

Mre11-Rad50–dependent activity of ATM/Tel1 at DNA breaks and telomeres in the absence of Nbs1

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ABSTRACT The Mre11-Rad50-Nbs1 (MRN) protein complex and ATM/Tel1 kinase protect genome integrity through their functions in DNA double-strand break (DSB) repair, checkpoint signaling, and telomere maintenance. Nbs1 has a conserved C-terminal motif that binds ATM/Tel1, but the full extent and significance of ATM/Tel1 interactions with MRN are unknown. Here, we show that Tel1 overexpression bypasses the requirement for Nbs1 in DNA damage signaling and telomere maintenance. These activities require Mre11-Rad50, which localizes to DSBs and bind Tel1 in the absence of Nbs1. Fusion of the Tel1-binding motif of Nbs1 to Mre11 is sufficient to restore Tel1 signaling in *nbs1Δ* cells. Tel1 overexpression does not restore Tel1 signaling in cells carrying the *rad50-I1192W* mutation, which impairs the ability of Mre11-Rad50 to form the ATP-bound closed conformation. From these findings, we propose that Tel1 has a high-affinity interaction with the C-terminus of Nbs1 and a low-affinity association with Mre11-Rad50, which together accomplish efficient localization and activation of Tel1 at DSBs and telomeres.

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INTRODUCTION

Double-strand breaks (DSBs) are one of the most dangerous DNA lesions, as they break chromosomes as well as the DNA backbone. An inability to properly repair DSBs can result in cell death or cancer (Hoeijmakers, 2001). The Mre11-Rad50-Nbs1 (MRN) protein complex acts as a primary responder to DSBs, quickly localizing to damage sites (Stracker and Petrini, 2011). The forkhead-associated (FHA) domain at the N-terminus of the Nbs1 subunit recruits CtlP/Ctp1, a DNA repair orthologue of *Saccharomyces cerevisiae* Sae2, which activates the intrinsic nuclease activity of Mre11 (Limbo *et al.*, 2007; Sartori *et al.*, 2007; Lloyd *et al.*, 2009; Williams *et al.*, 2009; Cannavo and Cejka, 2014). In mitotic cells, MRN-Ctp1 nucleolytically

displaces Ku from DNA ends, and then initiates resection of the 5' strand to generate a 3' single-stranded DNA (ssDNA) overhang (Garcia *et al.*, 2011; Langerak *et al.*, 2011; Shibata *et al.*, 2014; Lafrance-Vanasse *et al.*, 2015). Rad51 mediates the invasion and base pairing of the ssDNA tail into homologous DNA sequences, usually in the sister chromatid, which it uses as a template to carry out the error-free pathway of homology-directed repair (Stracker and Petrini, 2011).

In addition to CtlP/Ctp1, the MRN complex also recruits the ATM/Tel1 (ataxia telangiectasia mutated) serine/threonine kinase to damage sites (Uziel *et al.*, 2003). ATM is a member of the PI3K-like protein kinase (PIKK) family of proteins, whose members include DNA-PKcs, ATR, and mTor (Paull, 2015). In response to damage, ATM serves to initiate cell cycle arrest, stimulate repair factors, and activate senescence and apoptosis pathways through the phosphorylation of many substrates that include CHK2, H2AX, NBS1, BRCA1, and p53 (Paull, 2015). Mutations in the ATM gene are associated with ataxia telangiectasia (A-T or Louis-Bar syndrome). Patients with this neurodegenerative disorder present with ataxia, telangiectasia, sensitivity to ionizing radiation, immunodeficiency, and a predisposition to cancer (Shiloh, 1997). Mutations in Nbs1 are associated with Nijmegen breakage syndrome, an A-T-like syndrome that includes microcephaly (Carney *et al.*, 1998). Mre11 mutations are associated with an ataxia-telangiectasia-like disorder

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Abbreviations used: ChIP, chromatin immunoprecipitation; CPT, camptothecin; DSB, double-strand break; IR, ionizing radiation; MMS, methyl methanesulfonate; NLS, nuclear localization signal; SMC, structural maintenance of chromosomes; TAE, Tris acetic acid EDTA; TBD, Tel1-binding domain; UV, ultraviolet; wt, wild type.

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(ATLD), which resembles A-T with the exception that in most cases, ATLD does not result in cancer or immunodeficiency (Stewart *et al.*, 1999; Delia *et al.*, 2004; Fernet *et al.*, 2005; Uchisaka *et al.*, 2009).

The activities of the MRN complex are regulated by the Rad50 subunit, which provides a structural scaffold for the complex (Lafrance-Vanasse *et al.*, 2015). Rad50 is an ABC-ATPase with an extended coiled-coil domain that is typical of SMC proteins. In the ATP-bound form, the Mre11-Rad50 globular domains, comprised of the Mre11 nuclease and Rad50 ATPase, are in a closed conformation that promotes DNA binding/tethering and ATM activity. ATP hydrolysis leads to an opening of the complex, which exposes the nuclease sites of Mre11 (Lammens *et al.*, 2011; Lim *et al.*, 2011; Williams *et al.*, 2011; Mockel *et al.*, 2012; Lee *et al.*, 2013; Deshpande *et al.*, 2014).

We have previously shown that a Tel1-binding motif at the extreme C-terminus Nbs1 is critical for Tel1 activity in fission yeast (You *et al.*, 2005). This motif is evolutionarily conserved, being found in Nbs1 orthologues from *S. cerevisiae*, *Xenopus laevis*, mice, and humans (Nakada *et al.*, 2003; Falck *et al.*, 2005; You *et al.*, 2005; Cerosaletti *et al.*, 2006; Difilippantonio *et al.*, 2007; Difilippantonio and Nussenzweig, 2007; Stracker *et al.*, 2007). In most studies, the C-terminus of Nbs1 was found to be crucial for Tel1 activity, but two investigations with murine cells found that eliminating this motif only impairs a subset of ATM activities (Difilippantonio *et al.*, 2007; Stracker *et al.*, 2007), suggesting there may be additional interaction mechanisms involving Nbs1, or even direct interactions of ATM/Tel1 with the Mre11-Rad50 subcomplex. Here, we report studies in

the fission yeast *Schizosaccharomyces pombe* that uncover a mechanism for Tel1 activity at DSBs and telomeres that requires Mre11 but not Nbs1. From these investigations, we propose that in addition to binding Nbs1, Tel1 has direct interactions with Mre11-Rad50 that play an important role in DNA damage signaling at DSBs and telomeres.

RESULTS

The C-terminus of Nbs1 is critical for Tel1 activity

The N-terminus of Nbs1 contains an FHA domain fused to tandem BRCT domains. This region is flexibly linked to a C-terminal region containing motifs that bind Mre11 and ATM/Tel1 (Figure 1A; Lloyd *et al.*, 2009; Williams *et al.*, 2009; Lafrance-Vanasse *et al.*, 2015). These motifs are broadly conserved in eukaryotes, but there are inconsistent findings about whether the C-terminal ATM/Tel1-binding motif of Nbs1 is essential for ATM/Tel1 activity (Nakada *et al.*, 2003; Falck *et al.*, 2005; You *et al.*, 2005; Cerosaletti *et al.*, 2006; Difilippantonio *et al.*, 2007; Difilippantonio and Nussenzweig, 2007; Stracker *et al.*, 2007). We decided to reexamine this question in *S. pombe* using a sensitive immunoblot assay for formation of phosphorylated histone H2A, otherwise known as γ H2A. As both Tel1 and Rad3/ATR phosphorylate histone H2A in fission yeast (Figure 1B; Nakamura *et al.*, 2004), we performed these assays in a *rad3 Δ* background. γ H2A is formed at replication stress sites and at DSBs (Rozenzhak *et al.*, 2010), thus we assayed the basal level of γ H2A formed during S-phase as well as the increased level of γ H2A formed after exposure to ionizing radiation (IR). Basal and

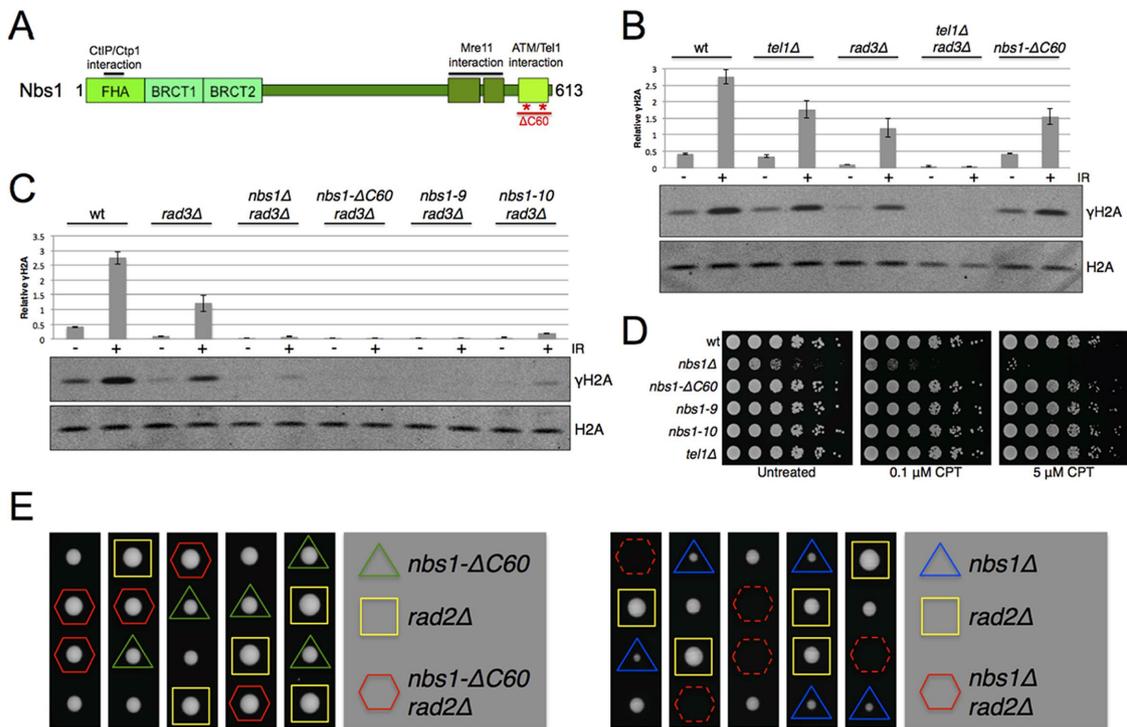


FIGURE 1: The C-terminus of Nbs1 is important for Tel1-mediated phosphorylation of histone H2A but dispensable for DNA repair. (A) Domain architecture of Nbs1. Asterisks denote the location of *nbs1-9* (D603N, D604N) and *nbs1-10* (F611E, F613E) alleles. (B) Both Rad3 and Tel1 contribute to phosphorylation of histone H2A (γ H2A) in response to ionizing radiation (IR). Cells lacking the last 60 residues of Nbs1 encompassing the entire Tel1-binding domain (*nbs1- Δ C60*) have similar levels of γ H2A as *tel1 Δ* . Error bars represent deviation of the mean from three independent experiments. An immunoblot from one of these experiments is shown. (C) *nbs1- Δ C60* and point mutations in the Tel1 interaction motif of Nbs1 (*nbs1-9* and *nbs1-10*) have greatly diminished Tel1-mediated γ H2A formation. (D) *tel1 Δ* and Nbs1 C-terminal mutants are insensitive to CPT. (E) In the *rad2 Δ* (FEN1) background, *nbs1- Δ C60* cells are viable (left), unlike *nbs1 Δ* (right), as assayed by tetrad dissection.

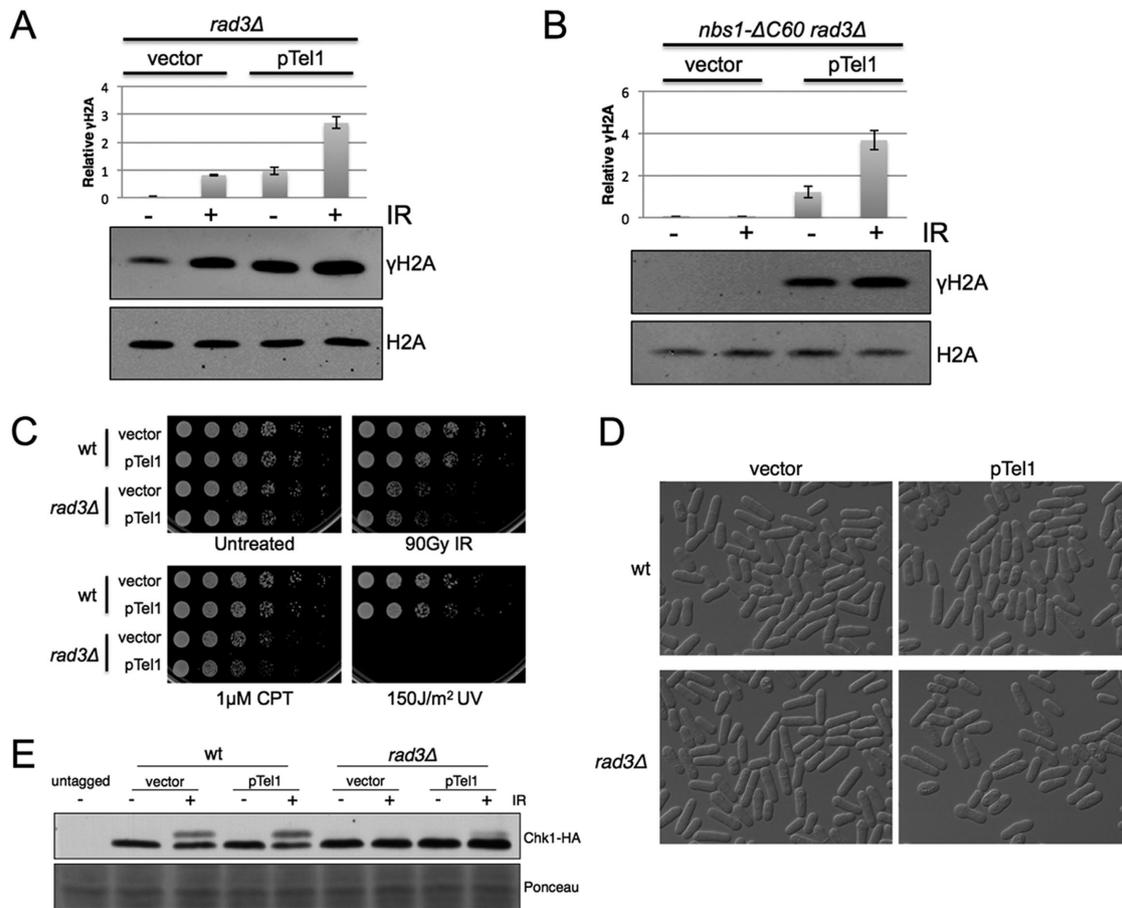


FIGURE 2: Overexpression of Tel1 bypasses the need for the C-terminus of Nbs1 in Tel1 signaling without deleterious effects. (A) Basal levels of γ H2A are increased in *rad3\Delta* cells transformed with a *tel1*⁺ overexpression plasmid. IR exposure increased γ H2A over basal levels. (B) *tel1*⁺ overexpression restores IR-induced γ H2A in *nbs1-ΔC60 rad3Δ* cells. Error bars represent deviation of the mean from three independent experiments. (C) Tel1 overexpression does not negatively affect the growth rate or increase sensitivity to IR, CPT, or UV. (D) Tel1 overexpression does not affect cell morphology or cause cell elongation associated with checkpoint arrest. (E) Tel1 overexpression does not spontaneously cause activating phosphorylation of Chk1. However, Tel1 overexpression does partially restore Chk1 phosphorylation in *rad3Δ* cells in response to IR.

IR-induced γ H2A were nearly abolished in *nbs1Δ rad3Δ* cells (Figure 1C), confirming that Nbs1 is crucial for Tel1 activity. Similarly, truncation of the C-terminal 60 residues of Nbs1 (*nbs1-ΔC60*) almost eliminated γ H2A in the *rad3Δ* background (Figure 1C). The same defects were observed for double missense alleles *nbs1-9* (D603N, D604N) and *nbs1-10* (F611E, F613E), which mutate conserved residues in the Tel1-binding motif (Figure 1C; You *et al.*, 2005). These data confirm the importance of the Tel1-binding motif at the C-terminus of Nbs1.

To confirm that Tel1 binding to Nbs1 is not required for efficient DNA repair, we plated these mutants on media containing camptothecin (CPT), which is a topoisomerase-1 inhibitor. The sensitivity of *nbs1-ΔC60*, *nbs1-9*, and *nbs1-10* mutants to CPT was not increased relative to wild type (Figure 1D), as previously observed for IR and the DNA alkylator methyl methanesulfonate (MMS; You *et al.*, 2005). Similarly, *nbs1-ΔC60* cells were highly viable in the absence of the Rad2 (FEN1) flap endonuclease, which is responsible for maturation of Okazaki fragments, whereas *nbs1Δ* caused lethality in the *rad2Δ* background (Figure 1E). Taken together, these data show that the C-terminal Tel1-binding motif of Nbs1 is crucial for Tel1 activity but plays no obvious role in DSB repair.

Overexpression of Tel1 bypasses the requirement for the C-terminus of Nbs1

In the *rad3Δ* background, the Nbs1 C-terminal mutants often had a weak γ H2A signal in response to IR (Figure 1C). Surprisingly, this signal was also detected in *nbs1Δ rad3Δ* cells (Figure 1C). We never observed γ H2A in *rad3Δ tel1Δ* cells (Figure 1B), showing that Tel1 catalyzed the weak γ H2A formation in *rad3Δ nbs1Δ* cells.

To explore how *nbs1Δ rad3Δ* cells retain weak γ H2A formation in response to IR, we investigated the effects of overexpressing Tel1, as we supposed this might enhance low-affinity interactions with the Mre11-Rad50 complex. We constructed a plasmid expressing *tel1*⁺ from the *nmt1* promoter, which is an attenuated version of the thiamine-repressible *nmt1* promoter. In *rad3Δ* cells, Tel1 overexpression increased the basal signal of γ H2A (Figure 2A). A similar effect was observed in *nbs1-ΔC60 rad3Δ* cells (Figure 2B). Importantly, the IR-induced increase of γ H2A was maintained in these cells, indicating that Tel1 activity remained responsive to DNA damage.

Tel1 overexpression caused no obvious growth defects or increased sensitivity to DNA-damaging agents (Figure 2C), suggesting that it does not cause arrest of the cell cycle. Indeed, *S. pombe rad3*⁺ and *rad3Δ* cells overexpressing Tel1 had normal cell morphologies,

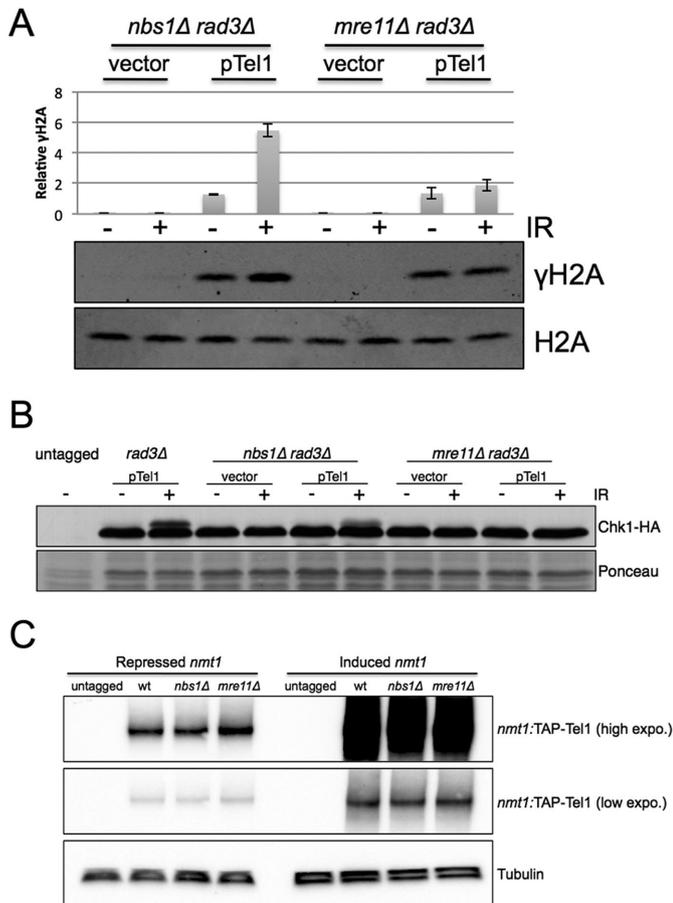


FIGURE 3: Overexpression of Tel1 bypasses the requirement of Nbs1, but not Mre11, in damage-induced Tel1 signaling. (A) IR-induced increase in γ H2A occurs in *nbs1Δ rad3Δ* but not *mre11Δ rad3Δ* background. Tel1 overexpression restores basal γ H2A formation to the same level in both *nbs1Δ rad3Δ* and *mre11Δ rad3Δ* backgrounds, indicating equal Tel1 expression between strains. Error bars represent deviation of the mean from three independent experiments. (B) Chk1 phosphorylation in response to IR is partially restored when Tel1 is overexpressed in the *nbs1Δ rad3Δ* background but not in *mre11Δ rad3Δ* cells. (C) TAP-Tel1 is overexpressed to the same amount in wild-type (wt), *nbs1Δ*, and *mre11Δ* backgrounds. In induced lanes, cells were grown for 24 h in media lacking thiamine to ensure full expression.

unlike the hyperelongated phenotype observed in cells that have activated the checkpoint (Figure 2D). These observations contrast with comparable studies of *S. cerevisiae* in which Tel1 overexpression arrests cell division (Clerici *et al.*, 2001). Our findings suggested that γ H2A formation by overexpressed Tel1 in nonirradiated cells was not accompanied by activation of Chk1 checkpoint kinase. Indeed, Chk1 phosphorylation as assayed by an immunoblot mobility shift was absent in Tel1-overexpressing nonirradiated cells (Figure 2E). Tel1 does not normally phosphorylate Chk1 (Limbo *et al.*, 2011), but Tel1 overexpression caused weak IR-dependent Chk1 phosphorylation in *rad3Δ* cells.

Tel1 overexpression bypasses the requirement of Nbs1 but not Mre11 in DNA damage signaling and telomere maintenance

Our data suggested that Tel1 interactions with MRN complex are not limited to the C-terminus of Nbs1. To investigate the nature of

these interactions, we examined the effect of overexpressing Tel1 in the absence of Nbs1. The *nmt41* promoter driving *tel1⁺* expression in these assays becomes fully derepressed after 18–22 h of growth in media lacking thiamine. Thus, cells were grown for a minimum of 24 h to ensure maximal expression. In *nbs1Δ rad3Δ* cells, Tel1 overexpression restored both basal and IR-induced γ H2A (Figure 3A). This observation suggested that overexpressed Tel1 might phosphorylate histone H2A without any assistance from the MRN complex. Indeed, the basal level of γ H2A was the same in *nbs1Δ rad3Δ* and *mre11Δ rad3Δ* cells that overexpressed Tel1. However, the IR-induced increase in γ H2A observed in *nbs1Δ rad3Δ* cells was largely absent in *mre11Δ rad3Δ* cells (Figure 3A). Similarly, Tel1 overexpression caused IR-induced phosphorylation of Chk1 in *nbs1Δ rad3Δ* cells but not in *mre11Δ rad3Δ* cells (Figure 3B). Immunoblots confirmed that overexpressed Tel1 abundance was nearly equal in wild-type, *nbs1Δ*, and *mre11Δ* strains (Figure 3C), which is consistent with the basal levels of γ H2A being nearly equal in *nbs1Δ rad3Δ* and *mre11Δ rad3Δ* cells that overexpress Tel1 (Figure 3A).

In *S. pombe*, Rad3 and Tel1 contribute to telomere maintenance by phosphorylating a subunit of the Shelterin complex, Ccq1, which leads to the recruitment of telomerase (Moser *et al.*, 2011; Yamazaki *et al.*, 2012). Telomeres erode in the absence of Rad3 and Tel1, which eventually leads to the appearance of survivors with circularized chromosomes (Naito *et al.*, 1998). Elimination of MRN subunits in *rad3Δ* cells leads to the same outcome, showing that Tel1 activity at telomeres requires the MRN complex (Nakamura *et al.*, 2002). Our findings suggested that Tel1 overexpression might also bypass the requirement for Nbs1 but not Mre11 in Tel1 function at telomeres. To test this prediction, we generated *mre11Δ rad3Δ* or *nbs1Δ rad3Δ* mutants with *tel1⁺* expressed from either its native promoter or the *nmt1* overexpression promoter. Genomic DNA was prepared from cells after each passage and Southern blotting was performed, probing for a telomere-associated sequence (TAS1). We found that Tel1 overexpression robustly prevented telomere loss in *nbs1Δ rad3Δ* cells (Figure 4). In contrast, Tel1 overexpression failed to prevent telomere loss in *mre11Δ rad3Δ* cells, although it did appear to delay the complete loss of TAS1 sequences (Figure 4). These effects correlated with the improved growth of *nbs1Δ rad3Δ* and *mre11Δ rad3Δ* cells in the presence of overexpressed Tel1 (Supplemental Figure S1). These results show that as seen for DNA damage signaling, Tel1 overexpression also bypasses the requirement for Nbs1, but not Mre11, in Tel1 activity at telomeres.

Mre11-Rad50 form a complex independently of Nbs1 that is capable of binding DSBs

The different consequences of Tel1 overexpression in *mre11Δ* and *nbs1Δ* backgrounds were surprising, given that both mutants are equally sensitive to DNA-damaging agents (Chahwan *et al.*, 2003). Our data suggested that Mre11 and Rad50 may form a protein complex independently of Nbs1 that facilitates Tel1 activity at DSBs and telomeres when Tel1 is overexpressed. To test this model, we first examined whether Mre11 and Rad50 form a stable protein complex in *nbs1Δ* cells. We performed coimmunoprecipitation (CoIP) experiments with Mre11-MYC and TAP-Rad50 expressed from their respective native promoters. As predicted, Mre11-MYC coprecipitated readily with TAP-Rad50 in *nbs1Δ* cells (Figure 5A). In fact, the coprecipitation was equally efficient in *nbs1Δ* and *nbs1⁺* strains.

We next performed a chromatin immunoprecipitation (ChIP) experiment, assaying Mre11 enrichment around a site-specific DSB generated by the HO endonuclease under the *nmt41* promoter. Mre11 was enriched 0.2 kb from the break site in both *nbs1⁺* and *nbs1Δ* backgrounds (Figure 5B). It is possible that Mre11 binds the

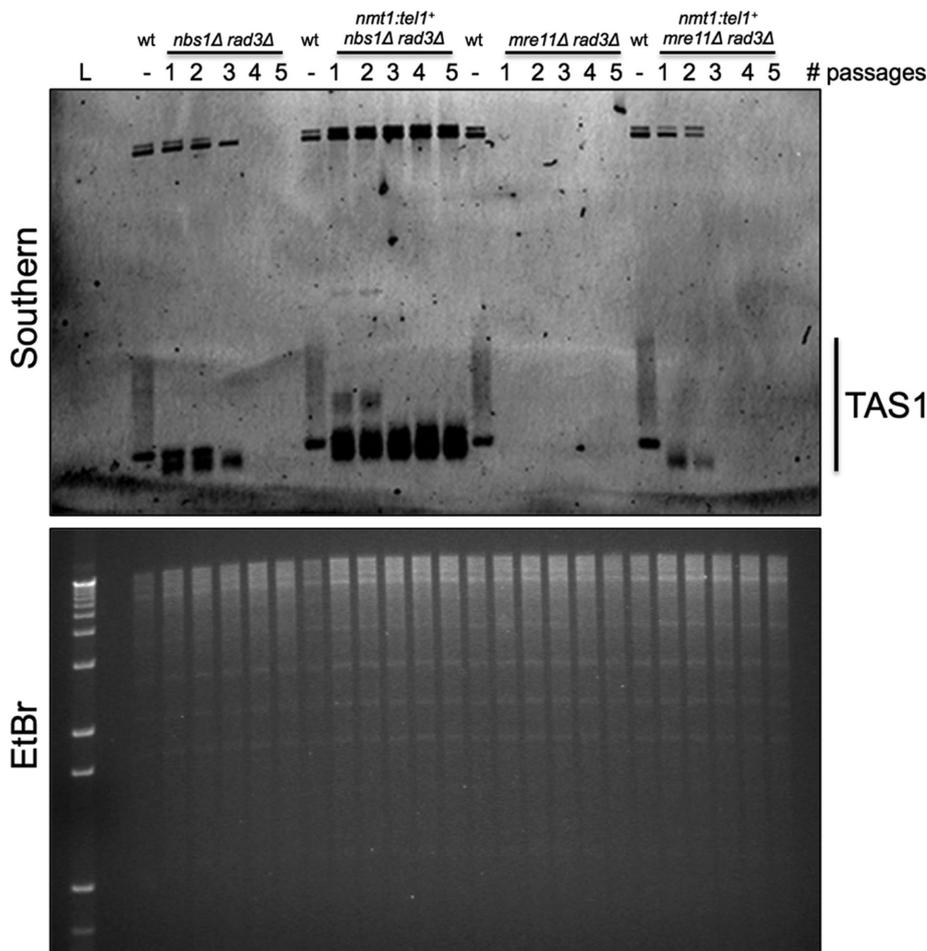


FIGURE 4: Overexpression of Tel1 bypasses the requirement of Nbs1, but not Mre11, in Tel1-mediated telomere maintenance. Southern blot of *EcoRI*-digested genomic DNA probing for telomere-associated sequences (TAS1). Telomere loss observed in *nbs1Δ rad3Δ* cells after successive passages is prevented by Tel1 overexpression. In the *mre11Δ rad3Δ* background, Tel1 overexpression only slightly delayed telomere erosion. Ethidium bromide (EtBr) stained gel serves as a loading control.

HO-induced DSB independently of Rad50. To address this, we performed similar ChIP experiments of Mre11 in the absence of Rad50. Our data shows that the Mre11 enrichment at DSBs in wild-type cells is ablated in the absence of Rad50 (Figure 5C). Similarly, TAP-Rad50 enrichment at the HO site is also dependent on Mre11. Thus, Mre11 and Rad50 are both required for their enrichment at DSBs

We extended our analyses to determine the stability of other subcomplexes of MRN subunits. Nbs1 did not coprecipitate with Rad50 in the absence of Mre11 (Figure 5D), which is consistent with structural data showing Mre11 interacts with both Rad50 and Nbs1, bridging the two subunits (Lammens *et al.*, 2011; Schiller *et al.*, 2012). Next, we looked at the stability of Mre11-Nbs1 in the presence or absence of Rad50 by reciprocal coimmunoprecipitation. The deletion of Rad50 reduces, but does not abolish, the interaction of Mre11-Nbs1 (Figure 5E), suggesting that the Mre11-Nbs1 interaction is stabilized by Rad50 *in vivo*. Taken together, our results show that Mre11 and Rad50 form a stable subcomplex that binds DSBs in absence of Nbs1.

Fusion of the C-terminus of Nbs1 to Mre11 restores Tel1 activity in *nbs1Δ* cells

Having shown that Tel1 overexpression bypasses the need for Nbs1 in Tel1 signaling and that Mre11-Rad50 alone can localize to

DSBs, we asked what role Nbs1 normally plays in this pathway. We postulated that Nbs1 might serve to recruit Tel1 to damage sites where, then, Mre11-Rad50 stimulates its activity. To address this question, we fused the last 60 residues of Nbs1 containing the Tel1-binding domain (TBD) to the C-terminus of full-length Mre11 (Figure 6A). This *mre11-TBD* construct replaced the endogenous *mre11+* gene. We then assayed histone H2A phosphorylation in the *mre11-TBD nbs1Δ rad3Δ* background with endogenous Tel1 levels. Interestingly, Mre11-TBD fusion fully restored phosphorylation of histone H2A to at least the level of *rad3Δ* cells, both basally and in response to IR treatment (Figure 6B). In fact, the basal level of γ H2A exceeded that of *rad3Δ* cells. Moreover, we found that the Mre11-TBD fusion prevented the telomere erosion observed in *nbs1Δ rad3Δ* cells (Figure 6C).

To test the effect of the Mre11-TBD fusion on Mre11 function, we performed spot dilution assays, exposing the strains to different DNA-damaging agents (Figure 6D). The fusion protein alone did not increase the sensitivity of cells to IR and CPT, suggesting that it did not impair Mre11 function. As expected, the Mre11-TBD fusion did not restore the DNA damage repair defect of *nbs1Δ* cells, because the DNA repair activity of the MRN protein complex requires Ctp1 binding to the FHA domain found at the N-terminus of Nbs1 (Lloyd *et al.*, 2009; Williams *et al.*, 2009). Taken together, these results demonstrate that the fusion of the C-terminal 60 residues of Nbs1 to Mre11 was sufficient to restore Tel1 signaling in *nbs1Δ* cells. DNA repair remained defective, however, sug-

gesting that Nbs1 has distinctly separable repair and checkpoint functions.

Tel1 interacts with the Mre11-Rad50 subcomplex

Given the requirement of Mre11-Rad50 in the stimulation of Tel1 activity, it is possible that in addition to a physical interaction with Nbs1, Tel1 may also have an interaction with Mre11-Rad50. Confirming previous results (You *et al.*, 2005), we were able to detect Nbs1-FLAG, expressed under its endogenous promoter, coprecipitating with overexpressed Tel1 (Figure 7A). Similarly, we were able to detect Mre11-MYC, also expressed from its native promoter, in pull downs of overexpressed Tel1, even in the *nbs1Δ* background (Figure 7B). These results suggest that Mre11-Rad50 likely stimulates Tel1 stimulation through direct interactions.

Nbs1-independent activity of Tel1 at DSBs requires the ATP-bound closed conformation of Mre11-Rad50

To further explore the role of Mre11-Rad50 on Tel1 activity, we repeated our γ H2A assay in cells with the *mre11-H134S* allele, which ablates Mre11 nuclease activity (Williams *et al.*, 2008). We found that Tel1 overexpression restored basal and IR-induced γ H2A formation in *mre11-H134S nbs1Δ rad3Δ* cells (Figure 8A), indicating that

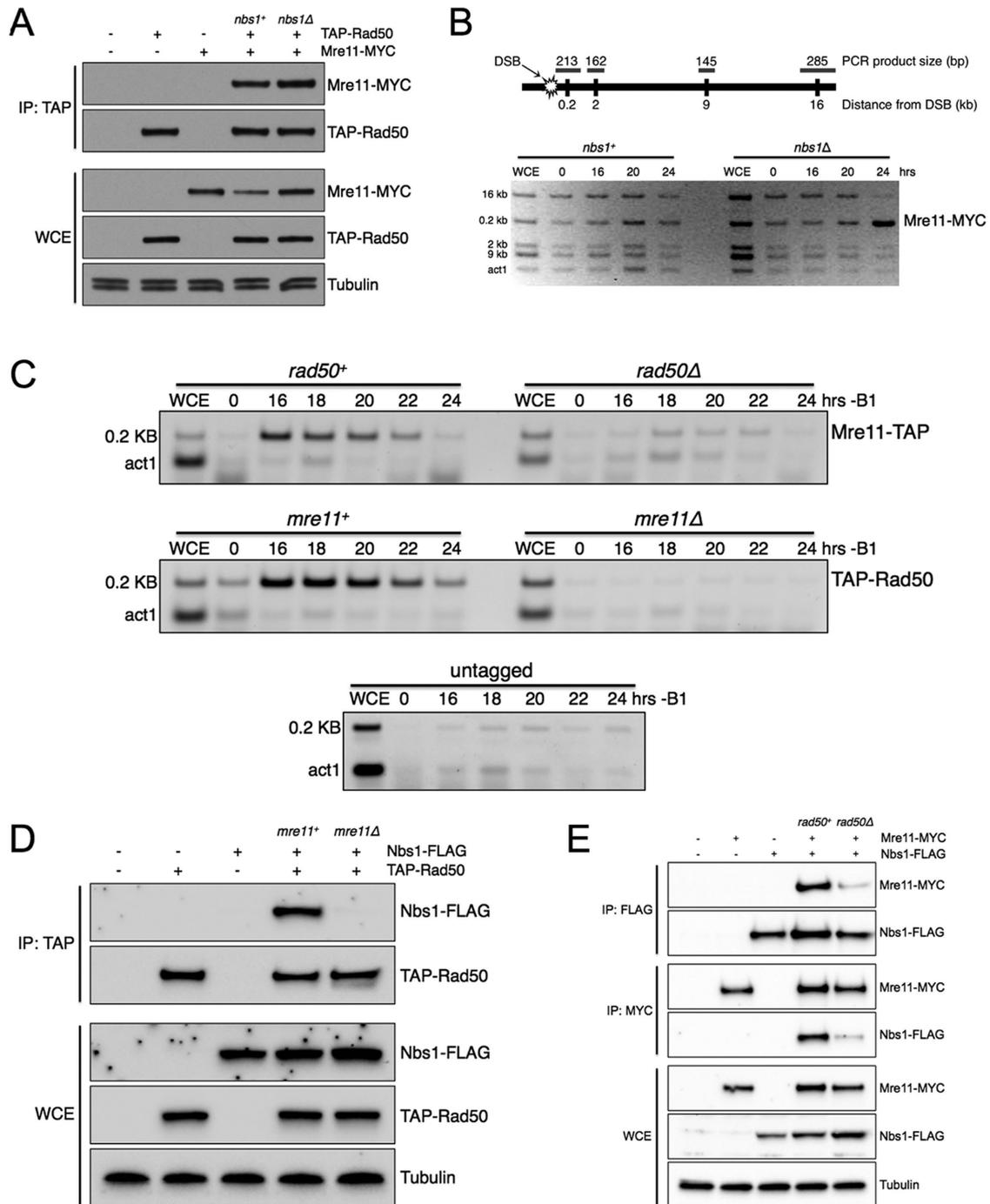


FIGURE 5: Mre11-Rad50 form a stable complex independently of Nbs1 that can localize to DNA double-strand breaks. (A) Mre11-MYC efficiently coprecipitates with TAP-Rad50 in the presence or absence of Nbs1. (B) Mre11-MYC is efficiently enriched at a DSB created by HO endonuclease with or without Nbs1 as assayed by ChIP. HO-endonuclease expression was regulated under the thiamine-repressible *nmt41* promoter, and growth time (in hours) after removal of thiamine (B1) from the media is indicated. Locations of primer sets relative to the HO site and expected PCR product sizes are noted in the schematic. Actin (*act1*), present in a separate locus, serves as a control. (C) Both Mre11 and Rad50 are required for their localization to DSBs as assayed by ChIP. (D) Rad50 does not form a stable complex with Nbs1 in the absence of Mre11. (E) Rad50 stabilizes Mre11-Nbs1 interactions.

the nuclease activity of Mre11 is not required for the Mre11-Rad50-dependent activity of Tel1.

Upon ATP binding, the Mre11-Rad50 subcomplex undergoes a conformational switch from an open state to a closed state (Lammens *et al.*, 2011; Lim *et al.*, 2011; Williams *et al.*, 2011; Mockel *et al.*, 2012; Lee *et al.*, 2013; Deshpande *et al.*, 2014). The Rad50-I1192W

mutation interferes with this conformational switch by obstructing a cavity in the dimer that accommodates the closed conformation (Deshpande *et al.*, 2014). We previously showed that TAP-tagged Rad50-I1192W is partially defective in DSB repair and nearly completely defective in Tel1 signaling (Deshpande *et al.*, 2014). Overexpression of Tel1 was unable to overcome the deficiency of the

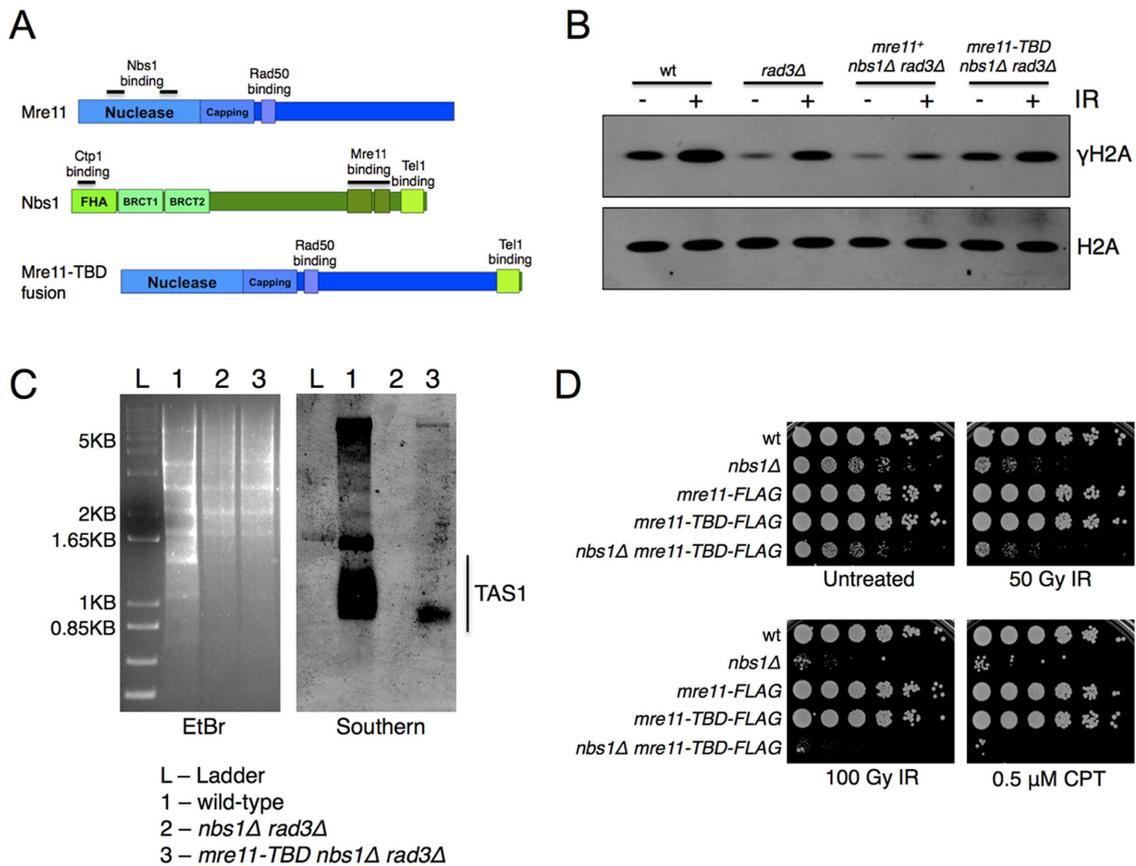


FIGURE 6: Fusion of the Nbs1 C-terminus to Mre11 is sufficient for Tel1 activity. (A) Schematic of Mre11, Nbs1, and fusion protein generated by the addition of C-terminal 60 amino acids encompassing the Tel1-binding domain of Nbs1 to the C-terminus of full-length Mre11. (B) The Mre11-TBD (Tel1-binding domain) fusion protein restores histone H2A phosphorylation in the *nbs1Δ rad3Δ* background under endogenous Tel1 levels. (C) The Mre11-TBD fusion protein prevents telomere loss in *nbs1Δ rad3Δ* cells under endogenous Tel1 levels. Strains were passaged 10 times, and *EcoRI*-digested genomic DNA was probed for telomere-associated sequences (TAS1). Ethidium bromide (EtBr) stained gel serves as a loading control. (D) The Mre11-TBD fusion protein does not noticeably affect Mre11 function in response to DNA-damaging agents IR or CPT. The fusion protein does not correct the DNA repair defect of *nbs1Δ* cells.

TAP-rad50-11192W rad3Δ mutant to generate IR-induced γ H2A (Figure 8B). Importantly, the mutant Rad50 protein was readily detected at an HO-induced DSB (Figure 8C), indicating that it maintained the ability to form a complex with Mre11 that binds DSBs. Thus, overexpression of Tel1 does not overcome the requirement for the proper conformation of the Mre11-Rad50 subcomplex in detecting Tel1 activity at DSBs.

DISCUSSION

Our data show that the Tel1-binding module at the C-terminus of Nbs1 that is critical for Tel1 function can be bypassed by increasing the cellular concentration of Tel1. Surprisingly, the entirety of Nbs1 protein is also dispensable for Tel1 signaling when Tel1 is overexpressed, but Mre11 remains essential. Mre11-Rad50 binds DSBs and interacts with Tel1 in the absence of Nbs1. From these results, we propose a dual mechanism of MRN interacting with Tel1/ATM at DSBs and telomeres in fission yeast. Tel1 recruitment to DNA ends is principally accomplished by binding to the C-terminus of Nbs1. Tel1 then interacts with Mre11-Rad50, which stimulates Tel1 activity.

Our observation of Mre11-Rad50 forming a complex independently of Nbs1 was not surprising, as the Mre11-Rad50 complex is well characterized and is conserved in all domains of life. On the other hand, the Nbs1 subunit has only been identified in eukaryotes (Stracker and Petrini, 2011). In mammalian cells and budding yeast,

Nbs1 is required for localization of the complex to the nucleus (Desai-Mehta *et al.*, 2001; Tsukamoto *et al.*, 2005). In mice, it was recently shown that only a minimal fragment of Nbs1 containing the Mre11-binding domain was required for stability of the complex, nuclear localization, DNA binding, and nuclease activities of Mre11-Rad50 (Kim *et al.*, 2017). This fragment lacked the C-terminal ATM binding domain, yet ATM activity was not completely abolished. In budding yeast, it was recently reported that fusion of a nuclear localization signal (NLS) to Mre11 was sufficient to retain nuclease functions of the Mre11 complex independent of Xrs2 (Nbs1), but not Tel1 activity (Oh *et al.*, 2016). Our data indicate that *S. pombe* Nbs1 is not required for Mre11-Rad50 localization to the nucleus, as the Mre11-Rad50 complex is efficiently enriched at a DSB produced by HO endonuclease. Moreover, in Tel1-overexpressing cells the Mre11-Rad50 complex facilitates phosphorylation of the chromatin-bound target histone H2A in response to DNA damage. In addition to binding DSBs, the Mre11-Rad50 complex likely retains some telomere interactions in the absence of Nbs1, as telomere erosion in *nbs1Δ rad3Δ* cells is delayed compared with *mre11Δ rad3Δ* cells. Mre11-Rad50 binding at telomeres may enact a weak DNA end protection function and/or facilitate Tel1 stimulation. However, Mre11-Rad50 is unable to catalyze DSB repair without Nbs1, presumably because Nbs1 is required to recruit Ctp1, which is essential for DNA end processing and resection by the MRN complex

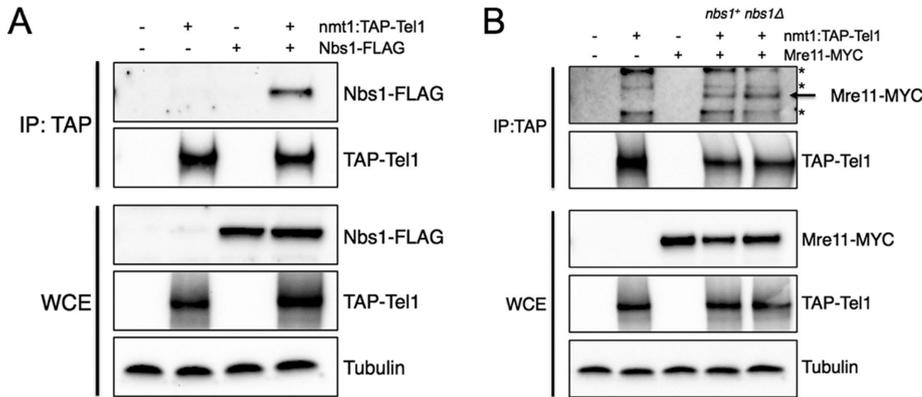


FIGURE 7: Tel1 Binds Mre11-Rad50 in the absence of Nbs1. (A) Nbs1 coprecipitates with overexpressed Tel1. (B) Mre11 coprecipitates with overexpressed Tel1 in the presence or absence of Nbs1. Asterisks indicate degraded TAP-Tel1 background bands.

(Lloyd *et al.*, 2009; Williams *et al.*, 2009). Interestingly, meiotic defects in fission yeast caused by *nbs1Δ* are less severe than those caused by *mre11Δ*, *rad50Δ*, or *ctp1Δ* (Milman *et al.*, 2009), suggesting that Mre11-Rad50 may retain a weak DNA end-processing activity in the absence of Nbs1, at least during meiosis.

We have demonstrated that overexpressed Tel1 interacts with Mre11 independently of Nbs1 *in vivo*. What remains unclear is whether this Mre11-Rad50-Tel1 interaction has a role under normal conditions. The first crystallographic structure of an Mre11-Nbs1 interface showed that two monomers of *S. pombe* Nbs1 bind the Mre11-Rad50 globular domain asymmetrically through a region at the C-terminus of Nbs1 (Schiller *et al.*, 2012). The Tel1-binding module of Nbs1 lies immediately downstream from the Mre11 interaction region, suggesting that Tel1 is normally in close proximity to the

Mre11-Rad50 globular domain. From this architecture, it would be reasonable to speculate that an Mre11-Rad50-Tel1 interface could exist in addition to the well-established Nbs1-Tel1 interface. Our evidence of phosphorylation of histone H2A by Tel1 overexpression requiring Mre11 but not Nbs1, suggests that Tel1 has a physiologically relevant interaction with Mre11-Rad50 at DSBs that is revealed by Tel1 overexpression. These data correspond with *in vitro* gel filtration experiments indicating that ATM has an affinity for Mre11-Rad50, and studies reporting Mre11-Rad50 stimulation of ATM-mediated p53 phosphorylation (Lee and Paull, 2004). It will be interesting to map the Mre11-Rad50-Tel1 interface and determine whether it is required for Tel1 signaling in *nbs1+* cells.

How Mre11-Rad50 stimulates Tel1 activity remains enigmatic. It was previously reported that Mre11 nuclease activity generated short ssDNA oligonucleotides that stimulated ATM activity in *Xenopus* egg extracts (Jazayeri *et al.*, 2008). However, ATM stimulated by ssDNA oligonucleotides alone was unable to phosphorylate histone H2AX, which likely requires the presence of MRN for phosphorylation of chromatin-bound targets. On the other hand, our data show that the nuclease activity of Mre11 is dispensable for Tel1 activity, which is consistent with data in mice and with purified human proteins (Lee and Paull, 2005; Lee *et al.*, 2013; Buis *et al.*, 2008; Limbo *et al.*, 2011). It is likely that ATM activity is stimulated by the presence of DNA ends regardless of their source—whether from the damage itself or from the by-products of DNA end resection. In other words, nuclease-defective MRN that can localize to

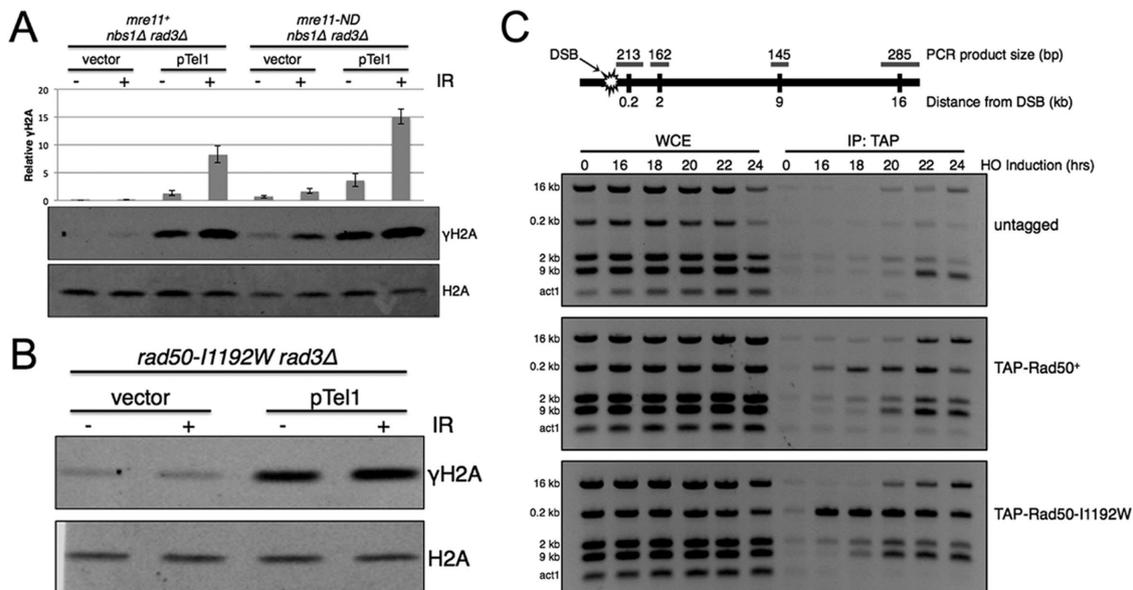


FIGURE 8: Stimulation of Tel1 activity by Mre11-Rad50 depends on conformational state but not nuclease activity. (A) The nuclease-dead Mre11 allele, *mre11-H134S*, does not reduce γ H2A formation in *nbs1Δ rad3Δ* cells when Tel1 is overexpressed. Error bars represent deviation of the mean from three independent experiments. An immunoblot from one of these experiments is shown. (B) The constitutive open-conformation *TAP-rad50-11192W* allele is unable to stimulate Tel1 activity in response to IR damage. (C) *TAP-Rad50-11192W* is enriched at DNA DSBs induced by the HO endonuclease. Locations of primer sets analyzed relative to the site of DSB are noted in the schematic. Actin (*act1*), present in a separate locus, serves as a control.

DSBs can still activate ATM, but its lack of nuclease activity may prevent the accumulation of ssDNA oligonucleotides that amplify the ATM signal to detectable levels. Because Rad3, the ATR homologue, is the primary kinase responsible for DNA damage signaling in *S. pombe* (Limbo *et al.*, 2011), it is unclear whether ssDNA oligo mediated stimulation of Tel1 occurs or whether a need for it even exists.

In the absence of DNA damage, ATM/Tel1 exists as an inactive homodimer. Exposure to IR induces monomerization, which exposes the kinase domain. In human cells, this monomerization is catalyzed by autophosphorylation of serine-1981 (Bakkenist and Kastan, 2003). The importance of this autophosphorylation is controversial, as mutation of the homologous residue in murine ATM (S1987) does not significantly impair ATM activity (Pellegrini *et al.*, 2006; Daniel *et al.*, 2008). Cryo-EM structures of *S. pombe* Tel1 homodimers demonstrated that this serine residue lies in a 32-amino acid insertion (termed INS32) that is absent in *S. pombe* Tel1 (Wang *et al.*, 2016). Thus, as with murine ATM (Pellegrini *et al.*, 2006), autophosphorylation at this site may be unnecessary for Tel1 activation in *S. pombe*. Regardless, ATM/Tel1 monomerization likely remains a crucial step in its activation.

Interestingly, the presence of Mre11-Rad50 at DSBs alone was insufficient to elicit Tel1 activity, as the open-conformation Rad50-L1192W mutant was unable to stimulate Tel1 activity in response to IR. The conformation of Mre11-Rad50 has been shown to greatly influence ATM activity, with ATM activation occurring largely before ATP hydrolysis, in the ATP-bound closed conformation (Williams *et al.*, 2011; Lee *et al.*, 2013). Our data suggest that the stimulatory role of Mre11-Rad50 on Tel1 activity is dependent on its proper conformation. It is tempting to speculate that *S. pombe* Mre11-Rad50, through direct interactions with Tel1 and ATP-dependent conformational changes, stimulates Tel1 activity through Tel1 monomerization.

In our study, cells overexpressing Tel1 had a significant increase in basal γ H2A levels when compared with the empty vector controls regardless of the genetic background. It remains to be determined whether this γ H2A formation occurs randomly in chromatin or in response to specific events such as replication fork collapse or telomere erosion. Whichever is the case, it is evident that Tel1 overexpression restores substantial Tel1 activity even in the absence of the MRN complex. Oxidative stress that does not involve DNA damage has been reported to cause MRN-independent ATM activation (Guo *et al.*, 2010). Interestingly, unlike the MRN-dependent pathway of ATM activation that requires monomerization, ATM activation from oxidative stress exists as a disulfide-linked covalent dimer formed through the C-terminus of ATM. Thus, MRN is not absolutely required for all ATM activity, but appears to be critical in the context of DSBs.

Nbs1 acts as a modular adapter that has clearly separable checkpoint and DNA damage signaling functions. Our study underscores the importance of MRN in Tel1 activity at DSBs and telomeres. We provide *in vivo* evidence of a critical stimulatory role of Mre11-Rad50 in the Tel1 signaling pathway. We propose that recruitment of Tel1 to DNA ends is principally dependent on high-affinity binding to Nbs1, where Mre11-Rad50 then provides a critical stimulatory function. This sequential mechanism of recruitment and activation of ATM/Tel1 may play an important role in coordinating its activity with DSB repair and telomere maintenance.

MATERIALS AND METHODS

General *S. pombe* methods used have been previously described (Forsburg and Rhind, 2006). The strains used are listed in Supple-

mental Table S1. For DNA damage sensitivity assays, fivefold serial dilutions of log-phase cells were spotted onto agar plates and treated with the indicated dose of DNA damage. Chromatin immunoprecipitation experiments were performed as previously described (Limbo *et al.*, 2007) and are representative of at least two independent experiments. HO-endonuclease expression was driven from the thiamine-repressible *nmt41* promoter and samples were taken at indicated time points after removal of thiamine. The primers used are listed in Supplemental Table S2. The Mre11-TBD fusion construct was generated by amplifying the 3' end of the *nbs1*⁺-FLAG-containing strain using primers containing homologous regions to the 3' end of *mre11*⁺. The PCR product was transformed into wild-type cells and checked for proper integration. Details and primer sequences are available upon request.

Western blots and coimmunoprecipitation experiments were performed as previously described (Limbo *et al.*, 2012). Experiments were performed with asynchronous cells grown to log phase. Where indicated, cells were treated with 90 Gy of IR from a Cs-137 source and harvested 30 min following exposure. The following antibodies were used: PAP (Sigma; P1291), MYC (Covance; MMS-150P), FLAG (Sigma; F3165), Tubulin (Sigma; T5168), HA (Roche; 11666606001), total H2A (Active Motif; 39235), γ H2A antibody (Rogakou *et al.*, 1999), anti-mouse HRP (Pierce; 31430), and anti-rabbit IR800CW (LI-COR; 926-32211). H2A Western blots were quantified on a LI-COR Odyssey imaging system and expressed as a ratio between γ H2A and total H2A. Error bars represent the deviation from the mean of at least three independent experiments.

For telomere Southern blots, *mre11* Δ or *nbs1* Δ strains were crossed to *rad3* Δ with *tel1*⁺ either under its endogenous promoter or the full-strength thiamine-repressible *nmt1* promoter. The strains were then streaked for single colonies sequentially, with a liquid culture grown at each passage for genomic DNA extraction. Strains were grown on minimal media lacking thiamine to ensure full expression of *tel1*⁺ for the duration of the experiment. For the Mre11-TBD fusion Southern blot, generated mutants were streaked 10 times sequentially before isolation of genomic DNA to allow for circularization of chromosomes. Southern blotting was performed as previously described (Limbo *et al.*, 2012). Briefly, genomic DNA was digested with *EcoRI* and resolved on 2% TAE agarose gels. DNA was transferred to a nylon membrane by capillary method and incubated with a TAS1 probe (Nakamura *et al.*, 1998) generated by PCR using biotinylated dCTP. The membrane was incubated with labeled streptavidin and scanned on a LI-COR Odyssey imaging system. An alternate pathway of Tel1 recruitment to telomeres independently of the C-terminus of Nbs1 has been previously described (Subramanian and Nakamura, 2010). In this alternate pathway, Tel1-mediated telomere maintenance was observed in a pathway that depended on the N-terminus of both Rad3 and Nbs1. To exclude this alternate pathway, we used full deletions of both of these proteins in both our DNA damage and telomere assays. Moreover, this alternate pathway appears to be specific to telomeres (Supplemental Figure S2).

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