and 10µl of tenfold serial dilutions were spot plated on control and drug containing YPD plates. Plates were incubated at 28^0C for 2-3 days.

2.9 Recombination rate assay:

Recombination assays were performed using the *ade2-NdeI::URA3::ade2-AatII* system as previously described (Huang & Symington 1994). Briefly, colonies were isolated onto YPD + Ade medium and grown for 3 days at 28°C. Seven single colonies per strain were re-suspended in 1 mL dH_20. Cells were plated to SC-ade, -uracil to select for recombinants and onto SC medium for total cell number. Plates were incubated at 30°C for 3–4 days. To determine rates, the Lea and Coulson method of the median with 95% confidence intervals was used (Lea & Coulson 1949). Data represent five independent experiments.

3. Results

3.1 Phosphorylation at Thr238 of Cdc5 is dispensable for viability but reduces the kinase activity of the protein

The activity of yeast polo kinase Cdc5 is restricted in G2/M phase and is strictly regulated by post translational modifications (Charles et al. 1998; Shirayama et al. 1998; Lee et al. 2005). The activation loop of the kinase domain of Cdc5 is phosphorylated at two sites namely T238 and T242, which are conserved in higher eukaryotes (Fig.1A). Of note, phosphorylation of the T242 by Cdc28/CDK1 is absolutely required for the activation of protein and viability of the cells, whereas phosphorylation of the T238 site has found to have variable effect (Qian et al. 1999; Kelm et al. 2002). By sequence alignment, we noted that T238 site correspond to the T210 site in human Plk1 (Figure 1A). Importantly, phosphorylation of T210 in Plk1 by Aurora A has been involved in DNA damage checkpoint recovery (Macůrek et al. 2008; Seki et al. 2008). Thus we decided to investigate specifically the role of phosphorylation of T238 in Cdc5, focusing on DNA damage response and genome stability maintenance.

Firstly, we mutagenized the T238 or T242 sites to non phosphorylable amino acid, Alanine in a plasmid carrying *CDC5*. Then, we analyzed the role of T242 and T238 phosphorylations in cell viability by assessing complementation of thermo sensitive allele *cdc5*-1 at restrictive temperature. We also tested the wild type *CDC5* and the kinase-dead *cdc5*-N209A alleles, as controls. As shown in Fig. 1B, at non permissive temperature, the cells carrying thermo sensitive allele *cdc5*-1, are inviable due to failure to complete mitotic transition (Hartwell et al. 1973). The thermo sensitivity was completely rescued by expressing either the wild type *CDC5* or the *cdc5*-T238A alleles on the plasmid. Importantly, the expression of the kinase-dead *cdc5*-N209A and *cdc5*-T242A alleles did not rescue the cell lethality of *cdc5*-

1 at 37°C, as described previously (F Hu et al. 2001; Mortensen et al. 2005). Therefore, our complementation assay supports previous finding (Mortensen et al. 2005), indicating that the phosphorylation of the T238 in the T-loop of Cdc5 seems to be dispensable for the fully activation of the kinase domain and cell viability.

To further address the effect of the -T238A mutation on Cdc5 kinase activity and cell cycle dependent protein level, we integrated the *cdc5*-T238A allele at its endogenous locus. Cells were kept blocked in G1 or G2 cell cycle phases with α-Factor or nocodazole treatment respectively, and protein samples were collected at indicated time points. In the same experiment, we also tested the *cdc5*-L251W (also called *cdc5*-ad) mutant cells, which are known to be defective in checkpoint adaptation (Toczyski et al. 1997). As shown in supplementary figure 1, the wild type Cdc5 protein and both the Cdc5-T238A and L251W protein variants are accumulated in G2 blocked cells, while they are degraded in G1 blocked cells. Then, we immunoprecipitated from the G2 blocked cells the Cdc5-3HA protein variants, using anti –HA antibodies. The in vitro kinase assay was performed as described previously, using Casein as substrate and γ-^{32}P-ATP (Charles et al. 1998). Interestingly, we found that the Cdc5-T238A variant had almost 60% reduction in its kinase activity compared to wild type protein (Figure-1C, D), while the Cdc5-L251W retained the wild type level of kinase activity, as previously shown (Charles et al. 1998).

3.2 *cdc5*-T238A mutation alters spontaneous mitotic recombination events and chromosome loss rate

Temperature sensitive allele *cdc5*-1, has been previously reported to alter genome stability by increasing the mitotic recombination rate, chromosome loss rate and also led to defect in mitochondrial transmission to

zygote (Dutcher 1982; Hartwell & Smith 1985; Aguilera & Klein 1988). Moreover, Cdc5 was recently shown to regulate key factors of the recombination process (Matos et al. 2011; Matos et al. 2013). Therefore, we decided to characterize the effect of *cdc5*-T238A allele in spontaneous mitotic recombination and chromosome loss rate. Firstly, we took advantage of a standard direct-repeat recombination assay (Huang & Symington 1994). This genetic background consists of two heteroalleles of the *ade2* gene in direct repeat orientation at the *ADE2* locus (*ade2-n::URA3::ade2-a*). The mutations in *ade2* leads to inviability of the parental strain on synthetic media lacking adenine. The recombination can occur by two major mechanisms, leading to the restoration of one copy of functional *ADE2* gene. First mechanism, which is referred to as gene conversion, maintains the *ade2* repeats intact and *URA3* marker, whereas in the second mechanism, called as pop out, either of the repeats with *URA3* marker is lost. As shown in Figure 2A, after quantifying recombination rate for Ade^+ prototrophy, we found that *cdc5*-T238A cells had almost 60% reduction in recombination rate ($2.09x10^{-6} \pm 0.3$) as compared to wild type cells ($5.04x10^{-6} \pm 0.4$). All the Ade^+ prototrophs were scored for presence of *URA3* marker for distinguishing the recombination pathway. As the percentage of Ura^+ prototrophs remained almost equal, both the pathways gene conversion and pop out appeared to be down regulated in *cdc5*-T238A cells (Fig. 2B). Then, we investigated chromosome loss rate in *cdc5*-T238A cells. To this aim, we used a modified genetic assay in which strain with stable Chromosome III fragment (CF) was created using CFV/D8B-tg as a result of break induced replication (Davis & Symington 2004). The presence of 110 kb CF was confirmed by Pulse-filed gel electrophoresis. In W303 cells, the presence of *SUP11* marker on CF suppresses the *ade2*-1 mutation leading to formation of white colonies, whereas the cells lacking the CF form red colonies (schematic Fig. 2C). In this genetic background, the wild type cells

have approximately ~1X10^{-3} chromosome loss rate per cell per generation. Interestingly, *cdc5*-T238A cells were found to increase the chromosome loss rate by three fold in unperturbed conditions (Fig. 2D). Taken together the results in figure 2, it becomes evident that the *cdc5*-T238A mutation affects recombination rate and chromosome stability, although it doesn't reduce cell growth in unperturbed cell cycle.

3.3 *cdc5*-T238A cells do not adapt to one irreparable DSB

Cdc5 has been found to promote checkpoint adaptation after one persistent DSB and telomere uncapping (Toczyski et al. 1997). In fact, *cdc5*-ad cells do not switch off checkpoint and do not re-start cell cycle after one irreparable HO-induced DSB (Toczyski et al. 1997; Pellicioli et al. 2001). So we asked if *cdc5*-T238A cells have any effect on checkpoint inactivation after persistent DSB. We took an advantage of yeast genetic background JKM139, in which an irreparable DSB is induced at *MAT* locus by the conditional over-expression of HO (White & Haber 1990). This is an ideal system to monitor checkpoint signalling and cell cycle progression, as it is unaffected by repair intermediates due to lack of homology sequences (White & Haber 1990; Lee et al. 1998). Thus, G1 unbudded cells were micro-manipulated in galactose containing medium to induce the HO-break. After DSB induction, the activation of the DNA damage checkpoint blocks cell cycle progression at the G2/M transition for several hours (Lee et al. 1998). However, wild type cells are known to undergo checkpoint adaptation, proceeding through 3-4 divisions after 24 hours, and are scored as the percent of cells forming micro-colonies. Strikingly, the number of cells underwent adaptation was severely reduced in *cdc5*-T238A mutant similarly to the previously characterized *cdc5*-ad [Fig. 3A and (Toczyski et al. 1997)]. Of note, the cells with phospho-mimicking mutant *cdc5*-T238D were able to adapt proficiently (Fig 3A), further supporting the

hypothesis that the phosphorylation of T238 site of Cdc5 is a prerequisite for Cdc5 activity during checkpoint adaptation.

To address checkpoint adaptation at the molecular level in *cdc5*-T238A cells, we analysed Rad53 phosphorylation by western blotting, after the induction of one HO-induced DSB. As previously shown (also in Fig. 3B), in wild type cells Rad53 is dephosphorylated after 12-15 hours after DSB induction (Pellicioli et al. 2001). In contrast to wild type cells, Rad53 dephosphorylation was severely impaired in *cdc5*-T238A cells till almost 20-22 hours, although the defect is less severe than in *cdc5*-ad cells (Fig. 3B). In particular, we noted that in *cdc5*-T238A cells the percentage of cells adapting to irreparable DSB still remained low, although Rad53 was significantly dephosphorylated at later time points. To further investigate this phenomenon, we monitored nuclear division accompanied by spindle elongation during checkpoint adaptation. Upon the induction of one irreparable DSB in logarithmically growing cells, the wild type cells switch-off checkpoint after 12-14 hours and undergo nuclear division accompanied by spindle elongation which can be seen under immunofluorescence microscope (Fig. 3C, D). Consistently with the defect in checkpoint adaptation and micro-colony formation, both the *cdc5*-T238A and *cdc5*-ad cells remained in metaphase arrest with undivided nuclei at the bud neck with short spindle (Fig. 3C, D).

3.4 Mutations in checkpoint factors bypass the permanent cell cycle block in *cdc5*-T238A cells after one irreparable DSB.

Further supporting that *cdc5*-T238A cells remained blocked in G2/M due to the hyper-activation of DDC, we analysed the *rad9Δ cdc5*-T238A double mutant cells, in which the DDC pathway is terminated upstream of Rad53 & Chk1. In the same assay, we decided to investigate other interesting checkpoint factors and their mitotic effectors, such as Tel1, Mad2 and Cdh1.

Indeed, it was previously shown that the maintenance of prolonged cell cycle block after the formation of one persistent DSB is mediated by the contributions of Chk1 and the spindle assembly checkpoint factor Mad2, in addition to the Rad53 activity (Dotiwala et al. 2010). Moreover, it has also been shown that Rad53 dependent inhibition of Cdc5 in G2/M phase keeps Cdh1 and Bfa1 in active state, thereby restricting mitotic spindle elongation and mitotic exit (Zhang et al. 2009; Valerio-Santiago et al. 2013), thus reinforcing the arrest. In addition, Rad52 and Tel1 were shown to be involved in distinct mechanisms to maintain checkpoint response after one irreparable DSB. Deletion of *RAD52* was shown to rescue checkpoint adaptation defect of *rad51Δ* mutant; whereas deletion of *TEL1* was shown to suppress a number of adaptation defective mutants (*mec1*-ad*, sae2Δ, sgs1Δ* and *dna2Δ*), which also had a defect in DSB resection (Lee et al. 2003; Clerici et al. 2014).

After micro-manipulating the cells in the presence of galactose to induce the HO-mediated irreparable DSB, we found that the permanent cell cycle block of *cdc5*-T238A cells was completely rescued by deleting either *RAD9, MAD2* or *CDH1* (Figure 3E). Interestingly, we also found that *CDH1* deletion did not rescue *cdc5*-ad. (Supplementary Fig. 2), whereas it was found to be suppressed by the deletion of SAC component *MAD2* (Dotiwala et al. 2010). Similarly to *cdc5*-ad, in addition we found that the permanent cell cycle block of *cdc5*-T238A was not rescued by deletion of recombination factor *RAD52* (Toczyski et al. 1997; Vaze et al. 2002) and neither it was suppressed by the deletion of *TEL1*. This genetic analysis suggests that Cdc5p acts in an independent pathway to promote checkpoint adaptation compared to Rad52, Rad51 and RPA mediated checkpoint signalling and adaptation (Lee et al. 1998; Lee et al. 2003). Moreover, these *cdc5*-ad and *cdc5*-T238A alleles, even though share similar adaptation defect phenotype, they apparently behave in

different ways, in term of genetic epistasis (Figure 3E & S2) and kinase activity of the corresponding protein (Figure 1C, D).

In conclusion, our data in Figure 3 suggest that the checkpoint adaptation defect of *cdc5*-T238A cells might be related to the inability to inactivate multiple checkpoint mitotic targets, such as Mad2 and Cdh1, in addition to other DNA damage checkpoint targets, such as Rad53 and Chk1.

3.5 Cdc5-T238A and Cdc5-ad protein variants show altered localization to spindle pole bodies after one irreparable DSB

Recent findings indicate that Cdc5 is nuclearized after DNA damage and Rad53 activation (Valerio-Santiago et al. 2013), thus preventing Bfa1 inactivation through Cdc5-dependent phosphorylation at Spindle Pole bodies (SPBs). Based on these observations, we speculated that Cdc5 might relocalize in to the cytoplasm and at SPBs to inactivate Bfa1 and promote mitotic exit, during checkpoint adaptation. Thus, to analyze how Cdc5 is localized during checkpoint adaptation we inserted an eGFP tag to the C-terminal of Cdc5, Cdc5-T238A and Cdc5-ad proteins, in JKM139 background. After 6 hours of induction of one irreparable DSB, we observed that almost 80% cells got arrested in metaphase, with strong signal of Cdc5-eGFP in the nucleus. In wild type cells, after 10-12 hours of induction Cdc5 signal was observed outside the nucleus and at SPBs. Consequently greater number of cells with divided nuclei were observed during later time points (Fig. 4A and B). Interestingly, even though the Cdc5-T238A protein variant was nuclearized after 6 hours of DSB induction, then we observed a prominent delay of its localization at SPB at 16 – 18 hours (Fig. 4A, B). This delay in Cdc5 localization may reflect the prolonged metaphase block with short spindle in *cdc5*-T238A cells, after one irreparable DSB. In the same experiment, we also investigated the localization of Cdc5-ad-eGFP protein variant, after HO induction. Surprisingly, we

observed anticipated and persistent GFP signal at SPBs in *cdc5*-ad cells. Although we do not have a clear explanation for this phenomenon, possibly it can correlate to the frequent nuclear excursion already documented in *cdc5*-ad cells, also in unperturbed cell cycle (Thrower et al. 2003; Dotiwala et al. 2007).

Altogether, our genetic and microscopic observations in Figure 3 and 4 indicate that both the Cdc5-T238A and Cdc5-ad protein variant can localize to SPBs after one irreparable DSB, even though the kinetics of the process is very different in the two *CDC5* mutants. However, our analysis does not explain if Cdc5-T238A and Cdc5-ad protein variants are defective in checkpoint adaptation and cell cycle re-start after one irreparable DSB as a consequence of defective functions at SPBs.

3.6 Phosphorylation of Cdc5 at Thr238 is crucial for regulating refractory DSB repair and timely checkpoint recovery.

Then, we asked if Cdc5 phosphorylation at T238 has any role to promote checkpoint recovery after repair of one DSB. To this aim, we used two specific genetic systems in which an HO-induced DSB can be slowly repaired either by ectopic gene conversion (EGC), or Single Strand Annealing (SSA). Importantly, in both the repair assays, the persistent DSB is relocalized to the nuclear periphery and directed for recombinational repair (Kalocsay et al. 2009; Nagai et al. 2008; Oza et al. 2009).

To test EGC, we took advantage of tGI354 strain, in which one DSB is induced by HO on chromosome V and is repaired by interchromosomal recombination using homologous MAT a-*inc* sequence on chromosome III (Fig. 5A). Importantly, the gene conversion and crossovers products and can be easily visualized by Southern blot analysis (Ira et al. 2003) . Interestingly, although the viability after 3 days of DSB induction was unaffected in *cdc5*-

T238A cells (Fig. 5B), by careful Southern blot analysis we observed a delay in total repair (as a sum of gene conversion and crossovers events) kinetics, starting from 6hrs after induction of DSB. (Fig. 5C, D). Consistently, with the delay in total repair, we found that *cdc5*-T238A cells retained a prolonged hyper-phosphorylated Rad53, which remained detectable by western blot till 12 hours as compared to wild type (Fig. 5E). Furthermore, by single cell micromanipulation in a plate and microscopic observation, we found that wild type cells restarted cell cycle after 6 hours of DSB induction, leading to almost 80% of micro-colonies at 12hours with at least 3 or more cells. Strikingly, almost 30% of *cdc5*-T238A cells were still blocked in G2/M dumbbell stage at 12hours, suggesting that the phosphorylation of Cdc5-T238 site plays a role to promote efficient DSB repair and checkpoint recovery during EGC.

We also characterized *cdc5*-T238A cells for cell cycle restart analysis in YMV80 genetic background, in which one HO induced DSB on chromosome III is predominantly repaired through SSA, after extensive DSB resection (Vaze et al. 2002). Similarly to our previous finding in the interchromosomal assay (Figure 5) we observed a delay in DSB repair kinetics by SSA and strong delay in cell cycle restart in *cdc5*-T238A cells, whereas the percent viability after 3 days of induction was unaffected as compared to wild type cells (Supplementary Figure S3).

3.7 *cdc5*-T238A reduces the activity of Mus81-Mms4 mediated resolution pathway

After characterizing the *cdc5*-T238A allele in response to DSB, we asked if it has any effect on the cell viability in response to various DNA damaging agents. To this aim, we plated serial dilution of cells in the presence of MMS (Methyl Methanesulfonate, an alkylating agent) and CPT (Camptothecin, a Topoisomerase I inhibitor), which cause DNA lesions

during replication. As shown in Figure 6A, at the high doses, *cdc5*-T238A cells were slightly sensitive to MMS and CPT. Interestingly, *cdc5*-T238D and *cdc5*-ad cells were not sensitive to the two compounds.

Considering that the Cdc5-T238A protein variant retained a lower kinase activity (Fig. 1C, D), we hypothesized that the mild sensitivity to MMS and CPT of the *cdc5*-T238A cells can be potentially explained by a failure to phosphorylate a critical target, which is required to repair replication-coupled DNA lesion. Indeed, it was previously shown that Cdc5 mediate the phosphorylation of Mms4, the regulatory subunit of the structure specific Mus81 nuclease. Thus, upon Cdc5-mediated activation, Mus81-Mms4 complex processes Holliday junctions (HJs), contributing to repair those DNA lesions generated during stressful replication in the presence of MMS or CPT (Matos et al. 2011; Schwartz et al. 2012).

Addressing this in more details, we decided to assess Mms4 phosphorylation in *cdc5*-T238A cells. To this aim, we inserted a 3xHA tag at the C-terminal of Mms4, both in wild type and *cdc5*-T238A strains. Cells were synchronized in G1 by α-Factor and released in fresh media containing 0.02% MMS. Sample were taken at the time points indicated in Figure 6B and analyzed by western blotting. We observed a robust hyper-phosphorylation of Mms4 in wild type cells starting from 120 minutes after the released in MMS. Instead, in *cdc5*-T238A cells the Mms4 hyper-phosphorylation was severely delayed and lowered. This *in vivo* result supports our previous finding, indicating a reduced kinase activity of the Cdc5-T238A protein variant by in vitro assay (Figure 1). We can speculate that the reduced phosphorylation of Mms4, compromising the activity of the Mus81-Mms4 complex, could explain the mild MMS sensitivity of *cdc5*-T238A cells, at least at high dosage of the drug (Figure 6A).

Supporting that, *cdc5*-T238A cells should have a reduced activity of the Mus81-Mms4 to process HJs, we hypothecated that *cdc5*-T238A mutation should increase the MMS sensitivity of *sgs1Δ* cells, which accumulate persistent dHJs (Liberi et al. 2005). Indeed, dHJs in replication stress due to MMS are primarily processed by the activity of dissolution complex (Sgs1-Top3-Rmi1) in S phase; then, persistent dHJ are resolved later on by the activity of Mus81-Mms4 (Szakal & Branzei 2013). Strikingly, we observed a severe hypersensitivity to very mild doses of MMS of *cdc5-T238A sgs1Δ* double mutant cells, even more than the single *sgs1Δ* mutant cells (Fig. 6D). Therefore, the genetic interaction between *sgs1Δ* and *cdc5*-T238A mutants described in Figure 6, further supports that the phosphorylation of the Cdc5 T238 site is an important prerequisite to fully activate Cdc5 in cells responding to DNA damage.

4. Discussion

PLKs are activated through phosphorylation of well-conserved Threonine sites in the T-loop of the kinase domain (Mortensen et al. 2005; Macůrek et al. 2008; Seki et al. 2008). In human Plk1, the T210 in the T-loop is phosphorylated by Aurora kinases in cooperation with Bora which is not only necessary to activate the kinase but also essential for cell cycle restart after DNA damage (Macůrek et al. 2008; Seki et al. 2008).

By sequence alignment (Fig. 1A), the T238 site of Cdc5 in *S. cerevisiae* corresponds to T210 of Plk1. Indeed, T238 residue of Cdc5 has been found phosphorylated in vivo by an unknown kinase (Mortensen et al. 2005). So far, the functional role of the T238 phosphorylation of Cdc5 has been controversial, because the *cdc5*-T238A mutation does not affect cell viability in unperturbed cell cycle [(Mortensen et al. 2005) and Fig. 1B]. Moreover, it was shown that the Cdc28-dependent phosphorylation of T242

in the T-loop of Cdc5 is responsible of the full activation of the kinase domain and becomes essential for cell viability (Mortensen et al. 2005).

According to previous findings, we show that T238A mutation does not affect cell viability in unperturbed condition, and it also rescues the thermo-sensitivity of *cdc5*-1 cells very well. However, we found that the Cdc5-T238A protein variant retains a significantly reduced kinase activity by in vitro assay (Fig. 1B-D). In addition, the phosphorylation of Mms4, a well-known target of Cdc5 (Matos et al. 2011), is severely compromised in *cdc5*-T238A cells treated with MMS (Figure 6), further supporting that the phosphorylation of T238 residue contributes to Cdc5 activation. As a consequence of reduced activity of the Mus81-Mms4 complex in processing HJs, *cdc5*-T238A cells are mild sensitive to high doses of MMS and CPT, and become extremely sensitive to MMS after combining with *SGS1* deletion, which abrogates the HJ dissolution pathway (Fig 6).

Interestingly, we also found that *cdc5*-T238A cells show 50% reduction in spontaneous mitotic recombination rate and threefold increase in chromosome loss rate in unperturbed conditions (Fig 2), which possibly can be explained by a lowered Cdc5 activity in G2/M phase in unperturbed cells, affecting resolution of recombination intermediates and chromosome segregation.

Furthermore, *cdc5*-T238A cells are defective in checkpoint adaptation after inducing one irreparable DSB, and remain blocked in G2/M phase with prolonged Rad53 phosphorylation and short spindle (Fig. 3C). This persistent checkpoint, even though inactivated at later time points in *cdc5*-T238A cells, is detrimental for the cells, which in fact do not restart cell division even after 24hours (Fig. 3A).

Recent studies indicated that Cdc5 is nuclearized in presence of DNA damage and it is speculated that it should relocalize to cytoplasm, specifically

to SPBs, to inactivate inhibitors of mitosis and cell cycle regulators i.e. Bfa1-Bub2 complex, Mad2 (component of Spindle Assembly checkpoint) and Cdh1 (inhibitor of spindle elongation) (Crasta et al. 2008; Zhang et al. 2009; Valerio-Santiago et al. 2013). Strikingly, we found that the permanent cell cycle block observed in *cdc5*-T238A cells after one irreparable DSB is rescued either by deletion of *RAD9*, *MAD2* or *CDH1* (Fig. 3E). This indicates that *cdc5*-T238A cells may have a defect in inactivating one or more factors involved in enforcing the cell cycle arrest.

Of importance, *cdc5*-T238A cells slightly affect the kinetics of DSB repair and cell cycle restart after the formation of one persistent DSB that can be slowly repaired by EGC or SSA (Fig. 5 and S3). In these assays, Rad53 de-phosphorylation is significantly delayed in *cdc5*-T238A cells, enlightening an unexplored role of Cdc5 in checkpoint recovery. Notably, our results differentiate the *cdc5*-T238A allele from previously reported adaptation-defective missense mutant *cdc5*-L251W (*cdc5*-ad), which was found to have no effect on checkpoint recovery (Vaze et al. 2002). Moreover, the Cdc5-T238A variant retains about 40% kinase activity by in vitro assay, whereas the Cdc5-ad variant has almost comparable kinase activity to wild type protein [(Charles et al. 1998) and (Fig. 1C,D)]. We also found a relevant difference between the two Cdc5 variants in localizing to SPBs. In fact, Cdc5 localization to SPBs after the formation of one irreparable DSB is delayed in *cdc5*-T238A cells, while it is miss-regulated and anticipated in *cdc5*-ad cells (Fig. 4). In the future, it will be important to test whether a defective regulation of critical factors, such as Bfa1 and others, at SPBs may explain some of the phenotypes described for *cdc5*-T238A and *cdc5*-ad cells.

In summary, we show that the phosphorylation of T238 residue in the T-loop domain of Cdc5 contributes to fully activate Cdc5, controlling multiple events for cell cycle re-restart after DNA damage response. At the molecular

level, similarly to what has been shown for the regulation of Plk1 (Jang et al. 2002; Xu et al. 2013; Archambault et al. 2015a), we can speculate that the phosphorylation T238 site may contribute to reduce the interaction between the kinase domain and the PBD, leading to the activation of Cdc5. This mechanism can be particularly important to activate Cdc5 when the phosphorylation of the T242 site in the T-loop is compromised, such as when the Cdc28 activity is kept low in the presence of DNA damage. Moreover, considering the multiple defects in the DNA damage response in the *cdc5*-T238A mutant, we hypothesize that this mutation may have a defect in either interacting or inactivating one or more of Cdc5 targets. Indeed, it would be interesting to analyse in more details the substrate specifically required for checkpoint adaptation and recovery in future studies.

Considering the intense research efforts to target PLKs activities in cancer therapy, we believe that our study of the regulation of Cdc5 in yeast may be of potential interest, stimulating novel strategies to target PLKs in near future.

Acknowledgements:

We would like to thank J. E. Haber, L. S. Symington and Gregory Ira for generously providing yeast strains. We also thank all the members of our lab for helpful discussions and critical comments.

Funding:

This work is supported by AIRC grant 15488 to AP, MF is supported by Fondazione Veronesi.

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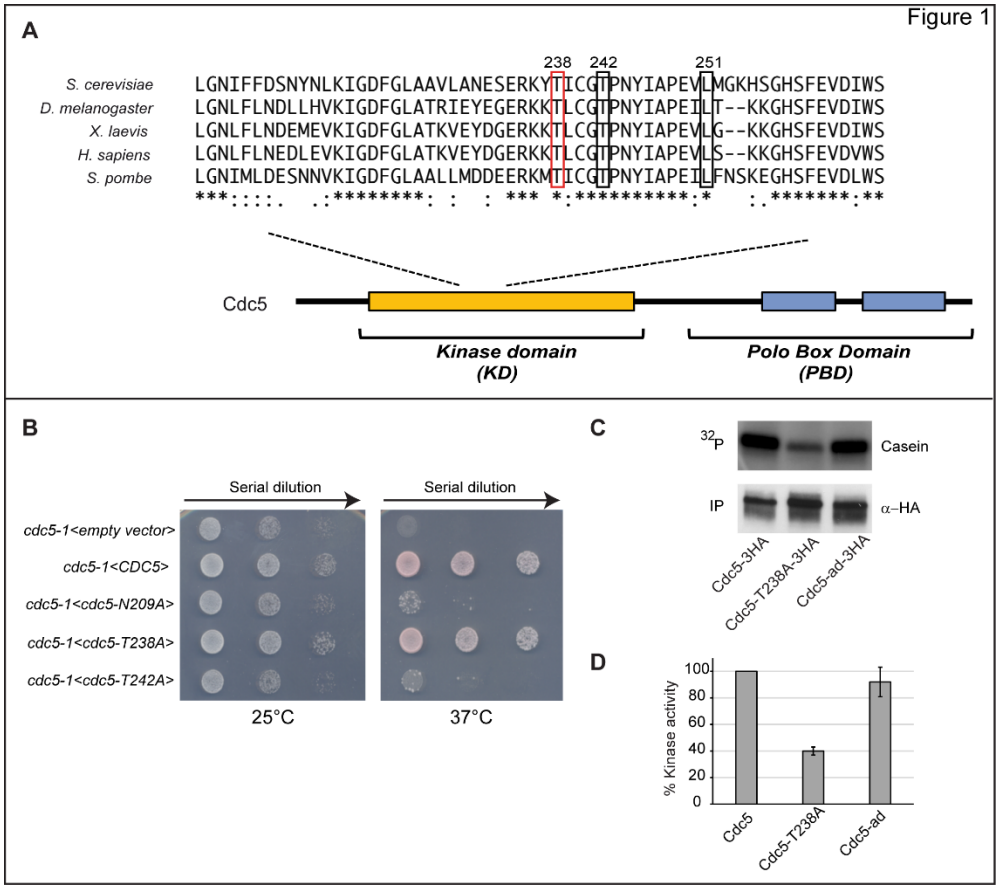


Figure 1: Mutagenesis of T238 of *CDC5* and effect on kinase activity

A) Multiple sequence analysis of activation/T-loop of polo-kinases in higher eukaryotes with conserved T238 and T242 in *S. cerevisiae*.

B) Complementation of ts allele *cdc5*-1 with plasmid carrying wild type and indicated mutants of *CDC5*.

C) & D) In vitro kinase assay and percent kinase activity of indicated mutants.

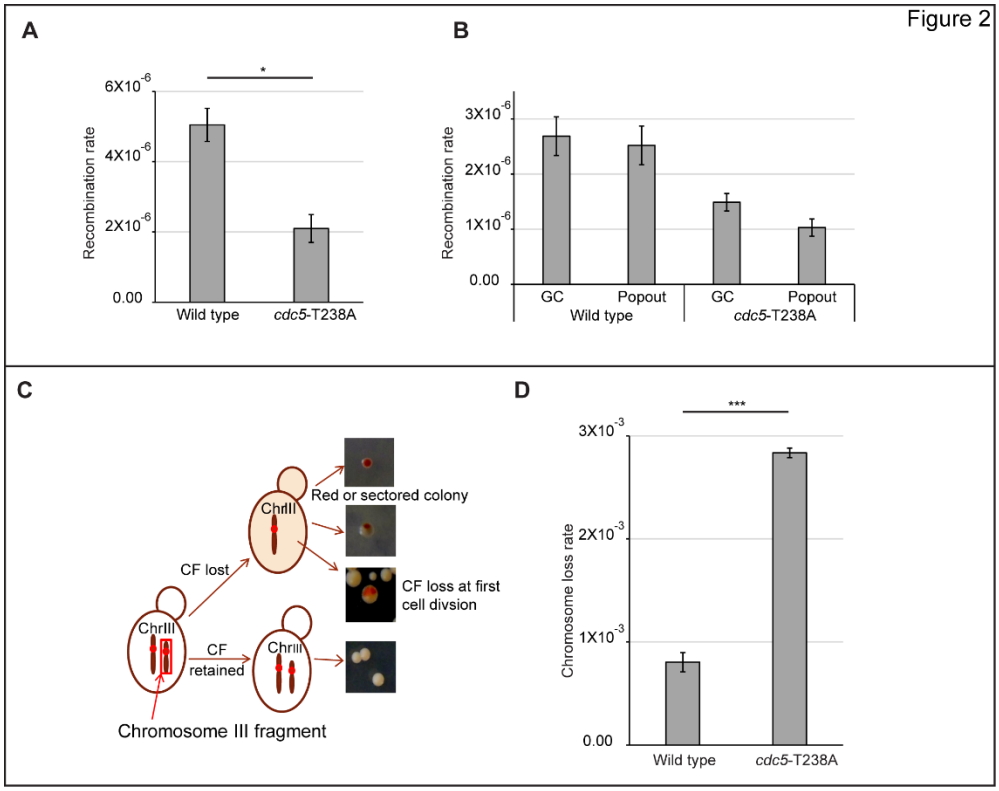


Figure 2: *cdc5*-T238A cells have altered genome stability

A) Spontaneous recombination rate measured by direct repeat assay (*ade2-NdeI::URA3::ade2-AatII* system) as described in (Huang & Symington 1994).

B) Recombination rate comprising pathway of gene conversion and popout in wild type and *cdc5*-T238A cells.

C) Schematic representation of chromosome loss assay.

D) Chromosome loss rate in wild type and *cdc5*-T238A cells (P value was calculated by two tailed student's t-test).

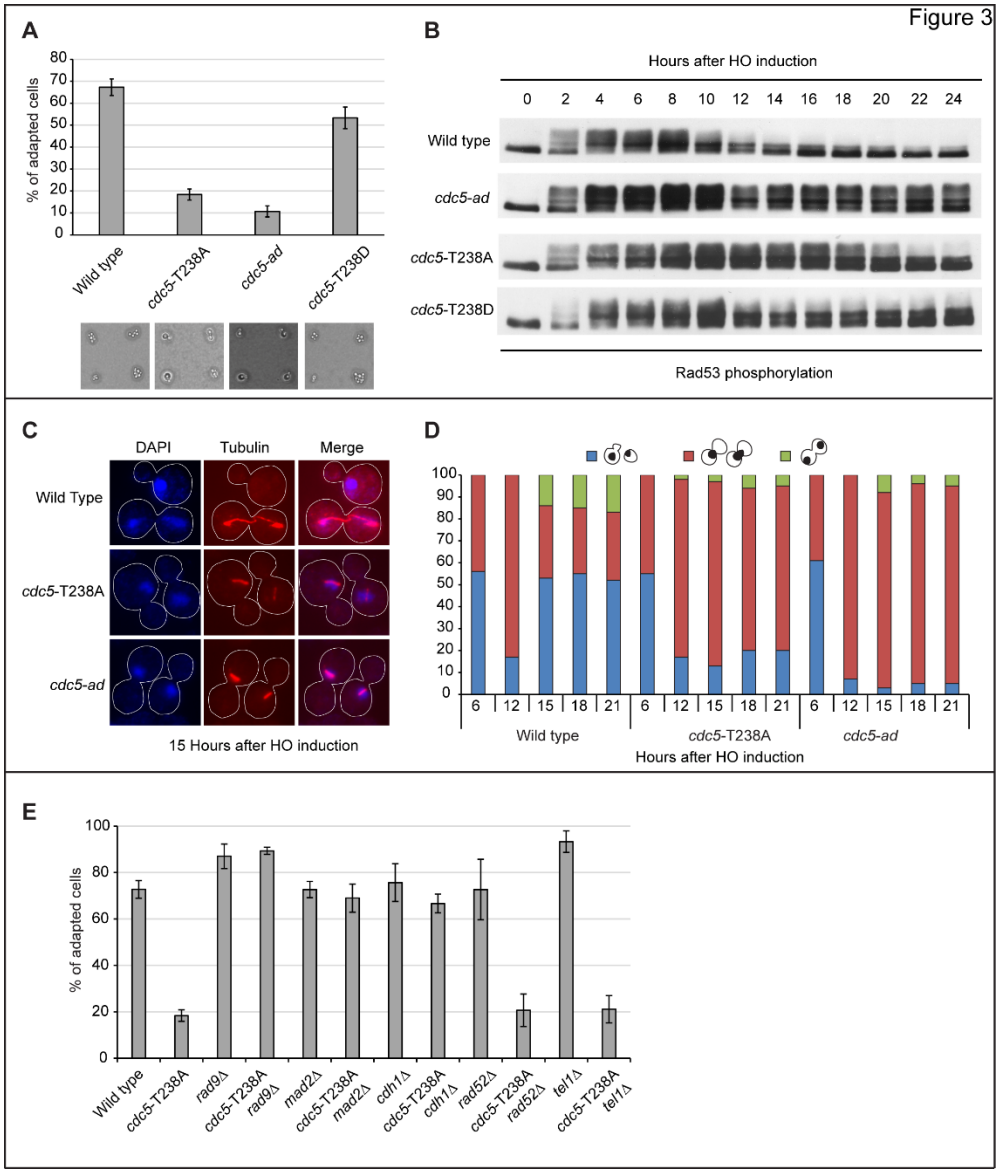


Figure 3: *cdc5*-T238A cells do no adapt to irreparable DSB

A) Percentage of cells undergoing checkpoint adaptation in JKM139 derived strains after induction of unrepairable DSB after 24hrs.

B) Analysis of Rad53 phosphorylation during checkpoint adaptation in indicated mutants.

C) & D) Analysis of nuclear division and spindle elongation during checkpoint adaptation and graphical analysis representing count of 100 cells at each time point for each strain.

E) Percentage of cells undergoing checkpoint adaptation in JKM139 derived strains after 24hours.

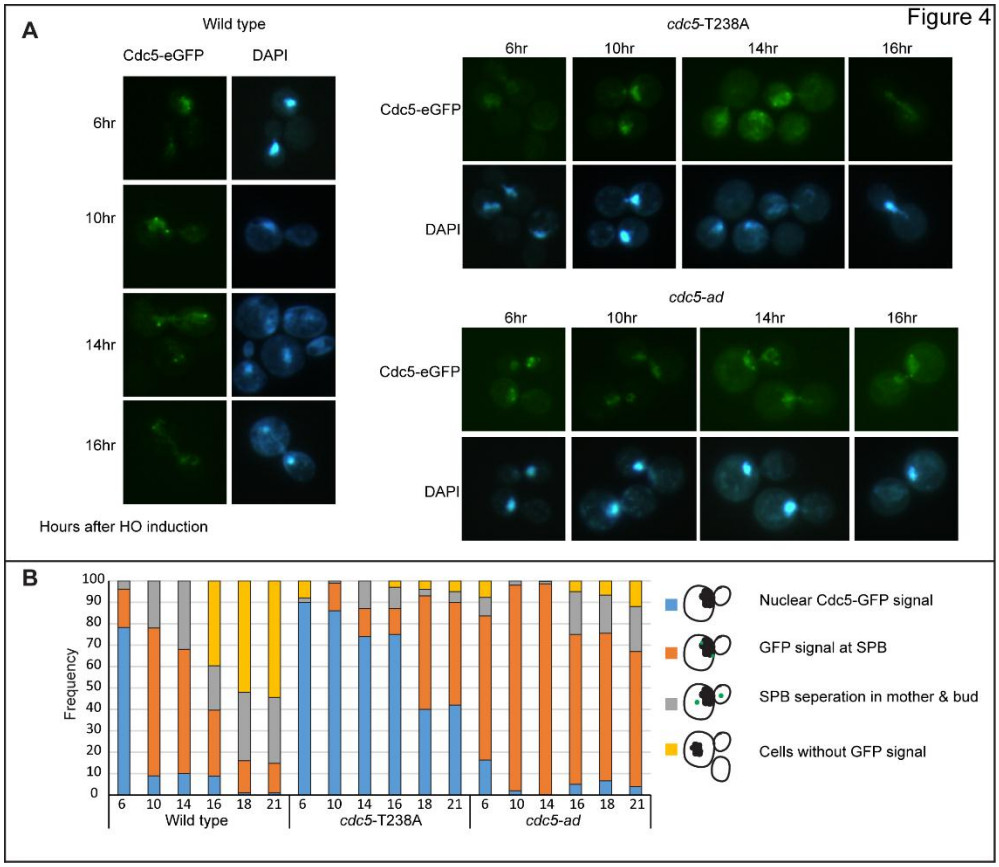


Figure 4: Analysis of Cdc5 localization in response to single irreparable DSB

A) & B) Analysis of Cdc5-eGFP strains in JKM139 background at indicated time points with graphical analysis representing count of 100 cells at each time point for each strain.

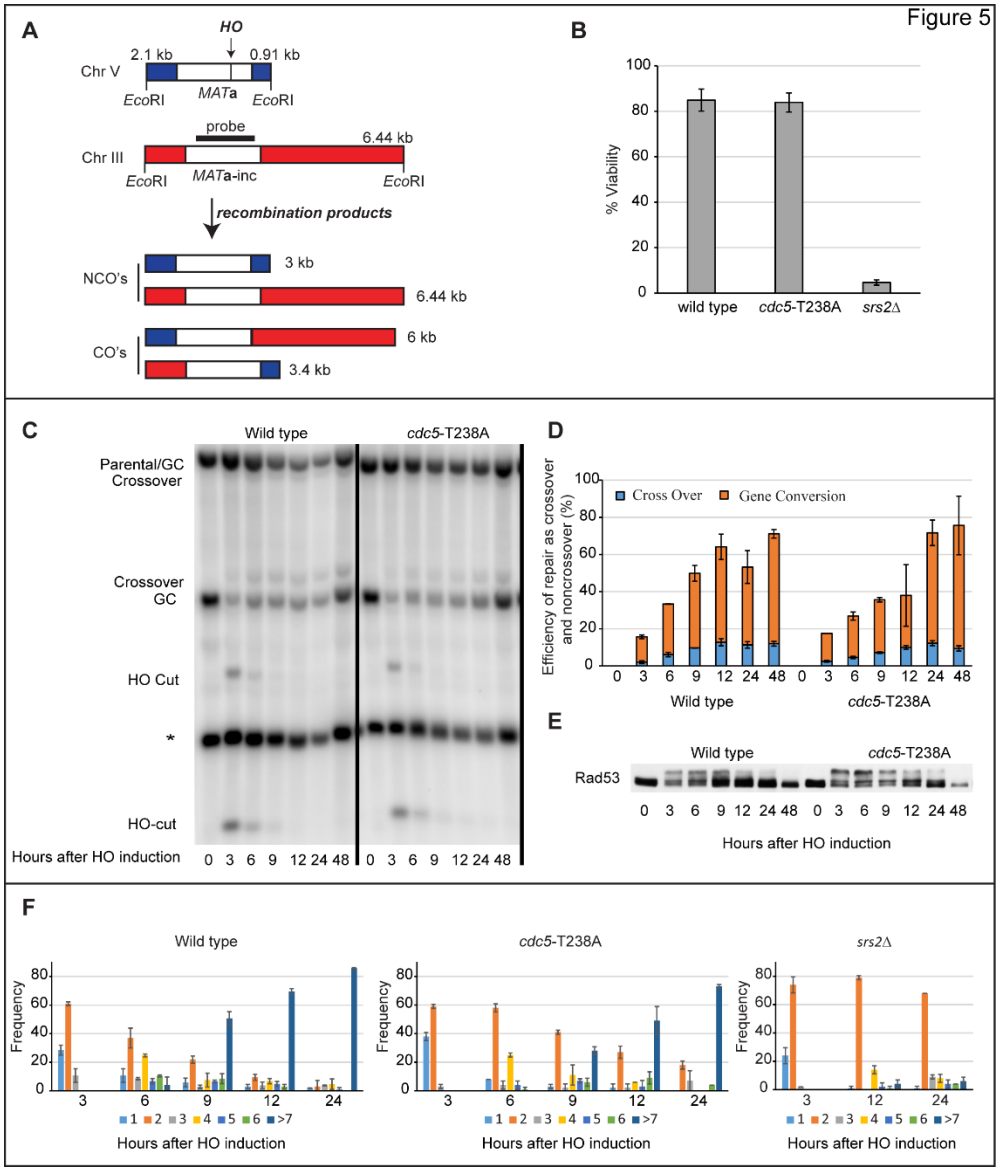


Figure 5
A
HO
2.1 kb
0.91 kb
Chr V
MATa
EcoRI
EcoRI
probe
6.44 kb
Chr III
EcoRI
MATa-inc
EcoRI
recombination products
NCO's
3 kb
6.44 kb
CO's
6 kb
3.4 kb
B
% Viability
100
80
60
40
20
0
wild type
cdc5-T238A
srs2Δ
C
Wild type
cdc5-T238A
Parental/GC Crossover
Crossover GC
HO Cut
*
HO-cut
Hours after HO induction
0 3 6 9 12 24 48 0 3 6 9 12 24 48
D
Cross Over
Gene Conversion
Efficiency of repair as crossover and noncrossover (%)
100
80
60
40
20
0
0 3 6 9 12 24 48 0 3 6 9 12 24 48
Wild type
cdc5-T238A
E
Wild type
cdc5-T238A
Rad53
0 3 6 9 12 24 48 0 3 6 9 12 24 48
Hours after HO induction
F
Wild type
cdc5-T238A
srs2Δ
Frequency
80
60
40
20
0
3 6 9 12 24
1 2 3 4 5 6 >7
Hours after HO induction

Figure 5: Phosphorylation of Cdc5 at Thr238 is crucial for regulating refractory DSB repair and timely checkpoint recovery.

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) Percentage of viability in tGI354 derived strain after induction of DSB. We also tested *srs2Δ* as positive control (Vaze et al. 2002).

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.

D) Percentage of crossovers and non-crossovers among all cells in the interchromosomal recombination assay described in (C).

E) Western blot analysis shows Rad53 phosphorylation of the same experiment.

F) Analysis of cell cycle progression by micromanipulation on YP + Raff + Gal plates at indicated time points by counting number of cell/cells in micro-colony in indicated mutants. We also tested *srs2Δ* as positive control (Vaze et al. 2002).

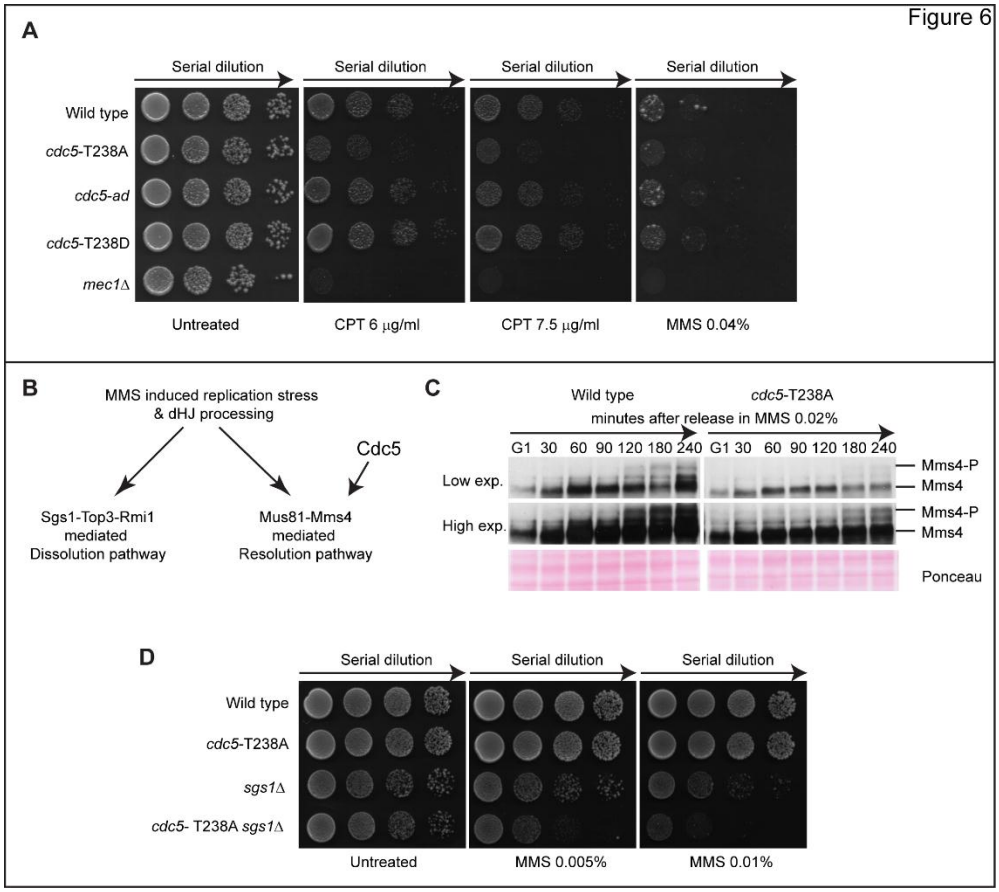


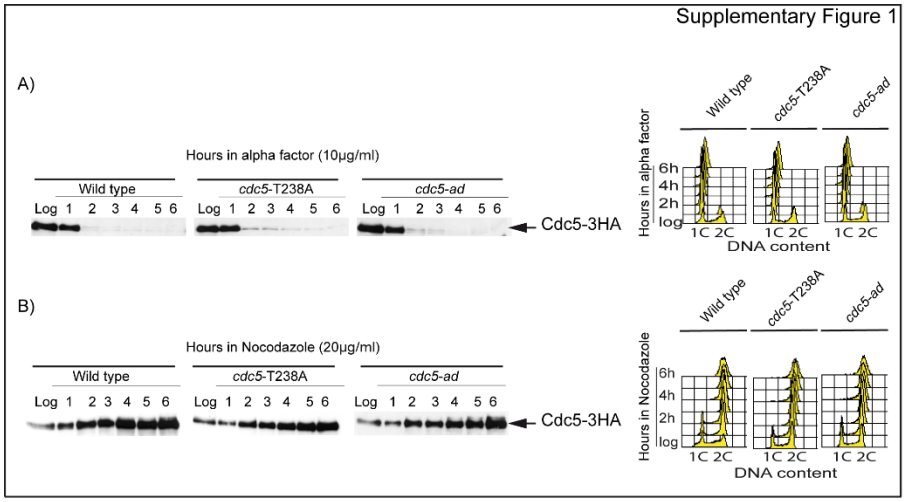
Figure 6: *cdc5*-T238A reduces the activity of resolution pathway

A) Drug sensitivity by spot test of serially diluted cultures (1:10) on YPD, YPD with either MMS or CPT.

B) Schematic representation of Sgs1-Top3-Rmi1 mediated dissolution pathway and Mus81-Mms4 mediated resolution pathway.

C) Mms4 phosphorylation analyzed by western blot.

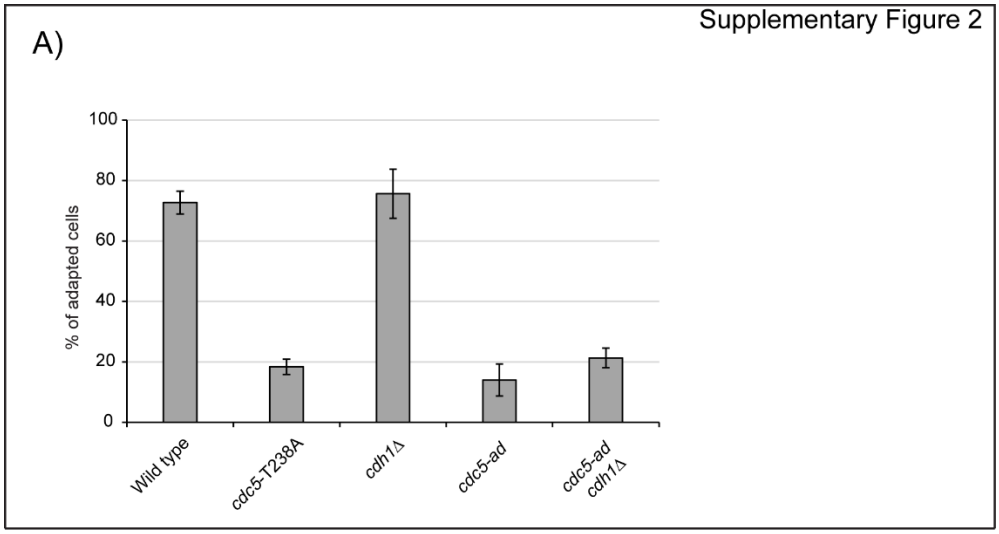
D) Genetic interaction between *SGS1* and *cdc5*-T238A cells in response to MMS.



Supplementary figure S1

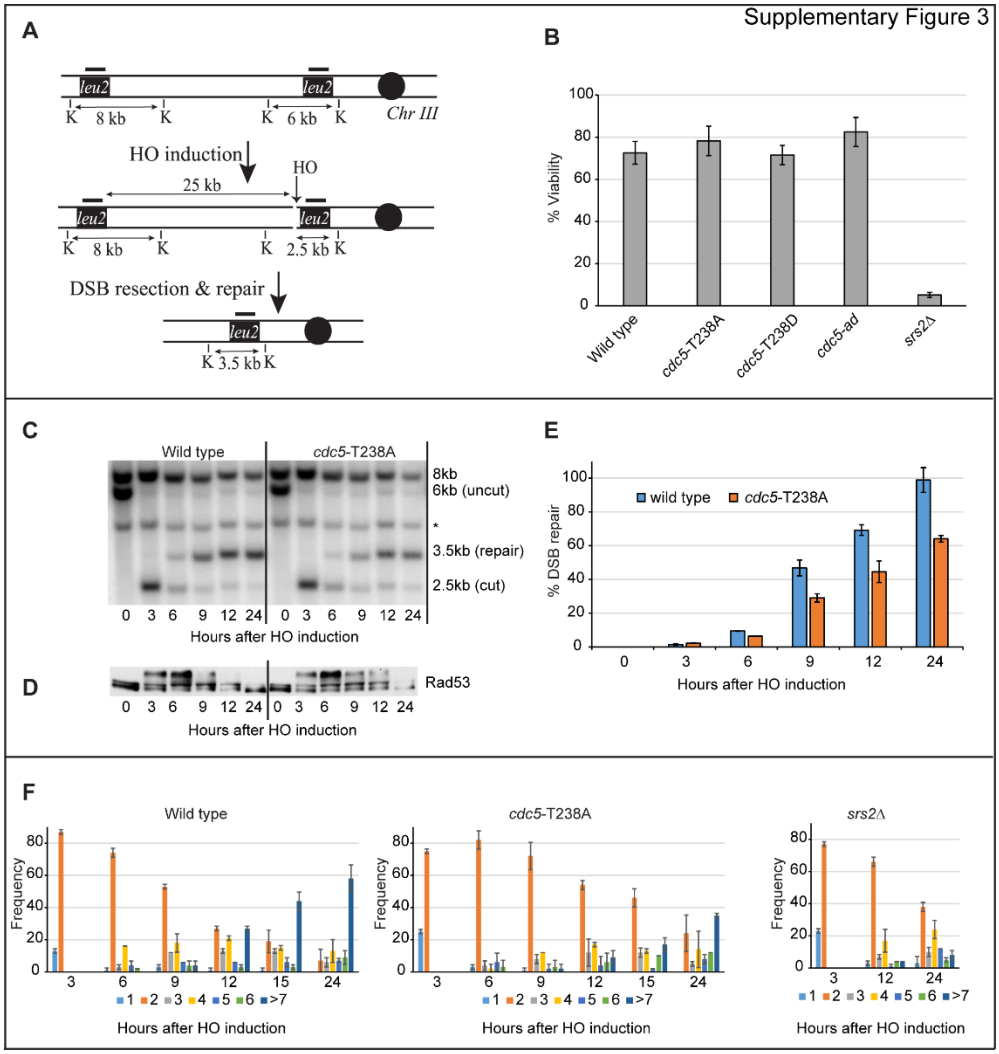
A) Analysis of Cdc5-3HA protein in G1 arrested cells by western blot in indicated mutants.

B) Analysis of Cdc5-3HA protein in G2 arrested cells by western blot in indicated mutants.



Supplementary figure S2

Percentage of cells undergoing checkpoint adaptation in JKM139 derived strains after 24hours.


Supplementary Figure 3
A
leu2
K 8 kb K
K 6 kb K
Chr III
HO induction
HO
25 kb
K 2.5 kb K
DSB resection & repair
K 3.5 kb K
B
% Viability
Wild type
cdc5-T238A
cdc5-T238D
cdc5-ad
srs2Δ
C
Wild type
cdc5-T238A
8kb
6kb (uncut)
*
3.5kb (repair)
2.5kb (cut)
0 3 6 9 12 24
Hours after HO induction
D
Rad53
E
% DSB repair
wild type
cdc5-T238A
F
Frequency
1 2 3 4 5 6 >7

Supplementary figure S3

A) Schematic representation of YMV80 Chr III region, containing the HO-cut site. The indicated vertical bars show *Kpn*I 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) Percent viability of YMV80 derived strains after induction of DSB. We also tested *srs2Δ* as positive control (Vaze et al. 2002).

C) Southern blotting analysis of the single strand annealing using the probe as described in (A), in indicated YMV80 derivatives after inducing HO in nocodazole-arrested cells. The intensity of each band was normalized respect to unprocessed *ATG5* locus (*).

D) Western blot analysis of Rad53 phosphorylation of the same experiment.

E) Densitometric analysis of product band signals normalized with respect to unprocessed *ATG5* locus.

F) Analysis of cell cycle progression by micromanipulation on YP + Raff + Gal plates at indicated time points by counting number of cell/cells in micro-colony in indicated mutants. We also tested *srs2Δ* as positive control (Vaze et al. 2002).

Table S1. List of yeast strains described in this work.

Strain name	Parental strain / background	Genotype	Source
Y1264	W303	*MATa ade2- 1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 Rad5+*	Lab stock
Y1126	JKM179	*MATalpha ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3, 112 lys5, trp1::hisG ura3-52 lys5::ade3::GAL10::HO* Generous gift from J. Haber	Lee et al., 1998
Y1600 / Y117	JKM139	*MATa ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3, 112 lys5, trp1::hisG ura3-52 lys5::ade3::GAL10::HO* Generous gift from J. Haber	Lee et al., 1998
Y603	tGI354	*ho hml*::*ADE1 MAT***a**-*inc hmr*::*ADE1 ade1 leu2-3,112 lys5 trp1*::*hisG ura3-52 ade3*::*GAL*::*HO* (*arg5,6*::*MAT***a**::*HPH)* Generous gift from G. Ira	Ira et al., 2003
Y1601	YMV80	*matΔ::hisG1, hmlΔ::ADE, hmrΔ::ADE1, lys5, ura3-52, leu2::HOcs, ade3::GAL::HO, his-URA3-5'Δleu2-is4* Generous gift from J. Haber	Vaze et al., 2002
Y8	YMV80	*matΔ::hisG1, hmlΔ::ADE, hmrΔ::ADE1, lys5, ura3-52, leu2::HOcs, ade3::GAL::HO, his-URA3-5'Δleu2-is4, cdc5 L251W*	Vaze et al., 2002
Y40D 4	YKH12α	*MATα,* ***ade2-n::URA3::ade2-a*** *leu2-3,112 his3-11,15 canl -100 ura3-1 trpl-l* Generous gift from L.S. Symington	Huang & Syminton , 1994
Y1666	Y1601	*cdc5*-T238A	This study
Y1509	Y1601	*cdc5*-T238D	This study
Y2790	Y603	*cdc5*-T238A	This study
Y152	Y117	*CDC5-3HA::KANMX6*	This study
Y1465	Y117	*cdc5*-L251W-*3HA::KANMX6*	This study
Y1466	Y117	*cdc5*-T238A-*3HA::KANMX6*	This study
Y1573	Y117	*cdc5*-T238D-*3HA::KANMX6*	This study
Y1398	Y117	*cdc5*-L251W	This study
Y1777	Y117	*cdc5*-T238A	This study

Y1908	Y1126	*cdc5*-T238A-*3HA::KANMX6*	This study
Y1743	Y1264	*cdc5*-T238A-*3HA::KANMX6*	This study
Y2554	Y1743 X Y40D4	*Mat a, cdc5*-T238A-*3HA::KANMX6,* ***ade2-n::URA3::ade2-a***	This study
Y1973	Y1264	*BIR induced chromosome – D8B CFV, Left arm Chr III*	This study
Y1979	Y1743	*cdc5*-T238A-*3HA::KANMX6*, *BIR induced chromosome – D8B CFV, Left arm Chr III*	This study
Y2147	Y1600	*MMS4-3HA::TRP1*	This study
Y2206	Y2147 X Y1908	*cdc5*-T238A-*3HA::KANMX6 MMS4-3HA::TRP1*	This study
Y286	Y117	*mad2Δ::KANMX6*	This study
Y1574	Y1466 X Y285	*cdc5*-T238A-*3HA::KANMX6 mad2Δ::KANMX6*	This study
Y601	Y117	*rad9Δ::KANMX6*	This study
Y1535	Y601 X Y1466	*cdc5*-T238A-*3HA::KANMX6 rad9Δ::KANMX6*	This study
Y792	Y117	*cdh1Δ::KANMX6*	This study
Y1579	Y792 X Y1466	*cdc5*-T238A-*3HA::KANMX6 cdh1Δ::KANMX6*	This study
Y2228	Y1600	*CDC5-eGFP::KANMX6*	This study
Y2230	Y1777	*CDC5*-T238A-*eGFP::KANMX6*	This study
Y2232	Y1398	*CDC5*-L251W-*eGFP::KANMX6*	This study
Y505	W303	*cdc5*-1 Kind gift from Marco Muzi-Falconi	Lab stock.
Y1327	Y505	*cdc5*-1 <*Ycplac22-TRP-CEN*>	This study
Y1329	Y505	*cdc5*-1 <*CDC5-HA-TRP-CEN*>	This study
Y1331	Y505	*cdc5*-1 <*cdc5-N209A-HA-TRP-CEN*>	This study
Y1333	Y505	*cdc5*-1 <*cdc5-T238A-HA-TRP-CEN*>	This study
Y1461	Y505	*cdc5*-1 <*cdc5-T242A-HA-TRP-CEN*>	This study