RESEARCH ARTICLE

Non-Smc element 5 (Nse5) of the Smc5/6 complex interacts with SUMO pathway components

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ABSTRACT

The Smc5/6 complex in Saccharomyces cerevisiae contains six essential non-Smc elements, Nse1-6. With the exception of Nse2 (also known as Mms21), which is an E3 small ubiquitin-like modifier (SUMO) ligase, very little is understood about the role of these components or their contribution to Smc5/6 functionality. Our characterization of Nse5 establishes a previously unidentified relationship between the Smc5/6 complex and factors of the SUMO pathway. Nse5 physically associates with the E2 conjugating enzyme, Ubc9, where contacts are stabilized by non-covalent interactions with SUMO. SUMO also mediates the interactions between Nse5 and the two PIAS family E3 SUMO ligases, Siz1 and Siz2. Cells carrying the nse5-ts1 allele or lacking either SIZ1 or SIZ2 exhibit a reduction in Smc5 sumoylation upon MMS treatment and demonstrate functional redundancy for SUMO mediated events in the presence of DNA damage. Overall, given the extensive connection between Nse5 and components of the SUMO pathway, we speculate that one function of the Smc5/6 complex might be as a scaffold center to enable sumoylation events in budding yeast.

KEY WORDS: Smc5/6 complex, SUMO, Ubc9, PIAS E3 SUMO ligases

INTRODUCTION

The Smc5/6 complex is a member of the structural maintenance of chromosomes (SMC) family of proteins that includes cohesin and condensin. These complexes function in part by providing organization to chromatin during multiple processes including transcription, chromosome condensation and segregation, and DNA repair (Jepsson et al., 2014; Tapia-Alveal et al., 2014). The Smc5/6 complex includes two SMC components, Smc5 and Smc6, and six non-Smc elements, Nse1-6, all of which are essential for survival in the budding yeast Saccharomyces cerevisiae (Zhao and Blobel, 2005; Ben-Aroya et al., 2008; Duan et al., 2009b). The binding location of Nse1-4 within the complex is conserved between organisms; however, the position of Nse5-Nse6, which forms a heterodimeric subcomplex, is more divergent. In Schizosaccharomyces pombe (fission yeast), Nse5 and Nse6 were mapped to bind the head region of Smc5 and Smc6 (Palecek et al., 2006), but in Saccharomyces cerevisiae (budding yeast), Nse5 and Nse6 were found to bind the hinge region of Smc5 and Smc6 (Duan et al., 2009b). High throughput yeast two hybrid (Y2H) studies in budding yeast identified a number of potential Nse5 binding partners including some components of the sumoylation machinery and the small ubiquitin modifier (SUMO) protein itself (Hazbun et al., 2003). In addition, we determined that even though Nse5 interacted with SUMO, it was not a target of sumoylation (Bustard et al., 2012). Here we generate additional mutant alleles of NSE5 with the aim of understanding the physiological significance of Nse5-SUMO interactions and determining a role for Nse5 within the Smc5/6 complex.

Nse2 (hereafter referred to as Mms21) is a component of the complex that binds the coiled-coil domain of Smc5 (Duan et al., 2009a). Mms21 is an E3 SUMO ligase with a diverse range of targets including Smc5, Yku70, and Smc2 (Zhao and Blobel, 2005; Takahashi et al., 2008), and thus it potentially regulates a range of nuclear functions. Disruption of the Smc5 binding domain in Mms21, rather than it ligase domain, results in lethality. Thus, the essential function of Mms21 is likely its involvement in maintaining the conformation of the Smc5/6 complex, and not its SUMO ligase activity (Duan et al., 2009a).

SUMO family members have different names and the homolog in budding yeast is called SMT3 (suppressor of mif two 3). Sumoylation is a posttranslational modification where SUMO is covalently attached to and detached from other proteins to modulate their functions. Prior to conjugation with target proteins, SUMO is first cleaved at its extreme C-terminus by Ulp1 to reveal a di-glycine motif (Johnson et al., 1997). Next, the E1-activating enzyme Aos1/Uba2 uses energy from ATP to form a SUMO-adenylate conjugate (Johnson et al., 1997). This SUMO-adenylate bond is necessary to form the thioester bond between SUMO and the E2 conjugating enzyme, Ubc9, which itself is able to conjugate SUMO to target proteins (Johnson and Blobel, 1997). Even though Ubc9 catalyzes sumoylation on its own, the process is greatly enhanced by the presence of an E3 SUMO ligase (Gareau and Lima, 2010). In budding yeast, there are four E3 SUMO ligases: PIAS family homologs, Siz1 and Siz2, which appear to catalyze the majority of sumoylation (Johnson and Gupta, 2001). Cst9 is a meiosis-specific ligase (Cheng et al., 2006), and Mms21, which as mentioned above, is a component of the Smc5/6 complex (Zhao and Blobel, 2005). Each of these ligases contain a Sp-RING domain that is essential for functionality, however the term ‘ligase’ is somewhat misinforming, as these E3 ligases do not actually perform an enzymatic reaction. Rather, it has been proposed that the role of the E3 is to orient the E2-thioester-SUMO complex in a conformation that favors the transfer of SUMO to the target protein (Geiss-Friedlander and Melchior, 2007). A SUMO acceptor site in targets has been mapped to be a lysine residue in the consensus ΨKXE where Ψ is an aliphatic residue (Mahajan et al., 1998; Matunis et al., 1998). Crystal structures revealed that the acceptor lysine sits in the catalytic site of...


harboring nse5 genetic interactions were also observed, for example sensitivity was also observed when cells were grown on plates harboring α proteins, such as Smc5, after MMS exposure. Investigate if Nse5 mediates the sumoylation of certain target pathway (Hazbun et al., 2003; Bustard et al., 2012), we wanted to (Zhao and Blobel, 2005) and Nse5 has potential ties to the SUMO pathway components and show that interactions between Nse5 and PIAS E3 ligases, Siz1 and Siz2, and with the E2 conjugating enzyme, Ubc9, are partially mediated by SUMO. Two temperature sensitive (ts) alleles, nse5-ts1 or nse5-ts2, show a marked decrease in Smc5 sumoylation and neither protein interacts with Smt3 in Y2H analysis. However unlike nse5-ts1, nse5-ts2 mutant cells did not show sensitivity to MMS, which uncouples a functional role for Smc5 sumoylation in response to MMS-induced DNA damage.

**RESULTS**

**Genetic interactions and Smc5 sumoylation in nse5-ts1 mutants during MMS treatment**

The Smc5/6 complex is involved in DNA replication and repair and characterizing the individual subcomponents of the complex will augment full understanding of how Smc5/6 works. To begin our characterization of the Nse5 component, we utilized the ts mutant, nse5-ts1, which is lethal at 37°C (Fig. S1A,B) (Bustard et al., 2012; Ben-Aroya et al., 2008). We combined this allele with other mutants in the Smc5/6 complex. Upon treatment with 0.03% MMS for 1 h, there was a loss in viability to ~7% when nse5-ts1 was combined with mms21-11 compared to 67% and 96% for the nse5-ts1 and mms21-11 single mutants, respectively (Fig. 1A). This synergistic loss of viability was not observed when nse5-ts1 was combined with two other complex mutants, smc5-6 or smc6-9 (Fig. 1B,C), suggesting a potential for overlap in the functionality of Nse5 and Mms21. This sensitivity was also observed when cells were grown on plates containing a low concentration of 0.001% MMS (Fig. 1D). Other genetic interactions were also observed, for example nse5-ts1/nse3-1 double temperature-sensitive mutants showed moderate sensitivity over the single alleles (Fig. S1C) and cells harboring nse5-ts1/nse4-2 double mutants exhibited MMS sensitivity similar to levels observed with nse5-ts1/mms21-11 (Fig. S1C). In contrast to Mms21, however, a defined role for Nse4 within the complex is currently unclear and therefore was not further investigated in this study. As Mms21 is SUMO ligase (Zhao and Blobel, 2005) and Nse5 has potential ties to the SUMO pathway (Hazbun et al., 2003; Bustard et al., 2012), we wanted to investigate if Nse5 mediates the sumoylation of certain target proteins, such as Smc5, after MMS exposure.

We measured Smc5 sumoylation by western blot analysis with α-Smt3 (SUMO) on immuno-precipitated Smc55My/ε-0.3% MMS treatment (Fig. 1E). Consistent with previous reports (Cremona et al., 2012; Bermudez-Lopez et al., 2015), sumoylation in wild-type cells, which is detected in unchallenged condition, markedly increases as after 1 h of exposure to MMS (Fig. 1E). Under conditions of MMS treatment, the level of Smc5 sumoylation was substantially reduced in nse5-ts1 mutants (Fig. 1E) and was even lower than what was observed in mms21-11 mutant cells (Fig. 1F, showing a lighter exposure for α-smt3 than in Fig. 1E to distinguish the levels sumoylation between WT and mms21-11). Due to limited antibodies, hereafter the status of sumoylation was detected using 8His-tagged Smt3 strains, where Smt3 is Ni-NTA purified under denaturing conditions. This method has been routinely used (Wohlschlegel et al., 2004; Psakhye and Jentsch, 2012; Ferreira et al., 2011; Bustard et al., 2012), and it was reassuring that the two approaches gave comparable results. The intensity of multiple bands migrating above 150 kDa, which represents the sumoylated form of Smc5, were reduced in the two mutants (Fig. 1G), with the reduction in Smc5 sumoylation in nse5-ts1 mutant cells appearing more pronounced than that observed with cells harboring the mms21-11 allele (Fig. 1G).

**Nse5 interacts with other components of the sumoylation pathway**

Nse5 was previously shown to interact with SUMO (Hazbun et al., 2003; Bustard et al., 2012) and from the same high throughput Y2H screen (Hazbun et al., 2003), Nse5 was also reported to potentially interact with the E3 SUMO ligase, Siz2, and the E2 conjugating enzyme Ubc9. To verify these observations and expand these analyses of Nse5, Y2H was performed between Nse5 and the four known E3 SUMO ligases in budding yeast. Statistically significant interactions were observed between Nse5 with all E3 ligases except Cts9, the meiosis-specific E3 (Fig. 2A) (Cheng et al., 2006).

The level of binding between Nse5 and Mms21 was significantly higher than background, (i.e. between Nse5 with vector alone), however it was ~5% of the levels measured with Siz1 and Siz2 (Fig. 2A). These data are consistent with Nse5 and Mms21 binding distinct sites of Smc5, where Nse5 interacts at the hinge and Mms21 binds the coiled-coil region (Duan et al., 2009a,b), and suggests that while Nse5 and Mms21 are in the same complex, a strong direct interaction between these subcomponents is not observed.

Given Nse5 interacts with SUMO, we addressed whether the Nse5-Siz1 or -Siz2 interactions depended on SUMO. To this end we incorporated into our Y2H reporter strain a randomly generated temperature sensitive mutant of SMT3, smt3-331, where general sumoylation is impaired (Biggins et al., 2001; Srikumar et al., 2013). The interactions between Nse5 with Siz1 and Siz2 were reduced in cells harboring smt3-311 by 77% and 57%, respectively (Fig. 2B), even when protein overexpression levels were similar (Fig. S2A). In smt3-331 cells, SUMO is not completely depleted as it is an essential protein, thus it is unclear if the residual interactions are dependent on SUMO or not, however these data clearly show there is a correlation between decreased SUMO levels and decreased interactions between Nse5 and both PIAS family E3 SUMO ligases, Siz1 and Siz2. Given that the integrity of Nse5 was important for the SUMO status of Smc5 (Fig. 1F,G), we wanted to determine if the two ligases that interacted with Nse5 contributed to Smc5 sumoylation. A pronounced reduction in Smc5 sumoylation was observed in cells lacking either SIZ1 or SIZ2 after MMS treatment, with only a faint lower-migrating band visible (Fig. 2C). A previous quantitative mass spectrometry (MS) approach determined that Smc5 was not a target for sumoylation by Siz1 or Siz2 under unchallenged conditions (Albuquerque et al., 2013). However upon MMS treatment, our results suggest that functional redundancy with the E3 ligases might arise in response to genotoxic stress, as Smc5 sumoylation is reduced in siz1Δ and siz2Δ mutant cells.

We next determined binding between Nse5 and the E2 conjugating enzyme, Ubc9. A very robust interaction between Nse5 and Ubc9 was observed that was ~5-fold greater than the interactions observed with Siz1 and Siz2 (Fig. 2D,E). As the interactions between Siz1 and Siz2 with Nse5 were partially dependent on SUMO, we wanted to address if the Nse5-Ubc9 interaction also depended on SUMO. We observed that the
Nse5-Ubc9 interaction in smt3-331 mutants was less than 10% of the level in SMT3+ cells (Fig. 2D; Fig. S2B), suggesting that SUMO is also critical for Ubc9 binding Nse5.

SUMO and Ubc9 interact in multiple ways, the most obvious is via a thioester bond at the catalytic site of Ubc9. Transfer of this thioester bond between Ubc9 to the target protein is the last step in sumoylation (Schwarz et al., 1998). In order to test if this thioester bond was important for the Nse5-Ubc9 (SUMO-mediated) interaction, the Ubc9 catalytic cysteine residue 93 was mutated to alanine (Ubc9-C93A), and two-hybrid analysis was performed. The interaction between Nse5 and Ubc9 was only mildly reduced (Fig. 2E). Another way in which Ubc9 and SUMO can interact is through the sumoylation of Ubc9, which has been found to promote SUMO chain formation on target proteins (Klug et al., 2013). In order to determine whether Nse5 was specifically interacting with sumoylated Ubc9, the SUMO-acceptor lysine in Ubc9 was mutated to an alanine (Ubc9-K153R). Again, the Nse5-Ubc-K153R interaction was only mildly reduced, and the double mutant Ubc9-C93A/K153R was comparable to the single amino acid substitutions (Fig. 2E). None of the Ubc9 mutants were determined to be statistically different than wild type for interacting with Nse5. Previous mutational
analysis of Ubc9 also determined that it can extensively interact with SUMO through non-covalent interactions (Bencsath et al., 2002), therefore it is plausible that non-covalent interactions between SUMO and Ubc9 support Nes5-Ubc9 binding, as mutations in the two characterized covalent SUMO-binding sites of Ubc9 had little impact on the Nes5-Ubc9 interaction(s).
Nse5 point mutants were generated to identify residues interacting with SUMO

As Nse5 interacts with SUMO, and its interactions with Siz1, Siz2, and Ubc9 are mediated by SUMO, we wanted to identify critical residues in Nse5 that regulate its interactions with SUMO. The nse5-ts1 allele was generated by PCR-based random mutagenesis and contains 4 amino acid substitutions (Fig. 3A) (Bustard et al., 2012). Here we performed site-directed mutagenesis on the Y2H vector containing NSE5 to generate the individual mutations collectively found in nse5-ts1 (Fig. 3A). Interactions between Smt3 (SUMO) with Nse5, wild-type and mutant proteins, were assessed after verification of expression levels (Fig. S3A). The Nse5-ts1 protein showed a marked reduction in its interaction with Smt3 to 0.5% of wild type (Fig. 3B) (Bustard et al., 2012). The individual point mutants showed varied interactions with Smt3. N183D and Y111H had the most severe defects, down to 1% and 18% of wild type respectively. There was also measurable, but less severe, reduction in the binding between Smt3 with H319Y to 40% of wild type (Fig. 3B), and downward trend, but a non-significant reduction, with Y123H (75%) compared to wild type (Fig. 3B). Next, we determined the ability of the point mutants to interact with Nse6, which is the binding partner of Nse5 within the complex. In contrast to Nse5-ts1, all mutants containing the individual amino acid substitutions interacted with Nse6 at levels comparable to wild type (Fig. 3C; Fig. S3B), indicating that the loss of Nse5-ts1 binding to Nse6 is not attributed to a single mutation. In a parallel set of experiments, we also characterized protein-protein interactions with nse5-ts2, another mutant that contains two substitutions, L70A and L247A (Fig. 3A) (Bustard et al., 2012).
et al., 2012). The point mutants and Nse5-ts2, which contains both L70A and L247A, showed a complete loss of interaction with Smt3 to the level of the vector control (Fig. 3D; Fig. S3A). In contrast, the individual alanine substitution, L70A and L247A, maintained a level of association with Nse6 that was indistinguishable from wild type (Fig. 3E; Fig. S3B) (Horigome et al., 2015). By comparison, the interaction of Nse5-ts2 with Nse6 was reduced to \( \sim 63\% \) of wild-type levels, which was more severe than the single mutants but was not as severe as Nse5-ts1 at only 1% of wild type (Fig. 3E). With Nse5 mutants that lose interaction with SUMO now identified, we next wanted to determine their impact in vivo on Smc5 sumoylation and cell survival upon MMS treatment.

**Mutants of nse5 were integrated into the genome and assessed for Smc5 sumoylation and cell survival**

We generated strains where the nse5 alleles, including the NSE5 promoter, were integrated into the genome at the URA3 locus and where endogenous NSE5 was subsequently deleted so that only the mutant form was present. This was performed for all possible double and triple mutant substitutions in nse5-ts1. Only when all four substitutions were made, and the nse5-ts1 sequence was reconstructed, that substantial MMS sensitivity was observed (Fig. 4B). We also monitored Smc5Myc sumoylation in strains containing 8His-tagged Smt3 by Ni-NTA purification. In contrast to nse5-ts1, which showed major defects in Smc5 sumoylation, only the nse5Y111H and nse5Y123H mutants displayed minor, but visible

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**Fig. 4. Individual point mutants in nse5-ts1 and nse5-ts2 exhibit only minor changes in Smc5 sumoylation and MMS sensitivity.** (A) The His-Smt3 Ni-NTA purification system allowed for purification of sumoylated Smc5 following 0.3% MMS treatment at 25°C in cells containing endogenously Myc-tagged Smc5 with un-tagged Smt3 wild-type (JC720), or His8-tagged Smt3 in wild-type (JC1157), nse5-ts1 (JC1156), nse5-Y111H (JC3797), nse5-Y123H (JC3798), nse5-N183D (JC3799), or nse5-H319Y (JC-3800) mutants. (B) Drop assays (1:10 serial dilutions) with exponentially growing cultures were performed on YPAD±medium containing 0.01% MMS for wild-type (JC470), nse5Y111H (JC2138), nse5Y123H (JC2195), nse5Y111H/Y123H (JC2192), nse5Y111H/Y123H/N183D (JC2193), nse5Y111H/Y123H/H319Y (JC2194), nse5Y111H/Y123H/N183D/H319Y (JC2196), nse5Y111H/Y123H/N183D/H319Y (JC2197), nse5Y111H/Y123H/N183D/H319Y (JC2198), nse5Y111H/Y123H/N183D/H319Y (JC2199), nse5Y111H/Y123H/N183D/H319Y (JC2200), nse5Y111H/Y123H/N183D/H319Y (JC2201), nse5Y111H/Y123H/N183D/H319Y (JC2202), nse5Y111H/Y123H/N183D/H319Y (JC2203), nse5Y111H/Y123H/N183D/H319Y (JC2204), nse5Y111H/Y123H/N183D/H319Y (JC2205), nse5Y111H/Y123H/N183D/H319Y (JC2206), nse5Y111H/Y123H/N183D/H319Y (JC2207), nse5Y111H/Y123H/N183D/H319Y (JC2208), nse5Y111H/Y123H/N183D/H319Y (JC2209), nse5Y111H/Y123H/N183D/H319Y (JC2210), nse5Y111H/Y123H/N183D/H319Y (JC2211), nse5Y111H/Y123H/N183D/H319Y (JC2212), nse5Y111H/Y123H/N183D/H319Y (JC2213), nse5Y111H/Y123H/N183D/H319Y (JC2214), and nse5-ts1 (JC1361) cells at 25°C. (C) Smc5 sumoylation was monitored as in A for un-tagged Smt3 wild-type (JC720), or His8-tagged Smt3 in wild-type (JC1157), nse5-ts1 (JC1156), nse5-ts2 (JC1884), nse5-L70A (JC2900), and nse5-L247A (JC3801) mutants. (D) Drop assays (1:10 serial dilutions) with exponentially growing cultures were performed on YPAD±medium containing 0.02% MMS for wild-type (JC1157), nse5-ts1 (JC1156), nse5-ts2 (JC1884), nse5-L70A (JC2900), and nse5-L247A (JC3801) cells at 25°C.
decreases in the slowest migrating band, indicating reduced sumoylation (Fig. 4A). However, the changes in Smc5 sumoylation showed no correlation with the changes in Smt3-Nse5 interactions. For example, Nse5-N183D showed reduced binding to Smt3, but in nse5N183D cells, Smc5 sumoylation and MMS sensitivity looked indistinguishable from wild type (Fig. 3B, Fig. 4A,B). Taken together, these data suggest that multiple, or all substitutions in the protein expressed from the nse5-ts1 allele are required to reproduce the loss of Smc5 sumoylation and MMS sensitivity.

The alleles of nse5-ts2, nse5L70A, and nse5L247A were also integrated into the genome and assessed for Smc5 sumoylation. Similar to nse5-ts1, nse5-ts2 mutant cells showed a marked loss in Smc5 sumoylation (Fig. 4C). Individually, the nse5L70A and nse5L247A alleles showed a decrease in sumoylation, however the reduction was not as severe as that measured in nse5-ts2 mutant cells (Fig. 4C). These results demonstrate that nse5 mutants can be generated, which have reduced binding to Smt3 and compromised Smc5 sumoylation, but which still interact with Nse6 (and by extension the Smc5/6 complex). However, and surprising to us, cells harboring nse5-ts2 or the individual point mutations exhibited no sensitivity to MMS (Fig. 4D), indicating Smc5 sumoylation is dispensable for cell survival after MMS exposure. These results do not question the importance of the Smc5/6 complex in DNA repair or genome maintenance, but rather the role of Smc5 sumoylation in these processes. Until a clear function for Smc5 sumoylation is identified via mutagenesis of the acceptor lysine(s) on Smc5 we note however, even though this work suggests that Smc5 sumoylation offers no survival advantage after MMS-induced DNA damage, we cannot exclude the possibility that Smc5 sumoylation shares redundancy with a compensatory factor or pathway. As such, the impact of losing Smc5 sumoylation would only be revealed through additional mutagenesis. Moreover, our results do not exclude a role for Smc5 sumoylation or Nse5-SUMO interactions in response to other types of damage (Horigome et al., 2015).

By two-hybrid analysis, Nse5 interacts with Smt3/SUMO, however Nse5, itself, is not a target of sumoylation (Bustard et al., 2012), and no canonical SUMO-interacting motifs (SIMs) are predicted in Nse5. SIMs are a short hydrophobic motif: (V/I)-x-(V/I)-(V/I) which may or may not be flanked by a stretch of acidic residues and located in unstructured, exposed areas of the protein where they insert into a hydrophobic groove on SUMO, facilitating hydrophobic interactions (Kerscher, 2007). However, it seems conceivable that Nse5 could contain non-canonical SUMO-interacting motifs, which have not yet been identified, that stabilize the non-covalent SUMO-Nse5 interaction. To date, there have been at least 20 ubiquitin-binding domains identified (Dikic et al., 2009), yet only one SUMO-interacting motif (SIM) has been described (Kerscher, 2007). It seems plausible that additional SUMO-interacting domains await identification.

Lastly, in human cells promyelocytic leukemia (PML) bodies form as a result of PML sumoylation and its colocalization with Smc5/6 (Brouwer et al., 2009; Potts and Yu, 2007). PML bodies function as ‘sumoylation centers’, however, S. cerevisiae lack PML bodies. Given the extensive interactions between the SUMO pathway components and Nse5 and Mms21, perhaps the Smc5/6 complex in budding yeast serves as a center for sumoylation in the absence of PML protein. We speculate that chromatin bound Smc5/6 facilitates the assembly of sumoylation enzymes at distinct loci to coordinate the sumoylation of target substrates.

MATERIALS AND METHODS

Yeast strains, plasmids, and antibodies

All experiments were performed at 25°C unless indicated otherwise. All strains used in these studies are listed in Table S1. Temperature sensitive strains nse1-5, nse3-1, nse4-2, nse5-ts1, smc5-6, and smc6-9 were derived from the Hieter lab (Ben-Aroya et al., 2008), and mms21-11 were derived from the Zhao lab (Zhao and Blobel, 2005). The smt3-3kr (Jc2996) and smt3-331 (Jc2758) were derived from alleles provided by the Raught lab.
of methyl methane sulfonate (MMS). Plates containing DNA damaging dilution was plated on YPAD plates with and without the indicated amounts were diluted in 10-fold serial dilutions (unless noted), and 4 to untreated cells (plated prior to MMS treatment). Percent survival was the average of three independent experiments (Bjergbaek et al., 2005).

Extracts were incubated with anti-Myc coupled Dynabeads for 2 h at 4°C. Extracts were prepared by lysing cells with glass beads in lysis buffer Myc-tagged Smc5 was used to detect Smc5 sumoylation were whole cell (Aushubel et al., 1994). Protein-protein interactions were detected by (JC2758), where the reporter plasmid pSH18034 was also transformed Liquid culture assays were also performed in wild type (JC470) or LEU2

Plasmids used for Y2H analysis are listed in Table S2. The Drop assays were performed by growing cells overnight, then counting and assays, cells were grown up and plated in fivefold serial dilutions on plates -galactosidase activity for permeabilized cells and represent the various mutants of NSE5, nse5+ (Table S2). These vectors were subsequently integrated into the URA3 locus by plasmid digestion with Apal (Invitrogen) and transformation into wild-type W303 RAD5+ MAFA (JC470). In a separate experiment, JC471, which is W303 RAD5+ MATa, the J-053 linearized vector was integrated at the trpl-1 locus after XbaI digestion, which created trpl-1::NSEC:TRP1. This was followed by deletion of the endogenous NSE5 via PCR-cassette based mutagenesis with a PCR product generated from primers containing the 5' (FW) and 3' (RV) UTR sequence of NSE5 and HIS3-pFA6a. The resulting MATa strain, containing trpl-1::NSEC::TRP1 and nse5A::HIS3, was then crossed to the various MATa strains containing the point mutants, ura3-1::nse5+::URA3, and NSE5+. Upon sporulation and tetrad dissection, haploid cells with the proper marker segregation, with growth on SC – Ura – His, but not SC – Ura – Trp, were verified by DNA sequencing at the University of Calgary sequencing facility to have NSE5, wild type or mutants, at the URA3 locus but not at the endogenous or TRP1 loci.

Viability and drop assays
Drop assays were performed by growing cells overnight, then counting and adjusting cell density to the same concentration (roughly 1×107 cells). Cells were diluted in 10-fold serial dilutions (unless noted), and 4 μl of each dilution was plated on YPAD plates with and without the indicated amounts of methyl methane sulfonate (MMS). Plates containing DNA damaging agents were always poured within 24 h of plating. Plates were incubated at 25°C (unless otherwise noted) for 4-5 days before photographing. Cells were grown overnight at 25°C, and adjusted to 5×106 cells/ml in the morning. Survival of transient MMS treatment was determined by treating cells with MMS (at the concentrations indicated) for one hour. 1000 cells were plated on YPAD, and percent survival was calculated by normalizing to untreated cells (plated prior to MMS treatment). Percent survival was calculated by normalizing to untreated cell growth.

Yeast 2-hybrid (Y2H) analysis
Plasmids used for Y2H analysis are listed in Table S2. The NSE5 genes were cloned into a pEG202-derived bait plasmid, creating Nse5-LexA fusion proteins, wild-type and mutant, which were under control of a galactose-inducible promoter (Aushubel et al., 1994). NSEC, SMT3, UBC9, SIZ1, SIZ2, MMS21 and CST9 were cloned into pG4-6-derived prey vectors, creating a B42-activating domain fusion protein under the control of a galactose-inducible promoter. The Ubc9 mutants C93A and K153R were created by performing site-directed mutagenesis on plasmid J042. All constructs were confirmed by sequencing, and expression was confirmed by western blot analysis using anti-LexA (2-12, sc7392, Lot# H1914) and anti-HA (F-7, sc7392, Lot# K2006) antibodies from Santa Cruz Biotechnology.

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