Sumoylation Influences DNA Break Repair Partly by Increasing the Solubility of a Conserved End Resection Protein

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Abstract

Protein modifications regulate both DNA repair levels and pathway choice. How each modification achieves regulatory effects and how different modifications collaborate with each other are important questions to be answered. Here, we show that sumoylation regulates double-strand break repair partly by modifying the end resection factor Sae2. This modification is conserved from yeast to humans, and is induced by DNA damage. We mapped the sumoylation site of Sae2 to a single lysine in its self-association domain. Abolishing Sae2 sumoylation by mutating this lysine to arginine impaired Sae2 function in the processing and repair of multiple types of DNA breaks. We found that Sae2 sumoylation occurs independently of its phosphorylation, and the two modifications act in synergy to increase soluble forms of Sae2. We also provide evidence that sumoylation of the Sae2-binding nuclease, the Mre11-Rad50-Xrs2 complex, further increases end resection. These findings reveal a novel role for sumoylation in DNA repair by regulating the solubility of an end resection factor. They also show that collaboration between different modifications and among multiple substrates leads to a stronger biological effect.

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Introduction

Efficient and accurate genome repair requires regulatory mechanisms that adjust DNA repair levels and pathway usage depending on the cellular context. For example, in response to increased lesion loads, DNA repair pathways are upregulated [1–3]. Additionally, DNA double-strand breaks (DSBs) are repaired by either homologous recombination (HR) or non-homologous end joining (NHEJ) depending on the cell cycle stage [4,5]. The regulatory changes in these situations occur rapidly, involve many targets, and are reversible [1–3]. They are often enabled by protein modifications that reversibly add modifier groups to multiple targets. The best-illustrated example of this is protein phosphorylation mediated by the DNA damage checkpoint and cyclin-dependent kinases, which occurs within minutes of changes in repair needs and affects hundreds of protein targets (e.g. [6–9]).

More recently, another protein modification, sumoylation, has emerged as a key regulator of genome repair [reviewed in (10–13)]. However, many important details of how sumoylation influences DNA repair have yet to be elucidated. For example, sumoylation is important for DSB repair in humans and yeast partly by promoting DNA end resection [14–18]. Yet, it has been unclear for which resection factor(s) sumoylation is relevant, how sumoylation influences specific attributes of such target(s), and how this modification is coordinated with phosphorylation-based regulation.

To address these questions, we used budding yeast as a model system to examine the sumoylation of a conserved DNA end resection factor, Sae2. Sae2 collaborates with the Mre11-Rad50-Xrs2 (MRX) complex in processing multiple kinds of DSBs, including those with clean ends and ends capped with proteins or hairpin structures [reviewed in (19,20)]. Sae2 and MRX can
Author Summary

Proper repair of DNA lesions is crucial for cell growth and organism development. Both the choice and capacity of DNA repair pathways are tightly regulated in response to environmental cues and cell cycle phase. Recent work has uncovered the importance of protein modifications, such as phosphorylation and sumoylation, in this regulation. Sumoylation is known to be critical for the efficient repair of highly toxic DNA double-strand breaks in both yeast and humans, and this is partly mediated by influencing DNA end resection. However, it has been unclear for which resection factor sumoylation is important, how sumoylation influences specific attributes of the relevant targets, and how this modification is coordinated with phosphorylation-based regulation. Here, we provide exciting new insights into these issues by revealing that 1) a conserved end resection factor is a SUMO target relevant to this process, 2) this regulation favors a specific repair pathway, 3) sumoylation collaborates with phosphorylation to promote protein solubility, and 4) sumoylation-based regulation of DNA repair via an “ensemble effect” that entails simultaneous small alterations of multiple substrates. Our work reveals both a novel mechanism and a general principle for SUMO-mediated regulation of DNA repair.

remove the capping structure and 100–300 bp of single-stranded DNA from DSBs in a process called end clipping [21–29]. This first stage of DSB end resection is followed by long-range resection via parallel pathways mediated by the Exo1 exonuclease and the Sgs1/Dna2 helicase-nuclease pair [22,24]. End clipping commits DSB repair to HR, as resected DNA ends are poor substrates for NHEJ. This commitment point is tightly regulated in conjunction with cell cycle phase [30–32], as NHEJ is more beneficial in G1 when sister chromatids are absent, whereas recombination constitutes more faithful repair during S and G2 phases when the synthesized sister chromatids provide accurate repair templates.

Previous studies have shown that kinases confer cell-cycle-dependent regulation of end clipping [31–37]. Both S phase cyclin-dependent kinase (CDK) and DNA damage checkpoint kinases phosphorylate Sae2 to promote end clipping [33,36,37]. This is achieved at least partly by dynamically increasing Sae2 protein solubility [37]. This form of regulation is critical as Sae2 is predominantly present as inactive aggregates in G1, presumably to limit resection in this phase [36,37]. Upon treatment with DNA damaging agents in S phase, phosphorylation of Sae2 facilitates the rapid release of active monomeric and dimeric forms from the inactive aggregates to promote end clipping, and thus HR [37].

Here, we show that the conserved sumoylation of Sae2 also promotes its functions in the processing and repair of multiple kinds of DSBs. Interestingly, like phosphorylation, sumoylation also increases the levels of soluble Sae2. We show that the two different modifications act in synergy to achieve a stronger effect on Sae2 function. Moreover, we present evidence that sumoylation of MRX also favors resection, suggesting that the coordinated sumoylation of multiple substrates leads to greater biological consequences.

Results

Sae2 sumoylation occurs on a single lysine in its self-association domain

We and others recently reported that five proteins involved in DNA end resection are sumoylated upon DNA damage in budding yeast [17,18]. Here we examined the sumoylation of the Sac2 protein. The Sac2 sumoylated form migrates ~20 kDa higher than the unmodified form upon SDS-PAGE, as expected from the typical up-shift caused by mono-sumoylation (Fig. 1A). The sumoylated form can be preferentially detected by an antibody against the SUMO moiety (Fig. 1A and [17]). In addition, this modification was abolished by the simultaneous removal of both the homologous Siz1 and Siz2 SUMO ligases, but not of either single ligase (Fig. 1A). These results validate Sac2 sumoylation and indicate that the Siz ligases redundantly sumoylate Sac2.

To evaluate the functional consequences of Sac2 sumoylation, we identified the lysine that is targeted for sumoylation. Sac2 possesses two sumoylation consensus motifs, KxK/E/D, where x is a large hydrophobic acid [38,39]. Mutating one of these sites, K97, to arginine abolished its sumoylation in vivo (Fig. 1B). This residue is conserved among Sac2 orthologs in closely related Saccharomycyes species (Fig. S1A). To confirm that K97 is the SUMO conjugation site, Sac2 was co-expressed with sumoylation enzymes in E. coli to enable its sumoylation (see Methods). A higher-migrating form of Sac2 in the purified protein prep was specifically eliminated by treatment with the desumoylase Ulp1, indicating that it is the sumoylated form of Sac2 (Fig. S1B). Consistent with the in vivo finding, Sac2-K97R mutant protein was not sumoylated in vitro (Fig. 1C). Together, our in vivo and in vitro data indicate that lysine 97 is the bona fide SUMO conjugation site on Sac2. K97 is located within the N terminal domain that is important for self-association in several organisms (Fig. 1D and [37,40-42]).

Sac2 orthologs are also sumoylated

As conserved modification of a protein in different organisms is indicative of functional importance, we examined whether Sac2 orthologs that share DNA resection functions are also targeted for sumoylation. To this end, we subjected recombinant Sac2 orthologs, namely fission yeast Ctp1 and human CtIP, to sumoylation in E. coli using reconstituted fission yeast and mammalian SUMO conjugating systems, respectively. Both SpCtp1 and hCtIP exhibited a slow migrating modified form only upon co-expression of SUMO and conjugating enzymes (Fig. 1E–1F), suggesting that they can be sumoylated. The conserved sumoylation of Sac2 orthologs supports the notion that this modification can be functionally relevant.

Sac2 sumoylation facilitates processing of complex DSB ends

Next, we investigated whether abolition of Sac2 sumoylation affects its functions by studying the sac2-K97R allele. *sae2-K97R* did not affect Sac2 protein levels in normal growth conditions or after genotoxic treatment at either 30°C or 37°C, excluding an effect of sumoylation on general protein stability (Fig. 2A and S1C Fig.). We then tested whether *sae2-K97R* affects the processing and repair of complex DNA ends, such as those capped by hairpin structures or covalently linked with proteins, using established assays. To query hairpin repair *in vivo*, we measured recombination that requires removal of hairpins formed through inverted Alu sequences at DSBs [27,43]. Consistent with a previous report, deleting SAE2 greatly reduced the recombination rate measured in this assay (Fig. 2B and [27,43]). *sae2-K97R* showed a 2-fold reduction (Fig. 2B), suggesting a moderate deficiency of Sac2 function in hairpin removal.

To examine processing of DSB ends that are covalently linked with proteins, we first examined Sac2-mediated processing of DSBs capped with Top1, which are induced upon camptothecin
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(CPT) treatment [29,44]. Consistent with previous reports, sae2Δ cells were sensitive to CPT (Fig. 2C and [29,44]). sae2-K97R cells exhibited an increase in sensitivity to CPT at 37°C (Fig. 2C), suggesting that sumoylation of Sae2 contributes to CPT repair. Consistent with this, Sae2 sumoylation is induced by CPT treatment and elevated temperature (S1D Fig.). Second, we examined sporulation efficiency, as Sae2-mediated removal of Spol1 conjugated to DSB ends in meiosis is required for sporulation (Fig. 2D and [28,45–47]). sae2-K97R homozygous diploid cells exhibited a reproducible 20% reduction in this assay, indicating a moderate defect (Fig. 2D, p<0.05). Taken together, these results show that sae2-K97R is partially defective in the processing and repair of complex DSBs.

Sae2 sumoylation promotes DNA end clipping

We proceeded to assess whether Sae2-mediated end clipping of clean DSB ends is affected by sae2-K97R. In yeast, end clipping can be directly assayed at DSBs induced by the endonuclease HO at the MAT locus [22]. As shown previously, because end clipping is an intermediate stage in end resection, it can be best monitored when the downstream extensive resection step is blocked by removing Sgs1 and Exo1 [22]. Both qPCR- and Southern blot-based assays can be used to assess Sae2 function in this setting. The two assays take advantage of the fact that single-stranded DNA generated by resection is resistant to restriction enzyme digestion. In the qPCR-based assay, PCR products amplified using primers flanking a StyI site located 700 bp distal to the DSB are compared between digested and undigested samples (Fig. 2E, left panel, and [24,30,48]). PCR products from a control locus, ADH1, are used for normalization (see Methods). Using this assay, we found that sae2-K97R exhibited 60–80% of the wild-type level of resection in a time course of 120 minutes in the sgs1Δ exo1Δ background (Fig. 2E, right). The lethality of sae2Δ sgs1Δ exo1Δ prevents comparison of sae2-K97R defects with sae2Δ in this setting [22].

In the Southern blot assay, end clipping products run as a smear of bands below the HO cut (unprocessed) fragment, and both types of bands are detected by a radio-labeled probe specific to a sequence flanking the DSB (S2A Fig. and [22]). In sgs1Δ exo1Δ cells, the intensity of the smear moderately increased as the unprocessed fragment diminished with time (S2A–S2C Fig.), signifying the progress of end clipping [22]. Introducing the sae2-K97R mutation reduced end clipping efficiency, as seen by the persistence of the unprocessed fragment and decreased intensity of the smear (Fig. 2F). Clone analysis indicated a reduction of up to 50% in the fraction of end clipping products among total cut fragments (Fig. S2C). Taken together, both the qPCR- and Southern blot-based
assays show that lack of Sae2 sumoylation impairs end clipping of clean DSBs.

Sae2 sumoylation suppresses NHEJ in the sgs1Δ exo1Δ background

As end clipping favors NHEJ, its impairment would promote NHEJ [36,49,50]. Accordingly, a prediction of the observed end clipping defect in sae2-K97R sgs1Δ exo1Δ cells compared with sgs1Δ exo1Δ cells (Fig. 2E and S2A–S2C Figs.) is that the former should have higher NHEJ levels. Indeed, we detected an ~30% increase in NHEJ in the triple mutant compared with the double, using a standard chromosomal NHEJ assay (S2D Fig.). As this assay was performed side-by-side with the Southern blot-based resection assay, equal efficiency of HO cleavage between genotypes was confirmed (S2A Fig.). We also used a well-established plasmid-based NHEJ assay in which cells are transformed with linearized or undigested plasmid DNA and plated on media selective for the plasmid. Percentage plasmid repair was calculated by dividing the number of colonies recovered from digested samples with undigested. See Methods for experimental details. For each genotype, values from at least three experiments were averaged and standard deviations were calculated. The difference between the two genotypes at indicated time points is statistically significant (p<0.05, asterisk).

Sumoylation and phosphorylation of Sae2 contribute separately to DNA damage resistance

As phosphorylation of Sae2 is required for its resection function and DNA damage resistance [33,36,37], we asked whether sae2-K97R interferes with this modification. The phosphorylated forms of Sae2 manifest as slower migrating bands, and mutating two Bmh1-digested or undigested plasmid DNA and plated on media selective for the plasmid. Percentage plasmid repair was calculated by dividing the number of colonies recovered from digested samples with undigested. See Methods for experimental details. For each genotype, values from at least three experiments were averaged and standard deviations were calculated. The difference between the two genotypes at indicated time points is statistically significant (p<0.05, asterisk).

To further elucidate the interplay between the two modifications, we assayed the sumoylation levels of Sae2 phosphorylation.
Sumoylation of Sae2 increases the levels of soluble Sae2
We proceeded to examine how sumoylation of Sae2 affects its function. As shown recently, an important means of regulating Sae2 by protein modification is through increasing its solubility [37]. Sae2 is primarily in inactive aggregate forms in G1, whereas the soluble and active fraction of Sae2 increases upon entering S phase in the presence of DNA damage [37]. Phosphorylation of Sae2 by Mec1 and S-CDK promotes this increase [37]. Considering the genetic interaction between the two types of Sae2 modifications, we examined if sumoylation also alters the levels of soluble Sae2.

Using an established solubility assay, we examined G1-arrested cells after release into 0.03% MMS [37]. Consistent with our genetic data, levels of soluble Sae2-K97R-3A protein were significantly lower than that of Sae2-3A after cells were released from G1 (Fig. 4A). We also observed a similar but smaller decrease in levels of soluble Sae2-K97R when compared with that of wild-type Sae2 (Fig. 4B). In this case, the level of soluble Sae2-K97R protein is decreased by ~25% compared to wild-type in a cell cycle-independent manner, suggesting that sumoylation by itself also affects Sae2 solubility. This reduction is less severe than that of Sae2-3A alone, which showed an S phase-specific decrease in Sae2 soluble levels by up to 50%, compared with wild-type Sae2 (Fig. 4C). These results suggest that while both phosphorylation and sumoylation promote Sae2 solubility, the former has a stronger effect. As the solubility difference between Sae2-3A and Sae2-K97R mutants is only 25%, yet their MMS sensitivities differ greatly, it is possible that the Sae2-3A has additional defects besides solubility.

Evidence supporting a role for MRX sumoylation in DSB processing
Because sae2-30R exhibited mild resection defects and genotoxin sensitivity (Fig. 2 and S2 Fig.), and not to the level that has been reported for SUMO E2 (e.g. [17]), we reasoned that sumoylation likely yields a strong influence on this process by additionally targeting other factor(s), such as MRX. As mapping sumoylation sites on the three subunits of MRX proved difficult, we devised a genetic strategy to reduce MRX sumoylation. It has been shown that the catalytic domain of the de-sumoylating enzyme Ulp1 (UD) when fused with a protein can lead to the targeted removal of SUMO conjugated to the protein or its interactors [52]. A fusion with mutations of four residues required for catalytic activity and SUMO interaction (UD*) was used to control for the effect of tagging with this domain [52].

MRX physically interacts with the Ku complex, which arrives at DSBs concomitantly with MRX [53,54]. We tested if fusing the UD domain to the Ku70 subunit (YKU70-UD) can specifically decrease MRX sumoylation. To this end, we introduced the YKU70-UD or YKU70-UD* constructs at the endogenous YKU70 locus with its native promoter. As shown in Fig. 5A, sumoylation of all three subunits of MRX was either abolished or strongly reduced in cells expressing YKU70-UD compared with YKU70-UD* control cells. To assess the specificity of desumoylation, we examined proteins that arrive at DSBs around the same time as MRX [53,55]. YKU70-UD did not affect the sumoylation of Sae2, or the Ku-interacting protein Lif1 (Fig. 5B). In addition, sumoylation of the downstream recombination proteins Rad1 and...
Saw1 was not affected by YKU70-UD (Fig. 5B). Together, these results suggest that YKU70-UD can limit MRX sumoylation with good specificity.

We then examined whether YKU70-UD has a phenotype indicative of defective MRX-mediated resection. As MRX deficiency can exacerbate sae2Δ sensitivity to DNA damaging agents, we performed resection assays in wild-type and YKU70-UD* cells. The results showed that resection was impaired in YKU70-UD compared to YKU70-UD*. qPCR-based resection assay was performed as described in Fig. 2E. At least three spore clones for each genotype were tested. Asterisks indicate statistically significant differences (p < 0.05).

**Fig. 5.** Sumoylation of MRX contributes to DNA end resection. A–B. Sumoylation of the three MRX subunits (Mre11, Rad50 and Xrs2), but not that of Sae2, Lif1, Rad1 or Saw1, is decreased in YKU70-UD cells compared to YKU70-UD*. Experiments were done as in Fig. 1A, and cells were treated with MMS. Triangles indicate the mono-sumoylated forms of the proteins examined. C. YKU70-UD, but not YKU70-UD*, sensitizes sae2Δ to MMS. 10-fold serial dilutions were used. D. Resection is impaired in YKU70-UD compared to YKU70-UD*. qPCR-based resection assay was performed as described in Fig. 2E. At least three spore clones for each genotype were tested. Asterisks indicate statistically significant differences (p < 0.05). E. sae2-K97R impairs resection in YKU70-UD, but not YKU70-UD* cells, Assay was performed as in Fig. 2E. At least three spore clones for each genotype were tested. Asterisks indicate statistically significant differences between YKU70-UD and YKU70-UD* at all time points (p < 0.05). F. Working model for the role of Sae2 sumoylation in DSB resection. MRX denotes the Mre11-Rad50-Xrs2 complex.

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agents in certain contexts [44], we tested whether reducing MRX sumoylation by YKU70-UD causes a similar phenotype. Indeed, we found that YKU70-UD worsened the MMS sensitivity of sae2Δ cells, while YKU70-UD* conferred suppression (Fig. 5C). The latter effect is likely due to the tag’s interference with Ku function, such as in inhibiting HR by Exo1 exclusion [44,56,57]. That YKU70-UD is additive with sae2Δ suggests that the defects caused by reduced MRX sumoylation overrides any suppression conferred by defective Ku function. This in turn suggests the possibility that reduced MRX sumoylation impairs its resection function. To test this idea, we examined resection dynamics in YKU70-UD vs. YKU70-UD* cells. In both qPCR- and Southern blot-based assays, DSB resection was decreased by 15-20% in YKU70-UD cells compared to YKU70-UD*, most obviously at early time points (Fig. 5D–5E and S3A–S3B Fig.), suggesting that sumoylation of MRX facilitates resection.

To assess if the sumoylation of MRX and Sae2 independently promotes resection, we measured end resection in YKU70-UD sae2-K97R cells. As shown in Fig. 5E, sae2-K97R further compromised resection in YKU70-UD, but not YKU70-UD*, cells. The moderate additivity in resection defects did not result in exacerbation of YKU70-UD’s MMS sensitivity by sae2-K97R (Fig. 5C), likely because it is insufficient to confer MMS sensitivity or YKU70-UD exerts compensatory effects due to impaired Ku function. Taken together, our results suggest that sumoylation of MRX, in addition to that of Sae2, contributes to resection.

**Discussion**

Regulation of DNA repair pathway levels and capacity in response to cell cycle changes and lesion loads is important for genome maintenance and damage resistance. Despite recent progress, many forms and targets of regulation are not yet identified or understood. One of the most highly regulated DNA repair proteins is the end resection factor, Sae2. Its solubility is tightly controlled such that its activity and other functions are limited in G1 phase and increased in S phase under damage conditions [37]. We reveal here that regulation of Sae2 solubility is partly mediated by sumoylation. We also show that sumoylation collaborates with checkpoint-dependent phosphorylation in facilitating Sae2 function in end clipping. Furthermore, we provide evidence that sumoylation promotes DNA end resection by additionally targeting the MRX nuclease. This work reveals a novel mechanism of SUMO-mediated regulation of DNA repair and uncovers an example wherein sumoylation and phosphorylation, as well as multiple sumoylation events, collaborate to promote nuclease function (see model in Fig. 5F).

Proteins that cleave DNA are double-edged swords, and unscheduled DNA cleavage has to be minimized. In general, upstream constraints such as limiting recruitment to lesions can restrict the activity of downstream factors (e.g. [58–61]). As Sae2 is one of the first resection proteins to arrive at DSBs without any upstream constraints such as limiting recruitment to lesions can restrict the activity of downstream factors (e.g. [58–61]). As Sae2 is one of the first resection proteins to arrive at DSBs without any known recruiters [55], it is not surprising that it is subjected to other forms of regulation. Our findings and previous reports strongly suggest that Sae2 regulation can be achieved by different protein modifications that collaborate to ensure timely availability of the active forms of the protein [37,62,63].

Thus far, regulation of protein solubility by sumoylation has been reported only for proteins involved in neuronal diseases (e.g. [64–66]). We now provide the first example wherein sumoylation regulates the solubility of a DNA metabolism protein. As protein aggregation is a widespread phenomenon caused by high intrinsic aggregation potential of the protein (e.g. [67,68]), it is conceivable that SUMO-mediated protein solubilization is a general effect. Such an effect by SUMO may be similar to its promotion of solubility in recombinant protein applications [69,70]. Thus, in addition to the previously proposed glue effect of sumoylation in bridging interactions in complexes [18,71,72], sumoylation can also have the opposite effect of “anti-glue” to disperse proteins from aggregates. As sumoylation occurs in the Sae2 self-association domain and at a region of high aggregation potential (SA Fig. and [41,73]), steric or charge changes conferred by sumoylation in these regions can disfavor aggregation.

Several independent analyses show that lack of Sae2 sumoylation moderately reduces end resection and increases NHEJ (Fig. 2B–2F and S2A–S2D Fig.). The correlation of these effects with changes in the levels of soluble Sae2 suggests that the decreased availability of active Sae2 can at least partly account for the end resection defects and NHEJ increase, though other possibilities such as defective DSB recruitment cannot be excluded. As the resection defect of sae2-K97R is less severe than that of the SUMO E2 mutant, sumoylation of additional resection factors also likely matters. Indeed, reduction of MRX sumoylation also impairs resection, and in a manner additive with sae2-K97R (Fig. 5). Although a thorough examination of MRX sumoylation awaits mapping of sumoylation sites on all three subunits, these results suggest that sumoylation achieves a large biological effect by simultaneously inducing small changes in multiple substrates (“ensemble effect”). This suggestion is consistent with the observations that several dozen repair proteins are sumoylated upon DNA damage [17,18,74], and individual non-sumoylatable mutants usually exhibit only mild phenotypes (e.g. [75–77]). We propose that the ensemble effect model is common in DNA repair regulation and other processes to confer robustness to a system. We also note that the usefulness of this strategy is also seen for other protein modifications (e.g. [32,62]), and that as in the case of sumoylation, the effects of a particular modification are unique to the substrate, rather than conforming to a general mechanism (e.g. [8,78–80]).

In summary, our work provides strong evidence for a new role for sumoylation in regulating DNA repair and its collaboration with phosphorylation-based regulation. Considering that only a few sumoylated substrates in DNA repair have been examined in detail thus far, future studies on additional substrates and the interplay between sumoylation and other forms of regulation will greatly expand our knowledge of how repair pathway levels and choice are determined in cells.

**Materials and Methods**

**Yeast strains and genetic manipulations**

Strains used are listed in Table 1. Only one strain per genotype is shown for simplicity, but at least two strains per genotype were tested in each assay. Standard yeast protocols were used for strain generation, growth and medium preparation. As siz1Δ siz2Δ results in amplification of the 2 micron plasmid [81], strains with siz1Δ siz2Δ mutations were cured of the plasmid as described [82].

**Protein preparation and detection of sumoylated proteins**

Detection of the sumoylated form of Sae2 was performed as described previously [17]. In brief, log phase cultures were treated with 0.3% methyl methanesulfonate (MMS, Sigma-Aldrich) or 50 µg/ml camptothecin (CPT, Sigma-Aldrich) or at 37°C for 2 h. Cells were lysed by bead beating under denaturing conditions, and TAP- or HA-tagged proteins were immunoprecipitated. These were then washed and eluted with loading dye, followed by SDS-PAGE and western blotting with antibodies against SUMO [83],
| Strain        | Genotype                                                                 | Source                        |
|--------------|---------------------------------------------------------------------------|-------------------------------|
| W1588-4A    | MATα leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RADS5             | R. Rothstein                  |
| T587        | MATa SAE2-TAP::HIS3                                                       | This study                    |
| X4086-10B   | SAE2-TAP::HIS3 mms21-11::HIS3                                            | This study                    |
| X6231-2A    | SAE2-TAP::HIS3 sae2::URA3                                                | This study                    |
| X3200-1C    | sae2-K97R-TAP::HIS3                                                      | This study                    |
| X4088-11B   | MATα leu2::GAL1-HO-LEU2 hmr1 hml1 sae2-K97R gsg1-1::HYG exo1-1::KAN     | This study                    |
| X3868-25C   | MATα leu2::GAL1-HO-LEU2 hmr1 hml1 sae2-K97R gsg1-1::HYG exo1-1::KAN     | This study                    |
| Z358-3      | MATα sae2-K97R                                                           | This study                    |
| X3293-1B    | sae2::KAN                                                                | This study                    |
| T954-1      | sae2-267A-TAP::HIS3                                                      | This study                    |
| T1237-2     | sae2-K97R-3HA::URA3                                                      | This study                    |
| T1240-1     | sae2-249A, S278A, T279A-3HA::URA3                                        | This study                    |
| X1259-1A    | SAE2-TAP::HIS3 (S288C)                                                   | This study                    |
| X1382-7B    | SAE2-TAP::HIS3 mec1-1::HYG sml1-1::KAN (S288C)                             | This study                    |
| T1184-5     | sae2-267A::KAN                                                           | This study                    |
| T1185-3     | sae2-K97R, S267A::KAN                                                    | This study                    |
| T1165-1B    | sae2-249A, S278A, T279A::KAN                                             | This study                    |
| T1166-15    | sae2-K97R, S249A, S278A, T279A::KAN                                      | This study                    |
| T1241-10    | MATα sae2-K97R, S249A, S278A, T279A-3HA::URA3                            | This study                    |
| G786        | MATa SAE2-3HA::URA3                                                      | J. Petrini                    |
| T1238-2     | MATalpha sae2-267A-3HA::URA3                                             | This study                    |
| T1239-29    | MATalpha sae2-K97R, S267A-3HA::URA3                                      | This study                    |
| X6006-1B    | YKU70-V5-UD::KAN MRE11-TAP::HIS3                                        | This study                    |
| X6005-2D    | YKU70-V5-UD::KAN MRE11-TAP::HIS3                                        | This study                    |
| X6025-3A    | YKU70-V5-UD::KAN RAD50-3HA::HIS3                                         | This study                    |
| X6024-2A    | YKU70-V5-UD::KAN RAD50-3HA::HIS3                                         | This study                    |
| X6039-3C    | YKU70-V5-UD::KAN XRS2-TAP::HIS3                                         | This study                    |
| X6038-2C    | YKU70-V5-UD::KAN XRS2-TAP::HIS3                                         | This study                    |
| X6023-1B    | YKU70-V5-UD::KAN SAE2-TAP::HIS3                                         | This study                    |
| X6022-4A    | YKU70-V5-UD::KAN SAE2-TAP::HIS3                                         | This study                    |
| X6112-1D    | YKU70-V5-UD::KAN LIF1-TAP::HIS3                                         | This study                    |
| X6111-2C    | YKU70-V5-UD::KAN LIF1-TAP::HIS3                                         | This study                    |
| X6057-9C    | YKU70-V5-UD::KAN RAD1-TAP::HIS3                                         | This study                    |
| X6056-5B    | YKU70-V5-UD::KAN RAD1-TAP::HIS3                                         | This study                    |
| T1653-12D   | YKU70-V5-UD::KAN                                                         | This study                    |
| T1655-4D    | YKU70-V5-UD::KAN                                                         | This study                    |
| X5945-4C    | YKU70-V5-UD::KAN sae2-3::HA::URA3                                      | This study                    |
| X5947-12D   | YKU70-V5-UD::KAN sae2-3::HA::URA3                                      | This study                    |
| X6040-1A    | YKU70-V5-UD::KAN sae2-K97R                                               | This study                    |
| X6041-2C    | YKU70-V5-UD::KAN sae2-K97R                                               | This study                    |
| X6482-9A    | MATa ade3::GAL1-HO hmr1 hml1 YKU70-V5-UD::KAN                             | This study                    |
| X6483-9A    | MATa ade3::GAL1-HO hmr1 hml1 YKU70-V5-UD::KAN                             | This study                    |
| X6480-5D    | MATalpha ade2::GAL1-HO hmr1 hml1 YKU70-V5-UD::KAN sae2-K97R               | This study                    |
| X6481-41B   | MATa ade3::GAL1-HO hmr1 hml1 YKU70-V5-UD::KAN sae2-K97R                   | This study                    |
| G938        | MATa ade5-1, hmr7-2, leu2-3,112::p305L3 (LEU2) trp1-289, ura3-3::HgrB   | K. Lobachev                   |
| G939        | G938 ade5-1, hmr7-2, leu2-3,112::p305L3 (LEU2) trp1-289, ura3-3::HgrB   | K. Lobachev                   |
| T1772-1     | G938 sae2-K97R::KAN in CGL                                                | This study                    |
| G784        | MATa/MATα sae2-K97R, lys2, leu2, ade4 in SK1                             | S. Keeney                     |
| X6556-1     | MATa/MATα sae2-K97R, lys2, leu2, ade4 in SK1                             | This study                    |
the protein A portion of the TAP tag (Sigma-Aldrich) or HA (12CA5). We note that as the F. portion of the SUMO antibody interacts with the Protein A part of TAP, it also detects the unmodified protein, but more strongly so for the sumoylated form because of additional high affinity for SUMO. Protein preparation for detecting Sae2 phosphorylation and protein levels was performed as described [33]; DNA damage treatment was performed as described above.

Assessment of soluble Sae2 protein levels

Assay was performed essentially as described [37] except that all Sae2 constructs were expressed from its own chromosomal locus. In brief, G1-arrested cells were released into 0.03% MMS and samples were harvested at the indicated time points for protein and FACS analyses. Upon complete cell lysis by bead beating and removal of DNA by DNaseI treatment, cell extract was centrifuged at high speed (14k rpm for 30 min) to separate the soluble fraction from the insoluble. The soluble fractions were analyzed by SDS-PAGE and western blotting against the tag. The housekeeping protein Adh1 was used as loading control as its levels are invariant during the time course. FACS analyses show proper arrest and release for all the strains examined. We note that Sae2 is sumoylated in both G1 and S phases during this procedure (S4B Fig.). To assess Sae2 soluble forms in different strains, two spore clones of each genotype were examined in at least two independent tests. For quantification, we compared solubility between the two strains for each time point. In brief, we first determined the soluble Sae2 protein level relative to loading control for each strain at each time point, and then calculated the ratio between the genotypes to represent it in Fig. 4. The student’s t test statistical analysis was performed for “Sae2 protein level relative to loading control” between the two genotypes from 6 repeats (2 trials with 3 spores).

In vitro sumoylation assay

Both GST-tagged Sae2 and hCtIP were sumoylated in E. coli by co-expression with E1 (Aos1-Uba2), E2 (Ubc9) and SUMO-1 [84] (the pT-E1E2S1 plasmid was a gift from Dr. Hisato Saitoh). Plasmids for expression of GST-tagged Sae2 and hCtIP are derivatives of pGEX-4T1 and were a gift from Dr. Stephen Jackson [36,85]. The plasmid expressing GST-Sae2K97R (pRS752) was made by site-directed mutagenesis of pGEX-4T1-Sae2. Plasmids were transformed into BL21 (DE3) cells, and single colonies were used to inoculate overnight cultures of LB (plus 100 μg/ml ampicillin or 25 μg/ml ampicillin and 17 μg/ml chloramphenicol), which were incubated at 30 °C with shaking at 250 rpm. These starter cultures were used to inoculate fresh LB (with ampicillin plus chloramphenicol added as required) cultures to an OD600 of 0.1, which were then grown at 30 °C with shaking at 250 rpm to an OD600 of 1.2. The temperature was then lowered to 25 °C and 250 μM IPTG added to induce expression of the proteins. The cultures were incubated for another 16 h at 25 °C before harvesting by centrifugation. The cell pellet was resuspended in PBS (pH 7.3) supplemented with 1 mM PMSF and 5 mM DTT. After sonication and centrifugation (47000 × g for 20 min), the soluble protein fraction was loaded onto an equilibrated Glutathione Sepharose Fast Flow column (GE Healthcare Life Sciences). The column was washed with 10 column volumes of PBS and the GST-Sae2 and GST-Sae2-SUMO proteins eluted with 2 column volumes of 50 mM Tris-HCl (pH 8.0), 30 mM reduced glutathione. Proteins were dialyzed against 20 mM Tris, pH 8.0 containing 150 mM NaCl at 4 °C. To perform SUMO cleavage reactions, recombinant purified Ulp1 (10 nM) [86] was added to 5 μM partially purified GST-Sae2/ GST-Sae2-SUMO and incubated at 23 °C for 30 min in buffer containing 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween-20, and 2 mM DTT. The proteins were separated on a 10% SDS PAGE gel and analyzed on a western blot probed with an anti-GST antibody (GE Healthcare Life Sciences). 3HA-tagged SpCtp1 was sumoylated in E. coli BL21 (DE3) cells by co-expression with the S. pombe E1 (Rad31+Puf2), E2 (Hus5 fused to a 6 × His-tag) and SUMO (Pmt3GG). The 6 × His-Hus5 fused to 3HA-SpCtp1 and Pmt3GG is tagged with GST. Full details of the plasmid constructs will be provided elsewhere. Transformed BL21 (DE3) cells were cultured and proteins were isolated as described above.

Inverted Alu recombination assay

These were performed as described [43]. Single colonies were picked from streakouts and allowed to grow for 3 days. Each single colony was resuspended in 0.25 ml water by vortexing (0th dilution) and ten-fold serial dilutions were prepared. 100 ul of the 5th dilution was plated onto complete medium, while 100 ul of the 2nd dilution (or 0th dilution for sae2Δ cells) was plated onto medium lacking lysine. Successful recombination by processing Alu-generated hairpin DSBs generates LYS+ colonies. Fourteen colonies were analyzed in this manner for each genotype, and the recombination rate was calculated by fluctuation analysis.

DSB resection assays

Both qPCR- and Southern blot-based assays were performed as described [17,48]. For both assays, a DSB at the MAT locus was introduced by galactose-induced expression of the HO endonuclease throughout the time course either in asynchronous (Figs. 2E and 5D, S2A–S2C and S3A–S3B Figs.), or G2-arrested cultures (Fig. 5E). Samples were collected at the indicated time points. Genomic DNA was isolated and an aliquot was subjected to digestion with XbaI and StyI. For Southern blot-based method, digested DNA was subjected to native agarose gel electrophoresis, transferred to Hybond XL (GE Healthcare) membranes, and
hybridized with radiolabeled DNA probes. Quantification of intensities of bands on the Southern blots was done using ImageGauge. DSB end resection at each time point was calculated as the ratio of the signal intensity at that time point to that at the first time point after HO induction. Note that as Sae2 sumoylation was strongly increased at 37°C (S1D Fig., sae2-97R phenotype in the above assays (except Fig. 5E) was examined at this temperature.

qPCR-based resection assay was performed as described [40]. In brief, 150 ng of genomic DNA isolated as above was subjected to restriction enzyme digestion with StyI or mock-digested in a reaction volume of 15 μl. DNA was diluted by addition of 35 μl of ice-cold dH2O. 8.8 μl of the diluted DNA was used for each qPCR reaction in a total volume of 20 μl. Primer sequences are specified in [40]. PCRs were performed using Bio-Rad Quantitative PCR System, Bio-Rad DNA Engine Chromo 4 system and corresponding software (Opticon). All reactions were amplified using the following program: 95°C for 10 min, 40 cycles of (95°C for 10 s, 55°C for 20 s, and melting curve 10 min. Reactions were set up in triplicates for all primer pairs and the resulting average threshold cycle (Ct) value was used for calculation. The percentage of DNA resolved to 0.7 kb in HO-cut DNA was calculated by \[\frac{Ct_{\text{HO-cut}} - C_{\text{mock}}}{C_{\text{mock}}} \times 100\%\], where \(\Delta C = C_{\text{HO-cut}} - C_{\text{mock}}\), and f is the fraction cut by HO as quantified by Southern blot analysis. All Ct values were corrected for DNA concentrations by comparing with values for amplification at the ADH1 locus. For both resection assays, at least two spore clones of each genotype were examined in two or more trials.

**NHEJ assays**

The analysis of chromosomal NHEJ levels was performed as previously described [87]. DSB induction was induced for 1.5 h, was performed side-by-side with the resection assay (compare cleavage efficiency at 1.5 h). DSBs were induced in cells that cannot repair the break by HR and rely on NHEJ for repair; thus, NHEJ proficiency can be discerned by comparing the numbers of colonies that survive transient DSB induction. Plasmid-based NHEJ assay was performed by transforming either 1 ng of undigested or 20 ng of BamHI-digested pRS416 plasmid carrying URA3 into competent cells, and plating on medium lacking uracil. For yka70A control cells, 100 ng of digested plasmid was used for transformation. Successful NHEJ repair results in ligation of the linearized plasmid and thus growth on -URA medium. Transformation efficiency was calculated as the number of colonies on -URA medium divided by the amount of DNA transformed. NHEJ repair for each genotype was calculated as the ratio of transformation efficiencies of digested to undigested samples. For both NHEJ assays, at least two spore clones of each genotype were examined in two or more independent trials.

**Other methods**

Spot assays were performed as described previously [17]. Briefly, log phase cells were diluted 10-fold and spotted onto YPD media with or without CPT or MMS. Plates were incubated at 30°C (Fig. 5C) or 37°C (Figs. 2D, 2G and 3E–3F), and photographed after 24–72 h. At least two spore clones of each genotype were examined in two or more independent trials. Sporulation assay was performed essentially as described [36]. Diploid SK1 cells were grown overnight in YPD medium, washed twice with warm sporulation medium, and left in sporulation medium for 36 h at 30°C. The percentage of sporulated cells was determined by light microscopy.

**Supporting Information**

**S1 Fig** Sae2 is sumoylated in vitro and in vivo, and sae2-97R does not affect protein levels. A. Sequence alignment of Sae2 and its orthologs from closely related *S. cerevisiae* species at the sumoylation site. The sumoylated lysine of Sae2 is indicated by the red arrow. B. Sae2 sumoylation in *E. coli* and validation by Ulp1 treatment. See Materials for details. C. Sumoylation of Sae2 does not affect its protein levels. Sae2-97R (KR) protein levels are similar to wild-type (WT) before (-) or after treatment with CPT, hydroxyurea (HU) or MMS at 37°C. Extracts from SAE2-TAP* cells exposed to the indicated agents were analyzed by western blotting with antibody recognizing TAP. D. Sae2 sumoylation is induced by CPT, MMS or elevated temperature. TAP-tagged Sae2 from cells treated with the indicated agents or elevated temperature was immuno-precipitated and analyzed as in Fig. 1A. (EPS)

**S2 Fig** sae2-97R impairs DSB end resection and increases NHEJ in *sgs1A exolA* cells. A–C. Formation of end clipping products is impaired by sae2-97R. A. Left: Diagram illustrating the resection assay. Induction of the HO endonuclease results in a double strand break at the *MATa* locus. Resection can be visualized by the disappearance of cut fragment released by HO and digestion with StyI (S). This 0.7 kb cut fragment (double arrowed line below the locus, middle panel) can be visualized with a probe (dark line above the construct) on Southern blots. Right: End clipping was examined in *sgs1A exolA* cells where extensive resection is blocked. The HO cut fragment (HO cut) and the end clipping fragments below them (bracket) were examined using the indicated probe (left) on Southern blots at indicated time points after HO induction. An example of this analysis is shown with the loading control. B. HO cut fragment persists longer in sae2-97R cells. The 0.7 kb cut fragment, i.e. the unprocessed fragment from (A) and two more trails of similar experiments was quantified for each time point, normalized to the loading control and compared to the value at 30 min. Note that HO cleavage efficiency was taken into account at each time point. C. *sae2-97R* reduces the levels of end clipping products. The percentage of end clipping products within total cut fragment was calculated for each genotype based on at least three experiments. The averages and standard deviations at each time point are shown. The difference between the two strains for each time point is statistically significant (p<0.05, asterisk). D. Lack of Sae2 sumoylation results in higher NHEJ levels in the *sgs1A exolA* background. Chromosomal NHEJ was assayed using the same construct as in A. As the homologous repair template is absent, cells can survive transient HO induction only if NHEJ seals the break. Thus, survival percentage is a readout of NHEJ efficiency. Asterisks indicate statistically significant differences (p<0.05, asterisk). (EPS)

**S3 Fig** Reduction of MRX sumoylation impairs DSB end resection. A–B. Resection efficiency is reduced in *YKU70-UD* compared to *YKU70-UD*° cells. A. A representative result of resection assay as in S2A Fig. is shown. B. Quantification of the ratio of unprocessed HO cut fragment in *YKU70-UD*° to that in *YKU70-UD* is plotted based on at least three independent trials. Asterisks indicate statistically significant differences (p<0.05). Three spore clones of each genotype were examined. (EPS)

**S4 Fig** Sumoylation time course and aggregation potential profile of Sac2. A. Sac2 sumoylation was examined in the experimental conditions for testing solubility of Sac2. Time course was performed as in Fig. 4 and sumoylation levels were examined
as in Fig. 1A. B. Aggregation potential profile of Sac2 with sumoylation site shown in red and phosphorylation sites indicated in purple. Aggregation potential for a protein was calculated by AGGREGSCAN [73], which is based on amino acid measurements from in vitro experiments and assumes that short and specific stretches of amino acids regulate protein aggregation.

[EPS]

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Author Contributions

Conceived and designed the experiments: PS RS MCW XZ. Performed the experiments: PS RS. Analyzed the data: PS RS MCW XZ. Contributed reagents/materials/analysis tools: QF TTP. Wrote the paper: PS RS MCW XZ. Edited the manuscript: RS MCW QT TP. Discussed results and communicated unpublished data: VA LK.

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