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Characterization of factors involved in DNA damage checkpoint recovery and adaptation in yeast

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Part I

Abstract

Genome maintenance and stability are essential goals for all the organisms in order to transfer the correct genetic information to the progeny and to keep fully functional the cellular metabolism. In eukaryotic cells, the presence of DNA lesions causes the activation of an evolutionary conserved mechanism called the DNA damage checkpoint that arrests the cell cycle and stimulates the repair pathways. Double strand breaks (DSBs) are deleterious lesions that can be a serious threat for the cell. In fact, the formation of only one DSB is enough to activate a robust checkpoint response. This DNA lesion is processed by several factors leading to the checkpoint factors recruitment and to the homologous recombination repair. After lesion repair the checkpoint is switched off through a process called recovery; however it has been demonstrated that damaged cells are able to inactivate the checkpoint and restart the cell cycle also in the presence of a persistent DNA lesion, through a checkpoint adaptation process. The reason why this process occurs is not understood, but it has been related to the unrestrained proliferation of cancer cell. In my laboratory we are interested in shedding light on the molecular mechanism of these checkpoint inactivation processes and in the characterization of the involved factors.

During the PhD I focused on the characterization of the functions and regulation of some factors already known to play a role in DSB ends processing and checkpoint switch off: the polo kinase Cdc5, the DNA translocase Tid1/Rdh54 and the nuclease-associated protein Sae2.

First of all we found that high levels of Cdc5 lead to checkpoint switch off and cell cycle re-enter. Relying on this data we decide to perform a biochemical screening in order to identify the Cdc5 targets in presence of DNA damage. This biochemical screening was based on a GST pulldown approach, coupled with tandem mass

spectrometry protein identification. As expected, we identify many interactors and among them we found the repair protein Sae2. Interestingly, we found that in presence of elevated levels of Cdc5, Sae2 is hyperphospholylated and binds strongly to the DSB ends. In order to understand the functional role of the Cdc5-Sae2 interaction, I mutagenized different putative Cdc5 binding sites in Sae2. It turned out that Cdc5 binds a C-terminal region of Sae2, which is conserved in other eukaryotes orthologs.

The obtained Sae2 mutants give us interesting results that can be useful for the proper comprehension of the Sae2 function in DNA damage response. Indeed in this thesis I will present preliminary results on the characterization of the Sae2 role in the recovery process.

I was also involved in a project with the aim to study the regulation of Tid1/Rdh54 in the presence of DSB. Tid1 belongs to the Swi2/Snf2 family of chromatin remodellers, is an ATP-dependent DNA translocase able to induce DNA structure remodelling, Rad51 removal from double strand DNA and promote D-loop formation during homologous recombination. Moreover this protein has also a puzzling function in checkpoint inactivation during adaptation since TID1 deletion causes a permanent G2/M block in the presence of one irreparable DSB. I found that Mec1 and Rad53 checkpoint kinases, through a process that requires also the recombination factor Rad51, phosphorylate Tid1 in the presence of DSBs. I also found that Tid1 is recruited on to the DSB site, and that its ATPase activity is dispensable both for the loading and the phosphorylation of the protein. We believe that Tid1 phosphorylation is important to stabilize the binding of the protein on the lesion and to regulate its functional role during checkpoint adaptation.

State of the Art

The DNA is a stable molecule but it may suffer different kind of damages caused by endogenous or exogenous agents. First of all the aqueous cell environment is essential to give the correct structure to DNA but, paradoxically, causes spontaneous hydrolysis of the molecule. These hydrolytic damages are deamination, that causes misappropriate base coupling, or depurination, that create an abasic site (Lindahl, 1993). The DNA replication, chromosomes segregation and cellular metabolism are other sources of endogenous genome instability. During cell lifespan DNA must be duplicated and divided between the two daughter cells. DNA replication can causes wrong base insertions, short DNA deletions or insertions (McCulloch & Kunkel, 2008) while replicative fork progression in zones containing particular DNA structures, transcribed genes or DNA binding proteins may lead to fork collapse and generation of recombinogenic intermediates, prerequisite of chromosomal rearrangements (Branzei & Foiani, 2010). After genome duplication cell divides, but errors in the chromosomes alignment on the metaphase plate lead to chromosomes missegregation and aneuploidy (Tanaka & Hirota, 2009). In addition also the cellular energetic metabolism synthetizes many dangerous side products like reactive oxygen or nitrogen species. These compounds attack the DNA molecule producing a variety of different DNA lesions like base and sugar modification, DNA intra-strand crosslinks and single or double strand breaks (SSBs or DSBs, respectively) (Dizdaroglu, 2012). Finally also exogenous physical or chemicals agents cause DNA damage. Examples of physical agent are ionizing radiations (IR) and ultraviolet light (UV), IR is mainly involved in the formation of SSBs and DSBs but creates also oxidative stress, while UV light induces 6-4 photoproducts or thymidine dimers. Many chemical agents cause a

plethora of DNA lesions: alkylating agents like methyl methanesulfonate (MMS) attack alkyl groups to DNA bases; crosslinking agents such as cisplatin, mitomycin C and psoralen link DNA strands together; topoisomerases inhibitors (camptothecin and etoposide) induce SSB or DSB formation; other cancer causing chemicals are those produced by smoking or contained in contaminated foods, an example of these dangerous compounds are aflatoxins (Ciccia & Elledge, 2010; Wogan et al., 2004).

All this DNA damages can lead to genome instability that is a serious threat for all the organisms and a hallmark of cancer cells, thereafter to preserve genome integrity, organisms evolved different molecular mechanisms that are able to recognize the lesions and activate the correct cellular response.

The DNA Damage Response (DDR)

During evolution, organisms develop an intricate system to cope with DNA lesions; this mechanism has been called DNA Damage Response (DDR). The DDR functions are essentially two: slow down the cell cycle progression and activate the proper repair pathway. However, in the case of large amount of irreparable DNA lesions, DDR induces apoptosis or senescence, leading to cell death. The fundamental cellular function of the DDR is highlighted by the fact that depletion and mutation of its components strongly increase cell sensitivity to DNA damage; moreover it is frequently deregulated in tumors and in several genetic diseases characterized by genetic instability (Ciccia & Elledge, 2010).

The DNA repair systems

According to the type of DNA lesion and the enzymes involved, the repair pathway is sub-divided in 5 main categories: direct damage reversal, base excision repair, nucleotide excision repair, mismatch repair and the double strand break repair. Below I will present the DNA double strand break repair system because it is the objective of my thesis.

The Double Strand Break Repair pathways

DNA double strand breaks (DSBs) are considered the most dangerous DNA lesion that can occurs. In fact, if not immediately repaired, DSBs may cause chromosomes rearrangements like translocations, chromosome arm loss and amplification, all typical features of cancer cells. DSBs can be created not only by exogenous source like ionizing radiations or chemical compounds, but also by fork collapse during replication, meiotic recombination and V(D)J rearrangements in the immunoglobulins genes. The cell has two different repair systems to cope with DSBs: Non Homologous End Joining (NHEJ) and Homologous Recombination (HR). These systems share different components and the choice between them is strongly regulated by the cell cycle phase and DNA structures. In fact NHEJ occurs mainly in the G_1 phase of the cell cycle, while HR occurs in G_2/M phases, when the donor sequence is available on the homologous chromosome.

The nucleolytic degradation of the 5' DSB end is at the basis of the molecular switch between NHEJ and HR. This process is called DSB ends resection and leaves a 3' DNA tail that mediates homologous sequence searching and annealing (Symington & Gautier, 2011).

Non Homologous Ends Joining (NHEJ)

NHEJ is a repair mechanism that acts rejoining the two DSB ends. The core proteins of the system are: the Ku heterodimer (Ku70 and Ku80), DNA ligase 4/Dnl4, XRCC4/Lif1, Nej1 (protein present only in yeast) and the MRN/X complex (formed by the Mre11, Rad50 and Nbs1/Xrs2 proteins) (Daley et al., 2005). After the formation of a DSB, the Ku complex and the MRN/X complex bind the DSB ends through an independent mechanism (Wu et al., 2008). The Ku complex acts as a barrier against exonucleases activity and protects DSB ends, whereas the MRN/X complex keeps the DSB ends in close proximity and, through the Mre11 nuclease activity, can remove some bases from the DSB ends when it is necessary in order to promote a correct end joining. This alternative NHEJ pathway is called microhomology-mediated end joining (MMEJ) and requires the nuclease activity of Mre11 and the CtIP/Sae2 protein. The nucleolytic process is not required in the presence of compatible ends formed by a 5'-Phosphate or a 3'-OH (McVey & Lee, 2008). Then, Ku complex and MRX recruit Dnl4 and Lif1, respectively. This two proteins form the DNA ligase 4 heterodimer (Palmbos et al., 2008). In yeast, Nej1 binds independently to the Ku complex and keeps the Ligase 4-Ku complex stable (Chen & Tomkinson, 2011). At this point DSB ligation occurs. It is important to observe that NHEJ is not an error free mechanism, in fact, errors in the annealing reaction can lead to deletion of few nucleotides and, moreover, NHEJ is responsible of chromosomes rearrangements like DSB-telomere fusions or chromosomes translocations (Myung et al., 2001).

Homologous Recombination (HR)

Homologous recombination is a process that allow DSB repair using another DNA homologous sequence as a template. The template is also called *donor sequence*. For the correct functionality of the HR machinery many factors are required and many of them are part of the Rad52 epistasis group. Different models and

mechanisms of HR repair have been proposed (see Figure 1 for a scheme): Single strand annealing (SSA), Synthesis dependent strand annealing (SDSA), double Holliday junction sub-pathway (dHJ) and break induced replication (BIR) (Heyer et al., 2010).
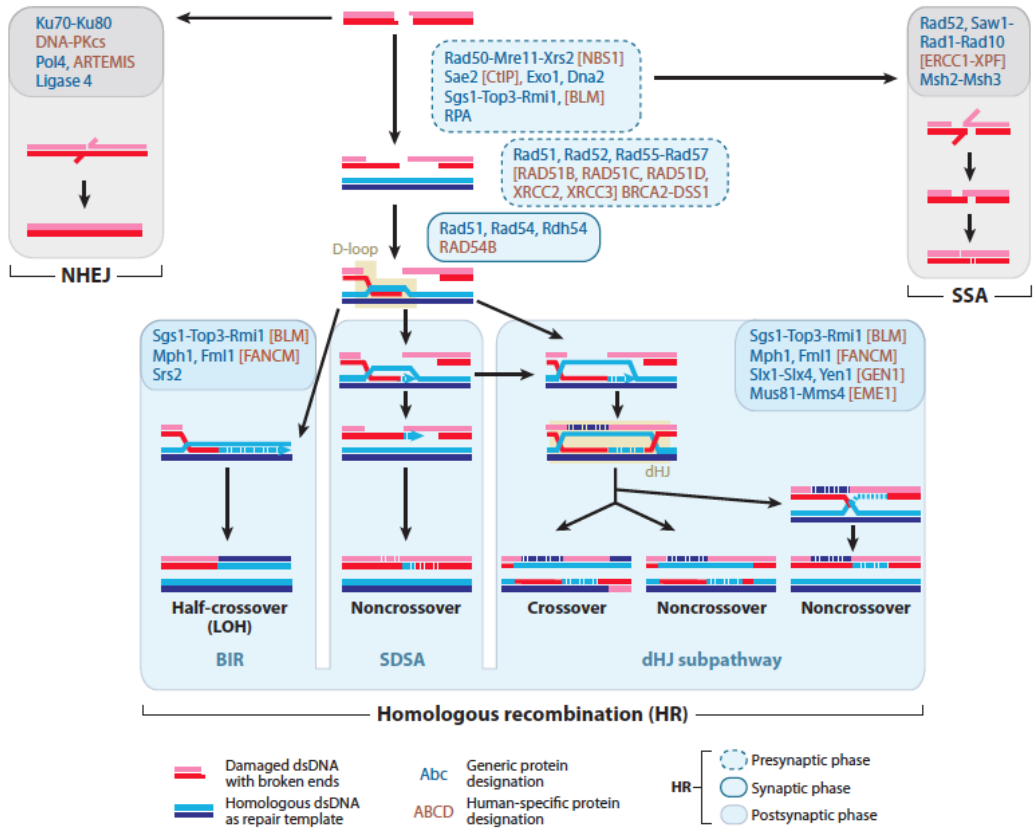
Figure 1: DSB repair mechanisms (from Heyer et al., 2010)

Whatever HR sub-pathways the cell use to repair, the starting event is the DSB ends resection. In order to avoid the unscheduled degradation of the single stranded DNA filament, the RPA heterotrimeric complex covers the 3' end tail. At this point, if a homolog sequence is found nearby the lesion, the repair can occurs by SSA. If this is not the case, RPA is substituted by Rad51 protein, leading to the formation

of a nucleoprotein recombinogenic filament required for the strand invasion process, homologous sequence recognition, pairing, and the HR process can proceed through the other pathways (Heyer et al., 2010).

Single Strand Annealing (SSA)

This system is preferentially used to repair a DSB arose between two direct repeats, which is a very common situation in higher eukaryotes, such as mammals, whose genomes contain a large amount of frequently repeated sequences. The presence of a donor sequence nearby the lesion lead to a direct reannealing reaction dependent only on the Rad52 protein (Ivanov et al., 1996), then specific flap endonuclease complex, formed by Slx4 and Rad1-Rad10, removes non-homologous flanking regions (Flott et al., 2007), and the gaps are filled by DNA repair synthesis (see Figure 1). This process causes the loss of the region between the direct repeats, leading to a genomic deletion as big as the distance between the two homologs sequences. SSA is the only HR repair pathway that doesn't require the Rad51 protein because strand invasion doesn't take place.

Synthesis dependent strand annealing and the dHJ subpathways

After resection and the formation of the Rad51 nucleoprotein filament, the 3' tail can invade the donor sequence. This step, which is also called *strand invasion*, requires several factors and mediates the formation of a DNA intermediate commonly named D-loop. The DNA helicase Srs2 can remove Rad51 from the nucleoprotein filament, counteracting the strand invasion, while two ATPases/translocases, Rad54 and Rdh54/Tid1, stimulate the D-loop formation and stabilization. After the stabilization of the D-loop, DNA polymerases are recruited and extend the 3' filament using the homolog strand as a template. At this point, according to the HR sub-pathways used, DSB repair will give different outcome. If the invading tail, after the elongation step, is displaced from the homolog and

annealed with the other complementary end tail, the resulting gap can be filled, leading to DSB repair without crossover events. This sub-pathway is called SDSA, and it is responsible for gene conversion events that occur in mitotic cells. The situation is more complicated if also the second end is captured, leading to the formation of a double Holliday Junction intermediate (dHJ). Indeed, the jointed DNA filaments require to be cut to complete the recombination process, and to allow chromosomes segregation.

There are multiple enzymes that can cut the cruciform structures at the dHJs, and they work through two distinct mechanisms. The first is called *dissolution*, and is mediated by the helicase/topoisomerase Sgs1-Top3-Rmi1 complex, leading to non-crossovers events (Bernstein et al., 2010). Alternatively, if the HJs are kept far away each other, specific nucleases, called *resolvases*, including Slx4-Slx1 (Andersen et al. 2009; Fekairi et al., 2009; Munoz et al., 2009; Saito et al., 2009; Svendsen et al., 2009), Mus81-Mms4 (Kaliraman et al., 2001) and Yen1 (Ip et al., 2008), cut single strand filaments, leading to both crossovers and non-crossovers events. Therefore from random dHJ resolution it is expected an equal number of crossovers and non-crossovers products (See Figure 1). This DSB-induced recombination process is particularly relevant in meiotic cell cycle to promote genetic variation.

Break Induced Replication

Sometimes it is possible that only one end of the DSB is able to anneal with a homologous sequence; in this case a replication fork is assembled and the repair synthesis can go till the end of the chromosome through a process called BIR. It has been implicated in replication restart after fork collapse at telomeres, and can mediate telomere elongation when telomerase is not functional or telomere capping is lost. It is important to note that if only one end of a DSB is engaged in a BIR process, the other chromosome region is lost, resulting in loss of heterozygosis

(LOH), and relevant phenotypes can arise (Llorente et al., 2008). In a study made in Haber's lab it has been shown that BIR requires only a 72 bp homology (Bosco & Haber, 1998); this short homologies requirement makes BIR prone to introduce chromosomes aberration. Because of this, it is believed that BIR is a backup system, used only when all the others DSB repair pathways fail (Malkova et al., 2005).

The DSB Ends Processing

Ends resection is a fundamental step in order to repair a DSB through HR pathways. The 5' end degradation is a nucleolytic process in which multiple factors are involved. Among them the MRN/X complex, CtIP/Sae2, Exo1 and Dna2 nucleases (Mimitou & Symington, 2009) are the main players but also other factors, such as helicases and chromatin remodelers are required. For example the Bloom/Sgs1 helicase (Mimitou & Symington, 2008; Zhu et al., 2008; Gravel et al., 2008) and the chromatin remodelers like the RSC complex (Shim et al., 2007) and SMARCAD1/Fun30 (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012). As mentioned above, the MRN/X complex and the Ku heterodimer are the first factors loaded on the DSB ends. Although both complexes protect DSB ends and are involved in NHEJ, the MRN/X complex plays an additional role in starting DSB ends resection through the endonuclease and 5'-to-3' exonuclease activities of the Mre11 subunit.

Both in yeast and man, it has been shown that the DSB resection is a finely regulated process. This is not surprising, if we consider that the initiation of the DSB resection is a fundamental step to channel a DSB into HR.

Initially, it was shown that CDK1 kinase activity promotes the DSB resection (Ira et al., 2004; Aylon et al., 2004), maybe counteracting a Ku-dependent inhibitory step (Clerici et al., 2008; Mimitou et al., 2010; Shim et al., 2010). Then, it has been demonstrated that CDK1 targets the MRN/X-associated factors CtIP/Sae2.

In G_2/M phase, CtIP/Sae2 is phosphorylated by CDK1 in a residue present in the evolutionary conserved C-terminal part of the protein, serine 267 in *S. cerevisiae* and threonine 847 in human. This phosphorylation primes DSB ends processing. Indeed, alanine substitution of the corresponding S267 or T847 causes severe DSB resection and repair defects (Sartori et al., 2007; Huertas et al., 2008; Huertas & Jackson, 2009). The effective role of CtIP/Sae2 in starting resection has not been

fully understood so far, the more promising hypothesis is that CtIP/Sae2 stimulates the MRN/X complex activity towards particular DNA structures but also, in a recent publication, it has been found that in yeast Sae2 has also an endonucleolytic activity *in vitro.* This can suggest a possible role of Sae2 in the DNA processing but at the moment no *in vivo* nuclease activity has been found and the human CtIP has no nuclease activity at all (Lengsfeld et al., 2007).

It's important to note that in presence of DSBs induced by an endonuclease like HO or I-Sce1 (that leaves clean ends) the nucleolytic activity of the MRN/X complex is not essential (Clerici et al., 2005; Llorente & Symington, 2004), whereas in presence of DSB end produced by IR or other drugs that create particular DNA structures (hairpins or proteins covalently bound to the DSB ends), the catalytic activity of the complex becomes determinant (Lobachev et al., 2002; Deng et al., 2005). In agreement with all these data, Neale and colleagues (Garcia et al., 2011), proposed a bidirectional model for DSB resection, based on their recent studies on meiotic DSB processing in yeast. In meiosis the MRX complex, together with Sae2, is essential; in fact, by an endonucleolytic cut, it mediates the removal of the specific endonuclease Spo11 from the DSB ends in order to allow meiotic recombination. MRX complex binds and introduces a nick at 300 nucleotides away from the 5' strand DSB ends. Then the MRX complex resects the break heading to the DSB ends in order to remove all the possible obstacles present at the DSB ends. This causes the removal of Spo11 from the ends and allows to all the other DSB resection factors to process the lesion (Garcia et al., 2011).
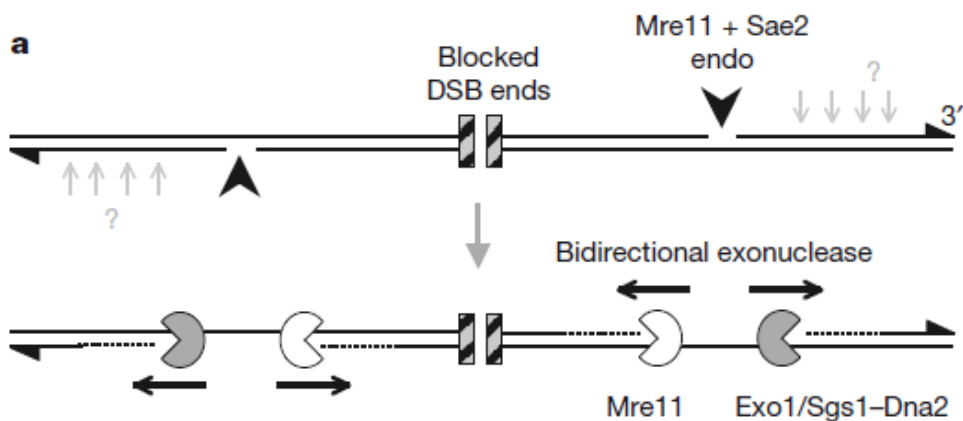
Figure 2: Model of the DSB resection initiation from Garcia et al., 2011

Therefore, the MRN/X complex is important for the resection early step and other nucleases and factors would be required to produce longer ssDNA tracts. A number of works have recently clarified this aspect. The two key factors for processive resection are the 5'-to-3' exonuclease Exo1 and the endonuclease Dna2 that acts together with the helicase Sgs1. In fact it has been demonstrated that a yeast strain carrying *EXO1* and *SGS1* or *DNA2* deletions accumulates only short ssDNA tracts of about 100-700 nucleotides (Mimitou & Symington 2008; Zhu et al., 2008; Gravel et al., 2008). These short tails are not enough to permit optimal homologous recombination events. In fact, normally, mitotic recombination requires a ssDNA tail length between 2 and 4 kb (Chung et al., 2010).

The Sgs1 helicase belongs to the RecQ family of 3'-to-5' DNA helicases and forms a complex with Top3 and Rmi1. This complex is very well conserved in human, where the Bloom helicase interacts with Top3α, Rmi1 and Rmi2. This complex processes the double Holliday Junction (dHJ) through the dissolution pathway (Heyer et al., 2010), but it is also necessary for the Sgs1 function in DSB resection. In this case the Top3 catalytic activity is not required, suggesting that the complex has only a structural function during resection (Niu et al., 2010). As mentioned

above, Dna2 is the BLM/Sgs1 partner in the resection process. Dna2 protein has a flap endonuclease activity that cuts the 5' DNA filament unwounded by BLM/Sgs1. Recently, it has been shown that CDK1 phosphorylates Dna2, mediating its loading on to the DSB lesion and its checkpoint-dependent phosphorylation. Considering that CDK1 phosphorylates also Sae2 factor (see above), it is believed that CDK1 plays a central role in the regulation of the DSB resection, influencing the process throughout the cell cycle (Chen et al., 2011).

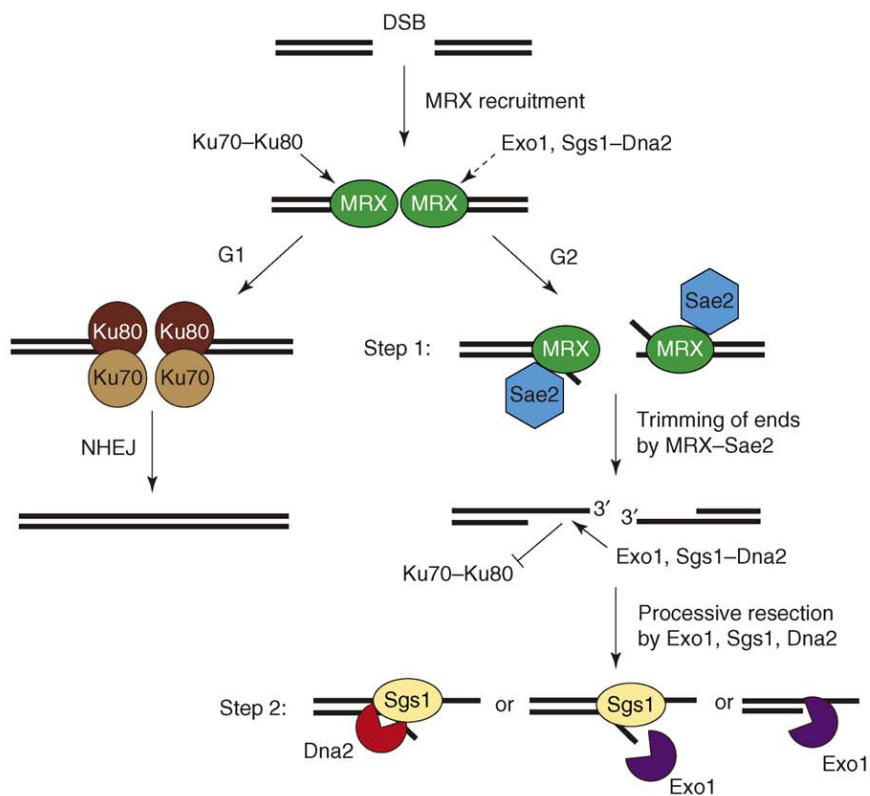**Figure 3:** Schematic representation of DSB repair in the different phases of the cell cycle, and of the dual steps resection process (from Mimitou & Symington, 2009)

DSB resection, as well as all other DNA metabolic processes, occurs within the context of chromatin. It is not surprising, therefore, that cells evolved a complex network of post-translational histone modifications and ATP-dependent chromatin

remodeling reactions to modulate chromatin structure and its accessibility with each step of DNA repair (reviewed in Van Attikum & Gasser, 2009; Soria et al., 2012; Seeber et al., 2013), following an "access-repair-restore" model (Smerdon, 1991). So far, three factors have been involved in DSB resection.

i) RSC (Remodels the Structure of Chromatin) is an ATP-dependent chromatin remodeler of the SWI/SNF-family, and it is necessary for the establishment of a normal nucleosome pattern in unperturbed conditions, suggesting that at least a small amount of RSC is constitutively present onto the chromatin (Kent et al., 2007). On the other hand, different subunits of the RSC complex are recruited at HO-induced DSB sites a few minutes after HO induction, suggesting a role in the early step of DSBR (Chai et al., 2005; Shim et al., 2005). It was later shown that RSC is required for nucleosome repositioning around the HO-induced break site, after break induction, suggesting that RSC might open the chromatin to the DSBR machinery (Kent et al., 2007; Shim et al., 2007). Indeed, ChIP analysis demonstrated that Mre11 and Ku70 recruitment to DSB are reduced in *rsc* mutants, as are ssDNA formation and loading of RPA. These findings indicate that loss of RSC activity likely leads to a resection defect. (Liang et al., 2007; Kent et al., 2007; Shim et al., 2007).

ii) Ino80 is a Swr1-like ATP dependent chromatin remodeler composed by 15 subunits in budding yeast, seven of which are conserved also in human INO80 (Chambers & Downs, 2012). Similarly to *rsc* mutants, *ino80* mutants accumulate less ssDNA compared to a wild type, consistently with a mild defect in resection (Van Attikum et al., 2004; van Attikum et al., 2007; Morrison et al., 2007), probably linked to a reduction in MRX loading at the break observed in this mutant (Van Attikum et al., 2007). How Ino80 modulates the recruitment of end resection machinery is still unknown, however, it has been proposed that, by catalyzing nucleosome sliding or histone exchange, it may increase accessibility to the break (Shen et al., 2003; Udugama et al., 2011; Papamichos-Chronakis et al., 2011).

Ino80 involvement in DSB processing seems to be conserved among eukaryotes. Indeed, in human cells, after ionizing radiation treatment, hINO80 localizes to damaged chromatin, and it is required for the homology-directed repair of a I-SceI induced break (Gospodinov et al., 2011).

iii) Fun30/SMARCAD1 is an ATP-dependent chromatin remodeler belonging to the Etl1 Snf2 family. As other family members, Fun30 binds nucleosomes and*,* thanks to its ATPase activity, facilitates the exchange of H2A-H2B dimers and the sliding of nucleosomes *in vitro* (Awad et al., 2010). The ATP-dependent chromatin remodeling activity is required *in vivo* to establish gene silencing in heterochromatic regions such as the HMR locus, rDNA repeats and telomeres (Neves-Costa et al., 2009), and for the formation of correct architecture at centromeres (Durand-Dubief et al., 2012). In the last year, three independent groups reported a role for Fun30 in resection of DNA ends. Deletion of Fun30 mildly affects nucleolytic processing in the close proximity of an HO-cut site, whereas it strongly impairs resection further from the break, suggesting an involvement of this protein in the control of long-range resection (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012). Consistently with a role in DSB processing, Fun30 is recruited to DSB sites and spreads along the chromatin in both directions, similarly to the DNA resection machinery. Moreover, epistasis analysis revealed that Fun30 promotes both Exo1- and Sgs1/Dna2- dependent resection pathways (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012). This would be consistent with the observation that in *fun30Δ* mutants Exo1 and Dna2 are recruited at the DSB site, but fail to spread along the chromatin. Fun30 seems to stimulate long-range resection by removing the barrier represented by nucleosome-bound Rad9 (see below for the discussion on the barrier).

Similarly, the human counterpart of Fun30, SMARCAD1, is recruited to laser- and FokI-induced DSBs, and its downregulation affects DSB ends processing, reducing the formation of RPA foci as well as of ssDNA. In agreement with a resection

defect, *SMARCAD1* knockdown cells are defective in recombinational repair (Costelloe et al., 2012).

Moreover, recent data showed that the checkpoint factor 53BP1/Rad9 plays and additional role in regulating DSB ends resection. In yeast, the Rad9 loading on the break slow down resection speed, in fact deletion of *RAD9* causes an increase in ssDNA tracts length that is not dependent on Exo1 (Lazzaro et al., 2008). Also in human 53BP1 depletion causes an increase in resection rate that is fundamental to stimulate HR instead of NHEJ (Bunting et al., 2010), this phenotype is made together with Rif1, a protein also involved in telomeres homeostasis (Zimmerman et al., 2013; Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013).

The Checkpoints

The genome integrity and the ability to transfer it correctly at the offspring is an essential goal for the cell. For this purpose the cell cycle progression is carefully controlled by different surveillance mechanisms called checkpoints.

This control mechanisms act in specific cell cycle phases checking that all the cell cycle events happen in the correct order and, in case of errors, they are able to slow down or even stop cell cycle progression, therefore activating the proper repair system. Different types of checkpoints exist, for example a morphogenetic checkpoint is activated in case of cell's shape problem (Lew, 2003), and a mitotic checkpoint, called Spindle Checkpoint, is activated in case of mitotic spindle assembly errors (Lara-Gonzalez et al., 2012). Accordingly with the aims of my projects, I will speak only about the DNA damage checkpoint.

In higher eukaryotes the alteration of this mechanisms lead to an increased genome instability that can be correlated with an increased risk of tumorigenesis (Nyberg et al., 2002).

The DNA Damage Checkpoint

Activation of the DNA damage checkpoint

In the presence of DNA lesions such as DSBs, the cell activates a surveillance mechanism, called the DNA damage checkpoint. Depending on the cell cycle phase in which the lesion occurs, three distinct DNA damage checkpoints can be induced: the G_1/S phase checkpoint blocks cell cycle before replication starts, the intra S phase checkpoint works during replication and the G_2/M phase checkpoint prevents chromosomes segregation, blocking cell cycle at the metaphase/anaphase transition (Branzei & Foiani, 2008).

By using the HO nuclease-based system in yeast (Sandell & Zakian, 1993; Moore & Haber, 1996), many details of the cellular response to DSB have been understood. First of all, it was shown that a single DSB is enough to trigger checkpoint activation, blocking the cell cycle progression in G2/M (Sandell & Zakian, 1993).

Moreover, it turned out that the checkpoint proteins are very well conserved from yeast to mammals (see a list of factors in table 1), thus suggesting *Saccharomyces cerevisiae* as an ideal system to study genetic and biochemical details of the checkpoint mechanisms.

Table 1: Homologous recombination and DNA damage checkpoint factors (from Mimitou & Symington, 2009)

S. cerevisiae	S. pombe	Human	Biochemical function
RPA	RPA	RPA	Heterotrimeric ssDNA-binding protein
Rad51	Rad51 (Rhp51)	RAD51	RecA homolog. Binds ssDNA and dsDNA, promotes homologous pairing and strand exchange
Mre11–Rad50–Xrs2 (MRX complex)	Mre11 (Rad32)–Rad50– Nbs1 (MRN complex)	MRE11–RAD50– NBS1 (MRN complex)	Mre11, ssDNA endonuclease and 3'–5' exonuclease; Rad50, DNA binding and tethering activity; Xrs2 (Nbs1), interacts with Tel1 (ATM)
Spo11	Rec12	SPO11	Catalyzes the formation of meiosis-specific DSBs by a topoisomerase-like transesterase mechanism
Sae2	Ctp1	CtIP	Interacts with MRX (MRN) complex on DNA; S. cerevisiae protein has ssDNA endonuclease
Exo1	Exo1	EXO1	5'–3' exonuclease and flap endonuclease
Sgs1	Rqh1	BLM	3'–5' helicase of the RecQ family
Top3	Top3	TopoIIIα	Type I topoisomerase
Rmi1	Rmi1	RMI1	Interacts with Sgs1–Top3 complex
Dna2	Dna2	DNA2	ssDNA endonuclease; ATPase; helicase
Mec1–Ddc2	Rad3–Rad26	ATR–ATRIP	PIKK required for DNA damage and replication checkpoint
Tel1	Tel1	ATM	PIKK required for DNA damage checkpoint and telomere maintenance
Rad53	Cds1	CHK2	Effector kinase
Rad9	Crb2	53BP1	DNA damage sensor; interacts with modified histones and Rad53
Ddc1–Rad17–Mec3 (9-1-1 complex)	Rad9–Rad1–Hus1	RAD9–RAD1–HUS1	PCNA-like clamp
Rad24	Rad17	RAD17	Alternative large subunit of the RFC clamp loader complex
Yku70–Yku80	Pku70–Pku80	KU70–KU80	Heterodimeric DNA end binding protein
Mus81	Mus81	MUS81	Catalytic subunit of Mus81–Mms4 (Eme1) heterodimeric nuclease
Mms4	Eme1	EME1	Interacts with Mus81
Yen1	Not identified	GEN1	Symmetrical cleavage of HJs

The upstream factors required for checkpoint activation are the two evolutionary conserved proteins kinases ATR/Mec1 and ATM/Tel1. In fact, the DNA damage checkpoint is a signal transduction cascade, based on phosphorylation events, and requires the activity of other protein kinases.

Generally speaking, we distinguish different classes of checkpoint factors: i) the DNA damage sensors, ii) the signal transducers and iii) the effector kinases that phosphorylate multiple targets (Table 1) (Harrison & Haber, 2006).

Supporting evidence in all the organisms correlates the formation of a ssDNA intermediate, covered by RPA, with the checkpoint activation. Indeed, it was shown that the upstream checkpoint kinases have been recruited on to DNA lesion through two distinct mechanisms. ATM/Tel1 arrives on the break through MRN/X complex binding, in particular through Nbs1/Xrs2 recruitment (Nakada et al., 2003; Falck et al., 2005), whereas the other kinase ATR/Mec1 recruitment requires different factors and a ssDNA-RPA structure. In different studies has been demonstrated that the main factor involved in ATR/Mec1 activation is the checkpoint factor

ATRIP/Ddc2 (Paciotti et al., 2001; Melo et al., 2001; Zou & Elledge, 2003) but, depending on the cell cycle phase and the nature of the DNA damage, other factors like TopBP1/Dpb11 (Puddu et al., 2008; Navadgi-Patil & Burgers, 2008; Mordes et al., 2008a; Mordes et al., 2008b), the 9-1-1 complex (Majka et al., 2006a) and the Dna2 nuclease (Kumar & Burgers, 2013) are involved.

Both the yeast protein Ddc2 and the corresponding human ATRIP bind tightly the catalytic subunit, Mec1 or ATR respectively, forming a protein dimer essential to interact with the ssDNA covered by RPA.

The ATR/Mec1 activation is dependent upon an additional complex of checkpoint factors. This complex is formed by Rad9/Rad17, Hus1/Mec3 and Rad1/Ddc1 proteins and shares structural homology with the PCNA complex; in fact it is also called PCNA-like complex (Kondo et al., 1999). The 9-1-1 is recruited on the lesion immediately after its formation in a Mec1 independent manner (Kondo et al., 2001), while it is dependent on the Rad24 protein together with the Rfc 2-5 proteins (Majka & Burgers, 2003), and the ssDNA-RPA structure (Majka et al., 2006b). The role of this complex in the ATR/Mec1 activation is dependent on the cell cycle phase. In G_1 the complex is necessary and sufficient for the ATR/Mec1 activation, while in S and G2/M phases the activation requires also TopBP1/Dpb11. The TopBP1/Dpb11 protein strongly stimulates the ATR/Mec1 activity together with the 9-1-1 complex (Puddu et al., 2008; Mordes et al., 2008a), in fact in yeast it has been demonstrated that Dpb11 coordinates a ternary complex formed by the 9-1-1 complex, Mec1 and Rad9, this complex is essential for the correct phosphorylation of Rad9 by Mec1 and the consequent checkpoint activation (Pfander & Diffley, 2011).

Recently, it has been demonstrated that also the endonuclease Dna2 is involved in the ATR/Mec1 activation, but its requirement is restricted only to the intra-S phase checkpoint and its role in the mechanism is not completely understood (Kumar & Burgers, 2013).
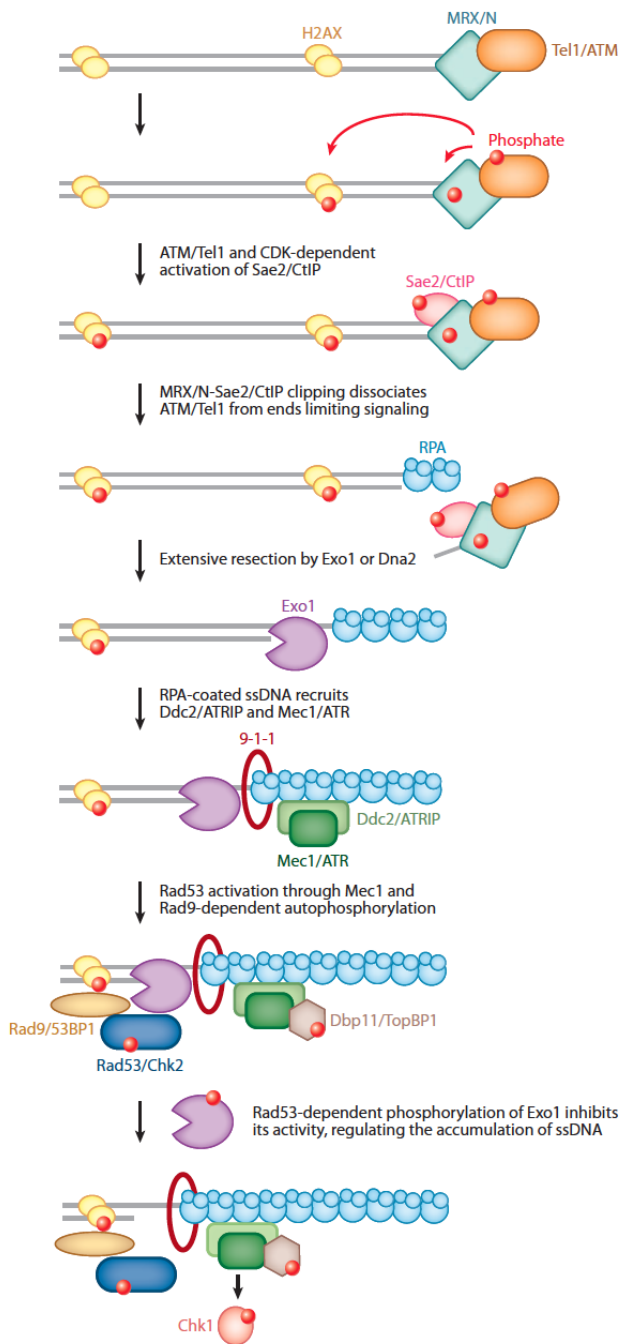
Figure 4: Model of the DNA damage checkpoint activation (from Symington & Gautier, 2010)

After the upstream kinases activation, the DNA damage signal transduction to the checkpoint targets occurs. Among the many factors that are targeted by the apical kinases, the two well conserved Chk1 and Chk2/Rad53 kinases are activated and, in turn, phosphorylate several proteins, triggering an intricate regulatory network. This transduction cascade rapidly amplifies and propagates the DNA damage signal.

In yeast the main role of Chk1 is the stabilization of the securin Pds1 (Wang et al., 2001). The Pds1 stabilization is essential to prevent the separase Esp1 activation, avoiding cohesins cleavage and chromosomes segregation (Ciosk et al., 1998). Also Chk2/Rad53 is able to block cell cycle progression, but through a completely different mechanism. Rad53 phosphorylates the Anaphase Promoting Complex (APC) regulatory subunit Cdc20, causing the inhibition of the complex (Agarwal et al., 2003). The APC^{Cdc20} is the ubiquitin ligase that degrade Pds1, therefore the checkpoint blocks the Pds1 degradation acting on two different mechanisms: directly, through Chk1 phosphorylation of Pds1, and, indirectly, through Rad53 phosphorylation of Cdc20.

In yeast, the two effectors kinases are activated by Mec1 or Tel1 through an activation mechanism that requires the contribution of other proteins called adaptors, Rad9 and Mrc1 (Vialard et al., 1998; Alcasabas et al., 2001). Rad9, is a protein of 150kDa containing different functional domains: two Breast Cancer C-Terminal (BRCT) domains that are required for the protein-protein interaction, a SCT domain rich in SQ/TQ clusters and a TUDOR domain that is able to bind directly the DNA (Lancelot et al., 2007). The SCT domain is phosphorylated by the apical kinases; and its mutation or deletion causes the inability to activate Rad53; in fact Rad53 has a phosphothreonine-binding FHA domain that binds the SCT domain of Rad9, mediating the Rad53 oligomerization (Sweeney et al., 2005). This process allows the full activation of Rad53 by *in trans* autophosphorylation (Pellicioli et al., 1999), at this point the Rad53 oligomers are disassembled and Rad53 is able to phosphorylate its targets (Ma et al., 2006). The Mec1-dependent

Rad9 SCT domain phosphorylation induces also the interaction between a Rad9 protein with the BRCT domain of another Rad9 molecule, inducing the formation of homo-oligomers. It was shown that the Rad9 oligomerization is essential for the maintenance of the checkpoint response but not for its activation, in fact Rad9 mutant variants defective in protein oligomerization are able to activate the checkpoint, but at a certain moment the checkpoint signal decays (Usui et al., 2009).

Interestingly, different studies in both human and yeast have demonstrated that Rad9 and its human counterpart 53BP1 are loaded on to a DSB through the binding to the phosphorylated serine 129 on the histone H_2A and the methylated lysine 79 on the histone H_3, made by Mec1 and the methyltransferase Dot1 respectively (Giannattasio et al., 2005; Wysocki et al., 2005; Toh et al., 2006; Hammet et al., 2007).

Furthermore, Smolka and colleagues showed that in presence of replication stress the Slx4-Rtt107 scaffold protein is recruited near the stalled replication forks and counteracts the Rad9 binding. This phenomenon leads to Rad53 signal damping and avoids a too much prolonged checkpoint block (Ohouo et al., 2013).

DNA damage checkpoint switch off and cell cycle restart

The DNA damage checkpoint inactivation is an essential process required to restart the cell cycle after DNA damage. Two genetically distinct finely regulated mechanisms are responsible for the cell cycle restart. They are called checkpoint recovery and adaptation. After successful repair cell cycle restarts through checkpoint recovery, however it has been demonstrated that also in presence of an irreparable DNA lesion cells are able to switch off the checkpoint and divide despite the damage still remains; this process is called checkpoint adaptation (Clemenson & Marsolier-Kergoat, 2009). Interestingly, this process occurs not only in unicellular organisms like yeast (Sandell & Zakian, 1993), where it can be

considered as an extreme attempt to survive, but it has been shown also in *Xenopus laevis* and human cells (Yoo et al., 2004; Syljuasen et al., 2006). In this case, the functional role of checkpoint adaptation is unclear; in fact on one hand it can be related to unrestrained proliferation, which is a main feature of cancer cells, but it may be also induced by cells in order to undergo a mitotic catastrophe and apoptosis, in the presence of high levels of unrepaired DNA lesions (Syljuasen, 2007). In yeast, checkpoint adaptation has been studied mainly in the presence of a single DSB induced by the endonuclease HO that activates the G_2/M phase checkpoint (Sandell & Zakian, 1993; Toczyski et al., 1997; Lee et al., 1998; Pellicioli et al., 2001); however in other organisms adaptation occurs in the presence of unrepaired DSBs induced by IR (Syljuasen et al., 2006), and following replication stress induced by aphidicolin (Yoo et al., 2004).

Different studies, mainly in yeast, have been made in order to identify the factors involved in these processes. From this analysis emerge that the key factors in both the checkpoint shutdown systems are phosphatases. In fact, since the checkpoint is a kinases-based signaling cascade, it was expected that dephosphorylation events would be involved to signal switch off. In yeast PP2C phosphatases Ptc2 and Ptc3 are involved in Rad53 dephosphorylation during checkpoint deactivation both through recovery and adaptation, whereas a PP2A like phosphatase, Pph3, dephosphorylates the serine 129 on the histone H_2A during checkpoint recovery. It is not understood why Pph3 is not involved in checkpoint adaptation too (Heideker et al., 2007).

However, additional factors, such as different kinases and repair proteins, are required to mediate the checkpoint recovery and adaptation processes. Among them, the DSB end binding factor Ku, the resection-involved factors Sgs1, MRX and Sae2, the chromatin remodelers Fun30, Tid1/Rdh54 and Ino80, the helicase Srs2, the recombination proteins Rad52 and Rad51, the polo-kinase Cdc5 and the casein kinase 2 (CKII) (Toczyski et al., 1997; Lee et al., 1998; Lee et al., 2001;

Vaze et al., 2002; Lee et al., 2003; Clerici et al., 2006; Papamichos-Chronakis et al., 2006; Eapen et al., 2012).

Interestingly, it is noticed that factors involved in the DSB resection, (the Ku complex, MRX complex, Sae2, Sgs1, Ino80 and Fun30) are also important to checkpoint switch off (Lee et al., 1998; Clerici et al., 2006; Papamichos-Chronakis et al., 2006; Eapen et al., 2012). The reasons why these proteins are implicated is not understood, although it was suggested that a fine regulation of the formation of the ssDNA covered by RPA is a fundamental step to get a proper checkpoint switch on and off.

Other works underline a possible role of Rad51 in modulating the checkpoint inactivation process. For instance, it turned out that the DNA helicase Srs2, which is involved to dismantle Rad51 from ssDNA (see the discussion on HR above), promotes both checkpoint recovery and adaptation. One possibility is that, in the absence of Srs2, Rad51 could remain bound to the filament, even after the repair process, keeping the checkpoint signal active (Vaze et al., 2002: Yeung & Durocher, 2011). Interestingly, mutations of *TID1/RDH54* gene, which codify for a DNA translocase involved in Rad51 removal from dsDNA cause a severe defect in checkpoint adaptation (Lee et al., 2001). I will discuss more details of this pathway in the main results section, as it was an issue of my PhD projects.

Finally also two kinases are important regulators of checkpoint inactivation: the Casein Kinase 2 (CK2) and the polo kinase Cdc5 (Toczyski et al., 1997). CKII phosphorylates and activates PP2C phosphatases, thus mediating both checkpoint recovery and adaptation (Guillemain et al., 2007). On the contrary, the involvement of Cdc5 is still not understood, although the corresponding factors in human cells (PLK1), has been implicated in checkpoint switch off (Smits et al., 2000; Macurek et al., 2008; Van Vugt et al., 2010), supporting the notion that polo kinases are central players of the checkpoint inactivation mechanism (Bahassi, 2011).

During my PhD, I will be involved in a project to further characterize Cdc5 role in cells responding to DNA damage (see Main Results section 1, 2 and 4).

In the following chapter I will briefly summarize the regulation and functions of the polo kinase Cdc5 and its pathways.

The Polo Kinase Cdc5

Polo Kinases are fundamental regulators of the cell cycle in almost all the eukaryotes, in fact, all of them, excluding plants, have at least one polo related kinase (Golsteyn et al., 1996). In budding yeast the *CDC5* gene was identified as a fundamental cell cycle player in the pioneering work of L. Harwell (Harwell, 1970). These proteins contain a N-terminal Ser/Thr kinase domain and a C-terminal characteristic polo box domain (PBD). This domain is specific for this class of kinases and it is required for their particular functions and cellular localization (Lee et al., 2005). In mammals cells 5 different polo kinases were found (Plk1, Plk2, Plk3, Plk4 and Plk5), whereas in *S. cerevisiae* and *S. pombe* only a polo kinase homolog is present, named Cdc5 and Plo1 respectively (de Carcer et al., 2011). A study performed in human cell lines reveals that Plk1 localized at the centrosome and at the kinetochore in late S phase, remaining at this structure till the metaphase/anaphase transition (see in figure 5); in yeast Cdc5 is closely related to Plk1, in fact starts to localize at the spindle pole bodies (SPBs) in G_1 and at the ring shaped structure that divides the mother and the daughter cells (bud neck) during the G_2/M phases.
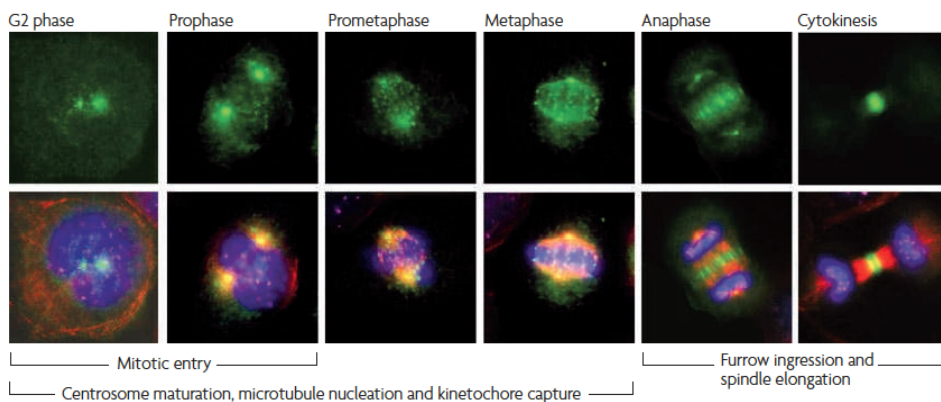

Figure 5: Localization of Plk1 during mitosis. Upper panel: Immunofluorescence of Plk1 (green) in HeLa cells during the main mitotic phases. Lower panel: staining of DNA (blue), kinetochores (purple) and α-tubulin (red) (From Strebhardt, 2010).

Mutagenesis experiments and crystallographic studies on the PBD structure were performed in order to understand the function of this domain. It was understood that the PBD is formed by two homodomains called polobox 1 and polobox 2, both necessary for the interaction with the substrates (Elia et al., 2003). Moreover this domain is able to bind specifically consensus sequences containing a serine or threonine that was previously phosphorylated by CDKs or MAPK (Elia et al., 2003), although in the last few years it has been demonstrated that this particular consensus motif is not always necessary in budding yeast (Chen & Weinreich, 2010).

CDK1 is not only involved in the polobox substrates recognition, but it is also necessary for Cdc5 activation. In fact CDK1 phosphorylates the threonine 242 located in the kinase domain of Cdc5, this causes the activation of the protein during the G_2/M phase of the cell cycle, exactly when the polo kinases are essential for the orchestration of the mitotic events (Mortensen et al., 2005). This residue is extremely conserved, suggesting a possible common activator mechanism in all PLKs. In fact, in human cells the phosphorylation of the corresponding residue T210 by the Aurora kinase is essential for the DNA damage checkpoint inactivation and recovery (Smits et al., 2000; Macurek et al., 2008).
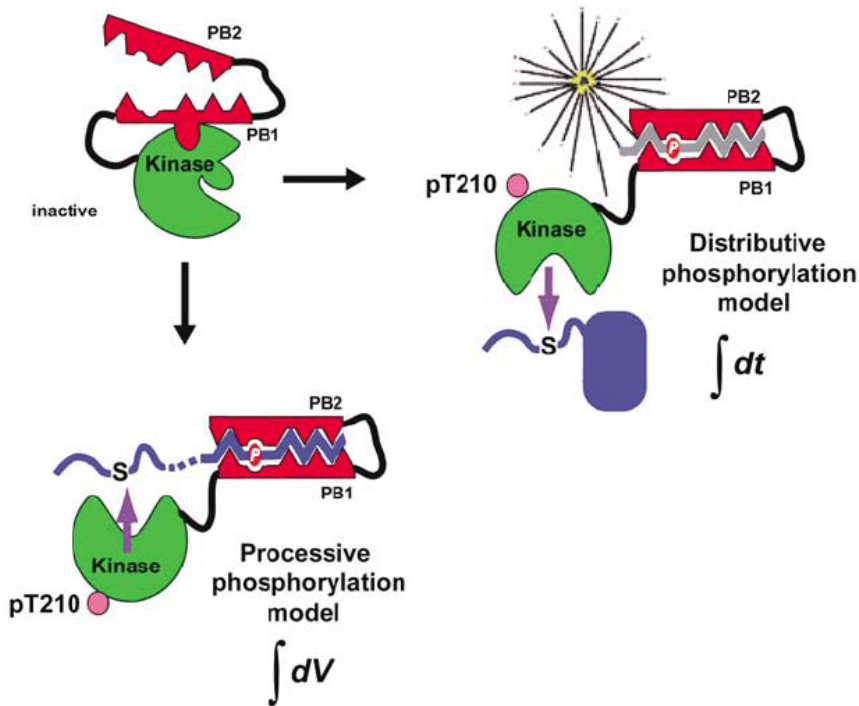
Figure 6: Two different models of PBD mediated substrate targeting. In the processive phosphorylation model the PLKs bind a protein for subsequent direct phosphorylation, while in the distributive phosphorylation model the PLKs bind to a protein and phosphorylate another nearby protein.

Thus, in yeast CDK1 is important to activate the polo kinase, which in turn, through a feedback loop, is required to keep active CDK1. Indeed Cdc5 phosphorylates Swe1, a CDK1 inhibitor, leading to Swe1 polyubiquitination and degradation by the APC complex and full activation of the CDK (Lee et al., 2005). During an unperturbed cell cycle, Cdc5 phosphorylates several targets. Among these factors, the cohesin subunit Scc1 is one of the most relevant. This event promotes the cohesins ring opening and, as a consequence, the chromosomes segregation (Alexandru et al., 2000). Moreover, Cdc5 is an activator of the Mitotic Exit Network (MEN), which promotes the release of the Cdc14 phosphatase from the nucleolus in order to dephosphorylate the CDK1-dependent targets at the latest

stage of the mitosis (Segal, 2011). Accordingly to all these evidences, Cdc5 is considered a master regulator of the mitosis. Furthermore, recent findings revealed a functional role for PLKs in cells responding to different types of DNA damage (Bahassi, 2011). As mentioned above, in yeast Cdc5 is involved in the checkpoint adaptation process, which is a role described for PLK1 in other eukaryotes too. In particular, in human and *X. laevis* during the recovery from DNA damage, PLK1 targets the checkpoint adaptor proteins Claspin and 53BP1, functional ortholog of Mrc1 and Rad9. During recovery from the intra-S phase checkpoint, induced by the replication stress agent aphidicolin, Claspin is phosphorylated by PLK1, this event causes Claspin removal from chromatin in *Xenopus*; in human cells, the PLK1-dependent phosphorylation of Claspin leads to its degradation, Chk1 inactivation and cell cycle restart (Mailand et al., 2006; Peschiaroli et al., 2006; Yoo et al., 2004). In G_2/M phase, PLK1 binds 53BP1 and *in trans* phosphorylates Chk2, causing its inactivation and checkpoint signaling switch off (Van Vugt et al., 2010).

Aim of the Project

Genome maintenance and stability are essential goals for all the organisms in order to transfer the correct genetic information to the progeny and to keep fully functional the cellular metabolism. In eukaryotic cells, the presence of DNA lesions causes the activation of an evolutionary conserved mechanism called the DNA damage checkpoint that arrests the cell cycle and stimulates the repair pathways. This control mechanism is organized as a signal transduction cascade that progressively activates different kinases that phosphorylate many downstream targets. After lesion repair the checkpoint is switched off through a process called recovery; however it has been demonstrated that after a prolonged checkpoint response, damaged cells are able to inactivate the checkpoint and restart the cell cycle despite the presence of dangerous DNA lesion, through a checkpoint adaptation process. The reason why this process occurs is not understood, but it has been related to the unrestrained proliferation of cancer cell. Therefore, it is expected that further comprehension of the mechanisms leading to checkpoint switch off will provide important information to identify novel targets for cancer therapy.

During the PhD I focused my attention on the characterization of the functions and regulation of some factors already known to play a role in DSB ends processing and checkpoint switch off: the Polo kinase Cdc5, the DNA translocase Tid1/Rdh54 and the nuclease-associated protein Sae2.

The Polo kinases are involved in the regulation of the checkpoint switch off and the cell cycle re-enter after DNA damage in different organisms. Moreover, it has been shown that the human Polo-like kinase Plk1 is overexpressed in many tumours and is essential for tumour growth, as well for normal cells. In fact an increasing number of chemotherapeutic compounds that target PLK1 are in clinical trial.

Despite PLKs are subjected to extensive studies; their role and targets regulated during the checkpoint switch off are not completely characterized. Thus, taking advantages of the simple yeast model system we decided to investigate the role of the yeast polo kinase Cdc5 in the checkpoint inactivation process. In order to identify the Polo kinase target in DNA damage condition, I performed a biochemical screen using the PBD domain as bait. As expected, I identified several factors, including Sae2, which is an important player involved in the checkpoint response. Sae2 is required for the DSB end processing and in the checkpoint inactivation by recovery or adaptation. The role of Sae2 in the checkpoint switch off is unclear, thus we expect to get novel information on this mechanism by the studying of the Cdc5-Sae2 crosstalk. Moreover, we believe that these finding will shed light on the checkpoint mechanism that regulates genome stability and protect from cancer. Checkpoint adaptation also requires the Tid1/Rdh54 factor. This protein belongs to the Swi2/Snf2 family of chromatin remodellers and it has been involved in different processes related to homologous recombination. Although several phenotypes related to DSBs have been described for *tid1* mutant cells, the regulation of the corresponding protein has been poorly studied. Thus, in our laboratory we started a project with the aim to characterize the *in vivo* regulation of Tid1 protein in cells responding to DSBs. We found that the Tid1 protein is a novel target of the DNA damage checkpoint, which is also involved in the checkpoint adaptation.

Main Results, Conclusion and Future Perspectives

1. Elevated Levels of the Polo Kinase Cdc5 Override the Mec1/ATR Checkpoint in Budding Yeast by Acting at Different Steps of the Signaling Pathway (Donnianni et al., 2010)

In this work, we took advantage of the yeast model organism to study the DSB-induced checkpoint when the polo kinase Cdc5 is overexpressed. Indeed, it was known that the polo kinase Plk1 is frequently overexpressed in cancer cells (Strebhardt & Ullrich, 2006), while Plk1 silencing by siRNA causes a fast apoptosis induction in different cancer cell lines. Thus, starting from this intriguing finding, we were interested to investigate how the Plk1 overexpression promotes the tumour cells growth. In previous works made in yeast, it has been shown that a Mec1 and Rad53-dependent phosphorylation inhibit Cdc5 in order to stop the cell cycle progression in the presence of DNA damage (Cheng et al., 1998; Sanchez et al., 1999; Zhang et al., 2009). Moreover, a point mutation in *CDC5* gene was responsible of a permanent G2/M cell cycle block in the presence of one irreparable DSB, and to a defect in switching off Rad53 kinase (Toczyski et al., 1997; Pellicioli et al., 2001). All these data prompts us to hypothesize that Cdc5 overexpression may cause a checkpoint bypass in presence of DNA damages. In fact we found that high levels of Cdc5 activity remove the block of the cell cycle after treatment with the alkylating agent MMS, and inhibit the activity of the Rad53 kinase after treatment with zeocin, an agent that causes DSBs, in G_2/M blocked cells (Donnianni et al., 2010, Fig. 1). The same occurs also after the formation of a single DSB through the

expression of the HO nuclease under the galactose promoter (Donnianni et al., 2010, fig. 2). Therefore we concluded that the overexpression of Cdc5 activity is able to switch off both the replicative and the mitotic DNA damage checkpoint, blocking the Rad53 kinase activity. Then, to test if Cdc5 might act upstream Rad53 activation, we decided to verify if also the Mec1-dependent *in trans* phosphorylation of Rad53 was abrogated. Using a specific kinase-dead allele of Rad53, which can be phosphorylated in trans by Mec1, but has lost the auto-phosphorylation activity, we found that also the Mec1 dependent phosphorylation of Rad53 is inhibited (Donnianni et al., 2010, fig.3). We analyse also the phosphorylation of the upstream checkpoint factors Ddc2 and Rad9, both depending on Mec1. As expected, the Ddc2 and Rad9 phosphorylation were strongly reduced, confirming our hypothesis that Cdc5 acts upstream in the checkpoint signal transduction cascade.

One possibility that we decide to investigate was that the overexpression of Cdc5 might affect the DSB resection process, reducing the checkpoint activation. Indeed this was the case, as we found that the overexpression of Cdc5 lead to a mild resection defect, which is dependent on Rad9. However, we also noted that the effect on DSB resection is likely too low to be considered the only mechanism through which Cdc5 counteract the activation of the checkpoint response (Donnianni et al., 2010 fig. 4-5). We also excluded that Cdc5 might block the recruitment of checkpoint activator factors, in fact, by ChIP analysis we found that Ddc1, Dpb11, Ddc2 and Rad9 have been loaded at the same amount with or without Cdc5 overexpression (Donnianni et al., 2010, fig. 6). Then, we surprisingly found that Sae2 loading near the DSB site increased when Cdc5 was overexpressed and, moreover, we also found that it was hyper-phosphorylated (Donnianni et al., 2010, fig. 7). As I discussed in the State of the Art section, Sae2 is phosphorylated by CDK1 in G_2/M (Huertas et al., 2008), raising the possibility that Cdc5, through its PBD domain, binds CDK1-dependent phosphorylated sites in Sae2. Indeed, we performed two hybrid and pulldown assays using the Cdc5 PBD domain as bait.

This domain is essential to target all the PLKs on the correct targets. Interestingly, the PBD interacts with the Sae2 protein in both the assays (Donnianni et al., 2010, fig.8), supporting the idea that Cdc5 binds and phosphorylates Sae2, regulating its binding activity to DSB sites.

In conclusion in this paper we showed that: 1) elevated levels of Cdc5 cause the inhibition of the DNA damage checkpoint acting at an apical step of the signaling; 2) Cdc5 overexpression affects the DSB end processing in a Rad9 dependent manner; 3) Cdc5 binds and likely phosphorylates Sae2 causing its accumulation on the break.

Sae2 was already involved in checkpoint switch off after the formation of a DSB and, interestingly, also the Sae2 overexpression, counteracts the checkpoint response (Clerici et al., 2006), as we found for Cdc5. This raises the intriguing possibility that the overexpressed Cdc5 targets Sae2, leading to an increased loading of Sae2 on the break, and a faster checkpoint switch off.

2. The RSC chromatin-remodeling complex influences mitotic exit and adaptation to the spindle assembly checkpoint by controlling the Cdc14 phosphatase (Rossio et al., 2010)

A prerequisite to correct chromosomes segregation is the binding between the mitotic spindle and kinetochores. After this event has been established accurately, cohesins cleavage and sister chromatids separation can occur, promoting cell division.

A surveillance mechanism, called the Spindle Assembly Checkpoint (SAC), monitors the correct sequence of these events. SAC is a well conserved pathway in all the eukaryotes. During the kinetochores-spindle attachment the mitotic checkpoint complex formed by Bub3, Mad2 and Mad3 inhibits the APC subunit Cdc20, arresting the cell cycle until all the kinetochores are properly attached to the mitotic spindle (Musacchio & Salmon, 2007). However it has been shown that cells do not arrest indefinitely in the presence of an active SAC. Indeed, after a prolong block, cells re-enter the cell cycle despite the presence of unattached kinetochores. This process is called SAC adaptation or mitotic slippage, and it has been linked with the ability of tumoral cells to survive to the treatment with chemotherapeutic compounds that affect the mitotic spindle stability like taxanes and vinca alkaloids (Rieder & Maiato, 2004). Different factors are required for this event and, collaborating with Dr. Piatti (CRBM, Montpellier, France) we showed that Cdc5 and the chromatin remodelled complex RSC have a role in the release of Cdc14 phosphatase from the nucleolus, leading to SAC adaptation. In this work, my experimental contribution was the pulldown approach to show the physical Cdc5-Rsc2 interaction, which was suggested by other genetic results previously obtained by Dr. Piatti laboratory. I found that the Cdc5 PBD domain interacts in vitro with the Rsc2 protein and, interestingly, the residues in the PBD domain responsible for the interaction with the target proteins through a canonical mechanism are

dispensable (Rossio et al., 2010, fig.9 panel B), suggesting that a priming phosphorylation of the Rsc2 protein is not required for the interaction The functional role of the Cdc5-Rsc2 interaction is still not understood, however it is tempting to speculate that it might influence the Cdc5 ability to regulate the Cdc14 release from the nucleolus. Further investigations are required to characterize the molecular mechanism and function of this novel pathway.

3. Tid1/Rdh54 translocase is phosphorylated through a Mec1- and Rad53-dependent manner in the presence of DSB lesions in budding yeast (Ferrari et al., 2013)

Tid1/Rdh54 is a recombination factor, member of the Swi2-like family of chromatin remodellers. These proteins have dsDNA ATP dependent translocase activity and, moving along dsDNA filament, remodel nucleosome positioning and chromatin state, and might also affect chromatin around a DSB lesion.

Moreover, these factors can supercoil and unwind DNA and promote D-loop formation and branch migration in homologous recombination processes (San Filippo et al., 2008). Different *in vitro* and *in vivo* data indicated that Tid1 dissociates Rad51 recombinase from dsDNA, thus preventing the accumulation of toxic Rad51-DNA intermediates and also ensuring that a sufficient amount of Rad51 will be available for DSB repair and recombination (Shah et al., 2010). Tid1 shares some molecular functions and mechanisms with the Swi2-like homologs Rad54 and Usl1. However, they likely have distinct functions, as indicated by the distinct phenotypes of the corresponding mutants (Shah et al., 2010). Tid1 plays major role in meiotic recombination, while it is involved in minor pathway in mitotic recombination, specifically in diploids (Shinohara et al., 1997; Klein, 1997). Interestingly, Tid1 has been involved in checkpoint adaptation from a G2/M arrest induced by an irreparable DSB (Lee et al., 2001). To further address the functional role of Tid1 in cells responding to DSB and in checkpoint adaptation, we tested whether Tid1 translocase activity is essential for the adaptation process and also if this protein is post-translationally regulated in the presence of DNA damages. We found that the Tid1 ATPase activity is required for the adaptation process; in fact the ATPase defective mutant *tid1*-K318R cells are not able to switch off the checkpoint after the formation of a single irreparable DSB (Ferrari et al., 2013, fig. 3). Then, by western blotting analysis we could not see any mobility shift of Tid1 protein during

a normal cell cycle or after treatment with drugs that activates the intra-S phase checkpoint but, strikingly, we are able to see a mobility shift of Tid1 when a single DSB is formed by the HO nuclease, or after treatment with zeocin and 4-NQO (Ferrari et al., 2013, fig. 1 and 3). This modification was completely reverted in vitro by phosphatase treatment, indicating that it is due to a phosphorylation event (Ferrari et al., 2013, fig. 1).

Both the wild type and the ATPase defective protein showed the DSB-induced phosphorylation, even if the Tid1-K318R variant has a slightly higher mobility shift respect to the wild type protein (Ferrari et al., 2013, fig. 3). We hypothesized that the DSB-induced phosphorylation was made by the checkpoint kinases, since Tid1protein sequence has several putative Mec1/Tel1 and Rad53 phosphorylation motifs. In fact, we found that the DSB-induced Tid1 phosphorylation and hyper-phosphorylation are affected in mec1 and rad53 mutants (Ferrari et al., 2013, fig. 1-3), similarly to other factors such as Srs2, Rad51, Sae2, and Cdc5 involved in turning off Rad53 during checkpoint adaptation (Liberi et al., 2000; Flott et al., 2011; Baroni et al., 2004; Cheng et al., 1998).

Then, we found that the ATPase activity is dispensable for Tid1 loading near a DSB site and, interestingly, the Tid1-K318R variant accumulates on to the DSB to a greater extend (Ferrari et al., 2013, fig. 5). Thus it is possible to suggest that the ATPase defective variant remains blocked onto the lesion, and this event enables us to see more clearly a very transient Rad53 and Rad51-dependent phosphorylation of Tid1. We also found that the recruitment of Tid1 near the DSB site is strongly reduced in *mec1* mutant cells (Ferrari et al., 2013, fig. 5), suggesting that the checkpoint-dependent phosphorylation might affect the binding of Tid1 to the DSB. The functional role of Tid1 onto the DSB lesion is still unclear, but we can hypothesize that it is involved in the regulation/removal of nucleosome or other proteins from the lesion, which might allow a correct DSB processing and checkpoint adaptation.

4. A biochemical screening to identify novel Cdc5 interactors in DNA damage condition.

In this part of the results, I'm presenting the work done during the last period of my Ph.D. In particular, the project on the DSB resection in *sae2* mutant cells is very promising and hopefully it will be ready soon for a publication manuscript.

4.1. The screening procedure

It has been shown that the polo kinases are involved in checkpoint switch off in different organisms but the molecular mechanism and the factors involved are unknown. Cdc5 is the only polo kinase in *S. cerevisiae*, which is the model organism I used in my Ph.D. project. In order to identify novel Cdc5 interacting factors during DNA damage checkpoint response, I performed a biochemical screening by GST pulldown coupled with mass spectrometry to obtain the interactors identification. This kind of approach has already been used for the identification of the PLK1 and Cdc5 interactors in undamaged condition (Lowery et al., 2007; Snead et al., 2007). First of all I prepared a plasmid expressing just the Polobox domain of Cdc5 (PBD, aa 357 to 705), carrying one N-terminal GST tag and a small C-terminal 6XHis tag (GST-PBD-His) (Supplementary Figure 1). The chimeric protein was produced and purified from *E. coli* in two sequential steps. By the first step, the *E. coli* crude extract was passed through a Ni-NTA resin column, in order purify only the full length GST-PBD-His polypeptides, discarding the unwanted truncated forms of the protein. Then, the GST-PBD-6His proteins were eluted and passed through a second column containing a glutathione resin. This tandem purification system allowed the production of a nearly clean bait protein, which can be used in the pulldown experiments. To that purpose, I prepared yeast crude extracts from cells suffering DNA damage. In particular, in one case I collected cells after 6 hours of induction of the HO nuclease, which causes the formation of one irreparable DSB in the genetic

system I was using (Moore & Haber, 1996); in the second case I collected cells after 12 hours from DSB formation, during the checkpoint adaptation process; in the third case I collected cells after 3 hours treatment with the alkylating agent MMS, which blocks DNA replication and activates the DNA damage checkpoint. In the three different treatments, Rad53 phosphorylation was monitored to test the activation of the DNA damage checkpoint (data not shown).

The yeast crude extracts were incubated with the GST-PBD-6His resin, and then the resin was boiled and run on a SDS-PAGE gel in order to separate the proteins from the resin. At this point the gel was stained with the coomassie and gel slices containing putative interacting proteins were cut, processed and analysed by mass spectrometry in the Dr. Santocanale laboratory (Galway, National University of Ireland). See Supplementary Figure 2 for a scheme of the procedure.

The mass spectrometer output was analysed with the online free software GPM (www.thegpm.org) in order to obtain the interactors identification; then a graphical representation of the interaction network was obtained for each experiments using the Cytoscape program (www.cytoscape.org) (Supplementary Figures 3, 4 and 5). As expected by previous genetic and biochemical findings (Snead et al., 2007), many proteins were found to interact with the Cdc5-PBD. These proteins are involved in cell cycle regulation, translation regulation, energetic metabolism and DNA damage response. The pulldown of some factors, including the cohesins complex, Dbf4, Cdc48 and Ivy1, already known to interact with Cdc5, validated the screening procedure.

In the following table I'm listing the more promising interactors involved in DNA damage response, and in the lab we are planning to further characterize their roles.

Protein	MW (KDa)	Peptides	Deletion	DDR	Function (from SGD database)
Gcn2	190.1	9	Viable	Si	Protein kinase, phosphorylates the alpha-subunit of translation initiation factor eIF2 (Sui2p) in response to starvation; activated by uncharged tRNAs and the Gcn1p-Gcn20p complex; contributes to DNA damage checkpoint control
Tom1	374,2	5	Viable	Si	E3 ubiquitin ligase of the hect-domain class; has a role in mRNA export from the nucleus and may regulate transcriptional coactivators; involved in degradation of excess histones
Mms1	161,2	1	Viable	Si	Subunit of an E3 ubiquitin ligase complex involved in resolving replication intermediates or preventing the damage caused by blocked replication forks; regulates Ty1 transposition; involved with Rtt101p in nonfunctional rRNA decay
Bre1	80,7	1	Viable	Si	E3 ubiquitin ligase, forms heterodimer with Rad6p to monoubiquinate histone H2B-K123, which is required for the subsequent methylation of histone H3-K4 and H3-K79; required for DSBR, transcription, silencing, and checkpoint control
Sae2	40	1	Viable	Si	Endonuclease that processes hairpin DNA structures with the MRX complex; involved in meiotic and mitotic double-strand break repair; phosphorylated in response to DNA damage and required for normal resistance to DNA-damaging agents
Fyv6	20	1	Viable	Si	Protein of unknown function, required for survival upon exposure to K1 killer toxin; proposed to regulate double-strand break repair via non-homologous end-joining
Sap190	125	4	Viable		Protein that forms a complex with the Sit4p protein phosphatase and is required for its function; member of a family of similar proteins including Sap4p, Sap155p, and Sap185p
Sap185	121,3	1	Viable		Protein that forms a complex with the Sit4p protein phosphatase and is required for its function; member of a family of similar proteins including Sap4p, Sap155p, and Sap190p
Sit4	35,5	1	Viable		Type 2A-related serine-threonine phosphatase that functions in the G1/S transition of the mitotic cycle; cytoplasmic and nuclear protein that modulates functions mediated by Pkc1p including cell wall and actin cytoskeleton organization

Interestingly, in all the analysis the Gcn2 kinase was scored with the larger number of the peptides found, suggesting that it can be a relevant Cdc5 interactor. Gcn2 has been already involved in the response to alkylating agent MMS (Menacho-Marchez et al., 2007), and its human homolog hGcn2 has a role in apoptosis induction in tumoral cells after chemotherapeutic treatment (Peidis et al., 2010). For these reasons, in the lab we are validating the *in vivo* interaction between Cdc5 and the kinase Gcn2 with other methods (Two Hybrid, CoIP), and we are investigating the

role of this protein in checkpoint adaptation and recovery from DNA damage. However in this thesis I will focus my attention only on the pulldown factor Sae2, that is involved in DSB ends resection process (See the State of the Art section).

4.2. Characterization of the Cdc5-Sae2 interaction

I have already found by 2H and pulldown assays that Sae2 interacts with Cdc5, and that Sae2 is hyper-phosphorylated when Cdc5 is overexpressed (see the result section R.2 and the accompanying paper Donnianni et al., 2010).

It is known that the PBD domain preferentially binds proteins previously phosphorylated by a priming kinase, which is often CDK1 (Elia et al., 2003). In particular, in the case of the Cdc5 PBD, the best consensus binding motif is a SSP, in which the second serine has been phosphorylated by CDK1. Interestingly, through the analysis of the Sae2 aminoacid sequence, we identified 3 putative CDK1 sites that can be bind by Cdc5: serine 134, serine 179 and serine 267 (Donnianni et al., 2010, fig. 8). Among these sites, serine 267 is in the evolutionary conserved C-terminal domain and it was already shown that the CDK1-mediated phosphorylation of this site primes Sae2 for the DSB resection (Huertas et al., 2008).

To verify if these sites were involved in the Cdc5 binding, I mutagenized the corresponding sites by substituting each serine with alanine (an aminoacid that cannot be phosphorylated), in *SAE2* gene cloned in to an integrative plasmid vector that I used to direct the gene replacement at the endogenous locus. I obtained the following mutant strains: *sae2*-S134A, *sae2*-S179A, *sae2*-S267A and the triple mutant called sae2-3Ala, in which all the three sites are mutagenized to alanine. First of all, I analyse the expression level and the phosphorylation state of the various protein variants by western blot after a SDS-PAGE (Supplementary Figure 6). I produced the protein extracts from yeast cells grown in untreated condition, or treated with nocodazole, to block cell cycle in G2/M phase, or in the presence of one irreparable HO-cut. I found that the different protein variants are expressed more or

less at the same level in the untreated conditions, while the wild type protein is phosphorylated in cells treated with nocodazole, and hyper-phosphorylated following DNA damage, according to previous data (Baroni et al., 2004). Interestingly, the mobility shift of the sae2-3Ala variant is severally impaired respect to the wild type protein, while the single protein variants do not show any clear differences.

Then I tested if the same phosphorylation sites were involved in the interaction with Cdc5-PBD. To address this question, I performed a GST-pulldown assay using the same GST-PBD-6XHis column described above and crude protein extract prepared from *sae2*-3Ala mutant cells. I found that the Cdc5-PBD is able to bind normally the Sae2-3Ala protein variant (Supplementary Figure 7), suggesting that the Cdc5-Sae2 interaction may follow unconventional role, as already described for other target proteins (Rahal & Amon, 2008; Rossio et al., 2010; Chen & Weinreich, 2011). Therefore, I decided to further investigate the Cdc5-Sae2 interaction and, in order to find the Sae2 region bound by the Cdc5-PBD, I prepared yeast strains expressing different truncated variants of Sae2 protein (Supplementary Figure 8). Then, I performed GST-pulldown experiments and I found that the region involved in the Cdc5-Sae2 interaction is probably localized at the C-terminal part of Sae2 (Supplementary Figure 8). Unfortunately, I also noted that the Sae2-Δ_{C170} variant is a very instable protein, thus I am not confident on the obtained result. However, the finding that the Cdc5-PBD interacting region is in the evolutionary conserved C-terminal domain of Sae2, suggests the intriguingly possibility that this interaction could be also conserved in other organisms, between PLK1 and CtIP.

4.3. The CDK1-mediated phosphorylation of Sae2 is required for checkpoint recovery

As I discussed in the State of the Art section, a CDK1-mediated phosphorylation activates Sae2 in the DSB resection process (Huertas et al., 2008). Moreover, it has been published by Longhese laboratory that *sae2Δ* cells do not recover from a DSB, leading to a prolonged Rad53 activation (Clerici et al., 2005).

I decided to test the possibility that the CDK1-mediated phosphorylation of Sae2 may be involved not only in the early step of the DSB processing, but also in the checkpoint recovery. To this aim, I took advantage of the YMV80 genetic background (Vaze et al., 2002), in which the conditional expression of the HO endonuclease under the galactose promoter causes the formation of one DSB in a specific locus in chromosome III. It was shown that this DSB could be repaired through SSA or BIR process (Jain et al., 2009), as summarized in the following scheme (fig. 6).
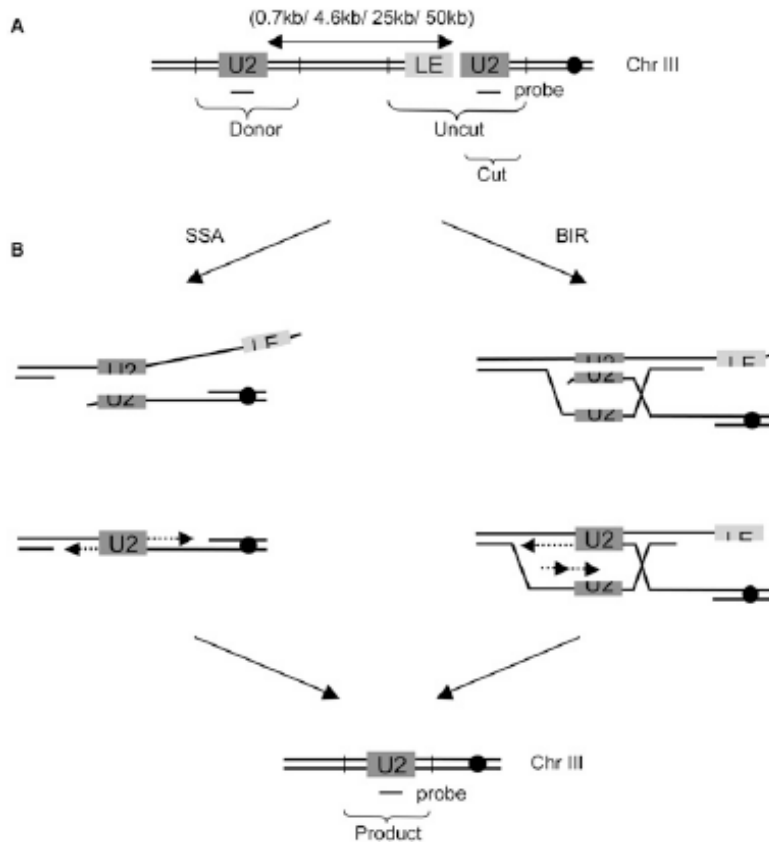
Figure 6: Schematic representation of the DSB repair by SSA or BIR in the YMV80 strain (from Jain et al., 2009).

First of all, I analysed the viability of the *sae2Δ* cells in YMV80 background, in plates containing raffinose or galactose. I also tested the contribution of the Rad51-dependent BIR pathway analysing double mutant *sae2Δ rad51Δ*. According to previous finding (Clerici et al., 2005), I found *sae2Δ* cells do not completely recover from a single DSB, moreover the viability of the double mutant *sae2Δ rad51Δ* cells is almost completely abolished (Supplementary Figure 9), supporting the hypothesis that the residual cell viability observed in the single *sae2Δ* mutant is likely due to BIR-mediated repair.

Then I introduced the various *sae2* mutations described above in the YMV80 background, and I tested cells viability on plates containing galactose or raffinose. I

found that the *sae2*-3Ala mutant cells show a reduced viability following DSB formation (galactose plate), mirroring the phenotype already observed in *sae2*Δ cells. Interestingly, I also found that while the *sae2*-S179A cells are able to grow as the wild type, the single *sae2*-S134A and *sae2*-S267A mutations show a mild sensitivity to the DSB, I also tested *sae2*-S134A-S267A double mutant, and I found that it had the same phenotype of the *sae2*-3Ala and the *sae2*Δ strains. Thus, I concluded that the CDK1-mediated phosphorylation of both the S134 and the S267 sites are required for the Sae2 function in DSB repair and checkpoint recovery (Supplementary Figure 10, panel A).

Then, to further characterize the functional role of the CDK1-dependent Sae2 phosphorylation, I checked the cells viability of the various *sae2* mutants in camptothecin (CPT). This drug is an alkaloid compound that blocks the topoisomerase 1 in a protein-DNA toxic complex, leading to the formation of an intermediate normally converted in to a DSB during replication. Sae2 has already been shown to be required to remove the DNA-Top1 adduct and promote DSB repair, and *sae2* Δ cells are sensible to CPT treatments (Deng et al., 2005). I found that the *sae*2-3Ala and the *sae2*-S134A-S267A double mutant showed the same sensibility to CPT as the *sae2* Δ , while the *sae2*-S134A and the *sae2*-S267A showed a minor sensitivity (Supplementary Figure 10, panel B).

4.4. DSB-related defects of *sae2* mutants are suppressed by the deletion of *RAD9* gene

Few years ago, in our laboratory we discovered a functional role of the checkpoint factor Rad9 in controlling DSB resection. In particular, it was showed that Rad9 counteracts the DSB processing, likely acting as resection barrier (Lazzaro et al., 2008). I also found that the deletion of *RAD9* gene rescues the resection delay caused by the overproduction of Cdc5 (Donnianni et al., 2010).

Therefore, I decided to test if the *rad9Δ* mutation may rescue the *sae2Δ* phenotype observed in the presence of one DSB in the YMV80 background. Interestingly, I found that it was the case: the double mutant *sae2Δ rad9Δ* cells are perfectly viable following DSB formation in the plates containing galactose (Supplementary Figure 11). Moreover, I also found that the deletion of *RAD9* rescues the lethality observed in the *sae2*-3Ala cells (Supplementary Figure 11).

At the same time I performed a Southern blot analysis of the repair process (as described in Vaze et al., 2002), and I confirmed that the increased viability of the *sae2Δrad9Δ* double mutant correlates with an increase in DSB repair (Supplementary Figure 12).

I have also tested the possibility that the Rad9 deletion in some way may channel the unprocessed DSB, generated in *sae2Δ*, in to the BIR repair pathway. However the triple mutant *sae2Δ rad9Δ rad51Δ* cells recovers very well respect the *sae2Δ rad51Δ* cells, indicating that BIR is not involved (Supplementary Figure 13).

Then, I tested if also the deletion of *DDC1*, which acts upstream Rad9 in the checkpoint signaling pathway (see the State of the Art section), and the kinase-defective *rad53*-K227A mutation, may rescue *sae2Δ* phenotype. Interestingly, I found that both the *ddc1Δ* and the *rad53*-K227A mutations do not rescue cell viability of the *sae2Δ* strain (Supplementary Figure 14). These results support the hypothesis that the suppression is due to a Rad9 function different than its role in the checkpoint signaling pathway, and we believed it is related to the DSB resection barrier.

It is known that Sae2 works together with the MRX complex in the first step of the DSB resection. Thus, if this model would be correct I was expected to find the same Rad9-dependent sensitivity to DSB in both the *sae2Δ* and *mre11* nuclease-defective mutants. Indeed I found that the two different *mre11* nuclease-defective alleles *mre11*-D16A and *mre11*-D56A show cells lethality following DSB formation, which is partially suppressed by the *rad9Δ* mutation (Supplementary Figure 15).

Theses results further confirm that the MRX complex, together with Sae2, plays a fundamental role to initiate DSB resection and trigger the extensive processing to allow SSA repair.

Moreover, taking all these data together I could hypothesize that Rad9 plays an inhibitory role in the formation of long ssDNA tracts, probably acting on the resection factors involved in this process.

The Exo1 nuclease and the Sgs1-Dna2 helicase-nuclease complex have been involved in the second step of the DSB resection (Mimitou & Symington, 2008; Zhu et al., 2008, and see also the State of the Art section).

Therefore, in order to test which nuclease mediate the DSB processing in *sae2Δ* cells in the absence of Rad9 barrier, I generated the triple mutant strains *sae2Δ rad9Δ exo1Δ* and *sae2Δ rad9Δ sgs1Δ*. It is important to mention that the generation of the triple mutant *sae2Δ rad9Δ sgs1Δ* was particularly difficult, because it is known that the deletion of *SGS1* gene is lethal in *sae2Δ* cells (Mimitou & Symington, 2010). However, we found that the deletion of RAD9 gene suppresses the *sae2Δ sgs1Δ* synthetic lethality, as it does for *dna2Δ* lethality (Budd et al., 2011). Then I plated the cells in galactose, and I interestingly found that Exo1 is not involved; in fact the triple mutant has the same cells viability of the double mutant *sae2Δ rad9Δ* and wild type (Supplementary Figure 16).

On the contrary, the viability of the *sae2Δ rad9Δ sgs1Δ* cells in the presence of the DSB is dramatically reduced (Supplementary Figure 17), strongly supporting the idea that Sgs1-Dna2 activity is required for the DSB processing and checkpoint recovery in *sae2Δ rad9Δ*.

At the moment I have in progress the experimental analysis by Southern blotting to test the DSB processing in these mutants and confirm the model.

Bibliography

Agarwal, R.; Tang, Z.; Yu, H. & Cohen-Fix, O. (2003), 'Two distinct pathways for inhibiting pds1 ubiquitination in response to DNA damage.', *J Biol Chem* **278**(45), 45027--45033.

Alcasabas, A. A.; Osborn, A. J.; Bachant, J.; Hu, F.; Werler, P. J.; Bousset, K.; Furuya, K.; Diffley, J. F.; Carr, A. M. & Elledge, S. J. (2001), 'Mrc1 transduces signals of DNA replication stress to activate Rad53.', *Nat Cell Biol* **3**(11), 958--965.

Alexandru, G.; Uhlmann, F.; Mechtler, K.; Poupart, M. A. & Nasmyth, K. (2001), 'Phosphorylation of the cohesin subunit Scc1 by Polo/Cdc5 kinase regulates sister chromatid separation in yeast.', *Cell* **105**(4), 459--472.

Andersen, S. L.; Bergstralh, D. T.; Kohl, K. P.; LaRocque, J. R.; Moore, C. B. & Sekelsky, J. (2009), 'Drosophila MUS312 and the vertebrate ortholog BTBD12 interact with DNA structure-specific endonucleases in DNA repair and recombination.', *Mol Cell* **35**(1), 128--135.

van Attikum, H.; Fritsch, O. & Gasser, S. M. (2007), 'Distinct roles for SWR1 and INO80 chromatin remodeling complexes at chromosomal double-strand breaks.', *EMBO J* **26**(18), 4113--4125.

van Attikum, H.; Fritsch, O.; Hohn, B. & Gasser, S. M. (2004), 'Recruitment of the INO80 complex by H2A phosphorylation links ATP-dependent chromatin remodeling with DNA double-strand break repair.', *Cell* **119**(6), 777--788.

van Attikum, H. & Gasser, S. M. (2009), 'Crosstalk between histone modifications during the DNA damage response.', *Trends Cell Biol* **19**(5), 207--217.

Awad, S.; Ryan, D.; Prochasson, P.; Owen-Hughes, T. & Hassan, A. H. (2010), 'The Snf2 homolog Fun30 acts as a homodimeric ATP-dependent chromatin-remodeling enzyme.', *J Biol Chem* **285**(13), 9477--9484.

Aylon, Y.; Liefshitz, B. & Kupiec, M. (2004), 'The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle.', *EMBO J* **23**(24), 4868--4875.

Bahassi, E. M. (2011), 'Polo-like kinases and DNA damage checkpoint: beyond the traditional mitotic functions.', *Exp Biol Med (Maywood)* **236**(6), 648--657.

Baroni, E.; Viscardi, V.; Cartagena-Lirola, H.; Lucchini, G. & Longhese, M. P. (2004), 'The functions of budding yeast Sae2 in the DNA damage response require Mec1- and Tel1-dependent phosphorylation.', *Mol Cell Biol* **24**(10), 4151--4165.

Bernstein, K. A.; Shor, E.; Sunjevaric, I.; Fumasoni, M.; Burgess, R. C.; Foiani, M.; Branzei, D. & Rothstein, R. (2009), 'Sgs1 function in the repair of DNA replication intermediates is separable from its role in homologous recombinational repair.', *EMBO J*(38).

Bosco, G. & Haber, J. E. (1998), 'Chromosome break-induced DNA replication leads to nonreciprocal translocations and telomere capture.', *Genetics* **150**(3), 1037--1047.

Branzei, D. & Foiani, M. (2010), 'Maintaining genome stability at the replication fork.', *Nat Rev Mol Cell Biol* **11**(3), 208--219.

Branzei, D. & Foiani, M. (2008), 'Regulation of DNA repair throughout the cell cycle.', *Nat Rev Mol Cell Biol* **9**(4), 297--308.

Budd, M. E.; Antoshechkin, I. A.; Reis, C.; Wold, B. J. & Campbell, J. L. (2011), 'Inviability of a DNA2 deletion mutant is due to the DNA damage checkpoint.', *Cell Cycle* **10**(10), 1690--1698.

Bunting, S. F.; Callén, E.; Wong, N.; Chen, H.-T.; Polato, F.; Gunn, A.; Bothmer, A.; Feldhahn, N.; Fernandez-Capetillo, O.; Cao, L.; Xu, X.; Deng, C.-X.; Finkel, T.; Nussenzweig, M.; Stark, J. M. & Nussenzweig, A. (2010), '53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks.', *Cell* **141**(2), 243--254.

de Cárcer, G.; Manning, G. & Malumbres, M. (2011), 'From Plk1 to Plk5: Functional evolution of polo-like kinases.', *Cell Cycle* **10**(14), 2255--2262.

Chai, B.; Huang, J.; Cairns, B. R. & Laurent, B. C. (2005), 'Distinct roles for the RSC and Swi/Snf ATP-dependent chromatin remodelers in DNA double-strand break repair.', *Genes Dev* **19**(14), 1656--1661.

Chambers, A. L. & Downs, J. A. (2012), 'The RSC and INO80 chromatin-remodeling complexes in DNA double-strand break repair.', *Prog Mol Biol Transl Sci* **110**, 229--261.

Chapman, J. R.; Barral, P.; Vannier, J.-B.; Borel, V.; Steger, M.; Tomas-Loba, A.; Sartori, A. A.; Adams, I. R.; Batista, F. D. & Boulton, S. J. (2013), 'RIF1 is essential for 53BP1-dependent nonhomologous end joining and suppression of DNA double-strand break resection.', *Mol Cell* **49**(5), 858--871.

Chen, X.; Cui, D.; Papusha, A.; Zhang, X.; Chu, C.-D.; Tang, J.; Chen, K.; Pan, X. & Ira, G. (2012), 'The Fun30 nucleosome remodeller promotes resection of DNA double-strand break ends.', *Nature* **489**(7417), 576--580.

Chen, X.; Niu, H.; Chung, W.-H.; Zhu, Z.; Papusha, A.; Shim, E. Y.; Lee, S. E.; Sung, P. & Ira, G. (2011), 'Cell cycle regulation of DNA double-strand break end resection by Cdk1-dependent Dna2 phosphorylation.', *Nat Struct Mol Biol*.

Chen, X. & Tomkinson, A. E. (2011), 'Yeast Nej1 is a key participant in the initial end binding and final ligation steps of nonhomologous end joining.', *J Biol Chem* **286**(6), 4931--4940.

Chen, Y.-C. & Weinreich, M. (2010), 'Dbf4 regulates the Cdc5 Polo-like kinase through a distinct non-canonical binding interaction.', *J Biol Chem* **285**(53), 41244--41254.

Cheng, L.; Hunke, L. & Hardy, C. F. (1998), 'Cell cycle regulation of the Saccharomyces cerevisiae polo-like kinase cdc5p.', *Mol Cell Biol* **18**(12), 7360--7370.

Chung, W.-H.; Zhu, Z.; Papusha, A.; Malkova, A. & Ira, G. (2010), 'Defective resection at DNA double-strand breaks leads to de novo telomere formation and enhances gene targeting.', *PLoS Genet* **6**(5), e1000948.

Ciccia, A. & Elledge, S. J. (2010), 'The DNA damage response: making it safe to play with knives.', *Mol Cell* **40**(2), 179--204.

Ciosk, R.; Zachariae, W.; Michaelis, C.; Shevchenko, A.; Mann, M. & Nasmyth, K. (1998), 'An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast.', *Cell* **93**(6), 1067--1076.

Clémenson, C. & Marsolier-Kergoat, M.-C. (2009), 'DNA damage checkpoint inactivation: adaptation and recovery.', *DNA Repair (Amst)* **8**(9), 1101--1109.

Clerici, M.; Mantiero, D.; Guerini, I.; Lucchini, G. & Longhese, M. P. (2008), 'The Yku70-Yku80 complex contributes to regulate double-strand break processing and checkpoint activation during the cell cycle.', *EMBO Rep* **9**(8), 810--818.

Clerici, M.; Mantiero, D.; Lucchini, G. & Longhese, M. P. (2006), 'The Saccharomyces cerevisiae Sae2 protein negatively regulates DNA damage checkpoint signalling.', *EMBO Rep* **7**(2), 212--218.

Clerici, M.; Mantiero, D.; Lucchini, G. & Longhese, M. P. (2005), 'The Saccharomyces cerevisiae Sae2 protein promotes resection and bridging of double strand break ends.', *J Biol Chem* **280**(46), 38631--38638.

Costelloe, T.; Louge, R.; Tomimatsu, N.; Mukherjee, B.; Martini, E.; Khadaroo, B.; Dubois, K.; Wiegant, W. W.; Thierry, A.; Burma, S.; van Attikum, H. & Llorente, B. (2012), 'The yeast Fun30 and human SMARCAD1 chromatin remodellers promote DNA end resection.', *Nature* **489**(7417), 581--584.

Daley, J. M.; Palmbos, P. L.; Wu, D. & Wilson, T. E. (2005), 'Nonhomologous end joining in yeast.', *Annu Rev Genet* **39**, 431--451.

Deng, C.; Brown, J. A.; You, D. & Brown, J. M. (2005), 'Multiple endonucleases function to repair covalent topoisomerase I complexes in Saccharomyces cerevisiae.', *Genetics* **170**(2), 591--600.

Di Virgilio, M.; Callen, E.; Yamane, A.; Zhang, W.; Jankovic, M.; Gitlin, A. D.; Feldhahn, N.; Resch, W.; Oliveira, T. Y.; Chait, B. T.; Nussenzweig, A.; Casellas, R.; Robbiani, D. F. & Nussenzweig, M. C. (2013), 'Rif1 prevents resection of DNA breaks and promotes immunoglobulin class switching.', *Science* **339**(6120), 711--715.

Dizdaroglu, M. (2012), 'Oxidatively induced DNA damage: mechanisms, repair and disease.', *Cancer Lett* **327**(1-2), 26--47.

Donnianni, R. A.; Ferrari, M.; Lazzaro, F.; Clerici, M.; Nachimuthu, B. T.; Plevani, P.; Muzi-Falconi, M. & Pellicioli, A. (2010), 'Elevated levels of the polo kinase Cdc5 override the Mec1/ATR checkpoint in budding yeast by acting at different steps of the signaling pathway.', *PLoS Genet* **6**(1), e1000763.

Durand-Dubief, M.; Will, W. R.; Petrini, E.; Theodorou, D.; Harris, R. R.; Crawford, M. R.; Paszkiewicz, K.; Krueger, F.; Correra, R. M.; Vetter, A. T.; Miller, J. R.; Kent, N. A. & Varga-Weisz, P. (2012), 'SWI/SNF-like chromatin remodeling factor Fun30 supports point centromere function in S. cerevisiae.', *PLoS Genet* **8**(9), e1002974.

Eapen, V. V.; Sugawara, N.; Tsabar, M.; Wu, W.-H. & Haber, J. E. (2012), 'The Saccharomyces cerevisiae chromatin remodeler Fun30 regulates DNA end resection and checkpoint deactivation.', *Mol Cell Biol* **32**(22), 4727--4740.

Elia, A. E. H.; Cantley, L. C. & Yaffe, M. B. (2003), 'Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates.', *Science* **299**(5610), 1228--1231.

Escribano-Díaz, C.; Orthwein, A.; Fradet-Turcotte, A.; Xing, M.; Young, J. T. F.; Tkáč, J.; Cook, M. A.; Rosebrock, A. P.; Munro, M.; Canny, M. D.; Xu, D. & Durocher, D. (2013), 'A cell cycle-dependent regulatory circuit composed of 53BP1-RIF1 and BRCA1-CtIP controls DNA repair pathway choice.', *Mol Cell* **49**(5), 872--883.

Falck, J.; Coates, J. & Jackson, S. P. (2005), 'Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage.', *Nature* **434**(7033), 605--611.

Fekairi, S.; Scaglione, S.; Chahwan, C.; Taylor, E. R.; Tissier, A.; Coulon, S.; Dong, M.-Q.; Ruse, C.; Yates, J. R.; Russell, P.; Fuchs, R. P.; McGowan, C. H. & Gaillard, P.-H. L. (2009), 'Human SLX4 is a Holliday junction resolvase subunit that binds multiple DNA repair/recombination endonucleases.', *Cell* **138**(1), 78--89.

Flott, S.; Alabert, C.; Toh, G. W.; Toth, R.; Sugawara, N.; Campbell, D. G.; Haber, J. E.; Pasero, P. & Rouse, J. (2007), 'Phosphorylation of Slx4 by Mec1 and Tel1 regulates the single-strand annealing mode of DNA repair in budding yeast.', *Mol Cell Biol* **27**(18), 6433--6445.

Flott, S.; Kwon, Y.; Pigli, Y. Z.; Rice, P. A.; Sung, P. & Jackson, S. P. (2011), 'Regulation of Rad51 function by phosphorylation.', *EMBO Rep* **12**(8), 833--839.

Garcia, V.; Phelps, S. E. L.; Gray, S. & Neale, M. J. (2011), 'Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1.', *Nature* **479**(7372), 241--244.

Giannattasio, M.; Lazzaro, F.; Plevani, P. & Muzi-Falconi, M. (2005), 'The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1 and H3 methylation by Dot1.', *J Biol Chem* **280**(11), 9879--9886.

Golsteyn, R. M.; Lane, H. A.; Mundt, K. E.; Arnaud, L. & Nigg, E. A. (1996), 'The family of polo-like kinases.', *Prog Cell Cycle Res* **2**, 107--114.

Gospodinov, A.; Vaissiere, T.; Krastev, D. B.; Legube, G.; Anachkova, B. & Herceg, Z. (2011), 'Mammalian Ino80 mediates double-strand break repair through its role in DNA end strand resection.', *Mol Cell Biol* **31**(23), 4735--4745.

Gravel, S.; Chapman, J. R.; Magill, C. & Jackson, S. P. (2008), 'DNA helicases Sgs1 and BLM promote DNA double-strand break resection.', *Genes Dev* **22**(20), 2767--2772.

Guillemain, G.; Ma, E.; Mauger, S.; Miron, S.; Thai, R.; Guérois, R.; Ochsenbein, F. & Marsolier-Kergoat, M.-C. (2007), 'Mechanisms of checkpoint kinase Rad53 inactivation after a double-strand break in Saccharomyces cerevisiae.', *Mol Cell Biol* **27**(9), 3378--3389.

Hammet, A.; Magill, C.; Heierhorst, J. & Jackson, S. P. (2007), 'Rad9 BRCT domain interaction with phosphorylated H2AX regulates the G1 checkpoint in budding yeast.', *EMBO Rep* **8**(9), 851--857.

Harrison, J. C. & Haber, J. E. (2006), 'Surviving the breakup: the DNA damage checkpoint.', *Annu Rev Genet* **40**, 209--235.

Hartwell, L. H.; Culotti, J. & Reid, B. (1970), 'Genetic control of the cell-division cycle in yeast. I. Detection of mutants.', *Proc Natl Acad Sci U S A* **66**(2), 352--359.

Heideker, J.; Lis, E. T. & Romesberg, F. E. (2007), 'Phosphatases, DNA damage checkpoints and checkpoint deactivation.', *Cell Cycle* **6**(24), 3058--3064.

Heyer, W.-D.; Ehmsen, K. T. & Liu, J. (2010), 'Regulation of homologous recombination in eukaryotes.', *Annu Rev Genet* **44**, 113--139.

Huertas, P.; Cortés-Ledesma, F.; Sartori, A. A.; Aguilera, A. & Jackson, S. P. (2008), 'CDK targets Sae2 to control DNA-end resection and homologous recombination.', *Nature* **455**(7213), 689--692.

Huertas, P. & Jackson, S. P. (2009), 'Human CtIP mediates cell cycle control of DNA end resection and double strand break repair.', *J Biol Chem* **284**(14), 9558--9565.

Ip, S. C. Y.; Rass, U.; Blanco, M. G.; Flynn, H. R.; Skehel, J. M. & West, S. C. (2008), 'Identification of Holliday junction resolvases from humans and yeast.', *Nature* **456**(7220), 357--361.

Ira, G.; Pellicioli, A.; Balijja, A.; Wang, X.; Fiorani, S.; Carotenuto, W.; Liberi, G.; Bressan, D.; Wan, L.; Hollingsworth, N. M.; Haber, J. E. & Foiani, M. (2004), 'DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1.', *Nature* **431**(7011), 1011--1017.

Ivanov, E. L.; Sugawara, N.; Fishman-Lobell, J. & Haber, J. E. (1996), 'Genetic requirements for the single-strand annealing pathway of double-strand break repair in Saccharomyces cerevisiae.', *Genetics* **142**(3), 693--704.

Jain, S.; Sugawara, N.; Lydeard, J.; Vaze, M.; Gac, N. T. L. & Haber, J. E. (2009), 'A recombination execution checkpoint regulates the choice of homologous recombination pathway during DNA double-strand break repair.', *Genes Dev* **23**(3), 291--303.

Kaliraman, V.; Mullen, J. R.; Fricke, W. M.; Bastin-Shanower, S. A. & Brill, S. J. (2001), 'Functional overlap between Sgs1-Top3 and the Mms4-Mus81 endonuclease.', *Genes Dev* **15**(20), 2730--2740.

Kent, N. A.; Chambers, A. L. & Downs, J. A. (2007), 'Dual chromatin remodeling roles for RSC during DNA double strand break induction and repair at the yeast MAT locus.', *J Biol Chem* **282**(38), 27693--27701.

Klein, H. L. (1997), 'RDH54, a RAD54 homologue in Saccharomyces cerevisiae, is required for mitotic diploid-specific recombination and repair and for meiosis.', *Genetics* **147**(4), 1533--1543.

Kondo, T.; Matsumoto, K. & Sugimoto, K. (1999), 'Role of a complex containing Rad17, Mec3, and Ddc1 in the yeast DNA damage checkpoint pathway.', *Mol Cell Biol* **19**(2), 1136--1143.

Kondo, T.; Wakayama, T.; Naiki, T.; Matsumoto, K. & Sugimoto, K. (2001), 'Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms.', *Science* **294**(5543), 867--870.

Kumar, S. & Burgers, P. M. (2013), 'Lagging strand maturation factor Dna2 is a component of the replication checkpoint initiation machinery.', *Genes Dev* **27**(3), 313--321.

Lancelot, N.; Charier, G.; Couprie, J.; Duband-Goulet, I.; Alpha-Bazin, B.; Quémeneur, E.; Ma, E.; Marsolier-Kergoat, M.-C.; Ropars, V.; Charbonnier, J.-B.; Miron, S.; Craescu, C. T.; Callebaut, I.; Gilquin, B. & Zinn-Justin, S. (2007), 'The checkpoint Saccharomyces cerevisiae Rad9 protein contains a tandem tudor domain that recognizes DNA.', *Nucleic Acids Res* **35**(17), 5898--5912.

Lara-Gonzalez, P.; Westhorpe, F. G. & Taylor, S. S. (2012), 'The spindle assembly checkpoint.', *Curr Biol* **22**(22), R966--R980.

Lazzaro, F.; Sapountzi, V.; Granata, M.; Pellicioli, A.; Vaze, M.; Haber, J. E.; Plevani, P.; Lydall, D. & Muzi-Falconi, M. (2008), 'Histone methyltransferase Dot1 and Rad9 inhibit single-stranded DNA accumulation at DSBs and uncapped telomeres.', *EMBO J* **27**(10), 1502--1512.

Lee, K. S.; Park, J.-E.; Asano, S. & Park, C. J. (2005), 'Yeast polo-like kinases: functionally conserved multitask mitotic regulators.', *Oncogene* **24**(2), 217--229.

Lee, S. E.; Moore, J. K.; Holmes, A.; Umezu, K.; Kolodner, R. D. & Haber, J. E. (1998), 'Saccharomyces Ku70, mre11/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage.', *Cell* **94**(3), 399--409.

Lee, S. E.; Pellicioli, A.; Malkova, A.; Foiani, M. & Habervaz, J. E. (2001), 'The Saccharomyces recombination protein Tid1p is required for adaptation from G2/M arrest induced by a double-strand break.', *Curr Biol* **11**(13), 1053--1057.

Lengsfeld, B. M.; Rattray, A. J.; Bhaskara, V.; Ghirlando, R. & Paull, T. T. (2007), 'Sae2 is an endonuclease that processes hairpin DNA cooperatively with the Mre11/Rad50/Xrs2 complex.', *Mol Cell* **28**(4), 638--651.

Lew, D. J. (2003), 'The morphogenesis checkpoint: how yeast cells watch their figures.', *Curr Opin Cell Biol* **15**(6), 648--653.

Liang, B.; Qiu, J.; Ratnakumar, K. & Laurent, B. C. (2007), 'RSC functions as an early double-strand-break sensor in the cell's response to DNA damage.', *Curr Biol* **17**(16), 1432--1437.

Liberi, G.; Chiolo, I.; Pellicioli, A.; Lopes, M.; Plevani, P.; Muzi-Falconi, M. & Foiani, M. (2000), 'Srs2 DNA helicase is involved in checkpoint response and its regulation requires a functional Mec1-dependent pathway and Cdk1 activity.', *EMBO J* **19**(18), 5027--5038.

Lindahl, T. (1993), 'Instability and decay of the primary structure of DNA.', *Nature* **362**(6422), 709--715.

Llorente, B.; Smith, C. E. & Symington, L. S. (2008), 'Break-induced replication: what is it and what is it for?', *Cell Cycle* **7**(7), 859--864.

Llorente, B. & Symington, L. S. (2004), 'The Mre11 nuclease is not required for 5' to 3' resection at multiple HO-induced double-strand breaks.', *Mol Cell Biol* **24**(21), 9682--9694.

Lobachev, K.; Vitriol, E.; Stemple, J.; Resnick, M. A. & Bloom, K. (2004), 'Chromosome fragmentation after induction of a double-strand break is an active process prevented by the RMX repair complex.', *Curr Biol* **14**(23), 2107--2112.

Lowery, D. M.; Clauser, K. R.; Hjerrild, M.; Lim, D.; Alexander, J.; Kishi, K.; Ong, S.-E.; Gammeltoft, S.; Carr, S. A. & Yaffe, M. B. (2007), 'Proteomic screen defines the Polo-box domain interactome and identifies Rock2 as a Plk1 substrate.', *EMBO J* **26**(9), 2262--2273.

Ma, J.-L.; Lee, S.-J.; Duong, J. K. & Sternu, D. F. (2006), 'Activation of the checkpoint kinase Rad53 by the phosphatidyl inositol kinase-like kinase Mec1.', *J Biol Chem* **281**(7), 3954--3963.

Mac?rek, L.; Lindqvist, A.; Lim, D.; Lampson, M. A.; Klompmaker, R.; Freire, R.; Clouin, C.; Taylor, S. S.; Yaffe, M. B. & Medema, R. H. (2008), 'Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery.', *Nature* **455**(7209), 119--123.

Mailand, N.; Bekker-Jensen, S.; Bartek, J. & Lukas, J. (2006), 'Destruction of Claspin by SCFbetaTrCP restrains Chk1 activation and facilitates recovery from genotoxic stress.', *Mol Cell* **23**(3), 307--318.

Majka, J.; Binz, S. K.; Wold, M. S. & Burgers, P. M. J. (2006), 'Replication protein A directs loading of the DNA damage checkpoint clamp to 5'-DNA junctions.', *J Biol Chem* **281**(38), 27855--27861.

Majka, J. & Burgers, P. M. J. (2003), 'Yeast Rad17/Mec3/Ddc1: a sliding clamp for the DNA damage checkpoint.', *Proc Natl Acad Sci U S A* **100**(5), 2249--2254.

Majka, J.; Niedziela-Majka, A. & Burgers, P. M. J. (2006), 'The checkpoint clamp activates Mec1 kinase during initiation of the DNA damage checkpoint.', *Mol Cell* **24**(6), 891--901.

Malkova, A.; Naylor, M. L.; Yamaguchi, M.; Ira, G. & Haber, J. E. (2005), 'RAD51-dependent break-induced replication differs in kinetics and checkpoint responses from RAD51-mediated gene conversion.', *Mol Cell Biol* **25**(3), 933--944.

McCulloch, S. D. & Kunkel, T. A. (2008), 'The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases.', *Cell Res* **18**(1), 148--161.

McVey, M. & Lee, S. E. (2008), 'MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings.', *Trends Genet* **24**(11), 529--538.

Melo, J. A.; Cohen, J. & Toczyski, D. P. (2001), 'Two checkpoint complexes are independently recruited to sites of DNA damage in vivo.', *Genes Dev* **15**(21), 2809--2821.

Menacho-Marquez, M.; Perez-Valle, J.; Ariño, J.; Gadea, J. & Murguía, J. R. (2007), 'Gcn2p regulates a G1/S cell cycle checkpoint in response to DNA damage.', *Cell Cycle* **6**(18), 2302--2305.

Mimitou, E. P. & Symington, L. S. (2010), 'Ku prevents Exo1 and Sgs1-dependent resection of DNA ends in the absence of a functional MRX complex or Sae2.', *EMBO J* **29**(19), 3358--3369.

Mimitou, E. P. & Symington, L. S. (2009), 'Nucleases and helicases take center stage in homologous recombination.', *Trends Biochem Sci* **34**(5), 264--272.

Mimitou, E. P. & Symington, L. S. (2008), 'Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing.', *Nature* **455**(7214), 770--774.

Moore, J. K. & Haber, J. E. (1996), 'Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae.', *Mol Cell Biol* **16**(5), 2164--2173.

Mordes, D. A.; Glick, G. G.; Zhao, R. & Cortez, D. (2008), 'TopBP1 activates ATR through ATRIP and a PIKK regulatory domain.', *Genes Dev* **22**(11), 1478--1489.

Mordes, D. A.; Nam, E. A. & Cortez, D. (2008), 'Dpb11 activates the Mec1-Ddc2 complex.', *Proc Natl Acad Sci U S A* **105**(48), 18730--18734.

Morrison, A. J.; Kim, J.-A.; Person, M. D.; Highland, J.; Xiao, J.; Wehr, T. S.; Hensley, S.; Bao, Y.; Shen, J.; Collins, S. R.; Weissman, J. S.; Delrow, J.; Krogan, N. J.; Haber, J. E. & Shen, X. (2007), 'Mec1/Tel1 phosphorylation of the INO80 chromatin remodeling complex influences DNA damage checkpoint responses.', *Cell* **130**(3), 499--511.

Mortensen, E. M.; Haas, W.; Gygi, M.; Gygi, S. P. & Kellogg, D. R. (2005), 'Cdc28-dependent regulation of the Cdc5/Polo kinase.', *Curr Biol* **15**(22), 2033--2037.

Muñoz, I. M.; Hain, K.; Déclais, A.-C.; Gardiner, M.; Toh, G. W.; Sanchez-Pulido, L.; Heuckmann, J. M.; Toth, R.; Macartney, T.; Eppink, B.; Kanaar, R.; Ponting, C. P.; Lilley, D. M. J. & Rouse, J. (2009), 'Coordination of structure-specific nucleases by human SLX4/BTBD12 is required for DNA repair.', *Mol Cell* **35**(1), 116--127.

Musacchio, A. & Salmon, E. D. (2007), 'The spindle-assembly checkpoint in space and time.', *Nat Rev Mol Cell Biol* **8**(5), 379--393.

Myung, K.; Datta, A. & Kolodner, R. D. (2001), 'Suppression of spontaneous chromosomal rearrangements by S phase checkpoint functions in Saccharomyces cerevisiae.', *Cell* **104**(3), 397--408.

Nakada, D.; Matsumoto, K. & Sugimoto, K. (2003), 'ATM-related Tel1 associates with double-strand breaks through an Xrs2-dependent mechanism.', *Genes Dev* **17**(16), 1957--1962.

Navadgi-Patil, V. M. & Burgers, P. M. (2008), 'Yeast DNA replication protein Dpb11 activates the Mec1/ATR checkpoint kinase.', *J Biol Chem* **283**(51), 35853--35859.

Neves-Costa, A.; Will, W. R.; Vetter, A. T.; Miller, J. R. & Varga-Weisz, P. (2009), 'The SNF2-family member Fun30 promotes gene silencing in heterochromatic loci.', *PLoS One* **4**(12), e8111.

Niu, H.; Chung, W.-H.; Zhu, Z.; Kwon, Y.; Zhao, W.; Chi, P.; Prakash, R.; Seong, C.; Liu, D.; Lu, L.; Ira, G. & Sung, P. (2010), 'Mechanism of the ATP-dependent DNA end-resection machinery from Saccharomyces cerevisiae.', *Nature* **467**(7311), 108--111.

Nyberg, K. A.; Michelson, R. J.; Putnam, C. W. & Weinert, T. A. (2002), 'Toward maintaining the genome: DNA damage and replication checkpoints.', *Annu Rev Genet* **36**, 617--656.

Ohouo, P. Y.; Bastos de Oliveira, F. M.; Liu, Y.; Ma, C. J. & Smolka, M. B. (2013), 'DNA-repair scaffolds dampen checkpoint signalling by counteracting the adaptor Rad9.', *Nature* **493**(7430), 120--124.

Paciotti, V.; Clerici, M.; Scotti, M.; Lucchini, G. & Longhese, M. P. (2001), 'Characterization of mec1 kinase-deficient mutants and of new hypomorphic mec1 alleles impairing subsets of the DNA damage response pathway.', *Mol Cell Biol* **21**(12), 3913--3925.

Palmbos, P. L.; Wu, D.; Daley, J. M. & Wilson, T. E. (2008), 'Recruitment of Saccharomyces cerevisiae Dnl4-Lif1 complex to a double-strand break requires interactions with Yku80 and the Xrs2 FHA domain.', *Genetics* **180**(4), 1809--1819.

Papamichos-Chronakis, M.; Krebs, J. E. & Peterson, C. L. (2006), 'Interplay between Ino80 and Swr1 chromatin remodeling enzymes regulates cell cycle checkpoint adaptation in response to DNA damage.', *Genes Dev* **20**(17), 2437--2449.

Papamichos-Chronakis, M.; Watanabe, S.; Rando, O. J. & Peterson, C. L. (2011), 'Global regulation of H2A.Z localization by the INO80 chromatin-remodeling enzyme is essential for genome integrity.', *Cell* **144**(2), 200--213.

Peidis, P.; Papadakis, A. I.; Rajesh, K. & Koromilas, A. E. (2010), 'HDAC pharmacological inhibition promotes cell death through the eIF2α kinases PKR and GCN2.', *Aging (Albany NY)* **2**(10), 669--677.

Pellicioli, A.; Lee, S. E.; Lucca, C.; Foiani, M. & Haber, J. E. (2001), 'Regulation of Saccharomyces Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest.', *Mol Cell* **7**(2), 293--300.

Pellicioli, A.; Lucca, C.; Liberi, G.; Marini, F.; Lopes, M.; Plevani, P.; Romano, A.; Fiore, P. P. D. & Foiani, M. (1999), 'Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase.', *EMBO J* **18**(22), 6561--6572.

Peschiaroli, A.; Dorrello, N. V.; Guardavaccaro, D.; Venere, M.; Halazonetis, T.; Sherman, N. E. & Pagano, M. (2006), 'SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response.', *Mol Cell* **23**(3), 319--329.

Pfander, B. & Diffley, J. F. X. (2011), 'Dpb11 coordinates Mec1 kinase activation with cell cycle-regulated Rad9 recruitment.', *EMBO J.*

Puddu, F.; Granata, M.; Nola, L. D.; Balestrini, A.; Piergiovanni, G.; Lazzaro, F.; Giannattasio, M.; Plevani, P. & Muzi-Falconi, M. (2008), 'Phosphorylation of the budding yeast 9-1-1 complex is required for Dpb11 function in the full activation of the UV-induced DNA damage checkpoint.', *Mol Cell Biol* **28**(15), 4782--4793.

Rahal, R. & Amon, A. (2008), 'The Polo-like kinase Cdc5 interacts with FEAR network components and Cdc14.', *Cell Cycle* **7**(20), 3262--3272.

Rieder, C. L. & Maiato, H. (2004), 'Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint.', *Dev Cell* **7**(5), 637--651.

Rossio, V.; Galati, E.; Ferrari, M.; Pellicioli, A.; Sutani, T.; Shirahige, K.; Lucchini, G. & Piatti, S. (2010), 'The RSC chromatin-remodeling complex influences mitotic exit and adaptation to the spindle assembly checkpoint by controlling the Cdc14 phosphatase.', *J Cell Biol* **191**(5), 981--997.

Saito, T. T.; Youds, J. L.; Boulton, S. J. & Colaiácovo, M. P. (2009), 'Caenorhabditis elegans HIM-18/SLX-4 interacts with SLX-1 and XPF-1 and maintains genomic integrity in the germline by processing recombination intermediates.', *PLoS Genet* **5**(11), e1000735.

Sanchez, Y.; Bachant, J.; Wang, H.; Hu, F.; Liu, D.; Tetzlaff, M. & Elledge, S. J. (1999), 'Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms.', *Science* **286**(5442), 1166--1171.

Sandell, L. L. & Zakian, V. A. (1993), 'Loss of a yeast telomere: arrest, recovery, and chromosome loss.', *Cell* **75**(4), 729--739.

Sartori, A. A.; Lukas, C.; Coates, J.; Mistrik, M.; Fu, S.; Bartek, J.; Baer, R.; Lukas, J. & Jackson, S. P. (2007), 'Human CtIP promotes DNA end resection.', *Nature* **450**(7169), 509--514.

Seeber, A.; Hauer, M. & Gasser, S. M. (2013), 'Nucleosome remodelers in double-strand break repair.', *Curr Opin Genet Dev*.

Segal, M. (2011), 'Mitotic exit control: a space and time odyssey.', *Curr Biol* **21**(20), R857--R859.

Shah, P. P.; Zheng, X.; Epshtein, A.; Carey, J. N.; Bishop, D. K. & Klein, H. L. (2010), 'Swi2/Snf2-related translocases prevent accumulation of toxic Rad51 complexes during mitotic growth.', *Mol Cell* **39**(6), 862--872.

Shen, X.; Ranallo, R.; Choi, E. & Wu, C. (2003), 'Involvement of actin-related proteins in ATP-dependent chromatin remodeling.', *Mol Cell* **12**(1), 147--155.

Shim, E. Y.; Chung, W.-H.; Nicolette, M. L.; Zhang, Y.; Davis, M.; Zhu, Z.; Paull, T. T.; Ira, G. & Lee, S. E. (2010), 'Saccharomyces cerevisiae Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks.', *EMBO J* **29**(19), 3370--3380.

Shim, E. Y.; Hong, S. J.; Oum, J.-H.; Yanez, Y.; Zhang, Y. & Lee, S. E. (2007), 'RSC mobilizes nucleosomes to improve accessibility of repair machinery to the damaged chromatin.', *Mol Cell Biol* **27**(5), 1602--1613.

Shim, E. Y.; Ma, J.-L.; Oum, J.-H.; Yanez, Y. & Lee, S. E. (2005), 'The yeast chromatin remodeler RSC complex facilitates end joining repair of DNA double-strand breaks.', *Mol Cell Biol* **25**(10), 3934--3944.

Shinohara, M.; Shita-Yamaguchi, E.; Buerstedde, J. M.; Shinagawa, H.; Ogawa, H. & Shinohara, A. (1997), 'Characterization of the roles of the Saccharomyces cerevisiae RAD54 gene and a homologue of RAD54, RDH54/TID1, in mitosis and meiosis.', *Genetics* **147**(4), 1545--1556.

Smerdon, M. J. (1991), 'DNA repair and the role of chromatin structure.', *Curr Opin Cell Biol* **3**(3), 422--428.

Smits, V. A.; Klompmaker, R.; Arnaud, L.; Rijksen, G.; Nigg, E. A. & Medema, R. H. (2000), 'Polo-like kinase-1 is a target of the DNA damage checkpoint.', *Nat Cell Biol* **2**(9), 672--676.

Snead, J. L.; Sullivan, M.; Lowery, D. M.; Cohen, M. S.; Zhang, C.; Randle, D. H.; Taunton, J.; Yaffe, M. B.; Morgan, D. O. & Shokat, K. M. (2007), 'A coupled chemical-genetic and bioinformatic approach to Polo-like kinase pathway exploration.', *Chem Biol* **14**(11), 1261--1272.

Soria, G.; Polo, S. E. & Almouzni, G. (2012), 'Prime, repair, restore: the active role of chromatin in the DNA damage response.', *Mol Cell* **46**(6), 722--734.

Strebhardt, K. (2010), 'Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy.', *Nat Rev Drug Discov* **9**(8), 643--660.

Strebhardt, K. & Ullrich, A. (2006), 'Targeting polo-like kinase 1 for cancer therapy.', *Nat Rev Cancer* **6**(4), 321--330.

Svendsen, J. M.; Smogorzewska, A.; Sowa, M. E.; O'Connell, B. C.; Gygi, S. P.; Elledge, S. J. & Harper, J. W. (2009), 'Mammalian BTBD12/SLX4 assembles a Holliday junction resolvase and is required for DNA repair.', *Cell* **138**(1), 63--77.

Sweeney, F. D.; Yang, F.; Chi, A.; Shabanowitz, J.; Hunt, D. F. & Durocher, D. (2005), 'Saccharomyces cerevisiae Rad9 acts as a Mec1 adaptor to allow Rad53 activation.', *Curr Biol* **15**(15), 1364--1375.

Syljuåsen, R. G. (2007), 'Checkpoint adaptation in human cells.', *Oncogene* **26**(40), 5833--5839.

Syljuåsen, R. G.; Jensen, S.; Bartek, J. & Lukas, J. (2006), 'Adaptation to the ionizing radiation-induced G2 checkpoint occurs in human cells and depends on checkpoint kinase 1 and Polo-like kinase 1 kinases.', *Cancer Res* **66**(21), 10253--10257.

Symington, L. S. & Gautier, J. (2011), 'Double-strand break end resection and repair pathway choice.', *Annu Rev Genet* **45**, 247--271.

Tanaka, K. & Hirota, T. (2009), 'Chromosome segregation machinery and cancer.', *Cancer Sci* **100**(7), 1158--1165.

Toczyski, D. P.; Galgoczy, D. J. & Hartwell, L. H. (1997), 'CDC5 and CKII control adaptation to the yeast DNA damage checkpoint.', *Cell* **90**(6), 1097--1106.

Toh, G. W.-L.; O'Shaughnessy, A. M.; Jimeno, S.; Dobbie, I. M.; Grenon, M.; Maffini, S.; O'Rorke, A. & Lowndes, N. F. (2006), 'Histone H2A phosphorylation and H3 methylation are required for a novel Rad9 DSB repair function following checkpoint activation.', *DNA Repair (Amst)* **5**(6), 693--703.

Udugama, M.; Sabri, A. & Bartholomew, B. (2011), 'The INO80 ATP-dependent chromatin remodeling complex is a nucleosome spacing factor.', *Mol Cell Biol* **31**(4), 662--673.

Usui, T.; Foster, S. S. & Petrini, J. H. J. (2009), 'Maintenance of the DNA-damage checkpoint requires DNA-damage-induced mediator protein oligomerization.', *Mol Cell* **33**(2), 147--159.

Vaze, M. B.; Pellicioli, A.; Lee, S. E.; Ira, G.; Liberi, G.; Arbel-Eden, A.; Foiani, M. & Haber, J. E. (2002), 'Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase.', *Mol Cell* **10**(2), 373--385.

Vialard, J. E.; Gilbert, C. S.; Green, C. M. & Lowndes, N. F. (1998), 'The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage.', *EMBO J* **17**(19), 5679--5688.

van Vugt, M. A. T. M.; Gardino, A. K.; Linding, R.; Ostheimer, G. J.; Reinhardt, H. C.; Ong, S.-E.; Tan, C. S.; Miao, H.; Keezer, S. M.; Li, J.; Pawson, T.; Lewis, T. A.; Carr, S. A.; Smerdon, S. J.; Brummelkamp, T. R. & Yaffe, M. B. (2010), 'A Mitotic Phosphorylation Feedback Network Connects Cdk1, Plk1, 53BP1, and Chk2 to Inactivate the G(2)/M DNA Damage Checkpoint.', *PLoS Biol* **8**(1), e1000287.

Wang, H.; Liu, D.; Wang, Y.; Qin, J. & Elledge, S. J. (2001), 'Pds1 phosphorylation in response to DNA damage is essential for its DNA damage checkpoint function.', *Genes Dev* **15**(11), 1361--1372.

Wogan, G. N.; Hecht, S. S.; Felton, J. S.; Conney, A. H. & Loeb, L. A. (2004), 'Environmental and chemical carcinogenesis.', *Semin Cancer Biol* **14**(6), 473--486.

Wu, D.; Topper, L. M. & Wilson, T. E. (2008), 'Recruitment and dissociation of nonhomologous end joining proteins at a DNA double-strand break in Saccharomyces cerevisiae.', *Genetics* **178**(3), 1237--1249.

Wysocki, R.; Javaheri, A.; Allard, S.; Sha, F.; Côté, J. & Kron, S. J. (2005), 'Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9.', *Mol Cell Biol* **25**(19), 8430--8443.

Yeung, M. & Durocher, D. (2011), 'Srs2 enables checkpoint recovery by promoting disassembly of DNA damage foci from chromatin.', *DNA Repair (Amst)* **10**(12), 1213--1222.

Yoo, H. Y.; Kumagai, A.; Shevchenko, A.; Shevchenko, A. & Dunphy, W. G. (2004), 'Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase.', *Cell* **117**(5), 575--588.

Zhang, T.; Nirantar, S.; Lim, H. H.; Sinha, I. & Surana, U. (2009), 'DNA damage checkpoint maintains CDH1 in an active state to inhibit anaphase progression.', *Dev Cell* **17**(4), 541--551.

Zhu, Z.; Chung, W.-H.; Shim, E. Y.; Lee, S. E. & Ira, G. (2008), 'Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends.', *Cell* **134**(6), 981--994.

Zimmermann, M.; Lottersberger, F.; Buonomo, S. B.; Sfeir, A. & de Lange, T. (2013), '53BP1 regulates DSB repair using Rif1 to control 5' end resection.', *Science* **339**(6120), 700--704.

Zou, L. & Elledge, S. J. (2003), 'Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes.', *Science* **300**(5625), 1542--1548.

Part II

Published Paper I

Elevated Levels of the Polo Kinase Cdc5 Override the Mec1/ATR Checkpoint in Budding Yeast by Acting at Different Steps of the Signaling Pathway

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Abstract

Checkpoints are surveillance mechanisms that constitute a barrier to oncogenesis by preserving genome integrity. Loss of checkpoint function is an early event in tumorigenesis. Polo kinases (Plks) are fundamental regulators of cell cycle progression in all eukaryotes and are frequently overexpressed in tumors. Through their polo box domain, Plks target multiple substrates previously phosphorylated by CDKs and MAPKs. In response to DNA damage, Plks are temporally inhibited in order to maintain the checkpoint-dependent cell cycle block while their activity is required to silence the checkpoint response and resume cell cycle progression. Here, we report that, in budding yeast, overproduction of the Cdc5 polo kinase overrides the checkpoint signaling induced by double strand DNA breaks (DSBs), preventing the phosphorylation of several Mec1/ATR targets, including Ddc2/ATRIP, the checkpoint mediator Rad9, and the transducer kinase Rad53/CHK2. We also show that high levels of Cdc5 slow down DSB processing in a Rad9-dependent manner, but do not prevent the binding of checkpoint factors to a single DSB. Finally, we provide evidence that Sae2, the functional ortholog of human CtIP, which regulates DSB processing and inhibits checkpoint signaling, is regulated by Cdc5. We propose that Cdc5 interferes with the checkpoint response to DSBs acting at multiple levels in the signal transduction pathway and at an early step required to resect DSB ends.

Introduction

Saccharomyces cerevisiae cells suffering a double stranded DNA break (DSB) activate a robust Mec1-dependent checkpoint response when DSB ends are processed to expose single-stranded DNA (ssDNA), and progression through the cell cycle is arrested prior to anaphase. Several well conserved factors are recruited at the DSB lesion, and contribute to the activation of a signaling pathway based on sequential phosphorylation events driven by the upstream kinases Tel1/ATM and Mec1/ATR which, in turn, activate the transducer kinases Rad53/Chk2 and Chk1 [1,2]. The checkpoint response is influenced at several levels by kinases such as CDK1, CKII and Polo-like Cdc5, all involved in promoting key events throughout an unperturbed cell cycle, supporting the notion that the cellular response to DNA damage is tightly linked to cell cycle events [3]. The intensity of the DSB-induced checkpoint response correlates to the amount of the ssDNA that is accumulated at DSB lesions [4]. 5′-to-3′ nucleolytic processing of DNA ends is dependent upon several factors, including CDK1 and the nucleases Mre11, Sae2, Dna2 and Exo1 [5]. Moreover, the checkpoint is a reversible signaling pathway which is turned off when DNA lesions are repaired, thus permitting the resumption of cell cycle progression [6]. Different types of phosphatases (Pph3,

Ptc2 and Ptc3) dephosphorylate and inactivate Rad53 and other checkpoint kinase targets [7]. Further, mutations in several DNA repair genes, including *SAE2*, *KU70/80*, *RAD51*, *RDH54*, *SRS2*, affect the inactivation of the DSB-induced checkpoint response [7,8]. These observations suggest that the attenuation, as well the activation, of the checkpoint pathway are related to the metabolism of DSB ends, in a way that is not yet completely understood. It is also known that the checkpoint response can be attenuated when an irreparable DNA lesion is formed in the cell, leading to adaptation to DNA damage. Checkpoint inactivation during recovery and adaptation to DNA damage is a phenomenon described also in higher eukaryotes [6]. The functional role of adaptation is not completely understood; however, it was suggested that it may be partly responsible for chromosomal rearrangements, genome instability and tumorigenesis [6,9]. Interestingly, the well conserved family of Polo-like kinases (Plks) has been involved in checkpoint adaptation and/or recovery both in budding yeast and vertebrates [10]. Cdc5 is the only polo kinase expressed in yeast, whereas higher eukaryotes usually express three or four Plks [11]. However, only Plk1, which is the most extensively studied, is a true mitotic kinase homolog to the *Drosophila* Polo kinase [11]. In yeast, *CDC5* is an essential gene and the point mutation *cdc5*-ad (a Leucine-to-Tryptophan substitution

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Author Summary

Double strand DNA breaks (DSBs) are dangerous chromosomal lesions that can lead to genome rearrangements, genetic instability, and cancer if not accurately repaired. Eukaryotes activate a surveillance mechanism, called DNA damage checkpoint, to arrest cell cycle progression and facilitate DNA repair. Several factors are physically recruited to DSBs, and specific kinases phosphorylate multiple targets leading to checkpoint activation. Budding yeast is a good model system to study checkpoint, and most of the factors involved in the DSBs response were originally characterized in this organism. Using the yeast *Saccharomyces cerevisiae*, we explored the functional role of polo kinase Cdc5 in regulating the DSB–induced checkpoint. Polo kinases have been previously involved in checkpoint inactivation in all the eukaryotes, and they are frequently overexpressed in cancer cells. We found that elevated levels of Cdc5 affect the cellular response to a DSB at different steps, altering DNA processing and overriding the signal triggered by checkpoint kinases. Our findings suggest that Cdc5 likely regulates multiple factors in response to a DSB and provide a rationale for a proteome-wide screening to identify targets of polo kinases in yeast and human cells. Such information may have a practical application to design specific molecular tools for cancer therapy. Two related papers published in *PLoS Biology*—by Vidanes et al., doi:10.1371/journal.pbio.1000286, and van Vugt et al., doi:10.1371/journal.pbio.1000287—similarly investigate the phenomenon of checkpoint adaptation/overriding.

at residue 251, within the kinase domain) causes the inability to adapt to one irreparable DSB lesion and to turn off Rad53 kinase [12,13]. However, *cdc5*-ad cells can recover from checkpoint when the DSB is repaired, suggesting that adaptation and recovery are two genetically separate processes [14]. A corresponding *cdc5*-ad mutation in Plks has not yet been isolated in mammals; however, it was found that Plk1 depletion severely blocks checkpoint recovery and adaptation [10,15,16], and rapidly causes cell death in cancer cells [17,18]. Based on the fact that the DNA damage checkpoint pathway is well conserved in all the eukaryotes, it is reasonable to expect that the functional role of Cdc5 in budding yeast and of Plk1 during adaptation (and perhaps in recovery) may be conserved. Polo-like kinases contain in the C-terminal region of the protein a polo box that mediates the interaction of Plks with substrates previously phosphorylated by CDK or MAPK kinases [19]. Indeed, Cdc5 targets multiple substrates during an unperturbed cell cycle [20] and could functionally interact with several checkpoint proteins as well. In vertebrates, polo kinases regulate the DNA damage checkpoint acting on multiple factors. They phosphorylate Claspin [21–24], a Chk1 kinase regulator, and the Fanconi-Anemia protein FANCM [25], promoting their degradation and checkpoint inactivation. Further, Plk1, Plk3 and Plk4 interact with and phosphorylate Chk2, the ortholog of Rad53 in human cells, likely influencing its activity [26–28]. Interestingly, yeast Cdc5 is phosphorylated and inhibited in a Mec1- and Rad53-dependent manner [29], and several studies indicate that in mammals Plk1 activity is inhibited by ATM/ATR-signaling in response to DNA damage [30–33]. Further, the DNA damage checkpoint regulates Plk1 protein stability in response to DNA damage in mitosis [34]. It was also shown that Aurora kinase A phosphorylates and re-activates Plk1 to promote recovery from DNA damage [35]. Altogether, these informations suggest that the DNA damage checkpoint inhibits Plk1, thus contributing to block

cell cycle progression in response to DNA damage; however, the re-activation of Plk1 is a crucial event of a feedback regulatory loop in the inactivation of the DNA damage checkpoint during recovery and adaptation.

Therefore, the activity of Plks must be finely regulated during the DNA damage response, and it is worth mentioning that the expression of a constitutively active Plk1 protein variant overrides the G2/M arrest induced by DNA damage [30]. Indeed, Plks are frequently overexpressed in tumor cells with uncontrolled proliferation and genome instability [36–39], and high level of Plk1 is predictive of a bad prognosis in several cancers [40–44].

To further characterize the functional link between Plks and the DNA damage checkpoint and, possibly, to understand why Plks are frequently overexpressed in cancer cells, we used budding yeast as a model system to study DNA damage related events in the presence of high levels of Cdc5.

Here, we show that overproduction of Cdc5 impairs the Mec1-signaling pathway in response to an inducible DSB lesion, altering phosphorylation of Ddc2, Rad9, Rad53 and other Mec1 targets. We also found that elevated levels of Cdc5 slow down DSB ends processing, although it does not prevent the formation of ssDNA, which triggers the recruitment of checkpoint factors. Consistently, we observed that overexpression of Cdc5 does not alter the loading of the apical Mec1 kinase checkpoint complex and recruitment of the checkpoint mediator Rad9, but surprisingly it physically interact with the checkpoint inhibitor Sae2, inducing its hyper-phosphorylation and an increased and persistent binding onto a DSB lesion.

We propose that high levels of polo kinase Cdc5 override Mec1-induced checkpoint response to DSB lesions, likely by regulating multiple factors, previously phosphorylated by CDK1, involved in both DSB processing and checkpoint signaling. Our work may represent a simple model to further understand why polo kinases are frequently overexpressed in cancer cells.

Results/Discussion

Elevated levels of Cdc5 override Mec1 signaling

DNA damage checkpoints represent a barrier to oncogenesis; in fact, loss of these surveillance mechanism is a characteristic of early tumor development [45]. Several evidences indicate that Plks are targets of the DNA damage checkpoint in all the eukaryotes [29–34], suggesting a functional model in which the DNA damage checkpoint inhibits Plks to maintain a cell cycle block at the metaphase to anaphase transition. Indeed, numerous cancer cells have been reported to display overexpression of Plks, and this may contribute to their transformed phenotype [36–39].

In budding yeast, overproduction of the polo kinase Cdc5 in *cdc13-1* mutant cells with uncapped telomeres has been reported to override the checkpoint-dependent cell cycle block in the G2 phase of the cell cycle [46,47]. We found that overproduction of Cdc5 impairs the replication checkpoint, which delays S phase in the presence of the alkylating agent MMS (methylmetane sulfonate, Figure 1A). Indeed, Figure 1A shows that MMS treated wild type cells accumulate in S phase for a very long period ($1C < DNA < 2C$), while Cdc5 overproducing cells rapidly go through the replication phase and reach a G2/M DNA content (2C). Moreover, the DNA damage-induced phosphorylation of Rad53 is essentially abolished in Cdc5 overproducing cells treated with zeocin, an agent causing DSBs (Figure 1B).

We have to assume that, although the DNA damage checkpoint inhibits Cdc5 [29,46], contribuiting to block cell cycle in the presence of DNA damage, when *CDC5* is placed under the control
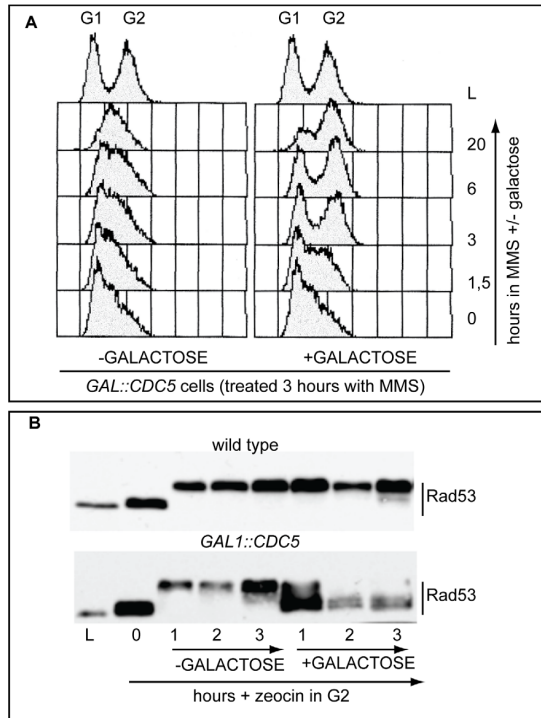
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Figure 1. Overproduction of Cdc5 overrides the DNA replication and DNA damage checkpoints. (A) Exponentially (L) growing culture of the strain Y114 (*GAL1::CDC5*) was grown in YEP+3%raffinose and treated for 3 hours with 0.02% MMS (time 0). Then the culture is split in two and 2% galactose was added to one half, while the other half was maintained in raffinose. Samples were taken at the indicated time and analysed by FACS. (B) Cultures of the strains Y79 (wild type), Y114 (*GAL1::CDC5*), exponentially (L) growing in YEP+3%raffinose were blocked in G2/M by nocodazole treatment (0). Zeocin (50 μg/ml) was then added to cause DSBs formation and after 30 minutes of treatment, 2% galactose was added. Samples were taken at the indicated time and Rad53 protein was analyzed by western blotting with Mab.EL7 antibody.
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of the *GAL1* promoter, the DNA damage-induced inhibition on overproduced Cdc5 is not complete. This is likely due to the elevated Cdc5 levels, which are higher than the endogenous amount (see also Figure S1), leading to the override of the checkpoint response. Indeed, it was previously shown that the overproduction of Cdc5, which is a finely regulated protein [29], causes severe phenotypes during an unperturbed cell cycle [48–51].

In order to expand the analysis on the crosstalk between polo kinases and checkpoint pathways, and possibly to understand why overexpression of Plks is often found in tumor cells characterized by uncontrolled proliferation and genome instability, we analysed the effects of elevated Cdc5 levels on the DSB-induced checkpoint cascade in *S. cerevisiae*. We took advantage of a standard yeast genetic system (JKM background) in which one irreparable DSB

can be induced at the *MAT* locus by expressing the site-specific HO nuclease [8]. We overexpressed wild-type *CDC5* and the two *cdc5*-ad and *cdc5*-kd mutant alleles (adaptation-defective and kinase-dead alleles, respectively [51]) from the galactose-inducible promoter and examined Rad53 phosphorylation and *in situ* auto-phosphorylation activity, which are routinely used as markers of DNA damage checkpoint activation [52]. To prevent variations due to cell cycle differences, we first arrested cells with nocodazole in mitosis, a cell cycle stage in which the DSB-depended checkpoint can be fully activated [12], and subsequently added galactose to induce Cdc5 overproduction and HO-break forma- tion, while maintaining the cell cycle block. Figure 2A shows the FACS profiles of the cell cultures. We observed that overproduc- tion of Cdc5 impairs the accumulation of hyper-phosphorylated Rad53 forms and prevents Rad53 auto-phosphorylation activity in
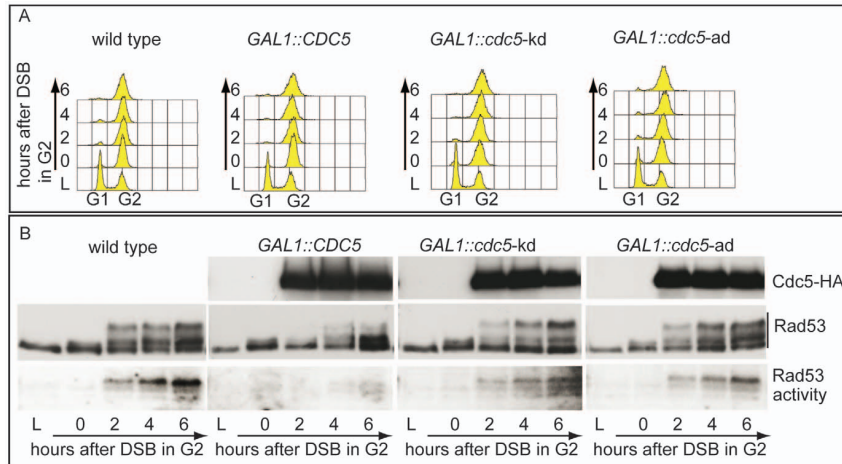
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Figure 2. Overproduction of Cdc5 affects DSB–induced Rad53 phosphorylation and activity. (A,B) YEP+raffinose nocodazole-arrested cell cultures of wild type JKM and isogenic *GAL1::CDC5*, *GAL1::cdc5*-kd (kinase dead, K110A mutation) and *GAL1::cdc5*-ad (adaptation defective, L251W mutation) strains were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). (A) Samples were taken at the indicated time points and analyzed by FACS. (B) Overproduced Cdc5 proteins have an additional HA epitope and their accumulation in galactose was analyzed by western blots using 12CA5 antibody. Rad53 was analyzed by western blots with Mab.EL7 antibodies. Rad53 in situ auto-phosphorylation activity was analyzed by ISA assay.
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response to DSB formation (Figure 2B). Interestingly, overproduction of the protein variants Cdc5-kd or Cdc5-ad did not significantly interfere with Rad53 phosphorylation and activation, suggesting that the kinase activity of Cdc5 and its capacity to interact with specific target(s) are required to override the DSB-induced Rad53 activation.

In vertebrates, polo kinases regulate the DNA damage checkpoint response by affecting the signal transduction pathway at different levels; interestingly, Chk2, the homologue of Rad53 in human cells, interacts with and is phosphorylated by the polo kinases Plk1, Plk3 and Plk4 [26–28].

Therefore, we tested whether the overproduction of Cdc5 might override Rad53 activation by targeting directly the Rad53 protein and/or by acting on other upstream checkpoint factors.

We failed to co-immunoprecipitate Rad53 and Cdc5, when expressed at endogenous levels or by using the polo box of Cdc5 in a standard GST pull down assay; however, we retrieved Rad53 with overproduced Cdc5 (Figure S2). Considering such physical interaction, we analyzed how overproduction of Cdc5 might affect the events leading to full activation of Rad53, which involves a two steps-based mechanism: an *in trans* phosphorylation event mediated by PIKKs, followed by auto-phosphorylation [53]. In theory, Cdc5 might affect any of these events required to activate Rad53. We analysed the effect of Cdc5 overexpression on the PIKKs-dependent phosphorylation of Rad53 by taking advantage of the catalytically inactive *rad53*-K227A mutant. Such protein can be phosphorylated *in trans* by the upstream kinases, but does not undergo auto-phosphorylation in the presence of DNA damage [52], allowing us to separate and discriminate the two steps.

In nocodazole blocked cells, induction of a single irreparable HO cut induced Mec1-dependent phosphorylation of the Rad53-K227A protein variant (Figure 3A). As expected, the corresponding phosphorylated bands of Rad53-K227A protein were not visualized by western blot using the monoclonal antibody (Mab.F9) which is specific for the auto-phosphorylated and active Rad53 isoform [54]. Moreover, the same phospho-specific antibody did not significantly detect Rad53 in wild type cells responding to DSB when Cdc5 is overproduced, confirming the results of the *in situ* kinase assay (Figure 2B). A residual shifted band of Rad53, visualized in *CDC5* overexpressing cells through the highly sensitive Mab.EL7 antibody (both in Figure 2B and Figure 3A, and in other figures below), could reflect low levels of Rad53 activation not detected by the antibody against the active form; this is consistent with the residual Rad53 activity in the *in situ* analysis in Figure 2B. In any case, it is unlikely that this remaining Rad53 activity is sufficient to maintain a full checkpoint response, since overproduction of Cdc5 functionally overrides the cell cycle block in the presence of DNA damage.

Significantly, Cdc5 overproduction abolished DSB-induced *in trans* phosphorylation of the Rad53-K227A variant (Figure 3A). This result rules out the hypothesis that Cdc5 may override the DSB-induced checkpoint acting only on the auto-phosphorylation step of Rad53 activation, and suggests that *CDC5* overexpression likely impairs the Mec1-dependent *in trans* phosphorylation and activation of Rad53.

The residual Rad53 phosphorylation and activity in the presence of high levels of Cdc5 might suggest that the upstream Mec1 kinase, which is mainly responsible of the Rad53 activation in the presence of a single DSB in wild type cells [55], is strongly
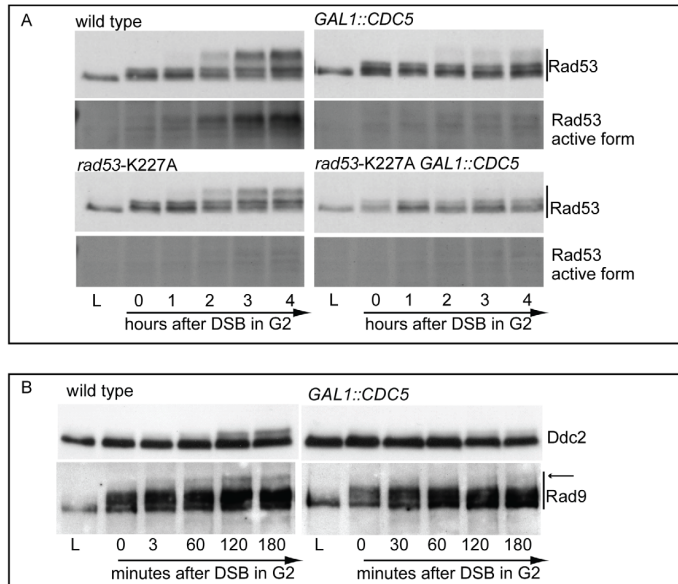
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Figure 3. Overproduction of Cdc5 overrides Mec1 checkpoint signaling. (A) YEP+raffinose nocodazole-arrested cell cultures of wild-type JKM and isogenic *rad53*-kd (kinase dead, K227A mutation) derivative strains, with or without *GAL1::CDC5*, were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). Samples were taken at the indicated time points and Rad53 was analyzed by western blots using monoclonal antibodies Mab.EL7 or Mab.F9, which recognized, respectively, all the forms of Rad53 or only the auto-phosphorylated and active forms. (B) YEP+raffinose nocodazole-arrested cell cultures of wild type JKM and isogenic *GAL1::CDC5* derivative strains, expressing *DDC2*-HA, were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). Ddc2 protein was analyzed by western blots using 12CA5 antibody; Rad9 protein was analyzed by polyclonal antibodies. An arrow denotes the hyper-phosphorylation band of Rad9 accumulated specifically in response to DNA damage.
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but not fully inhibited. Alternatively, Mec1 may still be functional as a kinase, but impaired in fully trans-activating Rad53. To test more directly the activity of the upstream kinase Mec1, we analysed the phosphorylation state of its interacting subunit Ddc2, the ortholog of human ATRIP, and that of the checkpoint mediator Rad9, which are known to be directly phosphorylated by Mec1 [1]. Cells were arrested with nocodazole and *CDC5* overexpression and induction of a single unrepairable DSB were induced by galactose addition (Figure 3B). Western blot analysis indicate that phosphorylated isoforms of Ddc2 and hyper-phosphorylated Rad9 (indicated by the arrow in Figure 3) accumulated after the formation of the HO cut in wild type cells, as expected; however, overexpression of Cdc5 reduced the DSB-induced hyper-phosphorylation form of both Ddc2 and Rad9, suggesting that the activity of Mec1 kinase is strongly impaired in the presence of high level of Cdc5. A careful analysis of the blot shown in Figure 3B or in analogous experiments indicates that reduced levels of phosphorylated Rad9 isoforms are present in *CDC5* overexpressing cells, suggesting that Mec1 could still retain a flebile activity toward Rad9 and Rad53, as discussed above. In addition, it is known that Rad9 is a target of multiple kinases [56] and we cannot rule out the possibility that the residual

phosphorylation of Rad9 observed in cells with elevated levels of Cdc5 may be due to other kinase(-s), including Cdc5 itself.

Taken together the results shown in Figure 1, Figure 2, and Figure 3 indicate that Cdc5 activity overrides the DSB-induced checkpoint by influencing an early step of the Mec1 signaling pathway, likely reducing the functionality of Mec1 activity. However, it is possible that Cdc5 may target multiple substrates, including the Mec1 interactor Ddc2, the checkpoint mediator Rad9, whose role in promoting Mec1-to-Rad53 signaling is well established, and Rad53 itself, thus counteracting the checkpoint signaling pathway at several levels.

High levels of Cdc5 affect DSB resection

Robust Mec1 and Rad53 activation is not triggered by the DSB itself, but requires multiple interconnected events following the formation of the lesion, including the generation of nucleolytic-dependent 5′-to-3′ processing of the DNA ends and recruitment of various DNA repair and checkpoint factors onto the long stretches of the generated ssDNA [4].

Therefore, we investigated whether Cdc5 may control Mec1 signaling by affecting DSB processing. We measured the kinetic of

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ssDNA formation after a single unrepairable DSB in cells overexpressing *CDC5*. Cells were arrested in mitosis, to prevent cell cycle-dependent effects on resection [57], and samples were collected at various time points after HO nuclease induction (Figure 4). The kinetic of production of ssDNA regions in genomic DNA was tested by the loss of restriction sites distal to the HO-cut site which leads to the accumulation of undigested ssDNA fragments detectable with a strand-specific probe after alkaline electrophoresis (see the scheme of the unprocessed and processed DNA locus in Figure 4A). *CDC5* overexpressing cells reproducibly
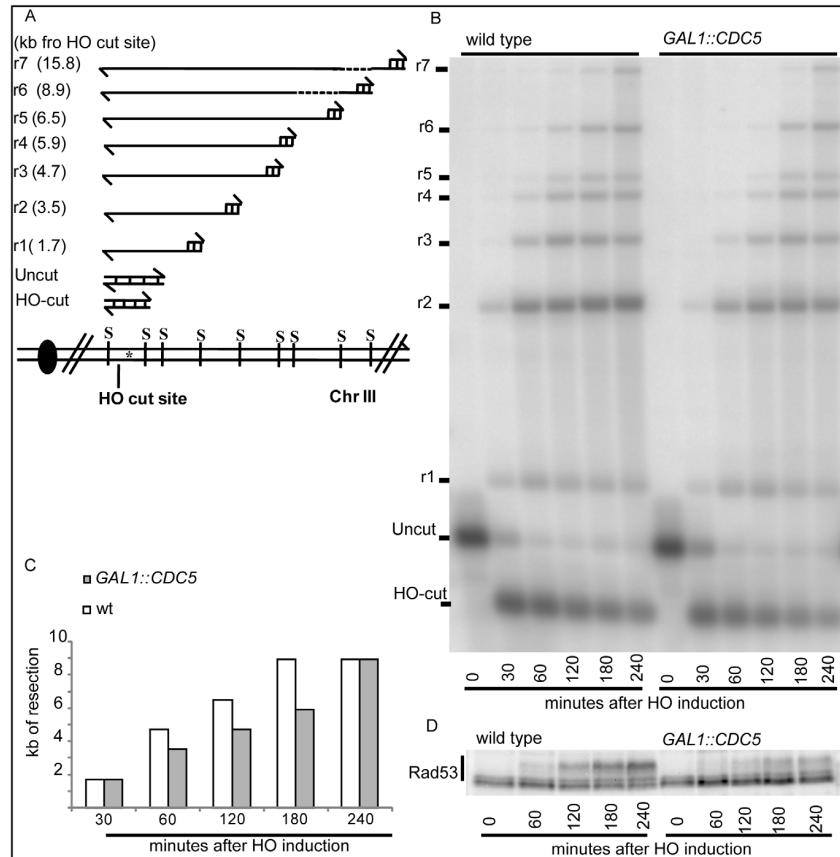
Figure 4. Overproduction of Cdc5 affects DSB processing. (A–D) YEP+raffinose nocodazole-arrested cell cultures of wild-type JKM MATα and isogenic *GAL1::CDC5* strain were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). (A) Schematic representation of the system used to detect DSB resection. Gel blots of *SspI*-digested genomic DNA separated on alkaline agarose gel were hybridized with a single-strand RNA probe specific for the un-resected strand at the MAT locus, which shows HO-cut and uncut fragments of 0.9 and 1.1 kb, respectively. 5'-to-3' resection progressively eliminates *SspI* sites located 1.7, 3.5, 4.7, 5.9, 6.5, 8.9, and 15.8 kb centromere-distal from the HO-cut site, producing larger *SspI* fragments (r1–r7) detected by the probe. (B) Analysis of ssDNA formation as described in (A). (C) The time of the first appearance over the background of each undigested band in the blot shown in (B) was graphically represented for both the wild type and *GAL1::CDC5* strains. (D) Western blot analysis of protein extracts with anti-Rad53 Mab.EL7 antibody.
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exhibited a slower DSB resection, measured by the kinetic of appearance of DNA fragments, which correlated with a reduced phosphorylation of Rad53 (Figure 4B–4D). However, we found that, although the kinetic of DSB ends resection was delayed, high levels of Cdc5 do not prevent the generation of a long ssDNA track (25 kb) which is required to repair the DSB in a specific yeast genetic background [14] by the single-strand annealing process (Figure S3).

We previously identified a role for the checkpoint mediator Rad9 in inhibiting the kinetic of DSB ends resection, likely by generating a non-permissive chromatin configuration around the DSB and/or interfering with the action of nucleases [58]. Therefore, we analyzed the Rad9 contribution in delaying DSB processing in *CDC5* overexpressing cells. Wild-type or *rad9Δ* cells, with or without *GAL1::CDC5*, were arrested in mitosis by

nocodazole treatment and the same experiment described in Figure 4B was performed. We found that the kinetic of appearance of ssDNA fragments was accelerated in *rad9Δ* strains, despite the high levels of Cdc5 kinase (Figure 5A and 5B). Moreover, the faster DSB resection in *CDC5* overexpressing *rad9Δ* cells also correlated with a modest increase in Ddc2 phosphorylation (Figure 5C); however, the phosphorylated state of Ddc2 did not reach the same level found in wild-type and *rad9Δ* cells, suggesting that overproduction of Cdc5 impaired Mec1-dependent signaling also in a *rad9Δ* background. These results suggest that elevated levels of Cdc5 may slow down DSB processing through the action of the Rad9-dependent barrier on resection [58], likely targeting Rad9 itself or other factors involved in this mechanism. Interestingly, many of the proteins involved in DSB ends processing (i.e. Rad9, Dna2, Xrs2 and Sae2) are phosphorylated
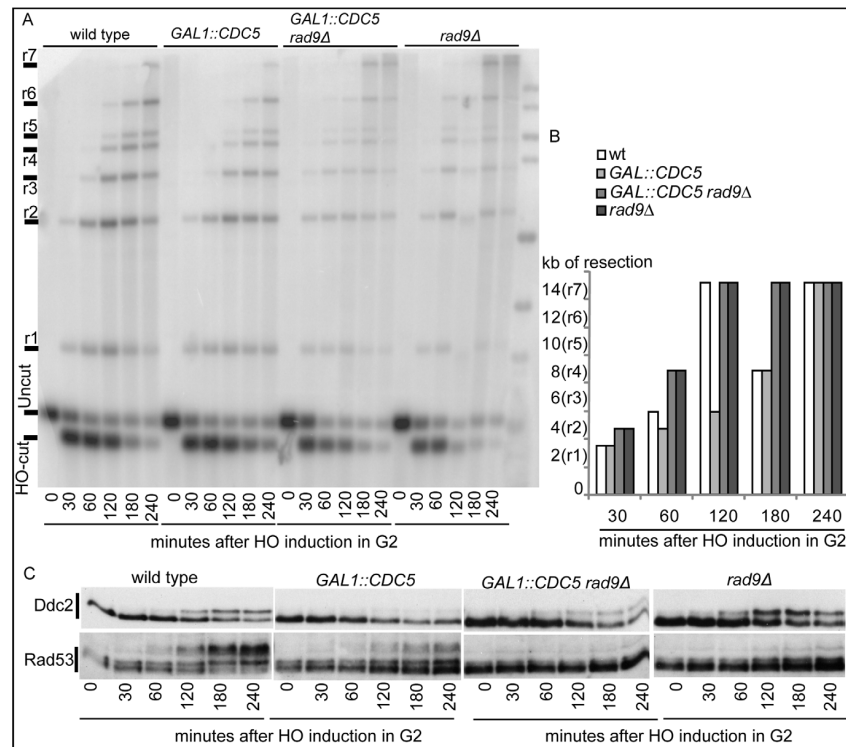

Figure 5. Deletion of *RAD9* gene accelerates DSB resection despites high Cdc5's levels. YEP+raffinose nocodazole-arrested cell cultures of wild type JKM MATa and isogenic *rad9Δ* strains, with or without *GAL1::CDC5*, were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). (A,B) Analysis of ssDNA formation as described in Figure 4. (C) Ddc2 protein was analyzed by western blots using 12CA5 antibody; Rad53 protein was analysed by monoclonal antibody Mab.EL7.
doi:10.1371/journal.pgen.1000763.g005

by CDK1 [59,60] and inspection of their protein sequence reveals that they may be potential targets of Cdc5.

Hence, Cdc5 may influence the DSB response acting on multiple factors, affecting DSB processing and Mec1-signaling; moreover, the possibility that Cdc5 might specifically regulate Rad53 by influencing its interaction with the checkpoint mediator Rad9 cannot be excluded.

Recruitment of checkpoint factors in CDC5-overexpressing cells

Since high levels of Cdc5 did not prevent the generation of long ssDNA regions but inhibit Mec1-signaling, we tested, by chromatin immunoprecipitation (ChIP), whether overexpression of CDC5 affected the recruitment of checkpoint factors onto the HO-induced DSB lesion in nocodazole-arrested cells. Sheared chromatin from formaldehyde crosslinked cells taken at different time-points after galactose addition was immunoprecipitated to recover checkpoint proteins (i.e. Ddc2, Ddc1, Dpb11, Rad9) carrying the MYC or HA epitope tags at their carboxyl-terminal end. Quantitative multiplex PCR was then used to monitor co-immunoprecipitation of DNA fragments located either 66 kb centromere-proximal to the *MAT* locus (CON) or 1 kb away from the HO-cut site (DSB) (Figure 6A).

Ddc2 and Ddc1 association at the DSB was not significantly affected in CDC5 overexpressing cells blocked by nocodazole treatment (Figure 6B and 6C). The Mec1 interacting factor Ddc2 and Ddc1, one of three subunits of the stable PCNA-like 9-1-1 checkpoint complex, are recruited early onto a DSB lesion [61–63]. We, therefore, assume that Cdc5 overproduction does not prevent the recruitment of upstream checkpoint protein complexes onto damaged DNA. This observation also confirms that elevated levels of Cdc5, while delaying resection, do not prevent the generation of ssDNA (see Figure 4, Figure 5, and Figure S3) which is required for the recruitment of checkpoint factors [4].

Similarly, we found that overproduction of Cdc5 did not prevent the localization near the DSB of Dpb11 (Figure 6D), the yeast ortholog of TopBP1, which, together with the 9-1-1 complex, stimulates the Mec1 kinase activity [64].

Moreover, when we tested by ChIP analysis the binding of the checkpoint mediator Rad9, we found that also its localization onto the DSB was not altered in CDC5 overexpressing cells (Figure 6E).

Taken together, the ChIP analyses of checkpoint factors at a DSB site indicate that high levels of Cdc5 kinase do not significantly interfere with the binding of checkpoint proteins to a processed DSB.

We then tested the DSB binding of Sae2, which is a protein regulated by CDK1 [60] and PIKKs [65] after DNA damage and is involved in DSB processing [5] and checkpoint inactivation [66,67]. Surprisingly, while in wild-type cells Sae2 loading was not significantly enriched at the HO-cut site (Figure 7A), likely because of its dynamic and transient binding to DSBs [67], Sae2 localization near the break greatly increased in CDC5 overexpressing cells (Figure 7A). To test whether Cdc5 may specifically target Sae2 influencing its binding onto DSBs, we analysed the level and modification of Sae2 by western blotting following DSB formation. In nocodazole-blocked cells, induction of the HO cut caused PIKKs-dependent phosphorylation of Sae2 at the same time-points at which Rad53 phosphorylation was observed (Figure 7B). Interestingly, although high levels of Cdc5 impair Rad53 phosphorylation, they seem to cause hyperphosphorylation of Sae2. Infact, in CDC5 overexpressing cells we observed the appearance of a ladder of slower migrating forms of Sae2 (Figure 7B), which are abolished by *in vitro* treatment with λ phosphatase (Figure 7C), indicating that they are due to

phosphorylation events of Sae2. We then found that overproduction of Cdc5 induces Sae2 hyper-phosphorylation in untreated cells and in nocodazole-blocked cells without the HO-cut formation (Figures 7D), supporting the idea that Sae2 might be a direct target of Cdc5. Indeed, as mentioned above, Sae2 protein sequence reveals several sites that could be bound and/or phosphorylated by Cdc5 (Figure 8A). The C-terminus of Cdc5, like other Polo-like kinases, contains a phospho-serine/phospho-threonine binding domain called the Polo-box Domain (PBD) [19]. The PBD is known to bind Plk substrates after they have been "primed" by a preliminary phosphorylation by another protein kinase [19]. Interestingly, the putative PBD binding motif of Sae2 has been previously shown to be phosphorylated by CDK1 [60], making it a perfect candidate for mediating the interaction between Sae2 and Cdc5. Indeed, by a 2-hybrid assay we found that the PBD of Cdc5 interacts with Sae2 (Figure 8B), and a recombinant GST-PBD fusion protein, purified from *E. coli*, precipitated Sae2-3HA from yeast extracts (Figure 8C).

Taken together, the results shown in Figure 7 and Figure 8 indicate that Cdc5, through its PBD, interacts with Sae2, causing its hyper-phosphorylation and accumulation at the DSB (see also a model in Figure 8D). It is interesting to point out that CtIP, the functional ortholog of Sae2 in human cells, was found to be associated to chromatin following DNA damage and its chromatin binding is promoted by phosphorylation and ubiquitination [68]. Indeed, recent evidences indicate that CtIP and Ctp1 (the CtIP counterpart in *S. pombe* [69]), are recruited to DSB sites through their interaction with Nbs1 [70–72], a subunit of Mre11 complex, and BRCA1 [73,74]. Moreover, CtIP is phosphorylated and regulated by CDK1 [74,75]. In yeast, Sae2 is involved both in promoting an early step of DSB ends resection [5] and in inactivating checkpoint signaling during recovery and adaptation [66,67], although the exact role of Sae2 in these processes is not yet fully understood. Interestingly, the overproduction of Sae2 also causes the overriding of the Mec1-signaling [66], while deletion of *SAE2* gene prevents switching off of the checkpoint [65,66].

One possible working model (Figure 8D), which needs to be verified, predicts that the increased and persistent binding of Sae2 to a DSB, induced by overproduction of Cdc5, may affect both DSB resection and Mec1-signaling. It is tempting to speculate that even physiological levels of Cdc5 may regulate Sae2 during recovery and adaptation, contributing to switch off the checkpoint signal. It is also possible that Sae2 is regulated by Cdc5 only when this kinase is expressed at elevated levels, leading to the checkpoint overriding. Indeed, such situation is frequently observed in cancer cells, when Plks are overexpressed [36–39], suggesting that what we found in yeast may represent a model for a pathological condition in human cells. Future works, requiring the analysis of *sae2* mutations in the sites regulated by Cdc5, may help to discriminate between the two possibilities.

In conclusion, in the present study we further explored the role of the polo kinase Cdc5 in attenuating the DNA damage checkpoint in budding yeast. We found that overproduction of Cdc5 affects different parameters of the cellular response to an inducible DSB: i) it overrides Mec1 signaling and prevents the phosphorylation of various Mec1 targets (Rad53, Rad9, Ddc2); ii) it causes a slower resection of DSB ends in a *RAD9*-dependent manner; iii) it binds Sae2 protein, causing its hyper-phosphorylation and leading to its increased and persistent binding onto DSB.

The emerging scenario suggests that Cdc5 may target multiple factors involved in various aspects of the cellular response to DSB lesions and DNA damage checkpoint signaling. Indeed, Cdc5 is a fundamental regulator of cell cycle progression and targets many
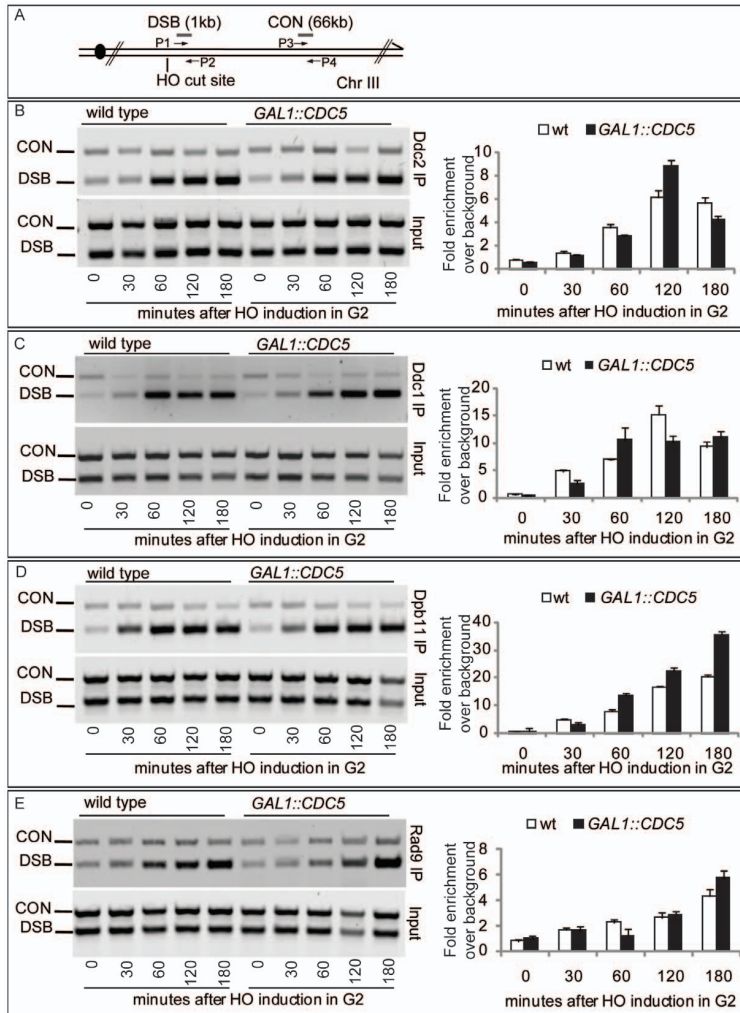
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Figure 6. Recruitment to DSB of checkpoint factors in *CDC5*-overexpressing cells. (A) Schematic representation of the HO cleavage site with the positions of the primers used to amplify regions 1 kb (DSB) and 66 kb (CON) from the HO cut site. PCR analysis at the CON site is used as a control of background signal. (B–E) YEP+raffinose nocodazole-arrested cell cultures of wild-type JKM and isogenic *GAL1::CDC5-MYC* or *GAL1-CDC5-HA* strains, expressing *DDC2*-HA, *DDC1*-MYC, *DPB11*-MYC, and *RAD9*-MYC alleles, were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). Cells were collected at the indicated times and then subjected to chromatin immunoprecipitation. Representative ChIP time-course analysis of protein-DSB association is shown for each protein tested before (Inputs) and after protein immunoprecipitation (IP).
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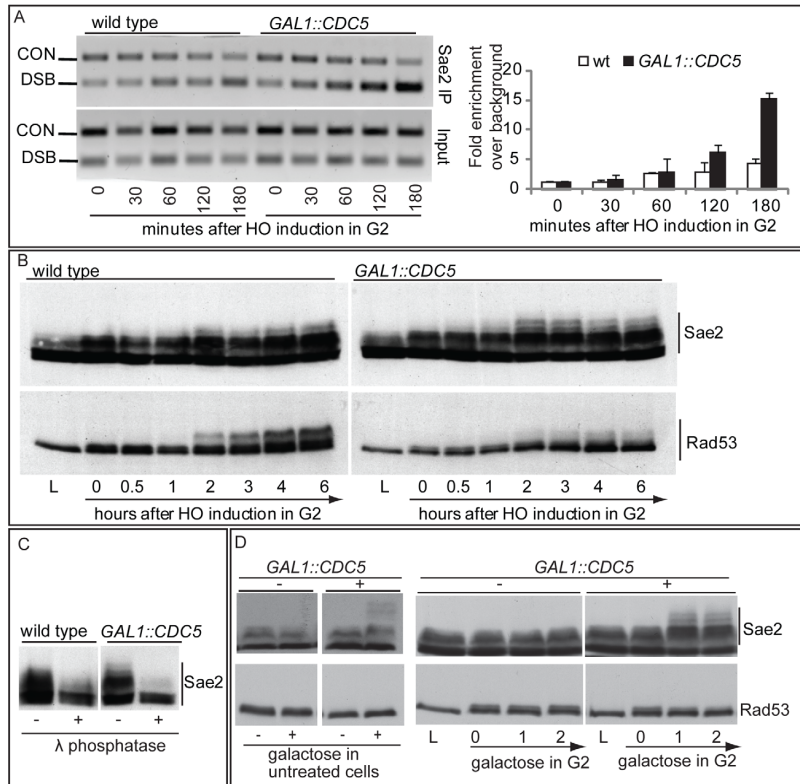
Figure 7. Analysis of Sae2 protein in *CDC5* overexpressing cells. (A,B) YEP+raffinose nocodazole-arrested cell cultures of wild type JKM and isogenic *GAL1::CDC5-MYC* strain, expressing *SAE2*-3HA allele, were transferred to nocodazole-containing YEP + raffinose + galactose (time zero). Cells were collected at the indicated times and then subjected to chromatin immunoprecipitation (ChIP) as described in Figure 6. Representative ChIP time-course analysis of protein-DSB association is shown before (Inputs) and after protein immunoprecipitation (IP). (B) Western blot analysis of protein extracts. (C) Western blot analysis of protein extracts prepared 3 hrs after HO induction and treated with or without λ phosphatase before gel electrophoresis. (D) YEP-raffinose growing cells of wild type and of wild-type JKM MATa-inc and isogenic *GAL1::CDC5-MYC* strains, expressing *SAE2*-3HA allele, were split in two. One half was treated with nocodazole to block cells in G2. Galactose was then added to the cultures to induce overproduction of Cdc5. Cells were collected at the indicated times after galactose addition. (B–D) Sae2-HA protein was analyzed by western blots using 12CA5 antibody; Rad53 protein was analysed by monoclonal antibody Mab.EL7.
doi:10.1371/journal.pgen.1000763.g007

proteins throughout a normal cell cycle [20]. Most of the Cdc5 substrates are proteins previously phosphorylated by CDK1, which is the principal regulator of the DSB-induced response, regulating DSB processing, recombination and checkpoint signaling [57]. Here we found that high levels of Cdc5 separately affected Mec1 signaling and DSB processing, leading us to speculate that Cdc5 may regulate multiple targets in response to DNA damage, including factors phosphorylated by CDK1. In support of such hypothesis, Plks phosphorylate, in vertebrates,

several proteins involved in various aspects of the DNA damage response, such as FANCM [25], Claspin [21–24], Chk2 [26–28], MCM5 [76], MCM7 [77] and others. Moreover, our findings on the functional role of Cdc5 in responding to a DSB in yeast rise the possibility that Plks may also regulate CtIP.

Recently, a proteome-wide screening led to the identification of novel Cdc5 targets in a normal cell cycle [20]; we believe that a similar approach is promising to identify Cdc5 targets regulated in response to DSBs. Good experimental evidence indicates that the
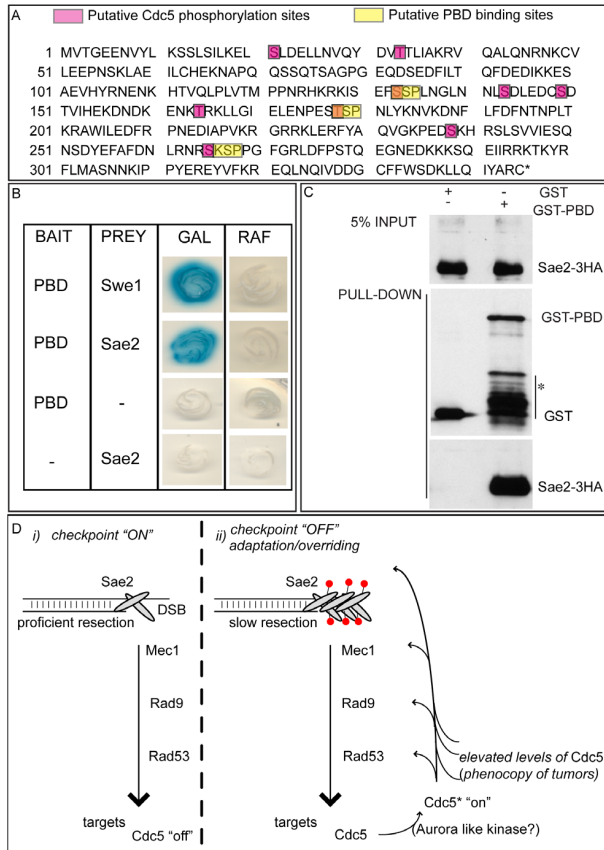
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Figure 8. Sae2 protein interacts with PBD of Cdc5. (A) Sae2 protein sequence. The putative Cdc5 phosphorylation sites and PBD binding sites are indicated. (B) Plasmid pEG202-PBD$_{340-705}$, carrying the polo box domain of Cdc5 (PBD, aa 340 to 705), and pJG4-5-SAE2, carrying the full length *SAE2* gene under the *GAL1* promoter, were co-transformed with pSH18-34, a β-galactosidase reporter plasmid in the wild type yeast strain EGY48. To assess two-hybrid interaction, these strains were patched on to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X Gal) plates containing either raffinose (RAF, prey repressed) or galactose (GAL, prey expressed). Accordingly to [50], the strain Y692 (PBD versus Swe1$_{173-400}$ protein fragment) was used as positive control. (C) Cells of the strain Y202, expressing *SAE2*-3HA gene, were blocked in G2/M by nocodazole treatment. Whole cell protein extract was prepared and incubated with glutathione-Sepharose beads carrying GST or GST-PBD$_{357-705}$. Input and pull-down samples were analyzed by western blotting with monoclonal antibody 12CA5 (αHA) or polyclonal antisera raised against GST (αGST). Asterisk denotes bands of GST-PBD degradation or expression of truncated proteins. (D) Schematic model to summarize the results presented in this work. (i) Sae2 transiently binds DSB, regulating ends resection and influencing Mec1-signaling. The checkpoint signal is amplified downstream, regulating several targets, including Cdc5. (ii) After a prolonged checkpoint response, adaptation to damage takes over and Cdc5 is re-activated, likely by an activating kinase (in human cells, it is aurora A [35]); Cdc5 then inhibits checkpoint signaling in a feedback regulatory loop, by likely targeting several factors, including Sae2 whose loading on the irreparable DSB increases, slowing down resection and contributing to counteract the checkpoint signaling (red circles denote phosphorylation). Alternatively, or in addition, Cdc5 function on several targets, including Sae2, is enhanced in the presence of elevated levels of Cdc5, a situation frequently found for Plks in tumor cells.
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functional role of Cdc5 in the DNA damage response is evolutionary conserved and the outputs of such a screening may provide important information for new cancer therapy strategies, targeting Plks and their substrates with specific tools.

Materials and Methods

Yeast strains and plasmids

Strains are listed in Table S1. All the strains were constructed during this study, and all were derivatives of JKM (*MAT*α, *hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO*), with the exception of strain Y38, which was generated from strain Y5 (YMV80, *matΔ::hisG1, hmlΔ::ADE, hmrΔ::ADE1, lys5, ura3-52, leu2::HOcs, ade3::GAL::HO, his-URA3-5′Δleu2-is4*). To construct strains, standard genetic procedures for transformation and tetrad analysis were followed. Y38 and Y210 were obtained by integration of ApaI-digested plasmid pJC57 (p*GAL1*::*CDC5*-3HA) at the URA3 locus. Y215 was derived by integration of ApaI-digested pJC59 (p*GAL1*::*CDC5*-3myc) at *URA3* locus. Y220 was obtained by integration of ApaI-digested plasmid pJC62 (p*GAL1*::*cdc5*-K110A-3HA) at *URA3* locus. Y222 was obtained by integration of ApaI-digested plasmid pJC69 (p*GAL1*::*cdc5*-L251W-3HA) at *URA3* locus. Deletions and tag fusions were generated by the one-step PCR system [78]. The yeast two-hybrid assay was performed using the B42/lexA system with strain EGY48 (*Mata his3 ura3 trp1 6lexAOP-LEU2*; *lexAOP-lacZ* reporter on plasmid pSH18-34) as the host strain [79]. Bait plasmid pEG202-PBD$_{340-705}$ for the two-hybrid assay, expressing lexA fusion with polo box domain of Cdc5, was obtained by amplifying the corresponding coding sequence of *CDC5* gene (aa 340 to 705) from genomic DNA and ligating the resulting fragment into pEG202 (kind gift from R. Brent). Prey plasmids pJG4-5-Swe1$_{173-400}$ and pJG4-5-SAE2, expressing B42 activating domain fusions, were obtained by amplifying the corresponding coding sequence of *SWE1* (aa 173 to 400) and *SAE2* (full length) from genomic DNA and ligating the resulting fragments into pJG4-5.

Western blot analysis

The TCA protein extraction and the western blot procedures have been previously described [29]. Rad53, Rad9, Sae2-HA, Ddc2-HA, Ddc1-myc, Dpb11-myc, Cdc5-HA, Cdc5-myc were analysed using specific monoclonal or polyclonal antibodies: anti-Rad53 Mab.EL7 and Mab.F9 monoclonal [54], anti-HA 12CA5 monoclonal, anti-myc 9E10 monoclonal, anti-Rad9 polyclonal (a kind gift from N Lowndes's lab).

In situ auto-phosphorylation assay

It was performed as previously described [52].

Immunoprecipitation analysis

Yeast whole cell extracts were prepared by FastPrep (MP Biomedicals) in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mm Tris (pH 7.5), 1 mM dithiothreitol (DTT), 60 mM β-glycerophosphate, 1 mM NaVO$_3$, cocktail proteases inhibitors (Roche)). HA-tagged proteins were immunoprecipitated using anti HA monoclonal antibody (12CA5) conjugated to protein G Agarose.

GST pulldown assay

GST and GST-PBD were induced in BL21 *E. coli* cells as previously described [80] and conjugated to glutathione-Sepharose 4B beads (GSH beads, Amersham). Yeast whole cell extracts, prepared as indicated above, were incubated with GST or GST-PBD GSH beads and rotated for 1 hour at 4°C. Samples were washed three times with NP-40 buffer, boiled in SDS-based sample buffer, and analyzed by Western blotting analysis.

In vitro dephosphorylation assay

Crude extracts were prepared as described [52], and resuspended in λ phosphatase buffer with or without 4000 U of λ phosphatase (Biolabs). Samples were incubated 30 min at 30°C and resuspended in Laemmli buffer.

Measurements of DNA resection and SSA at DSBs

Cells grown in YEP-raffinose 3% medium at 28°C to a concentration of 5×10^6 cells/ml were arrested with nocodazole (20μg/ml). A DSB was produced by adding 2% galactose and inducing the production of the HO endonuclease. The maintenance of the arrest was confirmed by FACS analysis and monitoring of nuclear division. Genomic DNA was isolated at intervals, and the loss of the 5′ ends of the HO-cleaved MAT locus was determined by Southern blotting [14,81,82]. To visualize the kinetics of resection, the graphs shown in Figure 4C and Figure 5B display, for each strain and for each ssDNA fragment (r1–r7), the time of the first appearance in the blot. In particular, since the appearance of a ssDNA fragment signal in the gel was due to the loss of the internal *SspI* sites, we represented the length of the minimal resection for each time point in the graph (see scheme in Figure 4A). All the experiments have repeated al least 3 times. In the corresponding figures, one representative example is shown with its graphic representation.

Chromatin immunoprecipitation analysis (ChIP)

ChIP analysis was performed as described previously [83,84]. Multiplex PCRs were carried out by using primer pairs complementary to DNA sequences located 1 kb from the HO-cut site at MAT (DSB) and to DNA sequences located 66 kb from MAT (CON). Gel quantitation was determined by using the NIH Image program. The relative fold enrichments of DSB-bound protein were calculated as follow: [DSB_IP/CON_IP]/[DSB_input/CON_input], where IP and Input represent the amount of PCR product in the immunoprecipitates and in input samples before immunoprecipitation, respectively.

Supporting Information

Figure S1 Cellular levels of endogenous and overproduced Cdc5 protein. (A) Exponentially (L) growing culture of the strain Y79 (wild type) and Y114 (*GAL1::CDC5*) were grown in YEP+3%raffinose. The cell cultures were treated with nocodazole to block and maintained the cells in G2/M. Galactose was then added to induce the overproduction of Cdc5 and sample have been taken at the indicated times. (A) The cell cycle block in G2/M was analyzed by FACS. (B) Cdc5 protein was analysed by western blotting with polyclonal antibody, which recognized both the endogenous Cdc5 and the overproduced Cdc5-myc tagged protein.
Found at: doi:10.1371/journal.pgen.1000763.s001 (0.96 MB TIF)

Figure S2 Overproduced Cdc5 co-immunoprecipitates with Rad53. (A) Cultures of the strains Y79 (wild type), Y114 (*GAL1::CDC5*-MYC), exponentially (L) growing in YEP+3%raffinose were blocked in G2/M by nocodazole treatment (N) and zeocin (50 μg/ml) was then added to cause DSBs formation. After 30 min. of treatment with zeocin, 2% galactose was added and samples were taken after 1 hour. Overproduced Cdc5 protein has been immunoprecipitated with anti MYC antibody. Cdc5-MYC

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and Rad53 proteins were analysed by western blotting with monoclonal antibodies 9E10 (αMYC) and Ma.EL7 (αRad53).
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Figure S3 Overproduction of Cdc5 does not prevent DSB repair by Single Strand Annealing (SSA). (A) Schematic representation of the YMV80 system used to detect DSB repair by SSA. Vertical bars show the relevant *KpnI* sites. After the HO cleavage, DNA is resected. When the left and right *leu2* sequences have been converted to ssDNA, repair by SSA can take place and can be monitored by the appearance of a SSA product in a Southern blot. (B,C) YEP+raffinose nocodazole-arrested cell cultures of wild type YMV80 and isogenic *GAL1::CDC5* strain were transferred to nocodazole-containing YEP+raffinose+galactose (time zero). (B) *KpnI*-digested genomic DNA, prepared from cells collected at the indicated times, was analysed by Southern blotting with a *LEU2* probe. Two fragments, 8 and 6 kb long (*his4::leu2*, *leu2::HOcs*) are evident in the absence of HO cut, whereas the HO-induced DSB causes the disappearance of the 6-kb species and the formation of a 2.5-kb fragment (HO-cut fragment). Repair by SSA converts such fragment to a repair product of 3.5-kb (SSA-product). (C) Western blot analysis of protein extracts with anti-Rad53 antibodies (Mab.EL7).

Found at: doi:10.1371/journal.pgen.1000763.s003 (0.81 MB TIF)

Table S1 Yeast strains used in this study.
Found at: doi:10.1371/journal.pgen.1000763.s004 (0.05 MB DOC)

Author Contributions

Conceived and designed the experiments: RAD MF FL AP. Performed the experiments: RAD MF FL BTN AP. Analyzed the data: RAD MF FL MC BTN AP. Contributed reagents/materials/analysis tools: MC. Wrote the paper: PP MMF AP.

References

1. Harrison JC, Haber JE (2006) Surviving the breakup: the DNA damage checkpoint. Annu Rev Genet 40: 209–235.
2. Harper JW, Elledge SJ (2007) The DNA damage response: ten years after. Mol Cell 28: 739–745.
3. Branzei D, Foiani M (2008) Regulation of DNA repair throughout the cell cycle. Nat Rev Mol Cell Biol 9: 297–308.
4. Lazzaro F, Giannattasio M, Puddu F, Granata M, Pellicioli A, et al. (2009) Checkpoint mechanisms at the intersection between DNA repair and damage. DNA Repair (Amst) 8: 1055–1067.
5. Mimitou EP, Symington LS (2009) Nucleases and helicases take center stage in homologous recombination. Trends Biochem Sci 34: 264–272.
6. Bartek J, Lukas J (2007) DNA damage checkpoints: from initiation to recovery or adaptation. Curr Opin Cell Biol 19: 238–245.
7. Clemenson C, Marsolier-Kergoat MC (2009) DNA damage checkpoint inactivation: adaptation and recovery. DNA Repair (Amst) 8: 1101–1109.
8. Lee SE, Pellicioli A, Demeter J, Vaze MP, Gasch AP, et al. (2000) Arrest, adaptation, and recovery following a chromosome double-strand break in Saccharomyces cerevisiae. Cold Spring Harb Symp Quant Biol 65: 303–314.
9. Galgoczy DJ, Toczyski DP (2001) Checkpoint adaptation precedes spontaneous and damage-induced genomic instability in yeast. Mol Cell Biol 21: 1710–1718.
10. van Vugt MA, Medema RH (2004) Checkpoint adaptation and recovery: beat with Polo after the break. Cell Cycle 3: 1383–1386.
11. van de Weerdt BC, Medema RH (2006) Polo-like kinases: a team in control of the division. Cell Cycle 5: 853–864.
12. Pellicioli A, Lee SE, Lucca C, Foiani M, Haber JE (2001) Regulation of Saccharomyces Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest. Mol Cell 7: 293–300.
13. Toczyski DP, Galgoczy DJ, Hartwell LH (1997) CDC5 and CKII control adaptation to the yeast DNA damage checkpoint. Cell 90: 1097–1106.
14. Vaze MB, Pellicioli A, Lee SE, Ira G, Liberi G, et al. (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell 10: 373–385.
15. van Vugt MA, Bras A, Medema RH (2004) Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells. Mol Cell 15: 799–811.
16. Syljuasen RG, Jensen S, Bartek J, Lukas J (2006) Adaptation to the ionizing radiation-induced G2 checkpoint occurs in human cells and depends on checkpoint kinase 1 and Polo-like kinase 1 kinases. Cancer Res 66: 10253–10257.
17. Liu X, Lei M, Erikson RL (2006) Normal cells, but not cancer cells, survive severe Plk1 depletion. Mol Cell Biol 26: 2093–2108.
18. Lane HA, Nigg EA (1996) Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. J Cell Biol 135: 1701–1713.
19. Lowery DM, Mohammad DH, Elia AE, Yaffe MB (2004) The Polo-box domain: a molecular integrator of mitotic kinase cascades and Polo-like kinase function. Cell Cycle 3: 128–131.
20. Snead JL, Sullivan M, Lowery DM, Cohen MS, Zhang C, et al. (2007) A coupled chemical-genetic and bioinformatic approach to Polo-like kinase pathway exploration. Chem Biol 14: 1261–1272.
21. Peschiaroli A, Dorrello NV, Guardavaccaro D, Venere M, Halazonetis T, et al. (2006) SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response. Mol Cell 23: 319–329.
22. Mamely I, van Vugt MA, Smits VA, Semple JI, Lemmens B, et al. (2006) Polo-like kinase-1 controls proteasome-dependent degradation of Claspin during checkpoint recovery. Curr Biol 16: 1950–1955.
23. Mailand N, Bekker-Jensen S, Bartek J, Lukas J (2006) Destruction of Claspin by SCFbetaTrCP restrains Chk1 activation and facilitates recovery from genotoxic stress. Mol Cell 23: 307–318.
24. Yoo HY, Kumagai A, Shevchenko A, Dunphy WG (2004) Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase. Cell 117: 575–588.
25. Kee Y, Kim JM, D'Andrea AD (2009) Regulated degradation of FANCM in the Fanconi anemia pathway during mitosis. Genes Dev 23: 555–560.
26. Bahassi el M, Conn CW, Myer DL, Hennigan RF, McGowan CH, et al. (2002) Mammalian Polo-like kinase 3 (Plk3) is a multifunctional protein involved in stress response pathways. Oncogene 21: 6633–6640.
27. Tsvetkov LM, Tsekova RT, Xu X, Stern DF (2005) The Plk1 Polo box domain mediates a cell cycle and DNA damage regulated interaction with Chk2. Cell Cycle 4: 609–617.
28. Petrinac S, Ganuelas ML, Bonni S, Nantais J, Hudson JW (2009) Polo-like kinase 4 phosphorylates Chk2. Cell Cycle 8: 327–329.
29. Cheng L, Hunke L, Hardy CF (1998) Cell cycle regulation of the Saccharomyces cerevisiae polo-like kinase cdc5p. Mol Cell Biol 18: 7360–7370.
30. Smits VA, Klompmaker R, Arnaud L, Rijksen G, Nigg EA, et al. (2000) Polo-like kinase-1 is a target of the DNA damage checkpoint. Nat Cell Biol 2: 672–676.
31. van Vugt MA, Smits VA, Klompmaker R, Medema RH (2001) Inhibition of Polo-like kinase-1 by DNA damage occurs in an ATM- or ATR-dependent fashion. J Biol Chem 276: 41656–41660.
32. Ando K, Ozaki T, Yamamoto H, Furuya K, Hosoda M, et al. (2004) Polo-like kinase 1 (Plk1) inhibits p53 function by physical interaction and phosphorylation. J Biol Chem 279: 25549–25561.
33. Tsvetkov L, Stern DF (2005) Phosphorylation of Plk1 at S137 and T210 is inhibited in response to DNA damage. Cell Cycle 4: 166–171.
34. Bassermann F, Frescas D, Guardavaccaro D, Busino L, Peschiaroli A, et al. (2008) The Cdc14B-Cdh1-Plk1 axis controls the G2 DNA-damage-response checkpoint. Cell 134: 256–267.
35. Macurek L, Lindqvist A, Lim D, Lampson MA, Klompmaker R, et al. (2008) Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery. Nature 455: 119–123.
36. Lu LY, Yu X (2009) The balance of Polo-like kinase 1 in tumorigenesis. Cell Div 4: 4.
37. Eckerdt F, Yuan J, Strebhardt K (2005) Polo-like kinases and oncogenesis. Oncogene 24: 267–276.
38. Takai N, Hamanaka R, Yoshimatsu J, Miyakawa I (2005) Polo-like kinases (Plks) and cancer. Oncogene 24: 287–291.
39. Smith MR, Wilson ML, Hamanaka R, Chase D, Kung H, et al. (1997) Malignant transformation of mammalian cells initiated by constitutive expression of the polo-like kinase. Biochem Biophys Res Commun 234: 397–405.

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40. Weichert W, Denkert C, Schmidt M, Gekeler V, Wolf G, et al. (2004) Polo-like kinase isoform expression is a prognostic factor in ovarian carcinoma. Br J Cancer 90: 815–821.
41. Tokumitsu Y, Mori M, Tanaka S, Akazawa K, Nakano S, et al. (1999) Prognostic significance of polo-like kinase expression in esophageal carcinoma. Int J Oncol 15: 687–692.
42. Knecht R, Oberhauser C, Strebhardt K (2000) PLK (polo-like kinase), a new prognostic marker for oropharyngeal carcinomas. Int J Cancer 89: 535–536.
43. Kneisel L, Strebhardt K, Bernd A, Wolter M, Binder A, et al. (2002) Expression of polo-like kinase (PLK1) in thin melanomas: a novel marker of metastatic disease. J Cutan Pathol 29: 354–358.
44. Yamada S, Ohira M, Horie H, Ando K, Takayasu H, et al. (2004) Expression profiling and differential screening between hepatoblastomas and the corresponding normal livers: identification of high expression of the PLK1 oncogene as a poor-prognostic indicator of hepatoblastomas. Oncogene 23: 5901–5911.
45. Bartek J, Lukas J, Bartkova J (2007) DNA damage response as an anti-cancer barrier: damage threshold and the concept of 'conditional haploinsufficiency'. Cell Cycle 6: 2344–2347.
46. Sanchez Y, Bachant J, Wang H, Hu F, Liu D, et al. (1999) Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. Science 286: 1166–1171.
47. Hu F, Wang Y, Liu D, Li Y, Qin J, et al. (2001) Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. Cell 107: 655–665.
48. Song S, Grenfell TZ, Garfield S, Erikson RL, Lee KS (2000) Essential function of the polo box of Cdc5 in subcellular localization and induction of cytokinetic structures. Mol Cell Biol 20: 286–298.
49. Song S, Lee KS (2001) A novel function of Saccharomyces cerevisiae CDC5 in cytokinesis. J Cell Biol 152: 451–469.
50. Bartholomew CR, Woo SH, Chung YS, Jones C, Hardy CF (2001) Cdc5 interacts with the Wee1 kinase in budding yeast. Mol Cell Biol 21: 4949–4959.
51. Charles JF, Jaspersen SL, Tinker-Kulberg RL, Hwang L, Szidon A, et al. (1998) The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in S. cerevisiae. Curr Biol 8: 497–507.
52. Pellicioli A, Lucca C, Liberi G, Marini F, Lopes M, et al. (1999) Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. EMBO J 18: 6561–6572.
53. Pellicioli A, Foiani M (2005) Signal transduction: how rad53 kinase is activated. Curr Biol 15: R769–771.
54. Fiorani S, Mimun G, Caleca L, Piccini D, Pellicioli A (2008) Characterization of the activation domain of the Rad53 checkpoint kinase. Cell Cycle 7: 493–499.
55. Mantiero D, Clerici M, Lucchini G, Longhese MP (2007) Dual role for Saccharomyces cerevisiae Tel1 in the checkpoint response to double-strand breaks. EMBO Rep 8: 380–387.
56. Toh GW, Lowndes NF (2003) Role of the Saccharomyces cerevisiae Rad9 protein in sensing and responding to DNA damage. Biochem Soc Trans 31: 242–246.
57. Ira G, Pellicioli A, Balijja A, Wang X, Fiorani S, et al. (2004) DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. Nature 431: 1011–1017.
58. Lazzaro F, Sapountzi V, Granata M, Pellicioli A, Vaze M, et al. (2008) Histone methyltransferase Dot1 and Rad9 inhibit single-stranded DNA accumulation at DSBs and uncapped telomeres. EMBO J 27: 1502–1512.
59. Ubersax JA, Woodbury EL, Quang PN, Paraz M, Blethrow JD, et al. (2003) Targets of the cyclin-dependent kinase Cdk1. Nature 425: 859–864.
60. Huertas P, Cortes-Ledesma F, Sartori AA, Aguilera A, Jackson SP (2008) CDK targets Sae2 to control DNA-end resection and homologous recombination. Nature 455: 689–692.
61. Kondo T, Wakayama T, Naiki T, Matsumoto K, Sugimoto K (2001) Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms. Science 294: 867–870.
62. Zou L, Elledge SJ (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science 300: 1542–1548.
63. Lisby M, Barlow JH, Burgess RC, Rothstein R (2004) Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins. Cell 118: 699–713.
64. Navadgi-Patil VM, Burgers PM (2009) A tale of two tails: Activation of DNA damage checkpoint kinase Mec1/ATR by the 9-1-1 clamp and by Dpb11/TopBP1. DNA Repair (Amst).
65. Baroni E, Viscardi V, Cartagena-Lirola H, Lucchini G, Longhese MP (2004) The functions of budding yeast Sae2 in the DNA damage response require Mec1- and Tel1-dependent phosphorylation. Mol Cell Biol 24: 4151–4165.
66. Clerici M, Mantiero D, Lucchini G, Longhese MP (2006) The Saccharomyces cerevisiae Sae2 protein negatively regulates DNA damage checkpoint signalling. EMBO Rep 7: 212–218.
67. Kim HS, Vijayakumar S, Reger M, Harrison JC, Haber JE, et al. (2008) Functional interactions between Sae2 and the Mre11 complex. Genetics 178: 711–723.
68. Yu X, Fu S, Lai M, Baer R, Chen J (2006) BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. Genes Dev 20: 1721–1726.
69. Limbo O, Chahwan C, Yamada Y, de Bruin RA, Wittenberg C, et al. (2007) Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination. Mol Cell 28: 134–146.
70. Yuan J, Chen J (2009) N terminus of CtIP is critical for homologous recombination mediated double-strand break repair. J Biol Chem.
71. Lloyd J, Chapman JR, Clapperton JA, Haire LF, Hartsuiker E, et al. (2009) A supramodular FHA/BRCT-repeat architecture mediates Nbs1 adaptor function in response to DNA damage. Cell 139: 100–111.
72. Williams RS, Dodson GE, Limbo O, Yamada Y, Williams JS, et al. (2009) Nbs1 flexibly tethers Ctp1 and Mre11-Rad50 to coordinate DNA double-strand break processing and repair. Cell 139: 87–99.
73. Chen L, Nievera CJ, Lee AY, Wu X (2008) Cell cycle-dependent complex formation of BRCA1.CtIP.MRN is important for DNA double-strand break repair. J Biol Chem 283: 7713–7720.
74. Yu X, Chen J (2004) DNA damage-induced cell cycle checkpoint control requires CtIP, a phosphorylation-dependent binding partner of BRCA1 C-terminal domains. Mol Cell Biol 24: 9478–9486.
75. Huertas P, Jackson SP (2009) Human CtIP mediates cell cycle control of DNA end resection and double strand break repair. J Biol Chem 284: 9558–9565.
76. Trenz K, Errico A, Costanzo V (2008) Plx1 is required for chromosomal DNA replication under stressful conditions. EMBO J 27: 876–885.
77. Tsvetkov L, Stern DF (2005) Interaction of chromatin-associated Plk1 and Mcm7. J Biol Chem 280: 11943–11947.
78. Longtine MS, McKenzie A, 3rd, Demarini DJ, Shah NG, Wach A, et al. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14: 953–961.
79. Gyuris J, Golemis E, Chertkov H, Brent R (1993) Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell 75: 791–803.
80. Miller CT, Gabrielse C, Chen YC, Weinreich M (2009) Cdc7p-dbf4p regulates mitotic exit by inhibiting Polo kinase. PLoS Genet 5: e1000498. doi:10.1371/journal.pgen.1000498.
81. Lee SE, Moore JK, Holmes A, Umezu K, Kolodner RD, et al. (1998) Saccharomyces Ku70, mre11/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage. Cell 94: 399–409.
82. Clerici M, Mantiero D, Lucchini G, Longhese MP (2005) The Saccharomyces cerevisiae Sae2 protein promotes resection and bridging of double strand break ends. J Biol Chem 280: 38631–38638.
83. Viscardi V, Bonetti D, Cartagena-Lirola H, Lucchini G, Longhese MP (2007) MRX-dependent DNA damage response to short telomeres. Mol Biol Cell 18: 3047–3058.
84. Clerici M, Mantiero D, Guerini I, Lucchini G, Longhese MP (2008) The Yku70-Yku80 complex contributes to regulate double-strand break processing and checkpoint activation during the cell cycle. EMBO Rep 9: 810–818.

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Published Paper II

The RSC chromatin-remodeling complex influences mitotic exit and adaptation to the spindle assembly checkpoint by controlling the Cdc14 phosphatase

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Upon prolonged activation of the spindle assembly checkpoint, cells escape from mitosis through a mechanism called adaptation or mitotic slippage, which is thought to underlie the resistance of cancer cells to antimitotic drugs. We show that, in budding yeast, this mechanism depends on known essential and nonessential regulators of mitotic exit, such as the Cdc14 early anaphase release (FEAR) pathway for the release of the Cdc14 phosphatase from the nucleolus in early anaphase. Moreover, the RSC (remodel the structure of chromatin) chromatin-remodeling complex bound to its accessory subunit Rsc2 is involved in this process as a novel component of the FEAR pathway. We show that Rsc2 interacts physically with the polo kinase Cdc5 and is required for timely phosphorylation of the Cdc14 inhibitor Net1, which is important to free Cdc14 in the active form. Our data suggest that fine-tuning regulators of mitotic exit have important functions during mitotic progression in cells treated with microtubule poisons and might be promising targets for cancer treatment.

Introduction

Chromosome segregation during anaphase requires the attachment of kinetochores to the mitotic spindle and removal of sister chromatid cohesion (Peters et al., 2008). In particular, cohesin must be cleaved by separase (Esp1 in yeast), which is kept in check by securin (Pds1 in yeast) until anaphase onset (Uhlmann, 2001). The ubiquitin ligase anaphase-promoting complex (APC) bound to its activator Cdc20 drives securin proteolysis and cohesin cleavage by separase at the metaphase-to-anaphase transition, thereby allowing sister chromatid separation (Nasmyth, 2002; Peters, 2006). Separase also contributes to mitotic exit and cyclin B proteolysis by acting in the Cdc14 early anaphase release (FEAR) pathway for nucleolar release and activation of the Cdc14 phosphatase.

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Abbreviations used in this paper: APC, anaphase-promoting complex; ChIP, chromatin immunoprecipitation; FEAR, Cdc14 early anaphase release; MCC, mitotic checkpoint complex; MEN, mitotic exit network; PBD, polo-box domain; rDNA, recombinant DNA; RENT, regulator of nucleolar silencing and telophase exit; RSC, remodel the structure of chromatin; SAC, spindle assembly checkpoint; tetO/tetR, tetracycline operator/repressor.

Indeed, Cdc14 is kept inactive in the nucleolus for most of the cell cycle as part of the regulator of nucleolar silencing and telophase exit (RENT) complex, which includes the Cdc14 inhibitor Net1/Cfi1 and the silencing protein Sir2 (Stegmeier and Amon, 2004). Besides separase, FEAR involves the polo kinase Cdc5, the Slk19 kinetochore protein, Spo12, and Bns1 (Stegmeier et al., 2002) and is negatively regulated by protein phosphatase 2A (Queralt et al., 2006), the replication fork block protein Fob1 (Stegmeier et al., 2004), and Tof2 (Waples et al., 2009). By promoting a first wave of partial Cdc14 release from the nucleolus in early anaphase, FEAR allows activation of the mitotic exit network (MEN), which leads to complete Cdc14 release and activation, thereby triggering cyclin B proteolysis and mitotic exit (Visintin et al., 1998).

The spindle assembly checkpoint (SAC) is a ubiquitous safety device ensuring the fidelity of mitotic chromosome segregation. During the process of microtubule capture by kinetochores in

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prophase and prometaphase, the SAC proteins Bub3, Mad2, and Mad3/BubR1 form the mitotic checkpoint complex (MCC), which inhibits the activity of Cdc20–APC, thereby preventing sister chromatid separation and mitotic exit until all chromosomes reach proper bipolar attachment to the mitotic spindle. Other SAC proteins, such as Mad1, Bub1, Mps1, and Ipl1/AuroraB, amplify the signal and regulate the rate of MCC formation (Musacchio and Salmon, 2007). Most SAC proteins accumulate at unattached kinetochores during prophase and prometaphase and generate from this location the stop anaphase signal leading to Cdc20–APC inhibition, possibly by accelerating the rate of MCC formation (Musacchio and Salmon, 2007).

Cells do not arrest indefinitely upon SAC activation, but they escape mitosis after a variable amount of time in the presence of unattached kinetochores. The process by which cells leak through the SAC-induced cell cycle arrest when the checkpoint is not satisfied is called adaptation or mitotic slippage (Rieder and Maiato, 2004). This process is largely responsible for the failure to efficiently block tumor progression with chemotherapeutic compounds targeting the mitotic spindle, such as taxanes and vinca alkaloids. In mammalian cells, mitotic slippage depends on progressive degradation of cyclin B, with SAC proteins being retained at kinetochores (Brito and Rieder, 2006; Gascoigne and Taylor, 2008). In yeast, inhibitory phosphorylation of cyclin B/Cdks has been proposed to accelerate adaptation to prolonged SAC activation (Minshull et al., 1996).

Here, we report a role for the budding yeast RSC (remodel the structure of chromatin) chromatin-remodeling complex in timely mitotic exit and adaptation to the SAC as a novel component of the FEAR network. The Rsc2-bound form of RSC appears to influence the rate of mitotic slippage by facilitating the nucleolar release of Cdc14, which then brings about cyclin B proteolysis and mitotic exit. Furthermore, our data suggest that Rsc2 regulates the FEAR function of the polo kinase Cdc5 in conditions that activate the SAC, but independently of SAC components, and provide a link between chromatin structure and the regulation of mitotic exit.

Results

MAD2 overexpression as a tool to study adaptation to the SAC

To study adaptation to the SAC, we set up conditions that lead to SAC hyperactivation without perturbing kinetochore attachment to the mitotic spindle. We cloned *MAD2* behind the strong galactose-inducible *GAL1* promoter (*GAL1-MAD2*) and integrated this construct in multiple copies in the yeast genome. We estimated that the levels of overexpressed Mad2 after 2 h in galactose are 20-fold higher than those of endogenous Mad2 (unpublished data). *GAL1-MAD2* cells released from G1 in the presence of galactose arrested transiently as large-budded cells with undivided nuclei, metaphase spindles, and high levels of nuclear Pds1 (Fig. 1 A). This metaphase arrest was caused by SAC hyperactivation as it was bypassed by *MAD1* and *MAD3* deletions (not depicted), by *PDS1* deletion (Fig. S1 C), and by expression of the dominant *CDC20-107* allele (Fig. S1, A and B), which is refractory to SAC inhibition (Hwang et al., 1998).

GAL1-MAD2 cells remained arrested for ∼4–5 h and then started to escape mitosis and enter in the next cycle, forming microcolonies of four or more cells on galactose-containing plates 6–8 h after release from G1 (Fig. 1 B) and eventually generating visible colonies (Fig. S1 B). Thus, Mad2-overproducing cells undergo mitotic slippage.

Characterization of SAC adaptation in yeast

In vertebrate cells, adaptation to the SAC takes place with SAC components still bound to kinetochores and is accompanied by cyclin B proteolysis (Brito and Rieder, 2006). As shown in Fig. 2 A, yeast *GAL1-MAD2* cells slipped out of mitosis and started reaccumulating in G1 7 h after release from G1 in the presence of galactose, with concomitant decrease of securin (Pds1) and cyclin B (Clb2) levels, whereas Mad2 levels remained constantly high. A similar independent experiment showed that *GAL1-MAD2* cells carrying the tetracycline operator/repressor (tetO/tetR)–GFP system to monitor sister chromatid separation (Michaelis et al., 1997) also started separating sister chromatids around the same time (Fig. 2 B). We then analyzed mitotic slippage in other conditions that engage the SAC by releasing G1-arrested wild-type cells carrying the aforementioned tetO/tetR-GFP system in the presence of the microtubule-depolymerizing drugs nocodazole or benomyl. Bipolar spindles did not assemble in either condition, although a fraction of benomyl-treated cells displayed cytoplasmic microtubules 4 and 6 h after release (see next paragraph). In spite of the complete absence of spindles, both nocodazole- and benomyl-treated cells underwent Pds1 and Clb2 degradation, separated sister chromatids, and slipped out of mitosis, although cells seemed to adapt faster in benomyl than in nocodazole (Fig. 2 C). In fact, benomyl-treated cells underwent almost complete Pds1 and Clb2 degradation, which resulted in cell division and reaccumulation of unbudded cells within 10 h after release. At the same time, a considerable fraction of nocodazole-treated cells was still arrested as large-budded cells with relatively high levels of Clb2 (Fig. 2 C).

To assess if adaptation in yeast correlates with silencing of SAC signaling, we monitored the levels of Mad1–Bub3 interaction, which takes place only in the presence of unattached kinetochores (Brady and Hardwick, 2000; Fraschini et al., 2001b) and therefore is a good readout for SAC signaling. G1-arrested cells expressing HA-tagged Bub3 (Bub3-HA3) were released in the presence of benomyl or nocodazole, followed by monitoring cell cycle progression by FACS analysis and Mad1–Bub3 interaction by coimmunoprecipitation. Again, 4 and 6 h after G1 release, a fraction of benomyl-treated cells (10 and 50%, respectively) displayed cytoplasmic microtubules (Fig. 2 E), which, in some cases, could drive an abnormal chromosome segregation (not depicted), but no bipolar spindles were detectable. Mad1–Bub3 interaction was stable up to 8 h after the G1 release in nocodazole-treated cells that were still arrested with 2C DNA content, whereas it started decreasing in the presence of benomyl after 4 h and was undetectable by 8 h, when most cells had exited mitosis (Fig. 2 D). The total levels of Mad1, but not of Bub3, also decreased in benomyl during the course of the
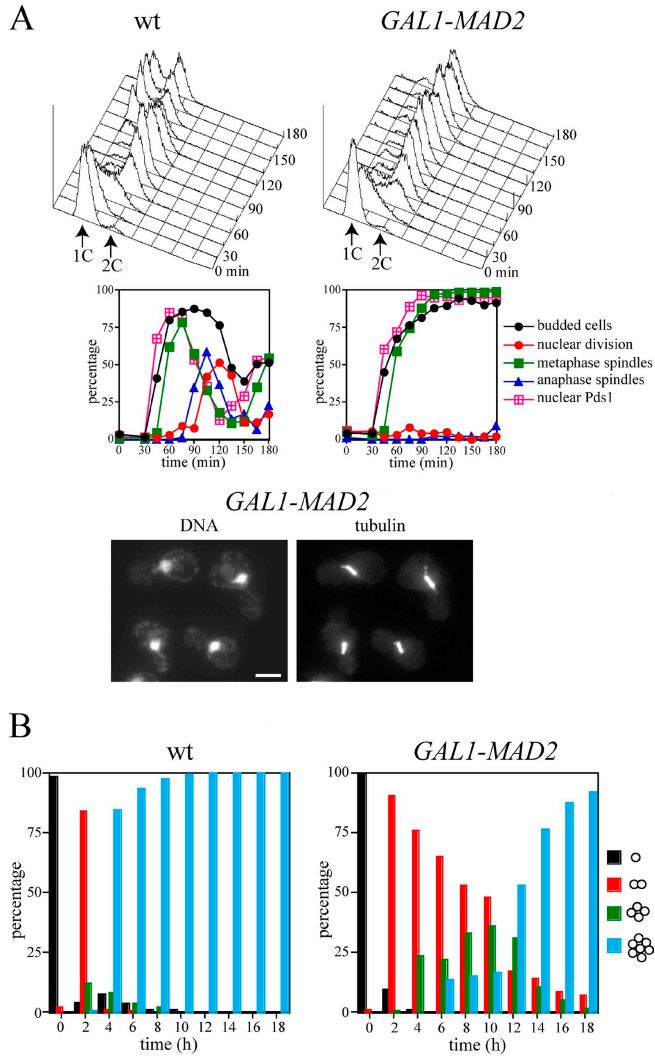
Figure 1. **MAD2 overexpression induces a transient metaphase arrest.** (A) Wild-type (wt; ySP4806) and *GAL1-MAD2* (ySP8526) cells were grown in YEPR, arrested in G1 with α-factor, and then released in YEPRG medium (t = 0). Samples were collected at the indicated times for FACS analysis of DNA contents and kinetics of budding, nuclear division, mitotic spindle formation/elongation, and Pds1 nuclear accumulation. Micrographs show examples of nuclear and microtubule staining (t = 150 min after release; bar, 5 µm). (B) Wild-type (W303) and *GAL1-MAD2* (ySP6170) cells were grown in YEPR, arrested in G1 with α-factor (unbudded cells), and spotted on YEPRG plates (t = 0). At the indicated times, 200 cells for each strain were scored to determine the frequency of single cells and of microcolonies of two, four, or more than four cells.
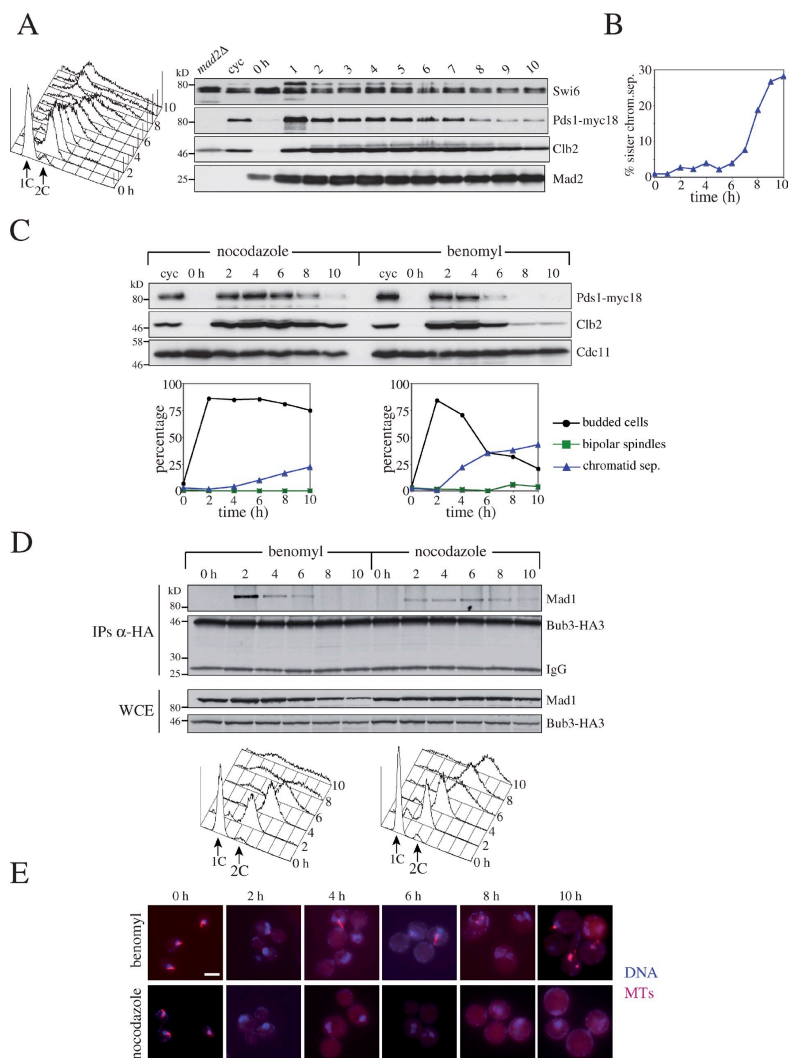
Figure 2. **Mitotic slippage upon prolonged treatment with microtubule destabilizers correlates with degradation of APC substrates and dissociation between Mad1 and Bub3.** (A) α-factor–arrested *GAL1-MAD2* (ySP8599) cells were released in YEPRG at 30°C (t = 0). α-factor was readded at 3 μg/ml after 2 h. Samples were collected at the indicated times for Western blot analysis of Pds1-myc18, Clb2, Mad2, and Swi6 (loading control). Cyc, cycling cells. (B) G1-arrested *GAL1-MAD2* cells carrying the tetO/tetR-GFP markers to score sister chromatid separation (ySP6699; Michaelis et al., 1997) were released in YEPRG at t = 0. (C) α-factor–arrested wild-type cells (ySP8534) were released in the presence of nocodazole or benomyl at t = 0. α-factor was readded at 3 μg/ml after 2 h, and samples were collected at the indicated times for Western blot analysis (top) of Pds1-myc18, Clb2, and Cdc11 (loading control),
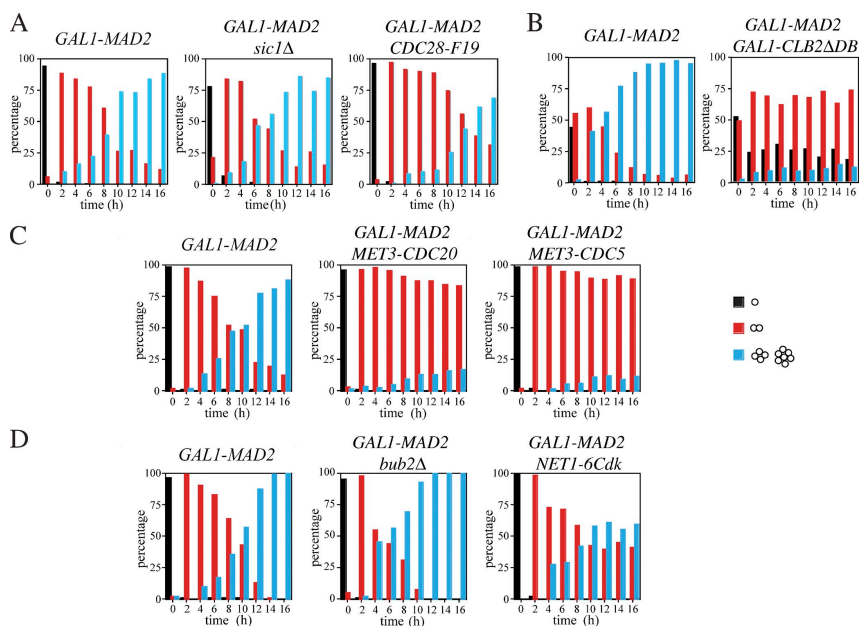
Figure 3. **SAC adaptation requires mitotic exit regulators.** (A) *GAL1-MAD2* (ySP6170), *GAL1-MAD2 sic1Δ* (ySP8706), and *GAL1-MAD2 CDC28-F19* (ySP8704) cells were grown in YEPR, arrested in G1 with α-factor (unbudded cells), and spotted on YEPRG plates (t = 0) at 30°C. 200 cells were scored at each time point for microcolony formation. (B) Cycling cultures of *GAL1-MAD2* (ySP3344) and *GAL1-MAD2 GAL1-CLB2ΔDB* (ySP8710) cells grown in YEPR were spotted on YEPRG plates (t = 0) at 30°C to follow microcolony formation. A fraction of *GAL1-MAD2 GAL1-CLB2ΔDB* cells remained unbudded because Clb2ΔDB inhibits budding (Surana et al., 1993). (C) *GAL1-MAD2* (ySP6170), *GAL1-MAD2 MET3-CDC20* (ySP8138), and *GAL1-MAD2 MET3-CDC5* (ySP8226) cells were grown in raffinose-containing medium lacking methionine, arrested in G1 with α-factor (unbudded cells), and spotted on YEPRG supplemented with 2 mM methionine (t = 0) to follow microcolony formation. (D) *GAL1-MAD2* (ySP6170), *GAL1-MAD2 bub2Δ* (ySP7677), and *GAL1-MAD2 NET1-6Cdk* (ySP7958) cells were treated as in A.

experiment but not as dramatically as in the Bub3 immunoprecipitates. Therefore, adaptation to the SAC in yeast is accompanied by silencing of checkpoint signaling.

Adaptation to the SAC requires cyclin B degradation, Cdc20, the polo kinase Cdc5, and Cdc14 nucleolar release

As SAC adaptation involves Clb2 proteolysis, we asked whether cyclin degradation, Cdk inhibitory phosphorylation, and/or Cdk inhibitors were required for mitotic slippage upon *MAD2* overexpression. As shown in Fig. 3 (A and B), microcolony formation of *GAL1-MAD2* cells on galactose plates was effectively delayed by expression of either the Cdk1 variant Cdc28-F19, which

cannot undergo Tyr19 inhibitory phosphorylation, or nondegradable Clb2. In contrast, deletion of the cyclin B/Cdk inhibitor Sic1 (Mendenhall, 1993; Schwob et al., 1994) had no effect.

We then asked whether SAC adaptation depends on cell cycle regulators that modulate mitotic exit and proteolysis of mitotic cyclins. Indeed, *CDC20* repression from the *MET3* promoter markedly prolonged the metaphase arrest of *GAL1-MAD2* cells (Fig. 3 C), suggesting that high levels of Mad2 are not sufficient to maintain Cdc20–APC inhibition for a long time. Inactivation of the polo kinase Cdc5 through a *MET3-CDC5* fusion yielded similar results (Fig. 3 C).

Adaptation to the SAC upon *MAD2* overexpression might also be influenced by advancing or delaying activation of the

as well as to monitor kinetics of budding, bipolar spindle formation, and sister chromatid separation (bottom graphs). (D and E). Cells expressing Bub3-HA3 (ySP8709) were treated as in C. At the indicated times, interaction between Bub3-HA3 and Mad1 was assessed by coimmunoprecipitation using anti-HA antibodies followed by Western blotting with anti-Mad1 and anti-HA antibodies. At the same times, DNA contents were measured by FACS analysis (D, histograms) and for tubulin staining by immunofluorescence (E). MTs, microtubules. WCE, whole cell extract. Bar, 5 μm.
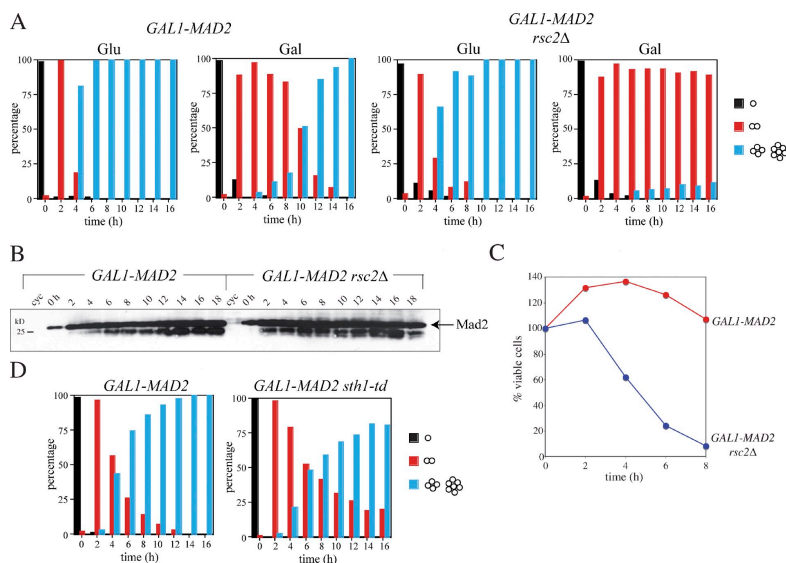
Figure 4. **The RSC complex is involved in SAC adaptation.** (A) *GAL1-MAD2* (ySP6170) and *GAL1-MAD2 rsc2Δ* (ySP6850) cells were grown in uninduced conditions (YEPR), arrested in G1 with α-factor (unbudded cells), and spotted on YEPD (Glu) and YEPRG (Gal) plates (t = 0) at 30°C. 200 cells were scored at each time point for microcolony formation. (B) The same strains as in A were grown in uninduced conditions, arrested in G1 with α-factor at 25°C, and released on YEPRG medium (t = 0) at 30°C. Samples were collected at the indicated times for Western blot analysis of Mad2 levels (arrow). Cyc, cycling cells. (C) The same strains as in A were treated as in B and plated at different times on YEPD plates to assess cell viability. Percentages represent mean values of three independent experiments. (D) *GAL1-MAD2* (ySP6170) and *GAL1-MAD2 GAL1-UBR1 CUP1-sth1[td]* (ySP7808) cells were grown in YEPR supplemented with 0.1 mM CuSO₄, arrested in G1 with α-factor (unbudded cells), and spotted on YEPRG plates (t = 0) at 37°C to follow microcolony formation. Data are representative of three independent repeats.

Cdc14 phosphatase that is necessary for mitotic exit. We thus forced unscheduled activation of the MEN and subsequent Cdc14 nucleolar release by eliminating the MEN inhibitor Bub2 (Piatti et al., 2006). Conversely, we delayed Cdc14 activation by expression of a nonphosphorylatable Net1 variant (Net1-6Cdks) that does not allow the transient release of Cdc14 from the nucleolus in early anaphase (Azzam et al., 2004). Notably, *BUB2* deletion accelerated microcolony formation of *GAL1-MAD2* cells on galactose plates, whereas *NET1-6Cdk* expression slowed it down (Fig. 3 D), suggesting that Cdc14 release from the nucleolus might be important for SAC adaptation.

The chromatin-remodeling RSC complex is involved in adaptation to the SAC

Because *MAD2* overexpression provides a good experimental setup to study the molecular bases of SAC adaptation in the absence of spindle/kinetochore defects, we used transposon mutagenesis of *GAL1-MAD2* cells to identify factors involved in adaptation and/or in fine tuning of mitotic exit. To this end, we screened for clones that were hypersensitive to *MAD2* overexpression and likely prolonged their cell cycle arrest under these conditions. We found that several clones with

this phenotype carried the transposon insertion 3′ to the *RSC2* gene, encoding an accessory subunit of the chromatin-remodeling complex RSC (Cairns et al., 1999). Indeed, the Rsc2-containing RSC complex seemed a good candidate for adaptation to the SAC because it had been previously implicated in chromosome segregation, mitotic progression, and regulation of sister chromatid separation (Hsu et al., 2003; Baetz et al., 2004; Huang and Laurent, 2004). Moreover, *RSC2* deletion was shown to have synthetic effects with mutations altering kinetochore components or cohesin (Baetz et al., 2004).

The latter observations were extended by analyzing the effects of *RSC2* deletion in a set of mutants in kinetochore components (Dam1 and Cep3) or microtubule-binding proteins (Stu2 and Cin8; Fig. S2 A). Besides confirming genetic interactions previously reported by others, *RSC2* deletion caused hypersensitivity to benomyl and decreased the maximal permissive temperature of the kinetochore mutants *dam1-11*, *cep3-10*, and *stu2-10*, as well as that of *cin8Δ* cells lacking the BimC family kinesin Cin8, which has a major role in spindle assembly (Hoyt et al., 1992). Because the aforementioned mutations and benomyl treatment engage the SAC, the deleterious effects of *RSC2* deletion in these conditions might be caused by prolonged

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SAC activation. Indeed, *RSC2* deletion turned out to be lethal for *GAL1-MAD2* cells in the presence of galactose (Fig. S2 B and Fig. 4 C). We then scored microcolony formation of *GAL1-MAD2* and *GAL1-MAD2 rsc2Δ* cells upon plating G1-synchronized cells on media containing either glucose (*GAL1-MAD2* off) or galactose (*GAL1-MAD2* on*)*. Deletion of *RSC2* slightly delayed cell cycle progression on glucose plates compared with otherwise wild-type cells in the presence of galactose (Fig. 4 A). Strikingly, the presence of galactose caused *GAL1-MAD2 rsc2Δ* cells to remain arrested in mitosis as large-budded cells for a longer time than *GAL1-MAD2* cells (Fig. 4 A), in spite of comparable levels of Mad2 (Fig. 4 B). This behavior paralleled with the dramatic lethal effect of *GAL1-MAD2* overexpression in *rsc2Δ* cells (Fig. 4 C).

Deletion of *RSC1*, encoding an RSC subunit alternative to Rsc2 (Cairns et al., 1999), had no effect on the mitotic escape of *GAL1-MAD2* cells on galactose plates (Fig. S3 A), suggesting that the Rsc2-containing form of RSC (RSC^{Rsc2}) is specifically implicated in this process. The lack of Rsc2 also prolonged the mitotic arrest of *MPS1*-overexpressing cells (Fig. S3 B), which transiently hyperactivate the SAC and eventually adapt (Hardwick et al., 1996), and of benomyl-treated cells (Fig. S3 C).

We then asked whether Rsc2 has a role in SAC adaptation as part of the RSC complex or independently of it. This was not trivial because all core RSC subunits are essential and must be inactivated by temperature-sensitive mutations, whereas the *GAL1* promoter required to overexpress *MAD2* is very inefficient at high temperatures. Indeed, *GAL1-MAD2* cells showed only a modest cell cycle arrest at 37°C, as almost 50% of the cells had escaped from the arrest and formed microcolonies of four or more cells on galactose within 4 h after plating (Fig. 4 D). However, RSC inactivation by the temperature-sensitive degron allele of *STH1* (*sth1^{td}*; Parnell et al., 2008), which encodes the RSC catalytic subunit, delayed adaptation of *GAL1-MAD2* cells by ∼2 h, suggesting that the whole RSC complex is involved in this process.

RSC^{Rsc2} inactivation prevents mitotic exit of SAC-deficient mutants in the presence of microtubule-depolymerizing drugs

As RSC inactivation might delay escape from mitosis by prolonging the SAC-dependent cell cycle arrest, we investigated its effects in SAC-deficient mutants treated with microtubule-depolymerizing drugs. To this end, wild-type, *mad2Δ*, *rsc2Δ*, and *mad2Δ rsc2Δ* cells were arrested in G1 by α-factor and released in the presence of nocodazole. As expected, *mad2Δ* cells rereplicated their DNA efficiently and accumulated DNA contents higher than 2C under these conditions, which instead caused the double *mad2Δ rsc2Δ* mutant to arrest in mitosis similarly to wild-type and *rsc2Δ* cells (Fig. 5 A). Deletion of *RSC2* prevented mitotic exit also of nocodazole-treated *mad1Δ*, *mad3Δ*, *bub1Δ*, *bub3Δ*, *cdc55Δ*, and *CDC20-107* cells (unpublished data). Moreover, rereplication of *mad2Δ* cells upon microtubule disruption was inhibited also by Sth1 inactivation through the *sth1^{td}* allele (Fig. 5 B), whereas it was not affected by *RSC1* deletion (Fig. S4 A). Altogether, these data suggest that RSC^{Rsc2} is required for the unscheduled mitotic exit of SAC mutants in the presence of spindle defects.

RSC2 deletion could prevent mitotic exit and rereplication of nocodazole-treated SAC mutants by either restoring Cdc20–APC inhibition or impinging on pathways controlling mitotic exit, such as the FEAR or MEN pathways for Cdc14 nucleolar release. In fact, whereas Cdc20–APC is required for degradation of securin and a fraction of cyclin B, Cdc14 triggers Cdh1/APC activation, which completes cyclin B degradation and drives accumulation of the Cdk inhibitor Sic1 (Visintin et al., 1998). To distinguish between these two possibilities, we first analyzed Pds1 and Clb2 degradation, as well as Sic1 accumulation, in wild-type, *mad2Δ*, *rsc2Δ*, and *mad2Δ rsc2Δ* cells that were released from G1 in the presence of nocodazole. As shown in Fig. 5 C, Pds1 was degraded in both *mad2Δ* and *mad2Δ rsc2Δ* cells, whereas a fraction of Clb2 was stabilized and Sic1 did not accumulate in *mad2Δ rsc2Δ* cells, in contrast to *mad2Δ* cells. These results are consistent with the role of RSC in the regulation of mitotic exit and, in particular, of Cdc14 nucleolar release (see next paragraph), rather than in Cdc20–APC activation. Like RSC mutations, mutations affecting the FEAR pathway, such as *esp1-1* (Fraschini et al., 2001a), *spo12Δ bns1Δ*, *slk19Δ* (Fig. S4 B), and *NET1-6Cdk* (not depicted) prevented rereplication of nocodazole-treated *mad2Δ* cells. In addition, simultaneous deletion of *SLK19*, *SPO12*, and *BNS1* retarded microcolony formation of *GAL1-MAD2* cells on galactose plates (Fig. S4 C). Similarly to FEAR mutations, *RSC2* deletion only modestly delayed mitotic exit both in unperturbed conditions (Fig. 6 A) and during recovery from nocodazole arrest (Fig. 6 B), as judged by the kinetics of spindle disassembly relative to spindle elongation and nuclear division. Conversely, lack of Rsc2 delayed the onset of anaphase (i.e., spindle elongation and nuclear division) relative to bipolar spindle assembly (Fig. 6, A and B), which is consistent with previous observations (Hsu et al., 2003; Baetz et al., 2004). Thus, RSC^{Rsc2} might regulate mitotic exit in a way similar to the FEAR pathway in conditions of SAC hyperactivation or in the presence of kinetochore/microtubule defects.

Lack of Rsc2 impairs Cdc14 release from the nucleolus at the metaphase-to-anaphase transition

The persistence of Clb2 and the lack of Sic1 accumulation in nocodazole-treated *mad2Δ rsc2Δ* cells, together with the similar effects caused by RSC and FEAR inactivation in SAC mutants upon microtubule disruption, suggested that RSC^{Rsc2} might be involved in the control of Cdc14 release from the nucleolus. We therefore analyzed Cdc14 nucleolar release in *mad2Δ*, *rsc2Δ*, and *mad2Δ rsc2Δ* cells released from G1 in the presence of nocodazole. Although *mad2Δ* cells transiently released Cdc14, all other strains retained it in the nucleolus (Fig. 7 A), suggesting that RSC^{Rsc2} is required for Cdc14 release in these conditions. Strikingly, expression of the Cdc14^{TAB6-1} dominant variant that associates loosely to its inhibitor Net1 (Shou et al., 2001) restored the ability of nocodazole-treated *mad2Δ rsc2Δ* cells to rereplicate DNA (Fig. 7 B), whereas it was not sufficient by itself to promote mitotic exit in these conditions (not depicted). These data support the notion that RSC^{Rsc2} inactivation interferes with Cdc14 nucleolar release and activation, prompting
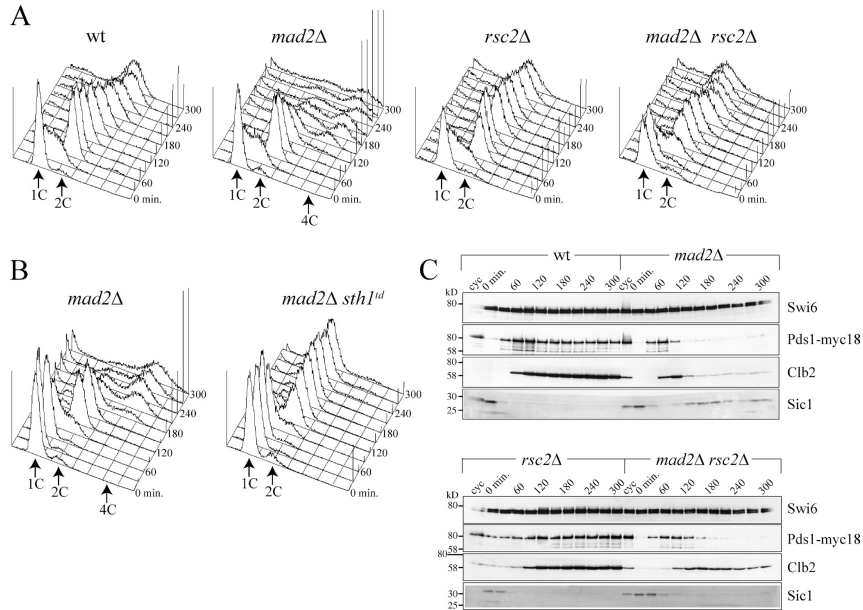
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Figure 5. **Lack of the RSC complex prevents mitotic exit of nocodazole-treated SAC-defective cells.** (A) Cultures of wild-type (wt), *mad2Δ*, *rsc2Δ*, and *mad2Δ rsc2Δ* cells (ySP4806, ySP1084, ySP6997, and ySP7543) were grown in YEPD, arrested in G1 by α-factor, and then released into medium containing nocodazole (t = 0). At the indicated times, cell samples were withdrawn for FACS analysis of DNA contents. (B) Cultures of *mad2Δ* (ySP1070) and *GAL1-UBR1 CUP1-sth1^{td} mad2::TRP1* (ySP7869) cells were grown in YEPR containing 0.1 mM CuSO₄ and arrested in G1 with α-factor at 27°C. 1 h after 2% galactose addition, cells were released in nocodazole-containing YEPRG at 37°C (t = 0), followed by FACS analysis of DNA contents at the indicated times. (C) The same strains and procedure as in A were used, but 10 µg/ml α-factor was readded to all cultures at t = 100 min after release (>90% of budded cells). At the indicated times, cells were collected for FACS analysis of DNA contents (not depicted) and for Western analysis of Pds1, Clb2, Sic1, and Swi6 (loading control). Cyc, cycling cells.

us to directly compare the kinetics of Cdc14 release from the nucleolus in *rsc2Δ* cells versus wild type and the FEAR mutant *spo12Δ bns1Δ*. To monitor only the partial Cdc14 release at the anaphase onset, we prevented MEN activation by overexpressing *BFA1* from the *GAL1* promoter (Li, 1999). Wild-type, *GAL1-BFA1*, *GAL1-BFA1 rsc2Δ*, and *GAL1-BFA1 spo12Δ bns1Δ* cells were synchronized in G1 and released in galactose-containing medium. We then followed partial and total Cdc14 release from the nucleolus during the cell cycle. As expected, wild-type cells started releasing Cdc14 after metaphase spindles had been assembled and concomitant to spindle elongation (Fig. 7 C). Nuclear division immediately followed, and Cdc14 was completely released into the nucleoplasm and cytosol before cytokinesis. Consistent with MEN inhibition, *GAL1-BFA1* cells arrested in telophase as large-budded cells with 2C DNA contents, divided nuclei, and elongated spindles. As expected, Cdc14 total release was abolished in these cells, and only the partial release in anaphase could be observed (Fig. 7 C). Like *GAL1-BFA1* cells, *GAL1-BFA1 rsc2Δ* and *GAL1-BFA1 spo12Δ*

bns1Δ cells arrested in telophase and showed no sign of total Cdc14 release. Moreover, Cdc14 partial release was abolished in *GAL1-BFA1 spo12Δ bns1Δ* cells and severely compromised in *GAL1-BFA1 rsc2Δ* cells (Fig. 7 C). Thus, Rsc2 and presumably the whole RSC^{Rsc2} complex contribute to the early anaphase release of Cdc14 from the nucleolus.

Deletion of *RSC2* has synthetic effects with mutations affecting the MEN

We analyzed the relationships between RSC and the FEAR or the MEN cascades by combining *RSC2* deletion with FEAR or MEN mutations. Deletion of *RSC2* caused little or no synthetic growth defects when combined with the FEAR mutations *slk19Δ*, *spo12Δ bns1Δ*, and *esp1-1* (unpublished data), suggesting that RSC^{Rsc2} works together with or in parallel to the FEAR pathway.

Inactivation of the FEAR pathway is known to be lethal for cells lacking the nonessential MEN activator Lte1 (Stegmeier et al., 2002). Similarly, *RSC2* deletion was found to be lethal with *LTE1* deletion (Ye et al., 2005). In fact, *rsc2Δ lte1Δ* cells
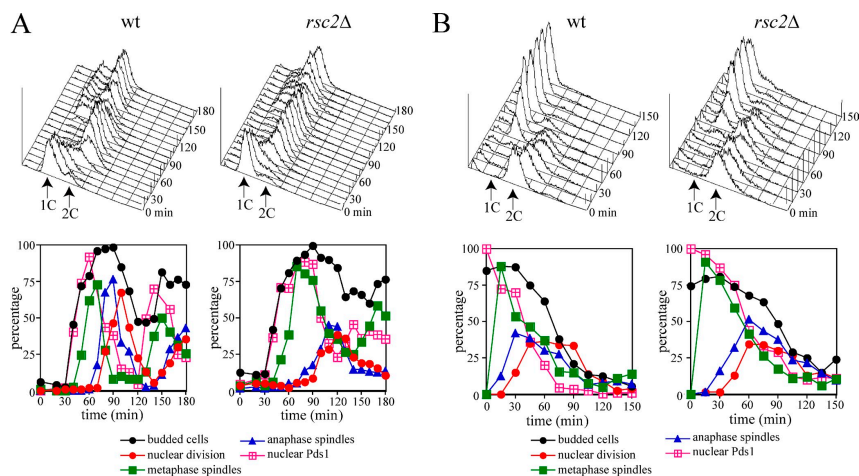
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Figure 6. Cell cycle progression of *rsc2Δ* cells and their recovery from SAC activation. (A) Cultures of wild-type (wt; ySP4806) and *rsc2Δ* (ySP6997) cells were grown in YEPD, arrested in G1 by α-factor, and then released in fresh medium (t = 0). At the indicated times, samples were analyzed as in Fig. 1 A. (B) The same strains as in A were grown in YEPD, arrested in mitosis by 5 µg/ml nocodazole treatment, and then released (t = 0) in 10 µg/ml YEPD containing α-factor, followed by the same analyses as in Fig. 1 A.

were, in most cases, inviable or extremely sick also in our genetic background (Fig. 8 A), and this lethality could be rescued by *BUB2* deletion (not depicted), suggesting that it was caused by constitutive trapping of Cdc14 in the nucleolus. *RSC2* deletion also caused sickness and lethality when combined with the temperature-sensitive alleles *cdc5-2*, affecting polo kinase, and *cdc14*-3, respectively (Fig. 8 A). In addition, it decreased the maximal permissive temperature of the *tem1-3, cdc15-2, dbf2-2,* and *cdc14-1* MEN mutants (Fig. 8 B), supporting the notion that RSC[Rsc2] regulates Cdc14 release from the nucleolus. Accordingly, *RSC2* overexpression suppressed *cdc15-2* lethality at 32°C (Fig. 8 C). Thus, RSC[Rsc2] controls Cdc14 release from the nucleolus at the metaphase/anaphase transition independently of MEN and in concert with the FEAR pathway.

Rsc2 interacts with the polo kinase Cdc5 and contributes to timely Net1 phosphorylation

FEAR components have been recently found to interact with the polo kinase Cdc5 (Rahal and Amon, 2008), which has a key role in Cdc14 nucleolar release acting in both the FEAR and the MEN pathways (Stegmeier and Amon, 2004). The *Xenopus laevis* homologue of Rsc2, polybromo-1/BAF180, was found to interact with polo kinase (Yoo et al., 2004), and Rsc2 itself was predicted to be a likely binding partner of Cdc5 (Snead et al., 2007). To investigate whether Rsc2 interacts with Cdc5, we expressed Flag-tagged Cdc5 (Cdc5-Flag3) in cells expressing either untagged Rsc2 or HA-tagged Rsc2 (Rsc2-HA3). Rsc2-HA3 immunoprecipitates from both cycling and nocodazole-arrested

cells contained Cdc5-Flag3, which was instead absent in the immunoprecipitates from the untagged Rsc2 strain (Fig. 9 A). Rsc2 could also bind the polo-box domain (PBD) of Cdc5, which normally binds substrates previously primed by phosphorylation by another kinase (Elia et al., 2003a). Indeed, Rsc2-HA3 bound to a recombinant GST-PBD fusion protein (Miller et al., 2009) but not to GST alone (Fig. 9 B). Surprisingly, this binding was not disrupted by mutating the critical $W^{517}V^{518}L^{530}$ residues (Elia et al., 2003b) into FAA, suggesting that it might be independent of preliminary phosphorylation.

Because Rsc2 binds to Cdc5 and is required for timely release of Cdc14 from the nucleolus, we evaluated whether *RSC2* deletion affected Net1 phosphorylation, which depends on Cdc5 and is required to release Net1-Cdc14 association (Shou et al., 2002; Yoshida and Toh-e, 2002). As shown in Fig. 9 D, a slow-migrating band corresponding to phosphorylated Net1 (Visintin et al., 2003; Queralt et al., 2006) appeared during anaphase in wild-type cells (80–90 min after release from G1 arrest; Fig. 9 C), whereas it was barely detectable in the absence of Rsc2, suggesting that the FEAR function of Cdc5 might require the RSC[Rsc2] complex.

RSC was previously involved in sister chromatid cohesion (Baetz et al., 2004; Huang and Laurent, 2004), and Cdc5 facilitates cohesin cleavage and sister chromatid separation besides promoting Cdc14 activation (Alexandru et al., 2001). We then asked whether Cdc5 distribution along chromosomes was altered in the absence of Rsc2 by studying Cdc5-Flag3 chromosomal distribution by ChIP (chromatin immunoprecipitation)-on-chip on the whole genome of yeast cells arrested in mitosis.
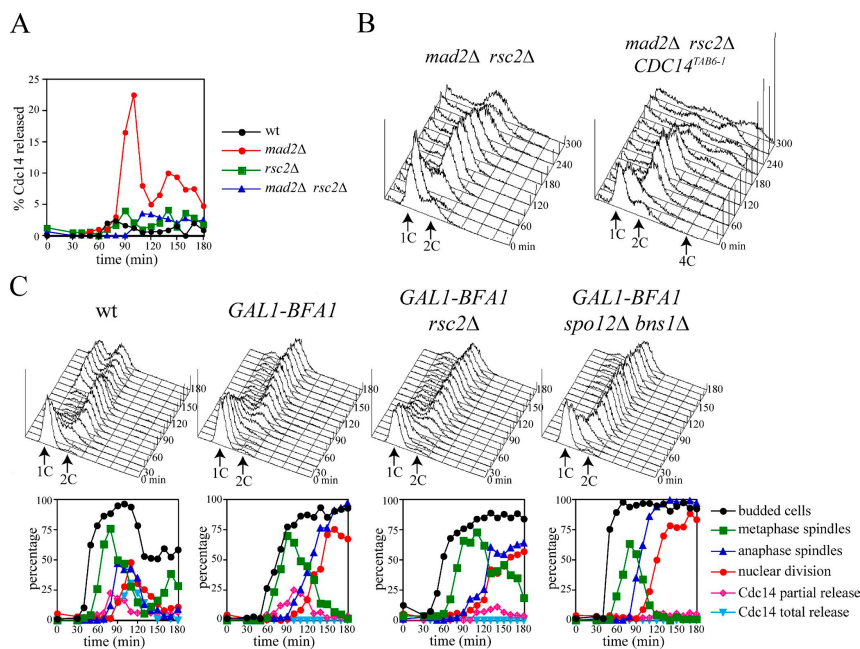
Figure 7. Rsc2 controls the early release of Cdc14 from nucleolus. (A) α-factor–arrested wild-type (wt; W303), *mad2Δ* (ySP1070), *rsc2Δ* (ySP6858), and *mad2Δ rsc2Δ* (ySP7088) cells were released in medium containing nocodazole (t = 0). At the indicated times, cells were collected for FACS analysis of DNA contents (not depicted) and for detecting Cdc14 release by immunofluorescence. (B) Cultures of *mad2Δ rsc2Δ* (ySP7088) and *mad2Δ rsc2Δ CDC14TAB6-1* (ySP7645) were treated as in A. At the indicated times after release (t = 0), cells were collected for FACS analysis of DNA contents. (C) Cultures of wild-type, *GAL1-BFA1*, *GAL1-BFA1 rsc2Δ*, and *GAL1-BFA1 spo12Δ bns1Δ* (W303, ySP1283, ySP7764, and ySP7803) cells were grown in YEPR, arrested in G1, and then released in YEPRG (t = 0). Samples were collected at the indicated times for FACS analysis of DNA contents and to follow the kinetics of budding, nuclear division, mitotic spindle formation/elongation, and Cdc14 partial/total release.

Cdc5 localized at centromeres and discrete sites along chromosome arms corresponding to cohesin-binding sites (see the left arm of chromosome VI as an example; Fig. 10, A and B), and it could be found also at recombinant DNA (rDNA; not depicted). *RSC2* deletion did not affect Cdc5 chromosomal distribution at any locus (Fig. 10 A and not depicted), suggesting that Rsc2 might regulate Cdc5 at levels other than its recruitment to specific chromosomal regions.

Discussion

Adaptation to the SAC depends on regulators of mitotic exit

Eukaryotic cells ultimately adapt to persistent SAC signaling and exit from mitosis, eventually leading to unbalanced chromosome segregation or cell death (Rieder and Maiato, 2004). Mitotic exit under these conditions is linked to a progressive decline in cyclin B/Cdk activity that, after reaching a threshold level, drives cells out of mitosis (Brito and Rieder, 2006; Gascoigne and Taylor, 2008). We show here that, similar to vertebrate cells, mitotic slippage in budding yeast, either in the presence of microtubule inhibitors or upon SAC hyperactivation in the absence of spindle damage, is accompanied by securin and cyclin B degradation and is delayed by expression of nondegradable cyclin B. As in mammalian cells (Brito and Rieder, 2006; Gascoigne and Taylor, 2008), the timing of mitotic slippage is highly variable depending on the conditions, ranging from ∼4 to 5 h in benomyl, 5 to 6 h upon *MAD2* overexpression, and 8 to 10 h in nocodazole. We also find that, as recently shown in mammalian cells (Lee et al., 2010), Cdc20 and other canonical regulators of cyclin B proteolysis and mitotic exit, such as the polo kinase Cdc5, are involved in SAC adaptation. In addition, the unphosphorylatable Cdc28-F19 variant delays mitotic slippage upon Mad2 overexpression consistently with the older proposal that inhibitory phosphorylation of cyclin B/Cdks accelerates adaptation to prolonged SAC activation (Minshull et al., 1996).
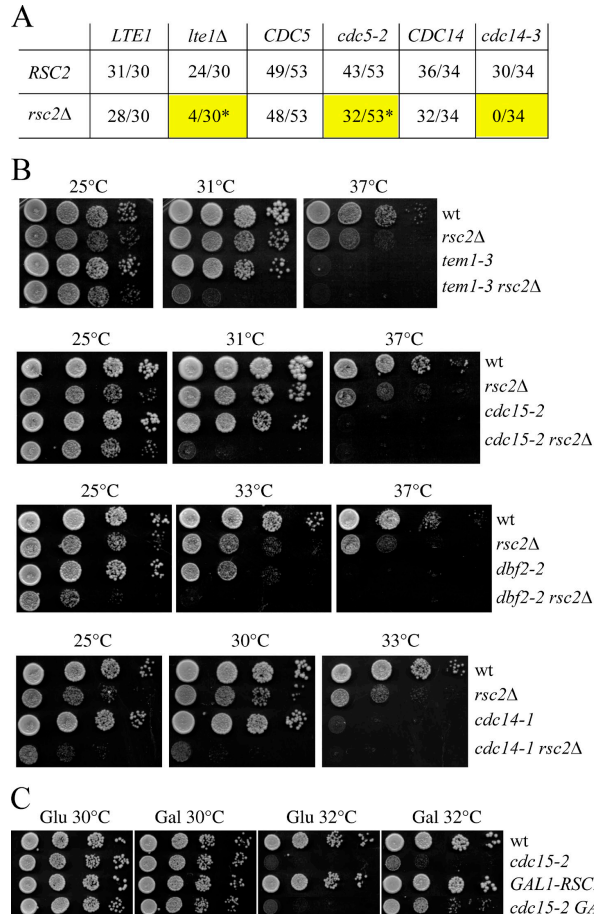
102

A

	LTE1	lte1Δ	CDC5	cdc5-2	CDC14	cdc14-3
RSC2	31/30	24/30	49/53	43/53	36/34	30/34
rsc2Δ	28/30	4/30*	48/53	32/53*	32/34	0/34

B

C

Figure 8. **Functional interactions between *RSC2* and MEN genes.** (A) Ratio of found/expected segregants observed over expected numbers of viable spores with the indicated genotypes after dissection of meiotic tetrads generated from diploid strains heterozygous for the *rsc2Δ* (ySP6859) and *lte1Δ* (ySP3418) alleles, the *cdc5-2* (ySP324) and *rsc2Δ* (ySP6859) alleles, or the *rsc2Δ* (ySP6859) and *cdc14-3* (ySP284) alleles. *, very sick viable spores. (B) Serial dilutions of strains with the indicated genotypes were spotted on YEPD plates and incubated at the indicated temperatures. (C) Serial dilutions of strains with the indicated genotypes were spotted on YEPD (Glu, *GAL1* promoter off) and YEPRG (Gal, *GAL1* promoter on) plates and incubated for 2 d at 30°C and 32°C. wt, wild type.

Cells expressing Cdc28-F19 were previously shown to be defective in Cdc20–APC activation (Rudner et al., 2000), thereby explaining their ability to retard adaptation to the SAC. All these data indicate that mitotic slippage requires conventional regulators of mitotic exit and are consistent with the proposal that it relies on the inability of the SAC to inhibit all Cdc20–APC complexes inside the cell (Brito and Rieder, 2006). Presumably, a fraction of Cdc20–APC remains active upon SAC activation and promotes cyclin B destruction until cyclin B/Cdk activity drops below a threshold level sufficient to drive cells out of mitosis.
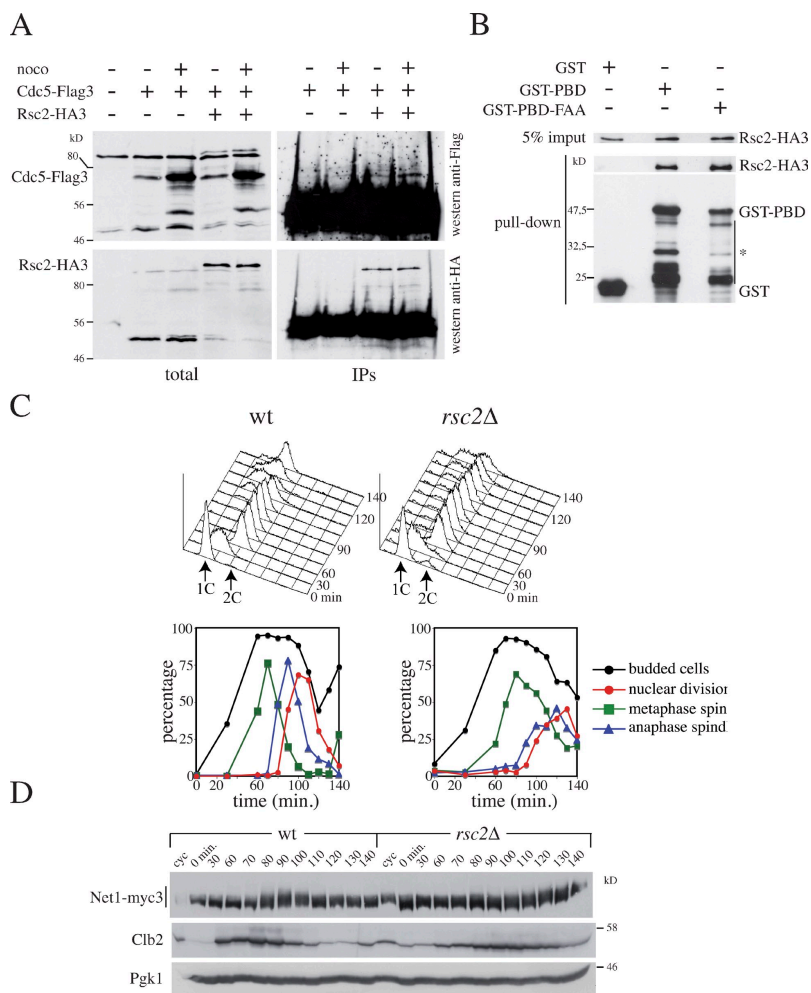
103

A

noco	–	–	+	–	+		–	+	–	+
Cdc5-Flag3	–	+	+	+	+		+	+	+	+
Rsc2-HA3	–	–	–	+	+		–	–	+	+

B

GST	+	–	–
GST-PBD	–	+	–
GST-PBD-FAA	–	–	+

C

wt

rsc2Δ

- budded cells
- nuclear division
- metaphase spindle
- anaphase spindle

D

Figure 9. **Rsc2 interacts physically with Cdc5 and is required for timely Net1 and Cdc14 phosphorylation.** (A) Wild type (wt; W303), *CDC5-FLAG3* (ySP7797), and *RSC2-HA3 CDC5-FLAG3* (ySP7814) were grown exponentially or arrested in nocodazole for 3 h. Protein extracts were analyzed by Western blotting with anti-HA (Rsc2) or anti-Flag (Cdc5) antibodies either directly (total) or after Rsc2 immunoprecipitation with anti-HA antibodies (IPs). (B) A protein extract prepared from nocodazole-arrested cells expressing Rsc2-3HA (ySP7092) was incubated with glutathione-Sepharose beads carrying GST, GST-PBD, or mutated GST-PBD-FAA. Input and pull-down samples were analyzed by Western blotting with anti-HA or anti-GST antibodies. The bar with an asterisk denotes truncated forms of GST-PBD. (C and D) α-factor–arrested wild-type (ySP8573) and *rsc2Δ* (ySP8596) cells expressing Net1-myc3 were released in fresh medium at 25°C (t = 0). At the indicated times, cell samples were collected for FACS analysis of DNA contents (C, histograms), to measure the kinetics of budding, spindle formation/elongation, and nuclear division (C, graphs), and for Western blot analysis (D) of Net1-myc3, Clb2, and Pgk1 (loading control).

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A
Cdc5-Flag3

B
Scc1-PK
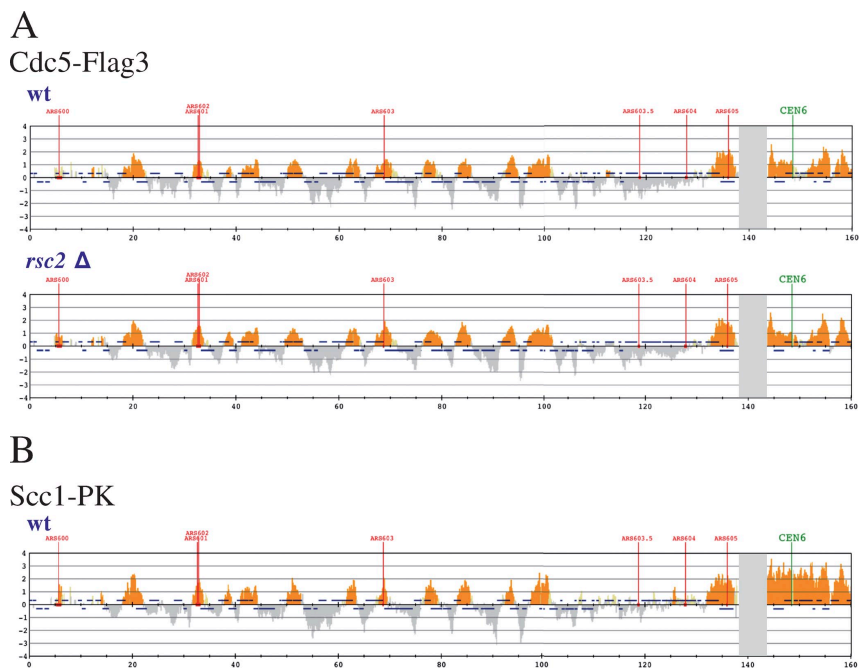
Figure 10. **RSC2 deletion does not affect Cdc5 chromosomal distribution.** Wild-type (wt; ySP7797) and *rsc2Δ* cells (ySP8200) expressing FLAG-tagged Cdc5 (A) and wild-type cells expressing PK-tagged Scc1 (B) were arrested in mitosis with benomyl and treated for ChIP-on-chip analysis. Enrichment of DNA fragments in the immunoprecipitate relative to a whole-genome DNA sample is shown along the first 160 kb (left arm and centromere) of chromosome VI. The y-axis scale is log2. Orange signals represent significant binding as previously described (Katou et al., 2003). The used statistical algorithm is identical to that for the GeneChip Operating Software (Affymetrix). The greenish signal indicates the centromere. Blue bars above and below the midline represent ORFs transcribed from left to right and opposite, respectively. A region around 140 kb masked by a gray box corresponds to Ty retrotransposon, which exists in multiple copies in the genome and was omitted from the analysis.

Upon prolonged treatment with nocodazole, adaptation in vertebrate cells takes place with SAC proteins still at kinetochores, leading to the proposal that it occurs through SAC signaling override (Brito and Rieder, 2006). We show that adaptation to the SAC in budding yeast coincides with Mad1 dissociation from Bub3, suggesting that the SAC is silenced. Microtubule-binding proteins, such as dynein and spindly, are involved in vertebrate SAC silencing through poleward transport of SAC proteins along microtubules (Howell et al., 2001; Wojcik et al., 2001; Gassmann et al., 2010). Therefore, it is likely that spindle disruption by nocodazole impairs this mechanism, thus accounting for the persistence of SAC proteins at unattached kinetochores during adaptation. In addition, Cdk activity is required to sustain the SAC (Li and Cai, 1997; Kitazono et al., 2003; Yamaguchi et al., 2003), and it drops during adaptation, suggesting that SAC signaling is likely to decline during mitotic slippage. In any case, whether silencing of

SAC signaling is a cause or a consequence of adaptation remains to be established.

A role for the RSC complex in the early anaphase release of Cdc14 from the nucleolus and in mitotic exit regulation

We provide experimental evidence of a novel role for the chromatin-remodeling complex RSC in regulation of Cdc14 nucleolar release and mitotic exit. Remarkably, histone posttranslational modifications have been recently implicated in the regulation of Cdc14 release from nucleolar chromatin in early anaphase (Hwang and Madhani, 2009), suggesting that multiple chromatin modifiers cooperate in this process.

The RSC complex regulates transcription mainly at PolII and PolIII promoters (Parnell et al., 2008) and has been implicated in several cell cycle processes, such as kinetochore function (Hsu et al., 2003) and sister chromatid cohesion (Baetz et al., 2004;

Huang and Laurent, 2004). However, transcriptional regulation of several classes of mitotic genes seems unaffected by RSC inactivation (Cao et al., 1997), suggesting that this complex might have additional and perhaps more direct functions in cell cycle progression. Other chromatin regulators have been involved in cell cycle processes unrelated to their transcriptional function. For example, chromatin-remodeling proteins were also found at human centrosomes, where they regulate the recruitment of centrosomal proteins, microtubule organization, and cytokinesis (Sillibourne et al., 2007).

Budding yeast RSC associates with two alternative and closely related subunits, Rsc1 and Rsc2 (Cairns et al., 1999), which were previously found to be differentially involved in mitotic processes, such as sister chromatid cohesion and 2-μm plasmid partitioning (Wong et al., 2002; Baetz et al., 2004). However, Rsc1 and Rsc2 bind to the same chromosomal regions (Ng et al., 2002), raising the possibility that differences in their abundance might account for their unique properties. Our data indicate that RSC^{Rsc2}, and not RSC^{Rsc1}, is specifically implicated in Cdc14 activation and adaptation to the SAC. The involvement of RSC^{Rsc2} in the control of mitotic exit is particularly apparent in conditions that activate the SAC, such as upon microtubule disruption or *MAD2* overexpression. Indeed, RSC impairment through *RSC2* deletion delays mitotic exit under these conditions but not during the unperturbed cell cycle. In this respect, RSC mutants behave similarly to FEAR mutants, which show a marked mitotic exit defect only when the MEN is partially inactive (Stegmeier et al., 2002). This raises the interesting possibility that RSC is itself part of the FEAR or acts in a parallel pathway. Indeed, *RSC2* deletion, like FEAR mutations (Stegmeier et al., 2002; Queralt and Uhlmann, 2008), impairs Net1 phosphorylation and prevents the partial nucleolar release of Cdc14 in early anaphase. Furthermore, it is lethal for *lte1Δ* cells and causes synthetic lethality/sickness to several MEN mutants. How RSC^{Rsc2} might regulate Cdc14 release from the nucleolus remains an open question, but our finding that Rsc2, like other FEAR components (Rahal and Amon, 2008), interacts physically with Cdc5 provides a possible mechanistic explanation. The Rsc2–Cdc5 interaction does not seem to require the critical residues in the PBD that are involved in phosphoepitope recognition (Song et al., 2000; Elia et al., 2003b), suggesting that it might be independent of prior Rsc2 phosphorylation and follow unconventional rules. Interestingly, the homologue of Rsc2 in higher eukaryotes, Baf180, interacts with the polo-like kinase in *X. laevis* (Yoo et al., 2004).

How could RSC regulate the FEAR function of Cdc5? Because RSC was found at numerous PolII and PolIII promoters (Ng et al., 2002) as well at centromeres (Hsu et al., 2003), we wondered whether RSC might regulate Cdc5 recruitment to specific chromosomal regions. However, our ChIP-on-chip data rule out this possibility. We found that Cdc5 binds to the rDNA, where it might interact with the RENT complex and promote Cdc14 release, but this chromosomal location is also unaffected by *RSC2* deletion (unpublished data). In addition, deletion of the whole rDNA region from chromosome XII did not rescue the ability of *mad2Δ rsc2Δ* cells to rereplicate DNA in the presence of nocodazole (unpublished data), suggesting that the control of

Cdc14 nucleolar release by RSC might be exerted at levels different from the rDNA. Several other possibilities can be envisioned: for example, RSC could have roles independent from its binding to chromatin, or it could locally regulate Cdc5 kinase activity and/or access to its substrates. Alternatively, because Cdc14 and Net1 bind to different sequences within the rDNA (Huang and Moazed, 2003; Stegmeier et al., 2004), and their binding is regulated by Cdc5 (Shou et al., 2002), changes in chromatin structure might affect interactions within the RENT complex and/or make it more susceptible to Cdc5-dependent regulation. Interestingly, sister chromatid cohesion at the transcriptionally silent mating type loci requires both Sir2, which is also part of the RENT complex (Shou et al., 1999), and RSC^{Rsc2} (Chang et al., 2005), suggesting that functional interactions between RSC and Sir2 may take place at other chromosomal locations.

Knowing the exact function of Cdc5 in the FEAR network and Cdc14 nucleolar release will certainly help addressing the role of RSC^{Rsc2} in Cdc5 regulation. The FEAR function of Cdc5 has been recently attributed primarily to Cdc5's ability to stimulate degradation of Swe1, the Wee1-like Cdk inhibitory kinase (Liang et al., 2009). However, *SWE1* deletion could not bypass the mitotic arrest of nocodazole-treated *mad2Δ rsc2Δ* cells (unpublished data), whereas the $CDC14^{TAB6-1}$ allele could do so, indicating that Cdc5 targets other substrates besides Swe1 to carry out its FEAR function. Interestingly, Cdc5 was recently shown to interact with Cdc14 (Snead et al., 2007; Rahal and Amon, 2008), suggesting that it might directly regulate its binding to Net1 and/or its phosphatase activity.

Budding yeast as a tool for the discovery of fine-tuning regulators of mitotic exit and candidate targets in cancer therapy

Recent data showed that cancer cells undergo two alternative and competing pathways after prolonged treatment to microtubule toxins: either they die by apoptosis or slip out of mitosis (Gascoigne and Taylor, 2008). Both the apoptotic and slippage pathways have thresholds, and the fate of the cell is dictated by which threshold is breached first. Importantly, inhibiting the cell death pathway by caspase inactivation commits cells to slip out of mitosis, whereas interfering with cyclin B degradation and mitotic exit channels cells into the apoptotic pathway. Thus, discovering the factors that influence the rate of adaptation to microtubule toxins in different organisms is clearly a crucial issue in cancer research. For example, the efficacy of antimitotic drugs could be markedly increased by inhibiting factors involved in mitotic slippage, thus favoring cell death.

Our data indicate that the molecular bases for adaptation to chronic SAC activation are likely conserved in all eukaryotic cells, making budding yeast a good model system to identify factors influencing the rate of mitotic slippage. Indeed, *MAD2*-overexpressing cells have proven to be a valuable tool to find novel factors involved in fine-tuning regulation of mitotic exit and SAC adaptation, which are potential targets for cancer treatment. Strikingly, mitotic exit has recently been proposed to be a better cancer therapeutic target than spindle assembly because Cdc20 inhibition efficiently kills cancer cells, preventing mitotic slippage and providing more time for apoptosis

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(Huang et al., 2009). Targeting essential regulators of mitotic exit during cancer treatment would have the drawback of killing also normally proliferating cells. Our finding that nonessential tuners of mitotic exit, such as the RSC complex, dramatically influence SAC adaptation opens important therapeutic perspectives that will be worth addressing in the future.

Materials and methods

Strains, media, and reagents

All yeast strains (Table S1) were derivatives of or were backcrossed at least three times to W303 (ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3, and ssd1). Cells were grown in synthetic complete–selective medium (6.7 g/liter yeast nitrogen base supplemented with the appropriate nutrients and sugar) to maintain selective pressure or YEP (yeast extract, peptone) medium (1% yeast extract, 2% bactopeptone, and 50 mg/liter adenine) supplemented with 2% glucose (YEPD), 2% raffinose (YEPR), or 2% raffinose and 1% galactose (YEPRG). Unless otherwise stated, α-factor was used at 2 µg/ml for BAR1 and 0.2 µg/ml for bar1 strains. Nocodazole was used at 15 µg/ml for prolonged mitotic arrests and 5 µg/ml for nocodazole washout experiments. Benomyl was used at 12.5 µg/ml to test the sensitivity of strains or at 80 µg/ml for adaptation experiments. For galactose induction of α-factor–synchronized cells, galactose was added 0.5 h before release.

Plasmid constructions and genetic manipulations

To clone MAD2 under control of the GAL1-10 promoter (plasmid pSP493), a BamHI PCR product containing the MAD2 coding region and 200 bp of downstream sequence was cloned into the BamHI site of a GAL1-10–bearing YIplac211 vector; pSP493 integration was directed to the URA3 locus by StuI digestion. To clone RSC2 behind the GAL1-10 promoter (plasmid pSP679), a PstI PCR product containing the RSC2 coding region and 200 bp of downstream sequence was cloned in the PstI site of a GAL1-10–bearing YIplac128 vector. pSP679 integration was directed to the LEU2 locus by AflII digestion. Copy number of the integrated plasmids was verified by Southern analysis. RSC2, LTE1, and RSC1 chromosomal deletion were generated by one-step gene replacement (Wach et al., 1994). RSC2 was tagged immediately before the stop codon by one-step gene tagging (Knop et al., 1999). CDC5-3Flag was a gift from E. Schwob (Institute of Molecular Genetics, Montpellier, France).

Screen for mutants hypersensitive to MAD2 overexpression

MATa and MATα GAL1-MAD2 strains (ySP6170 and ySP6273) were transformed with an mTn-lacZ/LEU2–mutagenized yeast library (Kumar et al., 2002). 3.2 × 10⁴ Leu⁺ transformants were then replica plated on synthetic complete–Leu galactose medium to identify slow-growing clones. To discard the clones that were slow growing because of defects in galactose metabolism, we streaked out the selected clones on 5-fluoro-orotic acid plates to select for their derivatives that had lost the GAL1-MAD2 construct marked URA3. The transposons were recovered and sequenced as previously described (Kumar et al., 2002) to identify their chromosomal insertion sites.

Immunoprecipitations, pull-downs, and Western blot analysis

For Rsc2–Cdc5 coimmunoprecipitation, cells were lysed with Zymolyase 20T at 30°C (1.2 M sorbitol, 0.1 M K-phosphate, pH 6.4, 0.5 mM MgCl₂, 0.6% β-mercaptoethanol, and 600 µg/ml Zymolyase). Spheroplasts were washed twice with the same buffer and incubated in immunoprecipitation buffer (50 mM Hepes, pH 7.4, 75 mM KCl, 1 mM MgCl₂, 1 mM sodium orthovanadate, 60 mM β-glycerophosphate, 1 mM EGTA, pH 8, 0.1% Triton X-100, and 1 mM DTT supplemented with a cocktail of protease inhibitors [Complete; Boehringer Ingelheim]) at 4°C for 30 min. 1–2 mg of cleared extracts were incubated for 30 min with protein A–Sepharose and 1 h with anti-HA antibodies (12CA5). Protein A–Sepharose was then added to the immunoprecipitations and incubated for 30 min. The slurry was washed four times with immunoprecipitation buffer and twice with PBS before loading. Mad1–Bub3 coimmunoprecipitations and pull-downs were performed as previously described (Brady and Hardwick, 2000; Donnianni et al., 2010). TCA protein extracts were prepared as previously described (Fraschini et al., 1999). Nondenaturing protein extracts were prepared according to Chiroli et al. (2003). Proteins transferred to Protran membranes (Schleicher and Schuell) were probed with 9E10 mAb for myc-tagged proteins, with 12CA5 or 16B12 mAb (Babco) for HA-tagged proteins, with anti-FLAG M2 mAb (Sigma-Aldrich) for FLAG-tagged proteins, or with polyclonal antibodies against Mad2, Clb2,

Cdc11 (Santa Cruz Biotechnology, Inc.), Pgk1 (Invitrogen), Sic1, and Swi6. Secondary antibodies were obtained from GE Healthcare, and proteins were detected by an enhanced chemiluminescence system (ECL; GE Healthcare) according to the manufacturer.

Other techniques

Flow cytometric DNA quantitation and in situ immunofluorescence were performed according to Fraschini et al. (1999). Nuclear division was scored with a fluorescence microscope (Eclipse E600; Nikon) on cells stained with propidium iodide. To detect spindle formation and elongation, β-tubulin immunostaining was performed with the YOL34 mAB (AbD Serotec) followed by indirect immunofluorescence using rhodamine-conjugated anti–rat antibody (Thermo Fisher Scientific). Cdc14 immunostaining was performed with sc-12045 polyclonal antibodies (Santa Cruz Biotechnology, Inc.) followed by indirect immunofluorescence using CY3-conjugated anti–goat antibody (GE Healthcare). Immunostaining of Pds1-myc18 was performed by incubation with the 9E10 mAb followed by indirect immunofluorescence using CY3-conjugated anti–mouse antibody (GE Healthcare). ChIP-on-chip analysis was performed as previously described (Sutani et al., 2009). Digital images were acquired at room temperature on a fluorescence microscope equipped with a charge-coupled device camera (DC350F; Leica) with an oil 100× 1.3 NA Plan Fluor objective (Nikon) using FW4000 software (Leica).

Online supplemental material

Fig. S1 shows that the mitotic arrest induced by MAD2 overexpression depends on SAC proteins and securin. Fig. S2 shows genetic interactions obtained combining RSC2 deletion with mutations in kinetochore components or microtubule-binding proteins. Fig. S3 shows the effects of RSC2 deletion on adaptation to the SAC upon MAD2 or MPS1 overexpression, as well as upon microtubule depolymerization by benomyl. Fig. S4 shows that FEAR components, but not Rsc1, are required for mitotic exit of mad2Δ cells treated with nocodazole, as well as for adaptation upon MAD2 overexpression. Table S1 contains the list of yeast strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201007025/DC1.

References

Alexandru, G., F. Uhlmann, K. Mechtler, M.A. Poupart, and K. Nasmyth. 2001. Phosphorylation of the cohesin subunit Scc1 by Polo/Cdc5 kinase regulates sister chromatid separation in yeast. Cell. 105:459–472. doi:10.1016/S0092-8674(01)00362-2

Azzam, R., S.L. Chen, W. Shou, A.S. Mah, G. Alexandru, K. Nasmyth, R.S. Annan, S.A. Carr, and R.J. Deshaies. 2004. Phosphorylation by cyclin B-Cdk underlies release of mitotic exit activator Cdc14 from the nucleolus. Science. 305:516–519. doi:10.1126/science.1099402

Baetz, K.K., N.J. Krogan, A. Emili, J. Greenblatt, and P. Hieter. 2004. The ctf13-30/CTF13 genomic haploinsufficiency modifier screen identifies the yeast chromatin remodeling complex RSC, which is required for the establishment of sister chromatid cohesion. Mol. Cell. Biol. 24:1232–1244. doi:10.1128/MCB.24.3.1232-1244.2003

Brady, D.M., and K.G. Hardwick. 2000. Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function. Curr. Biol. 10:675–678. doi:10.1016/S0960-9822(00)00515-7

Brito, D.A., and C.L. Rieder. 2006. Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. Curr. Biol. 16:1194–1200. doi:10.1016/j.cub.2006.04.043

Cairns, B.R., A. Schlichter, H. Erdjument-Bromage, P. Tempst, R.D. Kornberg, and F. Winston. 1999. Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH, and bromodomains. Mol. Cell. 4:715–723. doi:10.1016/S1097-2765(00)80382-2

Cao, Y., B.R. Cairns, R.D. Kornberg, and B.C. Laurent. 1997. Sfh1p, a component of a novel chromatin-remodeling complex, is required for cell cycle progression. *Mol. Cell. Biol.* 17:3323–3334.

Chang, C.R., C.S. Wu, Y. Hom, and M.R. Gartenberg. 2005. Targeting of cohesin by transcriptionally silent chromatin. *Genes Dev.* 19:3031–3042. doi:10.1101/gad.1356305

Chiroli, E., R. Fraschini, A. Beretta, M. Tonelli, G. Lucchini, and S. Piatti. 2003. Budding yeast PAK kinases regulate mitotic exit by two different mechanisms. *J. Cell Biol.* 160:857–874. doi:10.1083/jcb.200209097

Donnianni, R.A., M. Ferrari, F. Lazzaro, M. Clerici, B. Tamilselvan Nachimuthu, P. Plevani, M. Muzi-Falconi, and A. Pellicioli. 2010. Elevated levels of the polo kinase Cdc5 override the Mec1/ATR checkpoint in budding yeast by acting at different steps of the signaling pathway. *PLoS Genet.* 6:e1000763. doi:10.1371/journal.pgen.1000763

Elia, A.E., L.C. Cantley, and M.B. Yaffe. 2003a. Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science.* 299:1228–1231. doi:10.1126/science.1079079

Elia, A.E., P. Rellos, L.F. Haire, J.W. Chao, F.J. Ivins, K. Hoepker, D. Mohammad, L.C. Cantley, S.J. Smerdon, and M.B. Yaffe. 2003b. The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. *Cell.* 115:83–95. doi:10.1016/S0092-8674(03)00725-6

Fraschini, R., E. Formenti, G. Lucchini, and S. Piatti. 1999. Budding yeast Bub2 is localized at spindle pole bodies and activates the mitotic checkpoint via a different pathway from Mad2. *J. Cell Biol.* 145:979–991. doi:10.1083/jcb.145.5.979

Fraschini, R., A. Beretta, G. Lucchini, and S. Piatti. 2001a. Role of the kinetochore protein Ndc10 in mitotic checkpoint activation in *Saccharomyces cerevisiae*. *Mol. Genet. Genomics.* 266:115–125. doi:10.1007/s004380100533

Fraschini, R., A. Beretta, L. Sironi, A. Musacchio, G. Lucchini, and S. Piatti. 2001b. Bub3 interaction with Mad2, Mad3 and Cdc20 is mediated by WD40 repeats and does not require intact kinetochores. *EMBO J.* 20:6648–6659. doi:10.1093/emboj/20.23.6648

Gascoigne, K.E., and S.S. Taylor. 2008. Cancer cells display profound intra- and interline variation following prolonged exposure to antimitotic drugs. *Cancer Cell.* 14:111–122. doi:10.1016/j.ccr.2008.07.002

Gassmann, R., A.J. Holland, D. Varma, X. Wan, F. Civril, D.W. Cleveland, K. Oegema, E.D. Salmon, and A. Desai. 2010. Removal of Spindly from microtubule-attached kinetochores controls spindle checkpoint silencing in human cells. *Genes Dev.* 24:957–971. doi:10.1101/gad.1886810

Hardwick, K.G., E. Weiss, F.C. Luca, M. Winey, and A.W. Murray. 1996. Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. *Science.* 273:953–956. doi:10.1126/science.273.5277.953

Howell, B.J., B.F. McEwen, J.C. Canman, D.B. Hoffman, E.M. Farrar, C.L. Rieder, and E.D. Salmon. 2001. Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. *J. Cell Biol.* 155:1159–1172. doi:10.1083/jcb.200105093

Hoyt, M.A., L. He, K.K. Loo, and W.S. Saunders. 1992. Two *Saccharomyces cerevisiae* kinesin-related gene products required for mitotic spindle assembly. *J. Cell Biol.* 118:109–120. doi:10.1083/jcb.118.1.109

Hsu, J.M., J. Huang, P.B. Meluh, and B.C. Laurent. 2003. The yeast RSC chromatin-remodeling complex is required for kinetochore function in chromosome segregation. *Mol. Cell. Biol.* 23:3202–3215. doi:10.1128/MCB.23.9.3202-3215.2003

Huang, H.C., J. Shi, J.D. Orth, and T.J. Mitchison. 2009. Evidence that mitotic exit is a better cancer therapeutic target than spindle assembly. *Cancer Cell.* 16:347–358. doi:10.1016/j.ccr.2009.08.020

Huang, J., and B.C. Laurent. 2004. A Role for the RSC chromatin remodeler in regulating cohesion of sister chromatid arms. *Cell Cycle.* 3:973–975.

Huang, J., and D. Moazed. 2003. Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. *Genes Dev.* 17:2162–2176. doi:10.1101/gad.1108403

Hwang, L.H., L.F. Lau, D.L. Smith, C.A. Mistrot, K.G. Hardwick, E.S. Hwang, A. Amon, and A.W. Murray. 1998. Budding yeast Cdc20: a target of the spindle checkpoint. *Science.* 279:1041–1044. doi:10.1126/science.279.5353.1041

Hwang, W., and H.D. Madhani. 2009. Nonredundant requirement for multiple histone modifications for the early anaphase release of the mitotic exit regulator Cdc14 from nucleolar chromatin. *PLoS Genet.* 5:e1000588. doi:10.1371/journal.pgen.1000588

Katou, Y., Y. Kanoh, M. Bando, H. Noguchi, H. Tanaka, T. Ashikari, K. Sugimoto, and K. Shirahige. 2003. S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature.* 424:1078–1083. doi:10.1038/nature01900

Kitazono, A.A., D.A. Garza, and S.J. Kron. 2003. Mutations in the yeast cyclin-dependent kinase Cdc28 reveal a role in the spindle assembly checkpoint. *Mol. Genet. Genomics.* 269:672–684. doi:10.1007/s00438-003-0870-y

Knop, M., K. Siegers, G. Pereira, W. Zachariae, B. Winsor, K. Nasmyth, and E. Schiebel. 1999. Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast.* 15:963–972. doi:10.1002/(SICI)1097-0061(199907)15:10B<963::AID-YEA399>3.0.CO;2-W

Kumar, A., S. Vidan, and M. Snyder. 2002. Insertional mutagenesis: transposon-insertion libraries as mutagens in yeast. *Methods Enzymol.* 350:219–229. doi:10.1016/S0076-6879(02)50965-4

Lee, J., J.A. Kim, R.L. Margolis, and R. Fotedar. 2010. Substrate degradation by the anaphase promoting complex occurs during mitotic slippage. *Cell Cycle.* 9:1792–1801. doi:10.4161/cc.9.9.11519

Li, R. 1999. Bifurcation of the mitotic checkpoint pathway in budding yeast. *Proc. Natl. Acad. Sci. USA.* 96:4989–4994. doi:10.1073/pnas.96.9.4989

Li, X., and M. Cai. 1997. Inactivation of the cyclin-dependent kinase Cdc28 abrogates cell cycle arrest induced by DNA damage and disassembly of mitotic spindles in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 17:2723–2734.

Liang, F., F. Jin, H. Liu, and Y. Wang. 2009. The molecular function of the yeast polo-like kinase Cdc5 in Cdc14 release during early anaphase. *Mol. Biol. Cell.* 20:3671–3679. doi:10.1091/mbc.E08-10-1049

Mendenhall, M.D. 1993. An inhibitor of p34CDC28 protein kinase activity from *Saccharomyces cerevisiae*. *Science.* 259:216–219. doi:10.1126/science.8421781

Michaelis, C., R. Ciosk, and K. Nasmyth. 1997. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell.* 91:35–45. doi:10.1016/S0092-8674(01)80007-6

Miller, C.T., C. Gabrielse, Y.C. Chen, and M. Weinreich. 2009. Cdc7p-Dbf4p regulates mitotic exit by inhibiting Polo kinase. *PLoS Genet.* 5:e1000498. doi:10.1371/journal.pgen.1000498

Minshull, J., A. Straight, A.D. Rudner, A.F. Dernburg, A. Belmont, and A.W. Murray. 1996. Protein phosphatase 2A regulates MPF activity and sister chromatid cohesion in budding yeast. *Curr. Biol.* 6:1609–1620. doi:10.1016/S0960-9822(02)70784-7

Musacchio, A., and E.D. Salmon. 2007. The spindle-assembly checkpoint in space and time. *Nat. Rev. Mol. Cell Biol.* 8:379–393. doi:10.1038/nrm2163

Nasmyth, K. 2002. Segregating sister genomes: the molecular biology of chromosome separation. *Science.* 297:559–565. doi:10.1126/science.1074757

Ng, H.H., F. Robert, R.A. Young, and K. Struhl. 2002. Genome-wide location and regulated recruitment of the RSC nucleosome-remodeling complex. *Genes Dev.* 16:806–819. doi:10.1101/gad.978902

Parnell, T.J., J.T. Huff, and B.R. Cairns. 2008. RSC regulates nucleosome positioning at Pol II genes and density at Pol III genes. *EMBO J.* 27:100–110. doi:10.1038/sj.emboj.7601946

Peters, J.M. 2006. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat. Rev. Mol. Cell Biol.* 7:644–656. doi:10.1038/nrm1988

Peters, J.M., A. Tedeschi, and J. Schmitz. 2008. The cohesin complex and its roles in chromosome biology. *Genes Dev.* 22:3089–3114. doi:10.1101/gad.1724308

Piatti, S., M. Venturetti, E. Chiroli, and R. Fraschini. 2006. The spindle position checkpoint in budding yeast: the motherly care of MEN. *Cell Div.* 1:2. doi:10.1186/1747-1028-1-2

Queralt, E., and F. Uhlmann. 2008. Separase cooperates with Zds1 and Zds2 to activate Cdc14 phosphatase in early anaphase. *J. Cell Biol.* 182:873–883. doi:10.1083/jcb.200801054

Queralt, E., C. Lehane, B. Novak, and F. Uhlmann. 2006. Downregulation of PP2A(Cdc55) phosphatase by separase initiates mitotic exit in budding yeast. *Cell.* 125:719–732. doi:10.1016/j.cell.2006.03.038

Rahal, R., and A. Amon. 2008. The Polo-like kinase Cdc5 interacts with FEAR network components and Cdc14. *Cell Cycle.* 7:3262–3272.

Rieder, C.L., and H. Maiato. 2004. Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint. *Dev. Cell.* 7:637–651. doi:10.1016/j.devcel.2004.09.002

Rudner, A.D., K.G. Hardwick, and A.W. Murray. 2000. Cdc28 activates exit from mitosis in budding yeast. *J. Cell Biol.* 149:1361–1376. doi:10.1083/jcb.149.7.1361

Schwob, E., T. Böhm, M.D. Mendenhall, and K. Nasmyth. 1994. The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae*. *Cell.* 79:233–244. (published erratum appears in *Cell.* 1996. 84: following 174) doi:10.1016/0092-8674(94)90193-7

Shou, W., J.H. Seol, A. Shevchenko, C. Baskerville, D. Moazed, Z.W. Chen, J. Jang, A. Shevchenko, H. Charbonneau, and R.J. Deshaies. 1999. Exit from mitosis is triggered by Tem1-dependent release of the protein

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phosphatase Cdc14 from nucleolar RENT complex. *Cell.* 97:233–244. doi:10.1016/S0092-8674(00)80733-3

Shou, W., K.M. Sakamoto, J. Keener, K.W. Morimoto, E.E. Traverso, R. Azzam, G.J. Hoppe, R.M. Feldman, J. DeModena, D. Moazed, et al. 2001. Net1 stimulates RNA polymerase I transcription and regulates nucleolar structure independently of controlling mitotic exit. *Mol. Cell.* 8:45–55. doi:10.1016/S1097-2765(01)00291-X

Shou, W., R. Azzam, S.L. Chen, M.J. Huddleston, C. Baskerville, H. Charbonneau, R.S. Annan, S.A. Carr, and R.J. Deshaies. 2002. Cdc5 influences phosphorylation of Net1 and disassembly of the RENT complex. *BMC Mol. Biol.* 3:3. doi:10.1186/1471-2199-3-3

Sillibourne, J.E., B. Delaval, S. Redick, M. Sinha, and S.J. Doxsey. 2007. Chromatin remodeling proteins interact with pericentrin to regulate centrosome integrity. *Mol. Biol. Cell.* 18:3667–3680. doi:10.1091/mbc .E06-07-0604

Snead, J.L., M. Sullivan, D.M. Lowery, M.S. Cohen, C. Zhang, D.H. Randle, J. Taunton, M.B. Yaffe, D.O. Morgan, and K.M. Shokat. 2007. A coupled chemical-genetic and bioinformatic approach to Polo-like kinase pathway exploration. *Chem. Biol.* 14:1261–1272. doi:10.1016/j .chembiol.2007.09.011

Song, S., T.Z. Grenfell, S. Garfield, R.L. Erikson, and K.S. Lee. 2000. Essential function of the polo box of Cdc5 in subcellular localization and induction of cytokinetic structures. *Mol. Cell. Biol.* 20:286–298. doi:10.1128/MCB.20.1.286-298.2000

Stegmeier, F., and A. Amon. 2004. Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. *Annu. Rev. Genet.* 38:203–232. doi:10.1146/annurev.genet.38.072902.093051

Stegmeier, F., R. Visintin, and A. Amon. 2002. Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. *Cell.* 108:207–220. doi:10 .1016/S0092-8674(02)00618-9

Stegmeier, F., J. Huang, R. Rahal, J. Zmolik, D. Moazed, and A. Amon. 2004. The replication fork block protein Fob1 functions as a negative regulator of the FEAR network. *Curr. Biol.* 14:467–480. doi:10.1016/j.cub.2004.03.009

Surana, U., A. Amon, C. Dowzer, J. McGrew, B. Byers, and K. Nasmyth. 1993. Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J.* 12:1969–1978.

Sutani, T., T. Kawaguchi, R. Kanno, T. Itoh, and K. Shirahige. 2009. Budding yeast Wpl1(Rad61)-Pds5 complex counteracts sister chromatid cohesion-establishing reaction. *Curr. Biol.* 19:492–497. doi:10.1016/j.cub.2009 .01.062

Uhlmann, F. 2001. Secured cutting: controlling separase at the metaphase to anaphase transition. *EMBO Rep.* 2:487–492.

Visintin, R., K. Craig, E.S. Hwang, S. Prinz, M. Tyers, and A. Amon. 1998. The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. *Mol. Cell.* 2:709–718. doi:10.1016/S1097-2765(00)80286-5

Visintin, R., F. Stegmeier, and A. Amon. 2003. The role of the polo kinase Cdc5 in controlling Cdc14 localization. *Mol. Biol. Cell.* 14:4486–4498. doi:10.1091/mbc.E03-02-0095

Wach, A., A. Brachat, R. Pöhlmann, and P. Philippsen. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast.* 10:1793–1808. doi:10.1002/yea.320101310

Waples, W.G., C. Chahwan, M. Ciechonska, and B.D. Lavoie. 2009. Putting the brake on FEAR: Tof2 promotes the biphasic release of Cdc14 phosphatase during mitotic exit. *Mol. Biol. Cell.* 20:245–255. doi:10.1091/mbc .E08-08-0879

Wojcik, E., R. Basto, M. Serr, F. Scaërou, R. Karess, and T. Hays. 2001. Kinetochore dynein: its dynamics and role in the transport of the Rough deal checkpoint protein. *Nat. Cell Biol.* 3:1001–1007. doi:10.1038/ncb1101-1001

Wong, M.C., S.R. Scott-Drew, M.J. Hayes, P.J. Howard, and J.A. Murray. 2002. RSC2, encoding a component of the RSC nucleosome remodeling complex, is essential for 2 micron plasmid maintenance in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 22:4218–4229. doi:10.1128/MCB.22.12.4218-4229.2002

Yamaguchi, S., A. Decottignies, and P. Nurse. 2003. Function of Cdc2p-dependent Bub1p phosphorylation and Bub1p kinase activity in the mitotic and meiotic spindle checkpoint. *EMBO J.* 22:1075–1087. doi:10 .1093/emboj/cdg100

Ye, P., B.D. Peyser, X. Pan, J.D. Boeke, F.A. Spencer, and J.S. Bader. 2005. Gene function prediction from congruent synthetic lethal interactions in yeast. *Mol. Syst. Biol.* 1:2005.0026. doi:10.1038/msb4100034

Yoo, H.Y., A. Kumagai, A. Shevchenko, A. Shevchenko, and W.G. Dunphy. 2004. Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase. *Cell.* 117:575–588. doi:10.1016/S0092-8674(04)00417-9

Yoshida, S., and A. Toh-e. 2002. Budding yeast Cdc5 phosphorylates Net1 and assists Cdc14 release from the nucleolus. *Biochem. Biophys. Res. Commun.* 294:687–691. doi:10.1016/S0006-291X(02)00544-2

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Published paper III

DNA Repair

Tid1/Rdh54 translocase is phosphorylated through a Mec1- and Rad53-dependent manner in the presence of DSB lesions in budding yeast

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ABSTRACT

Saccharomyces cerevisiae cells with a single double-strand break (DSB) activate the ATR/Mec1-dependent checkpoint response as a consequence of extensive ssDNA accumulation. The recombination factor Tid1/Rdh54, a member of the Swi2-like family proteins, has an ATPase activity and may contribute to the remodelling of nucleosomes on DNA. Tid1 dislocates Rad51 recombinase from dsDNA, can unwind and supercoil DNA filaments, and has been implicated in checkpoint adaptation from a G2/M arrest induced by an unrepaired DSB.

Here we show that both ATR/Mec1 and Chk2/Rad53 kinases are implicated in the phosphorylation of Tid1 in the presence of DNA damage, indicating that the protein is regulated during the DNA damage response. We show that Tid1 ATPase activity is dispensable for its phosphorylation and for its recruitment near a DSB, but it is required to switch off Rad53 activation and for checkpoint adaptation. Mec1 and Rad53 kinases, together with Rad51 recombinase, are also implicated in the hyper-phosphorylation of the ATPase defective Tid1-K318R variant and in the efficient binding of the protein to the DSB site.

In summary, Tid1 is a novel target of the DNA damage checkpoint pathway that is also involved in checkpoint adaptation.

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1. Introduction

In *Saccharomyces cerevisiae* cells, formation of one irreparable DSB elicits a robust activation of Rad53 kinase, a central player of the DNA damage checkpoint pathway, and a transient cell cycle block in metaphase (reviewed in [1]). Rad53 is activated through phosphorylation by the upstream kinase Mec1, which is recruited to 5′–3′ resected DSB ends [1]. Rad53 phosphorylation can be analyzed by Western blotting, and the phosphorylation is commonly used as a biochemical marker to test activation of the Mec1-induced DNA damage checkpoint pathway. It has been observed that the checkpoint signalling is switched off 12–15 h after the formation of one irreparable DSB. Concomitantly, Rad53 becomes dephosphorylated and the cell cycle can restart in the presence of a damaged chromosome [2]. This phenomenon is called checkpoint adaptation and it has also been observed in other eukaryotic organisms in response to various types of DNA damage and replication stress [3]. Interestingly, checkpoint adaptation has been suggested to promote uncontrolled proliferation of cancer cells, and may play a role in the development of therapy-resistance tumours. Therefore, a better understanding of the mechanisms and factors involved in checkpoint adaptation is a relevant goal in cancer biology, and it may be useful to develop novel therapeutic strategies. Notably, PLK1-like kinases promote checkpoint adaptation in multicellular eukaryotes [3], and specific PLK1 inhibitors are in clinical trials for cancer therapy [4].

Budding yeast has proven to be an ideal system for the study of activation and inactivation of the DNA damage checkpoint and, in particular, for analysis of checkpoint adaptation in the presence of a single irreparable DSB lesion. A single DSB can be induced at a specific locus through the conditional overexpression of HO endonuclease. By using this genetic system, several proteins have been implicated in checkpoint adaptation in yeast [1]. Among these factors is Tid1 (also called Rdh54), a member of the Swi2-like family, which includes proteins having dsDNA-dependent ATPase activity that are able to translocate along a DNA molecule, thus contributing to nucleosome remodelling around the DSB

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site. Moreover, these factors can supercoil and unwind DNA and promote D-loop formation and branch migration in homologous recombination processes [5]. A number of *in vitro* and *in vivo* data indicate that Tid1 dissociates Rad51 recombinase from dsDNA, thus preventing the accumulation of toxic Rad51-DNA intermediates and also ensuring that a sufficient amount of Rad51 will be available for DSB repair and recombination [6].

Tid1 shares some molecular functions and mechanisms with the Swi2-like homologs Rad54 and Usl1. However, they likely have distinct functions, as indicated by the distinct phenotypes of the corresponding mutants [6]. Tid1 plays major role in meiotic recombination, while it is involved in minor pathway in mitotic recombination, specifically in a diploid [7,8]. Interestingly, Tid1 has been involved in checkpoint adaptation from a G2/M arrest induced by an irreparable DSB [2]. To further address the functional role of Tid1 in cells responding to DSB and in checkpoint adaptation, we tested whether Tid1 protein is post-translationally regulated in the presence of an irreparable DSB. We found that Tid1 is phosphorylated by the Mec1 and Rad53 kinases, similar to other factors such as Srs2, Rad51, Sae2, and Cdc5 involved in turning off Rad53 during checkpoint adaptation [9–12]. Therefore, Tid1 belongs to a heterogeneous family of factors which are targets of the DNA damage checkpoint pathway, and are involved in silencing the checkpoint response in the presence of one irreparable DSB.

2. Materials and methods

2.1. Yeast strains

All strains are derivatives of JKM background (*MATα* or *MATa, hmlΔ::ADE1, hmrΔ::ADE1 ade1-100, trp1Δ::hisG, leu2-3, leu2-112, lys5, ura3-52, ade3::GAL::HO*), generously provided by J. Haber (Brandeis University, Waltham, Boston). Y454 was obtained by integration of the 3XHA tag at the C-end of the *TID1* locus by the one-step PCR system [13]. Standard genetic procedures for transformation and tetrad analysis were followed to construct the various strains. Y841 was obtained by integrating of NvuI-digested pHK255 plasmid into the *TID1*-3XHA locus in Y454 strain. After pop-out by treatment with 5-FOA, the integration of the *tid1*-K318R mutation was confirmed by sequencing analysis. In previous papers [14,15], the K318R mutation was indicated as K352R due to the annotation of an upstream ATG start site. We verified by DNA sequencing the ATG start codon in the genetic background used here (data not shown). Y522 (*MATa, mec1Δ, TID1*-3XHA) was obtained by crossing Y454 with Y138 (*MATα, mec1Δ*); Y876 (*MATa, mec1Δ, tid1*-K318R-3XHA) was obtained by crossing Y841 with Y138 (*MATα, mec1Δ*); Y962 (*MATα, rad53*-K227A, *TID1*-3XHA) was obtained by crossing Y454 with Y677 (*MATα rad53*-K227A); Y966 (*MATa rad53*-K227A *tid1*-K318R-3XHA) was obtained by crossing Y841 with Y677 (*MATα rad53*-K227A); Y741 (*MATa, rad51Δ, TID1*-3XHA) was obtained by crossing Y623 (*MATα, TID1*-3XHA) with Y608 (*MATa, rad51Δ*); Y873 (*MATa, rad51Δ, tid1*-K318R-3XHA) was obtained by crossing Y841 with Y736 (*MATα, rad51Δ*); Y1768 (*MATα, dun1Δ, TID1*-3XHA) was obtained by crossing Y623 with Y1741 (*MATa, dun1Δ*); Y1769 (*MATα, dun1Δ, tid1-K318R-3XHA*) was obtained by crossing Y869 (*MATα tid1*-K318R-3XHA) with Y1741; Y967 (*MATa rad53*-K227A *rdh54*-K318R-3XHA) was obtained by crossing Y 869 with Y677; Y1771 (*MATα chk1Δ rad53*-K227A *rdh54*-K318R-3XHA) was obtained by crossing Y967 with Y1061 (*MATα chk1Δ*). Y811 was obtained by integration of the 3XHA tag at the C-end of the *TID1* locus by the one-step PCR system in YMV80 background [16]. All the strains used in this work are haploid; moreover, all the *mec1Δ* strains also have the *sml1Δ* mutation, to keep cells viable.

2.2. Western blot analysis

The TCA protein extraction and the Western blot procedures have been previously described [17]. Rad53 and Tid1-3XHA proteins were analysed using Mab.EL7 [17], and 12CA5 monoclonal antibodies, respectively.

2.3. Immunoprecipitation analysis

Tid1-3XHA protein was immunoprecipitated with the 12CA5 monoclonal antibody using a standard procedure. 2×10^9 cells were resuspended in 400 μl of lysis buffer (50 mM Tris–HCl, 50 mM NaCl, 60 mM β-glycerolphosphate, 1 mM DTT, 1 mM Sodium Orthovanadate, 1% NP40, supplemented with protease inhibitor cocktail (Roche)), and disrupted with glass beads by Fast Prep (MPBio). Crude extracts were incubated for 2 h at 4 °C in the presence of magnetic beads (Dynal), which were pre-incubated with the 12CA5 antibody. The beads were then washed with lysis buffer and resuspended with 30 μl of Laemmli sample buffer. The immunoprecipitates were analysed by Western blot using 12CA5 (anti-HA) and anti-phospho-S/T-Q motifs antibody (Cell Signalling).

2.4. 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 (15 μg/ml), respectively. DNA content was analyzed by FACSCalibur (Bekton-Dickinson) and Cell-Quest software (Bekton-Dickinson).

2.5. In vitro dephosphorylation assay

Crude extracts were prepared as described [18], and resuspended in λ phosphatase buffer with or without 4000 U of λ phosphatase (Biolabs). Samples were incubated 30 min at 30 °C and resuspended in Laemmli sample buffer.

2.6. Chromatin immunoprecipitation analysis (ChIP)

ChIP analysis was performed as described previously [19]. Multiplex PCRs were carried out by using primer pairs complementary to DNA sequences located 1 kb from the HO-cut site at MAT (DSB) and to DNA sequences located 66 kb from MAT (CON). Gel quantitation was determined using the Quantity One program (Biorad). The relative fold enrichments of DSB-bound protein were calculated as follow: [DSB_IP/CON_IP]/[DSB_input/CON_input], where IP and Input represent the amount of PCR product in the immunoprecipitates and in input samples before immunoprecipitation, respectively.

3. Results

3.1. Tid1 is phosphorylated in response to DSB formation

In *S. cerevisiae*, it has been shown that Srs2, Rad51, Sae2 and Cdc5 factors are involved in DNA damage checkpoint inactivation and adaptation, and that they are regulated through Mec1-dependent phosphorylation in the presence of DSB lesions [9–12]. We tested whether Tid1, a DNA translocase required for checkpoint adaptation, was specifically modified in response to a single irreparable DSB. To visualize Tid1 on Western blots of crude yeast protein extracts, we initially inserted a 3XHA tag sequence at the C-terminal of the *TID1* gene. The sensitivity of haploid cells carrying the *TID1::3XHA* allele to various DNA damaging agents (the alkylating agent methyl methanesulfonate (MMS), UV irradiation, bleocin, camptothecin) was similar to that of the wild type strain (data

not shown), indicating that the presence of the 3XHA tag at the C terminal of Tid1 does not affect the functionality of the protein.

We then tested the stability of Tid1 and the electrophoretic mobility of the protein throughout the cell cycle. Protein samples were prepared from yeast cells, blocked in G1 phase by α-factor treatment and released into fresh medium with or without the ribonucleotide reductase inhibitor hydroxyurea (HU, 0.2 M) or the DNA alkylating agent MMS, 0.02% (Fig. 1). As expected, the HU-treated cells remained blocked into S-phase because of the deprivation of dNTPs pools, and the MMS-treated cells delayed DNA replication because of the activation of the DNA damage checkpoint, while untreated cells progressed synchronously through S phase and the mitotic transitions, as shown by the FACS profiles (Fig. 1 A, C and E). In parallel, cells were grown for 6 h in medium containing galactose to overproduce the HO endonuclease and induce the formation of a single irreparable DSB at the MAT locus and, as a consequence, cells activated the DNA damage checkpoint and blocked in G2/M cell cycle phase [2]. Protein samples were prepared at the indicated time points and analysed by Western blotting using anti-HA antibodies (Abs) to visualize Tid1 and anti-Rad53 Abs to test activation of DNA damage checkpoint signalling. We found that Tid1 is a stable protein throughout the cell cycle, showing a modest increase in its level in early S-phase, likely linked to increased gene expression during this cell cycle phase. In the experimental conditions used here, we did not observe any Tid1 electrophoretic mobility shift either in untreated or in HU- and MMS-treated cells (Fig. 1B, D, F). Surprisingly, we found that Tid1 migrated as a doublet band in protein samples prepared from cells in which one irreparable DSB was induced by HO overexpression ([DSB] lane in Fig. 1B and D). The altered electrophoretic mobility of the upper band was reverted by *in vitro* treatment of the protein sample with phosphatase, indicating that Tid1 was modified by phosphorylation (Fig. 1G). Interestingly, we also found that Rad53 phosphorylation, which is used as a biochemical marker for Mec1-dependent checkpoint activation, did not always correlate with Tid1 phosphorylation; in fact, Rad53 is phosphorylated in HU- and MMS-treated cells, while Tid1 is not modified in such conditions (Fig. 1D and F). Tid1 has been identified in a proteome wide screening for proteins phosphorylated in the presence of MMS [20]; however, we can not see this modification by Western blotting (Fig. 1F); therefore, we believe that the Tid1 phosphorylation we observed in the presence of one irreparable DSB should be different in respect to the one identified by mass spectrometry in the presence of MMS, suggesting the idea that Tid1 would be regulated through distinct mechanisms in the presence of different types of DNA damage.

Moreover, we found that the Tid1 protein, immunoprecipitated from cells with one irreparable DSB, was recognized by anti-phosphorylated S/T-Q motifs specific antibodies (Fig. 1F). It is known that ATM/Tel1 and ATR/Mec1 kinases preferentially phosphorylate S/T-Q motifs in response to DNA damage [21], suggesting that they could be likely involved in Tid1 phosphorylation following a single irreparable DSB formation. Interestingly, in the presence of MMS it was reported that Tid1 is phosphorylated at the S51 residue [20], which is not a SQ motif, supporting the idea that MMS-induced and DSB-induced Tid1 phosphorylation could be different.

3.2. Analysis of the HO-induced Tid1 phosphorylation

It is known that one irreparable DSB is processed and activates a full Mec1-dependent checkpoint pathway in G2-blocked cells, but not in G1-blocked cells [22,23], thus we tested Tid1 and Rad53 phosphorylation in cells treated with α-factor or nocodazole to block the cell cycle in G1 or G2 phases, respectively. We found that Tid1 is not phosphorylated after one irreparable DSB induced in G1-blocked cells, while its phosphorylation is accumulated in G2-blocked cells, following the formation of one irreparable DSB and the activation of Rad53 (Fig. 2A). We then analysed Tid1 phosphorylation in a specific genetic background (YMV80), where the conditional overproduction of HO nuclease induces the formation of one DSB that can be repaired through a single strand annealing process (SSA). As previously reported [16], in these cells the accumulation of long ssDNA tails at the resected DSB elicits a robust Rad53 phosphorylation, which is reverted after the DSB has been completely repaired (Fig. 2B). Similarly to Rad53, we found that Tid1 is phosphorylated during the prolong SSA process (Fig. 2B), and its phosphorylation disappears during the checkpoint recovery, accordingly with the idea that Tid1 protein is a target of the DSB-induced checkpoint signalling.

3.3. Phosphorylation of the ATPase-inactive Tid1 protein variant

The ATPase activity of Tid1 is essential for its function during recombination and checkpoint adaptation [15,24]. We thus tested whether Tid1 ATPase activity was required for the DSB-induced phosphorylation of the protein. To this purpose, the site specific K318R mutation, which is known to abrogate the ATPase activity of the catalytic domain of Tid1 [15], was introduced into the *TID1*::3XHA locus. As expected [24,25], *tid1*-K318R cells were sensitive to MMS and did not adapt to a single irreparable DSB (data not shown).

HO endonuclease was overproduced in wild type and *tid1*-K318R cells to induce the formation of one irreparable DSB, and the phosphorylation state of both Tid1 and Rad53 proteins was analysed by Western blotting at several time points after the DSB formation (Fig. 3A). We found that in wild type cells, the HO-induced Tid1 phosphorylation parallels the Mec1-dependent activation of Rad53 and disappears at late time points during checkpoint adaptation. Similar to what was observed in wild type cells, the Tid1-K318R protein variant started to be phosphorylated 3 h after HO induction. However, at longer time points, essentially all the protein was modified and it remained phosphorylated until the end of the experiment, mirroring the extend of Rad53 activation, which was not dephosphorylated in the adaptation-defective *tid1*-K318R cells. Moreover, the electrophoretic mobility shift of the phosphorylated Tid1-K318R variant at 6–9 h after the HO induction appeared slightly higher than that observed for the wild type protein, while both proteins had the same electrophoretic mobility in undamaged conditions.

The results described above prompted us to test whether Mec1, the main protein kinase responding to DSB lesion in budding yeast, is required for Tid1 phosphorylation. We deleted the *MEC1* gene both in *TID1*::3XHA and *tid1*-K318R::3XHA strains, also carrying deletion of *SML1* gene to keep the cells viable. The HO gene was overexpressed in these strains to induce the formation of one irreparable DSB and samples were taken at various time points to analyse the phosphorylation state of both Tid1 and Rad53. As shown in Fig. 3B and C, the HO-induced modification of the wild type Tid1 and the Tid1-K318R variant are completely abrogated in *mec1Δ* cells, supporting the notion that Tid1 is regulated through Mec1-dependent phosphorylation in the presence of DSB lesions.

We then analysed Tid1 phosphorylation in G2-blocked cells treated with 4-Nitroquinoline-1-oxide (4NQO, an oxidizing agent that also acts as UV mimetic) and zeocin (a DSB inducing agent). Wild type and *mec1Δ* cells were treated with nocodazole to block cell cycle in G2 phase, then 4 μg/ml 4NQO or 10 μg/ml zeocin were added to the cultures, as indicated in Fig. 3D. We found that both Tid1 and Tid1-K318R variant were phosphorylated in a Mec1-dependet manner in the presence of 4NQO and zeocin in G2-blocked cells, suggesting that Tid1 is phosphorylated in the presence of multiple DNA lesions induced by zeocin and 4NQO treatments. Moreover, as 4NQO not only causes DNA adducts
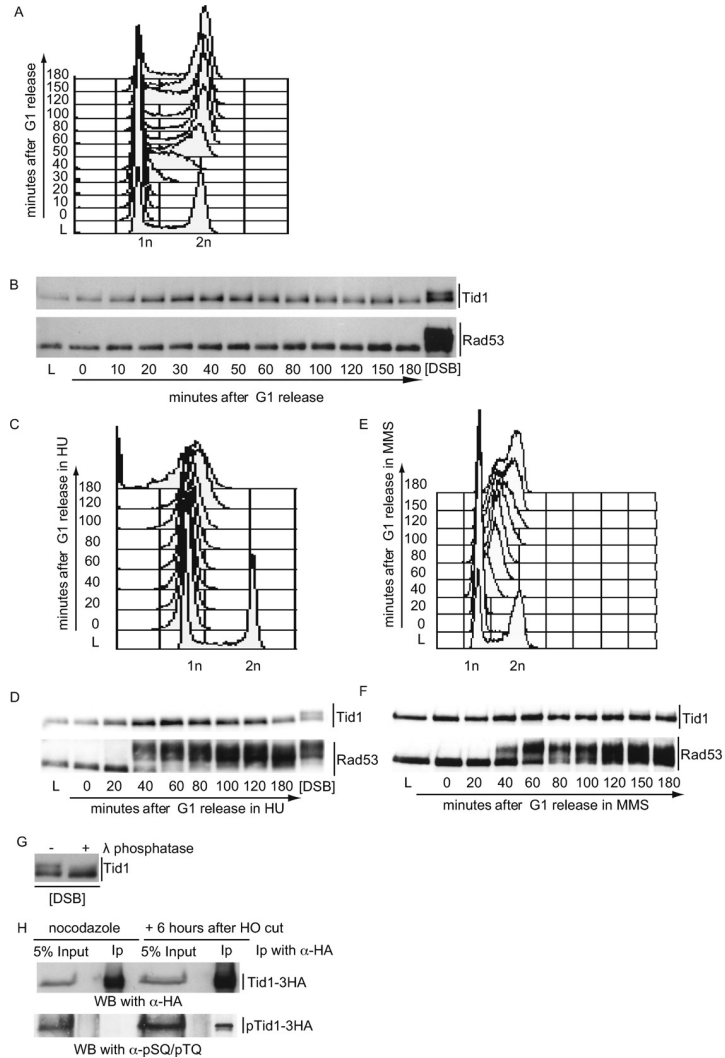
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Fig. 1. Analysis of Tid1 protein with or without DNA damage throughout the cell cycle. (A–F) An exponentially growing culture of the strain Y454 (wild type, *TID1*-3XHA) was grown in YEP+raffinose and pre-synchronized in G1 by α-factor (αF) treatment and released from the G1 block in fresh medium with/without 0.2 M HU or 0.02% MMS. (A, C, E) Samples were taken at the indicated time points to test FACS profiles. (B, D, F) Protein extracts were prepared and analysed by Western blot using 12CA5 (anti-HA) and Ma.EL7 (anti-Rad53) antibodies. Moreover, one part of the original culture was also split and treated with 2% galactose for 6 h to induce the overexpression of HO and the formation of one irreparable DSB; as a consequence, cells remained blocked in G2/M cell cycle phase (data not shown). Protein extract, labelled [DSB], was analysed in the same gels (B and D). (G) Protein extracts obtained after DSB formation (similarly to the lane [DSB] in B and D) were treated with λ phosphatase before gel electrophoresis and tested with 12CA5 (anti-HA) antibody. (H) Exponentially growing culture of the strain Y454 (wild type, *TID1*-3XHA) was grown in YEP+raffinose and synchronized at G2/M transition by nocodazole. Half of the culture was treated with galactose for 6 h to ensure DSB formation. Tid1-3XHA was isolated by immunoprecipitation with anti HA antibody and phosphorylation on SQ/TQ motifs tested by Western blot with anti pSQ/pTQ antibody (Cell Signalling).

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A

G1-blocked cells

Tid1

Rad53

0 30 60 90 120 150 180 210 240

minutes after HO cut in G1

G2/M-blocked cells

Tid1

Rad53

0 30 60 90 120 150 180 210 240

minutes after HO cut in G2/M

B

YMV80 wild type cells

Tid1

Rad53

0 3 6 9 12 15 18 21 24

hours after HO cut
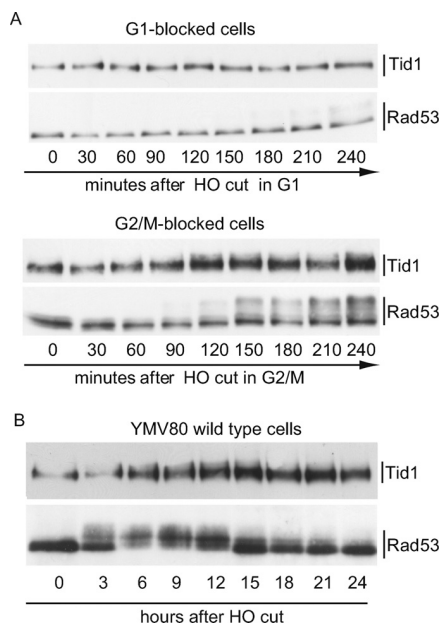
Fig. 2. Kinetic analysis of Tid1 phosphorylation in the presence of one HO-induced DSB throughout the cell cycle. (A) An exponentially growing culture of the JKM-background strain Y454 (wild type, *TID1*-3XHA) was grown in YEP + raffinose. Half of the culture was treated with α-factor (αF) to block cell cycle in G1 phase; the other part was treated with nocodazole (N) to block the cell cycle in G2 phase. 2% galactose was added (time zero) to induce the formation of one irreparable DSB and samples were taken at the indicated time points. (B) An exponentially growing culture of the YMV80-background strain Y811 (wild type, *TID1*-3XHA) was grown in YEP + raffinose. 2% galactose was added (time zero) to induce the formation of one repairable DSB and samples were taken at the indicated time points. Protein extracts were prepared and analysed by Western blot using 12CA5 (anti-HA) and Ma.EL7 (anti-Rad53) antibodies.

similarly to UV, but can also induce the formation of DSB lesions, it is possible that the Tid1 phosphorylation we observed in 4NQO-treated cells may be due to DSBs as well. Accordingly with previous results with the HO-induced DSB in Fig. 2A, we also found that the mobility shift of the Tid1-K318R variant in the presence of 4NQO and zeocin is higher than that seen in the wild type protein.

In summary, the Tid1 ATPase activity, although essential for its functional role in recombination and checkpoint adaptation, is not required to promote its DSB-induced phosphorylation. Moreover, in agreement with previous evidence showing that the ATPase activity of Tid1 is essential to restart cell cycle progression in the presence of one irreparable DSB [24], we found that this activity is required to mediate Rad53 inactivation during checkpoint adaptation.

3.4. Genetic requirement for the Tid1 phosphorylation

We further tested whether Rad53, which is one of the main protein kinases activated by Mec1, and Dun1, that is a protein kinase activated by Rad53 itself [1], were required for Tid1

phosphorylation. We deleted the *DUN1* gene and generated the kinase inactive *rad53*-K227A allele both in *TID1*::3HA and *tid1*-K318R::3HA strains. The HO gene was overexpressed in these strains to induce the formation of one irreparable DSB and samples were taken at various time points to analyse the phosphorylation state of both Tid1 and Rad53. As shown in Fig. 4A, the HO-induced modification of wild type Tid1 and the Tid1-K318R variant are not affected in *dun1*Δ cells. Interestingly, the *rad53*-K227A mutation does not affect the HO-induced phosphorylation of the wild type Tid1 protein (Fig. 4B). However, the hyper-phosphorylation state of the Tid1-K318R variant is not seen in the *rad53*-K227A mutant, and the phosphorylation state of the Tid1-K318R protein variant was similar to that observed for the wild type protein. These results, together with previous findings in Fig. 1, suggest that both Mec1 and Rad53 kinases are involved in the HO-induced Tid1 phosphorylation, but the Rad53 contribution becomes evident only when the ATPase-inactive Tid1-K318R variant is analyzed, raising specific questions on the functional regulation of Tid1, as it will be discussed below. We also tested whether Chk1, which is another kinase activated by Mec1 [1], was responsible for the residual Tid1 phosphorylation observed in the double mutant rad53-K227A tid1-K318R cells. To this aim, we generated the deletion of *CHK1* gene in the *rad53*-K227A cells, carrying the *tid1*-K318R::3HA allele, and induced one irreparable DSB as in previous experiments. We found that the residual phosphorylation of the Tid1-K318R variant is still present in the triple mutant *tid1*-K318R *rad53*-K227A *chk1*Δ (Fig. 4C), further supporting the idea that it could be mediated by Mec1.

It is known that Tid1 is recruited to a DSB through its interaction with Rad51 [14]. We thus tested whether Rad51 had any contribution to the HO-induced Rdh54 phosphorylation. Again we overexpressed the HO gene to induce the formation of one irreparable DSB in wild type and *rad51*Δ cells, carrying the *TID1*::3HA or *tid1*-K318R::3HA alleles. As shown in Fig. 4D, the DSB-induced phosphorylation of wild type Tid1 is not completely compromised in the absence of Rad51, although it appeared to be delayed. Interestingly, we found that hyper-phosphorylation of the Tid1-K318R variant is impaired in *rad51*Δ cells, with a residual phosphorylation migrating as the wild type protein (Fig. 4D). This result recapitulates what we found when Rad53 activity was impaired by the *rad53*-K227A mutation, suggesting that Rad53 and Rad51 may contribute to a common mechanism required for Tid1 hyper-phosphorylation. Moreover, we noticed that Rad53 phosphorylation is persistent in the double mutant *tid1*-K318R *rad51*Δ, accordingly with the notion that the deletion of *RAD51* gene does not rescue the checkpoint adaptation defect of *tid1* mutant cells [24,26].

3.5. Mec1-checkpoint mediates recruitment of Tid1 near to a DSB

We have shown that the Mec1-dependent checkpoint mediates Tid1 phosphorylation in the presence of one DSB. However, activation of the Mec1 and Rad53 kinases is not enough *per se* to induce such Tid1 phosphorylation since this protein is not modified in response to DNA replication stress induced by HU and MMS treatments (Fig. 1). Moreover, Tid1 hyper-phosphorylation requires the Rad51 recombinase (Fig. 4C), which also mediates Tid1 recruitment to the DSB lesion [14]. One possibility is that Tid1 is phosphorylated at the DSB site, and this modification may somehow reinforce the binding of the protein to DNA. We thus tested by chromatin immunoprecipitation (ChIP) whether the Mec1 kinase was also implicated in Tid1 loading near a single irreparable DSB. To this end, we recovered Tid1 protein from sheared chromatin prepared from formaldehyde crosslinked cells, at different time points after DSB formation (Fig. 5). Quantitative multiplex PCR was then used to monitor the co-immunoprecipitation of DNA
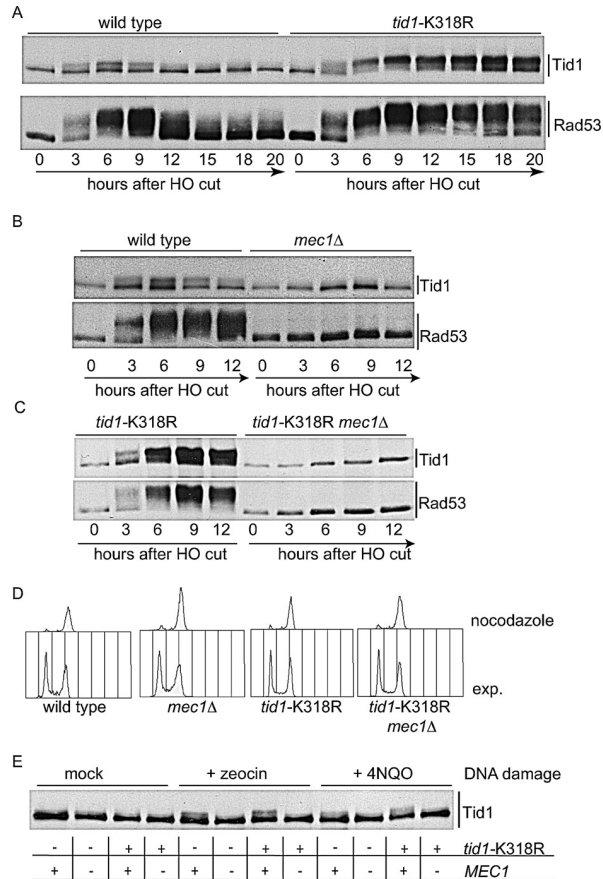
Fig. 3. Mec1-dependent phosphorylation of the wild type Tid1 and the ATPase-defective Tid1-K318R variant. (A) Exponentially growing cultures of the JKM-background strains Y454 (wild type, *TID1*-3XHA) and Y841 (*tid1*-K318R-3XHA) were grown in YEP + raffinose. 2% galactose was added (time zero) to induce the formation of one irreparable DSB and samples were taken at the indicated time points. (B) Exponentially growing cultures of the JKM-background strains Y454 (wild type, *TID1*-3XHA), Y841 (*tid1*-K318R-3XHA), Y522 (*mec1Δ, TID1*-3XHA) and Y876 (*mec1Δ, tid1*-K318R-3XHA), were grown in YEP + raffinose. 2% galactose was added (time zero) to induce the formation of one irreparable DSB and samples were taken at the indicated time points. (C) Exponentially growing cultures of the same strains described in (B), were grown in YPD and treated with nocodazole (N) to block cell cycle in G2 phase. Each culture was split in 3 parts and treated for 30 min with/without 4 μg/ml 4-Nitroquinoline-1-oxide (4NQO) or 10 μg/ml zeocin. Samples were taken at the indicated time points to determine FACS profiles. Protein extracts were prepared and analysed by Western blot using 12CA5 (anti-HA) and Ma.EL7 (anti-Rad53) antibodies.

fragments located either 1 Kb (DSB) or 66 Kb (CON) from the HO-cut site. PCR analysis of the CON site was used as a control of the background signal, as previously described [19]. Firstly, we found that the Tid1-K318R is recruited to the DSB site (Fig. 5), indicating that the ATPase activity is dispensable for recruitment of the protein. Interestingly, we noticed that the loading of the Tid1-K318R variant was significantly higher than that of the wild type protein, especially at late time points after the induction of the DSB, and this slight accumulation may be due to an impairment

of the catalytic defective protein variant to move along DNA molecules, as showed by previous *in vitro* assays [27].

Next we tested the binding to one DSB of both Tid1 and Tid1-K318R to a DSB in *mec1Δ* cells. As shown in Fig. 5, we found that loading of Tid1 near the DSB is significantly reduced in *mec1Δ* cells. This defect is particularly evident for the Tid1-K318R variant, whose binding is normally higher compared to the wild type protein. Therefore, the Mec1-dependent checkpoint contributes, together with Rad51, to mediate the Tid1 recruitment. Among

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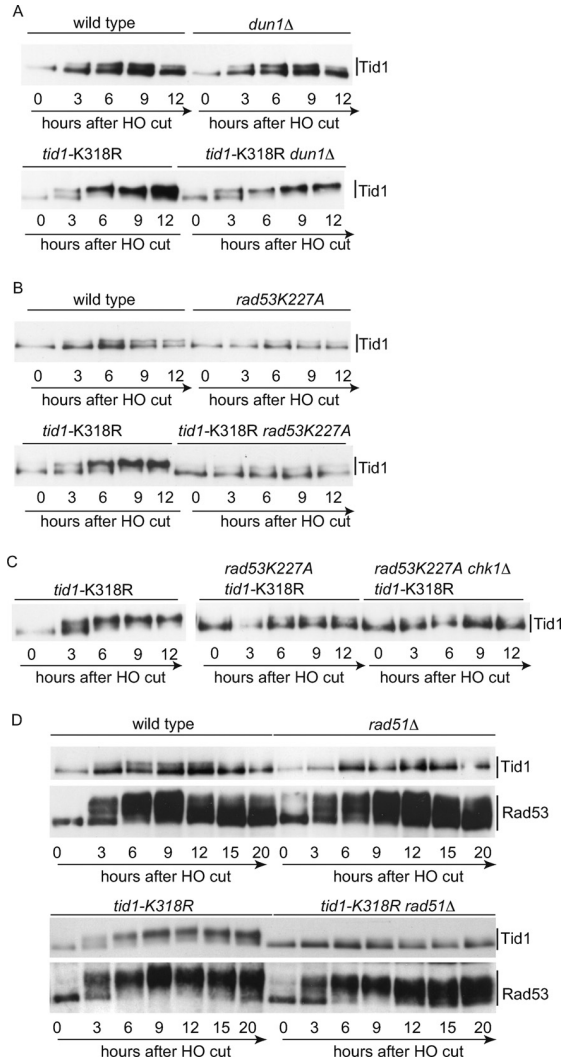
Fig. 4. Rad53 and Rad51 mediate Tid1 phosphorylation in the presence of one HO-induced persistent DSB. (A–D) Exponentially growing cultures of the JKM-background strains Y1768 (*dun1Δ, TID1*-3XHA), Y1769 (*dun1Δ, tid1*-K318R-3XHA), Y962 (*rad53*-K227A, *TID1*-3XHA), Y966 (*rad53*-K227A *tid*-K318R-3XHA), Y841 (*tid1*-K318R-3XHA), Y1771 (*rad53*-K227A, *chk1Δ tid1*-K318R-3XHA), Y741 (*rad51Δ, TID1*-3XHA), Y873 (*rad51Δ, tid1*-K318R-3XHA), were grown in YEP + raffinose. 2% galactose was added (time zero) and samples were taken at the indicated time points. Protein extracts were prepared and analysed by Western blot using 12CA5 (anti-HA) and Ma.EL7 (anti-Rad53) antibodies.
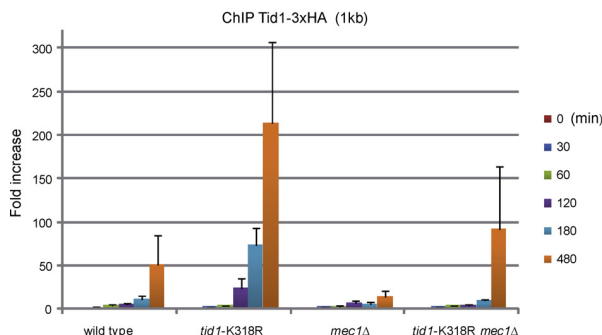
Fig. 5. Recruitment of Tid1 protein to a HO-induced persistent DSB. YEP + raffinose nocodazole-arrested cell cultures of the JKM-background strains Y454 (wild-type, *TID1*-3XHA), Y841 (*tid1*-K318R-3XHA), Y522 (*mec1Δ*, *TID1*-3XHA), Y876 (*mec1Δ*, *tid1*-K318R-3XHA), were transferred to nocodazole-containing YEP + raffinose + galactose (time zero), to induce the formation of one irreparable DSB. Cells were collected at the indicated time points and then subjected to chromatin immunoprecipitation analysis. The graph represents the results from four independent experiments.

various possibilities, we favour the idea that Tid1 protein is recruited to the DSB through its interaction with Rad51, as was previously showed by others [14]. Then the Tid1 function at the DSB site may be regulated through Mec1 and Rad53 phosphorylation events, which may affect its ATPase and translocase activities. Identification and characterization of the specific Tid1 phosphorylated sites will shed light on this mechanism.

4. Discussion

The Tid1 protein of *S. cerevisiae* cells has been implicated in several molecular pathways including DNA recombination, DSB repair and checkpoint adaptation. It has been reported that Tid1 is an *in vitro* target of the meiotic checkpoint kinase Mek1 [28], and that it is phosphorylated in the presence of DNA alkylating agent MMS [20]; however, regulation of Tid1 protein has been poorly studied. Here we show that Tid1 is phosphorylated in the presence of one DSB and, based on the different electrophoretic mobility shifts (see Fig. 1), this phosphorylation appears to be different from that observed in MMS-treated cells [20]. In our experimental conditions, the DSB ends are extensively resected and a long ssDNA filament is generated. As a consequence, Mec1 and Rad53 kinases are activated and the DNA damage checkpoint signalling contributes to block cell cycle progression in metaphase. Supporting this model, we did not observe Tid1 phosphorylation after one DSB induced in G1-blocked cells (Fig. 2A), in which DSB ends are not efficiently processed and Mec1-signalling is not fully active [22,23]. Moreover, Tid1 phosphorylation decreases both during checkpoint recovery and adaptation, in response to one repairable and irreparable DSB (Figs. 2B and 3A). Indeed, our genetic and biochemical evidences support the idea that the DSB-induced Tid1 phosphorylation is mediated by the Mec1 and Rad53 kinases. Accordingly to the protein sequence, Tid1 has several potential Mec1 and Rad53 phosphorylation motifs, suggesting that Tid1 protein can be directly phosphorylated by both Mec1 and Rad53. In agreement with this possibility, we found that in response to one irreparable DSB, immunoprecipitated Tid1 protein is recognized by specific antibodies directed against phosphorylated S/T-Q motifs (Fig. 1F), which are often phosphorylated by ATM/Tel1, ATR/Mec1 and Rad53 [20,21,29]. We do not know yet which Tid1 sites are phosphorylated, although we are trying to address this issue by

mass spectrometry analysis of the sites phosphorylated *in vivo* in response to DSBs.

It is known that ATPase activity is essential for the functional role of Tid1 in DSB repair, recombination and checkpoint adaptation [14,15,24]. However, here we have shown that the ATPase activity of Tid1 protein is dispensable for its initial recruitment near an irreparable DSB and that the same is true for Mec1-dependent Tid1 phosphorylation. We also observed that the ATPase defective Tid1-K318R variant is heavily hyper-phosphorylated, and this modification is abrogated in *rad53* and *rad51* mutants. It is tempting to speculate that the Tid1-K318R variant, after being loaded near the lesion through the interaction with Rad51, remains in a "frozen" and Rad53-mediated hyper-phosphorylated state. In this condition the catalytic-defective Tid1-K318R variant cannot translocate along the DNA filament [27], and we observed a slightly higher accumulation at the DSB site (Fig. 5). However, our results cannot exclude the possibility that the wild type Tid1 protein may become phosphorylated by Rad53-mediated reactions. In fact, because of its ATPase activity, Tid1 would likely translocate along the DNA in a highly dynamic way, perhaps undergoing a faster kinetic loading and dislodging from the DNA, making Rad53-dependent Tid1 phosphorylation very transient and, therefore, very difficult to be detected by Western blotting.

Our results raise the possibility that phosphorylation of Tid1 protein is relevant to mediate its efficient loading near the DSB site, and the identification and mutation of the specific Tid1 phosphorylated sites will shed light on this mechanism. Alternatively, or in addition, as the phosphorylation and binding to DSB of Tid1 protein are not completely abrogated in *rad51Δ* cells (Fig. 4 and [14]), we could also hypothesize a two-step Tid1 phosphorylation mechanism. Firstly Mec1 phosphorylates Tid1 at the DSB site and then after Tid1 has been engaged in a Rad51-dependent intermediate on the DNA, it is phosphorylated by Rad53. It is possible that Mec1- and/or Rad53-dependent phosphorylation mediates a functional interaction between Tid1 and Rad51, which, in turn, may mediate efficient binding of Tid1 near the DSB. Interestingly, in meiotic yeast cells, a Mek1-dependent phosphorylation of Rad54 (a Tid1 homologue), affects its interaction with Rad51 and meiotic recombination outputs [28], suggesting that a fine regulation of Rad51 protein complexes is a fundamental step to modulate DNA recombination and DSB repair. In addition, *rad51Δ* cells, as well as *tid1Δ* and *tid1Δ rad51Δ* mutants, do not adapt to a single

irreparable DSB [24], suggesting that the functional interaction between Tid1 and Rad51 is important to recruit Tid1 onto irreparable DSB, allowing the checkpoint adaptation process. Indeed, we found that the hyper-phosphorylation of Tid1-K318R variant is abrogated in *rad51Δ* cells, despite the fact that Rad53 phosphorylation is persistent (Fig. 4D), according to previous evidences [24,26], showing checkpoint adaptation defect in the *tid1Δ rad51Δ* double mutant cells. The functional role of Tid1 in checkpoint adaptation is mediated by its ATPase/translocase activity and may be linked to its capacity to remodel nucleosomes and dislocate Rad51 and/or other factors from dsDNA [5]. Indeed, it is known that specific nucleosome modifications mark several kilobases around a DSB and, surprisingly, a chromosome-wide spreading of Rad51 molecules was described starting from a persistent DSB [30].

In conclusion, we have shown that Tid1 is a stable protein throughout the cell cycle, and is phosphorylated in the presence of one irreparable DSB through Mec1 and Rad53 kinases. The accumulation of the phosphorylated state correlates with the binding of the protein to the DSB site, which is mediated by Mec1 and Rad53 kinases and Rad51 recombinase [14]. The ATPase activity of Tid1 is dispensable for the recruitment to the DSB and the phosphorylation of the protein, but it is necessary for the functional roles of Tid1 at the DSB site. Further biochemical and genetic analyses will be required to fully clarify the functional regulation of Mec1- and/or Rad53-dependent phosphorylation of Tid1, and to understand its role in checkpoint adaptation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

References

[1] J.C. Harrison, J.E. Haber, Surviving the breakup: the DNA damage checkpoint, Annu. Rev. Genet. 40 (2006) 209–235.
[2] A. Pellicioli, S.E. Lee, C. Lucca, M. Foiani, J.E. Haber, Regulation of Saccharomyces Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest, Mol. Cell 7 (2001) 293–300.
[3] J. Bartek, J. Lukas, DNA damage checkpoints: from initiation to recovery or adaptation, Curr. Opin. Cell Biol. 19 (2007) 238–245.
[4] K. Strebhardt, Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy, Nat. Rev. Drug Discov. 9 (2010) 643–660.
[5] J. San Filippo, P. Sung, H. Klein, Mechanism of eukaryotic homologous recombination, Annu. Rev. Biochem. 77 (2008) 229–257.
[6] P.P. Shah, X. Zheng, A. Epshtein, J.N. Carey, D.K. Bishop, et al., Swi2/Snf2-related translocases prevent accumulation of toxic Rad51 complexes during mitotic growth, Mol. Cell 39 (2010) 862–872.
[7] M. Shinohara, E. Shita-Yamaguchi, J.M. Buerstedde, H. Shinagawa, H. Ogawa, et al., Characterization of the roles of the *Saccharomyces cerevisiae* RAD54 gene and a homologue of RAD54, RDH54/TID1, in mitosis and meiosis, Genetics 147 (1997) 1545–1556.
[8] H.L. Klein, RDH54, a RAD54 homologue in *Saccharomyces cerevisiae*, is required for mitotic diploid-specific recombination and repair and for meiosis, Genetics 147 (1997) 1533–1543.
[9] S. Flott, Y. Kwon, Y.Z. Pigli, P.A. Rice, P. Sung, et al., Regulation of Rad51 function by phosphorylation, EMBO Rep. 12 (2011) 833–839.
[10] E. Baroni, V. Viscardi, H. Cartagena-Lirola, G. Lucchini, M.P. Longhese, The functions of budding yeast Sae2 in the DNA damage response require Mec1- and Tel1-dependent phosphorylation, Mol. Cell. Biol. 24 (2004) 4151–4165.
[11] G. Liberi, I. Chiolo, A. Pellicioli, M. Lopes, P. Plevani, et al., Srs2 DNA helicase is involved in checkpoint response and its regulation requires a functional Mec1-dependent pathway and Cdk1 activity, EMBO J. 19 (2000) 5027–5038.
[12] L. Cheng, L. Hunke, C.F. Hardy, Cell cycle regulation of the *Saccharomyces cerevisiae* polo-like kinase cdc5p, Mol. Cell. Biol. 18 (1998) 7360–7370.
[13] M.S. Longtine, A. McKenzie 3rd, D.J. Demarini, N.G. Shah, A. Wach, et al., Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*, Yeast 14 (1998) 953–961.
[14] Y. Kwon, C. Seong, P. Chi, E.C. Greene, H. Klein, et al., ATP-dependent chromatin remodeling by the *Saccharomyces cerevisiae* homologous recombination factor Rdh54, J. Biol. Chem. 283 (2008) 10445–10452.
[15] G. Petukhova, P. Sung, H. Klein, Promotion of Rad51-dependent D-loop formation by yeast recombination factor Rdh54/Tid1, Genes Dev. 14 (2000) 2206–2215.
[16] M.B. Vaze, A. Pellicioli, S.E. Lee, G. Ira, G. Liberi, et al., Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase, Mol. Cell 10 (2002) 373–385.
[17] S. Fiorani, G. Mimun, L. Caleca, D. Piccini, Pellicioli A, Characterization of the activation domain of the Rad53 checkpoint kinase, Cell Cycle 7 (2008) 493–499.
[18] A. Pellicioli, C. Lucca, G. Liberi, F. Marini, M. Lopes, et al., Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase, EMBO J. 18 (1999) 6561–6572.
[19] R.A. Donnianni, M. Ferrari, F. Lazzaro, M. Clerici, B. Tamilselvan Nachimuthu, et al., Elevated levels of the polo kinase Cdc5 override the Mec1/ATR checkpoint in budding yeast by acting at different steps of the signaling pathway, PLoS Genet. 6 (2010) e1000763.
[20] C.P. Albuquerque, M.B. Smolka, S.H. Payne, V. Bafna, J. Eng, et al., A multidimensional chromatography technology for in-depth phosphoproteome analysis, Mol. Cell. Proteomics 7 (2008) 1389–1396.
[21] M.B. Smolka, C.P. Albuquerque, S.H. Chen, H. Zhou, Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 10364–10369.
[22] G. Ira, A. Pellicioli, A. Balijja, X. Wang, S. Fiorani, et al., DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1, Nature 431 (2004) 1011–1017.
[23] R. Janke, K. Herzberg, M. Rolfsmeier, J. Mar, V.I. Bashkirov, et al., A truncated DNA-damage-signaling response is activated after DSB formation in the G1 phase of *Saccharomyces cerevisiae*, Nucleic Acids Res. 38 (2010) 2302–2313.
[24] S.E. Lee, A. Pellicioli, A. Malkova, M. Foiani, J.E. Haber, The Saccharomyces recombination protein Tid1p is required for adaptation from G2/M arrest induced by a double-strand break, Curr. Biol. 11 (2001) 1053–1057.
[25] P. Chi, Y. Kwon, C. Seong, A. Epshtein, I. Lam, et al., Yeast recombination factor Rdh54 functionally interacts with the Rad51 recombinase and catalyzes Rad51 removal from DNA, J. Biol. Chem. 281 (2006) 26268–26279.
[26] S.E. Lee, A. Pellicioli, M.B. Vaze, N. Sugawara, A. Malkova, et al., Yeast Rad52 and Rad51 recombination proteins define a second pathway of DNA damage assessment in response to a single double-strand break, Mol. Cell. Biol. 23 (2003) 8913–8923.
[27] T.K. Prasad, R.B. Robertson, M.L. Visnapuu, P. Chi, P. Sung, et al., A DNA-translocating Snf2 molecular motor: *Saccharomyces cerevisiae* Rdh54 displays processive translocation and extrudes DNA loops, J. Mol. Biol. 369 (2007) 940–953.
[28] H. Niu, L. Wan, V. Busygina, Y. Kwon, J.A. Allen, et al., Regulation of meiotic recombination via Mek1-mediated Rad54 phosphorylation, Mol. Cell 36 (2009) 393–404.
[29] S.H. Chen, C.P. Albuquerque, J. Liang, R.T. Suhandynata, H. Zhou, A proteome-wide analysis of kinase-substrate network in the DNA damage response, J. Biol. Chem. 285 (2010) 12803–12812.
[30] M. Kalocsay, N.J. Hiller, S. Jentsch, Chromosome-wide Rad51 spreading and SUMO-H2A.Z-dependent chromosome fixation in response to a persistent DNA double-strand break, Mol. Cell 33 (2009) 335–343.

Part III
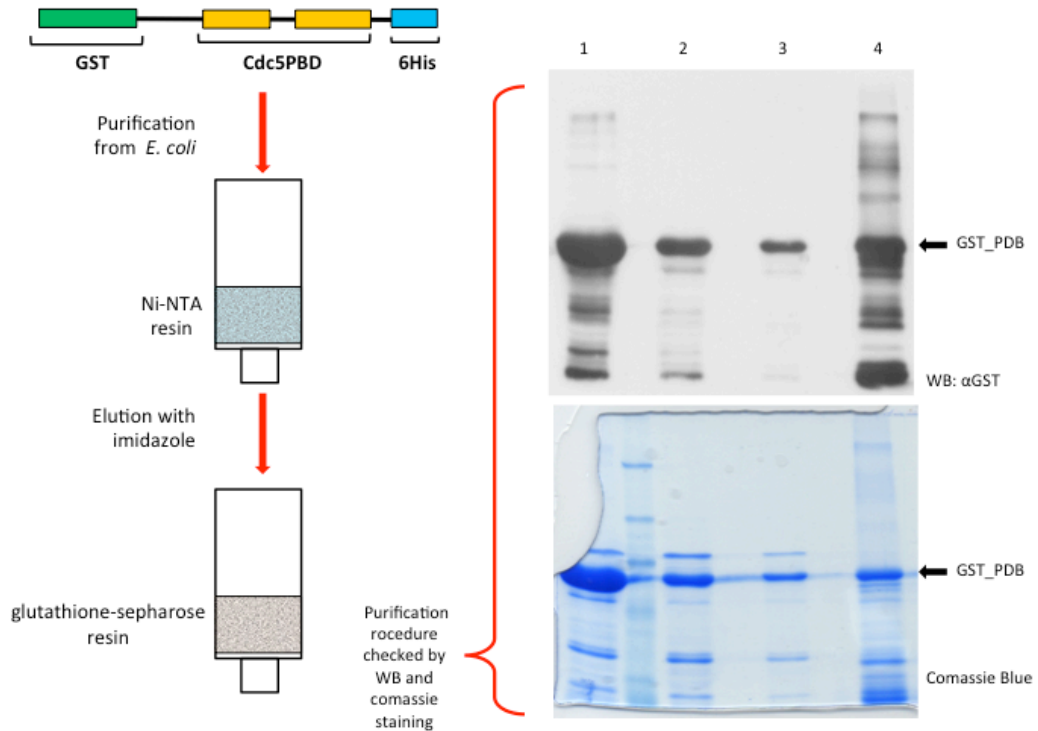
Supplementary Figure 1: Schematic representation of the GST-PBD-6XHis resin preparation. The fusion polypeptide was expressed in the *E. coli* strain BL21 CodonPlus, after induction with IPTG 0,5 mM, at 37°C for 2 hours. Cells were broken with lysozyme and sonication treatments, then the obtained cell crude extract was passed trough a Ni-NTA resin in a column (1). Then the polypeptides bound to the resin were eluted by imidazole treatment (2), and incubated with a glutathione-sepharose resin (3). The resin was checked by western blot with anti-GST antibody and Coomassie staining. In the lane 4 of the gel, a resin obtained only after glutathione-sepharose incubation was loaded in order to compare it with the two steps purification method.
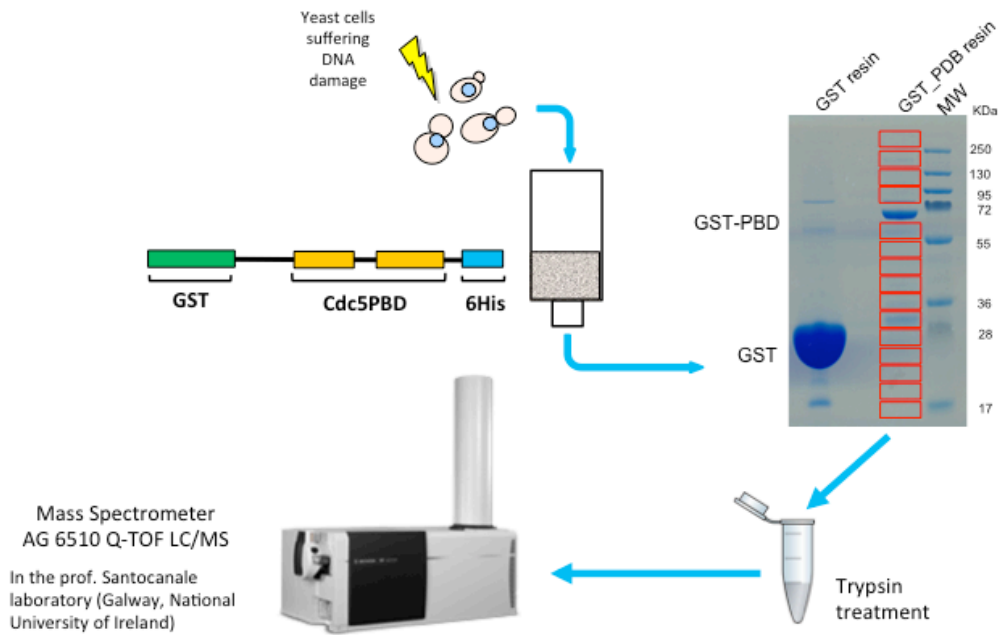
Supplementary Figure 2: Schematic representation of the screening procedure. The GST-PBD-6XHis resin was incubated for 2 hours at 4°C with yeast cell crude extract obtained by DNA damaged cells. After that the resin is boiled in Laemmly Buffer and run on a SDS-PAGE. Gel slices containing the putative interacting proteins were cut (red squares) and treated with trypsin in order to obtain the proteins peptides required for the mass spectrometry analysis.
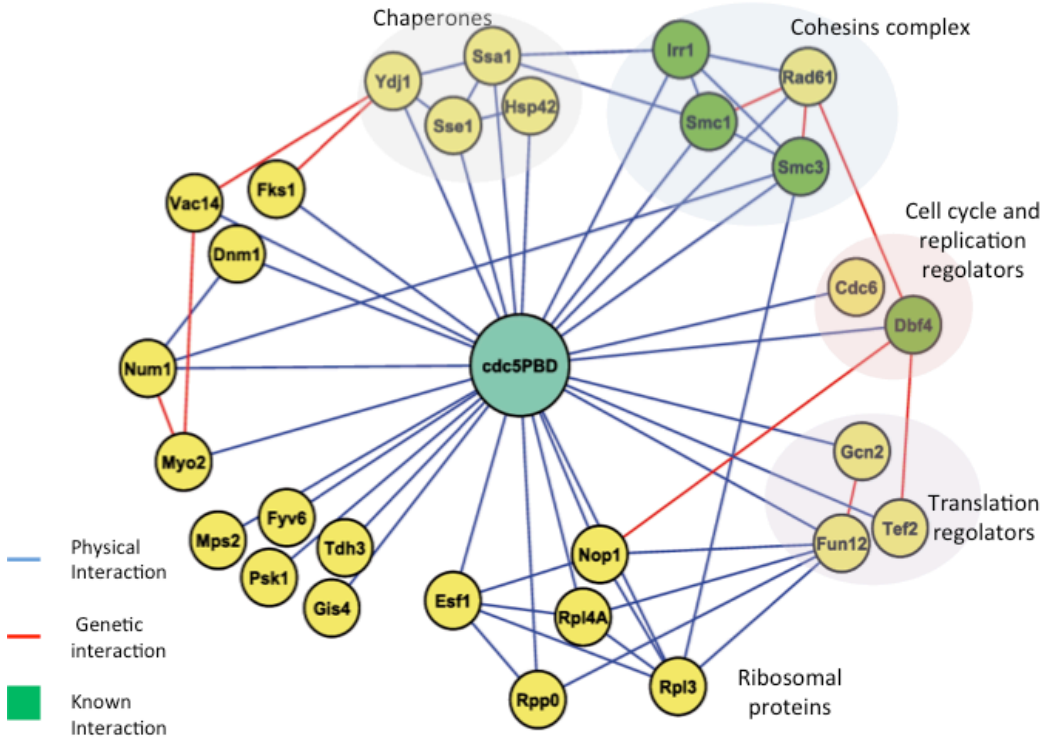
Supplementary Figure 6: Interactions network of the factors identified after 6 hours induction of one irreparable DSB by the HO endonuclease (see text for details).
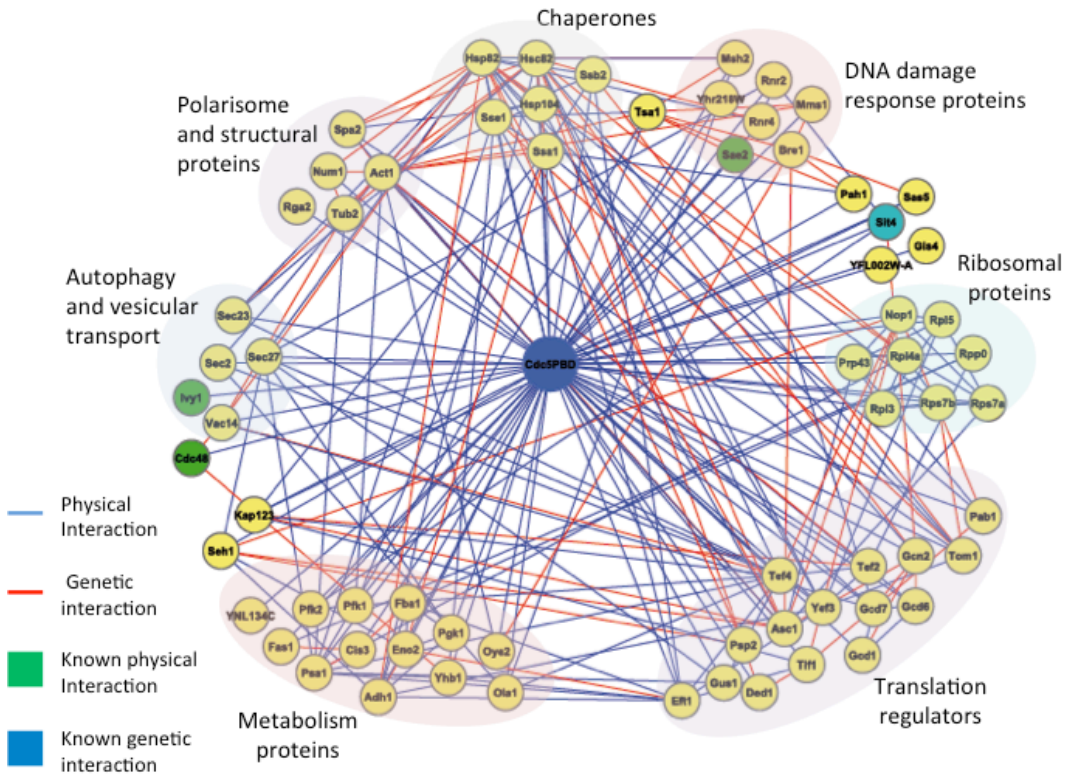
Supplementary Figure 4: Interactions network of the factors identified after 12 hours induction of one irreparable DSB by the HO endonuclease (see text for details).
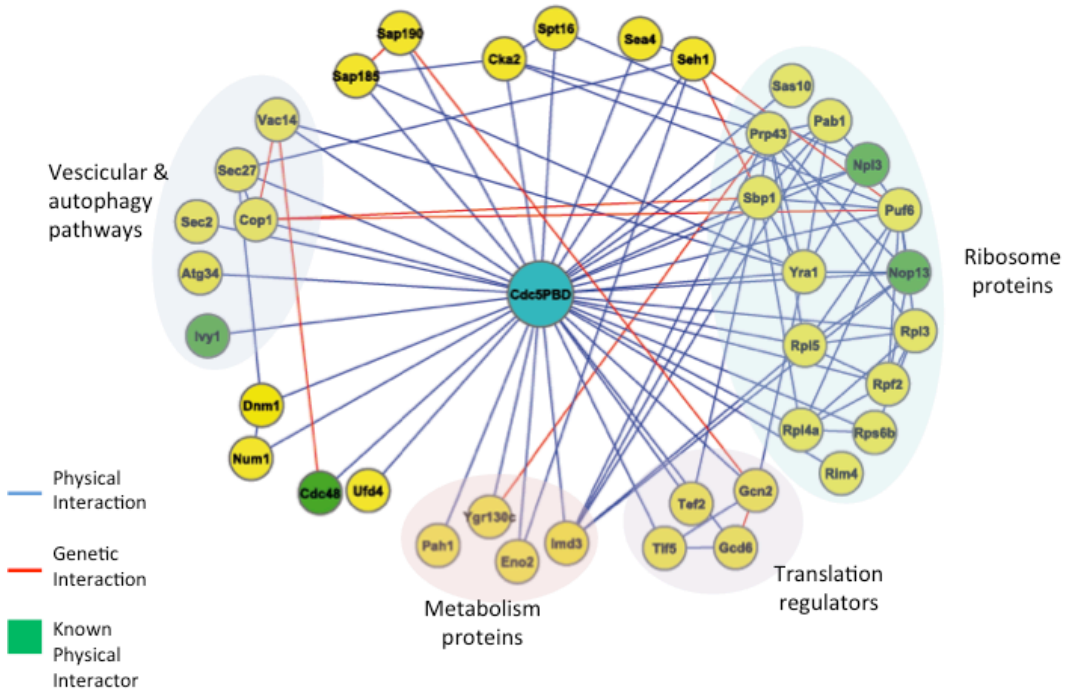
Supplementary Figure 5: Interactions network of the factors identified after a 3 hours treatment with the alkylating agent MMS (see text for details).
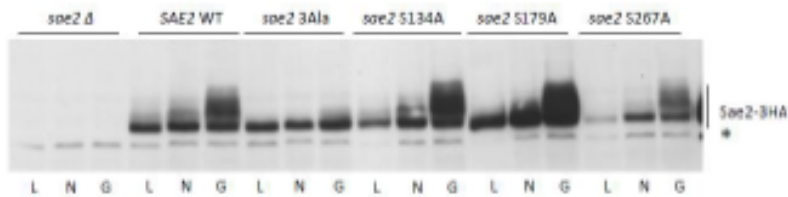
Supplementary Figure 6: Protein levels of various Sae2 variants. Cells of the strains: *sae2Δ*, *SAE2*-3HA, *sae2*-S134A-S179A-S267A-3HA (*sae2*-3Ala), *sae2*-S134A-3HA, *sae2*-S179A-3HA, *sae2*-S267A-3HA, were treated with Nocodazole (N) to induce a cell cycle arrest in G_2, or with galactose (G) to induce the formation of one irreparable DSB. The samples indicated with L (log) represent the protein extract obtained for exponentially growing cells. Crude protein extracts were done and analysed by monoclonal antibodies 12CA5 (αHA). The asterisk indicates an unspecific background detected by the 12CA5 Mab.
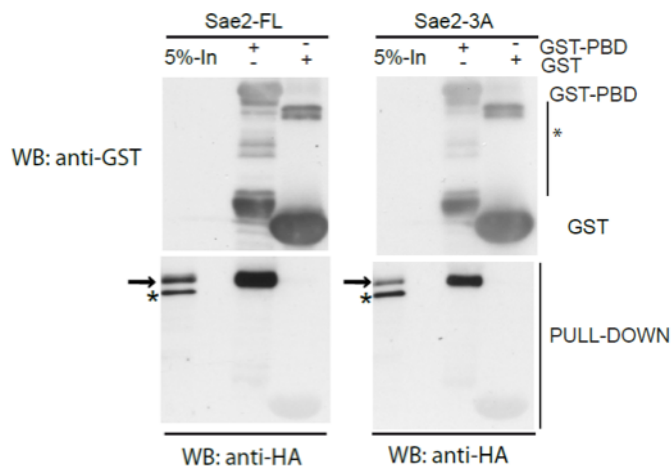
Supplementary Figure 7: The Sae2-3Ala protein variant still interacts by pulldown with the Cdc5-PBD. Crude extracts of the indicated strains were prepared from exponentially growing cells, and then incubated with the GST or GST-PBD-6XHis resins for 2 hours at 4°C. The Input and the eluate were analysed by Western Blot using monoclonal antibodies 12CA5 (αHA), and αGST. The arrows indicate the proteins of interest while the asterisks indicate an unspecific background detected by the 12CA5 Mab.
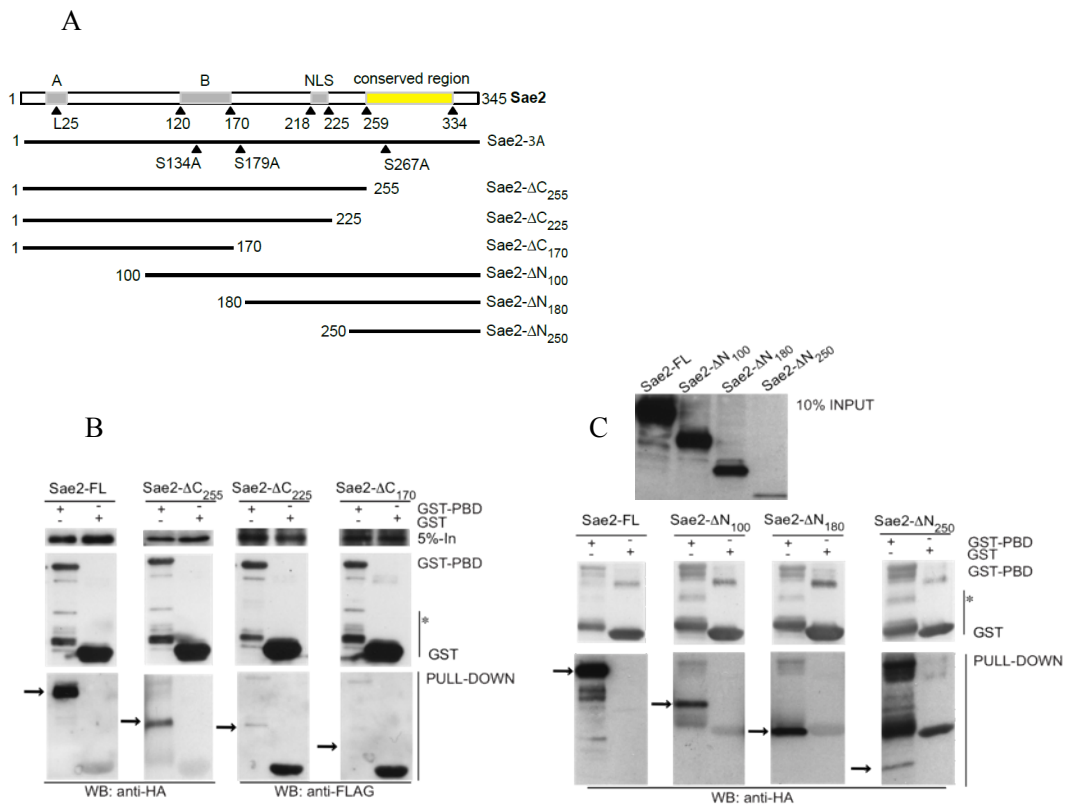
**Supplementary Figure 8: Western blot analysis of the Pulldown effectuated with the Sae2 truncated forms.** (A) Schematic representation of all the sae2 truncated form used in the pulldown experiments. (B) The protein extracts obtained from exponentially growing cells of the strains sae2ΔC170, sae2ΔC225, sae2ΔC255 and SAE2-3HA were incubated with GST and GST-Cdc5PBD resins for 2 hours at 4°C, then the eluates were analysed by SDS-PAGE and western blot using antibodies 12CA5 for the sae2ΔC255 or SAE2-3HA while for the sae2ΔC170 and sae2ΔC225 α-FLAG antibodies were used. (C) In order to produce the sae2 N-terminal truncated forms, cells of the strains sae2ΔN100, sae2ΔN180 and sae2ΔN250, in which the expression of the protein is induced under GAL1 promoter, and SAE2-3HA were grown in YP+Raffinose overnight, then cells were incubated with galactose for 2 hours and protein extracts were prepared. After that the extracts were incubated with GST or GST-Cdc5PBD resins and the obtained eluates were analysed by SDS-PAGE and western blot with α-HA (12CA5).
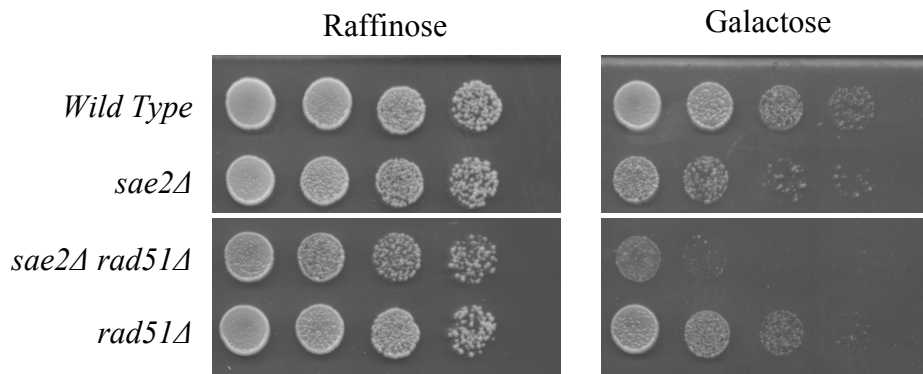
Supplementary Figure 9: **sae2Δ cells survive to a DSB through a Rad51-dependent pathway.** YMV80 and isogenic *sae2*Δ, *sae2*Δ*rad51*Δ and *rad51*Δ strains were grown overnight in YP+Raffinose. The cells culture concentrations were set to $1x10^7$ cells/ml, and the cells were spotted by a serial dilution on YP+Raffinose and YP+Galactose plates and incubated at 28 °C for 2 days.
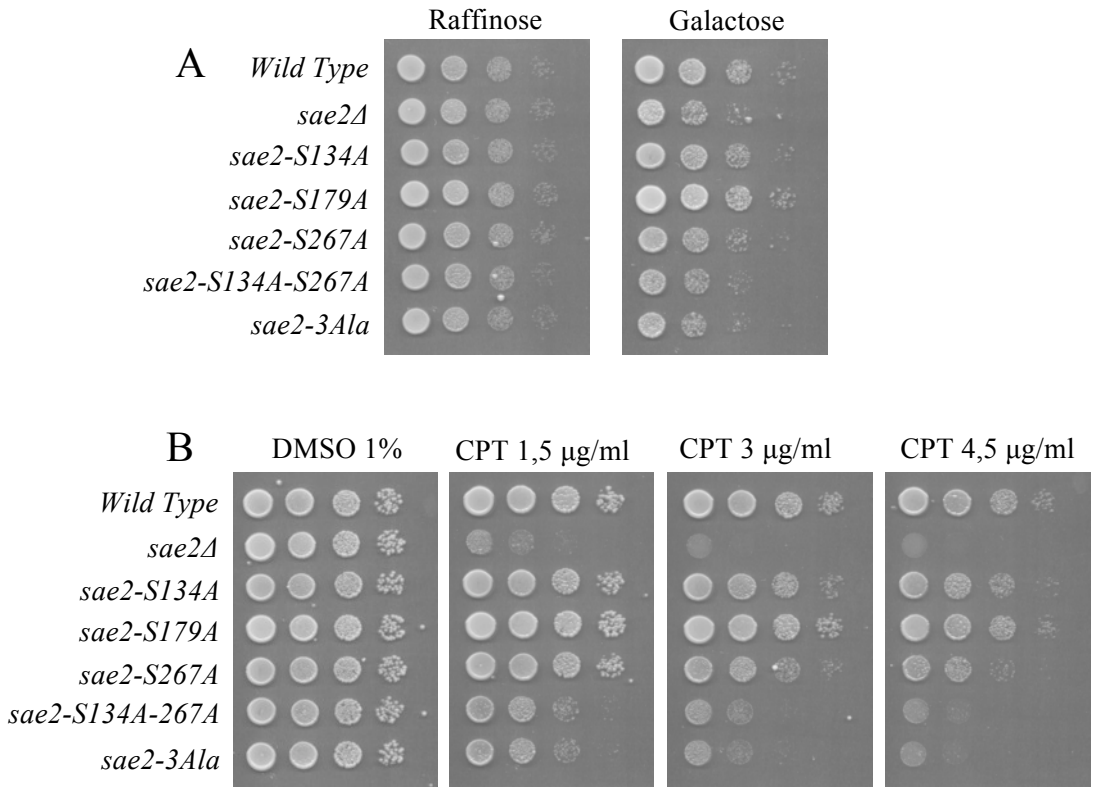
Supplementary Figure 10: Cells viability of various *sae2* mutants in the presence of one DSB or camptothecin. YMV80 and isogenic strains *sae2*Δ, *sae2*-S134A, *sae2*-S179A, *sae2*-S267A, *sae2*-S134A-S267A and *sae2*-3Ala were grown overnight in medium containing raffinose. The cells culture concentrations were set to 1×10^7 cells/ml, and the cells were spotted by a serial dilution on YP+Raffinose and YP+Galactose plates (A), or in plates containing the indicated concentration of camptothecin (CPT) (B). The plates were incubated at 28°C for 2 days.
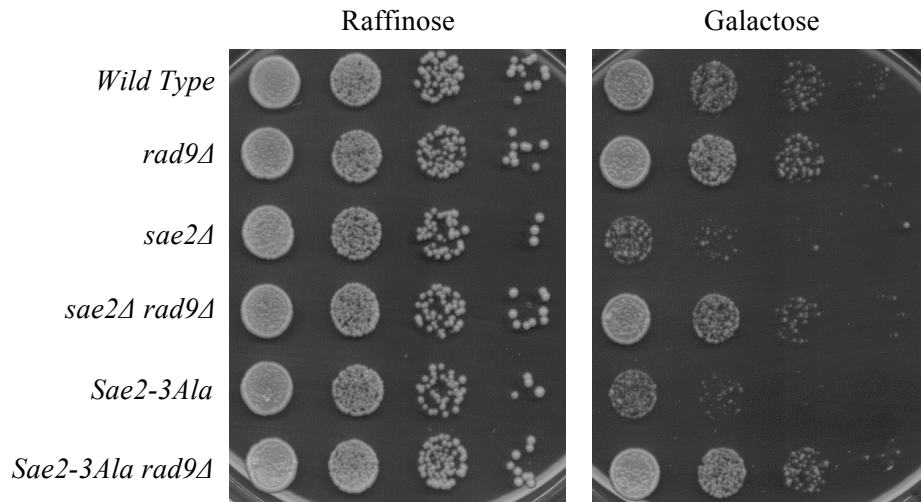
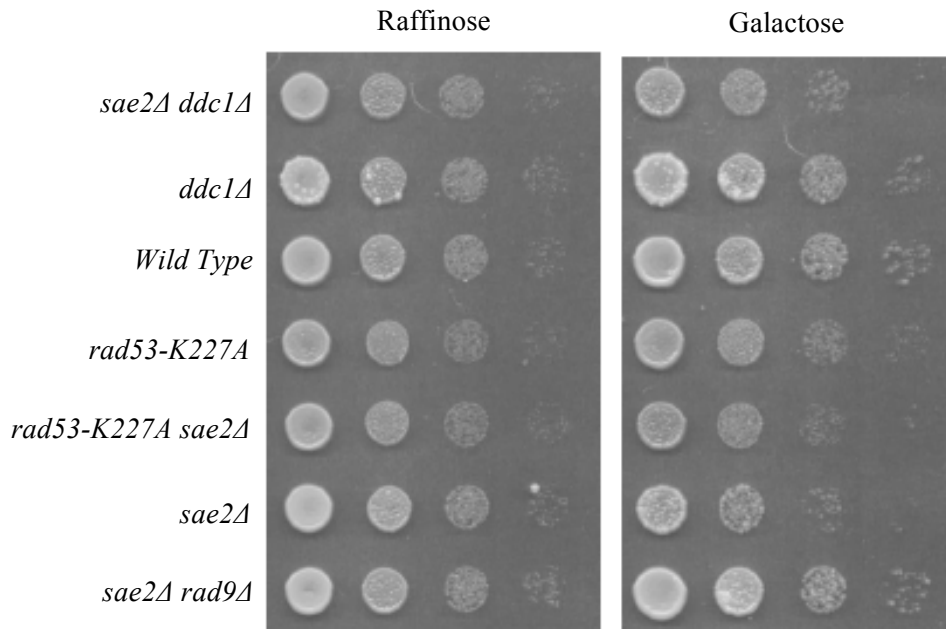**Supplementary Figure 11: Deletion of *RAD9* suppresses the *sae2Δ* and *sae2*-3Ala sensitivity to one DSB.**
YMV80 and isogenic *sae2*Δ, *sae2*Δ*rad9*Δ and *rad9*Δ strains were grown overnight in YP+Raffinose. The cells culture concentrations were set to 1×10^{7} cells/ml, and the cells were spotted by a serial dilution on YP+Raffinose and YP+Galactose plates and incubated at 28 °C for 2 days.
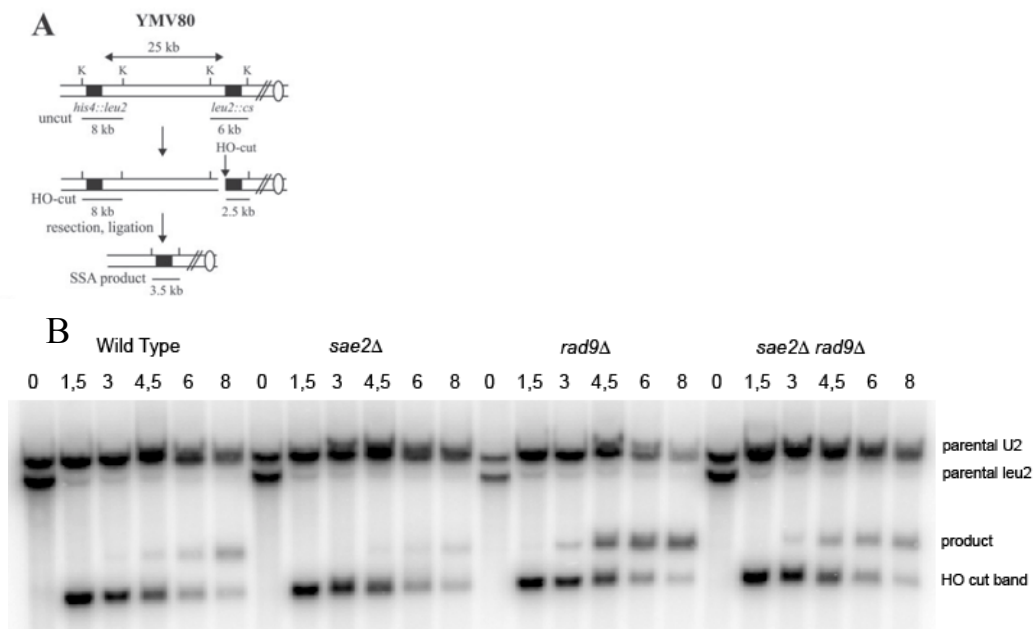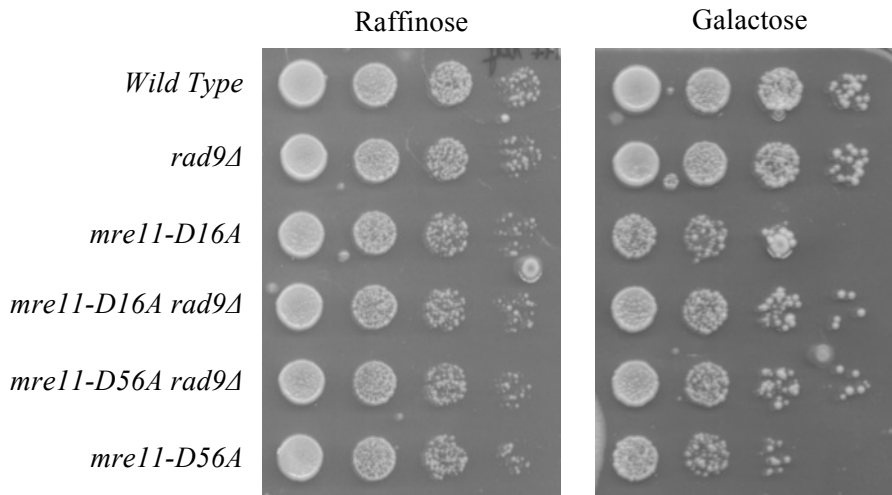
Supplementary Figure 12: Deletion of *RAD9* accelerates DSB repair through SSA in *sae2Δ* and wild type cells. (A) Map of the YMV80 chromosome 3 region containing the HO-cut sites flanked by the *LEU2* homologous sequence spaced 25 kb. The genomic DNA digested with Kpn I and analysed by Southern blotting with a specific *LEU2* probe shows 2 fragments before the HO cut (parental U2 and parental leu2). After DSB induction, the parental U2 band disappears and a faster migrating band appears at the bottom of the gel (HO cut band). Later the repair of the lesion by SSA or BIR leads to the appearance of the product band. (B) YP-Raffinose exponentially growing YMV80 and isogenic *sae2Δ*, *rad9Δ* and *sae2Δrad9Δ* strains were blocked in G_2 with nocodazole (time 0), then galactose was added to induce to HO cut and, at the indicated time point, cell samples were collected and genomic DNA prepared. Then the genomic DNA samples were subjected to Southern blotting analysis as described in (A).
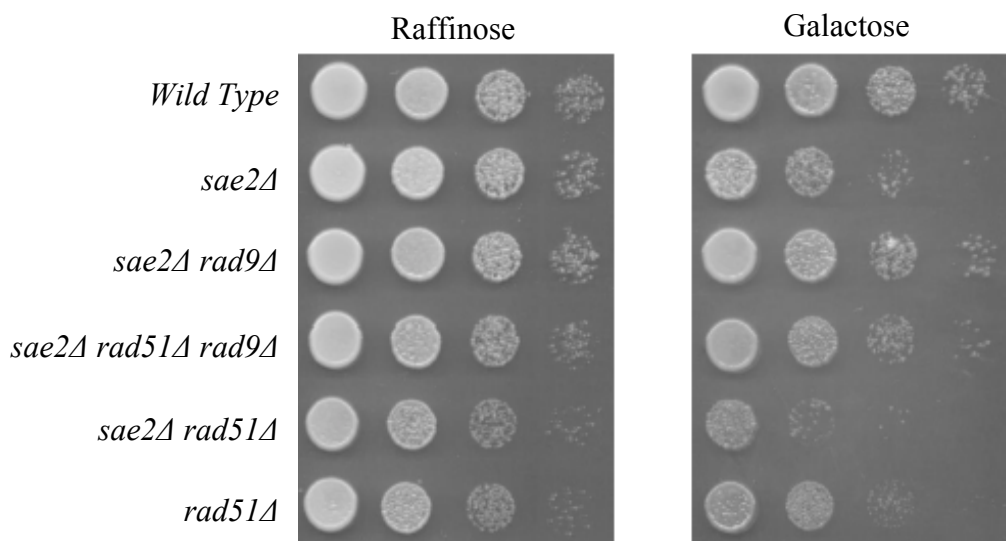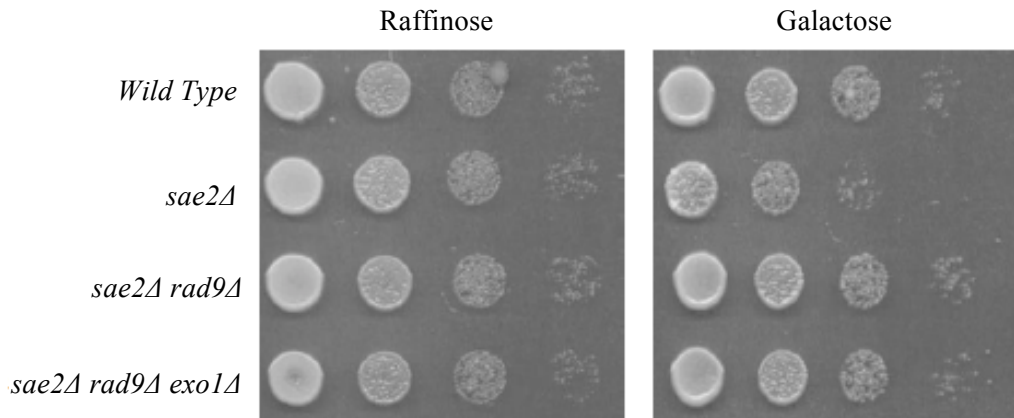
Supplementary Figure 13: *RAD51*-dependent pathway is not involved in the *sae2Δrad9Δ* cells viability in the presence of one DSB. YMV80 and isogenic *sae2Δ*, *sae2Δrad9Δ*, *sae2Δrad9Δrad51Δ*, *sae2Δrad51Δ* and *rad51Δ* strains were grown overnight in YP+Raffinose. The cells culture concentrations were set to $1x10^7$ cells/ml, and the cells were spotted by a serial dilution on YP+Raffinose and YP+Galactose plates and incubated at 28 °C for 2 days.

Supplementary Figure 14: Inactivation of Rad53 kinase activity or deletion of *DDC1* does not suppress the *sae2*Δ sensitivity to one DSB. YMV80 and isogenic *ddc1*Δ, *ddc1*Δ *sae2*Δ, *rad53*-K227A, *rad53*-K227A *sae2*Δ, *sae2*Δ and *sae2*Δ *rad9*Δ strains were grown overnight in YP+Raffinose. The cells culture concentrations were set to 1x10^7 cells/ml, and the cells were spotted by a serial dilution on YP+Raffinose and YP+Galactose plates and incubated at 28 °C for 2 days.

Supplementary Figure 15: Deletion of *RAD9* suppresses mre11 nuclease defective mutants sensitivity to one DSB. YMV80 and isogenic *rad9*Δ, *mre11*-D16A, *mre11*-D56A, *mre11*-D16A *rad9*Δ, *mre11*-D56A *rad9*Δ strains were grown overnight in YP+Raffinose. The cells culture concentrations were set to $1x10^7$ cells/ml, and the cells were spotted by a serial dilution on YP+Raffinose and YP+Galactose plates and incubated at 28 °C for 2 days.

Supplementary Figure 16: *EXO1*-dependent pathway is not involved in the sae2Δrad9Δ cells viability in the presence of one DSB. YMV80 and isogenic *sae2Δ, sae2Δ rad9Δ, sae2Δ rad9Δ exo1Δ* strains were grown overnight in YP+Raffinose. The cells culture concentrations were set to $1x10^7$ cells/ml, and the cells were spotted by a serial dilution on YP+Raffinose and YP+Galactose plates and incubated at 28 °C for 2 days.
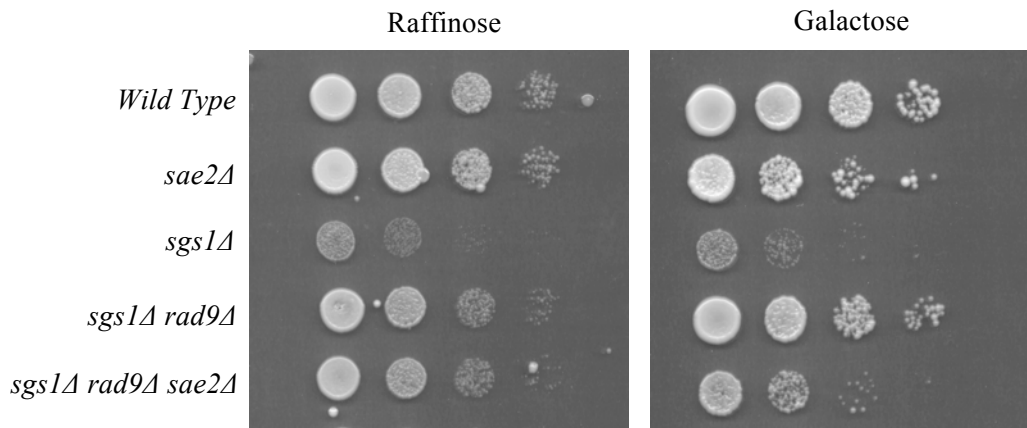
Supplementary Figure 17: *SGS1*-**dependent pathway is involved in the** *sae2Δrad9Δ* **cells viability in the presence of one DSB.** YMV80 and isogenic *sae2Δ, sgs1Δ, sgs1Δ rad9Δ, sae2Δ rad9Δ sgs1Δ* strains were grown overnight in YP+Raffinose. The cells culture concentrations were set to $1x10^7$ cells/ml, and the cells were spotted by a serial dilution on YP+Raffinose and YP+Galactose plates and incubated at 28 °C for 2 days.