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Recruitment, Loading and Activation of the Smc5-Smc6 SUMO Ligase

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Abstract

Duplication of the genome poses one of the most significant threats to genetic integrity, cellular fitness and organismal health. Therefore, numerous mechanisms have evolved that maintain replication fork stability in the face of DNA damage and allow faithful genome duplication. The fission yeast BRCT-domain containing protein Brc1, and its budding yeast orthologue Rtt107, has emerged as a “hub” factor that integrates multiple replication fork protection mechanisms. Notably, the cofactors and pathways through which Brc1, Rtt107 and their human orthologue (PTIP) act appeared largely distinct. This either represents true evolutionary functional divergence, or perhaps an incomplete genetic and biochemical analysis of each protein. In this regard we recently showed that like Rtt107, Brc1 supports key functions of the Smc5-Smc6 complex; including its recruitment into DNA repair foci, chromatin association and SUMO ligase activity. Furthermore, fission yeast lacking the Nse5-Nse6 genome stability factor were found to exhibit defects in Smc5-Smc6 function, similar to but more severe than those in cells lacking Brc1. Here we place these findings in context with the known functions of Brc1, Rtt107 and Smc5-Smc6, present data suggesting a role for acetylation in Smc5-Smc6 chromatin loading, and discuss wider implications for genome stability.

Keywords

Brc1; Smc5; Smc6; SUMO; Rad18; Nse5; Nse6; PTIP; Rtt107; replication stress

Introduction

The essential Smc5-Smc6 complex is related to the cohesin and condensin structural maintenance of chromosomes (SMC) complexes, but is uniquely equipped with E3 ligase activities for the post-translational modifiers SUMO and ubiquitin (Aragon 2018; Diaz and Pecinka 2018; Kakui and Uhlmann 2018; Matityahu and Onn 2018; Uhlmann 2016). Briefly, Smc5-Smc6 consists of an essential hexameric core complex assembled by the association of the stable sub-complexes Smc5/Smc6/Nse2 with Nse1/Nse3/Nse4 (Figure 1, (Aragon 2018; Diaz and Pecinka 2018; Uhlmann 2016)). In addition, we identified a third sub-complex of Smc5-Smc6 containing Nse5 and Nse6 (Pebernard et al. 2006), which

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appear functionally conserved across species despite extreme divergence in primary structure (Figure 1, (Duan et al. 2009; Raschle et al. 2015; Yan et al. 2013)).

Smc5-Smc6 remains the most mechanistically enigmatic of the SMC complexes, but it is of fundamental importance to cellular processes such as genome replication, mitotic and meiotic chromosome segregation, DNA repair through homologous recombination, and viral restriction (Aragon 2018; Diaz and Pecinka 2018; Hang and Zhao 2016; Rai and Laloraya 2017; Uhlmann 2016; Wani et al. 2018). Of note, the SUMO ligase activity of Smc5-Smc6 supports several of the foregoing processes by SUMOylating target proteins that include components of the Smc5-Smc6 complex ("auto-SUMOylation" (Andrews et al. 2005; Mahendrawada et al. 2017; Pebernard et al. 2008; Zhao and Blobel 2005)), the cohesin complex, the BLM/Sgs1 RecQ-like helicase, and telomere protection proteins e.g. TRF1/2 (Aragon 2018; Diaz and Pecinka 2018). The above functions of Smc5-Smc6 are amply described in excellent recent reviews (Aragon 2018; Diaz and Pecinka 2018; Hang and Zhao 2016; Uhlmann 2016), so will not be detailed herein. Instead, we discuss our data on the role of the Nse5-Nse6 sub-complex and its interacting partner Brc1 in the focal accumulation, chromatin association and SUMO ligase activity of Smc5-Smc6 at DNA lesions.

Materials and Methods

Yeast strains and growth conditions

Standard fission yeast methods were performed as described previously (Moreno et al. 1991). Strains used in this study are listed in Table 1.

Spot assays

Fission yeast were grown at 32 °C to logarithmic phase (optical density at 600 nm [OD_{600}] of 0.6 to 0.8), spotted on YES agar plates supplemented with the relevant drug in 5-fold dilutions from a starting OD_{600} of 0.5, and then incubated at 30 °C or 32 °C for 3 to 5 days.

Chromatin Immunoprecipitation

Method was adapted from (Nelson et al. 2006). Briefly, exponentially growing cell cultures (untreated, treated with 15 mM HU or 0.005% MMS 6 hrs) were fixed with 1% formaldehyde for 25 min, quenched with glycine. Cells were broken with beads in Lysis buffer (50 mM HEPES, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate; pH 7.6), supplemented with protease inhibitor cocktail (Roche) and 2 mM PMSF. Chromatin extract was sheared with the Bioruptor Pico (Diagenode). Immunoprecipitation was done with 10 μg of FLAG antibody (F3165, Sigma) for 2 hrs followed by incubation with protein G dynabeads (Invitrogen) for 1 hr at 4 °C. Dynabeads were washed 6 times with IP buffer (50 mM Tris-HCl, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, pH 7.5). Input and IP samples were incubated with 10% Chelex 100 (BioRad) and treated with Proteinase K. Quantitative PCR was performed with the resulting DNA samples using SensiFAST SYBR No-ROX Kit (Bioline). Primer sequences for telo2R and STE1 were as previously published (Hayashi et al. 2007; Pebernard et al. 2008; Tapia-Alveal and O'Connell 2011). DNA recovery was calculated using $2^{-\Delta Ct}$ method

and data, representative of 4 independent repeats, are presented as a percentage of input DNA.

Function of the Nse5-Nse6 Sub-Complex

We identified Nse5-Nse6 as an interactor and key cofactor of Smc5-Smc6 in both mitotic and meiotic nuclear divisions (Figure 1, (Pebernard et al. 2006; Wehrkamp-Richter et al. 2012)). The primary structures of Nse5 and Nse6 are not significantly conserved beyond closely related fission yeast species. However, based on their predicted structural composition, we proposed that they were functionally related to budding yeast YML023C (Nse5) and KRE29 (Nse6) that are also sequence orphans and components of the Smc5-Smc6 complex (Pebernard et al. 2006). Supporting functional conservation, we identified the SUMO-targeted Ubiquitin Ligase (STUbL) complex through its physical interaction with fission yeast Nse5 (Perry et al. 2008; Prudden et al. 2007), an interaction that is conserved in the highly diverged budding yeast (Horigome et al. 2016).

Recently, a human protein called FAM178A was found to interact with SMC5-SMC6 and exhibit weak similarity to yeast Nse6 (Raschle et al. 2015). Interestingly, FAM178A (renamed SLF2) interacts with the RAD18 cofactor BRCTx (renamed SLF1), forming a complex that is apparently orthologous to Nse5-Nse6 (Figure 1, (Raschle et al. 2015)).

Importantly, despite critically supporting Smc5-Smc6 functions, Nse5-Nse6 is non-essential in fission yeast but essential in other organisms where tested (Aragon 2018; Diaz and Pecinka 2018). Thus, phenotypic analysis of fission yeast lacking Nse5-Nse6 provides a unique clean separation-of-function within the Smc5-Smc6 complex. We hypothesized that Nse5-Nse6 supports the recruitment and/or loading of Smc5-Smc6 on chromatin, as described for the cohesin loader Scc2-Scc4 (Litwin and Wysocki 2018; Pebernard et al. 2006; Uhlmann 2016). We recently tested this hypothesis using ChIP-qPCR to determine Smc5-Smc6 chromatin association at some of its previously identified binding hotspots (Pebernard et al. 2008).

Strikingly, cells lacking Nse5-Nse6 showed an ~5–10 fold reduction in chromatin occupancy of Smc5-Smc6 at sites including centromeres, telomeres and tDNAs, in the presence or absence of replication stress (see Figure 2 and (Oravcova et al. 2018)). This provided the first unequivocal evidence for Nse5-Nse6 acting as an Smc5-Smc6 recruitment factor during both the unchallenged cell cycle, and in response to replication stress. Moreover, because Smc5-Smc6 is essential, and residual loading is detected in the absence of Nse5-Nse6, an alternative recruitment pathway likely exists.

In an attempt to identify such a pathway, we recently screened for specific dosage suppressors of the phenotypes caused by Nse5 or Nse6 deletion (Zilio et al. 2014). This approach returned Nts1 as a highly specific suppressor of *nse5*Δ or *nse6*Δ cells (Figure 2A and (Zilio et al. 2014)). Strikingly however, Nts1 expression aggravated the phenotypes of hypomorphic alleles of the essential Smc5-Smc6 core complex (e.g. *smc6*-74, Figure 2A and (Zilio et al. 2014)). This result underscores the separation of function between Nse5-Nse6 and the core Smc5-Smc6 complex.

Interestingly, Nts1 overexpression in wild-type cells significantly reduced Smc5-Smc6 chromatin association during replication stress, whereas in *nse6*Δ cells it had the opposite effect (Figure 2B,C). Whilst the mechanism is currently unknown, these data fit with the Nts1-induced sensitization of *smc6–74* cells but partial rescue of *nse6*Δ cells undergoing replication stress (Figure 2A and (Zilio et al. 2014)). Nts1 is part of a novel Clr6 (HDAC1)-based histone deacetylase complex (Zilio et al. 2014), suggesting that modulation of the acetylation state of chromatin or Smc5-Smc6 subunits could positively influence the loading of Smc5-Smc6 in the absence of Nse5-Nse6. Indeed, the association of condensin with chromosomes is impacted by local chromatin structure and, the acetylation state of cohesin's SMC coiled-coils affects its loading (Kulemzina et al. 2016; Robellet et al. 2017), both of which could be regulated by an Nts1-like HDAC.

Overall, Nse5-Nse6 is emerging as a functionally conserved cofactor for Smc5-Smc6 recruitment. Consistent with this, budding yeast hypomorphic for Nse5 (*nse5ts1*) show reduced localization of Smc5-Smc6 to stalled replication forks, but *nse5ts1* also appeared to impact the integrity of the Smc5-Smc6 complex (Bustard et al. 2016). The authors assessed complex stability by co-immunoprecipitation of Nse6 with Smc5 in *nse5ts1* cells (Bustard et al. 2012). Given that Nse5 and Nse6 form a sub-complex this is not surprising, and unlikely affects core complex stability, as previously determined using *in vitro* Smc5-Smc6 complex reconstitution (Pebernard et al. 2006).

In human cells, the SLF1-SLF2 complex recruits SMC5-SMC6 to stalled replication forks (Raschle et al. 2015). Whereas SLF2 contains a highly diverged Nse6-like domain, SLF1 contains ankyrin repeats and a BRCT domain, which are not found in Nse5 from either fission or budding yeasts. The SLF1 BRCT domain binds phosphorylated RAD18, which in turn uses its UBZ domain to recognize ubiquitin deposited at DNA lesions to drive SMC5-SMC6 recruitment (Figure 3, (Raschle et al. 2015)). As described below, this mechanism appears to be both an interesting "amalgam" of, and departure from, the mechanisms used in fission and budding yeasts.

Brc1 Supports Smc5-Smc6 Foci Formation at DNA Lesions

Brc1, a six BRCT domain-containing protein, critically supports resistance to replication stress in fission yeast (Reubens et al. 2017; Sheedy et al. 2005; Williams et al. 2010). It shares this role and its content of six BRCT domains with Rtt107 of budding yeast and human PTIP (Hang and Zhao 2016; Ray Chaudhuri et al. 2016). Furthermore, the C-terminal BRCTs of Brc1, PTIP and Rtt107 bind gamma-H2A enriched chromatin around DNA lesions, enabling them to recruit DNA repair factors through their amino terminal BRCTs (Li et al. 2012; Manke et al. 2003; Williams et al. 2010). Despite these similarities, Brc1, Rtt107 and PTIP appeared to "scaffold" distinct partner proteins to execute their replication fork protection roles (Hang and Zhao 2016).

Brc1 was initially isolated as a dosage suppressor of *smc6–74* (Verkade et al. 1999). Elegant genetic analyses showed that *smc6–74* suppression by Brc1 requires multiple nucleases and a non-catalytic "platform" function of the postreplication repair factor Rad18 (Lee et al. 2007; Sheedy et al. 2005). The combination of a BRCT domain factor (Brc1) and Rad18 in

supporting Smc5-Smc6 function presents an intriguing parallel to the human SMC5-SMC6 recruitment factors i.e. SLF1 and RAD18 (Figure 3, (Raschle et al. 2015)). However, the fission yeast genetic analyses of Brc1 indicated that it acted as a *bypass* suppressor of Smc5-Smc6 dysfunction.

Nonetheless, through biochemical and cell biology approaches we recently revealed that there is a more intimate connection between Brc1 and Smc5-Smc6 than thought. Smc5-Smc6 forms foci in response to replication stress such as hydroxyurea (HU) and methyl methanesulphonate (MMS, (Pebernard et al. 2008)). Brc1 also forms foci in response to replication stress (Lee et al. 2013; Williams et al. 2010) and of note; Brc1 is recruited to the same regions of the fission yeast heterochromatic centromeres as Smc5-Smc6 in the presence of HU (Lee et al. 2013; Pebernard et al. 2008). Moreover, *brc1*Δ and *nse6*Δ cells are hypersensitive to the microtubule poison thiabendazole, which together with their similar chromosome mis-seggregation phenotypes points to potentially overlapping roles for Brc1 and Smc5-Smc6 in centromere function (Lee et al. 2013; Pebernard et al. 2008).

Intriguingly, we found that *Smc5-Smc6 foci depend on both Brc1 and Nse5-Nse6*, and identified a physical interaction between these factors (Oravcova et al. 2018). Moreover, using ChIP-qPCR, Brc1 was shown to promote Smc5-Smc6 chromatin association (like Nse5-Nse6) in response to replication stress. Together with gamma-H2A binding by Brc1, this suggests a model for Smc5-Smc6 recruitment to DNA lesions (Figure 3). The role of Brc1 in Smc5-Smc6 chromatin association echoes that of Rtt107 in budding yeast (Leung et al. 2011), which was unforeseen given the largely distinct synthetic genetic interaction profiles of *brc1*Δ and *rtt107*Δ (Sanchez et al. 2015). Such conservation of Brc1 and Rtt107 function between these highly divergent species also raises the possibility that PTIP may have an SMC5-SMC6 related role that remains to be uncovered.

Surprisingly, in contrast to human SMC5-SMC6 recruitment we found that fission yeast Rad18 *is not required for Smc5-Smc6 foci formation or chromatin association*, despite recently being shown to form a complex with Brc1 (Reubens et al. 2017). Rad18 and Smc5-Smc6 both interact with Brc1's amino-terminal BRCT domains, so these interactions may be mutually exclusive (Oravcova et al. 2018; Reubens et al. 2017). If so, Brc1 acts through distinct pathways to promote Smc5-Smc6 activity during replicative stress. In this regard, Rtt107 scaffolds at least three distinct complexes through its amino-terminal BRCTs (Hang and Zhao 2016).

Activation of the Smc5-Smc6 SUMO Ligase During Replication Stress

The Smc5-Smc6 SUMO ligase is activated during replication stress, especially during MMS treatment, which can be detected as "auto-SUMOylation" of its subunits e.g. Nse4, and modification of certain replication proteins e.g. Mcm6 (Andrews et al. 2005; Hang et al. 2015; Pebernard et al. 2008). The mechanisms behind MMS-induced Smc5-Smc6 activation were not totally clear.

We had shown that Nse4 SUMOylation in MMS treated cells is dependent on Nse2 (Pebernard et al. 2008). Using Nse4 as a reporter, we recently showed that both Brc1 and

Nse5-Nse6 are also required for full activation of the Smc5-Smc6 SUMO ligase (Oravcova et al. 2018). We propose that MMS-induced foci formation facilitates the *trans*-SUMOylation of Nse4 and of other subunits due to the increased local concentration of the complex. Indeed, a recent *in vitro* analysis indicated that budding yeast Nse4 is SUMOylated by Nse2 in *trans*, as opposed to standard E3 ligase "auto-sumoylation" (Pichler et al. 2017; Varejao et al. 2018). Notably, Rtt107 also promotes the SUMOylation of Smc5-Smc6 targets in MMS treated budding yeast, revealing a conserved mechanism (Hang et al. 2015).

Interestingly, whereas deletion of either Brc1 or Nse5-Nse6 abrogates Smc5-Smc6 foci formation in MMS, Nse4 SUMOylation is *reduced* in *brc1*Δ cells but *abolished* in *nse6*Δ cells (Oravcova et al. 2018). This difference correlates well with the Smc5-Smc6 DNA binding defect, which is much more severe in *nse6*Δ versus *brc1*Δ cells (Oravcova et al. 2018). Of relevance, DNA binding by Smc5 was recently shown to activate the Nse2 SUMO ligase (Varejao et al. 2018). In addition, Smc5 ATPase activity is required for both the DNA association and activation of Smc5-Smc6's SUMO ligase (Bermudez-Lopez et al. 2015; Kanno et al. 2015).

In aggregate, the above data point to a sequential recruitment, loading, and activation model: (i) Brc1/Rtt107-mediated "concentration" around DNA lesions, (ii) Smc5-Smc6 ATPase mediated DNA loading assisted by Nse5-Nse6 and (iii) DNA and ATPase induced conformational changes in Smc5 that are transmitted to bound Nse2, promoting SUMOylation of its targets. This model provides a rationale for the selective SUMOylation of chromatin-associated targets by Smc5-Smc6 including cohesin subunits, telomere factors TRF1/2, the RecQ family helicase Sgs1, replication proteins and transcription machinery (Albuquerque et al. 2013; Aragon 2018; Diaz and Pecinka 2018; Hang et al. 2015).

The activation of Smc5-Smc6 SUMO ligase by Brc1/Rtt107 and Nse5-Nse6 will likely contribute to the recently described role of Smc5-Smc6 in regulating the Sgs1-Top3-Rmi1 (STR) complex (Bermudez-Lopez et al. 2016; Bonner et al. 2016). STR acts on DNA recombination structures to mitigate potential genome instability arising from a failure to remove covalent linkages between chromosomes and excessive crossover recombination outcomes, amongst other roles (Bermudez-Lopez and Aragon 2017; Mimitou and Symington 2011). SUMO-modified Smc5-Smc6 recruits STR into DNA repair foci via non-covalent SUMO-interacting motifs (SIMs) in STR, followed by Smc5-Smc5 dependent SUMOylation and "activation" of STR (Bermudez-Lopez et al. 2016; Bonner et al. 2016). Thus, given the defective foci formation and SUMOylation activity of Smc5-Smc6 in Brc1/Rtt107 and Nse5-Nse6 deleted cells, STR mis-regulation will likely contribute to their DNA repair defects.

In addition, by SUMOylating the cohesin subunit Scc1, Smc5-Smc6 promotes the *establishment* of cohesin-mediated sister chromatid cohesion and facilitates recombination-based repair (Almedawar et al. 2012; McAleenan et al. 2012; Wu et al. 2012). In a seemingly contrasting role, Smc5-Smc6 also promotes the prophase *removal* of cohesin from chromosome arms, but a role for SUMOylation in this function is untested (Tapia-Alveal et al. 2014; Tapia-Alveal et al. 2010). Clearly, the foregoing roles of Smc5-Smc6 in

the cohesin cycle appear contradictory i.e. establishment versus removal. This may be due to context specific interactions or additional posttranslational modifications, and requires further study. The role of Brc1/Rtt107 in supporting the chromatin recruitment and activation of the Smc5-Smc6 SUMO ligase further complicates the picture, but also provides additional avenues through which to dissect Smc5-Smc6 functions in chromosome dynamics.

Concluding Remarks

The SUMO ligase activity of Smc5-Smc6 is important during the cellular response to replication stress, and is promoted by Brc1/Rtt107 in the highly diverged fission and budding yeasts (Hang et al. 2015; Oravcova et al. 2018). Perhaps then, PTIP will play a similar role in human cells, but it is possible that alternative pathways have evolved that render this role redundant (Raschle et al. 2015). In this respect, although Nse5-Nse6 appears functionally conserved across species, the primary structures of its subunits have rapidly diverged beyond recognition by standard homology searches (Bustard et al. 2012; Leung et al. 2011; Oravcova et al. 2018; Pebernard et al. 2006; Raschle et al. 2015).

The formation of SUMO-dependent foci of Smc5-Smc6, which recruit and concentrate the DNA repair machinery via SUMO interactions e.g. the STR complex, is an intriguing parallel to PML nuclear bodies (PML NBs) of human cells (Bermudez-Lopez and Aragon 2017; Bonner et al. 2016; Hang et al. 2015; Oravcova et al. 2018; Pebernard et al. 2008). PML NBs are dynamic phase-separated sub-nuclear sites that also concentrate and integrate the SUMO and DNA repair pathways, including the human STR homologue, BTR (Chung et al. 2012; Dellaire and Bazett-Jones 2007; Nagai et al. 2011)). PML NB dynamics are regulated by the SUMO-targeted ubiquitin ligase (STUbL) RNF4, and in yeast, STUbL interacts with the Smc5-Smc6 complex through Nse5 (Hakli et al. 2005; Hazbun et al. 2003; Lallemand-Breitenbach et al. 2008; Prudden et al. 2007; Tatham et al. 2008). STUbL regulates the chromatin residence and/or stability of multiple poly-SUMOylated target proteins, which interestingly include the STR/BTR complex (Bohm et al. 2015; Nie and Boddy 2016).

Therefore, yeast, which do not have PML NBs, may form distinct phase-separated “repair foci” through Brc1/Rtt107 and SUMOylated Smc5-Smc6. Future analyses of Nse5-Nse6, Brc1, and Nts1 in relation to Smc5-Smc6 function will address this possibility, and also provide key insights into the regulation and disease suppressing activity of Smc5-Smc6 (Aragon 2018; Diaz and Pecinka 2018; van der Crabben et al. 2016).

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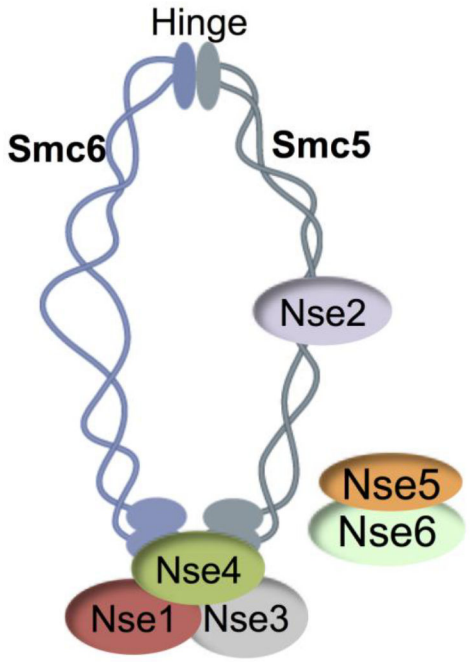

Yeast		Human
Smc5	– *SMC* –	SMC5
Smc6	– *SMC* –	SMC6
Nse2	– *SUMO E3* –	NSMCE2
Nse1	– *Ub E3* –	NSMCE1
Nse3	– *MAGE* –	NSMCE3
Nse4	– *Kleisin* –	NSMCE4
Nse5	– *Localization* –	SLF1
Nse6	– *Localization* –	SLF2

Figure 1. Smc5-Smc6 Subunits and Architecture.

Yeast Smc5-Smc6 subunits organized by known position in complex (Nse5-Nse6 position uncertain). Table shows yeast and human subunits grouped by the stable sub-complexes formed. Protein functions and families indicated: SMC (structural maintenance of chromosomes, SUMO/Ub E3 ligases tag proteins with SUMO or ubiquitin, respectively; MAGE (melanoma-associated antigen), and “kleisin” bridges Smc5/Smc6 ATPase head groups (reviewed in (Aragon 2018; Diaz and Pecinka 2018)).

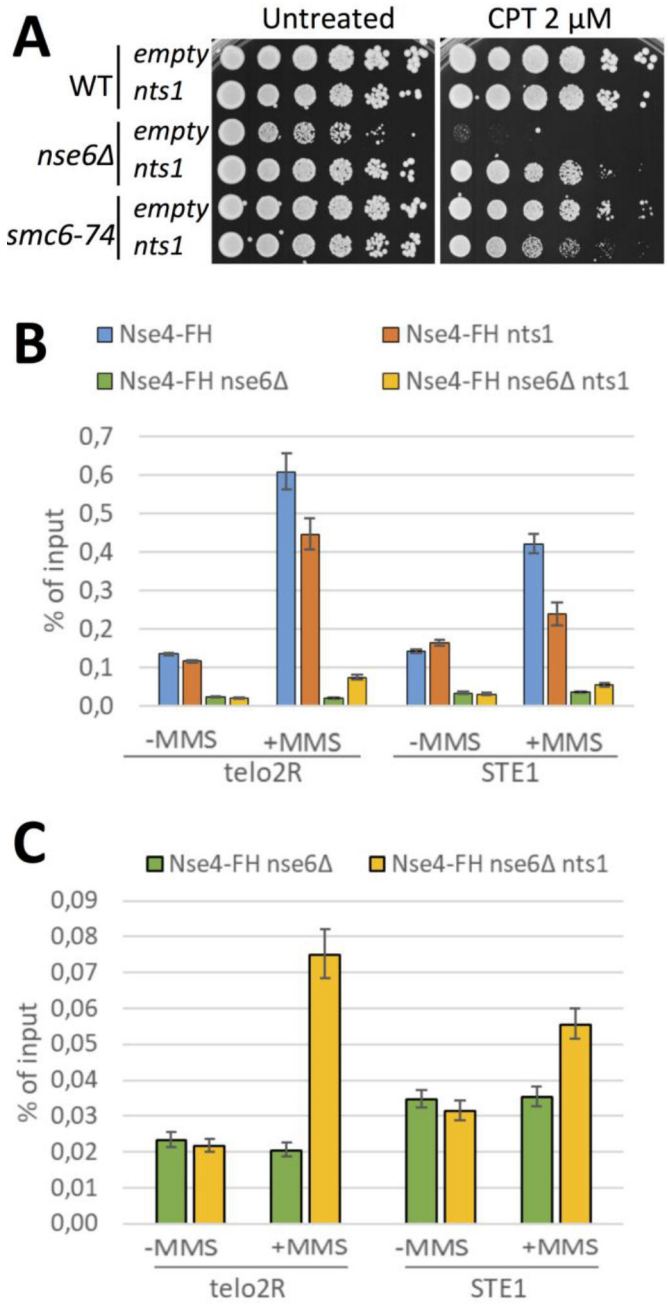

Figure 2. Nts1, a Potent and Specific Suppressor of *nse6*Δ.

A. *pAdh1-nts1* or empty vector (*pAdh1*) stably integrated at the *leu1* locus in the indicated strains. Cells were grown at 30°C for 3 days in the presence or absence of camptothecin (CPT). **B.** ChIP-qPCR against Nse4-FlagHis in the indicated strains at telomeric loci (as described in (Oravcova et al. 2018)). **C.** As in panel "B" but showing only *nse6*Δ background. Note 10-fold lower scale on Y-axis. Data represent ≥ 3 independent experiments.

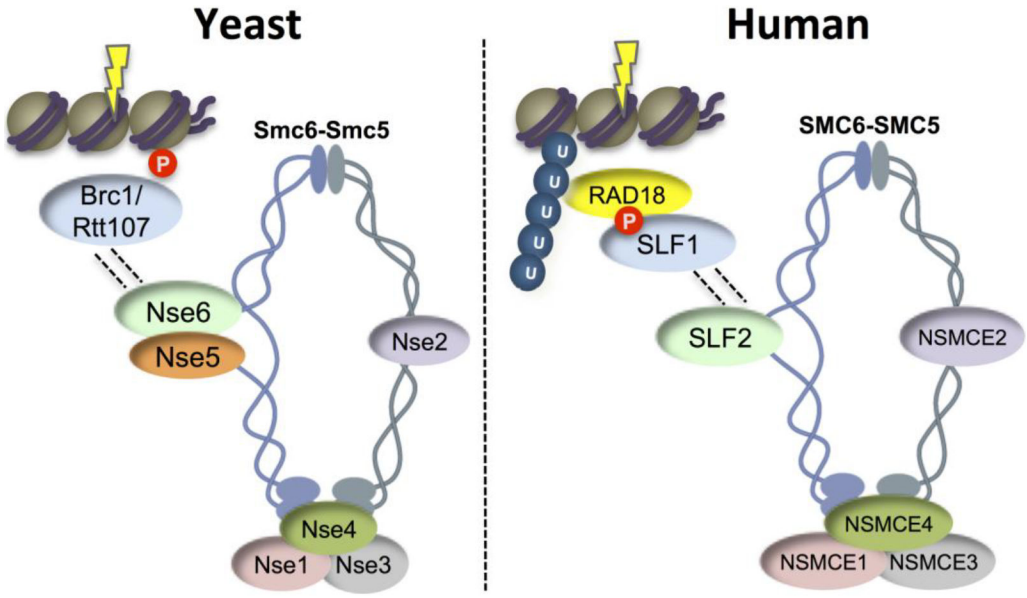

Figure 3. Smc5-Smc6 Recruitment Mechanisms.

In yeast, Brc1 and Rtt107 recruits Smc5-Smc6, likely through Nse5-Nse6, to DNA lesions decorated with gamma-H2A (Leung et al. 2011; Oravcova et al. 2018). Brc1 and Rtt107 use their BRCTs 1–4 to interact with Nse5-Nse6 and BRCTs 5–6 to bind gamma-H2A (Leung et al. 2011; Oravcova et al. 2018). In human cells, SLF2 (Nse6-like) binds SLF1, which binds phosphorylated RAD18 that in turn binds ubiquitin via its UBZ domain (Raschle et al. 2015).

Table 1.

List of yeast strains used in this study.

Strain	Genotype[i]	Source or reference
NB5931	*h+ nse4-Flag-His:kan^R*	(Zabrady et al. 2016)
NB5953	*h+ pJK148-Padh-ura4:leu1*	this study
NB5954	*h+ pJK148-Padh-nts1-ura4:leu1*	this study
NB5955	*h+ nse6::kanMX6 pJK148-Padh-ura4:leu1*	this study
NB5956	*h+ nse6::kanMX6 pJK148-Padh-nts1-ura4:leu1*	this study
NB5959	*h+ smc6-74*	(Verkade et al. 1999)
NB6009	*h- nse4-Flag-His:kan^R pJK148-Padh-ura4:leu1*	this study
NB6010	*h- nse4-Flag-His:kan^R pJK148-Padh-nts1-ura4:leu1*	this study
NB6004	*h- nse4-Flag-His:kan^R nse6::natMX6 pJK148-Padh-ura4:leu1*	this study
NB6005	*h- nse4-Flag-His:kan^R nse6::natMX6 pJK148-Padh-nts1- ura4:leu1*	this study
NB5963	*h- nse4-Flag-His:kan^R nse6::natMX6*	this study
NB5993	*h- smc6-74 pJK148-Padh-ura4:leu1*	this study
NB5994	*h- smc6-74 pJK148-Padh-nts1-ura4:leu1*	this study

[i] All strains are of *ura4-D18 leu1-32* background genotype. Double colons represent knockouts; single colons represent tagging.