

Ku prevents Exo1 and Sgs1-dependent resection of DNA ends in the absence of a functional MRX complex or Sae2

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In this study, we investigate the interplay between Ku, a central non-homologous end-joining component, and the Mre11–Rad50–Xrs2 (MRX) complex and Sae2, end-processing factors crucial for initiating 5′-3′ resection of double-strand break (DSB) ends. We show that in the absence of end protection by Ku, the requirement for the MRX complex is bypassed and resection is executed by Exo1. In contrast, both the Exo1 and Sgs1 resection pathways contribute to DSB processing in the absence of Ku and Sae2 or when the MRX complex is intact, but functionally compromised by elimination of the Mre11 nuclease activity. The ionizing radiation sensitivity of a mutant defective for extensive resection (*exo1Δ sgs1Δ*) cannot be suppressed by the *yku70Δ* mutation, indicating that Ku suppression is specific to the initiation of resection. We provide evidence that replication-associated DSBs need to be processed by Sae2 for repair by homologous recombination unless Ku is absent. Finally, we show that the presence of Ku exacerbates DNA end-processing defects established in the *sae2Δ sgs1Δ* mutant, leading to its lethality.

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Introduction

DNA lesions arise spontaneously during normal cell metabolism or after treatment with DNA-damaging agents. Among these lesions, DNA double-strand breaks (DSBs) are considered the most deleterious and if unrepaired or repaired inappropriately, they can lead to mutagenic events, such as chromosome loss, deletions, duplications or translocations. DSBs are repaired through non-homologous end joining (NHEJ), which directly rejoins DNA ends with no or limited homology, or by homologous recombination (HR), which requires a homologous template for repair and generally preserves genetic information at the break site. In

Saccharomyces cerevisiae, both pathways require the Mre11–Rad50–Xrs2 (MRX) complex, which is rapidly recruited to DSBs and signals checkpoint activation through the Tel1/ATM kinase, tethers DNA ends and regulates the initiation of 5′-3′ resection (Stracker *et al*, 2004; Mimitou and Symington, 2009). In addition, NHEJ requires Ku (a heterodimer encoded by the *YKU70* and *YKU80* genes in *S. cerevisiae*), Lif1, Nej1 and Dnl4 (DNA ligase IV), whereas HR requires proteins encoded by the *RAD52* epistasis group genes (Krogh and Symington, 2004; Daley *et al*, 2005).

The choice of the repair pathway used to repair DSBs is highly regulated to ensure that the cell engages the most appropriate one, thus optimizing genome stability. This is corroborated by the finding that certain types of DSB repair such as V(D)J recombination and meiotic recombination are linked to specific repair pathways (Keeney, 2001; Lee *et al*, 2004). The types of ends generated and the cell cycle stage are critical determinants governing the choice between repair pathways. NHEJ is the predominant pathway in G1, whereas HR is activated during S/G2 (Moore and Haber, 1996; Karathanasis and Wilson, 2002; Aylon *et al*, 2004; Ira *et al*, 2004; Barlow *et al*, 2008). One step where cell cycle control is exerted by cyclin-dependent kinases (CDK) is the 5′-3′ nucleolytic degradation of DNA ends, which generates 3′ single-stranded DNA (ssDNA) tails, the substrate for binding by the Rad51 protein to initiate HR (Aylon *et al*, 2004; Ira *et al*, 2004; Zierhut and Diffley, 2008). In *S. cerevisiae*, end resection takes place by a two-step mechanism. Initially, the MRX complex with Sae2 endonuclease catalyse the removal of a short oligonucleotide(s) from the 5′ ends of the break. In the second step, the short 3′ overhangs created are further processed by two alternative pathways, one dependent on the 5′-3′ exonuclease Exo1 and the other dependent on the Sgs1 helicase and Dna2 helicase/endonuclease (Gravel *et al*, 2008; Mimitou and Symington, 2008; Zhu *et al*, 2008). Sae2 is directly phosphorylated by CDK activating the initiation of end processing; in addition, nuclear entry of Dna2 during S-phase is regulated by CDK (Huertas *et al*, 2008; Kosugi *et al*, 2009).

The MRX complex and Ku rapidly, and almost simultaneously, bind independently to DNA ends after DSB formation (Wu *et al*, 2008). Mre11 exhibits exo- and endonuclease activities that are required for processing of meiotic DSBs, a subset of ionizing radiation (IR)-induced DSBs and DNA hairpins, but are dispensable for NHEJ, telomere maintenance and processive 5′-3′ resection of DNA ends generated by HO endonuclease (Bressan *et al*, 1998; Moreau *et al*, 1999; Rattray *et al*, 2001; Lobachev *et al*, 2002; Llorente and Symington, 2004). Ku requires a free DSB end for binding and once bound protects ends and mediates recruitment of downstream NHEJ factors (Daley *et al*, 2005). The dissociation of Ku from DSB ends *in vivo* is dependent on MRX and the timing correlates with bulk resection in preparation of HR (Wu *et al*, 2008). Several lines of evidence suggested that Ku

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dissociation is not merely a *de facto* result of resection, but instead is required to allow resection to occur. Deletion of *YKU70* was shown to increase resection initiation both at DSBs and telomeres (Lee *et al.*, 1998; Maringele and Lydall, 2002; Clerici *et al.*, 2008), partially rescue the IR and methylmethane sulphonate (MMS) hypersensitivity observed in *mre11Δ* and *rad50Δ* mutants (Bressan *et al.*, 1999; Wasko *et al.*, 2009) and increase Rfa1 foci formation in response to I-SceI-induced DSBs during G1 (Barlow *et al.*, 2008). Similarly, *sae2Δ* and *mre11* nuclease-defective mutants exhibit persistent Mre11 and Sae2 foci at DSBs, supporting a more general mechanism by which MRX-Sae2 regulate protein turnover at the DNA ends (Lisby *et al.*, 2004).

These observations suggest that the first step of end resection executed by MRX-Sae2 serves to create a substrate less suitable for Ku binding thus committing cells to extensive resection and HR. To test this hypothesis, we combined genetic and physical assays to determine whether the loss of the first step in DSB resection can be rescued by concomitant loss of Ku. Indeed, we show that the DNA damage sensitivity of mutants defective for resection initiation, but not bulk resection, is suppressed in the absence of Ku. Exo1 and Sgs1, which are required for extensive resection, are responsible for this suppression. Finally, we show that the lethality of the *sae2Δ sgs1Δ* mutant can be bypassed by the *yku70Δ* mutation or by high-copy expression of *EXO1*, but not by the *dnl4Δ* mutation. These findings suggest that Ku inhibits growth by blocking access to Exo1 preventing resection in strains lacking Sae2 and Sgs1, and not by promoting lethal end-joining events.

Results

Suppression of the radiation sensitivity of *mre11Δ* mutants by deletion of *YKU70*

Null mutation of any of the three genes encoding members of the MRX complex renders the cells highly sensitive to IR (Ivanov *et al.*, 1992; Tsubouchi and Ogawa, 1998; Bressan *et al.*, 1999; Moreau *et al.*, 2001). Notably, the *mre11Δ* IR sensitivity was shown to be suppressed by concomitant deletion of *YKU70* (Bressan *et al.*, 1999). The increased IR resistance of *mre11Δ yku70Δ* mutants is thought to originate from the loss of end protection by Ku allowing DSB ends to be processed even in the absence of Mre11. Given the redundancy of DSB resection pathways, we asked whether the *yku70Δ* suppression of the *mre11Δ* IR sensitivity is dependent on *SGS1* and/or *EXO1* by determining the plating efficiency of various mutant strains after IR exposure. In agreement with previous studies, we found that *mre11Δ* mutants exhibit high IR sensitivity (100-fold decrease in survival at 200 Gy), which is suppressed by deletion of *YKU70* (Figure 1). The suppression does not apply to all HR mutants, as *rad51Δ* cannot be suppressed by *yku70Δ*, supporting the link between increased end processing and loss of DSB end protection. The IR sensitivity of the *mre11Δ yku70Δ sgs1Δ* mutant is comparable with the sensitivity of the *mre11Δ yku70Δ* double mutant, indicating that the suppression is independent of Sgs1. Conversely, *exo1Δ* negated the suppression, suggesting that in the absence of the MRX complex, Ku blocks access to Exo1 (Figure 1). In agreement with this hypothesis, *EXO1* overexpression also suppressed the *mre11Δ* IR sensitivity (Figure 1) (Chamankhah *et al.*, 2000; Tsubouchi and Ogawa,

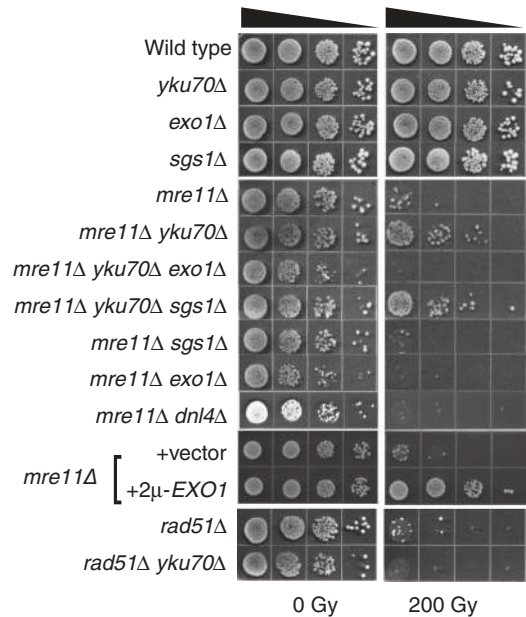


Figure 1 Suppression of the *mre11Δ* IR sensitivity by *YKU70* deletion. Exponentially growing cells of the indicated genotypes were 1:10 serially diluted, spotted onto YPD or selective plates and exposed to the indicated IR dose.

2000; Moreau *et al.*, 2001; Lewis *et al.*, 2002). Deletion of *DNL4* did not suppress the *mre11Δ* IR sensitivity, supporting the hypothesis that it is the loss of end protection by Ku that allows increased 5'-3' end processing (Figure 1). Analogous findings were reported in *Schizosaccharomyces pombe* in which deletion of *pku70* suppressed the IR and MMS sensitivity of *rad50* or *rad32* mutants in an *exo1⁺*-dependent manner (Tomita *et al.*, 2003; Williams *et al.*, 2008).

To determine whether the presence of Ku at DSB ends interferes with end processing in the presence of a structurally but not functionally competent MRX complex, we used an allele of *MRE11* (*mre11-H125N*) encoding a protein lacking endo- and exonuclease activities (Moreau *et al.*, 1999; Krogh *et al.*, 2005). For simplicity, this nuclease-defective allele is referred to as *mre11-nd*. In agreement with the previous studies, the *mre11-nd* mutant exhibited IR sensitivity only at high doses, with a 17-fold decrease in survival at 800 Gy (Figure 2A and B) (Moreau *et al.*, 1999). Deletion of *YKU70* in the *mre11-nd* mutant increased the IR resistance at 800 Gy by seven-fold ($P=0.01$) (Figure 2A and B). Interestingly, this increased resistance is dependent on both Exo1 and Sgs1. High-copy expression of *EXO1* increased the *mre11-nd* resistance at 800 Gy by only two-fold ($P=0.03$) (Figure 2C), consistent with a previous study (Moreau *et al.*, 2001). Our findings suggest that in the presence of a defective MRX complex, Ku provides a partial block to processing DSB ends by Exo1 and Sgs1.

The *mre11-nd* defect was further characterized using a physical assay that monitors resection of an HO-induced DSB at the *MAT* locus in strains with an integrated *P_{GALI}-HO* fusion. The assay was performed in *rad51Δ* mutants in which the processed ends do not engage in repair facilitating their detection. Following synchronous HO cleavage by addition of galactose, resection at different distances from the break can be monitored by detecting restriction enzyme

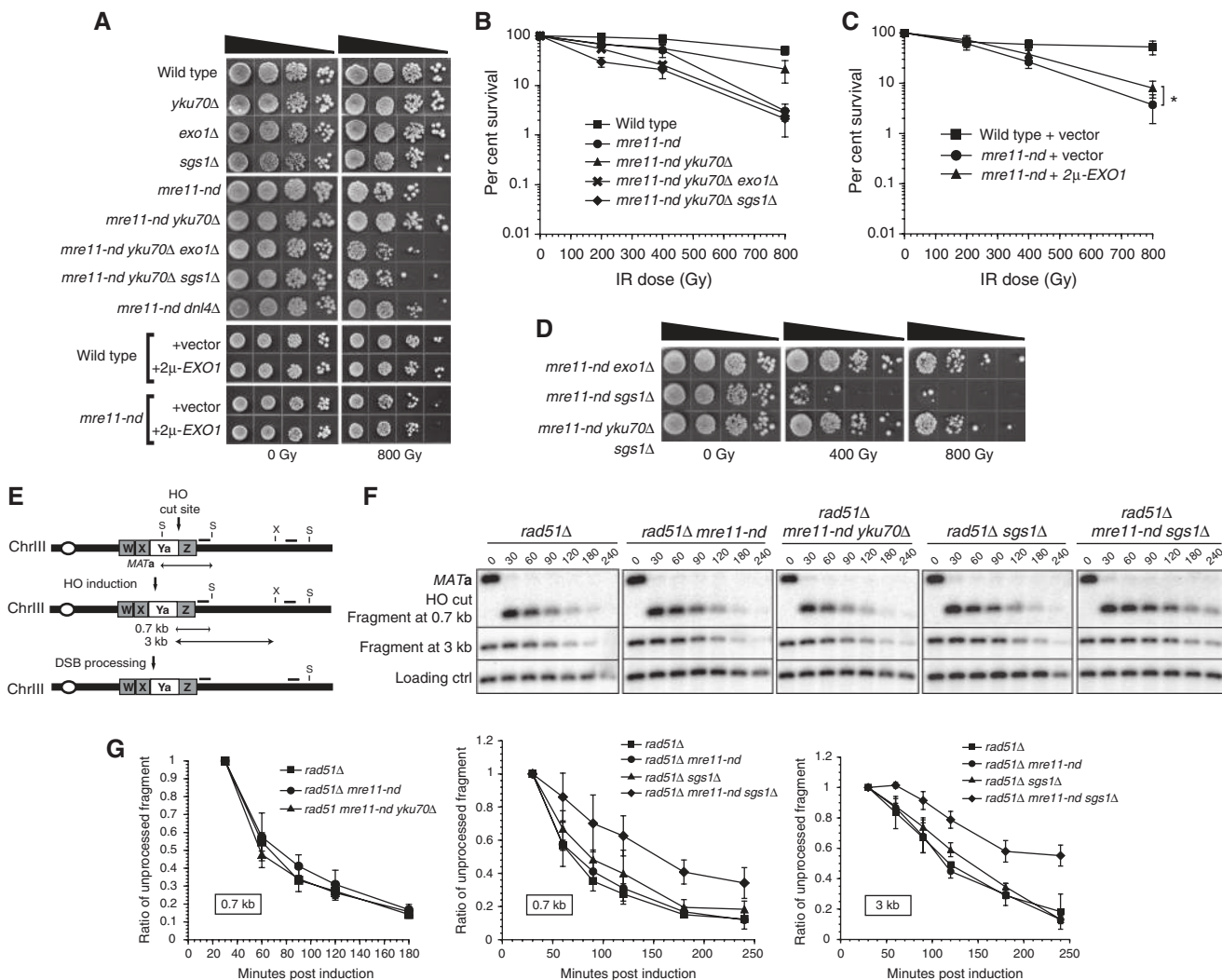


Figure 2 Phenotype of *mre11-nd* and *mre11-nd sgs1Δ* mutants. (A) Suppression of the *mre11-nd* IR sensitivity by the *yku70Δ* mutation (quantitation in (B)) or high-copy expression of *EXO1* (quantitation in (C), **P* = 0.03, unpaired *t*-test). (D) Radiation sensitivity of *mre11-nd* mutants in conjunction with *sgs1Δ* or *exo1Δ* mutations. (E) Schematic representation of the chromosome III *MAT* locus used in the physical assay to assess resection of an HO-induced DSB. The 5'-3' degradation destroys the *StyI* (S) and *XbaI* (X) recognition sites, which translates into the disappearance of the *StyI/XbaI* digestion fragments. (F) Southern blot analysis and (G) cut fragment intensity plots showing the kinetics of the cut fragment intensity disappearance as a ratio of the intensity 30 min after induction. The means from four experiments are presented, error bars indicate s.d.

fragments with probes specific for that region. As 5'-3' resection proceeds, a *StyI* site at 0.7 kb distal to the break and an *XbaI* site at 3 kb distal to the break are sequentially rendered ss; therefore, resistant to digestion (Figure 2E). As a result, after digesting genomic DNA with *StyI/XbaI*, the intensity of the bands corresponding to the DNA fragments diminishes over time. This analysis revealed that the disappearance of the fragment indicative of resection past the *StyI* site at 0.7 kb (Figure 2F and G) or the *XbaI* site at 3 kb (Figure 2F) is not altered in the *mre11-nd* strain, consistent with a previous study (Llorente and Symington, 2004). Moreover, deletion of *YKU70* in the *mre11-nd* background did not increase processing of the cut fragment. Although the increased requirement for the Mre11 nuclease in response to high IR doses could reflect a dosage effect, we have previously shown that the *mre11-nd* mutant is proficient for resection of multiple HO-induced DSBs (Llorente and Symington, 2004). Thus, we favour the hypothesis that the differential phenotype

and suppression of *mre11-nd* mutants by deletion of *YKU70* in IR sensitivity and resection assays reflects the different requirements for processing IR ('dirty') versus endonuclease ('clean')-induced DSBs. For IR-induced breaks, the requirement for the Mre11 nuclease to process some ends is increased, making the suppression by the loss of Ku more obvious.

***Sgs1* becomes important in the absence of the nuclease activity of Mre11**

Given that the suppression of the sensitivity to IR of *mre11-nd* by *yku70Δ* requires *Sgs1*, we hypothesized that in the presence of a defective MRX complex the helicase and nuclease activities of *Sgs1-Dna2* provide some redundant activity to initiate end processing. Consistent with this idea, the *mre11-nd sgs1Δ* double mutant exhibited a synergistic defect in IR resistance, with a 1700-fold decrease in survival at 800 Gy compared with the wild-type strain (Figure 2D). A similar

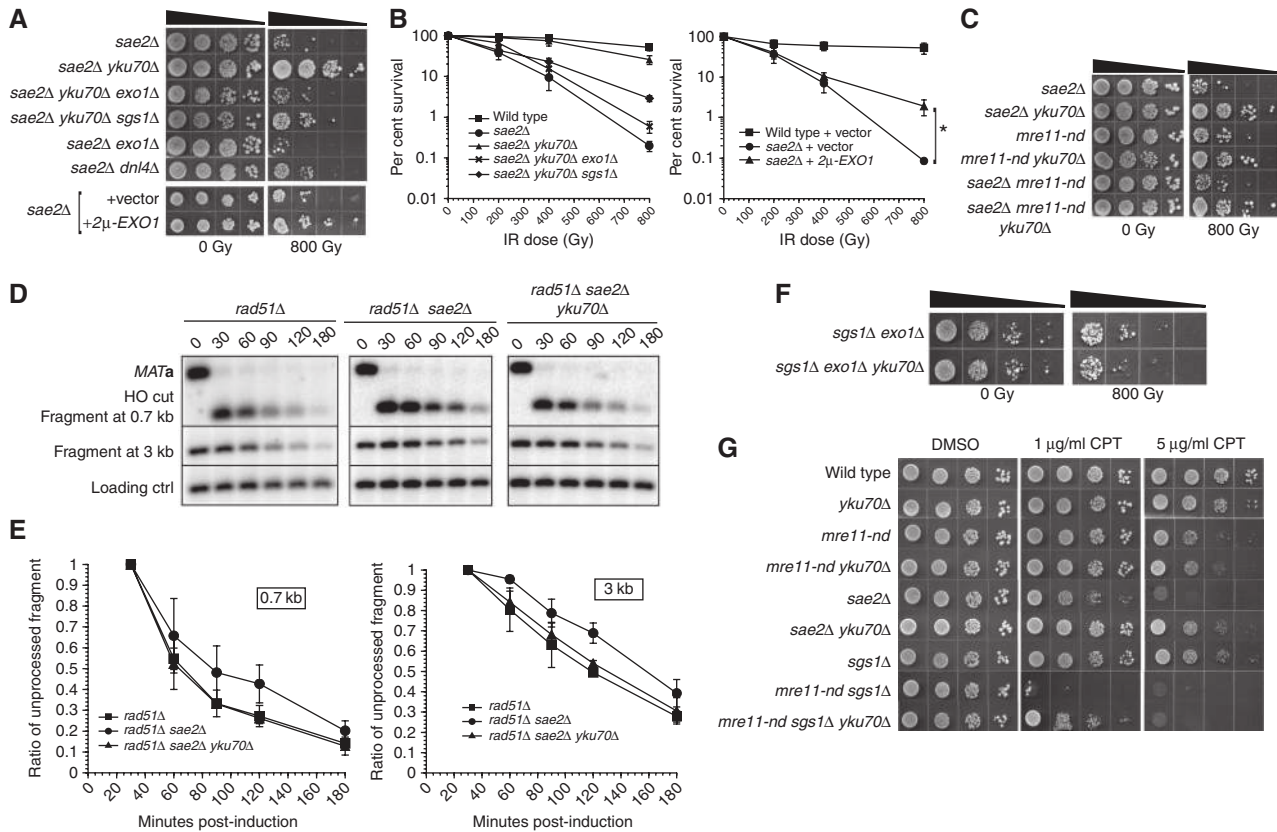


Figure 3 Suppression of the *sae2Δ* mutant phenotype by *YKU70* deletion. Radiation sensitivity of *sae2Δ* mutants: (A) spot assays and (B) survival plots as described in Figure 2C. * $P = 0.01$ (unpaired *t*-test). (C) Epistatic relationship between *sae2Δ* and *mre11-nd* mutants, as shown by IR spot assays. Resection physical assay: (D) Southern blot analysis and (E) cut fragment intensity plots as described in Figure 2G. (F) Radiation sensitivity of *sgs1Δ exo1Δ* mutants, as indicated by spot assays. (G) CPT sensitivity of *mre11-nd* and *sae2Δ* mutants. Exponentially growing cells in SC minimal medium were 1:10 serially diluted and spotted on SC plates containing the indicated concentration of camptothecin in DMSO.

finding was reported by Budd and Campbell (2009). The *mre11-nd exo1Δ* mutant exhibited higher resistance to IR than the *mre11-nd sgs1Δ* mutant, suggesting that Exo1 is less important for the initial processing of ends than Sgs1-Dna2 when Ku is present (Figure 2D). The *yku70Δ* mutation conferred a significant increase in the IR resistance of the *mre11-nd sgs1Δ* mutant, again consistent with the view that Ku prevents access of ends to Exo1. The *exo1Δ mre11-nd sgs1Δ* triple mutant is inviable preventing analysis of end processing in the absence of these overlapping functions (Mimitou and Symington, 2008).

For a more quantitative measure of DSB end processing, the disappearance of restriction fragments indicative of resection 0.7 and 3 kb from the HO-cut site was monitored in *rad51Δ mre11-nd*, *rad51Δ sgs1Δ* and *rad51Δ mre11-nd sgs1Δ* strains (Figure 2F). As noted above, the *rad51Δ* and *rad51Δ mre11-nd* mutants exhibited similar kinetics in the disappearance of the cut fragments over time. The *rad51Δ sgs1Δ* appeared slightly more defective in resection at both 0.7 and 3 kb, but the difference is not statistically significant. We consistently found a resection defect at both 0.7 and 3 kb in the *rad51Δ mre11-nd sgs1Δ* mutant (Figure 2F and G). At 120 min after HO induction, there is a two-fold decrease in the amount of ends resected at 0.7 kb ($P = 0.006$) and a 2.5-fold decrease at 3 kb ($P = 0.0003$) compared with *rad51Δ*. These results suggest that Sgs1 provides some redundant activity to

initiate end resection in the absence of the Mre11 nuclease activity. This requirement for Sgs1 is more pronounced for IR-induced breaks than endonuclease-induced ends as evidenced by the high IR sensitivity of the *mre11-nd sgs1Δ* mutants compared with the subtle HO end-resection defect.

sae2Δ* mutants exhibit high IR sensitivity that is suppressed by deletion of *YKU70

MRE11 and *SAE2* belong to the same epistasis group with respect to DSB resection and *sae2Δ* mutants exhibit a similar phenotype to *mre11-nd* mutants (Rattray *et al.*, 2001; Lobachev *et al.*, 2002; Clerici *et al.*, 2005). When tested for IR sensitivity, the *sae2Δ* mutant showed a 255-fold decrease in survival at 800 Gy. The IR sensitivity of the *sae2Δ* mutant is higher than *mre11-nd* and is exhibited at lower IR doses (Figure 3A and B). At 800 Gy, the *mre11-nd* mutant is 15-fold more resistant than the *sae2Δ* mutant ($P = 0.0026$) (Figures 2B and 3B).

The IR sensitivity of the *sae2Δ* mutant is highly suppressed by the *yku70Δ* mutation, resulting in almost equivalent survival to wild type. Similarly to the *mre11-nd* mutant, this effect is dependent on both *SGS1* and *EXO1*. Note that the *sae2Δ sgs1Δ yku70Δ* mutant is viable, whereas the *sae2Δ sgs1Δ* mutant is not (discussed later). This suggests that loss of end protection by Ku allows Exo1 and Sgs1 to initiate 5'-3' resection of DSBs, which normally requires Sae2. The

requirement for *exo1*⁺ in the suppression of *ctp1* (the functional counterpart of *SAE2*) by *pku70Δ* was previously reported in *S. pombe* (Limbo *et al.*, 2007). High-copy expression of *EXO1* also resulted in a significant suppression of the IR sensitivity of the *sae2Δ* mutant (24-fold increase in survival at 800 Gy, *P* = 0.01) (Figure 3A and B), supporting the model that Exo1 competes with Ku at ends to initiate resection of a subset of breaks. The suppression conferred by *yku70Δ* in *sae2Δ* mutants could also be attributed to defects in NHEJ that allow time for redundant resection factors to act. Indeed, we found that *sae2Δ dnl4Δ* mutants are slightly more resistant to IR than *sae2Δ* (10-fold increase in survival at 800 Gy, *P* = 0.0061), but still more sensitive than *sae2Δ yku70Δ* (Figure 3A). This suggests that both the end protection and NHEJ functions of Ku contribute to compromise initiation of DSB resection in *sae2Δ* mutants, in agreement with studies of resection of HO-induced DSBs in G1 cells (Clerici *et al.*, 2008). However, we cannot exclude the possibility that the slight suppression rendered by *dnl4Δ* is due to the decreased stability of Ku binding at DSBs (Zhang *et al.*, 2007). Notably, this suppression could not be detected in *mre11-nd* cells that are more resection proficient than *sae2Δ* (Figure 2A).

The differential IR sensitivity of *sae2Δ* and *mre11-nd* mutants prompted us to test their epistatic relationship (Figure 3C). In the presence of Ku, the more severe phenotype conferred by *sae2Δ* is observed for the double mutant, but in the absence of Ku, the more severe phenotype conferred by *mre11-nd* is evident. This suggests that once the inhibitory function of Ku is bypassed, Sae2 is no longer required and a functional MRX complex is able to initiate resection, whereas the MRX complex remains compromised in the *sae2Δ mre11-nd yku70Δ* mutant.

The resection of sequences 0.7 and 3 kb distal to the HO DSB was monitored by the disappearance of restriction fragments in *sae2Δ* strains as described above (Figure 2E). Unlike *mre11-nd*, the *sae2Δ* mutant exhibited a slight delay in the disappearance of both fragments (Figure 3D and E). More specifically, at 0.7 kb from the DSB, 43% of the fragment remains unprocessed 120 min after HO induction compared with 27% in *rad51Δ* (*P* = 0.02). A similar difference was observed at 3 kb from the break in which 69% remained unprocessed after 120 min compared with 49% observed in *rad51Δ* (*P* = 0.0003). Deletion of *YKU70* in the *rad51Δ sae2Δ* mutant allowed increased processing of both fragments with kinetics similar to those observed for *rad51Δ* control cells (Figure 3E). The *sae2Δ* resection defect is less pronounced than the IR sensitivity, which is likely due to the increased requirement for Sae2-dependent cleavage of dirty ends. However, we cannot rule out the possibility that *sae2Δ* cells can repair one or two DSBs, but not the large number of DSBs created by the doses of IR used to observe sensitivity.

To further validate the hypothesis that the suppression observed by the loss of Ku is due to increased initiation of resection, we tested whether mutants defective for extensive resection can be suppressed by *yku70Δ*. End resection is initiated in the *exo1Δ sgs1Δ* mutant, but only proceeds for about 100–700 nt (Mimitou and Symington, 2008; Zhu *et al.*, 2008). The *exo1Δ sgs1Δ* mutant is sensitive to IR, but the sensitivity cannot be suppressed by *yku70Δ* (Figure 3F), suggesting that the block to resection established by Ku takes place at the first step of end processing.

To ensure that the phenotypes observed for resection-defective mutants are not specific to IR-induced breaks, we also used camptothecin (CPT), which creates replication-associated DSBs. Similar to the findings with IR, the *mre11-nd* mutant was sensitive only at high CPT doses and was slightly suppressed by *yku70Δ* (Figure 3G). The *sae2Δ* mutant exhibited a higher CPT sensitivity than *mre11-nd*, and this was suppressed in the absence of Ku (Figure 3G). The synergistic sensitivity of combining *mre11-nd* with *sgs1Δ* was also observed for CPT, and, similar to IR, could be suppressed by *yku70Δ*. The phenotypes described are reminiscent of those observed after treating cells with IR, consistent with the idea that Ku protects DSB ends from degradation and this becomes limiting to resection when the MRX-Sae2 initial processing is compromised.

The suppression of the *sae2Δ* IR sensitivity by *yku70Δ* led us to test whether the sporulation defect of *sae2Δ/sae2Δ* diploids might be similarly suppressed. We made a diploid homozygous null for *SAE2* and *YKU70* (*sae2Δ/sae2Δ yku70Δ/yku70Δ*) and compared its sporulation efficiency to *SAE2/SAE2* and *sae2Δ/sae2Δ* diploids. As previously reported for W303, wild-type sporulation efficiency was approximately 50%, whereas *sae2Δ/sae2Δ* and *sae2Δ/sae2Δ yku70Δ/yku70Δ* failed to sporulate (data not shown). This indicates that during meiotic DSB processing, the absence of Ku is not enough to allow processing of the Spo11-bound ends by Sgs1 and/or Exo1.

Increased levels of Ku sensitize *mre11-nd* and *sae2Δ* mutants to IR

If Ku blocks resection in mutants compromised for the initial clipping of DNA ends, then one would predict that increased expression of Ku would render *mre11-nd* and *sae2Δ* mutants more sensitive to IR. This was tested by measuring the IR resistance of wild type, *mre11-nd* and *sae2Δ* mutants transformed with a high-copy number (2 μ) plasmid expressing *YKU70* and *YKU80*. There was no effect on the survival of wild-type cells over-expressing Ku, suggesting that in the presence of functional MRX-Sae2, the initial cleavage is not compromised (Figure 4A and B). However, high-copy expression of *YKU70-YKU80* sensitized the *mre11-nd* and *sae2Δ* mutants to 800 Gy by 30-fold (*P* = 0.01) and four-fold (*P* = 0.009), respectively (Figure 4B). It is notable that the survival of *mre11-nd* mutants over-expressing Ku at 800 Gy dropped to a level comparable with that seen by the *sae2Δ* mutant transformed with the empty vector, 0.11 and 0.08%, respectively. We conclude that, under conditions where the initial processing of the DSB ends cannot take place, increased/prolonged presence of Ku at DNA ends further impedes initiation of resection.

Suppression of the synthetic lethality of *rad27Δ sae2Δ* and *sgs1Δ sae2Δ* mutants by *yku70Δ*

The *RAD27* gene encodes a nuclease that functions to process Okazaki fragments during lagging strand DNA synthesis (Tishkoff *et al.*, 1997). Deletion of *RAD27* is lethal in combination with mutation of any one of the *RAD52* group genes, including *mre11Δ*, *sae2Δ* and *mre11-H125N*, suggesting that MRX-Sae2 are required to process lesions generated in a *rad27Δ* strain (Symington, 1998; Moreau *et al.*, 1999; Debrauwere *et al.*, 2001). To determine whether deletion of *YKU70* can suppress the *rad27Δ* synthetic lethality with

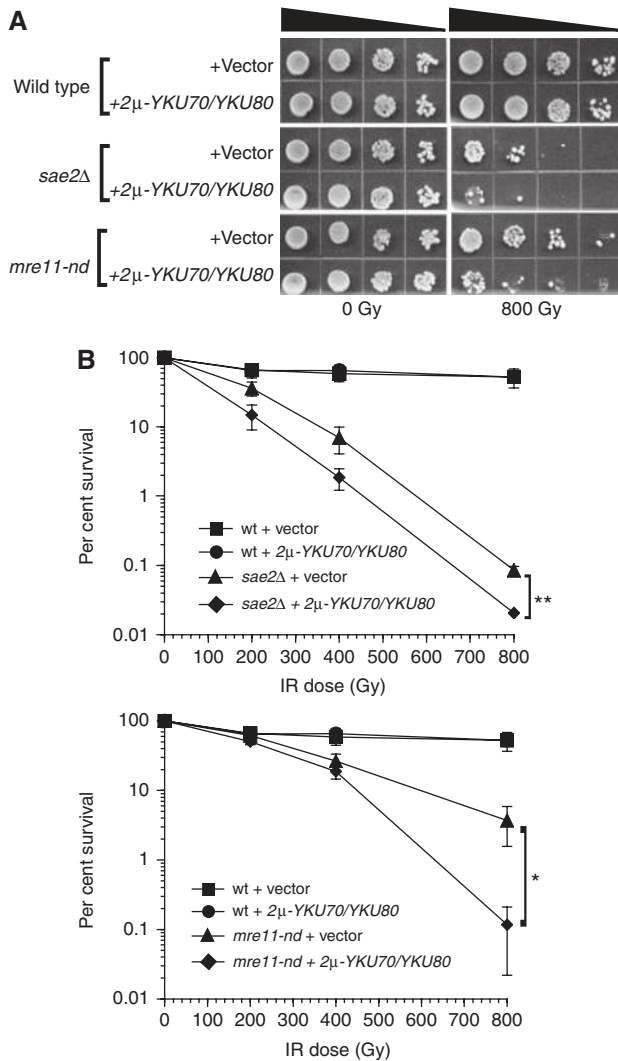


Figure 4 YKU70/YKU80 over-expression sensitizes *sae2Δ* and *mre11-nd* mutants to IR. (A) Spot assays and (B) survival plots of wild type, *sae2Δ* and *mre11-nd* mutants transformed with empty or YKU70/YKU80 over-expressing vectors. Exponentially growing cells in SC-Ura to maintain selection of the plasmids were 1:10 serially diluted, spotted onto SC-Ura plates and exposed to IR. The means from at least three experiments are presented, error bars indicate s.d.; * $P = 0.01$, ** $P = 0.009$.

sae2Δ or *mre11-nd*, diploids heterozygous for *RAD27* and *MRE11* or *SAE2* were generated, and after sporulation, tetrads were dissected to determine whether viable *rad27Δ mre11-nd yku70Δ* or *rad27Δ sae2Δ yku70Δ* spores could be obtained (Figure 5A). After dissection, the plates were incubated at 23°C because the *rad27Δ* growth defect is suppressed at lower temperature. Even at 23°C, the *rad27Δ sae2Δ* double mutant was found to be lethal, but the lethality was suppressed by *yku70Δ* (Figure 5A). Some viable *rad27Δ mre11-nd* spore colonies were recovered that grew very slowly. Nevertheless, *yku70Δ* suppressed this growth defect to some extent, as *rad27Δ mre11-nd yku70Δ* mutants consistently survived after dissection (Figure 5A). To ensure that the suppression of the synthetic lethality is related to DNA end processing, we also made diploids heterozygous for YKU70, *RAD27* and *RAD55*, and after sporulation found that no viable *rad27Δ rad55Δ yku70Δ* spores could be recovered (Figure 5A). Finally, deletion of YKU70 could not

suppress the synthetic lethality of *rad27Δ mre11Δ* (data not shown). Although elimination of Ku partially suppresses the IR sensitivity of the *mre11Δ* mutant, the *mre11Δ yku70Δ* double mutant is still highly sensitive to IR with no survivors at 500 Gy (data not shown). Thus, it seems likely that the large number of lesions generated in the *mre11Δ rad27Δ* mutant cannot be repaired even in the absence of Ku.

As *yku70Δ* completely suppresses the *rad27Δ sae2Δ* growth defect, we tested whether *yku70Δ* can also suppress the *sae2Δ sgs1Δ* synthetic lethality (Tong *et al.*, 2001; Ooi *et al.*, 2003). The majority of the *sae2Δ sgs1Δ* mutants were inviable, but some small spore colonies formed (<20% were viable). However, the *sae2Δ sgs1Δ yku70Δ* triple mutant grew remarkably well (Figure 5B). We were unable to derive the *sae2Δ sgs1Δ yku70Δ exo1Δ* quadruple mutant from crosses, indicating that the survival of the triple mutant is dependent on Exo1 (Figure 5B). Furthermore, by introducing a high-copy *EXO1* plasmid into a diploid heterozygous for *SAE2* and *SGS1* viable *sae2Δ sgs1Δ* spores that inherited the plasmid were recovered (Figure 5B). These data suggest that lethality of the *sae2Δ sgs1Δ* mutants is caused by their inability to process some physiological DNA intermediates that are also substrates for Ku, most likely DSBs.

Telomere phenotypes associated with *sae2Δ sgs1Δ*

Telomeres are specialized DNA-protein complexes that define the physical ends of linear chromosomes and protect them from end degradation. The protruding ssDNA 3' overhang, the G tail, has a central function in modulating telomere length, as it serves as a substrate for extension by telomerase and it is formed by active resection of the C-strand after completion of DNA synthesis (Gilson and Geli, 2007). Ku is one of the factors that bind to telomeres protecting them from degradation (Polotnianka *et al.*, 1998). As telomeres are physiological DNA structures that share important similarities with DSBs, it seemed possible that an alteration of the natural chromosome ends might be responsible for the *sae2Δ sgs1Δ* lethality.

The *sae2Δ sgs1Δ* double mutant was recently shown to have short telomeres, a defect in telomere addition in G2-arrested cells and no detectable G tails (Bonetti *et al.*, 2009). As described above, the *sae2Δ sgs1Δ* double mutant shows poor viability in the W303 strain background, but some rare viable spore colonies could be recovered. Analysis of the telomeres in these rare survivors indicated that their telomeres had normal length with occasional wider distribution (Figure 6A and B). Analysis of the *mre11-nd sgs1Δ* double mutant also revealed normal telomere length (Figure 6B). The *yku70Δ* mutant is known to exhibit short telomeres (Porter *et al.*, 1996), and, as expected, the *sae2Δ sgs1Δ yku70Δ* strain was also shown to have short telomeres. The normal telomere length observed for the slow growing *sae2Δ sgs1Δ* double mutants could mean that they have already escaped from senescence and elongated their telomeres (Bonetti *et al.*, 2009).

As the survival of the *sae2Δ sgs1Δ* mutant depends on the absence of YKU70, and the telomeres have no detectable ssDNA tails (Bonetti *et al.*, 2009), it seemed possible that Ku-dependent telomere fusions might contribute to the lethality. To test this, and in order to bypass the difficulties imposed by the *sae2Δ sgs1Δ* synthetic lethality, an indirect approach was used. The viable *sae2Δ sgs1Δ yku70Δ* mutant was

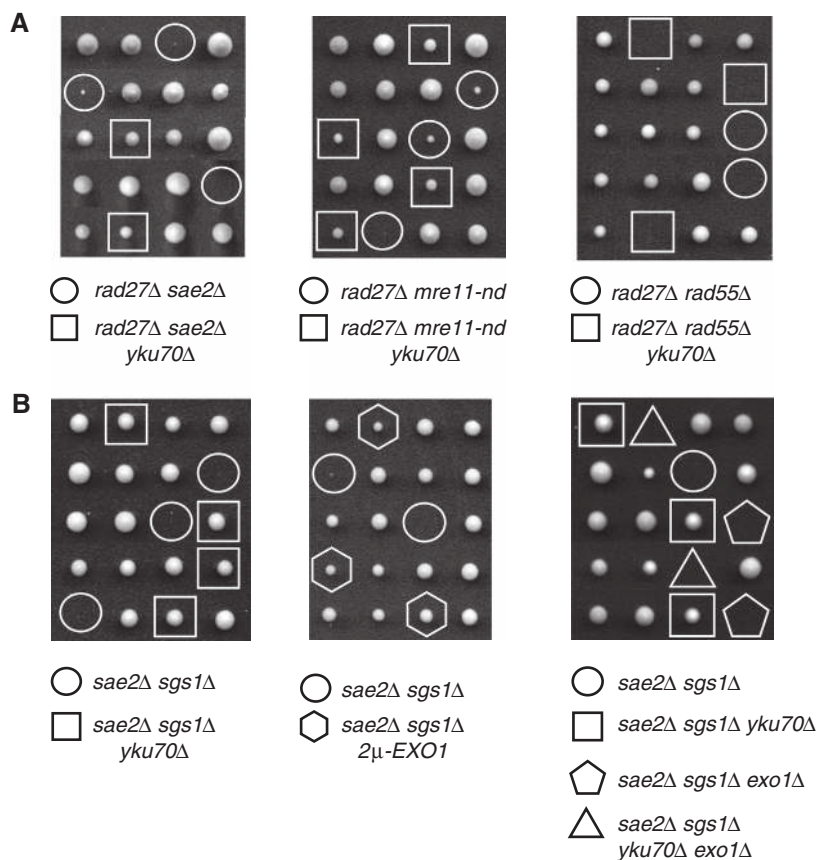


Figure 5 Genetic interactions between *rad27Δ*, *mre11-nd*, *sae2Δ* and *sgs1Δ* mutants. Viability and genotypes of spores derived from diploids heterozygous for the indicated mutations. (A) Deletion of *YKU70* suppresses the synthetic lethality/growth defect of *rad27Δ* mutants with *sae2Δ* and *mre11-nd*, but not with *rad55Δ*. (B) Loss of Yku70 or *EXO1* over-expression rescues the *sae2Δ sgs1Δ* synthetic lethality.

transformed with a plasmid expressing *YKU70* under a galactose-inducible promoter. The strain was maintained in glucose-containing medium to retain viability. Addition of galactose to the medium to induce *YKU70* expression led to a gradual telomere lengthening as shown by Southern blot analysis (Figure 6C). Notably, the *sae2Δ sgs1Δ* mutant exhibited slower and less efficient elongation of its telomeres after *YKU70* expression was restored, suggesting that the telomeres in *sae2Δ sgs1Δ* mutants are less accessible to telomerase. To detect whether telomere–telomere fusions (T-TF) are formed in the *sae2Δ sgs1Δ* mutant, a PCR-based assay was used (Mieczkowski *et al*, 2003). A *mec1Δ tel1Δ sml1Δ* mutant, which was previously shown to form T-TF, was used as a positive control (Mieczkowski *et al*, 2003). T-TF were readily detected in the *mec1Δ tel1Δ sml1Δ* mutant, but not in wild type or *sae2Δ sgs1Δ yku70Δ* after induction of *YKU70* with galactose (data not shown). Moreover, we noticed that the cells maintained their ability to divide when *YKU70* was over-expressed, albeit slowly. These data suggest that T-TF are probably not the reason for the *sae2Δ sgs1Δ* lethality. This is supported by the observation that deletion of *DNL4*, the ligase responsible for T-TF formation, does not suppress the *sae2Δ sgs1Δ* lethality (Figure 6D).

Discussion

The 5′-3′ processing of DSB ends leads to the formation of 3′ ss tails that serve as precursors for HR and as sensors for the

DNA damage checkpoint (Zou and Elledge, 2003; Krogh and Symington, 2004). Initiation of DSB processing is tightly regulated and provides a critical step in which the choice between NHEJ and HR repair pathways is made. The 5′-3′ resection of DSBs is under the control of CDK1 and to date, Sae2 and Dna2 are the only resection factors known to be cell cycle regulated (Huertas *et al*, 2008; Kosugi *et al*, 2009). CDK1-dependent phosphorylation of Dna2 is required for its nuclear localization during S-phase and phosphorylation of Sae2 at a conserved CDK site (Ser 267) was shown to correlate with initiation of resection and commitment to HR (Huertas *et al*, 2008; Kosugi *et al*, 2009). Similar findings were reported for the human and *S. pombe* Sae2 counterparts, CtIP and Ctp1, respectively, with the latter being controlled at the transcriptional level (Limbo *et al*, 2007; Huertas and Jackson, 2009; Yun and Hiom, 2009).

The initiation of end processing requires the MRX complex and Sae2, but as both Mre11 and Sae2 have nuclease activity and mutation in either results in a similar phenotype, it is not clear which nuclease is responsible for clipping 5′ ends (Lengsfeld *et al*, 2007; Mimitou and Symington, 2008; Zhu *et al*, 2008). Unfortunately, there are no recognizable nuclease motifs present in Sae2, and an *sae2* nuclease-defective mutant awaits identification. During meiosis, the initial endonucleolytic processing by MRX and Sae2 is obligatory to remove Spo11, which is covalently bound to the 5′ ends of DSBs at hotspots (Neale *et al*, 2005; Mimitou and Symington, 2009). However, it is unclear whether this two-step mechanism

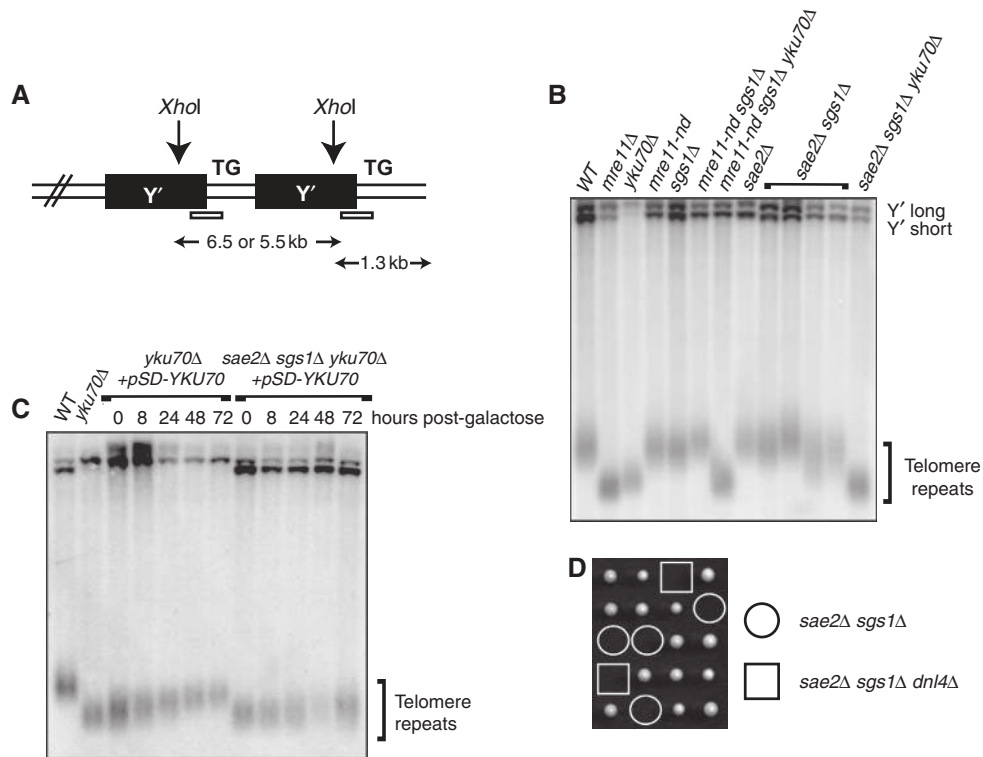


Figure 6 Telomere-associated phenotypes of *sae2Δ sgs1Δ yku70Δ* mutants. **(A)** Schematic representation of the telomeric Y' elements and TG repeats. *XhoI* liberates a wide band of ~1.3 kb in wild-type cells, which is used to evaluate telomere repeat length in other genetic backgrounds. **(B)** Southern blot analysis of *XhoI*-digested genomic DNA as detected with a Y' probe. **(C)** Telomere length analysis after induction of *YKU70* expression with galactose in the *yku70Δ* and *sae2Δ sgs1Δ yku70Δ* mutant. **(D)** Viability and genotypes of spores derived from diploids heterozygous for the indicated mutations.

is needed to resect DSB ends formed by different means. Here, we sought to investigate what purpose this initial processing might serve by testing whether competition is established by NHEJ when the MRX-Sae2-cleavage step is compromised. We found that the IR hypersensitivity of *mre11Δ* mutants is partially suppressed by *yku70Δ* in an Exo1-dependent manner, suggesting that when ends are not bound by MRX, Ku acts as a block to resection by Exo1. It is also possible that MRX bound to ends is inhibitory to Exo1-initiated resection (Farah *et al*, 2009). The observation that Sgs1 is not required for the suppression suggests that the MRX complex recruits Sgs1 to ends, consistent with the finding that Sgs1 associates with Mre11 upon DNA damage-induced checkpoint activation (Chiolo *et al*, 2005).

Unlike *mre11Δ*, the *yku70Δ* suppression of *mre11-nd* and *sae2Δ* was dependent on Exo1 and Sgs1, suggesting that in the presence of a structurally competent but functionally compromised MRX complex, binding of Ku limits access to both proteins. These results are also consistent with the MRX recruiting Sgs1 to DSBs. Surprisingly, the extent of the *mre11-nd* and *sae2Δ* IR sensitivity, as well as their suppression by *yku70Δ*, differs significantly (Figures 2 and 3). More specifically, after 800 Gy, *sae2Δ* survival was 0.2%, whereas *mre11-nd* was 3%, and *yku70Δ* restored survival to 26% in *sae2Δ* and 21% in *mre11-nd* mutants. Furthermore, overexpression of *YKU70-YKU80* resulted in greater IR sensitivity in the *mre11-nd* strain than in *sae2Δ* (Figure 4), and overexpression of *EXO1* conferred higher resistance in the *sae2Δ* strain than in *mre11-nd*. Similarly, we could detect a subtle

defect in endonuclease-induced DSB processing in *sae2Δ*, but not *mre11-nd* mutants.

Several hypotheses could account for this behaviour. First, Sae2 could have a dual function: as a regulator of Mre11 nuclease function and as a nuclease itself. In the presence of Sae2 and the nuclease-defective Mre11, some processing might take place, which would be more efficient for endonuclease than IR-induced DSBs (Bressan *et al*, 1998; Moreau *et al*, 1999; Llorente and Symington, 2004). On the other hand, complete absence of Sae2 would be expected to result in a more severe defect in initial processing because of loss of both nucleases. A second hypothesis is that the physical presence of Sae2 at the DNA ends is crucial to avoid NHEJ. In support of this, a previous study showed a 60-fold increase in NHEJ in *sae2Δ* mutants, compared with a seven-fold increase in *mre11-nd* mutants (Lee and Lee, 2007). It is possible that the initial processing step by MRX and Sae2 functions to remove Ku from DNA ends, analogous to the removal of Spo11 during meiosis. Thus, in the absence of clipping by the Mre11 nuclease and Sae2 Ku remains bound to DNA ends, preventing resection by Exo1 and promoting NHEJ. The third hypothesis is supported from studies in *S. pombe*, in which the Rad32^{Mre11} nuclease activity was shown to be involved in the removal of Top2 from 5' DNA ends as well as Top1 from 3' DNA ends, whereas Ctp1 is involved in the removal of covalently bound Top2, but inhibits Top1 removal (Hartsuiker *et al*, 2009). This suggests that Ctp1 protects the 3' end from processing, and if conserved, could mean that Sae2 is important in protecting the integrity of the 3' end for productive Rad51 filament formation.

It is also notable that even though deletion of *YKU70* significantly suppressed the end-processing defect of *sae2Δ*, it failed to suppress its meiotic defect. As the IR suppression requires *EXO1* and *SGS1*, it could mean that (i) neither Exo1 nor Sgs1-Dna2 can process Spo11 bound ends or (ii) one of them is inactive for processing during meiosis. A recent study showed that Exo1 has the major function in meiotic DSB processing after Spo11 removal with Sgs1-Dna2 providing some residual activity in *exo1Δ* cells, indicating that both activities are functional in meiotic cells (Manfrini *et al.*, 2010). These results suggest that Exo1 and Sgs1-Dna2 can process DSBs subsequent to Spo11 removal, but are ineffective in removal of the Spo11 adduct from 5' ends.

We found that viability of *rad27Δ sae2Δ* mutants depends on the absence of Ku, suggesting the accumulation of DNA intermediates in *rad27Δ* mutants that are substrates for both Sae2 and Ku. One hypothesis is that these substrates are replication intermediates, which need to be resected to allow repair by HR. These intermediates are probably collapsed replication forks caused by the presence of unrepaired lagging strand lesions from the preceding S-phase. HR was shown to be the major DSB repair pathway at collapsed replication forks (Saleh-Gohari *et al.*, 2005). The *SAE2* requirement in the *rad27Δ* background could reflect the need to initiate resection of replication-associated DSBs to channel repair by HR. Indeed, *rad27Δ* is lethal in combina-

tion with mutations in the genes of the *RAD52* epistasis group. Moreover, the sensitivity of *sae2Δ* mutants to CTP is suppressed by elimination of Ku, suggesting that DSBs formed after replication fork collapse can be bound by Ku and the presence of Sae2 is important to antagonize this binding. In agreement with our results, two recent studies show that DNA repair defects observed in *Caenorhabditis elegans*, mammalian or chicken cells deficient for Fanconi anaemia components, can be suppressed by loss of Ku (Adamo *et al.*, 2010; Pace *et al.*, 2010). This suggests that during replication cells use specialized factors to antagonize Ku, whose engagement with DSB intermediates during S-phase may inhibit the processing of DSBs required for loading of HR factors, while also promoting illegitimate repair.

Similarly, the ability to restore resection and enable HR-mediated repair of replication-associated DSBs could be the basis of the *yku70Δ* suppression of the *sae2Δ sgs1Δ* mutant lethality. The *sgs1* mutants exhibit aberrant DNA replication phenotypes, including increased frequency of replication fork collapse after fork stalling (Cobb *et al.*, 2003, 2005; Liberi *et al.*, 2005), and such events could increase the need for Sae2 to prevent illegitimate repair of DSBs by NHEJ. However, unlike *rad27Δ*, *sgs1Δ* mutations are not synthetically lethal with mutations in genes of the *RAD52* epistasis group, suggesting that there is something more contributing to the *sae2Δ sgs1Δ* lethality. Telomeres are logical candidates

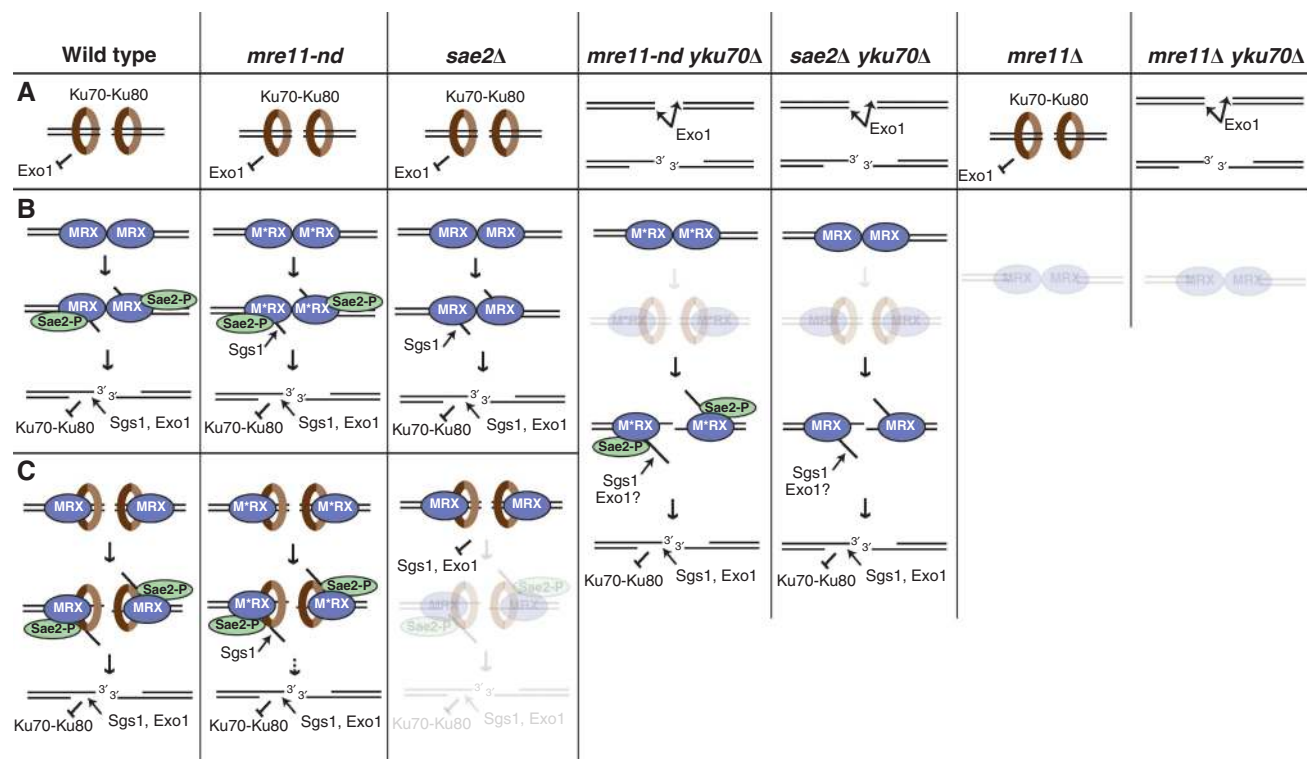


Figure 7 Models for the interplay between resection machinery and Ku at DSB ends. In wild-type cells, DSBs can exist in three dynamic states: (A) Ku-bound, which blocks access to Exo1, (B) MRX-bound, which can initiate end processing and (C) MRX-Ku-bound, which can initiate NHEJ and removal of Ku is required to allow resection initiation. Recruitment of Sae2 in G2 and clipping of the ends allows access to the processive resection machinery and creates an intermediate that can no longer convert states and commit to HR. In nuclease-defective mutants of Mre11, though compromised for initial processing, the presence of Sae2 channels ends to HR and redundant activity from Sgs1-Dna2 allows initiation of resection. In the absence of *SAE2*, the MRX-bound ends can still initiate resection, presumably with some assistance by Sgs1, whereas the Ku-bound and MRX-Ku-bound ends are blocked. When the end protection by Ku is lost, in *mre11-nd yku70Δ* and *sae2Δ yku70Δ* for example, MRX-naked ends can be resected by Exo1. For the MRX-bound ends even if compromised for the initial clipping, the absence of Ku allows Sgs1 (and maybe Exo1) to assist in initiating resection of the DSB. Finally, in the absence of Mre11, where the only state present is the Ku-bound state, access of Exo1 is blocked in a Ku-dependent manner.

because they are actively resected and bound by Ku, similar to DSBs. It was recently shown that *sae2Δ sgs1Δ* mutants fail to form the 3' G tails implicated in recruiting telomerase to maintain normal telomere length (Bonetti *et al*, 2009). Elimination of Ku at telomeres allows access to Exo1 (Maringele and Lydall, 2002), and this could rescue the formation of 3' G tails in the *sae2Δ sgs1Δ* mutant. Although this could explain the *yku70Δ* suppression of the *sae2Δ sgs1Δ* lethality, it is important to note that the *mre11Δ* mutant is equally defective in G-tail formation, but is viable with *sgs1Δ* (although growth is slow). In addition, the *mre11Δ yku70Δ* double mutant grows poorly because two independent pathways for telomerase recruitment are defective (Nugent *et al*, 1998). It is possible that MRX inhibits recruitment of Exo1 to DSBs and the *sae2Δ sgs1Δ* double mutant lethality is due to the block to Exo1 recruitment imposed by both Ku and MRX. In contrast, Exo1 might still be recruited, albeit inefficiently, in the *mre11Δ sgs1Δ* double mutant.

On the basis of our findings, we suggest the following models (Figure 7). DSBs can exist in three different dynamic states: Ku bound (Figure 7A), MRX bound (Figure 7B) and MRX-Ku bound (Figure 7C). The Ku-bound state, which is the only state present in *mre11Δ* mutants, blocks access to Exo1. Loss of Ku allows Exo1 to initiate resection at a subset of ends contributing to the Exo1-dependent suppression observed in *mre11-nd yku70Δ*, *sae2Δ yku70Δ* and *mre11Δ yku70Δ* mutants. The MRX-bound state is competent to initiate resection, unless Sae2 is absent or the nuclease activity of Mre11 is compromised. In this case, some redundancy from Sgs1 allows resection to initiate creating an intermediate committed to HR. The MRX-Ku-bound state presents the cells with a choice: HR or NHEJ? The fast and simultaneous recruitment of MRX and Ku at DSB ends presents an intermediate that can be readily used for NHEJ, and this is likely to be the default in G1. When cells transit through S/G2 and have a template for HR, Sae2 is recruited to ends in order to override the block to resection imposed by Ku. Mre11 with Sae2 catalyses the removal of oligonucleotides from the DSB ends resulting in short 3' overhangs (Jazayeri *et al*, 2008; Mimitou and Symington, 2008; Zhu *et al*, 2008), a step that serves to create more favourable substrates for Sgs1 and Exo1 and remove Spo11-bound or chemically modified ends present at DSBs after IR. In the context of this work, the MRX-Sae2 cleavage may provide a substrate less suitable for Ku (and maybe MRX) binding; thus, preventing NHEJ and committing repair by HR. Ku preferentially binds double-stranded DNA ends over ssDNA and would be expected to show lower affinity for the clipped ends (Mimori and Hardin, 1986; Tuteja and Tuteja, 2000). Moreover, the short ssDNA tails formed after MRX-Sae2 cleavage could be bound by RPA, which in turn could promote DSB resection through its interaction with Dna2 (Bae *et al*, 2001). In the absence of Sae2, the cells fail to override the Ku barrier and instead use NHEJ, a pathway competent for repair of endonuclease induced, but less so for IR-induced DSBs. In *mre11-nd* cells, the physical/functional presence of Sae2 provides some line of defence against Ku and NHEJ, and even though the MRX-Sae2 cleavage is defective, Sgs1-Dna2 recruitment allows resection to initiate. The *yku70Δ* suppression of *mre11-nd* and *sae2Δ* is also dependent on Exo1, but at this point, we cannot distinguish between Exo1 degradation of ends unbound or bound by MRX.

Materials and methods

Media, growth conditions and genetic methods

Rich medium (yeast extract-peptone-dextrose, YPD), synthetic complete (SC) medium lacking the appropriate amino acids or nucleic acid bases, sporulation medium and genetic methods were as described previously (Sherman *et al*, 1986). YPLactate, rich medium containing 2% lactate (pH 5.5) and supplemented with adenine, was used for the galactose induction of HO in DSB end-resection assays. Sporulation medium contained 1% potassium acetate and the appropriate amino acids or nucleic acid bases at 1/5 of the concentration used in SC medium. Where indicated CPT dissolved in DMSO was added to liquid SC agar before pouring into plates. Hygromycin B (Sigma) to 300 µg/ml was used for selection of the *hphMX4* cassette and G418 (Sigma) to 200 µg/ml was used for selection of the *kanMX6* cassette. Transformation of yeast cells was performed by the lithium acetate method (Ito *et al*, 1983). Yeast cells were grown at 30°C, unless otherwise indicated.

Yeast strains and plasmids

The strains used were derived from W303 and are listed in the Supplementary Table 1. Most of the strains were constructed by crossing isogenic strains present in our laboratory collection to produce haploid progeny of the indicated genotypes. Strain LSY1091 was made by one-step gene replacement of W1588-4C with *EcoRI*/*SphI*-digested pMJ536 (a gift of M Lichten) to replace the *SAE2* coding region with the *kanMX6* cassette. Strains containing the integrated *P_{GALI}-HO* cassette were generated by crossing LSY1009-1 to strains within the laboratory collection. LSY1009-1 was constructed by transforming W1588-4A with the *BstEII* and *PvuII* linearized *YIPade3-HO* as previously described (Sandell and Zakian, 1993).

The 2 µ-plasmid over-expressing *YKU70/YKU80*, pML550.46, was kindly provided by MP Longhese. The plasmid over-expressing *EXO1*, pEM-EXO1, was created by moving the *EXO1* containing *XhoI/SacII* fragment from pSM502 (Moreau *et al*, 2001) to the corresponding sites of pRS426. pSD-YKU70 was obtained by cloning a *BamHI/NheI* PCR fragment (coordinates 838176–840023 on chromosome XIII) into the corresponding sites of pESC-URA (Stratagene). Plasmid *YIPade3HO* was kindly provided by V Zakian.

Gamma irradiation survival assays

Cells were grown in liquid YPD or SC-Ura medium to mid-log phase. The cultures were serially 1:10 diluted and spotted onto solid YPD or SC-Ura plates. The plates were irradiated in a Gammacell-220 irradiator containing ⁶⁰Co for the designated dose. The plates were incubated for 3 days at 30°C before survivors were counted. For quantitations, the mean per cent survival from at least three independent experiments is presented.

Analysis of telomere lengths

Genomic DNA was isolated from 5 ml cultures and digested with *XhoI*. The products were examined by Southern blot analysis with pYT14 as a hybridization probe. Wild-type strains yield a terminal restriction fragment of 1.3 kb, which includes 400 bp of the G₁₋₃T telomeric repeat (Porter *et al*, 1996).

Physical analysis of HO-induced DSB end resection

For the end-resection experiments, yeast cells were initially grown in 5 ml of YPD medium for 18 h and then transferred to YPLactate. Cultures were grown to an optical density at 600 nm (OD₆₀₀) of 0.3–0.5 and then HO was induced by addition of galactose to a final concentration of 2%. Cell samples were removed before and after induction, harvested by centrifugation and the cell pellets were washed with H₂O and stored at –80°C. DNA isolated by glass bead disruption and phenol extraction protocol was digested with *XbaI* and *StyI* and separated by electrophoresis through 1% agarose gels. DNA fragments were transferred to nylon membranes and hybridized with multiple radiolabelled DNA probes. For the detection of the HO-cut fragment next to the break site, the MAT-0.7 kb probe was used. This probe was generated by PCR amplification of MAT sequences distal to the HO-cut site (coordinates 201176–201580 on chromosome III sequence). For the detection of the fragment 3 kb distal to the HO-cut site, the MAT-3 kb probe was used (coordinates 204184–204893 on chromosome III). Quantities of DNA loaded at each time point were normalized using a *DNL4* probe (coordinates 334672–335378 on chromosome

XV). Intensities of bands on Southern blots corresponding to probed DNA fragments were analysed using ImageJ. DSB end resection for each time point was estimated as a ratio of the signal intensity corresponding to the fragment of interest 30 min after induction and represents the mean of two independent experiments in each of the two different inductions (four trials total).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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