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Dma1 and Dma2: novel regulators of double-strand break response in *S. cerevisiae*

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Abstract

Double-strand breaks (DSBs) are extremely cytotoxic DNA lesions. If not properly repaired, they cause pathological alterations either in the structure or in the sequence of chromosomes, significantly increasing genome instability and predisposition to cancer development in higher eukaryotes. As a consequence, cells evolved an elaborate network of mechanisms that rapidly recognize and repair strand breaks, protecting genome integrity. This process, globally known as DNA Damage Response (DDR), consists mainly in the activation of both the DNA damage checkpoint and specific repair pathways (*i.e.* Non Homologous End Joining (NHEJ) and Homologous Recombination (HR), in the case of DSBs), coupled with chromatin remodeling and modulation of gene expression. In the last few years, many studies uncovered the essential contribution of ubiquitination and SUMOylation in the control of the DDR.

I report a new role for the two budding yeast FHA-RING ubiquitin ligases Dma1 and Dma2 in regulating the response to DSB. In particular, I show that these proteins are required for the repair of specific DNA lesions characterized by dirty ends, such as those generated by bleocin. According to my data, Dma1 and Dma2 are recruited to damaged chromatin and, through ubiquitination, promote the removal from DNA of the MRX complex, which is otherwise persistently blocked at Bleocin-induced modified ends. Stable accumulation of the MRX complex at the break site causes severe defects in the repair of broken chromosomes and a strong Tel1-dependent DNA damage checkpoint hyperactivation.

In conclusion, I propose that Dma1 and Dma2 play a critical role in the resolution of DNA-protein crosslink at DSB ends, probably through the direct ubiquitination and degradation via proteasome of the crosslinked factor.

State of the Art

Genome stability maintenance

Maintenance of genome integrity is a crucial process during the life cycle of all organisms. Indeed, cells have to cope with a range from 10^4 to 10^5 DNA lesions per day that, if not rapidly repaired, can give rise to deleterious chemical and structural alteration of the DNA molecule. These damages may be generated by both endogenous and exogenous sources. The intrinsic instability of the DNA double helix in the aqueous environment can result in spontaneous hydrolysis of residues, yielding abasic site formation and possible mispairing bases[1]. In addition, the normal cellular metabolism releases toxic oxidant intermediates that can directly hit DNA sequence, leading to high mutagenic modifications[2]. Replication itself contributes to threatening genome integrity by ribonucleotide incorporation, mismatches and indels[3]. On the other hand, cells are daily exposed to different chemical and physical agents, as UV light and cigarette smoke, which are able to induce structural distortion and breaks, leading to impairment of the basic DNA function[4].

To prevent the accumulation of dangerous lesions and their inheritance to the progeny, cells elaborated a sophisticated network of mechanisms, called DNA Damage Response (DDR), which coordinates multiple specialized pathways: from damage recognition to cell cycle arrest and activation of the specific repair process. In addition, all these processes are associated with significant changes in gene expression and chromatin remodeling[5].

The physiological relevance of DDR is highlighted by the fact that, in higher eukaryotes, the loss of function of genes responding to DNA damage results in severe genetic disorders often associated with high cancer predisposition[6,7].

Moreover, many of the chemotherapy compounds used for the clinical treatment of solid tumors are DNA damage-inducing agents that have therapeutic efficiency thanks to the inability of many cancer cells to rapidly sense and repair lesions[8–10].

DNA Double Strand Break Repair

Among all, DNA Double Strand Breaks (DSBs) are the most cytotoxic lesions for the cell. The accumulation of even a single unrepaired DSB can lead to cell death. Indeed, in contrast with almost all the other type of damages that may count on the presence of a faithful strand to repair the lesion, once DSB occurs, both the DNA strands are damaged at the same time. This, not only, makes DSB more difficult to repair, but strongly increases the risk of gross chromosome rearrangements and cancer transformation. As a consequence, repair of DSBs is a rapidly and tightly regulated process.

Two are the main and highly conserved pathways for DSB repair: Non Homologous End Joining (NHEJ) and Homologous Recombination (HR). These processes are in dynamic competition and the balancing depends upon many different factors, among all the phase of the cell cycle is the most significant. Indeed, while NHEJ is predominant in G0 and G1, in S and G2 phases cells preferentially repair the break through HR, exploiting to the presence of the undamaged homologous sequence on the sister chromatid that guides the repair. Moreover, also the type of DSB ends may influence the choice between the two pathways, pushing the recombination process in presence of modified ends[5,11].

Recently, many studies highlight the importance of a third independent repair pathway, dubbed as microhomology-mediated end joining (MMEJ). Indeed, this mechanism uses a 5-25 base pair (bp) homology to seal the DNA break.

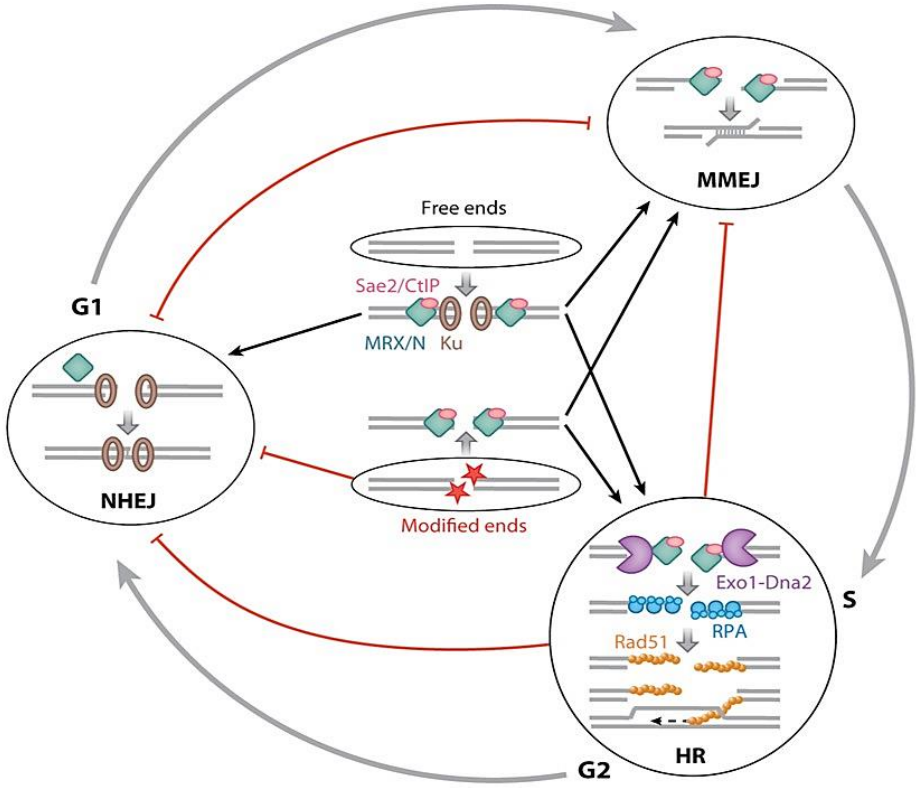


Figure 1. Schematic representation of the cell-cycle regulation of DSB repair mechanisms[11]

Non Homologous End Joining

Non Homologous End Joining (NHEJ) repair mechanism mediates the simple re-ligation of broken DNA ends. As mentioned before, this pathway does not require a template with a homologous sequence and, for this reason, it is not restricted to a specific phase of the cell cycle.

However, NHEJ is widely considered an error-prone repair process. Indeed, very often, DSB ends are not immediately compatible and have to be shortly nucleolytically processed before ligation takes place, thus increasing the probability to introduce small insertions or deletions. Despite this mutagenicity, NHEJ is the most favored DSB repair pathway in vertebrates, because of the high abundance of repetitive elements and non-coding genomic regions.

Moreover, due to its intrinsic capacity of inserting mutations at breakpoints, in the last few years, NHEJ became a powerful tool for genome editing by the CRISPR-Cas9 technology[12,13].

The NHEJ machinery consists of coordinated action of three main complexes: Ku complex, MRX complex (MRN in human cells) and DNA ligase IV[14,15].

Ku is the core complex, a Ku70 and Ku80 protein heterodimer with strong DNA affinity. It forms a sliding ring on DSB ends, preventing their extensive degradation by nucleases[16].

Independently from Ku, also the MRX complex is recruited at the break site immediately after DSB induction. This complex, formed by the three subunits Mre11, Rad50 and Xrs2 (homolog of human Nbs1), provides a flexible tether required to keep DSB ends together[17,18].

Finally, the ligation reaction is carried out by DNA ligase IV (Dln4) and its functional partner Lif1 (XRCC4 in mammalian cells)[19,20].

In humans, the core complex requires DNA-PK as an additional component. The role of this factor is essential. Indeed its artificial inhibition causes a strong impairment in NHEJ efficiency, sensitizing cells to different DSB inducing agent[21–23]. For this reason, DNA-PK inhibitors start to be used in cancer therapy to treat HR-deficient tumors (*e.g.* in BRCA1/2 mutated patients)[24].

Furthermore, altered level of DNA-PK were find in different types of solid tumors, highlighting how misregulation of NHEJ pathway strongly affects genome stability[25]. In addition, since this mechanism has also a physiological role in the immune system development, mutations in the NHEJ genes predispose to severe immunodeficiency-associated syndromes[26].

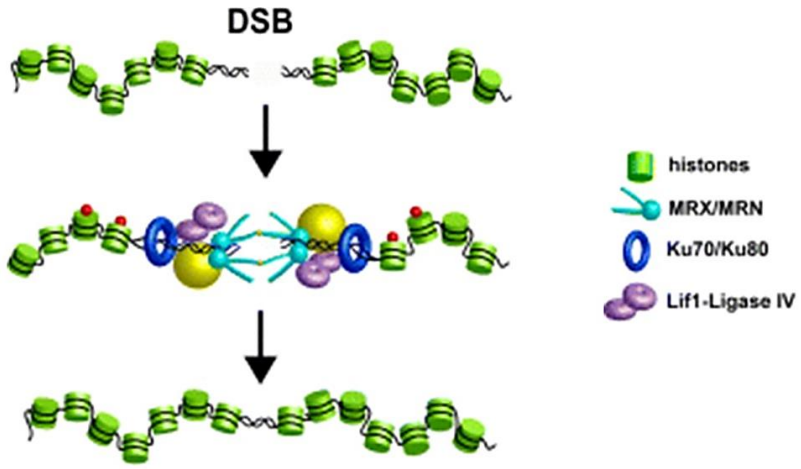


Figure 2. Non-Homologous End Joining repair system[5]

Homologous Recombination

Homologous Recombination (HR) pathway is a more complex repair mechanism compared to NHEJ and involves the activity of several enzymes. Even though is significantly slower, it is a largely more accurate process, due to the use of a homologous template to patch the break. In somatic cells, sister chromatid commonly provides DNA homologous sequence for the repair. However, HR has an essential role also during controlled meiotic diversification. For this reason, homologous recombination is a timely restricted mechanism, occurring only in S and G2 cell cycle phases[27].

The molecular switch that regulates the choice between NHEJ and HR is DSB processing. While end-joining requires a minimal end processing, extensive processing of DNA ends generating long 3'-ssDNA overhangs commits the cells to HR repair pathway[11,28]. This mechanism, generally known as *resection*, consists in the nucleolytic degradation of the 5'-terminated strand in 5'-3' direction from the break site, resulting in the exposure of 3'-OH ssDNA protruding filaments competent for the homology search. Therefore, accordingly with the relevance of its role, resection is strictly controlled preventing HR activation when a repair

template is not available (out from S or G2 phases). This regulation is achieved through the CDK-dependent phosphorylation of key resection factors[28–30].

Resection starts with the nuclease activity of the MRX complex (MRN complex in human). This complex, as already discussed before, is one of the first factors recruited on DNA after damage induction. It is composed of three different subunits: the Mre11 catalytic subunit with endo- and 3'-5'-exonuclease activity; the Rad50 structural subunit, an ATPase with essential role in end tethering and Mre11 activation; finally, Xrs2 subunit, homolog of NBS1 in human cells, the regulatory subunit of the complex[31–34].

Once DSB occurs, the complex rapidly localizes at DSB ends together with its functional partner Sae2 (CtIP in mammalian cells), which stimulates the endonuclease activity of Mre11. This leads to the formation of a nick a hundred bases away from the break. Starting from this point, the MRX complex exonuclease activity shortly processes DBS ends with 3'-5' polarity[35–38]. This first step of resection is essential in presence of modified ends, where the Mre11 nuclease activity is strictly required to clean "dirty" ends, break induced-DNA termini with particular chemical or steric (*i.e.* protein blockage) alterations [39–43].

The Sae2 protein not only regulates the Mre11 nuclease activity, directly stimulating the resection process, but it has a crucial role in controlling the MRX complex turnover at the break site. Indeed, yeast cells deleted for *SAE2* show a toxic accumulation of the MRX complex at the damage loci. The persistence of the complex on DNA negatively influences the proper repair of the lesion, increasing *sae2*Δ mutant sensitivity to DSB inducing agents and causing a misregulation of the checkpoint signaling[44,45]. Interestingly, the same phenotypes are shared by particular Rad50 hypomorphic alleles, collectively known as *rad50s* mutants, that have been proposed to impair the Rad50-Sae2 interaction[46–48]. Recently, two distinct research groups identified specific Mre11 alleles (H37R, H37Y, P110L), which rescue the *sae2*Δ and *rad50s* defects, simply forcing the MRX complex

detachment from DNA, highlighting the importance of a correct timely modulation of the MRX activity[49,50]. However, despite these mutations are known to physical destabilize the DNA binding of the complex, all the chemical and molecular details of the Sae2-dependent regulation of the MRX removal from DSB ends remain still elusive.

After MRX processing, the highly conserved Exo1 and Dna2-Sgs1/BLM/WRN nucleases/helicases remove DNA from the 5'-end extending the ssDNA region[37,51,52].

Simultaneously, many other enzymes cooperate to coordinate and regulate the resection process, including kinases and chromatin remodelers[53,54].

The newly formed ssDNA tails are immediately covered by the RPA complex, preventing the formation of detrimental secondary structures or nucleolytic degradation of the ends[55,56]. Moreover, the ssDNA-RPA intermediate recruits Mec1 (ATR in human cells), the main yeast apical kinase of the DNA damage checkpoint cascade.

As resection is ongoing, RPA is exchanged with the recombinase protein Rad51, creating the nucleoprotein filament competent for homology search and pairing. This step, in yeast, depends upon the mediator protein Rad52[57–59]. In contrast, human RAD52 has no remarkable mediator activity, and this role is carried out by the essential repair protein BRCA2, together with other enzymes. Indeed, cells depleted for BRCA2 exhibit a significant decrease in RAD51 foci formation after IR irradiation[60–62].

Finally, once the presynaptic filament is formed, strand invasion of the homologous sequence takes place, generating a displacement-loop (D-loop). In mammals, this step is directly promoted by the other BRCA-related tumor suppressor BRCA1[63]. After pairing, Rad54 catalyzes the removal of Rad51 from dsDNA in order to stabilize the heteroduplex and finalize the repair[64,65]. Most of the DNA synthesis during HR is performed by DNA polymerases δ and ε[66,67].

Finally, recombination can terminate through three alternative subpathways: Break Induced Replication (BIR), Synthesis Dependent Strand Annealing (SDSA) or canonical HR. In BIR, DNA synthesis proceeds until the end of the template chromosome, resulting in a loss of heterozygosity (LOH)[68]. For this reason, BIR is used only in particular conditions, when there are no other possibilities to fill the break. Instead, the balance between SDSA and canonical HR depend upon D-loop stability. Indeed, in SDSA, after a short repair synthesis, the invading DNA strand is displaced and re-anneals with the complementary broken end to terminate the repair, with no crossover product. In contrast, canonical HR is favored by D-loop stabilization. In this condition, also the second damaged strand pairs with the displaced ssDNA from the D-loop, forming a double Holliday Junction (dHJ) structure. Finally, different enzymes, generating respectively crossover or non-crossover outcomes, can process this intermediate[69].

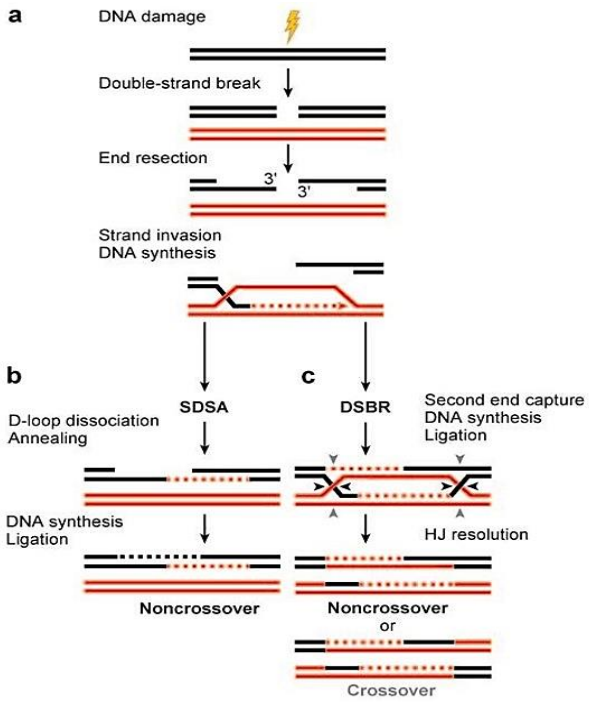


Figure 3. Synthesis Dependent Strand Annealing and canonical HR repair pathways[70]

Microhomology-Mediated End Joining

The Microhomology(MH)-Mediated End Joining (MMEJ) repair system is an alternative end joining mechanism, concerning the annealing of a short homologous sequence (from 5 to 25 bp) during the pairing of broken ends. It has been initially discovered as a backup repair system in cells deficient for NHEJ[71–74]. Recently, several studies highlight a substantial contribution of MMEJ in the physiological response to DNA damage, in particular during immune system development[75–77].

However, MMEJ repair mechanism is an extremely mutagenic event. Indeed, the nucleolytic cleavage of DNA flaps flanking the MH always results in nucleotide deletions. Given this high mutation rate and its strong association with other complex DNA aberrations, MMEJ contributes to the oncogenic accumulation of chromosome rearrangements in humans. Indeed, microhomologies are frequently found at the level of recurrent chromosome translocation in several types of hematological malignancies and human carcinomas[78–82]. According to this, in the last few years, studying the molecular mechanism of MMEJ acquires a great interest in cancer and immune research.

MMEJ shares the initial step of end processing with the homology directed repair systems. For this reason, as for HR, it has a predominant role out from the G1-phase, where the resection process is strongly inhibited, and it is favored in presence of modified ends. The proper exposition of the microhomology is totally dependent upon the Mre11 nuclease activity of the MRX complex and it is significantly favored by the Sae2 resection regulator and by the checkpoint apical kinase Tel1[72,83].

Following the short end resection, microhomology anneals to form an intermediate structure presenting 3'-ssDNA flaps on both the two side of the HM pairing. Despite all the details of the MH annealing mechanism still remain partially unknown, it is reported that high GC content in the homologous sequence facilitates the anneal, stimulating the MMEJ *in vitro*; conversely, nucleotide mispairing inside the MHs

negatively regulates the efficiency of the process[84].

Once the MH intermediate is formed, the Rad1-Rad10 endonuclease complex, and its human counterpart XPF-ERCC1, trims the overhanging ssDNA tails, giving the opportunity to the Pol4 and Polδ DNA polymerases to extend the damaged DNA and fill the break[72,83,85]. Finally, the Ligase I and Ligase IV enzymes (Ligase I and Ligase III in human cells) achieve the ligation step of the DNA joints[72,83,86].

The DNA Damage Checkpoint

Together with the activation of a specific repair system, the other essential branch of DDR is the DNA Damage Checkpoint (DDC) pathway. It may be considered as a cellular alarm that constantly monitors chromosome integrity and, in presence of DNA damage, arrests cell cycle progression providing the required time to repair the lesion[87].

As for many repair factors, depletion of checkpoint genes strongly increases genome instability and cancer predisposition, highlighting its crucial role in tumor suppression[88,89].

In yeast, there are three main DNA damage checkpoints, according to the cell cycle phases. Indeed, the G1/S checkpoint monitors chromosome integrity before DNA replication; the intra-S checkpoint slows down S-phase ensuring faithful genome replication; finally, the G2/M checkpoint prevents segregation of damaged chromosomes[90].

All these checkpoint pathways consist of highly conserved signal transduction cascades, characterized by several phosphorylation events, which amplify and relay the damage signal from local sensor proteins to downstream effectors[91]. Indeed, apical kinases are able to recognize specific structure at the damaged loci and activate the pathway. In yeast, Mec1 and Tel1 are the two main apical kinases, belonging to the PIKK kinase family and corresponding to ATR and ATM, respectively, in human cells[92].

Tel1 is recruited at the break site through a direct interaction with the Xrs2 subunit of the MRX complex, where it phosphorylates the histone H2A on serine 129[93–95]. Even though Tel1 plays a crucial role in telomere maintenance, it has only a marginal function in DDR and its depletion does not sensitize cell to genotoxic agents. This is not true for its human counterpart ATM that has, on the contrary, a predominant role.

As already anticipated, Mec1 is the main responsible for checkpoint activation in yeast cells. This essential sensor protein, together with its functional partner Ddc2, detects ssDNA-RPA stretches derived from almost all type of lesion processing[96]. However, full activation of Mec1 apical kinase depends on a series of positive feedback loops, coordinated by several other checkpoint factors. Indeed, once recruited at the break site, Mec1 binds and phosphorylates the Ddc1 subunit of the PCNA-like 9-1-1 complex (heterotrimeric complex, together with Mec3 and Rad17). This complex, deposited at the dsDNA-ssDNA junction, strongly stimulates the Mec1 kinase activity[97,98]. Moreover, Mec1-dependent Ddc1 phosphorylation facilitates recruitment on DNA of Dpb11 that interacts with Mec1 further increasing its activation[99,100]. In addition, recently was reported that also the Dna2 resection nuclease positively influences Mec1 functionality[101]. Once Mec1 is fully active, it phosphorylates the histone H2A and Dpb11 itself, recruiting adaptor proteins to amplify the signal[94,95,102].

The most important adaptor in *S. cerevisiae* is Rad9. Besides binding to the histone H2A and Dbp11, Rad9 is recruited near the lesion also through a constitutive interaction with methylated histone H3[103–107]. After a robust Mec1-dependent phosphorylation, Rad9 oligomerizes creating a platform for the loading of the effector kinase Rad53[108–111]. The same mechanism of action is conserved in mammals for 53BP1, the functional homolog of Rad9: it is highly phosphorylated by ATM and this modification is required for its essential role in both checkpoint signaling and repair process[112–115].

As Rad9, also Rad53 undergoes to multiple Mec1-dependent phosphorylations (CHK2 and ATR, respectively in human cells) that stimulates its ability of auto-trans-phosphorylation[116]. Due to the linear correlation between phosphorylation level and kinase activity, Rad53 is used as a principal marker for checkpoint activation. A second effector kinase, Chk1/CHK1, in yeast plays a role more restricted to G2/M phases[117].

Finally, once Rad53 or Chk1 are fully active, they leave Rad9 platform determining the phosphorylation of the final targets, depending on the cell cycle stages.

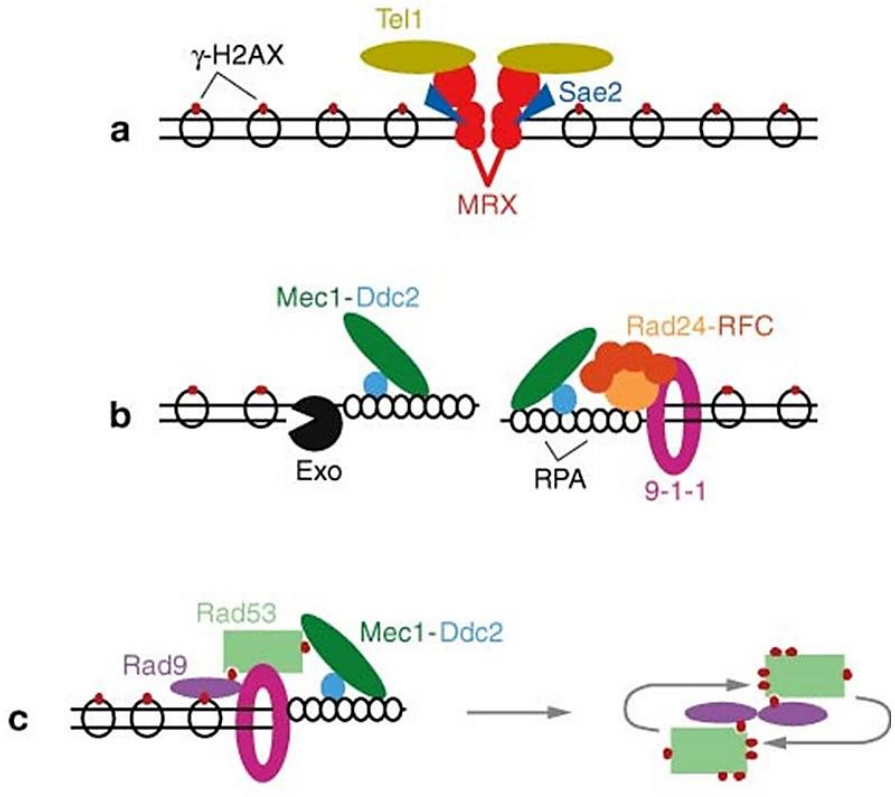


Figure 4. The DNA Damage Checkpoint cascade[91]

Ubiquitination and SUMOlyation in DSB response

Despite phosphorylation being the most abundant protein modification in DSB response, several studies uncovered the importance of other types of post-translational modifications (PTMs) in the regulation of DNA damage signaling. In particular, recent evidence highlighted an essential role for ubiquitination and SUMOylation in controlling many aspects of the cellular response to double-strand breaks[118,119]. Indeed, a high-throughput proteomic analysis revealed a global damage-induced ubiquitination and SUMOylation[120–122].

Different from all the other PTMs, both ubiquitination and SUMOylation consists of a three-step modification mechanism, carried out by the activating (E1), conjugating (E2) and ligase (E3) enzymes. Ubiquitin can be attached to substrates as a monomer or as polyubiquitin chains, conferring different structural properties and function to this modification. Indeed, while the K48-linked ubiquitin chain is a well established degradative signal, both mono- or K63-linked poly-ubiquitination are involved in regulation of many cellular processes. In contrast, only one type of SUMO chain seems to be predominant[123,124].

Many key factors of the DSB repair machinery, including the histones, that are recruited to the region surrounding the lesion, were found to be ubiquitylated or SUMOylated after damage induction in yeast and mammalian cells.

MRX/MRN complex

Proteomic studies in yeast reported a global 'SUMO response' that accelerates DSB repair in a MRX-dependent manner[120,122]. Consistently, the Mre11 subunit of the complex is SUMOylated, as it strongly interacts with both Ubc9 (E2) and Siz2 (E3) enzymes. This modification seems to be essential for correct complex assembly[122,125].

In human cells, several potential ubiquitination sites are identified in all the three subunits of the MRN complex[121,126,127]. Until now, only NBS1 was confirmed to be effectively ubiquitinated *in vivo*, promoting the recruitment of the complex at the

break site and the correct activation of HR repair system and checkpoint[128,129].

Sae2/CtIP and Ku complex

Both Sae2 and its human homolog CtIP are SUMOylated and this PTM is critical for their function in end processing[130].

Moreover, CtIP is also ubiquitinated in response to DSB by both BRCA1 and RNF138 promoting resection and homologous recombination[131,132]. Along with the CtIP ubiquitination, the evidence that RNF138 also targets Ku80 for degradation strongly supports a pro-resection function for this E3 enzyme[133]. In addition, Ku80 degradation is also promotes by the RNF8-dependent ubiquitination[134].

Exo1/EXO1, Dna2/DNA2 and Sgs1/BLM

In humans, EXO1 and BLM functions in DNA damage response are regulated by both ubiquitination and SUMOylation[135–138]. Moreover, also in *S.cerevisiae* Sgs1 recruitment and activity at the break site is controlled by the SUMO-targeted ubiquitin ligase complex Slx5-Slx8[139].

Finally, more than 20 ubiquitination sites for DNA2 were identified, but no one of these have been yet characterized[126,127].

Histones H1 and H2A

In human cells, another essential ubiquitin-dependent point of regulation of DSB response consists in H1 and H2A histone ubiquitination, carried out by the two E3 ligase enzyme RNF8 and RNF168, respectively. These modifications are strictly required for the correct recruitment of many downstream repair factors. Indeed, upon DSB induction, RNF8 is recruited at the damaged site through the recognition of the ATM-dependent phosphorylation on MDC1[140]. Once near the break, RNF8 ubiquitinates the linker histone H1, leading to the successive recruitment of RNF168 that ubiquitinates the histone H2A[141]. This modification provides an

important platform for the recruitment of several others E3 enzymes and many repair proteins, such as 53BP1 and BRCA1, leading to a correct orchestration of the DNA damage signaling[140,142–145].

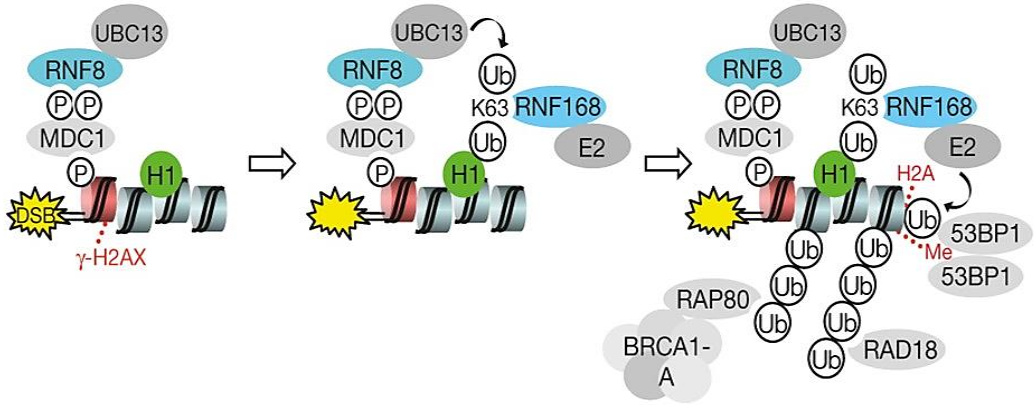


Figure 5. RNF8 and RNF168 dependent ubiquitination of histones in response to damage induction[141]

DNA-protein crosslink

A high number of interactions between proteins and DNA are physiologically required to carry out all the essential processes involved in DNA metabolism, from replication and transcription to DNA repair. Normally, these interactions are tightly regulated and highly dynamic. However, in particular conditions, a protein transiently binding to DNA can be trapped, through the formation of a stable covalent crosslink.

DNA-Protein Crosslinks (DPCs) are extremely cytotoxic lesions, which may significantly alter cell viability. Indeed, they are large adducts that constitute a deleterious steric blockage for all the chromatin-based processes, globally interfering with normal DNA metabolism.

DPCs are classified into two categories, enzymatic or non-enzymatic DPCs, based

on the origin of the DNA-protein adduct[146]. Interestingly, many of the chemotherapeutic agents actually used in clinic generate both enzymatic and non-enzymatic DPCs[8,147].

Enzymatic DPCs arise when transient covalent DNA-enzyme complexes, generated as a reaction intermediates, remain trapped to DNA. In both yeast and human cells, the most common of these is the crosslink of the Topoisomerase I (Top1) protein upon inhibitor treatment[148]. Another interesting mechanism for enzymatic DPC formation derives from BER-dependent oxidative damage repair. Indeed, some oxidative DNA damage inducing agents generate oxidized abasic sites characterized by a 2-deoxyribonolactone chemical modification at the 3'-termini. Removal of this particular modification specifically requires the lyase activity of DNA Polymerase β, which remains crosslinked to DNA end[149–151].

Non-enzymatic DPCs are formed after a non-specific DNA crosslink of chromatin-binding proteins, caused by either endogenous metabolites or exogenous agents. For instance, reactive aldehyde species are normally released in the cell as a result of various metabolic processes[152,153]. One of the most cytotoxic and studied DPC-inducing aldehyde is formaldehyde, derived from histone and DNA demethylation[154–156]. For its high capability to generate DPCs, formaldehyde is commonly used as a crosslinking agent in many experimental procedures. On the other hand, several exogenous chemicals can cause DPC accumulation. Platinum derivatives, such as cisplatin and carboplatin, as well as several other chemotherapeutics (*i.e.* PARP inhibitors), stabilize DNA-protein interactions, covalently trapping the enzyme on DNA[147,157].

Repair of DPCs may consist of nucleolytic cleavage of DNA, proteolytic degradation of the trapped enzyme or direct hydrolysis of the covalent bond, depending on the type and the origin of the adduct.

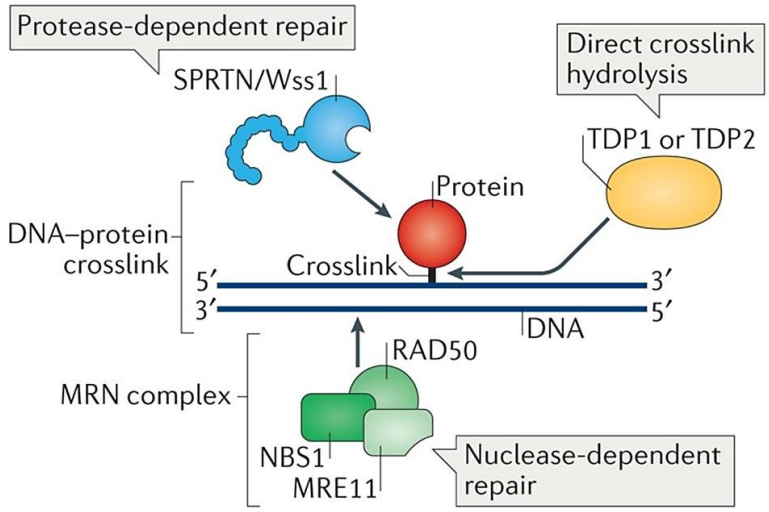


Figure 6. Repair mechanisms for DPCs[248]

Nuclease-dependent DPC repair: the MRX complex

The MRX complex (MRN in human cells) is an evolutionary conserved DPC repair system. Indeed, T4 bacteriophages use their MRX counterpart to resolve Topoisomerase II protein adducts[158,159]. Similar processes occur also in *Escherichia coli*, where the SbcCD complex, the homolog of the MRX complex, is involved in the removal of protein permanently trapped on DNA[160]. In eukaryotic cells, from yeast to mammals, the same repair strategy is activated in the presences of DNA-protein crosslinks[161–165].

The endonuclease activity of the Mre11 subunit of the complex is strictly required for its function in DPCs removal. Hence, it is already reported that either 5'- or 3'-protein adducts directly stimulate the Mre11 endonucleolytic cleavage *in vivo* and the Tel1 activity[38,165,166]. The incision can occur equally on the DPC-containing DNA strand or on the complementary one. The primary cut is followed by a 3'-5' resection event until the removal of the adduct and by a second cut on the opposite strand, generating a blunt DSB ends. Finally, the break can be repaired through the

canonical repair mechanisms. It is interestingly to note that the cleavage by MRX is entirely dependent on the presence of the blocked protein, but not affected by the identity and the origin of this adduct[38,167].

Protease-dependent DPC repair: Wss1/SPRTN protease and proteasome

Recent evidence highlight proteolytic repair of DPCs as a crucial mechanism for maintenance of genome stability[168]. In particular, mutations in the SPRTN protease dramatically increase cancer predisposition in mouse and human[169,170].

The Wss1/SPRTN family of proteases is a highly versatile system that can target almost every type of protein crosslink. Indeed, Wss1 is involved in the resolution of both enzymatic and non-enzymatic DPCs and is essential for replication machinery progression in presence of DNA-protein adducts[171]. DPC proteolysis ends up with an almost complete protein degradation, with the exception of a small peptide fragment that remains anchored to DNA. However, switching to a translesion synthesis (TLS) polymerases that can bypass the peptide presence easily restores replication progression[171]. Finally, the remnant is supposed to be excised by the nucleotide excision repair (NER) system, but, until now, there are no direct evidence for this mechanism.

In addition to this proteolytic mechanism, many DPCs are resolved by the proteasome-dependent degradation of the protein portion. Indeed, for instance, both Top1 and Polβ adducts are ubiquitinated and targeted by the proteasome[149,172]. However, even though many reports showed that proteasome inhibition strongly increases cell sensitivity to DPC inducing agents, the precise mechanism of DPC protein degradation remains still unclear.

Repair by direct crosslink hydrolysis: Tdp1

Direct hydrolysis of the DNA-protein crosslink is not an easy strategy to remove DPCs accumulation. Indeed, the chemical nature of the bond drastically differs

depending on the crosslinked molecule and the specific aminoacidic residue. However, some kinds of DPCs are so frequent to require specialized enzymes to quickly and easily remove such lesions.

The tyrosyl-DNA-phosphodiesterase 1 (Tdp1) enzyme hydrolyzes the covalent bond between the Top1 active site tyrosine residue and the 3'-end of a SSB[173]. Tdp1 orthologues are present in all eukaryotic cells and germline mutation in TDP1 human gene causes a rare and severe neuropathic syndrome[174].

Upon hydrolysis of the Top1-DNA bond, Tdp1 produces a 3' phosphate end that has to be further processed before gap filling. Thus, the bifunctional polynucleotide kinase 3´-phosphatase (PNKP) is recruited to DNA to remove the 3' phosphate and, at the same time, phosphorylate the 5' hydroxyl group[175]. At this point, the canonical SSB repair machinery is able to seal the nick.

Similarly to the Tdp1 mechanism, a Tdp2 enzyme is required to specifically remove the Topoisomerase II (Top2) protein adducts[173].

Dma1 and Dma2

Protein structure

In eukaryotic cells, there are two main families of E3 ubiquitin ligase enzymes, depending on the type of the catalytic domain. The first one contains the HECT (Homologous to the E6AP Carboxyl Terminus) domain, formed by two flexibly tethered lobes (N- and C-lobes). The N-lobe contain the E2 docking site, while the C-lobe presents the active-site cysteine[176]. The second class of enzymes is characterized by the presence of a RING (Really Interesting New Gene) domain. This domain contains a specific sequence, formed by many conserved cysteine and histidine residues (Cys-X_2-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys-X_2-Cys-$_{(4-48)}$-Cys-X_2-Cys; X represents any type of aminoacid) that bind two zinc atoms[177]. These two type of domains mainly differ in the mechanism of ubiquitin conjugation. Indeed, RING E3s catalyze the direct transfer of ubiquitin from the E2 enzyme to

the substrate. On the contrary, HECT family required an intermediate step in which the ubiquitin chain is first transferred from the E2 to the cysteine in the active-site of the E3 enzyme and then conjugated to the target[176].

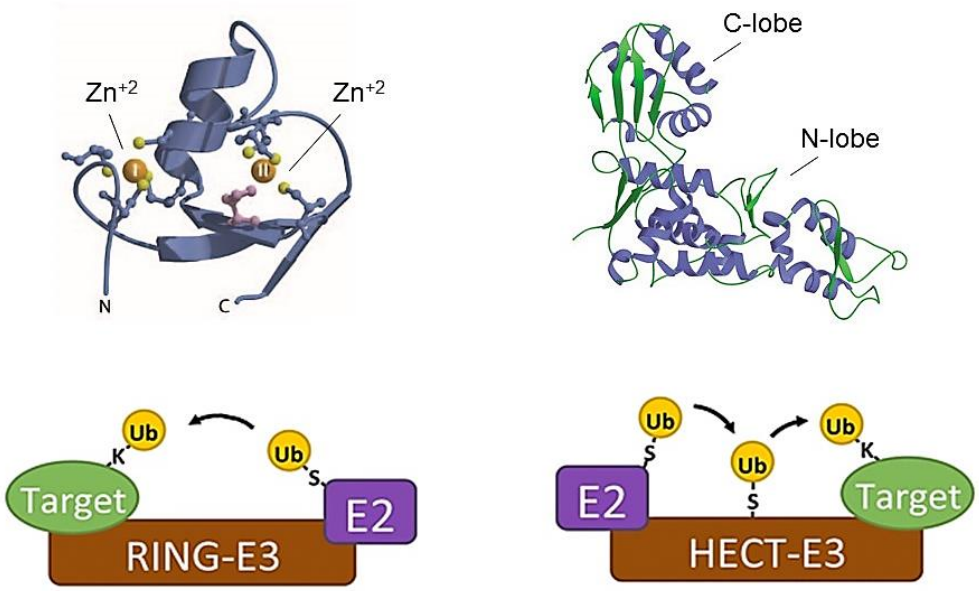


Figure 7. Catalytic domains and molecular mechanisms of the two RING and HECT families of E3 ubiquitin ligase enzymes[176,177]

Dma1 and Dma2 are two members of the *S.cerevisiae* RING family of E3 ubiquitin ligase enzymes. These proteins are the only two in budding yeast characterized by harboring both a C-terminal RING domain and a Fork-Head Associated (FHA) domain[178]. The FHA domain is a phosphothreonine-binding module present in thousands of proteins involved in DNA repair and checkpoint signaling[179].

Phylogenetic analysis revealed that the FHA-RING proteins are a conserved family of enzymes. Dma1 and Dma2 paralogs have been identified in the fission yeast *S. pombe* and in human cells (Dma1 and RNF8 and CHFR, respectively)[178]. However, the structural homology between yeast and human proteins may not reflect also a functional similarity. Indeed, Dma1 and Dma2 have been shown to be principally

involved in regulation of mitosis, while their human homologs RNF8 and CHFR mainly play roles in the DNA damage response.

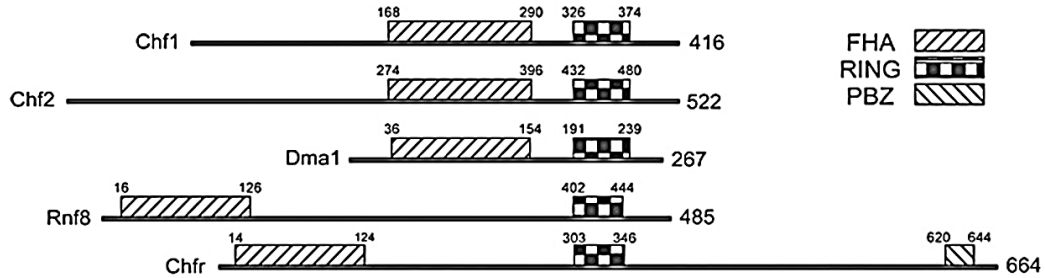


Figure 8. Domain localization of the yeast and human FHA-RING ligases[178]

Mitotic functions

As already anticipated, Dma1 and Dma2 functions are mainly implicated in the regulation of several key steps of mitosis. Indeed, several studies reported redundant roles for these proteins in controlling septin organization, spindle positioning, formin distribution, vacuole inheritance and, finally, cytokinesis[180–184]. However, in many cases, the molecular mechanism of Dma1 and Dma2 action remains yet to be clarified.

Role of Dma1 and Dma2 in the morphogenesis checkpoint

In budding yeast, mitotic entry is strictly controlled by the morphogenesis checkpoint. This pathway monitors cell dimension, bud formation and the correct actin cytoskeleton organization and septin ring deposition[185–188]. The activation of the checkpoint in presence of morphogenetic perturbations leads to a delay in the G2/M transition, preventing cell division in unfit conditions. In *Saccharomyces cerevisiae*, the whole pathway depends on the temporal regulation of Swe1 a protein kinase that inhibits the Cdc28 CDK[189–191]. Indeed, in unperturbed condition, Swe1 starts to accumulate in late G1 and S phases and it localizes at the budneck through

the interaction with its regulators Hsl1, Hsl7, Cla4 and the polo kinase Cdc5[192–195]. Here, it is hyperphosphorylated by some of these proteins and this modification leads to its subsequent degradation in G2 phase[196]. Therefore, Cdc28 is fully active and the cell can proceed to mitosis.

Dma1 and Dma2 have been identified as the responsible for the proteasome targeting of Swe1. Indeed, in Dma lacking cells Swe1 is not correctly ubiquitinated and this, under replicative stress conditions, causes the cell cycle arrest before mitotic entry[197].

Interestingly, morphogenesis checkpoint activation is mediated not only by the presence of altered morphogenetic stimuli, but also in response to replicative stress. Indeed, even though the molecular mechanism is still unknown, Swe1 degradation is regulated by the Rad53 effector kinase[198,199].

Role of Dma1 and Dma2 in septin dynamics and spindle positioning checkpoint

In *S. cerevisiae*, septins play an essential role in the bud site selection and growth, correct spindle positioning and cytokinesis. They localize early in G1 at the incipient bud site and, in G1/S phases, before the bud starts to grow, they form a ring structure around the bud neck until the cytokinesis is completed[200,201]. Moreover, the septin ring acts as a platform for the recruitment of many other mitotic factors at the bud neck[202].

Dma1 and Dma2 are directly implicated in the correct septin ring assembly and deposition. Indeed, cells lacking Dma activity show severe defects in ring stabilization and a genetic interaction with mutations in the gene coding for the Cla4 protein, essential in septin structure formation[180]. The Dma-dependent regulation of septin ring dynamics occurs through the control of the Elm1 protein localization at the bud neck, which is required for septin phosphorylation, and by the direct ubiquitination of the two septins Shs1 and Cdc11[182,203].

Moreover, the Elm1 misregulation in absence of Dma proteins not only causes

defects in septin stabilization, but also in activation of the spindle positioning checkpoint (SPOC). Indeed, in Dma1 and Dma2 depleted cells, Elm1 is not correctly localized, leading to the lack of Kin4 phosphorylation[203]. Therefore, upon alterations in the spindle positioning, cells fail to prevent the activation of the Mitotic Exit Network (MEN) and undergo aberrant cell divisions.

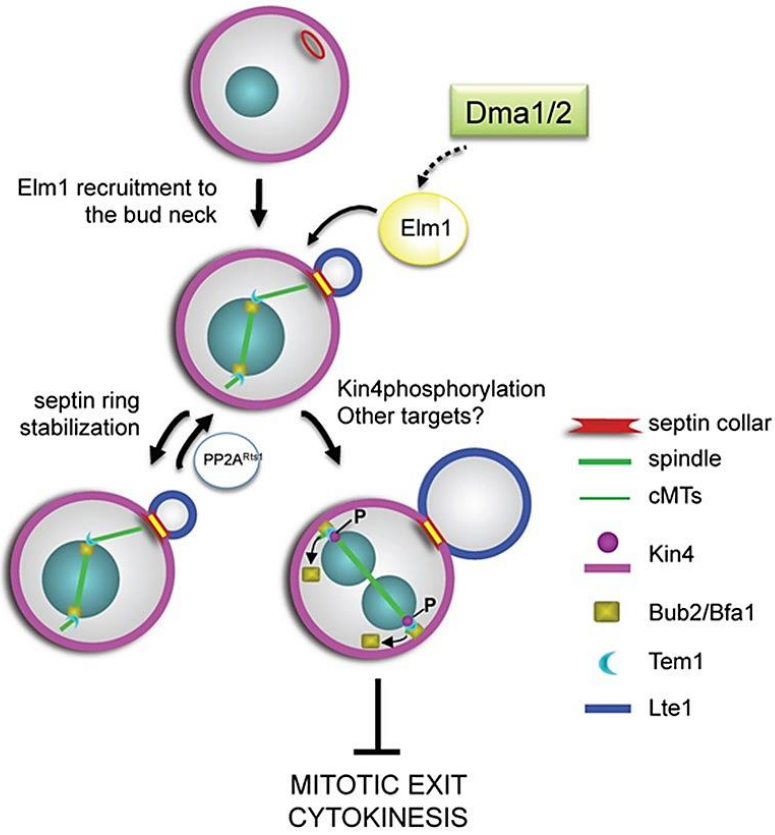


Figure 9. Dma1 and Dma2 role in septin ring stabilization and SPOC activation[203]

Role of Dma1 and Dma2 in formin distribution

Polarization is a critical function for all cells. Indeed, it is required for correct cell proliferation and differentiation, and alterations in the polarized pattern growth increase predisposition to developmental disorders and tumorigenesis[204].

Cell polarity depends upon actin cytoskeleton organization. Therefore, actin

network is a tightly regulated process, driven by several protein regulators, among which the Rho GTPase family, formins and the polarisome complex have a pivotal role[205–208]. All these factors work together to coordinate the spatial and temporal assembly and remodeling of the actin cables.

In particular, formins are the universal actin-polymerizing proteins that are able to catalyze the actin filament formation. The localization and the activity of these proteins are directly controlled by the Rho GTPases[209]. In *S. cerevisiae*, there are two formins, Bni1 and Bnr1, which assemble different sets of actin cables[210]. Indeed, Bni1 localizes at the budtip throughout the cell cycle until mitotic exit where it orchestrates the actin polarization toward the bud cortex. On the contrary, Bnr1 is recruited at the budneck through the interaction with septins and is responsible for the assembly of actin filaments toward the budneck[211,212]. Dma1 and Dma2 directly interact with both the two formins, controlling their abundance and polarized distribution. Indeed, in absence of Dma proteins, cells show a decrease in Bnr1 protein levels and a Bni1 mislocalization, maybe due to problems in its membrane anchorage, that reflect severe actin defects[181]. However, albeit the E3 activity is strictly required, how molecularly Dma1/2 act on formins still remains an open question.

Role of Dma1 and Dma2 in cytokinesis

In budding yeast, two interconnected and partially redundant pathways regulate cytokinesis completion[213]. The first mechanism consists in the formation of a functional actomyosin ring (AMR) at the level of budneck that contracts in a symmetric manner[214,215]. The construction of this structure is timely regulated by the MEN network and requires the coordinated action of several factors, such as Tem1, Iqg1, Bni1, Myo1 and actin[216,217]. Along with the AMR assembly, there is the deposition of the primary septum (PS), made by the Chs2, Cyk3, Hof1 and Inn1, that definitely separates mother and daughter cells[218–220].

Dma proteins participate in the control of both the two pathways. Indeed, upon Dma2 overexpression, cells show defects in AMR contraction[183]. However, no direct ubiquitination of these proteins by the Dma enzymes have been found and the mechanism remain still poorly understood. Nevertheless, in this context, a very recent study had better clarify the contribution of Dma proteins in this process. Indeed, Dma1 and Dma2 seem to be involved in the regulation of MEN's factor localization at the Spindle Pole Bodies (SPBs) during cytokinesis, through their direct ubiquitination of the SPB scaffold protein Nud1[249]. Finally, Dma1 and Dma2 interfere with Chs2 and Cyk3 PS deposition, leading to an asymmetric septum formation[183].

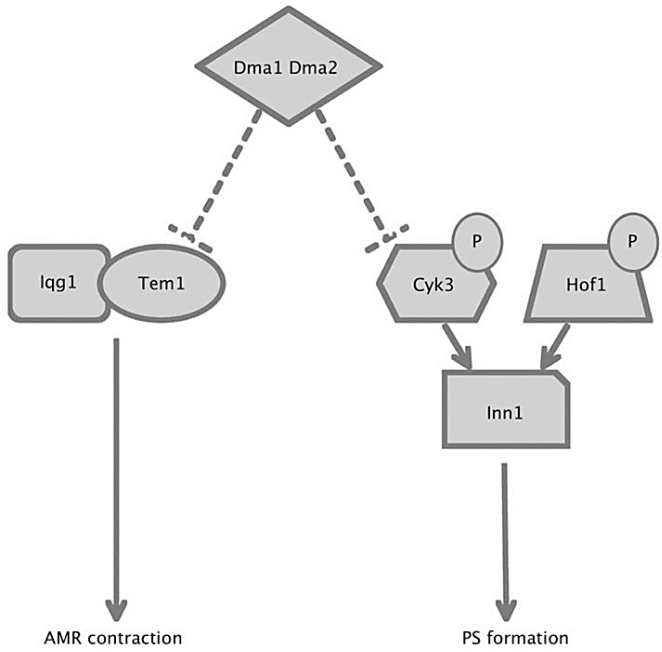


Figure 10. Dma1 and Dma2 control cytokinesis[183]

Other functions

In addition to their multiple roles in mitosis, a recent study identified Dma1 and Dma2 as regulative factors in the licensing-to-firing transition during replication[221].

Indeed, evidence in budding yeast supports a model in which the canonical CDK-dependent mechanism for licensing and firing is supported by a physical separation of the two phases[222–227]. Indeed, Dma proteins were found to control the G2/M temporal gap through the early ubiquitin-dependent degradation of the key firing factor Sld2[221].

Finally, Dma1 and Dma2 were also found to interact with the Sae2 repair machinery component. Indeed, a large-scale proteomic analysis reveals a FHA-mediated interaction between Dma1/2 and the phosphorylated Sae2[228]. However, a direct role for Dma in DNA damage response remains still elusive.

Functions of RNF8 and CHFR human homologs

The RNF8 and CHFR human homologs of Dma1 and Dma2 are mainly involved in the control of DNA damage response and genome integrity maintenance. Indeed, as already discussed above (see Ubiquitination and SUMOylation in DSB response), RNF8 plays an essential role in the activation of the damage signaling through the initial H1 histone ubiquitination[141]. This chromatin modification surrounding the DNA lesion leads to the consequent correct recruitment of several repair and checkpoint factors.

Moreover, in addition to histone H1, RNF8 directly targets two other essential repair proteins, the NBS1 subunit of the MRX complex and the NHEJ key player KU80[129,134]. Indeed, the RNF8-dependent ubiquitination of the MRN complex is strictly required for its binding at the damaged site and RNF8 depleted cells show severe defects in the Mre11 foci formation after damage induction[129]. On the contrary, the KU80 ubiquitination mediates its displacement from DBS ends through a proteasome degradation of this factor[134].

Together with RNF8, also CHFR regulates the first wave of ubiquitination in response to DNA damage. Indeed, RNF8 and CHFR are synergistically involved in chromatin modification (histone ubiquitination but also acetylation) at the damaged

loci[229]. Moreover, CHFR ubiquitinates the poly(ADP-ribose) polymerase 1 (PARP1), an essential enzyme in ssDNA repair[230].

However, even though they have predominant roles in the DNA damage response, RNF8 and CHFR also conserve some mitotic functions. In particular, as Dma1 and Dma2 in *S. cerevisiae*, RNF8 localizes at centrosomes and at the division site where it ubiquitinates septins[182]. Finally, CHFR is known to be implicated in the activation of stress induced-mitotic checkpoint[231–233].

Aim of the Project

Maintenance of genome stability is a tightly regulated mechanism, concerning the spatial and temporal coordination of several essential cellular processes. Indeed, accumulation of unrepaired lesions might cause toxic alteration either in the structure or in the sequence of the DNA molecule. In higher eukaryotes, all these genomic rearrangements strongly increase predisposition to cancer transformation and the loss of specific repair factors is at the base of severe genetic diseases.

For this reason, a detailed molecular characterization of the DNA damage response (DDR) pathway is an essential goal for biomedical research. Several groups in the last decades focused their attention on studying these mechanisms and a high number of the key factors involved in DDR have been identified. Nevertheless, many aspects of DDR regulation need to be further investigated.

In this view, the aim of my project was to clarify a new molecular role of the two yeast ubiquitin ligases Dma1 and Dma2 in controlling double-strand break response. Indeed, I identified these E3 enzymes as new players in the coordination of both repair systems and checkpoint activation.

Results & Conclusions

Identification of Dma1 and Dma2 as novel factors in the double-strand break response.

Double-strand breaks (DSBs) are considered one of the most cytotoxic DNA lesion. Indeed, as already discussed in the State of the Art paragraph, the presence of even a single unrepaired DSB can lead to the formation of gross chromosome rearrangements and cell death. The accumulation of these detrimental genomic alterations drastically increases the probability to develop cancer diseases in high eukaryotes. For this reason, in the recent past, the molecular mechanisms of DSB response have been intensively studied by several research groups and most of the key factors involved are now well characterized. However, several regulators remain still unknown.

In this view, I decided to look for new players involved in DSB processing, using *Saccharomyces cerevisiae* as a model organism. I started screening the sensitivity of several mutant yeast strains to bleocin, a drug commonly used in chemotherapy for its ability to kill cancer cells generating DSBs randomly in the genome.

I found that simultaneous deletion of the *DMA1* and *DMA2* genes drastically decrease cell viability after either a chronic or an acute treatment with increasing doses of bleocin (Figure 1, A and B). *DMA1* and *DMA2* genes encode for the two FHA-RING ubiquitin ligase enzymes identified as the yeast orthologues of the human RNF8 and CHFR factors[178]. Despite the fact that some roles of the human counterparts in DSB response have already been reported, I decided to further investigate the possible function of Dma1 and Dma2 in DNA damage response regulation in order to find new conserved roles also for RNF8 or CHFR.

Interestingly, aside from bleocin, we did not observe any significant sensitivity of

the *dma1*Δ*dma2*Δ mutant to any other of the DNA-damage inducing agents we tested (camptothecin and UV irradiation, Figure 1, C and D, respectively). *dma1*Δ*dma2*Δ mutant were also found to be sensitive to hydroxyurea (Figure 1E), as previously described[180,197].

The hydroxyurea (HU) sensitivity of Dma lacking cells is linked to their function in the mitotic metabolism. Indeed, Dma1 and Dma2 had been implicated in the regulation of spindle positioning, septin organization, formin distribution and cytokinesis[180–183,197,203]. In particular, Merlini et al. demonstrated that Dma proteins directly control localization of Elm1 at the bud neck and that its artificial recruitment through a *Bni4-Elm1*Δ*420* chimera rescues the HU sensitivity and bypasses other mitotic defects of *dma1*Δ*dma2*Δ cells[203,234]. I used the same chimeric protein in *dma1*Δ*dma2*Δ background to verify whether the bleocin sensitivity correlates to the Dma mitotic roles. While the chimera rescues the HU sensitivity, similarly to what was reported by Merlini et al., it does not ameliorate bleocin-induced cell lethality (Supplementary Figure 1).

Altogether, these data strongly suggest a possible involvement of Dma1 and Dma2 in the coordination of DSB response.

The role of Dma1 and Dma2 in DSB response requires their E3 ubiquitin ligase activity.

Dma1 and Dma2 are two E3 enzymes belonging to the RING family of ubiquitin ligases. Indeed, they contain a high conserved RING catalytic domain, requires for target specificity and ubiquitin transfer[178].

Since all the reported Dma functions depend on their E3 activity, I tested whether a fully functional RING domain is essential also for the Dma-dependent DSB tolerance. To do this I created two catalytic dead genomic mutants for Dma1 and Dma2 carrying two point mutations inside their RING domains (*dma1*$^{C345S;H350A}$*dma2*Δ and *dma1*Δ*dma2*$^{C451S;H456A}$, respectively). These mutations

had already been shown by different groups to totally abrogate the Dma1 and Dma2 ubiquitin ligase activity[182,235,236]. As shown in Figure 2, the inactivation of both Dma1 and Dma2 RING domains clearly sensitized cell to bleocin treatment similarly to the complete gene deletions. In light of this result, I concluded that the ubiquitin ligase activity of Dma1 and Dma2 is strictly required for their role in the DNA damage response.

The lack of Dma proteins causes severe defects in DBS repair.

In order to investigate how Dma1 and Dma2 affect DSBs repair, first, I tried to verify whether the decrease in cell viability after bleocin treatment correlates with repair defects in cells deleted for *DMA1* and *DMA2* genes.

To evaluate the efficiency of repair in wild type and *dma1*Δ*dma2*Δ cells, I started taking advantage of a particular genetic background (YMV80), in which a single DSB can be generated in a specific site of the chromosome III by the galactose-dependent expression of the HO nuclease. Repair of this lesion mainly occurs through the Single Strand Annealing (SSA) recombination pathway, using two homologous *leu2* sequences flanking the HO cut site, and it can be easily followed by Southern blot analysis[237]. Surprisingly, the lack of Dma1 and Dma2 activity does not sensitize cells to the presence of a single DSB and the repair of this lesion proceeds as control cells (Figure 3, A and B). This result probably derives from the blunt nature of the break caused by the enzymatic cleavage of the HO nuclease.

As a consequence, for my repair analysis I then decided to utilize Pulse-Field Gel Electrophoresis (PFGE) to follow chromosome repair for several hours after short bleocin treatment. This particular DNA electrophoresis permits to separate long DNA molecules (more than 30 kilobases), and to visualize all the entire yeast chromosomes[238].

Figure 4 shows that cells lacking Dma proteins fail to repair DSBs and exhibit a

persistence of broken chromosomes, even 28h after break induction. On the other hand, wild type cells are in a clearly advanced state of repair at 14 hours after the bleocin pulse. These results indicate that Dma factors are essential to promote repair of bleocin-induced DSBs.

To exclude the possibility that the bleocin sensitivity of *dma1*Δ*dma2*Δ cells may be due to a higher permeability of the drug, leading to the formation of a higher number of DSBs, I titrated bleocin concentration in order to determine the minimum amount of drug that induces chromosome fragmentation in wild type and double mutant cells (Supplementary Figure 2). As a positive control I used the *mnn10*Δ mutant (Supplementary Figure 3), which is known to import more bleocin than wild type cells, due to severe defects in cell wall formation[239,240]. Comparing the profile line obtained through quantification of each lane, it is fairly clear that the amount of DNA DSBs caused by low levels of bleocin in wild type and *dma1*Δ*dma2*Δ cells is the same. On the other hand, the equal amounts of bleocin have a much greater impact on chromosomes fragmentation in the *mnn10*Δ positive control. This result definitely supports the idea that Dma1 and Dma2 play a critical role in DSB repair.

In eukaryotic cells two main pathways are responsible for repairing double-strand breaks, Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR)[241,242]. To understand whether Dma1 and Dma2 specifically participate in one of these two repair mechanisms, I performed an epistatic analysis for bleocin survival, analyzing *rad52*Δ for HR and *ku70*Δ for NHEJ. In yeast, most of the bleocin-induced lesions were repaired via homologous recombination system as seen in Figure 5, where Rad52 deleted cells are much more sensitive to very low doses of bleocin compared to cells defective in NHEJ. However, *dma1*Δ*dma2*Δ shows an additive effect both with *rad52*Δ and with *yku70*Δ (Figure 5A). Moreover, removal of Dma1 and Dma1 from *rad52*Δ*yku70*Δ cells did not have any effect (Figure 5B). These results suggest two possible scenarios: i) Dma1 and Dma2 independently control both NHEJ and HR or, ii) they work in the control of an

upstream common step.

DMA1 and *DMA2* deletion hyperactivates the DNA Damage Checkpoint in a Tel1 dependent manner.

In parallel to the repair analysis, I performed some experiments to evaluate the efficiency of the DNA damage checkpoint in absence of Dma1 and Dma2.
I treated with bleocin wild type and *dma1*Δ*dma2*Δ cells, arrested either in G1- or in M-phase. Rad53 phosphorylation was monitored by SDS-PAGE at different time points after the treatment as a marker of checkpoint activation[243–245]. Figure 6A shows that loss of Dma1 and Dma2 did not prevent checkpoint activation and instead caused an anticipated and stronger phosphorylation of the Rad53 kinase compared to control cells. This result is in agreement with the previous observation of a strong accumulation of unrepaired DSBs in cells depleted for Dma1 and Dma2. To further characterize this effect, I investigated which of the two yeast apical kinases, Mec1 and Tel1, was responsible for the robust checkpoint activation observed in the absence of Dma proteins. To this aim, I analyzed the Rad53 phosphorylation after bleocin treatment in *dma1*Δ*dma2*Δ mutant lacking either the Mec1 or Tel1 activity or both. As expected, Mec1 contribution to checkpoint activation was substantial (Figure 6B). Surprisingly, when Dma1 and Dma2 are not present, the Mec1 inactivation alone did not completely turn off the checkpoint signal, and the removal of Tel1 was also required (Figure 6B). This indicates that in *dma1*Δ*dma2*Δ cells Tel1 plays an unexpected role in activating the DNA damage checkpoint cascade.

Depletion of Dma proteins correlates with the MRX complex persistence at the damaged site.

Both in yeast and in human cells, it was previously shown that Tel1/ATM activation

relies on the presence at broken ends of the MRX/MRN complex, which physically recruits Tel1/ATM to DSBs through the interaction with the Xrs2/NBS1 subunit[93]. The unconventional Tel1-dependent checkpoint signaling observed in the absence of Dma proteins might suggest that in these conditions the MRX complex persists at the damaged sites, activating Tel1. Moreover, this hypothesis can also explain the contribution of Dma proteins to both the NHEJ and HR. Indeed, a fully functional and transiently loaded MRX complex is strictly required for the correct activation of both DSB repair systems (for more details see the "State of the Art").
To test this hypothesis, I assayed the efficiency of the MRX turnover at the damaged sites in wild type and Dma depleted cells by looking at the kinetics of Mre11-YFP foci formation and disappearance after DSB induction. As shown in Figure 7, in wild type cells, after a quick recruitment, the Mre11 reaches a peak and then start disappearing after 4 hours from treatment. On the contrary, in *dma1*Δ*dma2*Δ cells, while the recruitment of Mre11 follows a superimposable kinetics and the signal reaches a similar peak, Mre11 persists at the break sites for the whole time window analyzed (Figure 7). This result confirms that Dma1 and Dma2 are involved in the removal of the MRX complex from the break site.
The Sae2 resection factor is already known to be essential for the correct disassembly of the MRX complex at DNA breaks. Indeed, cells deleted for the *SAE2* also show Mre11 foci persistence following an HO endonuclease-induced DSB[45,246]. Moreover, recently Liang et al. reported a damaged-dependent interaction between the Sae2 factor and the FHA domains of both Dma1 and Dma2[228]. This evidence led me to hypothesize that Dma1/2 might work upstream of Sae2 in the same MRX removal pathway. To verify this, I analyzed bleocin sensitivity, checkpoint activation and MRX localization in the triple mutant *dma1*Δ*dma2*Δ*sae2*Δ. However, cells carrying the simultaneous deletion of these three genes show an additive effect compared to both *sae2*Δ and *dma1*Δ*dma2*Δ mutants for all phenotypes (Figure 8A-C), genetically excluding Sae2 as a relevant

Dma target.

Moreover, *sae2*Δ cells can be rescued by a *mre11*^{H37R} allele, which destabilize MRX binding to DNA[49]; conversely, when I combined *mre11*^{H37R} with *dma1*Δ*dma2*Δ I did not rescue the phenotype (Figure 9A), suggesting once again that the Dma proteins are not regulating Sae2. Intriguingly, while the Mre11 foci are rapidly lost in the *mre11*^{H37R} mutant after bleocin treatment, removal of *dma1*Δ*dma2*Δ converts these destabilized Mre11 subunits to a persistent form (Figure 9B). These results are consistent with an essential role for Dma1/2 in the removal of the MRX complex from bleocin-induced DSBs.

The MRX complex is not ubiquitinated by Dma1 and Dma2.

The results shown above indicate that Dma1 and Dma2 are required to remove a persistent form of MRX from the DNA end. Since these factors are ubiquitin ligases, I investigated how loss of Dma1 and Dma2 affects the ubiquitination status of the three subunits of the MRX complex after bleocin treatment. His-tagged ubiquitin was recovered from a crude cell extract and Figure 10 suggest that both Mre11 and Rad50 may be ubiquitinated. Unfortunately, the modifications seem to be not dependent upon DNA damage or upon the Dma factors. The apparent increase in ubiquitination in a *dma1*Δ*dma2*Δ background is a known artefact due to a higher expression of the plasmid carrying the His-tagged version of ubiquitin in the mutant compared to control cells (Supplementary Figure 4).

Dma1 and Dma2 play a role in DSB end cleaning.

Since I demonstrated that Dma1 and Dma2 do not act directly on the MRX complex regulation, I tried to understand which could be the molecular hindrance for its removal from the damaged site. A reasonable explanation came out thinking on the

nature of the breaks generated by bleocin. Indeed, this drug is an oxidative agent that generates a high number of DSBs, characterized by a wild range of end modifications ("dirty ends"). As already discussed in the State of the art, both chemical and structural alterations in DSB ends have to be specifically processed and removed before repair can efficiently take place.

One hypothesis is that Dma1 and Dma2 may have a crucial role in cleaning of bleocin-induced dirty ends. In this case, depletion of these factors causes the accumulation of irreversibly bound MRX complex at the end of the DNA filament, leading to unscheduled Tel1 activation and to the inability to complete DSB repair. Another possibility is that the MRX complex, while processing bleocin-induced "dirty ends" becomes crosslinked to the DNA end. A similar situation has been reported for Polβ at lesions containing 2-deoxyribonolactone, an oxidized derivative of deoxyribose[150].

Tdp1 is an enzyme specialized in hydrolyzing the covalent bond between Topoisomerase 1 (Top1) and the 3'-end of SSB[173]. The formation of this DNA-protein adduct is stimulated by camptotecin (CPT) inhibition of Top1. In the absence of Tdp1, Top1 remains crosslinked to the DNA end, converting it in a "dirty end". Interestingly, while *dma1*Δ*dma2*Δ are not particularly sensitive to CPT, loss of Dma factors strongly increases the sensitivity to CPT of a *tdp1*Δ mutant (Figure 11A). This result confirms a crucial role for Dma1 and Dma2 in processing modified ends to allow repair.

Finally, Tdp1 also plays a role in the cleaning of bleocin-induced modified DSB ends[247] and *tdp1*Δ is indeed additive with *dma1*Δ*dma2*Δ for what concerns bleocin sensitivity (Figure 11B).

Discussion

Dma1 and Dma2 are well characterized factors involved in the regulation of several aspects of mitosis and cell polarity. Despite their human orthologs RNF8 and CHFR have a predominant role in double-strand break repair and genome maintenance, no function for Dma proteins in the DNA damage response have been yet reported. Here I described for the first time the involvement of these proteins in DSB processing and checkpoint signaling. Indeed, my results clearly indicate a direct role for Dma1 and Dma2 in chromosome repair and Tel1 signaling regulation following bleocin-induced DNA damage. Interestingly, the yeast and human functions in break response of these E3 enzymes are mostly divergent and, in some cases, totally opposite, opening a new scenario for a possible discovery of novel RNF8 conserved roles.

My data highlight a direct contribution of Dma1 and Dma2 in the repair of a particular family of DNA breaks, where the DNA ends are chemically modified ("dirty ends"). On the other end, the exact mechanism through which Dma proteins promote repair is still elusive. In particular, here I reported that Dma1 and Dma2 are required for cell survival in presence of end modifying genotoxins, suggesting a model where Dma1 and Dma2 promote DSB end cleaning allowing repair of the lesion. Indeed, intriguingly, *tdp1*Δ cells treated with camptothecin accumulate DNA ends with covalently bound Topoisomerase 1 (Top1) and these cells need Dma factors for survival. This result strongly indicates a role for Dma1 and Dma2 in cleaning protein-blocked DNA ends. Moreover, it was already shown by several research groups that the hydrolysis of the covalent Top1 bond to chromatin by the Tdp1 enzyme requires an initial step of protein degradation. According to this, one intriguing hypothesis is that Dma1 and Dma2 coordinate specifically the repair of

protein crosslinked to break ends, promoting the proteasomal degradation of the peptide through their ubiquitination activity. This idea is firstly supported by the evidence that, after break induction, catalytic dead mutant for both Dma1 and Dma2 behaves as a *dma1*Δ*dma2*Δ cells. In addition, cells lacking Dma proteins show a persistent irreversible block of the MRX complex at the site of damage, independently from the structural destabilization of its DNA binding. Finally, the repair analysis of a HO-induced DSB did not reveal any contribution of Dma1 and Dma2 in response to this type of enzymatic blunt lesion.

Up to now, there is no reported evidence of bleocin-dependent DNA-protein adduct accumulation. However, several other oxidative DNA damage inducing agent commonly used in chemotherapy (*i.e.* Neocarzinostatin and Tirapazamine) generate a wide range of DPCs. In particular, these reagents induce a chemical modification of the break ends called 2-deoxyribonolactone (dL) that, in mammals, traps the POLβ enzyme at the DNA termini of the lesion. Despite bleocin does not seem to induce formation of dL, chemically similar alterations are abundantly produced as intermediates of bleocin-dependent DNA oxidation and, probably, they might cause the same blockage of specific repair proteins. This scenario could also easily explain why the *dma1*Δ*dma2*Δ mutant is not sensitive to ionizing radiation (data not shown), which generate DSBs but with different ends.

A future experimental validation of this theory will be essential to clarify the mechanism of Dma1 and Dma2 regulation of DNA damage response, giving a new opportunity for the identification of protein targets. Indeed, since the POLβ mechanism is a conserved system for DPC repair, its yeast homologs Pol4 and Trf4 could be good candidates as Dma substrates. However, many data strongly suggest the MRX complex as a direct target of Dma1 and Dma2 activity. Indeed, even though I was not able to observe a Dma-dependent ubiquitination of the complex, I cannot definitely exclude this possibility because my *in vivo* ubiquitination assay probably leads to the complete loss of the chromatinic fraction. Moreover, the

physical interaction between Dma1 and Dma2 and the master regulator of the MRX complex Sae2 is another important indication of a possible direct contribution of Dma factors in controlling the MRX turn-over at the damage site.

Finally, a deeper characterization of the chemical alterations induced by bleocin at the level of DNA breaks and the detailed molecular comprehension of the specific repair pathways, could significantly enhance its therapeutic applications.

References

1. Lindahl T. Instability and decay of the primary structure of DNA. *Nature*. 1993. 362(6422):709-715.

2. Marnett LJ. Oxyradicals and DNA Damage. *Carcinogenesis.* 2000. 21(3):361-370.

3. Arana ME, Kunkel TA. Mutator phenotypes due to DNA replication infidelity. *Semin Cancer Biol*. 2010. 20(5):304-311.

4. Lindahl T, Wood RD. Quality Control by DNA Repair. *Science.* 1999. 286:1897-1906.

5. Longhese MP, Mantiero D, Clerici M. The cellular response to chromosome breakage. *Mol Microbiol*. 2006. 60(5):1099-1108.

6. Eyfjord JE, Bodvarsdottir SK. Genomic instability and cancer: Networks involved in response to DNA damage. *Mutat Res*. 2005. 592(1-2):18-28.

7. Negrini S, Gorgoulis VG, Halazonetis TD. Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol*. 2010. 11(3):220-228.

8. Pommier Y, Leo E, Zhang H, Marchand C. DNA Topoisomerases and Their Poisoning by Anticancer and Antibacterial Drugs. *Chem Biol*. 2010. 17(5):421-433.

9. Samadder P, Aithal R, Belan O, Krejci L. Cancer TARGETases: DSB repair as a pharmacological target. *Pharmacol Ther*. 2016. 161:111-131.

10. Schärer OD. DNA interstrand crosslinks: Natural and drug-induced DNA adducts that induce unique cellular responses. *ChemBioChem*. 2005. 6(1):27-32.

11. Symington LS, Gautier J. Double-strand break end resection and repair pathway choice. *Annu Rev Genet*. 2011. 45:247-271.

12. Doudna J a., Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. *Science*. 2014. 346(6213):12580-12590.

13. Jasin M, Haber JE. The democratization of gene editing: Insights from site-specific cleavage and double-strand break repair. *DNA Repair (Amst)*. 2016. 44:6-16.

14. Dudášová Z, Dudáš A, Chovanec M. Non-homologous end-joining factors of *Saccharomyces cerevisiae*. *FEMS Microbiol Rev*. 2004. 28:581-601.

15. Daley JM, Palmbos PL, Wu D, Wilson TE. Nonhomologous end joining in yeast. *Annu Rev Genet*. 2005. 39:431-451.

16. Walker JR, Corpina RA, Goldberg J. Structure of the Ku Heterodimer Bound to DNA and Its Implications for Double-Strand Break Repair. *Nature*. 2001. 412(6847):607-614. 2001.

17. Palmbos PL, Daley JM, Wilson TE. Mutations of the Yku80 C Terminus and Xrs2 FHA Domain Specifically Block Yeast Nonhomologous End Joining. *Mol Cell Biol*. 2005. 25(24):10782-10790.

18. Wu D, Topper LM, Wilson TE. Recruitment and dissociation of nonhomologous end joining proteins at a DNA double-strand break in Saccharomyces cerevisiae. *Genetics*. 2008.178(3):1237-1249.

19. Wilson TE, Grawunder U, & Liebe MR. Yeast DNA ligase IV mediates non-homologous DNA end joining. *Nature*. 1997. 388(6641):495-498.

20. Herrmann G, Lindahl T, Schä P. *Saccharomyces Cerevisiae* LIF1: A Function Involved in DNA Double-Strand Break Repair Related to Mammalian XRCC4. *EMBO J.* 1998. 17(14):4188-4198.

21. Davis AJ, Chen BPC, Chen DJ. DNA-PK: a dynamic enzyme in a versatile DSB repair pathway. *DNA Repair (Amst)*. 2014. 17:21-29.

22. Kim C-H, Park S-J, Lee S-H. A Targeted Inhibition of DNA-Dependent Protein Kinase Sensitizes Breast Cancer Cells Following Ionizing Radiation. *J Pharmacol Exp Ther*. 2002. 303:753-759.

23. Ciszewski WM, Tavecchio M, Dastych J, Curtin NJ. DNA-PK inhibition by NU7441 sensitizes breast cancer cells to ionizing radiation and doxorubicin. *Breast Cancer Res Treat*. 2014. 143:47-55.

24. Albarakati N, Abdel-Fatah TMA, Doherty R, et al. Targeting BRCA1-BER deficient breast cancer by ATM or DNA-PKcs blockade either alone or in combination with cisplatin for personalized therapy. *Mol Oncol*. 2015. 9(1):204-217.

25. Goodwin JF, Knudsen KE. Beyond DNA repair: DNA-PK function in cancer. *Cancer Discov*. 2014. 4(10):1126-1139.

26. Malu S, Malshetty V, Francis D, Cortes P. Role of non-homologous end joining in V(D)J recombination. *Immunol Res*. 2012. 54(1-3):233-246.

27. Zhao X, Wei C, Li J, et al. Cell cycle-dependent control of homologous recombination. *Acta Biochim Biophys Sin*. 2017. 49(8):655-668.

28. Shibata A. Regulation of repair pathway choice at two-ended DNA double-strand breaks. *Mutat Res*. 2017. 803-805:51-55.

29. Ira G, Pellicioli A, Balijja A, et al. DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. *Nature*. 2004. 431:1011-1017.

30. Chen X, Niu H, Chung W-H, et al. Cell cycle regulation of DNA double-strand break end resection by Cdk1-dependent Dna2 phosphorylation. *Nat Struct Mol Biol*. 2011. 18(9):1015-1029.

31. Trujillo KM, Yuan S-SF, Y-H Lee EP, Sung P. Nuclease Activities in a Complex of Human Recombination and DNA Repair Factors Rad50, Mre11, and p95. *J. Biol Chem*. 1998. 273(34):21447-21450.

32. Usui T, Ohta T, Oshiumi H, Tomizawa J, Ogawa H, Ogawa T. Complex Formation and Functional Versatility of Mre11 of Budding Yeast in Recombination. *Cell.* 1998. 95(5):705-716.

33. Paull TT, Gellert M. unwinding and endonuclease cleavage by the Mre11 / Rad50 complex. *Genes Dev*. 1999. 13(10):1276-1288.

34. Trujillo KM, Roh DH, Chen L, Van Komen S, Tomkinson A, Sung P. Yeast Xrs2 binds DNA and helps target Rad50 and Mre11 to DNA ends. *J Biol Chem*. 2003. 278(49):48957-48964.

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

36. Kim H-S, Vijayakumar S, Reger M, et al. Functional interactions between Sae2 and the Mre11 complex. *Genetics*. 2008. 178(2):711-723.

37. Garcia V, Phelps SEL, Gray S, Neale MJ. Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1. *Nature*. 2011. 479(7372):241-244.

38. Cannavo E, Cejka P. Sae2 promotes dsDNA endonuclease activity within Mre11–Rad50–Xrs2 to resect DNA breaks. *Nature*. 2014. 514(7520):122-125.

39. Cannavo E, Cejka P. Sae2 promotes dsDNA endonuclease activity within Mre11–Rad50–Xrs2 to resect DNA breaks. *Nature*. 2014. 514(7520):122-125.

40. Hartsuiker E, Mizuno K, Molnar M, Kohli J, Ohta K, Carr AM. Ctp1 CtIP and Rad32 Mre11 Nuclease Activity Are Required for Rec12 Spo11 Removal, but Rec12 Spo11 Removal Is Dispensable for Other MRN-Dependent Meiotic Functions. *Mol Cell Biol*. 2009. 29(7):1671-1681.

41. Lobachev KS, Gordenin DA, Resnick MA. The Mre11 Complex Is Required for Repair of Hairpin-Capped Double-Strand Breaks and Prevention of Chromosome Rearrangements. *Cell*. 2002. 108(2):183-193.

42. Hartsuiker E, Neale MJ, Carr AM. Distinct Requirements for the Rad32 Mre11 Nuclease and Ctp1 CtIP in the Removal of Covalently Bound Topoisomerase I and II from DNA. *Mol Cell*. 2009. 33(1):117-123.

43. Neale MJ, Pan J, Keeney S. Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. *Nature*. 2005. 436(7053):1053-1057.

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

45. Lisby M, Barlow JH, Burgess RC, Rothstein R. Choreography of the DNA Damage Response: Spatiotemporal Relationships among Checkpoint and Repair Proteins. *Cell*. 2004. 118(6):699-713.

46. Lisby M, Barlow JH, Burgess RC, Rothstein R. Choreography of the DNA Damage Response: Spatiotemporal Relationships among Checkpoint and Repair Proteins. *Cell*. 2004. 118(6):699-713.

47. Usui T, Ogawa H, Petrini JHJ. A DNA Damage Response Pathway Controlled by Tel1 and the Mre11 Complex. *Mol Cell*. 2001. 7(6):1255-1266.

48. Usui T, Ohta T, Oshiumi H, Tomizawa J, Ogawa H, Ogawa T. Complex Formation and Functional Versatility of Mre11 of Budding Yeast in Recombination. *Cell*. 1998. 95(5):705-716.

49. Puddu F, Oelschlaegel T, Guerini I, et al. Synthetic viability genomic screening defines Sae 2 function in DNA repair. *EMBO J*. 2015. 34(11):1509-1522.

50. Chen H, Donnianni RA, Handa N, et al. Sae2 promotes DNA damage resistance by removing the Mre11–Rad50–Xrs2 complex from DNA and attenuating Rad53 signaling. *PNAS USA*. 2015. doi:10.1073/pnas.1503331112.

51. Mimitou EP, Symington LS. Sae2 , Exo1 and Sgs1 collaborate in DNA double-strand break processing. *Nature*. 2008. 455(7214):770-774.

52. Zhu Z, Chung W-H, Shim EY, Lee SE, Ira G. Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell*. 2008. 134(6):981-994.

53. Longhese MP, Bonetti D, Manfrini N, Clerici M. Mechanisms and regulation of DNA end resection. *EMBO J*. 2010. 29(17):2864-2874.

54. Granata M, Panigada D, Galati E, et al. To trim or not to trim Progression and control of DSB end resection. *Cell cycle*. 2013. 12(12):1848-1860.

55. Chen H, Lisby M, Symington L. RPA Coordinates DNA End Resection and Prevents Formation of DNA Hairpins. *Mol Cell*. 2013. 50(4):589-600.

56. Deng SK, Yin Y, Petes TD, Symington LS. Mre11-Sae2 and RPA Collaborate to Prevent Palindromic Gene Amplification. *Mol Cell*. 2015. 60:500-508.

57. Shinohara A, Shinohara M, Ohta T, Matsuda S, Ogawa T. Rad52 forms ring structures and co-operates with RPA in single-strand DNA annealing. *Genes to Cells*. 1998. 3(3):145-156.

58. New JH, Sugiyama T, Zaitseva E, Kowalczykowski Stephen C. Rad52 protein stimulates DNA strand exchange by Rad51 and replication protein A. *Nature*. 1998. 391(6665):407-410.

59. Song B SP. Functional Interactions among Yeast Exchange Functional Interactions among Yeast Rad51 Recombinase , Rad52 Mediator , and Replication Protein A in DNA Strand Exchange. *J Biol Chem*. 2000. 275(21):15895-15904.

60. Yuan SSF, Lee SY, Chen G, Song M, Tomlinson GE, Lee EYHP. BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex in vivo. *Cancer Res*. 1999. 59(15):3547-3551.

61. Liu J, Doty T, Gibson B, Heyer W-D. Human BRCA2 protein promotes RAD51 filament formation on RPA-covered single-stranded DNA. *Nat Struct Mol Biol*. 2010. 17(10):1260-1262.

62. Liu J, Heyer W-D. Who's who in human recombination: BRCA2 and RAD52. *PNAS*. 2011.108(2):441-442.

63. Zhao W, Steinfeld J, Liang F, et al. BRCA1-BARD1 promotes RAD51-mediated homologous DNA pairing. *Nature*. 2017. 550(7676):360-365.

64. Solinger JA, Kiianitsa K, Heyer W-D. Rad54, a Swi2/Snf2-like Recombinational Repair Protein, Disassembles Rad51:dsDNA Filaments. *Mol Cell*. 2002. 10(5):1175-1188.

65. Wright WD, Heyer W-D. Rad54 Functions as a Heteroduplex DNA Pump Modulated by Its DNA Substrates and Rad51 during D Loop Formation. *Mol Cell*. 2014. 53(3):420-432.

66. Li X, Stith CM, Burgers PM, Heyer W-D. PCNA Is Required for Initiation of Recombination-Associated DNA Synthesis by DNA Polymerase d. *Mol Cell*. 2009. 36:704-713.

67. Wilson MA, Kwon Y, Xu Y, et al. Pif1 helicase and Pold promote recombination-coupled DNA synthesis via bubble migration. *Nature*. 2013. 502(7471):393-396.

68. Llorente B, Smith CE, Symington LS. Break-induced replication: what is it and what is it for? *Cell cycle*. 2008. 7(7):859-864.

69. Haber JE. DNA Repair: The Search for Homology. *BioEssays*. 2018. 40(5):1-12.

70. Filippo JS, Sung P, Klein H. Mechanism of Eukaryotic Homologous Recombination. *Annu Rev Biochem*. 2008. 77:229-257.

71. Yu X, Gabriel A. Ku-Dependent and Ku-Independent End-Joining Pathways Lead to Chromosomal Rearrangements During Double-Strand Break Repair in Saccharomyces cerevisiae. *Genetics*. 2003. 163(3):843-856.

72. Ma J-L, Kim EM, Haber JE, Lee SE. Yeast Mre11 and Rad1 Proteins Define a Ku-Independent Mechanism To Repair Double-Strand Breaks Lacking Overlapping End Sequences. *Mol Cell Biol*. 2003;23(23):8820-8828.

73. Boulton SJ, Jackson SP. *Saccharomyces Cerevisiae* Ku7O Potentiates Illegitimate DNA Double-Strand Break Repair and Serves as a Barrier to Error-Prone DNA Repair Pathways. *EMBO J*. 1996. 15(18):5093-5103.

74. Wang H, Perrault AR, Takeda Y, Qin W, Wang H, Iliakis G. Biochemical evidence for Ku-independent backup pathways of NHEJ. *Nucleic Acids Res*. 2003. 31(18):5377-5388.

75. Designed Research; L XWNT. Microhomology-mediated End Joining and Homologous Recombination share the initial end resection step to repair DNA double-strand breaks in mammalian cells. *PNAS*. 2013. 110(19):7720-7725.

76. Corneo B, Wendland RL, Deriano L, et al. Rag mutations reveal robust alternative end joining. *Nature*. 2007. 449(7161):483-486.

77. Yan CT, Boboila C, Souza EK, et al. IgH class switching and translocations use a robust non-classical end-joining pathway. *Nature*. 2007. 449(7161):478-482.

78. Stephens PJ, Mcbride DJ, Lin M-L, et al. Complex landscapes of somatic rearrangement in human breast cancer genomes. *Nature*. 2009. 462(7276):1005-1010.

79. Simsek D, Jasin M. Alternative end-joining is suppressed by the canonical NHEJ component Xrcc4–ligase IV during chromosomal translocation formation. *Nat Struct Mol Biol*. 2010. 17(4):410-416.

80. Zhang Y, Jasin M. An essential role for CtIP in chromosomal translocation formation through an alternative end-joining pathway. *Nat Struct Mol Biol*. 2010. 18(1):80-85.

81. Zhang Y, Gostissa M, Hildebrand DG, et al. The Role of Mechanistic Factors in Promoting Chromosomal Translocations Found in Lymphoid and Other Cancers. *Adv Immunol*. 2010. 106:93-133.

82. Weinstock DM, Jasin M. Alternative Pathways for the Repair of RAG-Induced DNA Breaks Downloaded from. *Mol Cell Biol*. 2006. 26(1):131-139.

83. Lee K, Lee SE. Saccharomyces cerevisiae Sae2-and Tel1-Dependent Single-Strand DNA Formation at DNA Break Promotes Microhomology-Mediated End Joining. *Genetics*. 2007. 176(4):2003-2014.

84. Daley JM, Wilson TE. Rejoining of DNA Double-Strand Breaks as a Function of Overhang Length. *Mol Cell Biol*. 2005. 25(3):896-906.

85. Ahmad A, Robinson AR, Duensing A, et al. ERCC1-XPF Endonuclease Facilitates DNA Double-Strand Break Repair. *Mol Cell Biol*. 2008. 28(16):5082-5092.

86. Liang L, Deng L, Nguyen SC, et al. Human DNA ligases I and III, but not ligase IV, are required for microhomology-mediated end joining of DNA double-strand breaks. *Nucleic Acids Res*. 2008. 36(10):3297-3310.

87. Lazzaro F, Giannattasio Michele, Puddu F, et al. Checkpoint mechanisms at the intersection between DNA damage and repair. *DNA Repair (Amst)*. 2009. 8:1055-1067.

88. Broustas CG, Lieberman HB. DNA Damage Response Genes and the Development of Cancer Metastasis. *Radiat Res*. 2014. 181(2):111-130.

89. Nojima H. Cell cycle checkpoints, chromosome stability and the progression of cancer. *Hum Cell*. 1997. 10(4):221-230.

90. Hartwell LH, Weinert TA. Checkpoints : Controls that Ensure the Order of Cell Cycle Events. *Science*. 1989. 264(4930):629-634.

91. Harrison JC, Haber JE. Surviving the Breakup: The DNA Damage Checkpoint. *Annu Rev Genet*. 2006. 40:209-235.

92. Gobbini E, Cesena D, Galbiati A, Lockhart A, Longhese MP. Interplays between ATM/Tel1 and ATR/Mec1 in sensing and signaling DNA double-strand breaks. *DNA Repair (Amst)*. 2013. 12:791-799.

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

94. Lee C-S, Lee K, Legube G, Haber JE. Dynamics of yeast histone H2A and H2B phosphorylation in response to a double-strand break. *Nat Struct Mol Biol*. 2014. 21(1):103-109.

95. Shroff R, Arbel-Eden A, Pilch D, et al. Distribution and Dynamics of Chromatin Modification Induced by a Defined DNA Double-Strand Break. *Curr Biol*. 2004. 14:1703-1711.

96. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science*. 2003. 300(5625):1542-1548.

97. Majka J, Niedziela-Majka A, Burgers PMJ. The Checkpoint Clamp Activates Mec1 Kinase during Initiation of the DNA Damage Checkpoint. *Mol Cell*. 2006. 24:891-901.

98. Majka J, Binz SK, Wold MS, Burgers PMJ. Replication Protein A Directs Loading of the DNA Damage Checkpoint Clamp to 5-DNA Junctions. *J Biol Chem*. 2006. 281(38):27855-27861.

99. Puddu F, Granata M, Di Nola L, et al. 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*. 2008. 28(15):4782-4793.

100. Mordes DA, Nam EA, Cortez D. Dpb11 activates the Mec1-Ddc2 complex. *PNAS*. 2008. 105(48):1830-1834.

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

102. Mordes D a, Nam E a, Cortez D. Dpb11 activates the Mec1-Ddc2 complex. *PNAS*. 2008. 105(48):18730-18734.

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

104. Granata M, Lazzaro F, Novarina D, et al. Dynamics of Rad9 chromatin binding and checkpoint function are mediated by its dimerization and are cell cycle-regulated by CDK1 activity. *PLoS Genet*. 2010. 6(8). pii: e1001047.

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

106. Pfander B, Diffley JFX. Dpb11 coordinates Mec1 kinase activation with cell cycle-regulated Rad9 recruitment. *EMBO J*. 2011. 30(24):4897-4907.

107. Wysocki R, Javaheri A, Sha F, Co J. Role of Dot1-Dependent Histone H3 Methylation in G 1 and S Phase DNA Damage Checkpoint Functions of Rad9. *Mol Cell Biol*. 2005. 25(19):8430-8443.

108. Vialard JE, Gilbert CS, Green CM, Lowndes NF. The Budding Yeast Rad9 Checkpoint Protein Is Subjected to Mec1/Tel1-Dependent Hyperphosphorylation and Interacts with Rad53 after DNA Damage. *EMBO J*. 1998. 17(19):5679-5688.

109. Schwartz MF, Duong JK, Sun Z, Morrow JS, Pradhan D, Stern DF. Rad9 Phosphorylation Sites Couple Rad53 to the *Saccharomyces cerevisiae* DNA Damage Checkpoint. *Mol Biol Cell*. 2002. 9:1055-1065.

110. Sun Z, Hsiao J, Fay DS, Stern DF. Rad53 FHA Domain Associated with Phosphorylated Rad9 in the DNA Damage Checkpoint. *Science*. 1998. 281(5374):272-274.

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

112. Xia Z, Morales JC, Dunphy WG, Carpenter PB. Negative Cell Cycle Regulation and DNA Damage-inducible Phosphorylation of the BRCT Protein 53BP1. *J Biol Chem*. 2000. 276(4):2708-2718.

113. Anderson L, Henderson C, Adachi Y. Phosphorylation and Rapid Relocalization of 53BP1 to Nuclear Foci upon DNA Damage. *Mol Cell Biol*. 2001. 21(5):1719-1729.

114. Rappold I, Iwabuchi K, Date T, Chen J. Tumor Suppressor p53 Binding Protein 1 (53BP1) Is Involved in DNA Damage-signaling Pathways. *J Cell Biol*. 2001. 153(3):613-620.

115. Ditullio RA, Mochan TA, Venere M, et al. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. *Nat Cell Biol*. 2002. 4(12):998-1002.

116. Pellicioli A, Foiani M. Signal Transduction: How Rad53 Kinase Is Activated. *Curr Biol*. 2005. 15(18):769-771.

117. Sanchez Y, Bachant J, Wang H, et al. Control of the DNA Damage Checkpoint by Chk1 and Rad53 Protein Kinases Through Distinct Mechanisms. *Science*. 1999. 286(5442):1166-1171.

118. Jackson SP, Durocher D. Regulation of DNA Damage Responses by Ubiquitin and SUMO. *Mol Cell*. 2013. 49(5):795-807.

119. Schwertman P, Bekker-Jensen S, Mailand N. Regulation of DNA double-strand break repair by ubiquitin and ubiquitin-like modifiers. *Nat Rev Mol Cell Biol*. 2016. 17(6):379-394.

120. Cremona CA, Sarangi P, Yang Y, Hang LE, Rahman S, Zhao X. Extensive DNA Damage-Induced Sumoylation Contributes to Replication and Repair and Acts in Addition to the Mec1 Checkpoint. *Mol Cell*. 2012. 45(3):422-432.

121. Elia AEH, Boardman AP, Wang DC, Koren I, Gygi SP, Correspondence SJE. Quantitative Proteomic Atlas of Ubiquitination and Acetylation in the DNA Damage Response. *Mol Cell*. 2015. 59(5):867-881.

122. Psakhye I, Jentsch S. Protein Group Modification and Synergy in the SUMO Pathway as Exemplified in DNA Repair. *Cell*. 2012. 151:807-820.

123. Swatek KN, Komander D. Ubiquitin modifications. *Cell Res*. 2016. 26:399-422.

124. Hay RT. Decoding the SUMO signal. *Biochem Soc Trans*. 2012. 41(2):463-473.

125. Chen Y, Chuang Y, Chuang C, et al. *S. cerevisiae* Mre11 recruits conjugated SUMO moieties to facilitate the assembly and function of the Mre11-Rad50-Xrs2 complex. *Nucleic Acids Res.* 2016. 44(5):2199-2213.

126. Kim W, Bennett EJ, Huttlin EL, et al. Systematic and Quantitative Assessment of the Ubiquitin-Modified Proteome. *Mol Cell*. 2011. 44(2):325-340.

127. Wagner SA, Beli P, Weinert BT, et al. A Proteome-wide, Quantitative Survey of In Vivo Ubiquitylation Sites Reveals Widespread Regulatory Roles. *Mol Cell Proteomics*. 2011. 10(10):M111.013284.

128. Wu J, Zhang X, Zhang L, et al. Skp2 E3 Ligase Integrates ATM Activation and Homologous Recombination Repair by Ubiquitinating NBS1. *Mol Cell*. 2012. 46(3):351-361.

129. Lu C-S, Truong LN, Aslanian A, et al. The RING Finger Protein RNF8 Ubiquitinates Nbs1 to Promote DNA Double-strand Break Repair by Homologous Recombination. *J Biol Chem*. 2012. 287(52):43984-43994.

130. Sarangi P, Steinacher R, Altmannova V, et al. Sumoylation Influences DNA Break Repair Partly by Increasing the Solubility of a Conserved End Resection Protein. Jinks-Robertson S, ed. *PLoS Genet*. 2015. 11(1):e1004899.

131. Yu X, Fu S, Lai M, Baer R, Chen J. BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. *Genes Dev*. 2006. 20(13):1721-1726.

132. Schmidt CK, Galanty Y, Sczaniecka-Clift M, et al. Systematic E2 screening reveals a UBE2D-RNF138-CtIP axis promoting DNA repair. *Nat Cell Biol*. 2015. 17(11):1458-1470.

133. Ismail IH, Gagné J-P, Genois M-M, et al. The RNF138 E3 ligase displaces Ku to promote DNA end resection and regulate DNA repair pathway choice. *Nat Cell Biol*. 2015. 17(11):1446-1457.

134. Feng L, Chen J. The E3 ligase RNF8 regulates KU80 removal and NHEJ repair. *Nat Struct Mol Biol*. 2012. 19(2):201-206.

135. Bologna S, Altmannova V, Valtorta E, et al. Sumoylation regulates EXO1 stability and processing of DNA damage. *Cell Cycle*. 2015. 14:2439-2450.

136. Blackford AN, Nieminuszczy J, Jackson SP, et al. TopBP1 Interacts with BLM to Maintain Genome Stability but Is Dispensable for Preventing BLM Degradation. *Mol Cell*. 2015. 57(6):1133-1141.

137. Tikoo S, Madhavan V, Hussain M, et al. Ubiquitin-dependent recruitment of the Bloom Syndrome helicase upon replication stress is required to suppress homologous recombination. *EMBO J*. 2013. 32:1778-1792.

138. Eladad S, Ye T-Z, Hu P, et al. Intra-nuclear trafficking of the BLM helicase to DNA damage-induced foci is regulated by SUMO modification. *Hum Mol Genet*. 2005. 14(10):1351-1365.

139. Böhm S, Mihalevic MJ, Casal MA, Bernstein KA. Disruption of SUMO-targeted ubiquitin ligases Slx5–Slx8/RNF4 alters RecQ-like helicase Sgs1/BLM localization in yeast and human cells. *DNA Repair (Amst)*. 2015. 26:1-14.

140. Mailand N, Bekker-Jensen S, Faustrup H, et al. RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. *Cell*. 2007. 131(5):887-900.

141. Thorslund T, Ripplinger A, Hoffmann S, et al. Histone H1 couples initiation and amplification of ubiquitin signalling after DNA damage. *Nature*. 2015. 527(7578):389-393.

142. Huen MSY, Grant R, Manke I, et al. RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly. *Cell*. 2007. 131(5):901-914.

143. Kolas NK, Chapman JR, Nakada S, et al. Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase. *Science*. 2007. 318(5856):1637-1640.

144. Doil C, Mailand N, Bekker-jensen S, et al. RNF168 Binds and Amplifies Ubiquitin Conjugates on Damaged Chromosomes to Allow Accumulation of Repair Proteins. *Cell*. 2009. 136(3):435-446.

145. Stewart GS, Panier S, Townsend K, et al. The RIDDLE Syndrome Protein Mediates a Ubiquitin-Dependent Signaling Cascade at Sites of DNA Damage. *Cell*. 2009. 136(3):420-434.

146. Stingele J, Jentsch S. DNA–protein crosslink repair. *Nat Rev Mol Cell Biol*. 2015. 16(8):455-460.

147. Chválová K, Brabec V, Kašpárková J. Mechanism of the formation of DNA-protein cross-links by antitumor cisplatin. *Nucleic Acids Res*. 2007. 35(6):1812-1821.

148. Pommier Y, Barcelo JM, Rao VA, et al. Repair of Topoisomerase II Mediated DNA Damage. *Prog Nucleic Acid Res Mol Biol*. 2006. 81:179-229.

149. Quiñones JL, Thapar U, Yu K, et al. Enzyme mechanism-based, oxidative DNA–protein cross-links formed with DNA polymerase β in vivo. *PNAS*. 2015. 112(28):8602-8607.

150. Demott MS, Beyret E, Wong D, et al. Covalent Trapping of Human DNA Polymerase by the Oxidative DNA Lesion 2-Deoxyribonolactone. *J Biol Chem*. 2002. 277(10):7637-7640.

151. Sung J-S, Demott MS, Demple B. Long-patch Base Excision DNA Repair of 2-Deoxyribonolactone Prevents the Formation of DNA-Protein Cross-links with DNA Polymerase. *J Biol Chem*. 2005. 280(47):39095-39103.

152. Swenberg JA, Lu K, Moeller BC, et al. Endogenous versus Exogenous DNA Adducts: Their Role in Carcinogenesis, Epidemiology, and Risk Assessment. *Toxicol Sci*. 2011. 120(Supplement 1):S130-S145.

153. Bandaru V, Sunkara S, Wallace SS, Bond JP. A novel human DNA glycosylase that removes oxidative DNA damage and is homologous to Escherichia coli endonuclease VIII. *DNA Repair (Amst)*. 2002. 1(7):517-529.

154. Conaway CC, Whysner J, Verna LK, Williams GM. Formaldehyde mechanistic data and risk assessment: endogenous protection from DNA adduct formation. *Pharmacol Ther*. 1996. 71(1-2):29-55.

155. Walport LJ, Hopkinson RJ, Schofield CJ. Mechanisms of human histone and nucleic acid demethylases. *Curr Opin Chem Biol*. 2012. 16(5-6):525-534.

156. Trewick SC, Henshaw TF, Hausinger RP, Lindahl T, Sedgwick B. Oxidative demethylation by Escherichia coli AlkB directly reverts DNA base damage. *Nature*. 2002. 419(6903):174-178.

157. Murai J, Huang SN, Das BB, et al. Trapping of PARP1 and PARP2 by Clinical PARP Inhibitors. *Cancer Res*. 2012. 72(21):5588-5599.

158. Woodworth DL, Kreuzer KN. Bacteriophage T4 Mutants Hypersensitive to an Antitumor Agent That Induces Topoisomerase-DNA Cleavage Complexes. *Genetics*. 1996;143(3):1081-1090.

159. Stohr BA, Kreuzer KN. Repair of Topoisomerase-Mediated DNA Damage in Bacteriophage T4. *Genetics*. 2001. 158:19-28.

160. Connelly JC, de Leau ES, Leach DRF. Nucleolytic processing of a protein-bound DNA end by the E. coli SbcCD (MR) complex. *DNA Repair (Amst)*. 2003. 2(7):795-807.

161. Neale MJ, Pan J, Keeney S. Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. *Nature*. 2005. 436(7053):1053-1057.

162. Hartsuiker E, Neale MJ, Carr AM. Distinct Requirements for the Rad32Mre11 Nuclease and Ctp1CtIP in the Removal of Covalently Bound Topoisomerase I and II from DNA. *Mol Cell*. 2009. 33(1):117-123.

163. Lee KC, Padget K, Curtis H, et al. MRE11 facilitates the removal of human topoisomerase II complexes from genomic DNA. *Biol Open*. 2012. 1:863-873.

164. Aparicio T, Baer R, Gottesman M, Gautier J. MRN, CtIP, and BRCA1 mediate repair of topoisomerase II–DNA adducts. *J Cell Biol*. 2016. 212(4):399-408.

165. Deshpande RA, Lee J-H, Arora S, Paull Correspondence TT. Nbs1 Converts the Human Mre11/Rad50 Nuclease Complex into an Endo/Exonuclease Machine Specific for Protein-DNA Adducts. *Mol Cell*. 2016. 64:593-606.

166. Fukunaga K, Kwon Y, Sung P, Sugimoto K. Activation of protein kinase Tel1 through recognition of protein-bound DNA ends. *Mol Cell Biol*. 2011. 31(10):1959-1971.

167. Deshpande RA, Lee J-H, Arora S, Paull TT. Nbs1 Converts the Human Mre11/Rad50 Nuclease Complex into an Endo/Exonuclease Machine Specific for Protein-DNA Adducts. *Mol Cell*. 2016. 64(3):593-606.

168. Stingele J, Schwarz MS, Bloemeke N, Wolf PG, Jentsch S. A DNA-Dependent Protease Involved in DNA-Protein Crosslink Repair. *Cell*. 2014. 158:327-338.

169. Maskey RS, Flatten KS, Sieben CJ, et al. Spartan deficiency causes accumulation of Topoisomerase 1 cleavage complexes and tumorigenesis. *Nucleic Acids Res*. 2017. 45(8):4564-4576.

170. Lessel D, Vaz B, Halder S, et al. Mutations in SPRTN cause early onset hepatocellular carcinoma, genomic instability and progeroid features. *Nat Genet*. 2014. 46(11):1239-1244.

171. Duxin JP, Dewar JM, Yardimci H, Walter JC. Repair of a DNA-Protein Crosslink by Replication-Coupled Proteolysis. *Cell*. 2014. 159(2):346-357.

172. Desai SD, Liu LF, Vazquez-Abad D, D'Arpa P. Ubiquitin-dependent destruction of topoisomerase I is stimulated by the antitumor drug camptothecin. *J Biol Chem*. 1997. 272(39):24159-24164.

173. Pommier Y, Huang S-YN, Gao R, Das B, Murai J, Marchand C. Tyrosyl-DNA-phosphodiesterases (TDP1 and TDP2). *DNA Repair (Amst)*. 2014. 19:114-129.

174. Takashima H, Boerkoel CF, John J, et al. Mutation of TDP1, encoding a topoisomerase I–dependent DNA damage repair enzyme, in spinocerebellar ataxia with axonal neuropathy. *Nat Genet*. 2002. 32(2):267-272.

175. El-Khamisy SF. To live or to die: a matter of processing damaged DNA termini in neurons. *EMBO Mol Med*. 2011. 3:78-88.

176. Sluimer J, Distel B. Regulating the human HECT E3 ligases. *Cell Mol Life Sci*. 2018. 75(17):1-21.

177. Deshaies RJ, Joazeiro C a P. RING domain E3 ubiquitin ligases. *Annu Rev Biochem*. 2009. 78:399-434.

178. Brooks L, Heimsath EG, Loring GL, Brenner C. FHA-RING ubiquitin ligases in cell division cycle control. *Cell Mol Life Sci*. 2008. 65(21):3458-3466.

179. Durocher D, Jackson SP. The FHA domain. *FEBS Lett*. 2002. 513(1):58-66.

180. Fraschini R, Bilotta D, Lucchini G, Piatti S, Scienza P. Functional Characterization of Dma1 and Dma2 , the Budding Yeast Homologues of *Schizosaccharomyces pombe* Dma1 and Human Chfr. *Mol Biol Cell*. 2004. 15:3796-3810.

181. Juanes MA, Piatti S. Control of Formin Distribution and Actin Cable Assembly by the E3 Ubiquitin Ligases Dma1 and Dma2. *Genetics*. 2016. 204:205-220.

182. Chahwan R, Gravel S, Matsusaka T, Jackson SP. Dma/RNF8 proteins are evolutionarily conserved E3 ubiquitin ligases that target septins. *Cell cycle*. 2013. 12(6):1000-1008.

183. Cassani C, Raspelli E, Santo N, Chiroli E, Lucchini G, Fraschini R. *Saccharomyces cerevisiae* Dma proteins participate in cytokinesis by controlling two different pathways. *Cell cycle*. 2013. 12(17):2794-2808.

184. Yau RG, Peng Y, Valiathan RR, Birkeland SR, Wilson TE, Weisman LS. Release from Myosin V via Regulated Recruitment of an E3 Ubiquitin Ligase Controls Organelle Localization. *Dev Cell*. 2014. 28:520-533.

185. Mcmillan JN, Sia RAL, Lew DJ. A Morphogenesis Checkpoint Monitors the Actin Cytoskeleton in Yeast. *J Cell Biol*. 1998. 142(6):1487-1499.

186. Barral Y, Parra M, Bidlingmaier S, Snyder M. Nim1-related kinases coordinate cell cycle progression with the organization of the peripheral cytoskeleton in yeast. *Genes Dev*. 1999. 13(2):176-187.

187. Harvey SL, Kellogg DR. Conservation of Mechanisms Controlling Entry into Mitosis: Budding Yeast Wee1 Delays Entry into Mitosis and Is Required for Cell Size Control. *Curr Biol*. 2003. 13(4):264-275.

188. Theesfeld CL, Zyla TR, Bardes EGS, Lew DJ. A Monitor for Bud Emergence in the Yeast Morphogenesis Checkpoint. *Mol Biol Cell*. 2003. 14(8):3280-3291.

189. Lew DJ, Reed SI. Morphogenesis in the Yeast Cell Cycle: Regulation by Cdc28 and Cyclins. *J Cell Biol*. 1993. 120(5):1305-1320.

190. Sia RA, Herald HA, Lew DJ. Cdc28 tyrosine phosphorylation and the morphogenesis checkpoint in budding yeast. *Mol Biol Cell*. 1996. 7(11):1657-1666.

191. Sia RAL, Bardes ESG, Lew DJ. Control of Swe1p Degradation by the Morphogenesis Checkpoint. *EMBO J*. 1998. 17(22):6678-6688.

192. Asano S, Park J-E, Sakchaisri K, et al. Concerted mechanism of Swe1/Wee1 regulation by multiple kinases in budding yeast. *EMBO J*. 2005. 24:2194-2204.

193. Sakchaisri K, Asano S, Yu L-R, et al. Coupling morphogenesis to mitotic entry. *PNAS* . 2004. 101(12):4124-4129.

194. Shulewitz MJ, Inouye CJ, Thorner J. Hsl7 Localizes to a Septin Ring and Serves as an Adapter in a Regulatory Pathway That Relieves Tyrosine Phosphorylation of Cdc28 Protein Kinase in *Saccharomyces Cerevisiae*. *Mol Cell Biol*. 1999. 19(10):7123-7137.

195. Mcmillan JN, Longtine MS, Sia RAL, et al. The Morphogenesis Checkpoint in *Saccharomyces Cerevisiae*: Cell Cycle Control of Swe1p Degradation by Hsl1p and Hsl7p. *Mol Cell Biol*. 1999. 19(10):6929-6939.

196. Lee KS, Asano S, Park J-E, Sakchaisri & Raymond K, Erikson LL. Monitoring the Cell Cycle by Multi-Kinase-Dependent Regulation of Swe1/Wee1 in Budding Yeast. *Cell Cycle*. 2005. 4(10):1346-1349.

197. Raspelli E, Cassani C, Lucchini G, Fraschini R. Budding yeast Dma1 and Dma2 participate in regulation of Swe1 levels and localization. *Mol Biol Cell*. 2011. 22(13):2185-2197.

198. Smolka MB, Chen S, Maddox PS, et al. An FHA domain–mediated protein interaction network of Rad53 reveals its role in polarized cell growth. *J Biol Chem*. 2006. 175(5):743-753.

199. Enserink JM, Smolka MB, Zhou H, Kolodner RD. Checkpoint proteins control morphogenetic events during DNA replication stress in *Saccharomyces cerevisiae*. *J Biol Chem*. 2006. 175(5):729-741.

200. Longtine MS, Bi E. Regulation of septin organization and function in yeast. *Trends Cell Biol*. 2003. 13(8):403-409.

201. Versele M, Thorner J. Some assembly required: yeast septins provide the instruction manual. *Trends Cell Biol*. 2005. 15(8):414-424.

202. Weirich CS, Erzberger JP, Barral Y. The septin family of GTPases: architecture and dynamics. *Nat Rev Mol Cell Biol*. 2008. 9(6):478-489.

203. Merlini L, Fraschini R, Boettcher B, Barral Y, Lucchini G, Piatti S. Budding yeast dma proteins control septin dynamics and the spindle position checkpoint by promoting the recruitment of the Elm1 kinase to the bud neck. *PLoS Genet*. 2012. 8(4):e1002670.

204. Wodarz A, Näthke I. Cell polarity in development and cancer. *Nat Cell Biol*. 2007. 9(9):1016-1024.

205. Sheu Y-J, Santos B, Fortin N, Costigan C, Snyder M. Spa2p Interacts with Cell Polarity Proteins and Signaling Components Involved in Yeast Cell Morphogenesis. *Mol Cell Biol*. 1998. 18(7):4053.4069.

206. Pruyne D, Legesse-Miller A, Gao L, Dong Y, Bretscher A. Mechanisms of polarized growth and organelle segregation in yeast. *Annu Rev Cell Dev Biol*. 2004. 20(1):559-591.

207. Wallar BJ, Alberts AS. The formins: active scaffolds that remodel the cytoskeleton. *Trends Cell Biol*. 2003. 13(8):435-446.

208. Perez P, Rincon SA. Rho GTPases: regulation of cell polarity and growth in yeasts. *Biochem J*. 2010. 426:243-253.

209. Imamura H, Tanaka K, Kamei T, Takahashi K, Sasaki T, Takai Y. Bni1p and Bnr1p: Downstream Targets of the Rho Family Small G-Proteins Which

Interact with Profilin and Regulate Actin Cytoskeleton in *Saccharomyces Cerevisiae*. *EMBO J*. 1997. 16(10):2745-2755.

210. Sagot I, Klee SK, Pellman D. Yeast formins regulate cell polarity by controlling the assembly of actin cables. *Nat Cell Biol*. 2002. 4(1):42-50.

211. Pruyne D, Gao L, Bi E, Bretscher A. Stable and Dynamic Axes of Polarity Use Distinct Formin Isoforms in Budding Yeast. *Mol Biol Cell*. 2004. 15:4971-4989.

212. Buttery SM, Yoshida S, Pellman D. Yeast Formins Bni1 and Bnr1 Utilize Different Modes of Cortical Interaction during the Assembly of Actin Cables. *Mol Biol Cell*. 2007. 18:1826-1838.

213. Bi E. Cytokinesis in Budding Yeast: The Relationship between Actomyosin Ring Function and Septum Formation. *Cell Struct Funct*. 2001. 26(6):529-537.

214. Lippincott J, Li R. Sequential Assembly of Myosin II, an IQGAP-like Protein, and Filamentous Actin to a Ring Structure Involved in Budding Yeast Cytokinesis. *J Cell Biol*. 1998. 140(2):355-366.

215. Bi E, Maddox P, Lew DJ, et al. Involvement of an Actomyosin Contractile Ring in *Saccharomyces cerevisiae* Cytokinesis. *J Cell Biol*. 1998. 142(5):1301-1312.

216. Shannon KB, Li R. The Multiple Roles of Cyk1p in the Assembly and Function of the Actomyosin Ring in Budding Yeast. *Mol Biol Cell*. 1999. 10(2):283-296.

217. Lippincott1 J, Shannon1 KB, Shou W, Deshaies RJ, Li R. The Tem1 small GTPase controls actomyosin and septin dynamics during cytokinesis. *J Cell Sci*. 2001. 114(7):1379-1386.

218. Chin CF, Bennett AM, Ma WK, Hall MC, Yeong FM. Dependence of Chs2 ER export on dephosphorylation by cytoplasmic Cdc14 ensures that septum formation follows mitosis. *Mol Biol Cell*. 2012. 23(1):45.58.

219. Lesage G, Shapiro J, Specht CA, et al. An interactional network of genes involved in chitin synthesis in *Saccharomyces cerevisiae*. *BMC Genet*. 2005. 6(8).

220. Vallen EA, Caviston J, Bi E. Roles of Hof1p, Bni1p, Bnr1p, and Myo1p in Cytokinesis in *Saccharomyces Cerevisiae*. *Mol Biol Cell*. 2000. 11(2):593-611.

221. Reusswig K-U, Zimmermann F, Galanti L, Correspondence BP, Pfander B. Robust Replication Control Is Generated by Temporal Gaps between Licensing and Firing Phases and Depends on Degradation of Firing Factor Sld2. *Cell Rep*. 2016. 17(4):556-569.

222. Tanaka S, Umemori T, Hirai K, Muramatsu S, Kamimura Y, Araki H. CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. *Nature*. 2007. 445(7125):328-332.

223. Tanaka S, Araki H. Multiple Regulatory Mechanisms to Inhibit Untimely Initiation of DNA Replication Are Important for Stable Genome Maintenance. *PLoS Genet*. 2011. 7(6):e1002136.

224. Labib K, Diffley JFX, Kearsey SE. G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. *Nat Cell Biol*. 1999. 1(7):415-422.

225. Drury LS, Perkins G, Diffley JFX. The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. *Curr Biol*. 2000. 10(5):231-240.

226. Siddiqui K, On KF, Diffley JFX. Regulating DNA replication in eukarya. *Cold Spring Harb Perspect Biol*. 2013. 5(9):a012930.

227. Blow JJ, Dutta A. Preventing re-replication of chromosomal DNA. *Nat Rev Mol Cell Biol*. 2005. 6(6):476-486.

228. Liang J, Suhandynata RT, Zhou H. Phosphorylation of Sae2 Mediates Forkhead-associated (FHA) Domain-specific Interaction and Regulates Its DNA Repair Function. *J Biol Chem*. 2015. 290(17):10751-10763.

229. Wu J, Chen Y, Lu L-Y, et al. Chfr and RNF8 synergistically regulate ATM activation. *Nat Struct Mol Biol*. 2011. 18(7):761-768.

230. Liu C, Wu J, Paudyal SC, You Z, Yu X. CHFR is important for the first wave of ubiquitination at DNA damage sites. *Nucleic Acids Res*. 2013. 41(3):1698-1710.

231. Summers MK, Bothos J, Halazonetis TD. The CHFR mitotic checkpoint protein delays cell cycle progression by excluding Cyclin B1 from the nucleus. *Oncogene*. 2005. 24(16):2589-2598.

232. Matsusaka T, Pines J. Chfr acts with the p38 stress kinases to block entry to mitosis in mammalian cells. *J Cell Biol*. 2004. 166(4):507-516.

233. Chaturvedi P, Sudakin V, Bobiak ML, et al. Chfr regulates a mitotic stress pathway through its RING-finger domain with ubiquitin ligase activity. *Cancer Res*. 2002. 62(6):1797-1801.

234. Moore JK, Chudalayandi P, Heil-Chapdelaine RA, Cooper JA. The spindle position checkpoint is coordinated by the Elm1 kinase. *J Cell Biol*. 2010. 191(3):493-503.

235. Bieganowski P, Shilinski K, Tsichlis PN, Brenner C. Cdc123 and Checkpoint Forkhead Associated with RING Proteins Control the Cell Cycle by Controlling eIF2 Abundance. *J Biol Chem*. 2004. 279:44656-44666.

236. Loring GL, Christensen KC, Gerber SA, Brenner C. Yeast Chfr homologs retard cell cycle at G1 and G2/M via Ubc4 and Ubc13/Mms2-dependent ubiquitination. *Cell Cycle*. 2008. 7(1):96-105.

237. Vaze MB, Pellicioli A, Lee SE, et al. Recovery from Checkpoint-Mediated Arrest after Repair of a Double-Strand Break Requires Srs2 Helicase. *Mol Cell*. 2002. 10(2):373-385.

238. Schwartz DC, Cantor CR. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell*. 1984. 37(1):67-75.

239. Bartkevičiūtė D, Sasnauskas K. Disruption of the MNN10 gene enhances protein secretion in Kluyveromyces lactis and Saccharomyces cerevisiae. *FEMS Yast Res*. 2004. 4:833-840.

240. Aouida M, Pagé N, Leduc A, Peter M, Ramotar D. A Genome-Wide Screen in Saccharomyces cerevisiae Reveals Altered Transport As a Mechanism of Resistance to the Anticancer Drug Bleomycin. *CANCER Res*. 2004. 64:1102-1109.

241. Lieber MR. The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End-Joining Pathway. *Annu Rev Biochem*. 2010. 79(1):181-211.

242. San Filippo J, Sung P, Klein H. Mechanism of Eukaryotic Homologous Recombination. *Annu Rev Biochem*. 2008. 77(1):229-257.

243. Sun Z, Fay DS, Marini F, Foiani M, Stern DF. Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. *Genes Dev*. 1996. 10(4):395-406.

244. Sanchez Y, Desany BA, Jones WJ, Liu Q, Wang B, Elledge SJ. Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. *Science*. 1996. 271(5247):357-360.

245. Pellicioli A, Lucca C, Liberi G, 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*. 1999. 18(22):6561-6572.

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

247. Liu C, Pouliot JJ, Nash HA. The role of TDP1 from budding yeast in the repair of DNA damage. *DNA Repair (Amst)*. 2004. 3:593-601.

248. Stingele J, Bellelli R, Boulton SJ. Mechanisms of DNA-protein crosslink repair. *Nat Rev Mol Cell Biol*. 2017. 18(9):563-573.

249. Tamborrini D, Juanes MA, Ibanes S, Rancati G, Piatti S. Recruitment of the mitotic exit network to yeast centrosomes couples septin displacement to actomyosin constriction. *Nat. Com.* 2018. 9:1-15.

Part II

Figures & Supplementary Figures

Figure contents

Supplementary Figure contents

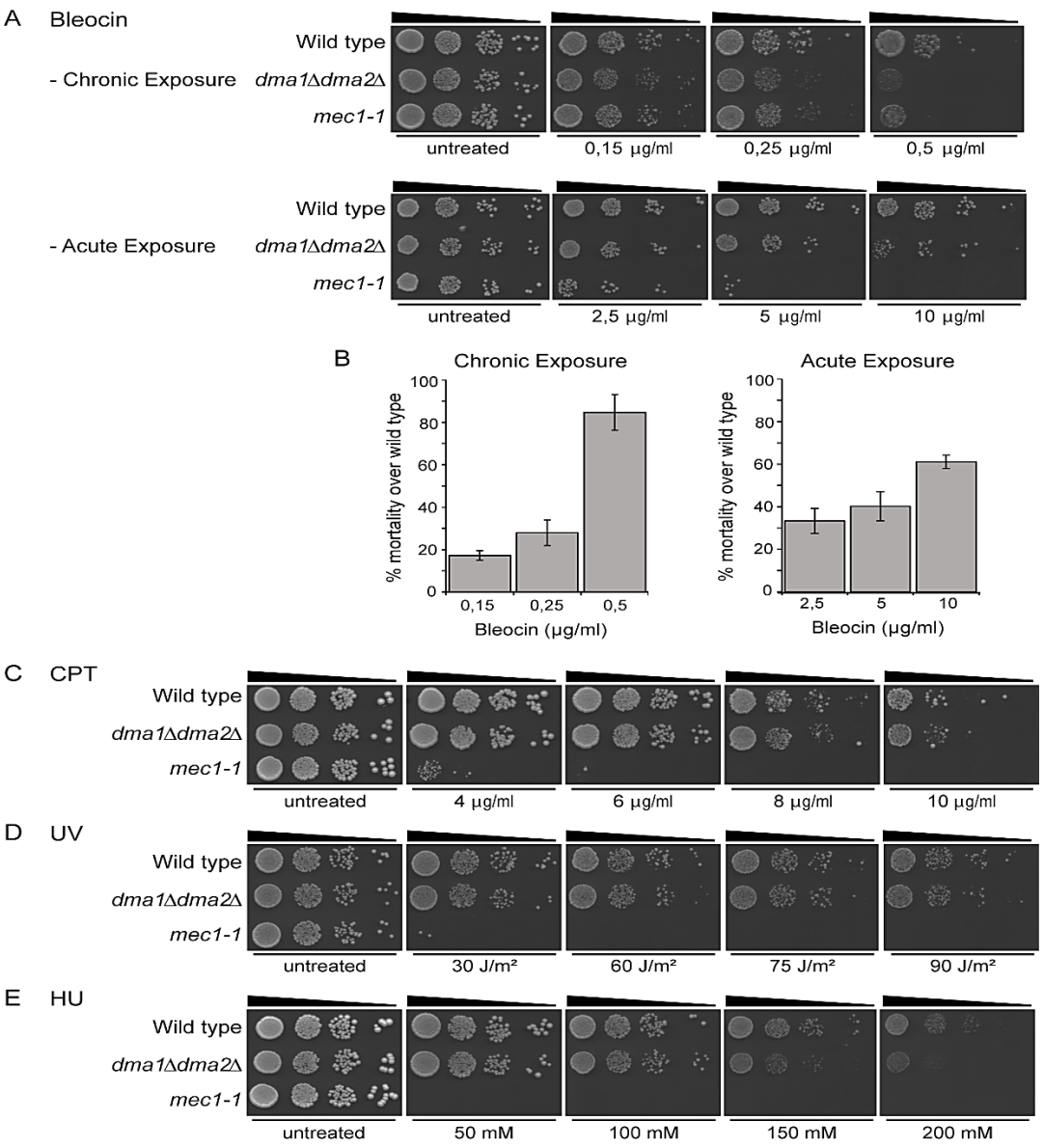


Figure 1. Sensitivity of *dma1*Δ*dma2*Δ mutant to different DNA damage inducing agents. (**A**, **C**, **E**) Serial dilutions of exponentially growing cell cultures were spotted onto YPD plates, after 30' incubation with the indicated doses of bleocin (**A**, lower panel), or plates containing the indicated doses of bleocin (**A**, upper panel), CPT (**C**) and HU (**E**). Plates were incubated 3 days at 28°C. (**D**) Serial dilutions of exponentially growing cell cultures were spotted onto YPD plates and irradiated with the indicated doses of UV. (**B**) Graphs show the percentage of mortality for the *dma1*Δ*dma2*Δ cells, compared to wild type cells, after plating on bleocin containing medium (left panel) or on YPD after acute exposure to indicated doses of bleocin (right panel).

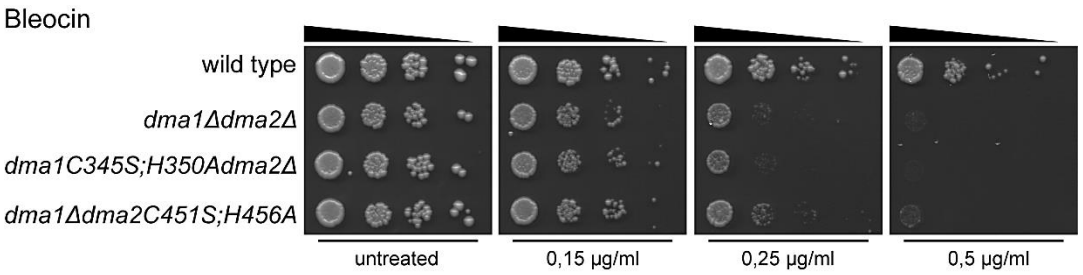


Figure 2. Tolerance to a Bleocin treatment requires the E3 ubiquitin ligase catalytic activity of the Dma1 and Dma2. Serial dilutions of exponentially growing cell cultures were spotted onto YPD or bleocin containing plates. Plates were incubated 3 days at 28°C.

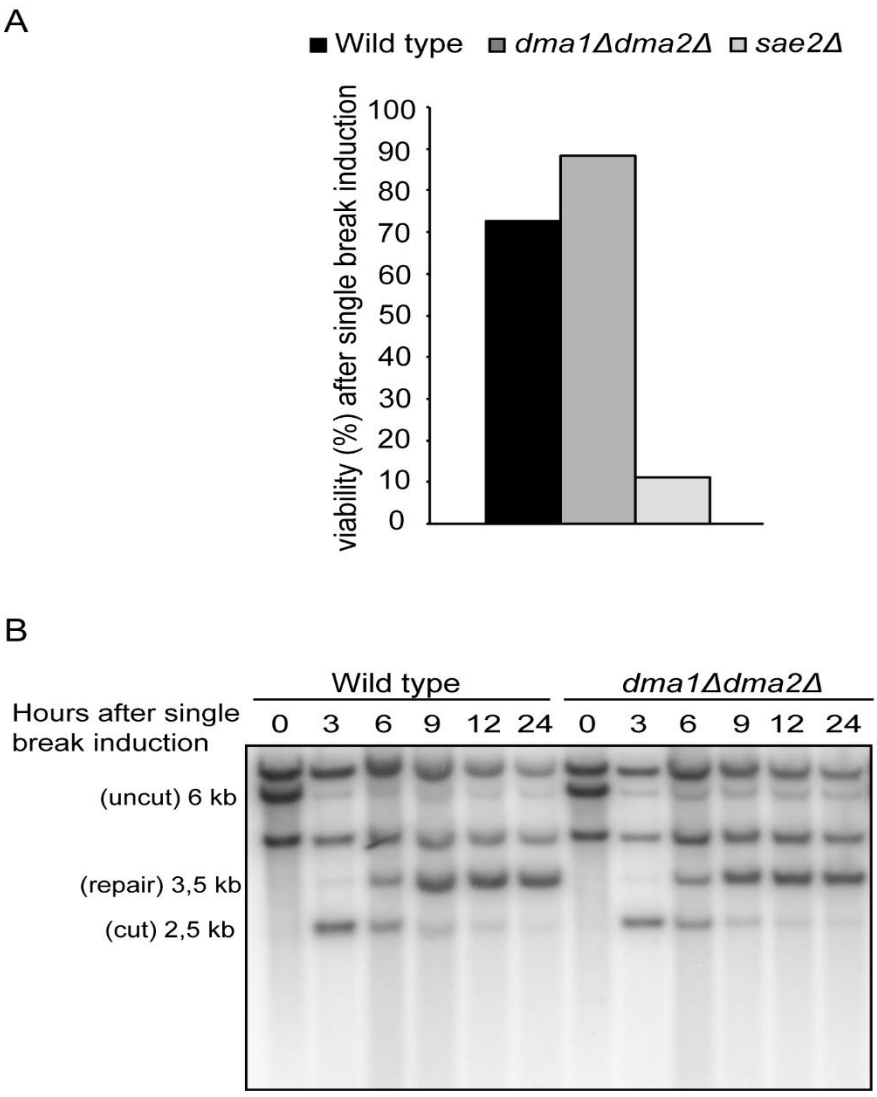


Figure 3. *dma1*Δ*dma2*Δ cells are not sensitive to a single HO-induced DSB. (**A**) exponentially growing cells were plated on both raffinose and raffinose+galactose containing medium. After 3 days at 28°C, surviving colonies on raffinose+galactose plates were manually counted and normalized respect to raffinose. (**B**) Repair analysis by Southern blot in M arrested cells. Galactose was added at time 0 to induced HO-cleavage and the appearance of the repair product was monitored by using a *LEU2* probe.

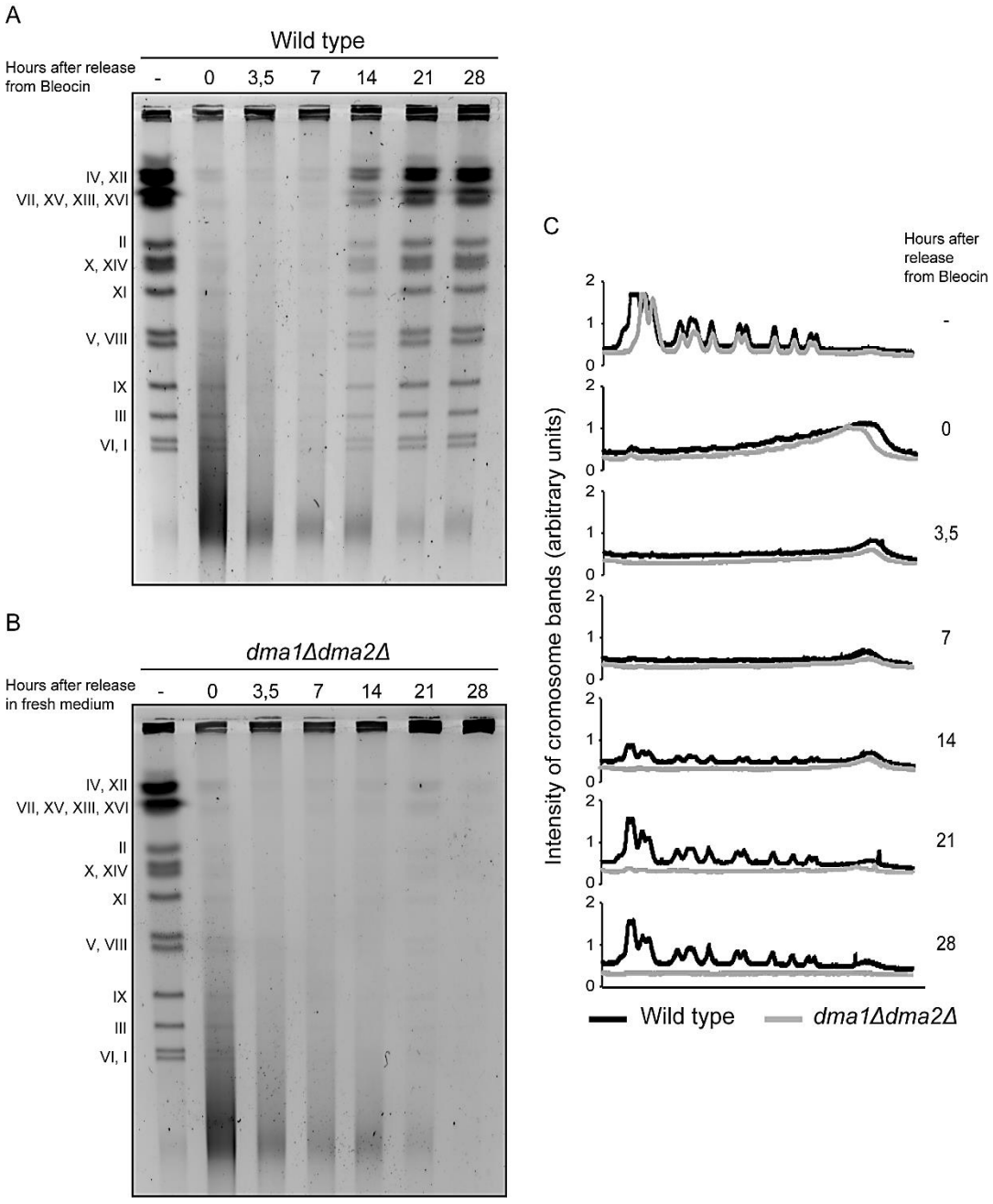


Figure 4. *dma1*Δ*dma2*Δ cells are not able to correctly repair chromosomes after bleocin treatment. (**A**-**B**) Chromosome analysis by PFGE in exponentially growing cell cultures at the indicated time point after bleocin treatment. Cells were treated with 100 µg/ml bleocin for 30', washed and then released in fresh media (time 0). (**C**) Graphs represent intensity picks corresponding to each chromosome bands.

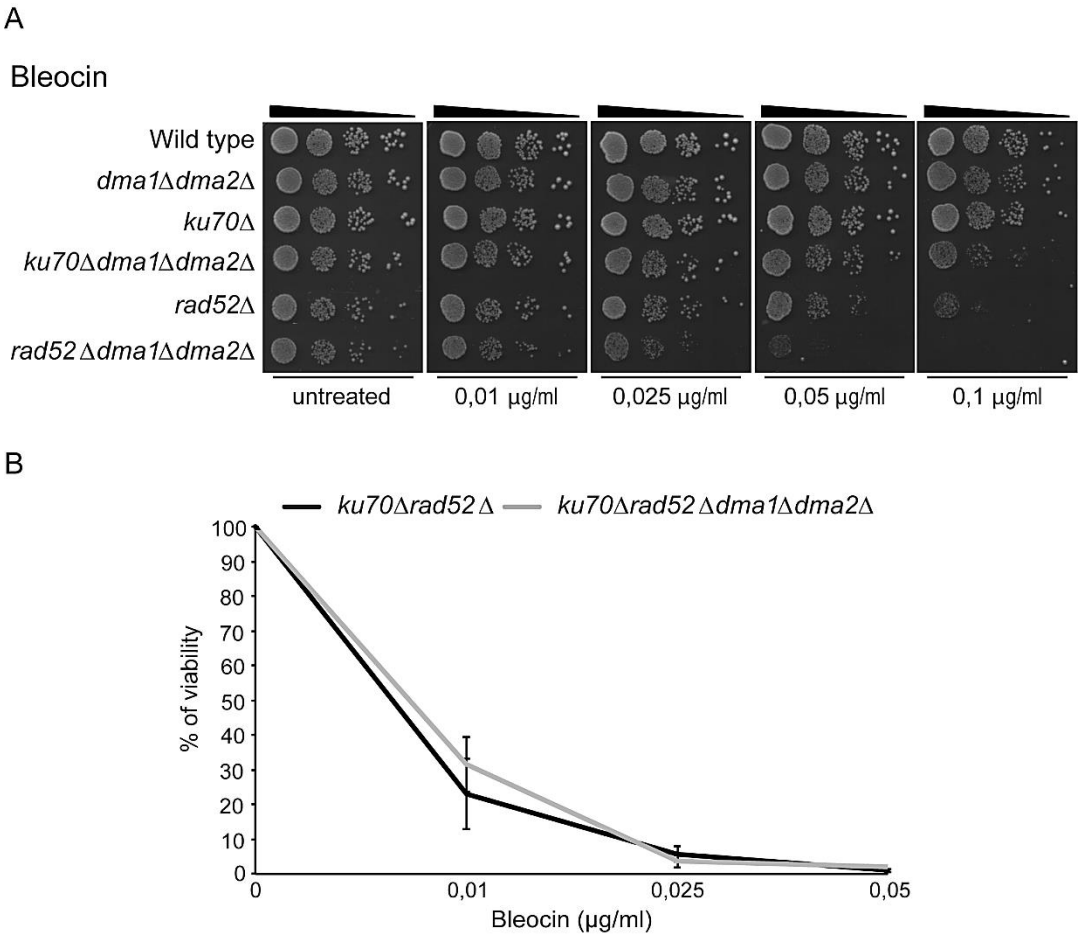


Figure 5. Double deletion of *DMA1* and *DMA2* genes increases bleocin sensitivity of both *rad52*Δ and *ku70*Δ cells. (**A**) Serial dilutions of exponentially growing cell cultures were spotted onto YPD plates or plates containing bleocin. Plates were incubated 3 days at 28°C. (**B**) Graph shows the percentage of cell viability after plating on bleocin containing medium. Error bars represent SEM, based on three independent experiments.

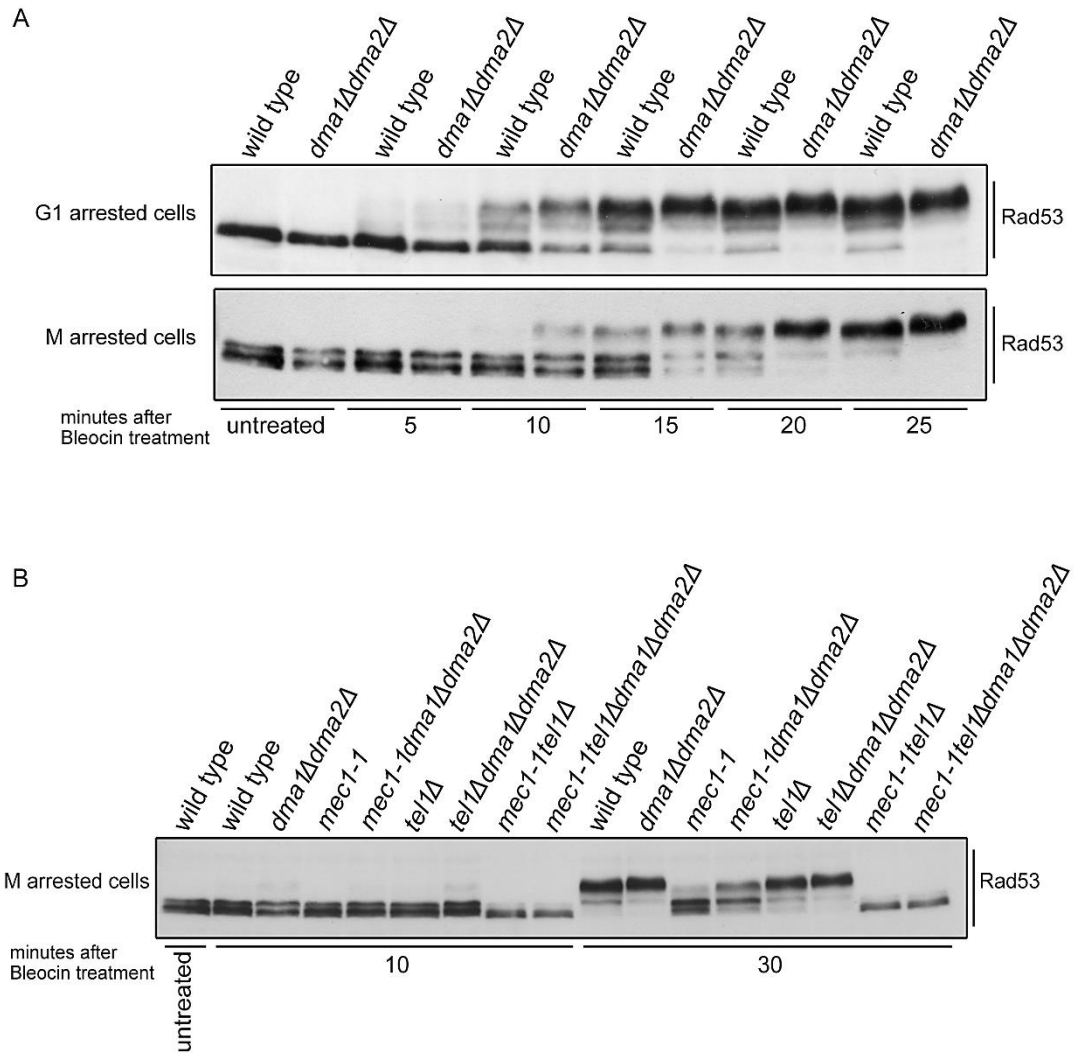


Figure 6. Double deletion of *DMA1* and *DMA2* genes causes a hyperactivation of DNA damage checkpoint, dependent on a hyperactivation of the apical kinase Tel1. (**A**-**B**) Rad53 phosphorylation analysis by Western Blot in G1 arrested cells by α factor (**A**, upper panel) or M arrested cells by nocodazole (**A**, lower panel, and **B**) after bleocin (100 µg/ml) treatment.

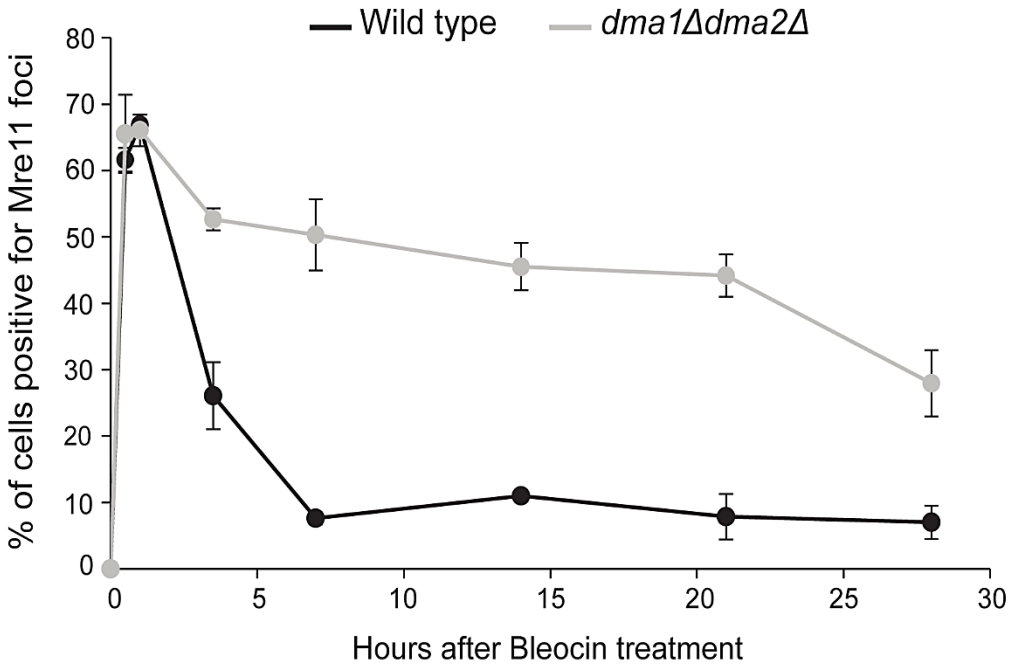


Figure 7. In *dma1*Δ*dma2*Δ cells there is a persistence of Mre11 foci after bleocin treatment. Graph shows the kinetics of Mre11 foci formation after bleocin (100 µg/ml) treatment, obtained scoring around 150 cells for each strains at each time points. Error bars represent SEM, based on three independent experiments.

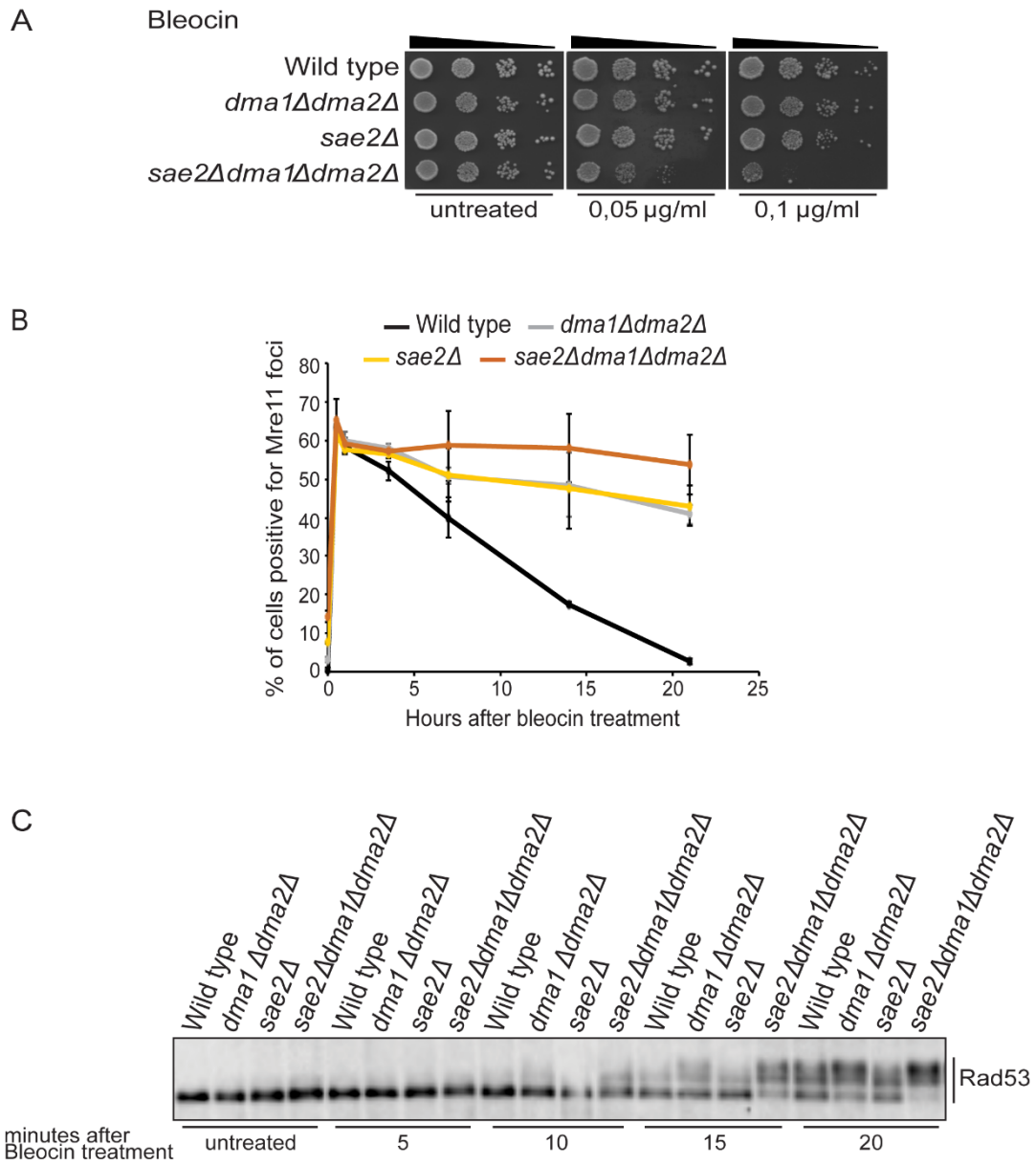


Figure 8. Dma1 and Dma2 depletion shows an additive effect with the *SAE2* gene deletion. (**A**) Serial dilutions of exponentially growing cell cultures were spotted onto YPD or bleocin containing plates. Plates were incubated 3 days at 28°C. (**B**) Graph shows the kinetics of Mre11 foci formation after bleocin (100 µg/ml) treatment, obtained scoring around 150 cells for each strains at each time points. Error bars represent SEM, based on three independent experiments. (**C**) Rad53 phosphorylation analysis by Western Blot after bleocin (100 µg/ml) treatment.

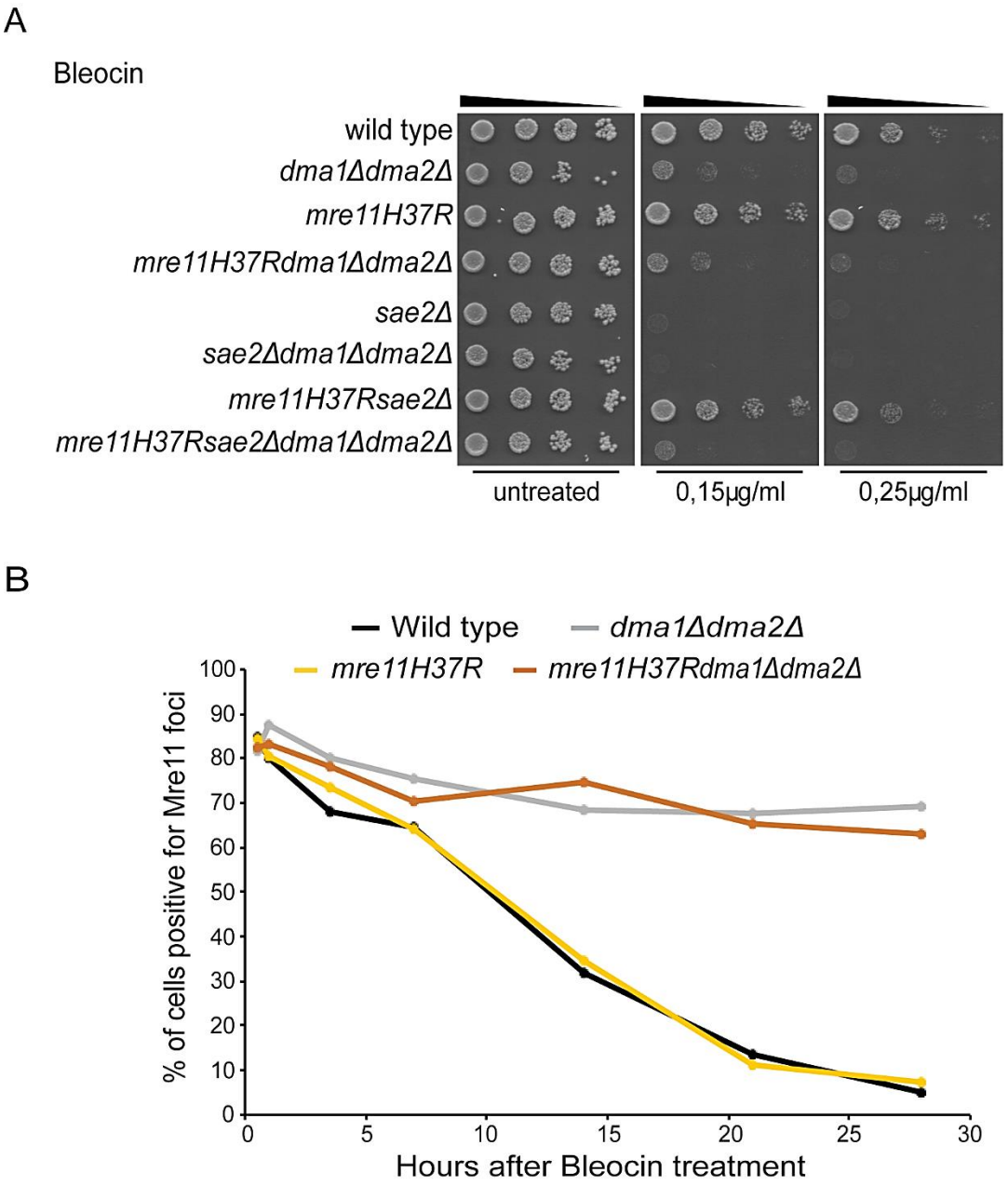


Figure 9. The *mre11*^{H37R} does not recover *dma1*Δ*dma2*Δ phenotypes. (**A**) Serial dilutions of exponentially growing cell cultures were spotted onto YPD or bleocin containing plates. Plates were incubated 3 days at 28°C. (**B**) Graph shows the kinetics of Mre11 foci formation after bleocin (100 µg/ml) treatment, obtained scoring around 150 cells for each strains at each time points.

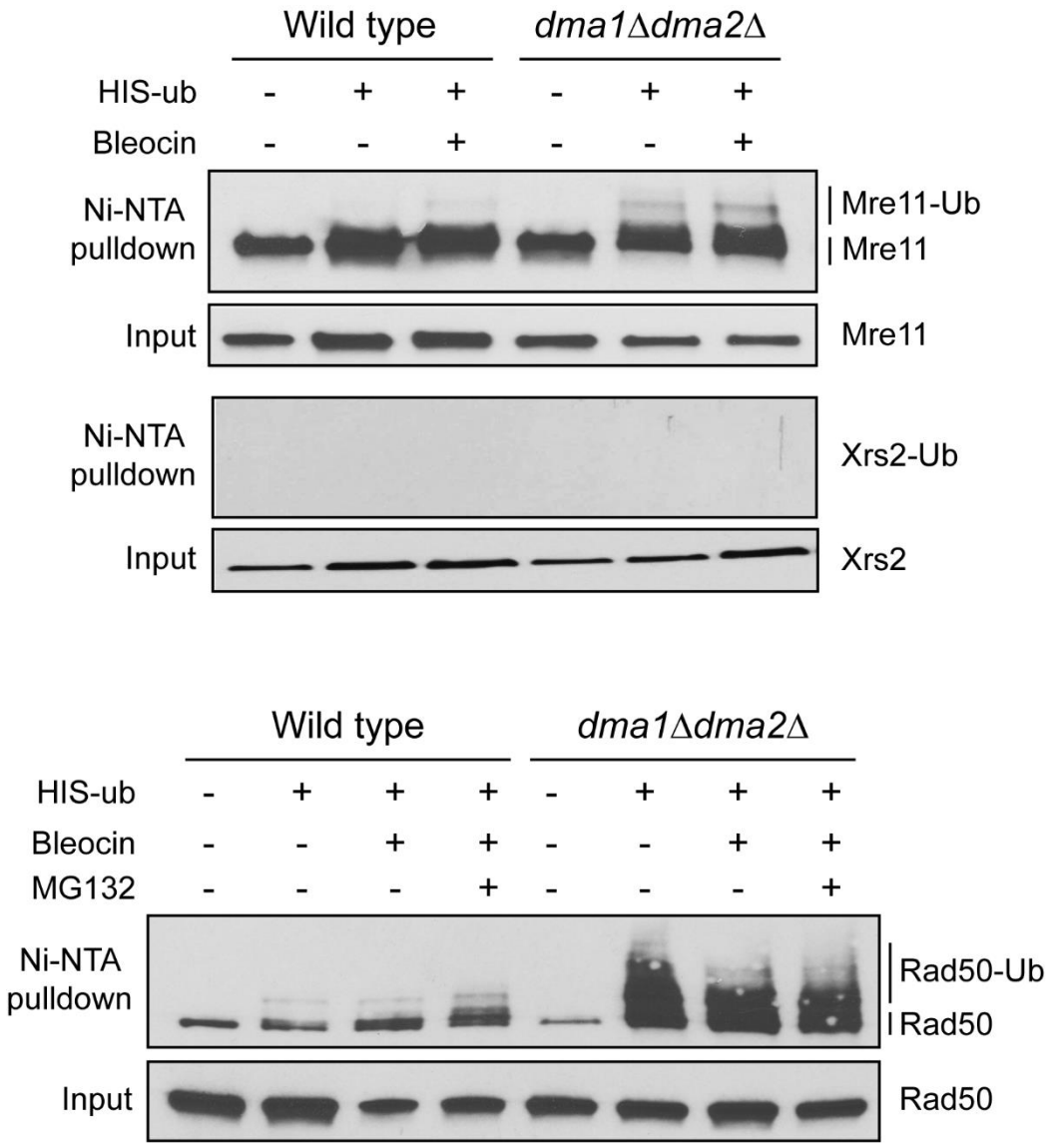


Figure 10. ***In vivo*** **ubiquitination assay of three MRX complex subunits**. Exponentially growing cells containing a high-copy number plasmid carrying untagged or HIS-tagged ubiquitin version were treated for 3 hours with 250 µM $CuSO_4$, to induce ubiquitin overproduction, and with or without 100 µg/ml bleocin and 75 µM proteasome inhibitor MG132. Denaturing protein extracts were prepared and incubated with Ni-NTA agarose beads to pulldown all the protein bound to HIS-ubiquitin. Protein eluates were separated by SDS-PAGE and MRX complex ubiquitination levels were visualized by Western blot analysis.

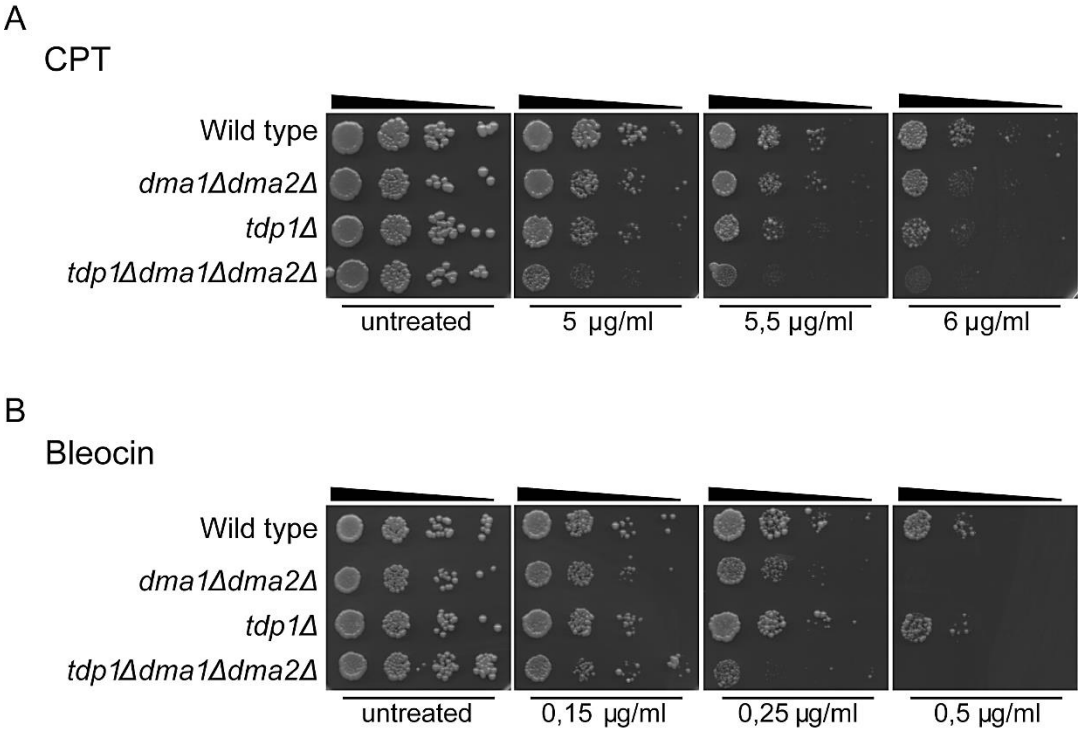
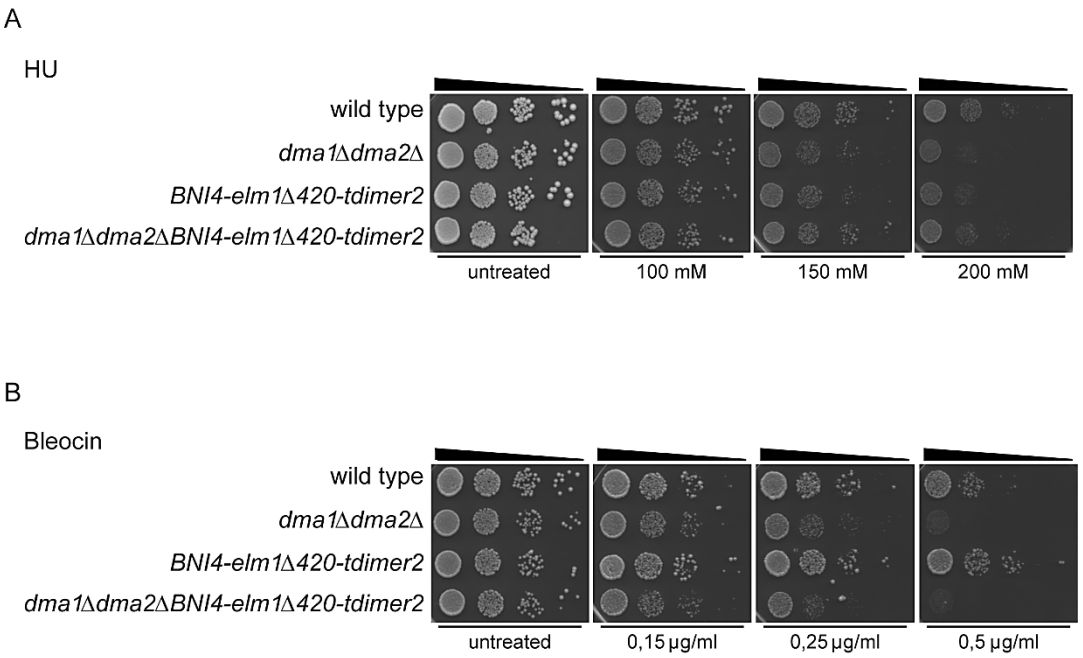
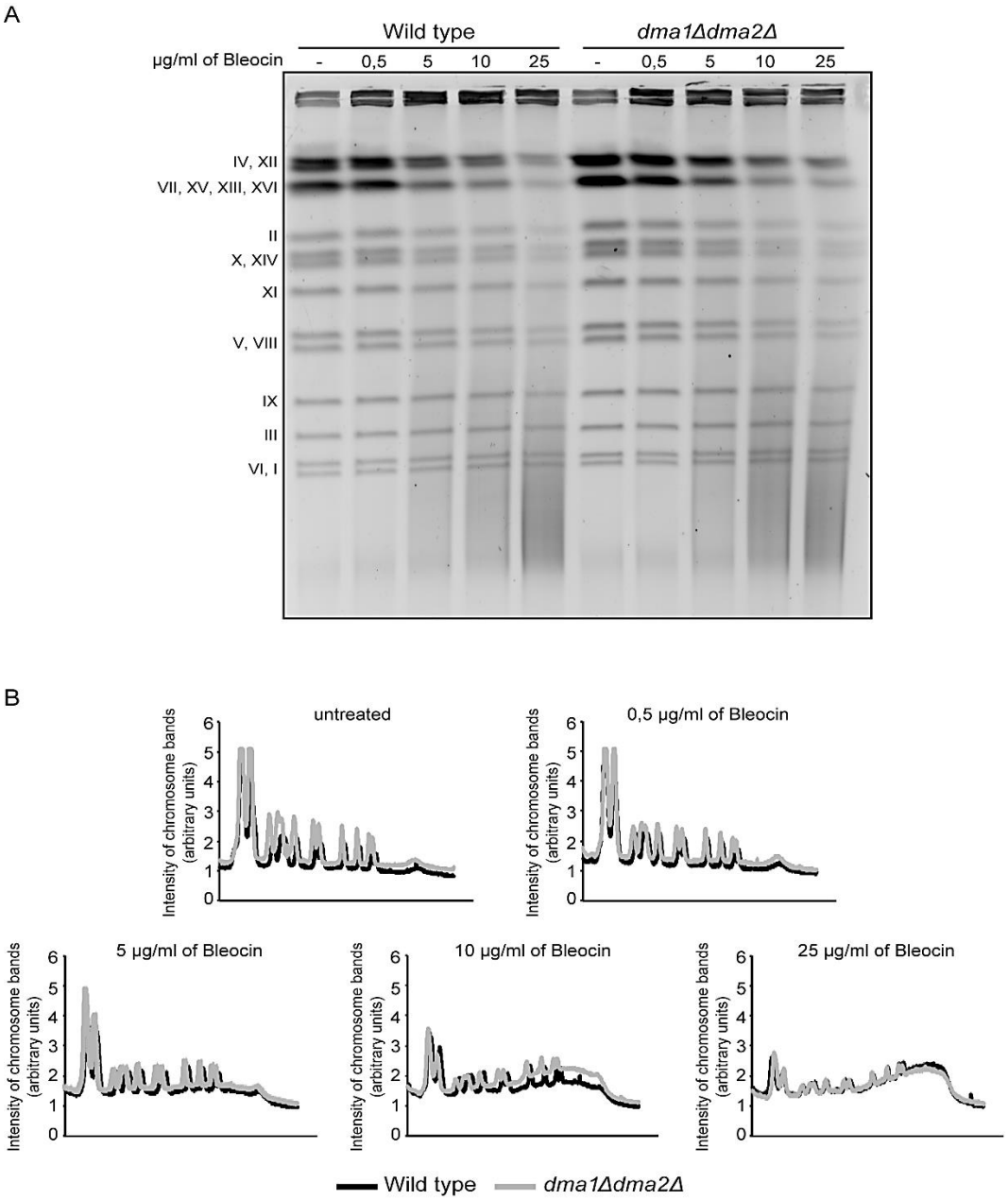


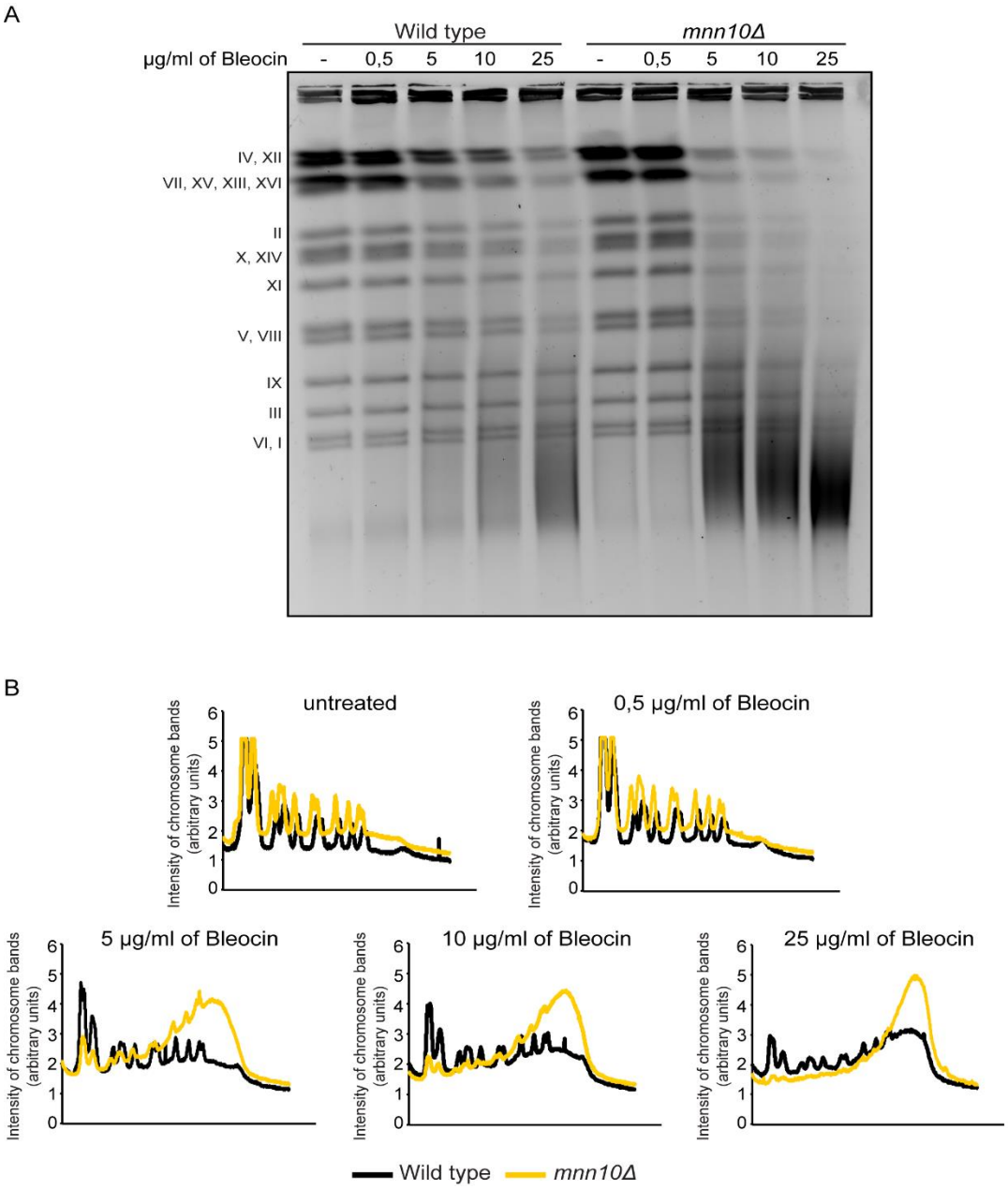
Figure 11. Dma1 and Dma2 cooperate with Tdp1 in dirty end cleaning. (**A** and **B**) Serial dilutions of exponentially growing cell cultures were spotted onto YPD plates or plates containing CPT (**A**) or bleocin (**B**). Plates were incubated 3 days at 28°C.



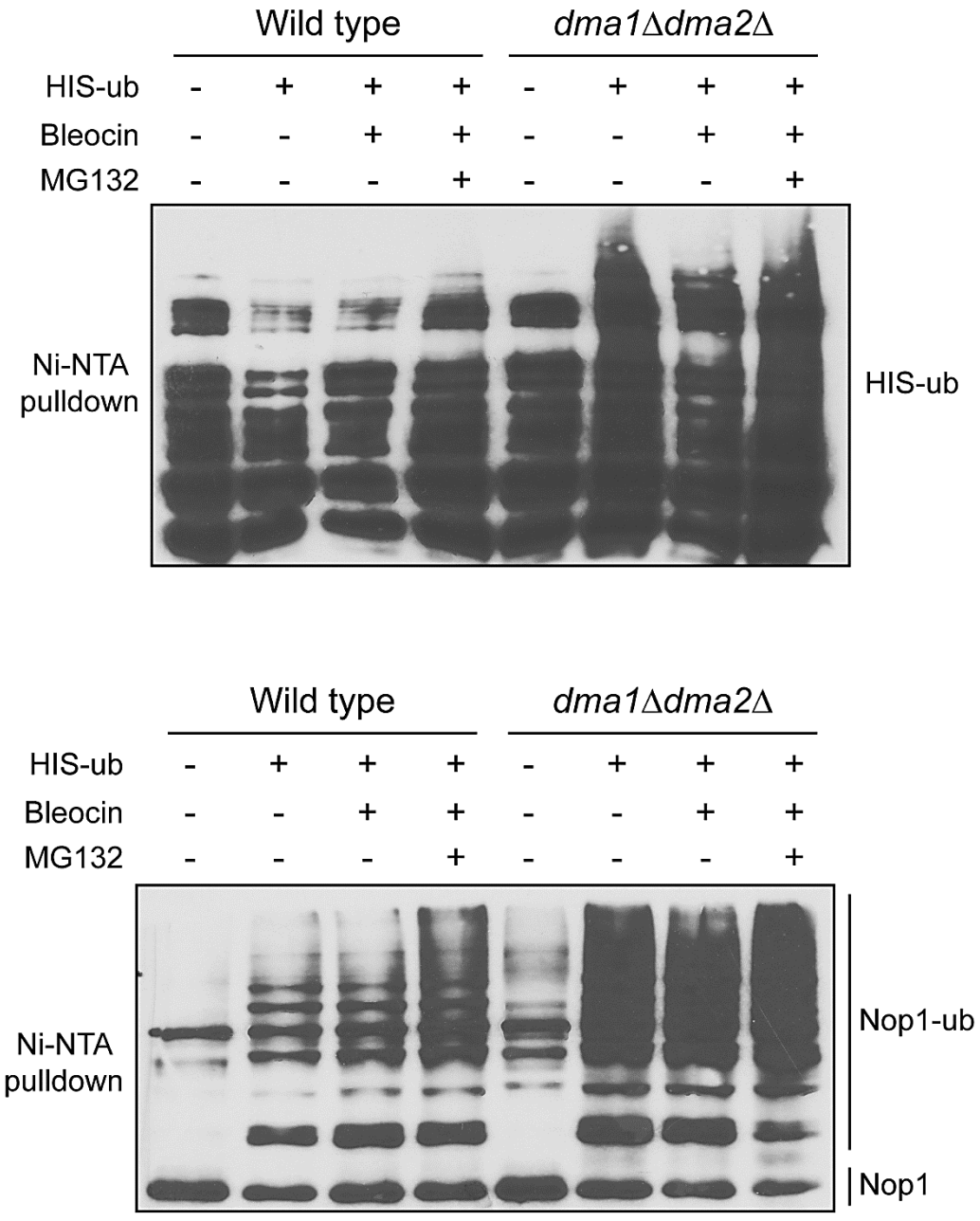
Supplementary Figure 1. Bleocin sensitivity of *dma1*Δ*dma2*Δ cells is not rescued bypassing its defects in spindle position checkpoint. (**A** and **B**) Serial dilutions of exponentially growing cell cultures were spotted onto YPD plates or plates containing HU (**A**) or bleocin (**B**). Plates were incubated 3 days at 28°C.



Supplementary Figure 2. The *dma1*Δ*dma2*Δ mutant shows same levels of chromosome fragmentation as wild type cells after treatment with low doses of bleocin. (**A**) Chromosome analysis by PFGE in exponentially growing cell cultures after treatment with the indicated doses of bleocin. (**B**) Graphs represent intensity peaks corresponding to each chromosome bands.



Supplementary Figure 3. Cells lacking Mnn10 enzyme have significantly higher chromosome fragmentation compared to control cells after treatment with the same low doses of bleocin. (**A**) Chromosome analysis by PFGE in exponentially growing cell cultures after treatment with the indicated doses of bleocin. (**B**) Graphs represent intensity peaks corresponding to each chromosome bands.



Supplementary Figure 4. Cells lacking Dma proteins shows a general increase in the HIS-ubiquitin expression. The membrane of the Ni-NTA pulldown performed for the Rad50 subunit of the MRX complex were incubated with both αHIS and αNop1 antibodies as a control for HIS-ubiquitin production and protocol success.

Materials & Methods

Table of yeast strains

Strain	Genotype
SY2080	MATa
YMAG380/1C	MATa *dma1::TRP dma2::LEU*
YMAG800	MATa *dma1::KanMX dma2::LEU*
YMAG482/2C	MATa *BNI4elm1-420tdimer::KanMX*
YMAG484/1D	MATa *BNI4elm1-420tdimer::KanMX dma1::TRP dma2::LEU*
YMAG463	MATa *ku70::KanMX*
YMAG481/3B	MATa *rad52::HPH*
YMAG515/2B	MATa *ku70::KanMX rad52::HPH*
YMAG465	MATa *ku70::KanMX dma1::TRP dma2::LEU*
YMAG444/8B	MATa *rad52::HPH dma1::TRP dma2::LEU*
YMAG511/4A	MATa *ku70::KanMX rad52::HPH dma1::TRP dma2::LEU*
YNOV188/3A	MATa *mec1-1 sml1*
YMAG572/6B	MATa *tel1::NAT*
YMAG875/4C	MATα *mec1-1 sml1 tel1::NAT*
YMAG712/2B	MATa *mec1-1 sml1 dma1::TRP dma2::LEU*
YMAG581/2A	MATa *tel1::NAT dma1::TRP dma2::LEU*

YMAG874/30A	MATa *mec1-1 sml1 tel1::NAT dma1::TRP dma2::LEU*
YMAG1073/2A	MATa *dma1C$^{345S;H350A}$*
YMAG1193/2B	MATa *dma2C$^{451S;H456A}$*
YMAG1069/1A	MATa *dma1$^{C345S;H350A}$ dma2::LEU*
YMAG1197/1B	MATa *dma1::TRP dma2C$^{451S;H456A}$*
YFP1177/2D	MATa *ADE2 bar1::LEU trp1-1 LYS2 RAD5 mre11::MRE11-LLAKKRKG-YFP*
YMAG878	MATa *ADE2 bar1::LEU trp1-1 LYS2 RAD5 mre11::MRE11-LLAKKRKG-YFP dma1::KanMX dma2::HPH*
YMAG1297	MATa *ADE2 bar1::LEU trp1-1 LYS2 RAD5 mre11:: MRE11^{H37R}-LLAKKRKG-HIS::KanMX-YFP*
YMAG1298	MATa *ADE2 bar1::LEU trp1-1 LYS2 RAD5 mre11:: MRE11^{H37R}-LLAKKRKG-HIS::KanMX-YFP dma1::KanMX dma2::HPH*
YMAG1308	MATa *ADE2 bar1::LEU trp1-1 LYS2 RAD5 mre11::MRE11-LLAKKRKG-YFP sae2::HIS*
YMAG1305	MATa *ADE2 bar1::LEU trp1-1 LYS2 RAD5 mre11:: MRE11^{H37R}-LLAKKRKG-HIS::KanMX-YFP sae2::HIS*
YMAG1310	MATa *ADE2 bar1::LEU trp1-1 LYS2 RAD5 mre11::MRE11-LLAKKRKG-YFP dma1::KanMX dma2::HPH sae2::HIS*
YMAG1307	MATa *ADE2 bar1::LEU trp1-1 LYS2 RAD5 mre11:: MRE11^{H37R}-LLAKKRKG-HIS::KanMX-YFP dma1::KanMX dma2::HPH sae2::HIS*
YMAG1109/8C	MATa *sae2::HPH*
YMAG1106	MATa *dma1::TRP dma2::LEU sae2::HPH*
YMAG1128/1A	MATa *tdp1::URA*
YMAG1135/2B	MATa *tdp1::URA dma1::TRP dma2::LEU*
YMAG854	MATa *MRE11-13Myc::HIS [2μ, TRP1-CUP1-UBI4]*

YMAG856	MATa *MRE11-13Myc::HIS [2μ, TRP1-CUP1-6xHIS-UBI4]*
YMAG858	MATa *dma1::KanMX dma2::LEU MRE11-13Myc::HIS [2μ, TRP1-CUP1-UBI4]*
YMAG860	MATa *dma1::KanMX dma2::LEU MRE11-13Myc::HIS [2μ, TRP1-CUP1-6xHIS-UBI4]*
YMAG862	MATa *RAD50-13Myc::HIS [2μ, TRP1-CUP1-UBI4]*
YMAG864	MATa *RAD50-13Myc::HIS [2μ, TRP1-CUP1-6xHIS-UBI4]*
YMAG866	MATa *dma1::KanMX dma2::LEU RAD50-13Myc::HIS [2μ, TRP1-CUP1-UBI4]*
YMAG868	MATa *dma1::KanMX dma2::LEU RAD50-13Myc::HIS [2μ, TRP1-CUP1-6xHIS-UBI4]*
YMAG986	*XRS2-13Myc::HIS [2μ, TRP1-CUP1-UBI4]*
YMAG988	*XRS2-13Myc::HIS [2μ, TRP1-CUP1-6xHIS-UBI4]*
YMAG990	MATa *dma1::KanMX dma2::LEU XRS2-13Myc::HIS [2μ, TRP1-CUP1-UBI4]*
YMAG992	MATa *dma1::KanMX dma2::LEU XRS2-13Myc::HIS [2μ, TRP1-CUP1-6xHIS-UBI4]*
Y1601	*ho hml ::ADE1 mata ::hisG hmr ::ADE1 his4::NatMXleu2-(XhoI-to Asp718) leu2::MATa ade3::GAL::HO ade1 lys5 ura3-52 trp1*
YSP13	*ho hml ::ADE1 mata ::hisG hmr ::ADE1 his4::NatMXleu2-(XhoI-to Asp718) leu2::MATa ade3::GAL::HO ade1 lys5 ura3-52 trp1 dma1::KanMX dma2::HPH*
Y78	*ho hml ::ADE1 mata ::hisG hmr ::ADE1 his4::NatMXleu2-(XhoI-to Asp718) leu2::MATa ade3::GAL::HO ade1 lys5 ura3-52 trp1 sae2::KanMX*

Most strains are derivatives of W303 background (*trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112 ade2-1 RAD5*). The full genotypes are shown for all of the non-W303 strains.

Yeast strains

Strains used in this study are listed in the Yeast strain table. Deletion and tag fusions were generated using one-step PCR system followed by standard genetic procedures of transformation or tetrad analysis. YMAG1073/2A, YMAG1193/2B, YMAG1069/1A and YMAG1197/1B were created using the Delitto Perfetto approach (Storici et al, 2001). The correct insertion of mutations were controlled by sequencing. YMAG1297, YMAG1298, YMAG1305 and YMAG1307 were obtained integrating the HpaI-digested pFP118.1 plasmid (Puddu et al, 2015) at the *MRE11* locus. Pop-out events were selected on FOA plates and the insertion of H37R mutation were checked by sequencing.

Bleocin, CPT, HU and UV sensitivity assay

To assess cellular sensitivity after a chronic exposure to different DNA damage inducing agents, serial dilutions of exponentially growing cultures were spotted on either YPD or CPT, Bleocin, HU containing media at the indicated doses; for UV treatment cells were spotted on YPD plates and then irradiated with different UV doses. Instead, sensitivity assay after acute exposure were performed treating exponentially growing cells with different bleocin doses for 30 minutes and then spotting serial dilutions of treated cells on YPD plates.

For mortality graphs and viability curve, approximately 500 cells/plate of exponentially growing cultures, untreated or exposed to a bleocin acute treatment, were plated on bleocin containing media at the indicated doses or on YPD plates, respectively. The total number of colonies were manually counted after 3 days at 28°C.

Chromosome analysis by Pulsed-Field Gel Electrophoresis

Exponentially growing cells were treated with 100 µg/ml of bleocin for 30 minutes

and, then, were washed and released in fresh YPD medium. At each time points, 40 ml of cells at $3x10^6$ cells/ml were collected and processed for chromosome separation by PFGE as described in Giannattasio et al, 2010.

Checkpoint experiments

Exponentially growing cells were arrested in G1 (2 µg/ml α-factor) or in M (20 µg/ml nocodazole) phases and treated with 100 µg/ml of bleocin. At the indicated time points after bleocin treatment, 15 ml of cells at $1,3x10^7$ were collected and TCA protein extracts were prepared as described in Muzi-Falconi et al, 1993. TCA extracts were separated by SDS-PAGE in 10% poly-acrylamide gels (acrylamide/bis-acrylamide, 75:1). Western blot analysis were performed using αRad53 antibodies (rabbit monoclonal antibodies, diluted 1:4500 in 5% milk in PBSt), after an incubation of 3 hours.

Mre11 foci visualization

Exponentially growing cells carrying wild-type or mutant Mre11-YFP were treated for 30 minutes with 100 µg/ml of bleocin, washed and, then, resuspended in fresh YPD medium. At the indicated time points, samples were taken and fixed with 3,7% formaldehyde for 15 minutes. Cells were subsequently washed twice with PBS and immediately mounted on a glass slide.

In vivo ubiquitination assay

Cells carrying an untagged or 6xHIS-tagged ubiquitin version were grown in selective medium to reach the concentration of $6x10^6$. The 6xHIS-ubiquitin overexpression were induced for 3 hours by adding 250 µM $CuSO_4$. At the end of incubation, cells were collected, washed and shifted to YPD fresh medium. Then, all the yeast cultures were treated for 30 minutes with 100 µg/ml of bleocin and,

were indicated, added with 75 µM MG132 (Sigma-Aldrich). After treatment, cells were washed in water and protein extracts were prepared under denaturing conditions as described in Yaffe and Schatz, 1984. Protein precipitations were solubilized in Buffer A (6 M Guanidine HCl, 100 mM $NaPO_4$ pH8, 10 mM Tris-HCl pH8) for 4 hours and cell debris were then removed by centrifugation. A Ni-pulldown were performed incubating protein extracts with Ni-NTA agarose beads (Qiagen) over-night at room temperature, in presence of 15 mM Imidazole and 0,005% Tween 20. Agarose beads were recovered the day after by slow centrifugation and washed two times with Buffer A and three times with Buffer C (8 M Urea, 100 mM $NaPO_4$ pH6,3, 10 mM Tris-HCl pH6,3), both added with 0,005% Tween 20. Proteins were eluted using 30 µl of HU Buffer (8 M Urea, 200 mM Tris-HCl pH 6,8, 1 mM EDTA, 5% SDS, 0,1% bromophenol blue, 1,5% DTT) and separated by SDS-PAGE in a 4-20% gradient poly-acrylamide gel (Novex). MRX subunit modifications were visualized by Western blot analysis using αMyc antibodies (mouse monoclonal antibodies, diluted 1:30 in 5% milk in PBSt). 6xHIS-ubiquitin expression and protocol success were controlled by incubation with both αHIS and αNop1 antibodies.

Single break analysis

YMV80 derivative strains were grown O/N at 28°C in raffinose containing medium. Cells were then normalized and approximately 500 cells/plate were plated on raffinose or galactose containing plates, respectively. The total number of colonies were manually counted after 3 days at 28°C.

For monitoring of the HO-induced DSB repair, 10^7 cell/ml cultures were arrested in M phase (20 µg/ml nocodazole) and 2% galactose was added to induce HO nuclease expression. Starting from galactose induction, 50 ml of cells were collected at the indicated time points and the purified genomic DNA was analyzed as described in Vaze et al., 2002.