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3 # Sequential interactions with Mre11-Rad50-Nbs1 activate

4 # ATM/Tel1 at DNA double-strand breaks and telomeres

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18 Running Title: MRN-mediated recruitment and activation of ATM/Tel1

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23 **Abstract**

24 The Mre11-Rad50-Nbs1 (MRN) protein complex, CtIP/Ctp1/Sae2 and ATM/Tel1 kinase
25 protect genome integrity through their functions in DNA double-strand break (DSB)
26 repair, checkpoint signaling, and telomere maintenance. Nbs1 has a conserved C-terminal
27 motif that binds ATM, but the full extent of ATM interactions with MRN are unknown.
28 Here, we show that Tel1 overexpression in *Schizosaccharomyces pombe* restores Tel1
29 activity at DSBs and telomeres in the absence of Nbs1. This activity requires Mre11,
30 indicating that Tel1 overexpression drives low affinity binding to the Mre11-Rad50
31 subcomplex. Mre11-Rad50 binds DSBs in *nbs1*Δ cells, and fusing the Tel1-binding motif
32 of Nbs1 to Mre11 fully restores Tel1 signaling in these cells. Tel1 overexpression does
33 not restore Tel1 signaling in cells carrying the *rad50-I1192W* mutation, which impairs
34 the ability of Mre11-Rad50 to form the ATP-bound closed conformation. From these
35 findings, we propose that Tel1 activation at DNA ends proceeds by a sequential
36 mechanism initiated by high affinity binding to Nbs1 which recruits Tel1, followed by a
37 low affinity interaction with Mre11-Rad50 in the closed conformation to activate Tel1.

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40 ## Introduction

41 DNA Double-strand breaks (DSBs) are one of the most dangerous lesions, as they break
42 chromosomes as well as DNA. An inability to properly repair DSBs can result in cell
43 death or cancer (Hoeijmakers, 2001). The Mre11-Rad50-Nbs1 (MRN) protein complex
44 acts as a primary responder to DSBs, quickly localizing to damage sites (Stracker &
45 Petrini, 2011). The FHA domain at the N-terminus of the Nbs1 subunit recruits
46 CtIP/Ctp1, a DNA repair ortholog of *Saccharomyces cerevisiae* Sae2, which activates the
47 intrinsic nuclease activity of Mre11 (Cannavo & Cejka, 2014, Limbo et al., 2007, Lloyd
48 et al., 2009, Sartori et al., 2007, Williams et al., 2009). MRN-Ctp1 first nucleolytically
49 displaces Ku or other proteins from DNA ends, and then initiates resection of the 5'
50 strand to generate a 3' single-stranded DNA (ssDNA) overhang (Garcia et al., 2011,
51 Lafrance-Vanasse et al., 2015, Langerak et al., 2011, Shibata et al., 2014). Rad51
52 mediates the invasion and base pairing of the ssDNA tail into homologous DNA
53 sequences, usually in the sister chromatid, which it uses as a template to carry out the
54 error-free pathway of homology-directed repair (Stracker & Petrini, 2011).

55 In addition to CtIP/Ctp1, the MRN complex also recruits the ATM/Tel1 (Ataxia
56 telangiectasia mutated) serine/threonine kinase to damage sites (Uziel et al., 2003). ATM
57 is a member of the PI3K-like protein kinase (PIKK) family of proteins, whose members
58 include DNA-PKcs, ATR, and mTor (Paull, 2015). In response to damage, ATM serves
59 to initiate cell cycle arrest, stimulate repair factors, and activate senescence and apoptosis
60 pathways through the phosphorylation of many substrates that include CHK2, H2AX,
61 NBS1, BRCA1, and p53 (Paull, 2015). Mutations in the ATM gene are associated with
62 ataxia telangiectasia (A-T or Louis-Bar syndrome). Patients with this neurodegenerative

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63 disorder present with ataxia, telangiectasia, sensitivity to ionizing radiation,
64 immunodeficiency, and a predisposition to cancer (Shiloh, 1997). Mutations in Nbs1 are
65 associated with Nijmegen breakage syndrome, an A-T-like syndrome that includes
66 microcephaly (Carney et al., 1998). Mre11 mutations are associated with an ataxia-
67 telangiectasia-like disorder (ATLD), which resembles A-T with the exception that in
68 most cases, ATLD does not result in cancer or immunodeficiency (Delia et al., 2004,
69 Fernet et al., 2005, Stewart et al., 1999, Uchisaka et al., 2009).

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

78 We have shown previously that a conserved domain in the C-terminus of the
79 fission yeast and *Xenopus laevis* Nbs1 subunits binds ATM/Tel1 (You et al., 2005). This
80 Tel1/ATM interaction mechanism is conserved in budding yeast Xrs2 and human Nbs1
81 (Falck et al., 2005). In human cells, elimination of the C-terminal 20-amino acids of Nbs1
82 impairs ATM localization into DNA repair foci and abrogates phosphorylation of some
83 key substrates such as CHK2, (Falck et al., 2005). However, this motif is not required for
84 ATM function in mice (Stracker et al., 2007). Purified human MRN was shown to
85 stimulate ATM activity and DNA binding, but these also occur in the absence of Nbs1,

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86 albeit to a lesser extent (Lee & Paull, 2004, Lee & Paull, 2005). In *Xenopus* extracts, the
87 Nbs1-ATM interaction is essential for both the recruitment of ATM to damage sites and
88 its activity (You et al., 2005). Collectively, these data suggest that the C-terminal
89 ATM/Tel1 binding motif of Nbs1 is highly conserved but there may be additional
90 interaction mechanisms involving Nbs1 or even direct interactions of ATM/Tel1 with the
91 Mre11-Rad50 subcomplex.
92 Here, we use fission yeast to probe for undiscovered interactions involving Tel1
93 and MRN *in vivo*. Our data suggests that high affinity binding to Nbs1 is principally
94 responsible for localizing Tel1 at DSBs and telomeres, whereupon it is activated by a
95 mechanism requiring the ATP-bound closed conformation of Mre11-Rad50.
96

97 ## Results

98 **The C-terminus of Nbs1 is critical for Tel1 activity**

99 The N-terminus of *S. pombe* Nbs1 contains an FHA domain fused to tandem BRCT
100 domains. This region is flexibly linked to a C-terminal region containing Mre11 and
101 ATM/Tel1 binding domains (Lafrance-Vanasse et al., 2015, Lloyd et al., 2009, Williams
102 et al., 2009) (Fig 1A). These domains are conserved in mammalian Nbs1 proteins, but
103 truncation of the C-terminal 24-amino acids in murine Nbs1 only partially impaired ATM
104 activity (Stracker et al., 2007). This result might be explained if there is an additional
105 MRN-ATM/Tel1 interaction mechanism. As *S. pombe* Nbs1 appears to have only a single
106 Tel1-binding motif, which is located at the extreme C-terminus of the Nbs1 polypeptide,
107 we decided to re-examine the importance of this motif for Tel1 function using a sensitive
108 immunoblot assay for formation of phosphorylated histone H2A, known as γH2A. As

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109 DNA is resected following DSB formation, the initial checkpoint signaling mediated by
110 MRN-Tel1/ATM switches to Rad26/ATRIP-Rad3/ATR, correlating with the appearance
111 of a 3' ssDNA overhang (Limbo et al., 2011, Shiotani & Zou, 2009). Thus, both Rad3
112 and Tel1 contribute to phosphorylation of key substrates such as histone H2A, which is
113 equivalent to H2AX in mammalian cells. Accordingly, we observed that ionizing
114 radiation (IR)-induced γH2A formation is maintained in *tel1*Δ and *rad3*Δ single mutants
115 but abolished in the double mutant (Fig 1B), as previously reported (Nakamura et al.,
116 2004). To specifically assay the activity of Tel1, we performed subsequent assays in a
117 *rad3*Δ background. γH2A formation was nearly abolished in *nbs1*Δ *rad3*Δ cells (Fig 1C),
118 confirming the critical requirement of Nbs1 for Tel1 activity. Similarly, truncation of the
119 C-terminal 60 residues of Nbs1 (*nbs1-ΔC60*) containing the Tel1-binding motif almost
120 ablated γH2A formation specifically in the *rad3*Δ background (Fig 1C). The same defects
121 were observed for double missense alleles *nbs1-9* (D603N, D604N) and *nbs1-10* (F611E,
122 F613E) that mutate conserved residues in the Tel1 binding domain of Nbs1 (You et al.,
123 2005). Interestingly, both *tel1Δ* and *nbs1-ΔC60* mutants had similar levels of γH2A (Fig
124 1B), underscoring the importance of the C-terminus of Nbs1 in Tel1 activity.
125 In addition to its roles in Tel1 signaling, Nbs1 is also critical for DNA repair,
126 recruiting the resection cofactor Ctp1 to damage sites through interactions with the N-
127 terminal FHA domain (Lloyd et al., 2009, Williams et al., 2009). Indeed, cells lacking
128 Nbs1 show severe growth defects and sensitivity to DNA damaging agents (Chahwan et
129 al., 2003). To see if deletion or mutation of the Tel1 binding domain in the C-terminus
130 affected the DNA damage repair function of Nbs1, we plated cells on plates containing
131 the topoisomerase inhibitor, camptothecin (CPT). The sensitivity of *nbs1-ΔC60*, *nbs1-9*

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132 and *nbs1-10* mutants to CPT was not increased relative to wild-type (Fig 1D), as
133 previously observed for IR and the alkylating agent, methyl methanesulfonate (MMS)
134 (You et al., 2005). Similarly, *nbs1-ΔC60* cells were viable in the absence of the Rad2
135 (FEN1) flap endonuclease that is responsible for the maturation of Okazaki fragments,
136 whereas *nbs1Δ* cells were lethal in this background (Fig 1E). Taken together, these data
137 show that the C-terminal region of Nbs1 that is critical for Tel1 activity at DSBs is
138 completely dispensable for the DNA repair functions of Nbs1.
139
140 **Overexpression of Tel1 bypasses the requirement for the C-terminus of Nbs1**
141 Although the Tel1 interaction motif at the C-terminus of Nbs1 is critical for Tel1 activity
142 at DSBs, we noted that in a *rad3*Δ background there was often a very weak but detectable
143 IR-induced stimulation of γH2A in Nbs1 C-terminal mutants (Fig 1C). Surprisingly, we
144 could also detect a weak IR-stimulated increase of γH2A in *nbs1*Δ *rad3*Δ cells.
145 Importantly we never observed γH2A in *rad3*Δ *tel1*Δ cells (Fig 1B), confirming that Tel1
146 catalyzed the small amount of γH2A formed in *rad3*Δ *nbs1*Δ cells.
147 We suspected that the Nbs1-independent activity of Tel1 might involve a low
148 affinity interaction of Tel1 with Mre11-Rad50 (MR). To address this possibility, we first
149 investigated whether overexpression of Tel1 bypasses the requirement for the Tel1
150 binding domain of Nbs1. We transformed *nbs1-ΔC60 rad3Δ* cells with a plasmid
151 expressing $tel1^{+}$ driven from the attenuated thiamine-repressible *nmt1* promoter, or an
152 empty vector control, and found that Tel1 overexpression restored both basal and IR-
153 induced phosphorylation of H2A (Fig 2A).

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154 Overexpression of Tel1 in *S. cerevisiae* caused prolonged cell-cycle arrest via
155 Rad53, even in the absence of exogenous DNA damage (Clerici et al., 2001). To address
156 whether Tel1 overexpression alone triggered the DNA damage checkpoint in *S. pombe*,
157 we first assayed phosphorylation of the effector checkpoint kinase, Chk1. In the absence
158 of damage, Tel1 overexpression did not cause a detectable mobility shift in Chk1 in
159 either wild type or *rad3Δ* backgrounds (Fig 2B). Rad3 mediates phosphorylation of Chk1
160 in most contexts (Limbo et al., 2011). Interestingly, overexpression of Tel1 partially
161 restored IR-dependent Chk1 phosphorylation in *rad3Δ* cells (Fig 2B). Cells
162 overexpressing Tel1 had normal cell morphology and were not hyper-elongated,
163 indicating that Tel1 overexpression did not activate Cds1/Rad53 or Chk1 (Fig 2C).
164 Moreover, Tel1 overexpression did not cause obvious growth defects nor increased
165 sensitivity to DNA damaging agents (Fig 2D), suggesting that overexpression of Tel1 had
166 no overt negative consequences to the cells.
167

168 **Tel1 overexpression bypasses the requirement of Nbs1, but not Mre11, in DNA**
169 **damage-induced Tel1 activity and telomere maintenance**

170 Having found that Tel1 overexpression can bypass the requirement for the C-terminus of
171 Nbs1, we next examined whether the same was true in cells lacking Nbs1 or Mre11. In
172 *nbs1Δ rad3Δ* cells, Tel1 overexpression rescued both basal and IR-induced
173 phosphorylation of histone H2A (Fig 3A). However, IR-induced formation of γH2A was
174 defective in *mre11Δ rad3Δ* cells overexpressing Tel1, with only basal γH2A levels being
175 restored (Fig 3A). Similarly, Tel1 overexpression partially restored IR-induced Chk1
176 phosphorylation in *nbs1Δ rad3Δ* cells, but not *mre11Δ rad3Δ* cells (Fig 3B).

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177 In *S. pombe,* both Rad3 and Tel1 contribute to maintenance of telomeres through
178 phosphorylation of Ccq1, a subunit of the Shelterin complex. Phosphorylated Ccq1 then
179 promotes recruitment of telomerase (Moser et al., 2011, Yamazaki et al., 2012). Thus, in
180 the absence of Rad3 and Tel1, cells undergo telomere erosion with a small subset
181 surviving through circularization of chromosomes after successive passages (Nakamura
182 et al., 2002). A similar relationship was observed when MRN null mutants were
183 combined with *rad3Δ* mutants, underscoring the importance of the MRN complex in Tel1
184 activity at telomeres. We asked whether Tel1 overexpression could prevent telomere loss
185 in cells lacking Rad3 and subunits of MRN complex. To address this question, we
186 generated *mre11Δ rad3Δ* or *nbs1Δ rad3Δ* mutants with *tel1*$^{+}$ either driven from its native
187 promoter or the *nmt1* overxpression promoter. Genomic DNA was prepared from cells
188 after each passage and Southern blotting was performed probing for telomere-associated
189 sequence (TAS1). We found overexpression of Tel1 prevented the loss of telomeres in
190 *nbs1Δ rad3Δ* cells (Fig 4). Tel1 overexpression also had an effect in *mre11Δ rad3Δ* cells,
191 although in this case it only delayed the loss of telomeres (Fig 4). These effects correlated
192 with the improved growth of *nbs1Δ rad3Δ* and *mre11Δ rad3Δ* cells in the presence of
193 overexpressed Tel1 (Fig EV1). Taken together, our results show that Tel1 overexpression
194 can bypass the requirement for Nbs1, but not Mre11, in both DNA damage signaling and
195 telomere maintenance.
196

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

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199 The different consequences of Tel1 overexpression in *mre11Δ* and *nbs1Δ* backgrounds
200 were surprising, given they are part of the same protein complex and both mutants are
201 equally sensitive to DNA damaging agents (Chahwan et al., 2003). These results
202 prompted us to examine whether Mre11 and Rad50 can form a stable protein complex
203 without Nbs1. We performed co-immunoprecipitation experiments using strains with
204 Mre11-MYC and TAP-Rad50 expressed from their endogenous loci and under their
205 native promoters. Interestingly, Mre11-MYC co-precipitated readily with TAP-Rad50,
206 both in the presence and absence of Nbs1 (Fig 5A).
207 We next performed a chromatin immunoprecipitation (ChIP) experiment,
208 assaying Mre11 enrichment around a site-specific DSB generated by the HO
209 endonuclease. Mre11 was enriched immediately adjacent (0.2kb) from the break site in
210 both wild-type and *nbs1Δ* backgrounds (Fig 5B). In fact, there was a greater enrichment
211 of Mre11 in the *nbs1Δ* background, which we previously observed in *ctp1Δ* and Mre11
212 nuclease-deficient mutants, which are defective in resection but maintain the integrity of
213 the MRN protein complex (Limbo et al., 2011, Williams et al., 2008). Taken together,
214 our results indicate that Mre11-Rad50 can form a stable subcomplex in the absence of
215 Nbs1 that can localize to DSBs.
216

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

218 Our results suggested that Tel1 has a high-affinity interaction with the C-terminus of
219 Nbs1, which can be bypassed by promoting a low affinity interaction with Mre11-Rad50
220 through Tel1 overexpression. However, our results did not exclude a model in which
221 Nbs1 also has a role in activating Tel1 that is overcome by Tel1 overexpression. To

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222 address this question, we fused the last 60 residues of Nbs1 containing the Tel1 binding
223 domain (TBD) to the C-terminus of full-length Mre11 (Fig 6A). This *mre11-TBD*
224 construct replaced the endogenous *mre11*$^{+}$ gene. We then assayed Tel1 activity in the
225 *mre11-TBD nbs1Δ rad3Δ* background. The Mre11-TBD fusion fully restored
226 phosphorylation of histone H2A to at least the level of *rad3Δ* cells, both basally and in
227 response to IR treatment (Fig 6B). In fact, the basal level of γH2A exceeded that of
228 *rad3Δ* cells. Moreover, we found that the Mre11-TBD fusion prevented the telomere
229 erosion observed in *nbs1Δ rad3Δ* cells (Fig 6C).
230 To test the effect of the Mre11-TBD fusion on Mre11 function, we performed
231 spot dilution assays, exposing the strains to different DNA damaging agents (Fig 6D).
232 The fusion protein alone did not increase sensitivity of cells to IR and CPT, suggesting it
233 did not impair Mre11 function. As expected, the Mre11-TBD fusion did not restore the
234 DNA damage repair defect of *nbs1Δ* cells, because the DNA repair activity of MRN
235 protein complex requires Ctp1 binding to the FHA domain found at the N-terminus of
236 Nbs1. Taken together, these results show that fusion of the C-terminal 60 residues of
237 Nbs1 to Mre11 was sufficient to restore Tel1 signaling in *nbs1Δ* cells, but the DNA repair
238 defect remained.
239
240 **Nbs1-independent activity of Tel1 at DSBs requires ATP-bound closed**
241 **conformation of Mre11-Rad50**
242 Our results showed that the functions of Nbs1 in DNA damage-induced Tel1 activation
243 could be largely bypassed by overexpression of Tel1 or fusion of the Tel1-binding
244 domain of Nbs1 to Mre11. However, Tel1 overexpression had no effects in the absence

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245 of Mre11. Thus, it is likely that Mre11-Rad50, which can bind damage sites
246 independently of Nbs1, has a low affinity interaction with Tel1 that is sufficient to restore
247 Tel1 activity to *nbs1Δ* cells when Tel1 is overexpressed. To explore if this role of Mre11-
248 Rad50 in Tel1 activity required Mre11 nuclease activity, we repeated our γH2A assay in
249 cells with the *mre11-H134S* allele, which ablates Mre11 nuclease activity (Williams et
250 al., 2008). Tel1 overexpression restored basal and IR-induced γH2A formation in *mre11-*
251 *H134S nbs1Δ rad3Δ* cells (Fig 7A), indicating that the nuclease activity of Mre11 is not
252 required for the MR-dependent activity of Tel1.

253 Upon binding ATP, the Mre11-Rad50 subcomplex undergoes a conformational
254 switch from an open to a closed state (Lammens et al., 2011, Lim et al., 2011, Mockel et
255 al., 2012). The Rad50-I1192W mutation interferes with the conformational switch by
256 obstructing a cavity in the dimer that accommodates the closed conformation. We
257 previously showed that TAP-tagged Rad50-I1192W is partially defective in DSB repair
258 and nearly completely defective in Tel1 signaling (Williams et al., 2011). Overexpression
259 of Tel1 was unable to overcome the deficiency of the *TAP-rad50-I1192W rad3Δ* mutant
260 to generate IR-induced γH2A (Fig 7B). Importantly, the mutant Rad50 protein was
261 readily detected at an HO-induced DSB (Fig 7C), indicating that it maintained the ability
262 to form a complex with Mre11 that binds DSBs. Thus, overexpression of Tel1 does not
263 overcome the requirement for the proper conformation of MR complex in detecting Tel1
264 activity at DSBs.
265

266 **Discussion**

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267 In this study, we have uncovered evidence for a mechanism of sequential recruitment and
268 activation of Tel1/ATM at DSBs and telomeres. In fission yeast, the Tel1-binding module
269 at the C-terminus of Nbs1 is critical for Tel1 function, but this recruitment mechanism
270 can be bypassed by increasing the cellular concentration of Tel1. Indeed, the entirety of
271 Nbs1 protein is dispensable for Tel1 signaling when Tel1 is overexpressed. However,
272 Mre11 remains essential for Tel1 activity at DSBs and telomeres, even when Tel1 is
273 overexpressed. This result implies that Mre11-Rad50 localizes in the nucleus, binds
274 DSBs, and maintains a low affinity interaction with Tel1 in the absence of Nbs1. Indeed,
275 we detected strong enrichment of Mre11 at a DSB in *nbs1Δ* cells. From these results, we
276 propose that for Tel1 activity at DSBs and telomeres, Nbs1 principally serves a
277 recruitment or enrichment role, whereas Mre11-Rad50 plays a critical stimulatory role
278 (Fig 8).

279 Mre11-Rad50 is conserved in all domains of life, whereas the Nbs1 subunit has
280 only been identified in eukaryotes (Stracker & Petrini, 2011). In mammalian cells and
281 budding yeast, Nbs1 is required for localization of the complex to the nucleus (Desai-
282 Mehta et al., 2001, Tsukamoto et al., 2005). In mice, it was recently shown that only a
283 minimal fragment of Nbs1 containing the Mre11 binding domain was required for
284 stability of the complex, nuclear localization, DNA binding, and nuclease activities of
285 Mre11-Rad50 (Kim et al., 2017). This fragment lacked the C-terminal ATM binding
286 domain, yet ATM activity was not completely abolished. In budding yeast, it was
287 recently reported that fusion of a nuclear localization signal (NLS) to Mre11 was
288 sufficient to retain nuclease functions of the Mre11 complex independent of Xrs2 (Nbs1),
289 but not Tel1 activity (Oh et al., 2016). Our data presented here demonstrate that *S. pombe*

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290 Nbs1 is completely dispensable for the formation of Mre11-Rad50 protein complex and
291 its localization at DSBs. This complex is unable to catalyze DSB repair, presumably
292 because Nbs1 is required to recruit Ctp1, which is essential for DNA end processing and
293 resection by Mre11 complex (Lloyd et al., 2009, Williams et al., 2009). However, the
294 meiotic defects caused by *nbs1*Δ are less severe than those caused by *mre11Δ, rad50Δ* or
295 *ctp1*Δ (Milman et al., 2009), suggesting that Mre11-Rad50 retains a weak Ctp1-
296 dependent DNA end processing activity in the absence of Nbs1, at least during meiosis.
297 The first crystallographic structure of an Mre11-Nbs1 interface showed that two
298 monomers of Nbs1 bind the Mre11-Rad50 globular domain asymmetrically through a
299 region at the C-terminus of Nbs1 (Schiller et al., 2012). The Tel1-binding module of
300 Nbs1 lies immediately downstream of the Mre11 interaction region, which suggests Tel1
301 localizes near the Mre11-Rad50 globular domain. This architecture, along with the
302 absolute requirement for Mre11-Rad50 in DNA damage-induced Tel1 activity, as shown
303 here, strongly suggests that in addition to the well-established Nbs1-Tel1 interaction
304 interface, another interface also exists between Mre11-Rad50 and Tel1. This model is
305 supported by *in vitro* gel filtration evidence indicating that ATM has an affinity for
306 Mre11-Rad50, as well as *in vitro* studies reporting that Mre11-Rad50 stimulates ATM-
307 mediated p53 phosphorylation (Lee & Paull, 2004).
308 How Mre11-Rad50 stimulates Tel1 activity remains enigmatic. Our data shows
309 that Mre11 endonuclease activity is dispensable for Tel1 activity, which is consistent
310 with previous data in mice and with purified human proteins (Buis et al., 2008, Lee et al.,
311 2013, Lee & Paull, 2005, Limbo et al., 2011). Despite the dispensability of Mre11
312 nuclease activity, the presence of Rad50 at DSBs alone was insufficient to elicit Tel1

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313 activity. TAP-Rad50-I1192W, which is unable to efficiently form the closed
314 conformation of Mre11-Rad50, was unable to stimulate Tel1 activity towards H2A in
315 response to IR, even when Tel1 was overexpressed. The conformation of Mre11-Rad50
316 greatly influences ATM activity, with ATM activation occurring in the ATP-bound,
317 closed conformation (Lee et al., 2013, Williams et al., 2011). Our data provide *in vivo*
318 evidence that both the recruitment of Tel1 by Nbs1 and the stimulatory role of Mre11-
319 Rad50 occur prior to ATP hydrolysis.

320 In the absence of DNA damage, ATM exists as an inactive homodimer. Exposure
321 to ionizing radiation induces monomerization, which exposes the kinase domain and
322 allows ATM to phosphorylate its substrates. In human cells, ATM monomerization is
323 catalyzed by autophosphorylation at the serine residue at position 1981 (Bakkenist &
324 Kastan, 2003). However, the importance of this autophosphorylation is controversial, as
325 mutation of the homologous residue in murine ATM (S1987) does not significantly
326 impair ATM activity (Daniel et al., 2008, Pellegrini et al., 2006). Cryo-EM structures of
327 *S. pombe* Tel1 homodimers demonstrated that this serine residue lies in a 32-amino acid
328 insertion (termed INS32) that is absent in *S. pombe* Tel1 (Wang et al., 2016). As with
329 murine ATM (Pellegrini et al., 2006), autophosphorylation may be unnecessary for Tel1
330 activation in *S. pombe.* However, as seen with other members of the PIKK family, its
331 activity may be inhibited through the blockage of the kinase domain of one molecule of
332 Tel1 with another. Thus, the most conserved property of ATM/Tel1 activation appears to
333 be the disengagement of the homodimer. It is tempting to speculate that Mre11-Rad50
334 mediates ATM/Tel1 activity through ATM/Tel1 monomerization.

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335 Although we failed to detect an IR-induced increase in γH2A formation when
336 Tel1 was overexpressed in *mre11Δ rad3Δ* or *rad50-I1192W rad3Δ* backgrounds, the
337 untreated samples had a significant increase in basal γH2A levels when compared to the
338 empty vector controls (Figs 3A and 7B). It remains to be determined whether this γH2A
339 formation occurs randomly in chromatin or in response to specific events such as
340 replication fork collapse or telomere erosion. Whichever is the case, it is evident that Tel1
341 overexpression restores substantial Tel1 activity even in the absence of MRN complex.
342 Oxidative stress was reported to cause MRN-independent ATM activation by a
343 pathway that does not involve DNA damage (Guo et al., 2010). Interestingly, unlike the
344 MRN-dependent pathway of human ATM activation, in which an inactive ATM dimer
345 disengages into active monomers after autophosphorylation, ATM activation from
346 oxidative stress exists as a disulfide-linked covalent dimer formed through the C-
347 terminus of ATM. Mutation of this C-terminal region specifically abolished ATM
348 activation caused by oxidative stress but retained activation stimulated by DNA damage.
349 Moreover, Nbs1-independent ATM activation has been observed by assaying
350 phosphorylation of p53 in postmitotic neural tissue (Frappart et al., 2005, Li et al., 2012).
351 Thus, MRN is not absolutely required for all ATM activity, but appears to be critical in
352 the context of DSBs and telomeres.
353 When ATM binds MRN at DSBs, it phosphorylates histone H2AX in surrounding
354 chromatin, which then binds the C-terminal BRCT domains of the DNA damage
355 mediator protein, MDC1. MDC1 binds the FHA/BRCT domains of NBS1 and
356 autophosphorylated ATM, which increases ATM signaling at DSBs (Lou et al., 2006,
357 Stucki et al., 2005). *S. pombe* has an MDC1-like protein known as Mdb1, which binds

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358 γH2A through its C-terminal BRCT domains (Wei et al., 2014). An FHA-like structure at
359 the N-terminus of Mdb1 mediates homodimerization, analogous to MDC1 (Luo et al.,
360 2015). Mdb1 might mediate the MRN-independent activity of Tel1 reported here.
361 In summary, our study underscores the importance of MRN, and particularly
362 Nbs1, in Tel1 activity at DSBs and telomeres. In addition, we provide *in vivo* evidence of
363 a critical stimulatory role of Mre11-Rad50 in the Tel1 signaling pathway. We propose
364 that recruitment of Tel1 to DNA ends is principally dependent on high affinity binding to
365 Nbs1, whereas activation of Tel1 after it binds Nbs1 involves a lower affinity interaction
366 with Mre11-Rad50. This sequential mechanism of recruitment and activation of
367 ATM/Tel1 may play an important role in coordinating its activity with DSB repair and
368 telomere maintenance.
369

370 **Materials and Methods**

371 General *S. pombe* methods used have been previously described (Forsburg & Rhind,
372 2006). Strains used are listed in Supplementary Table S1. For DNA damage sensitivity
373 assays, 5-fold serial dilutions of log-phase cells were spotted onto agar plates and treated
374 with the indicated dose of DNA damage. Chromatin immunoprecipitation experiments
375 were performed as previously described (Limbo et al., 2007) and are representative of at
376 least 2 independent experiments. HO-endonuclease expression was driven from the
377 thiamine repressible *nmt41* promoter. Samples were taken at indicated time points after
378 removal of thiamine. The Mre11-TBD fusion construct was generated by amplifying the
379 3’ end of *nbs1*$^+$ using primers containing homologous regions to 3’ end of *mre11*$^+$. The

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380 PCR product was transformed into wild-type cells and checked for proper integration.
381 Details and primers sequences are available upon request.
382 Western blots and co-immunoprecipitation experiments were performed as
383 previously described (Limbo et al., 2012). Experiments were done with asynchronous
384 cells grown to log-phase. Where indicated, cells were treated with 90 Gy of ionizing
385 radiation from a Cs-137 source and harvested 30 minutes after exposure. Membranes
386 were blotted with one of the following antibodies: PAP (Sigma P1291), MYC (Covance
387 MMS-150P), FLAG (Sigma F3165), Tubulin (Sigma T5168), HA (Roche 11666606001),
388 and total H2A (Active Motif 39235). The anti-γH2A antibody was previously described
389 (Rogakou et al., 1999).
390 For telomere Southern blots, *mre11Δ* or *nbs1Δ* strains were crossed to *rad3Δ* with
391 *tel1*$^+$ either under its endogenous promoter or the full-strength thiamine-repressible *nmt1*
392 promoter. Confirmed strains were then streaked for single colonies sequentially, with a
393 liquid culture grown at each passage for genomic DNA extraction. Strains were grown
394 and maintained on minimal media lacking thiamine to ensure full expression of *tel1*$^+$ for
395 the duration of the experiment. For the Mre11-TBD fusion Southern blot, generated
396 mutants were streaked 10 times sequentially prior to isolation of genomic DNA to allow
397 for circularization of chromosomes. Southern blotting was performed as previously
398 described (Limbo et al., 2012). Briefly, DNA was digested with *Eco*RI and resolved on
399 2% TAE agarose gels. DNA was transferred to a nylon membrane by capillary method
400 and incubated with TAS1 probe (Nakamura et al., 1998) generated by PCR with
401 biotinylated dCTP. The membrane was incubated with dye-labeled streptavidin and
402 scanned on a LI-COR Odyssey imaging system. An alternate pathway of Tel1

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403 recruitment to telomeres independently of the C-terminus of Nbs1 has been previously
404 described (Subramanian & Nakamura, 2010). In this alternate pathway, Tel1-mediated
405 telomere maintenance was observed in a pathway that depended on the N-terminus of
406 both Rad3 and Nbs1. To exclude this alternate pathway, we used full deletions of both of
407 these proteins in both our DNA damage and telomere assays. Moreover, this alternate
408 pathway appears to be specific to telomeres (Fig EV2).
409

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416

417 **Author Contributions**

418 OL, YY, and PR designed experiments and analyzed results. OL and YY performed
419 experiments. OL and PR prepared manuscript.
420

421 **Conflict of Interest**

422 The authors declare they have no conflict of interest.
423

424 **References**

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597 ## Figure Legends

598 **Figure 1. The C-terminus of Nbs1 is important for Tel1-mediated phosphorylation**
599 **of histone H2A but dispensable for DNA repair.**

600 A) Domain architecture of Nbs1. Asterisks denote location of *nbs1-9* (D603N, D604N)
601 and *nbs1-10* (F611E, F613E) alleles. B) Both Rad3 and Tel1 contribute to
602 phosphorylation of histone H2A. Cells lacking the last 60 residues of Nbs1 encompassing
603 the entire Tel1 binding domain (*nbs1-ΔC60*) have approximately the same level of γH2A
604 as *tel1Δ*. C) *nbs1-ΔC60* and point mutations in the Tel1 interaction motif of Nbs1 (*nbs1-9*

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605 and *nbs1-10*) have greatly diminished Tel1-mediated γH2A formation. D) *tel1Δ* and Nbs1
606 C-terminal mutants are insensitive to CPT. E) In the *rad2Δ* (FEN1) background, *nbs1-*
607 *ΔC60* cells are viable (left), unlike *nbs1Δ* (right), as assayed by tetrad dissection.
608
609 **Figure 2. Overexpression of Tel1 bypasses the need for the C-terminus of Nbs1 in**
610 **Tel1 signaling without deleterious effects.**
611 A) *nbs1-ΔC60 rad3Δ* cells were transformed with a plasmid containing *tel1*$^{+}$ expressed
612 from the *nmt41* promoter, or an empty vector, and assayed for γH2A formation following
613 IR exposure. B) Tel1 overexpression does not spontaneously cause activating
614 phosphorylation of Chk1. However, Tel1 overexpression does partially restore Chk1
615 phosphorylation in *rad3Δ* cells in response to IR. C) Tel1 overexpression does not affect
616 cell morphology or cause cell elongation. D) Tel1 overexpression does not negatively
617 affect growth rate nor increase sensitivity to IR.
618
619 **Figure 3. Overexpression of Tel1 bypasses the requirement of Nbs1, but not Mre11,**
620 **in damage-induced Tel1 signaling.**
621 A) Tel1 overexpression in *nbs1Δ rad3Δ* restores basal and IR-induced γH2A formation.
622 In the *mre11Δ rad3Δ* background, only basal levels of γH2A are restored. B) Chk1
623 phosphorylation in response to IR is partially restored when Tel1 is overexpressed in the
624 *nbs1Δ rad3Δ* background but not in *mre11Δ rad3Δ* cells.
625
626 **Figure 4. Overexpression of Tel1 bypasses the requirement of Nbs1, but not Mre11,**
627 **in Tel1-mediated telomere maintenance.**

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628 Southern blot of *Eco*RI digested genomic DNA probing for Telomere Associated
629 Sequences (TAS1). Telomere loss observed in *nbs1Δ rad3Δ* cells after successive
630 passages is prevented by Tel1 overexpression. In the *mre11Δ rad3Δ* background, Tel1
631 overexpression only slightly delayed telomere erosion. Ethidium bromide (EtBr) stained
632 gel serves as a loading control.
633
634 **Figure 5. Mre11-Rad50 form a complex independently of Nbs1 that can localize to**
635 **DNA double-strand breaks.**
636 A) Mre11-MYC efficiently co-precipitates with TAP-Rad50 in the presence or absence
637 of Nbs1. B) Mre11-MYC is efficiently enriched at a DSB created by HO endonuclease
638 with or without Nbs1 as assayed by chromatin immunoprecipitation. Note that
639 derepression of HO endonuclease expression under the control of the *nmt1* promoter
640 occurs at about 16-22 hours after removal of thiamine from the growth media (Forsburg
641 & Rhind, 2006).
642
643 **Figure 6. Fusion of the Nbs1 C-terminus to Mre11 is sufficient for Tel1 activity.**
644 A) Schematic of Mre11, Nbs1, and fusion protein generated by addition of C-terminal 60
645 amino acids encompassing the Tel1 binding domain of Nbs1 to the C-terminus of full-
646 length Mre11. B) The Mre11-TBD (Tel1 Binding Domain) fusion protein restores histone
647 H2A phosphorylation in the *nbs1Δ rad3Δ* background under endogenous Tel1 levels. C)
648 The Mre11-TBD fusion protein prevents telomere loss in *nbs1Δ rad3Δ* cells under
649 endogenous Tel1 levels. Strains were passaged 10 times and EcoRI-digested genomic
650 DNA was probed for Telomere Associated Sequences (TAS1). Ethidium bromide (EtBr)

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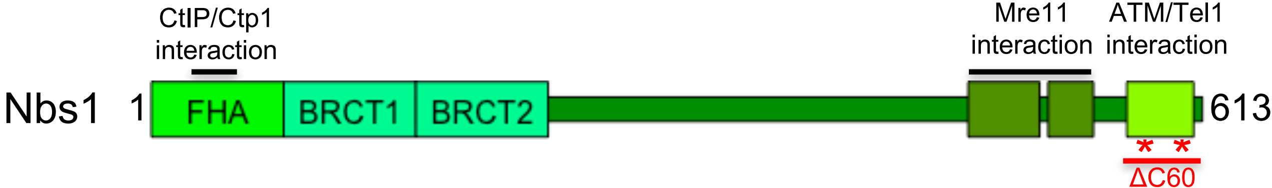
651 stained gel serves as a loading control. D) The Mre11-TBD fusion protein does not
652 noticeably affect Mre11 function in response to IR or CPT. The fusion protein does not
653 correct the DNA repair defect of *nbs1Δ* cells.
654
655 **Figure 7. Stimulation of Tel1 activity by Mre11-Rad50 depends on conformational**
656 **state but not nuclease activity.**
657 A) Nuclease-dead, *mre11-H134S,* does not reduce γH2A formation in *nbs1Δ rad3Δ* cells
658 when Tel1 is overexpressed. B) The open-conformation *TAP-rad50-I1192W* allele is
659 unable to stimulate Tel1 activity in response IR damage. C) TAP-Rad50-I1192W is
660 enriched at DNA double-strand breaks induced by the HO-endonuclease.
661
662 **Figure 8. Model for MRN interactions with Tel1.** In wild type, Tel1 localizes at DSB
663 and telomeres by binding the Tel1/ATM interaction module at the C-terminus of Nbs1.
664 This binding facilitates a lower affinity interaction with the closed conformation of
665 Mre11-Rad50, which stimulates Tel1 activity, resulting in phosphorylation of substrates
666 at DSBs and telomeres. Overexpression of Tel1 in *nbs1*Δ cells promotes the low affinity
667 interaction of Tel1 with Mre11-Rad50, which partially bypasses the requirement for Nbs1
668 in Tel1 activity.

Figure 1

A



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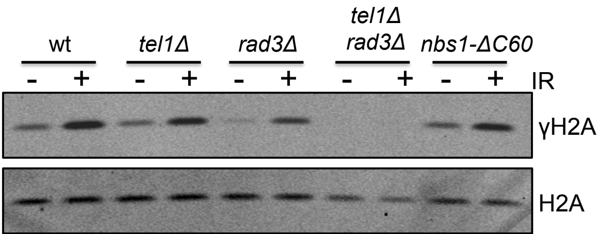

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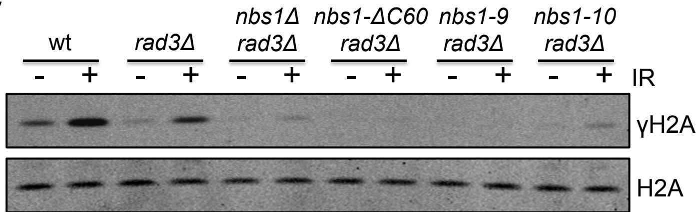

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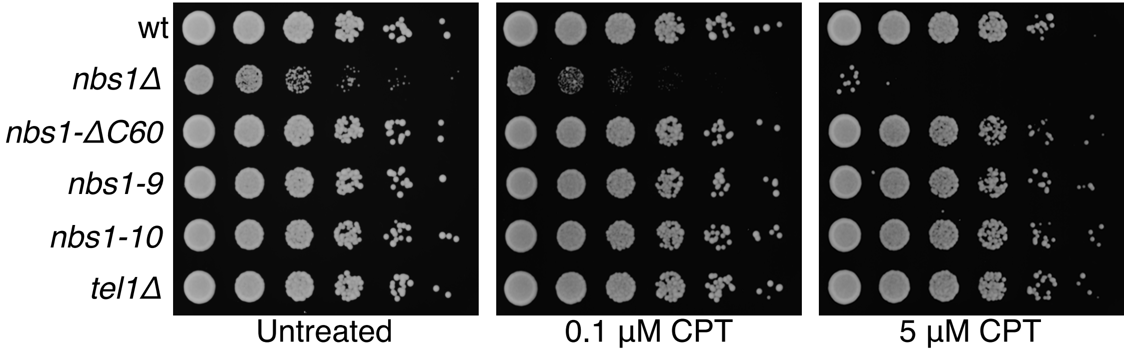

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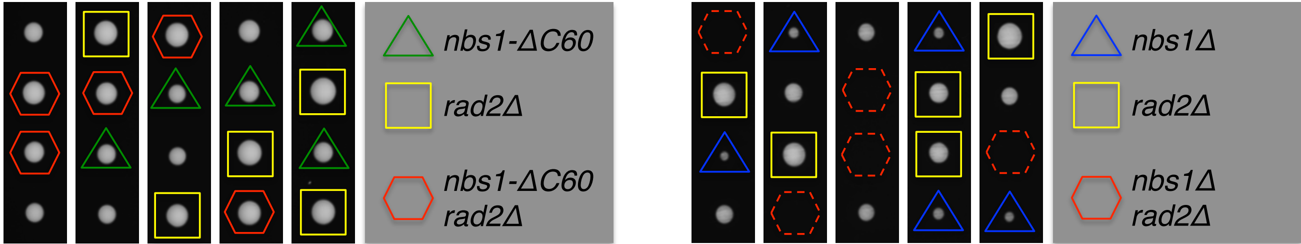

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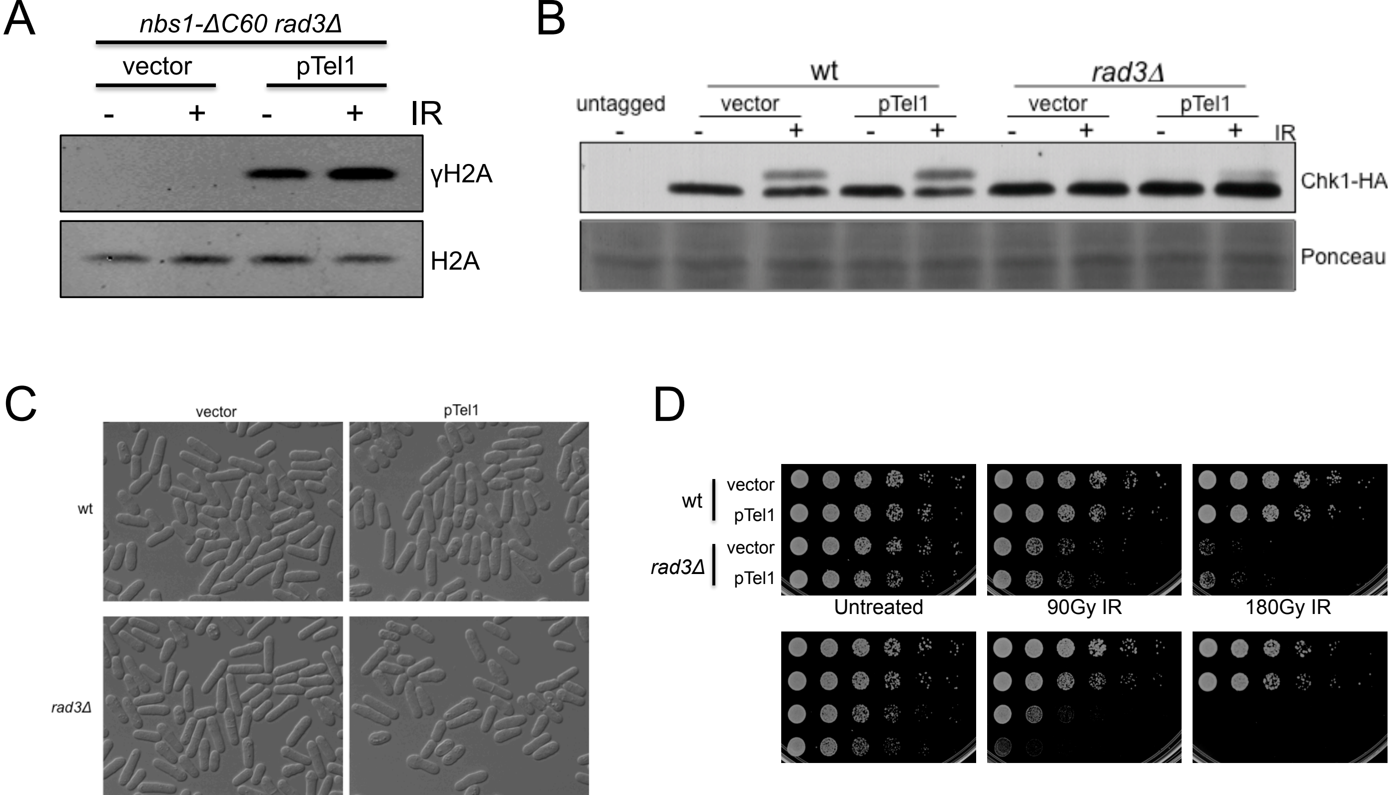

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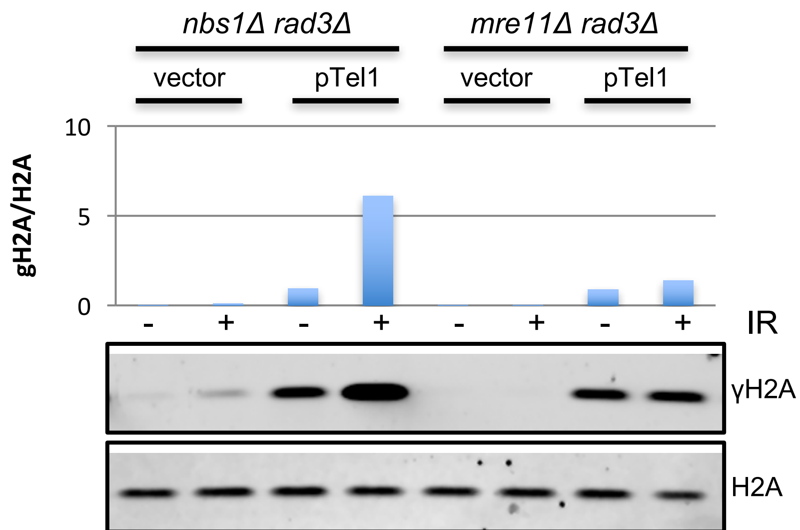

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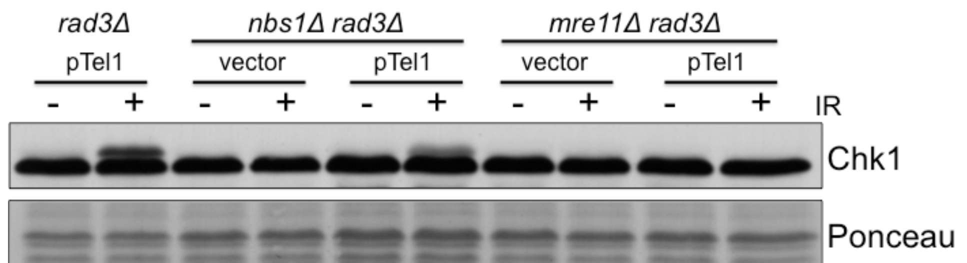

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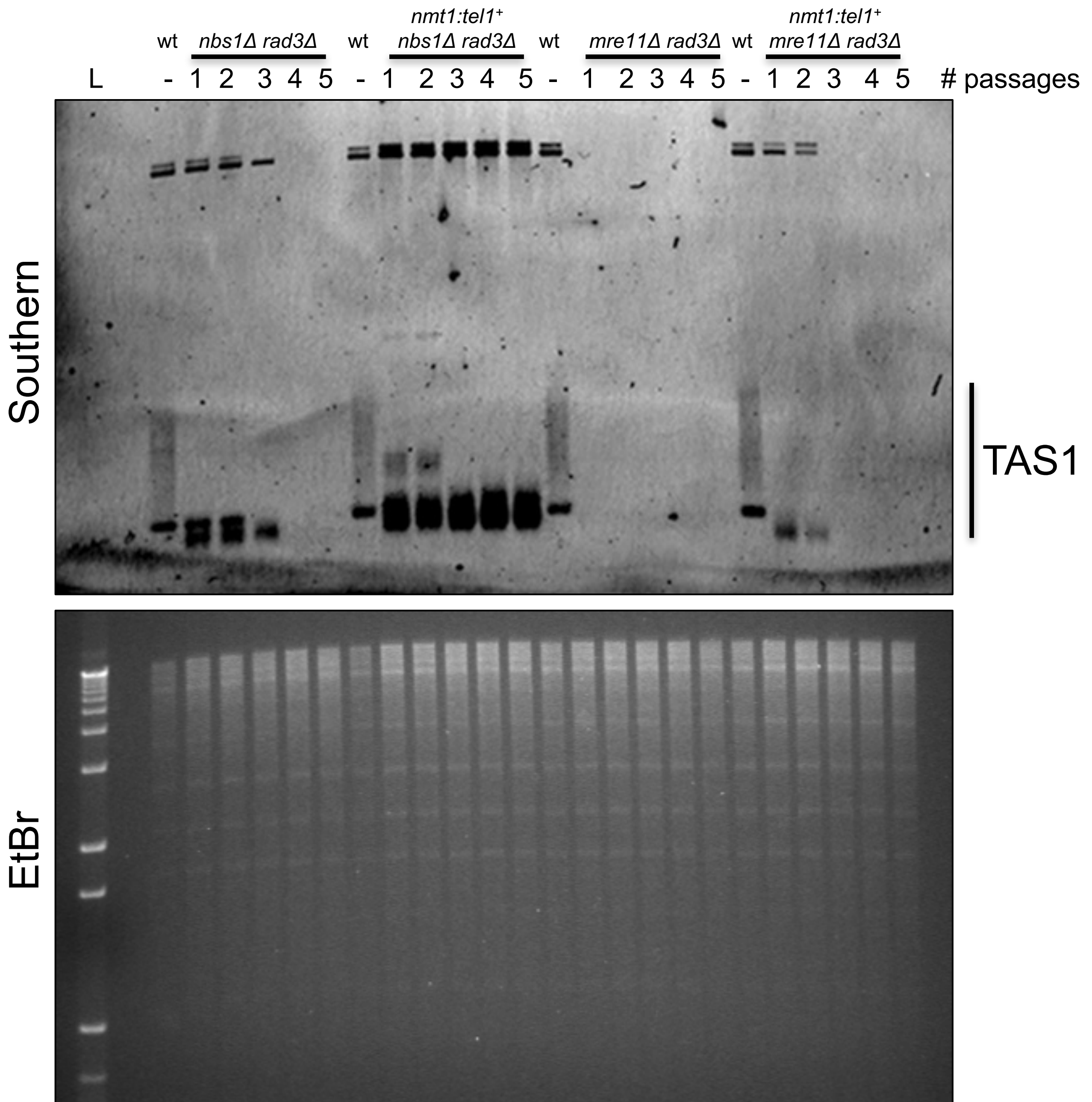

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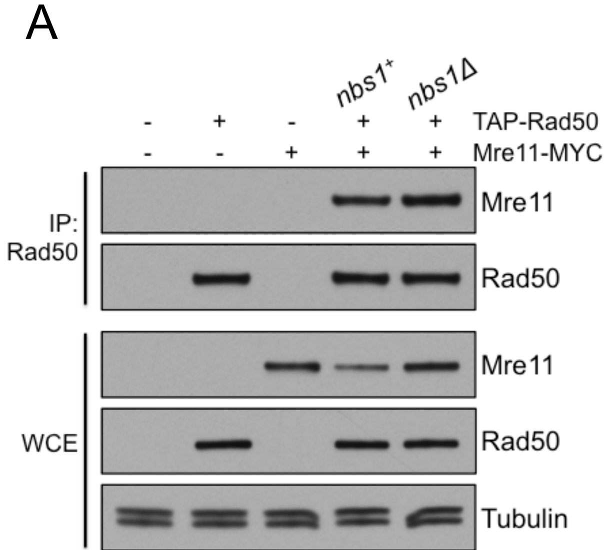

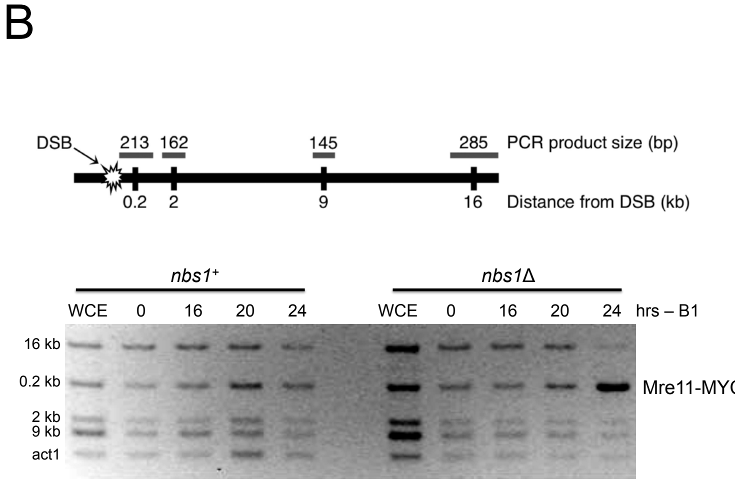

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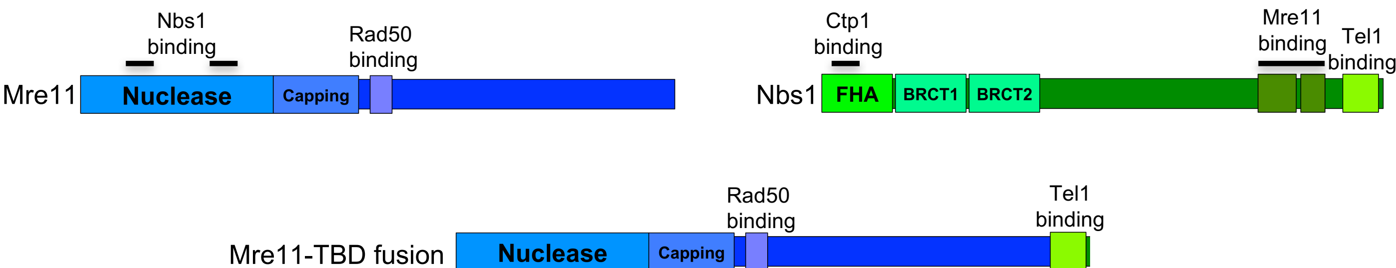
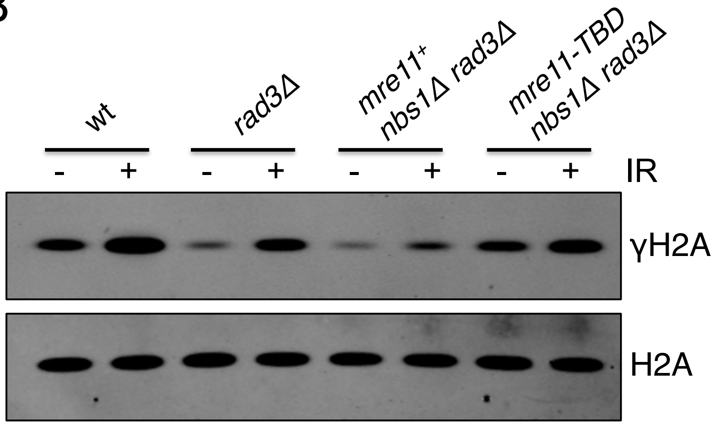
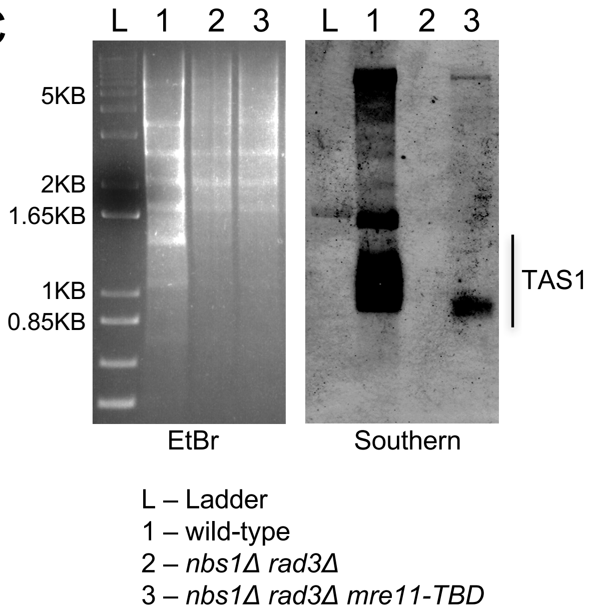






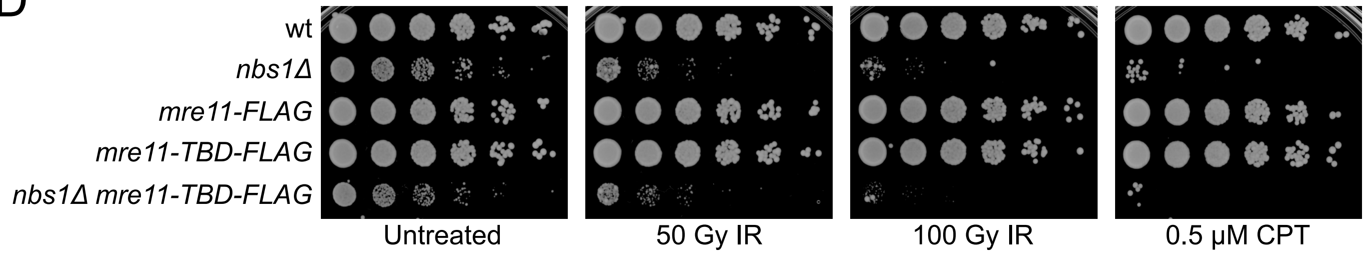
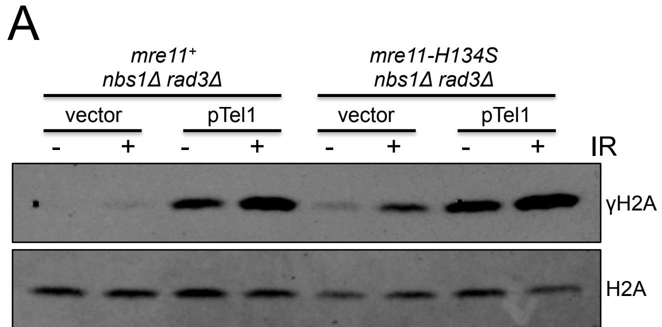
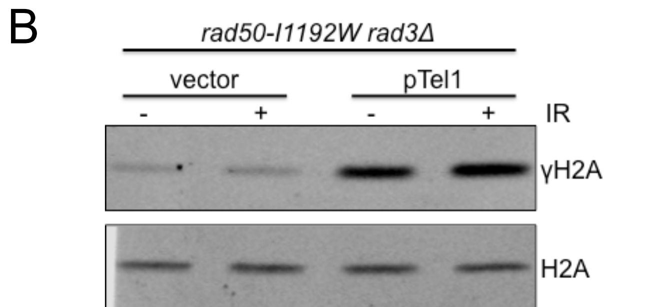


Figure 7





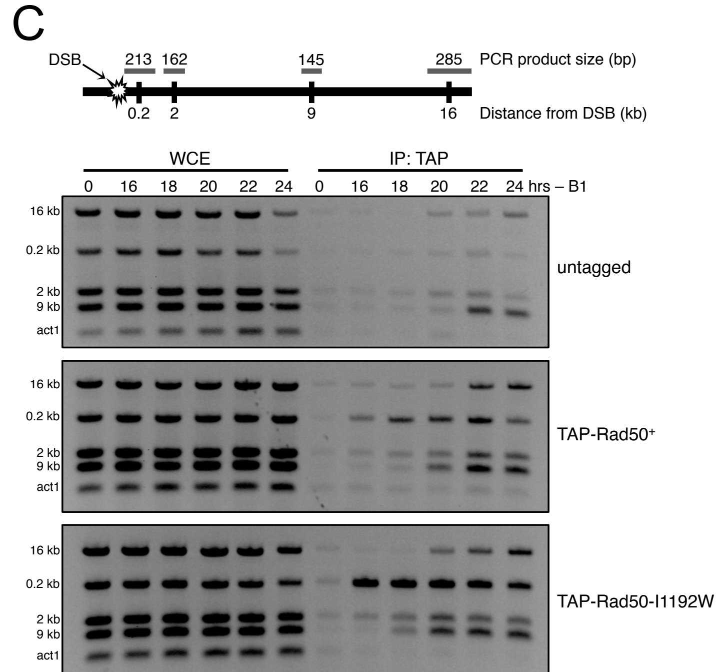
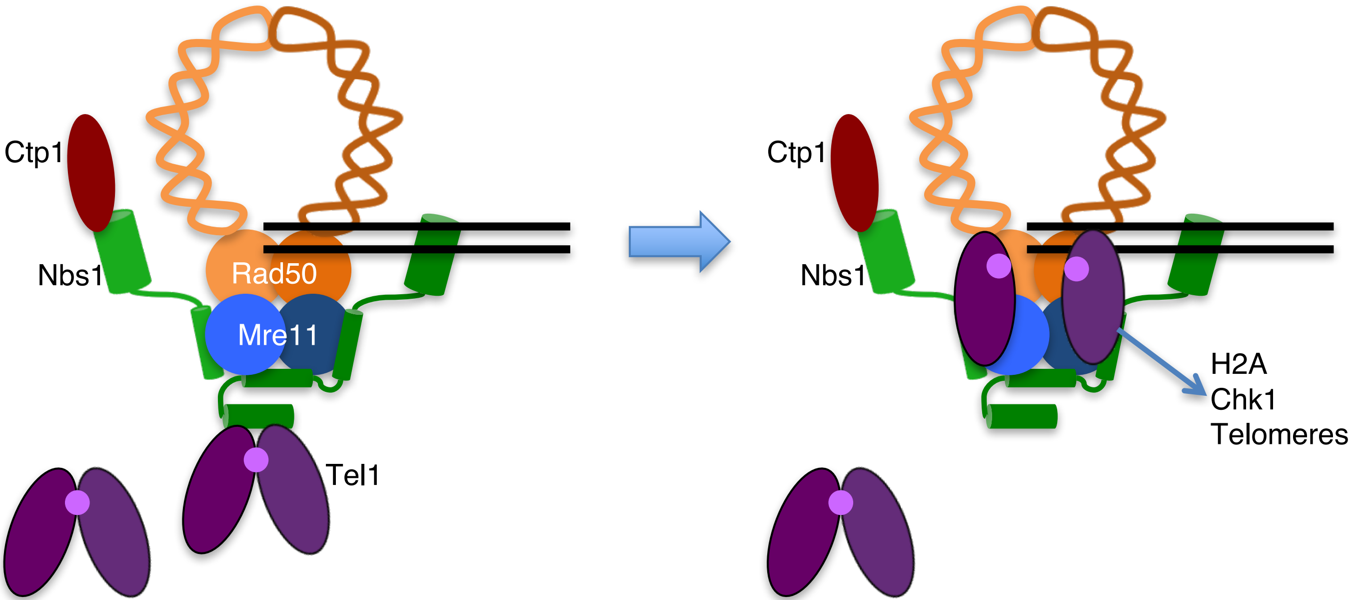


Figure 8

Wild-type



nbs1Δ + Tel1 overexpression

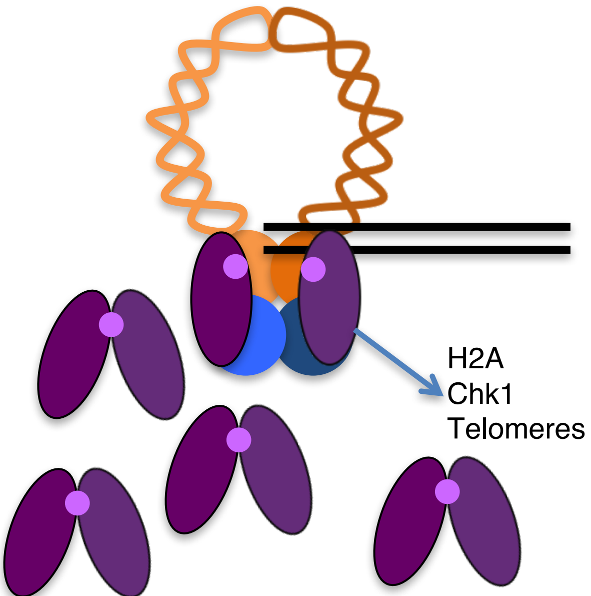


Figure EV1

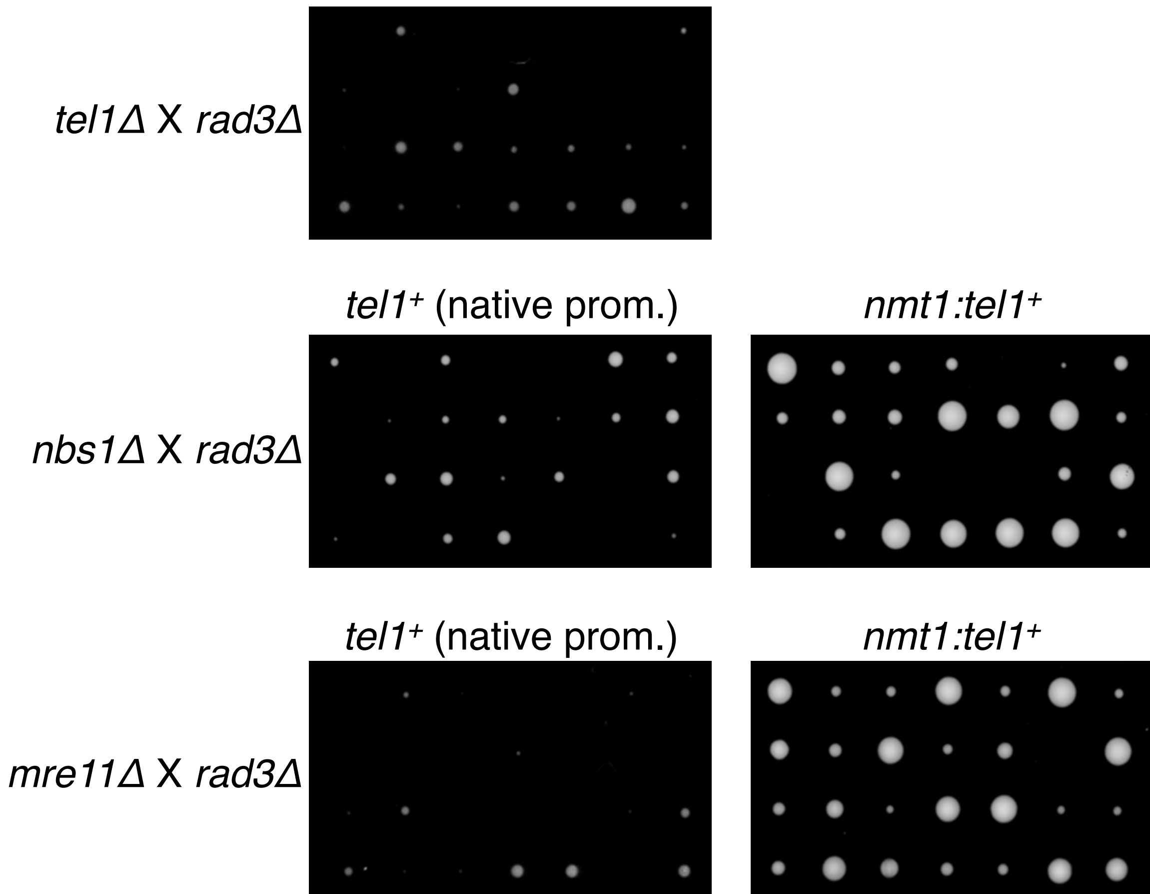


Figure EV1: Cells overexpressing Tel1 grow faster than cells with endogenous levels.
Cells with the indicated genotype were crossed with either *tel1⁺* under its native or the *nmt1* overexpression promoter. Tetrad dissection was performed on the same day with pictures taken after 4 days of growth at 30°C

Figure EV2

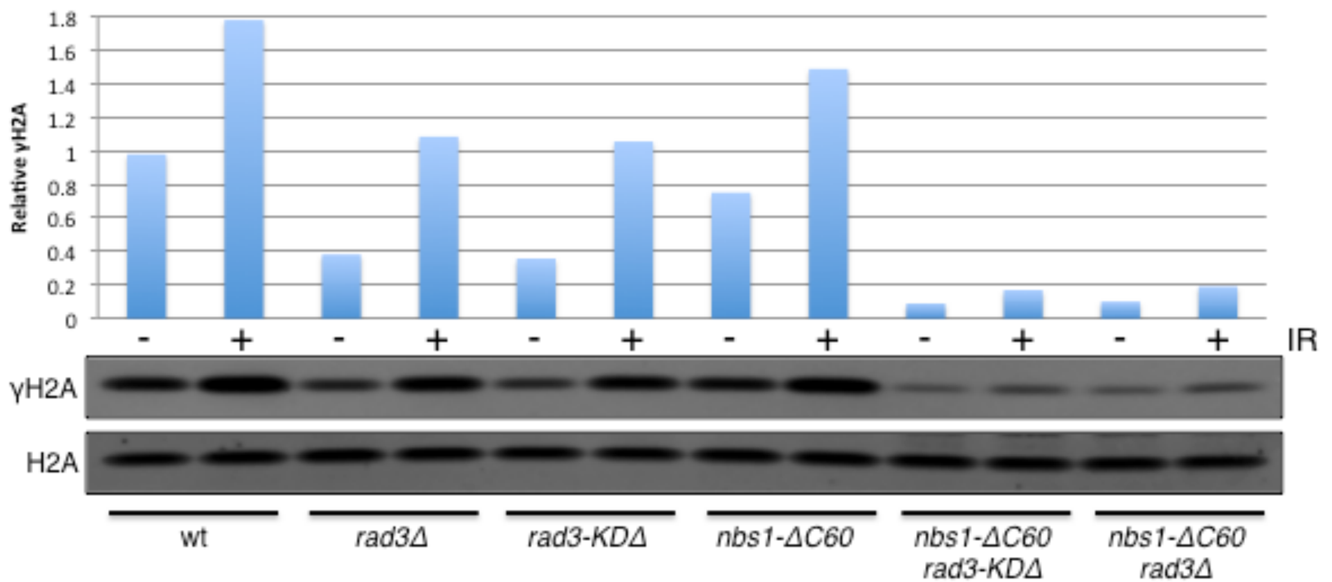


Figure EV2: The alternative method of Tel1 recruitment to telomeres dependent on the N-terminus of both Rad3 and Nbs1 is not sufficient for Tel1 activity towards γH2A in response to ionizing radiation

Rad3-KDΔ truncates the C-terminus of Rad3 containing the kinase domain. The *nbs1-ΔC60 rad3-KDΔ* strain was previously shown to be sufficient for Tel1 activity at telomeres.