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

Oliver Limbo, Yoshiki Yamada, and Paul Russell*
Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA 92037

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.

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 CtIP/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 CtIP/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 including 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 ataxia-telangiectasia-like disorder
(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; Ceresaletti 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; Ceresaletti 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 γH2A. As both Tel1 and Rad3/ATR phosphorylate histone H2A in fission yeast (Figure 1B; Nakamura et al., 2004), we performed these assays in a rad2α background. γH2A is formed at replication stress sites and at DSBs (Rozenzhak et al., 2010), thus we assayed the basal level of γH2A formed during S-phase as well as the increased level of γH2A formed after exposure to ionizing radiation (IR). Basal and

![Figure 1](image-url)

**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 (γH2A) in response to ionizing radiation (IR). Cells lacking the last 60 residues of Nbs1 encompassing the entire Tel1-binding domain (nbs1-ΔC60) have similar levels of γH2A as tel1Δ. Error bars represent deviation of the mean from three independent experiments. An immunoblot from one of these experiments is shown. (C) nbs1-ΔC60 and point mutations in the Tel1 interaction motif of Nbs1 (nbs1-9 and nbs1-10) have greatly diminished Tel1-mediated γH2A formation. (D) tel1Δ and Nbs1 C-terminal mutants are insensitive to CPT. (E) In the rad2Δ (FEN1) background, nbs1-ΔC60 cells are viable (left), unlike nbs1Δ (right), as assayed by tetrad dissection.

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In the rad3Δ background, the Nbs1 C-terminal mutants often had a weak γH2A signal in response to IR (Figure 1C). Surprisingly, this signal was also detected in nbs1Δ rad3Δ cells (Figure 1C). We never observed γH2A in rad3Δ tel1Δ cells (Figure 1B), showing that Tel1 catalyzed the weak γH2A formation in rad3Δ nbs1Δ cells.

To explore how nbs1Δ rad3Δ cells retain weak γH2A formation in response to IR, we investigated the effects of overexpressing Tel1, as we supposed this might enhance low-affinity interactions with Mre11-Rad50 complex. We constructed a plasmid expressing tel1+ from the nmt41 promoter, which is an attenuated version of the thiamine-repressible nmt1 promoter. In rad3Δ cells, Tel1 overexpression increased the basal signal of γH2A (Figure 2A). A similar effect was observed in nbs1-ΔC60 rad3Δ cells (Figure 2B). Importantly, the IR-induced increase of γH2A 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 rad3Δ and rad3Δ cells overexpressing Tel1 had normal cell morphologies,
Tel1 overexpression bypasses the requirement of Nbs1, but not Mre11, in damage-induced Tel1 signaling. (A) IR-induced increase in γH2A occurs in nbs1Δ rad3Δ but not mre11Δ rad3Δ background. Tel1 overexpression restores basal γH2A 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.

FIGURE 3: Overexpression of Tel1 bypasses the requirement of Nbs1, but not Mre11, in damage-induced Tel1 signaling. (A) IR-induced increase in γH2A occurs in nbs1Δ rad3Δ but not mre11Δ rad3Δ background. Tel1 overexpression restores basal γH2A 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 γH2A 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 tel1Δ 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 γH2A (Figure 3A). This observation suggested that overexpressed Tel1 might phosphorylate histone H2A without any assistance from the MRN complex. Indeed, the basal level of γH2A was the same in nbs1Δ rad3Δ and mre11Δ rad3Δ cells that overexpressed Tel1. However, the IR-induced increase in γH2A 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 γH2A 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 tel1Δ 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 nbs1Δ+ 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 nbs1Δ- and nbs1Δ+ backgrounds (Figure 5B). It is possible that Mre11 binds the
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 rad2Δ cells, both basally and in response to IR treatment (Figure 6B). In fact, the basal level of 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, suggesting 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 γH2A assay in cells with the mre11-H134S allele, which ablates Mre11 nuclease activity (Williams et al., 2008). We found that Tel1 overexpression restored basal IR-induced γH2A formation in mre11-H134S nbs1Δ rad3Δ cells (Figure 8A), indicating that


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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 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.
TAP-rad50-I1192W rad3Δ mutant to generate IR-induced γH2A (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.
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 nbs1Δ 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 the

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**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.

**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 γH2A 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-I1192W allele is unable to stimulate Tel1 activity in response to IR damage. (C) TAP-Rad50-I1192W 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 *mre11Δ-rad50* at DSBs alone was insufficient to elicit Tel1 activity, as the open-conformation *rad50-i1192w* mutant was unable to stimulate Tel1 activity in response to IR. The conformation of *mre11Δ-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 *mre11Δ-rad50* on Tel1 activity is dependent on its proper conformation. It is tempting to speculate that *S. pombe* *mre11Δ-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 γH2A levels when compared with the empty vector controls regardless of the genetic background. It remains to be determined whether this γH2A 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 *mre11Δ-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 *mre11Δ-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 Supplemental 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 *mre11Δ-tbd* fusion construct was generated by amplifying the 3′ end of the *nbs1Δ-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), γH2A 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 γH2A and total H2A. Error bars represent the deviation from the mean of at least three independent experiments.

For telomere Southern blots, *mre11Δ* or *nbs1Δ* strains were crossed to *rad3Δ* with tel1+ either under its endogenous promoter or the full-strength thiamine-repressible *nmt41* 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 *mre11Δ-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 EcoRl and resolved on 2% TAE agarose gels. DNA was transferred to a nylon membrane by capillary method and incubated with a TASI 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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