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Mre11-Rad50 oligomerization promotes DNA double-strand break repair

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1 **Abstract**

2 The conserved Mre11-Rad50 (MR) complex is crucial for the detection, signaling, end tethering
3 and processing of DNA double-strand breaks (DSBs). While it was known for decades that MR foci
4 formation at DSBs accompanies repair, the underlying molecular assembly mechanisms and
5 functional implications remained unclear. Combining pathway reconstitution in electron
6 microscopy, biochemical assays and genetic studies, we show that *S. cerevisiae* MR oligomerizes
7 via a conserved Rad50 beta-sheet to higher-order assemblies, which bind DNA with positive
8 cooperativity. We designed Rad50 point mutants with enhanced or disrupted MR oligomerization,
9 and demonstrate that MR oligomerization facilitates foci formation, DNA damage signaling and
10 repair *in vivo*. MR oligomerization does not affect its exonuclease activity but drives
11 endonucleolytic cleavage at multiple sites on the 5'-terminated DNA strand near DSBs.
12 Interestingly, mutations in the human Rad50 beta-sheet are linked to hereditary cancer
13 predisposition and our findings might provide new insights into their potential role in
14 chemoresistance.

15 Introduction

16 Misfunction of the MRE11-RAD50-NBS1 (MRN) complex has been linked to various
17 pathologies, including ataxia-telangiectasia-like disorder (ATLD), Nijmegen break syndrome
18 (NBS), NBS-like disorder, and various hereditary and sporadic cancers¹⁻³. Furthermore, aberrant
19 degradation of nascent DNA by MRE11 has been observed in tumor suppressor BRCA1/2-deficient
20 cells, and abrogation of MRE11 activity is linked to resistance to PARP inhibitors in cancer
21 chemotherapy⁴⁻⁹. Therefore, MRN components emerged as promising biomarkers for disease
22 detection and prognosis, especially due to the complex's dual role in cancer prevention and tumor
23 chemoresistance^{1,2,10,11}. At the molecular level, human MRN and yeast Mre11-Rad50-Xrs2 (MRX)
24 promote DNA double-strand break (DSB) repair *via* non-homologous end joining (NHEJ) and
25 homologous recombination (HR) by means of DNA tethering, activation of downstream pathway
26 components and ATP-dependent resection of the 5'-terminated strands at DSBs^{12,13}. The latter
27 function is particularly relevant for HR as well as a variant of the end-joining pathway termed
28 alternative end-joining (A-EJ)¹³. The MRN/X complex also activates the DNA damage checkpoint
29 kinase ATM/Tel1, which in turn phosphorylates targets to orchestrate cell cycle arrest and DNA
30 repair¹⁴.

31 Nucleolytic processing of DSBs by MRN/X is restricted to the vicinity of the breaks, and
32 hence termed short-range DNA end resection¹⁵⁻¹⁷. The activity of MRN/X is versatile, as it can
33 process DSBs with various secondary DNA structures and protein blocks, both covalently bound,
34 such as topoisomerase cleavage complexes, or non-covalently associated, such as the NHEJ factor
35 Ku¹⁸⁻²¹. This preference for cleaving protein-blocked DNA ends by MR homologs is conserved in
36 evolution from archaea and bacteria to yeast and humans²²⁻²⁵.

37 In budding yeast *S. cerevisiae*, the nuclease activity of Mre11 is essential for processing
38 protein-blocked DNA ends. This activity can be bypassed in case of free DNA ends by long-range
39 nucleases normally acting downstream, including Exo1 and Dna2¹⁵. NBS1/Xrs2 is essential for
40 resection in humans, but largely dispensable in yeast. It is, however, needed for Tel1 activation and
41 nuclear import^{25,26}. Due to the requirement of a sister chromatid as a homologous template for repair
42 in HR, resection is limited to the S/G2 phase of the cell cycle, which is achieved through cell cycle-
43 regulated stimulation of Mre11 endonuclease activity by phosphorylated Sae2 (pSae2)^{27,28}. *In vitro*,
44 MR first incises the 5'-terminated strand ~ 15-35 bp away from the DSB end with its endonuclease
45 function, and then exonucleolytically degrades DNA backwards to the end in 3'-5' direction^{19,22,29}.
46 In cells, Mre11-dependent resection can generate 3'-overhangs of up to ~ 300 nt in length^{16,29,30}. It
47 is unclear whether the short-range resection tracts are generated by DNA cleavage that proceeds
48 stepwise away from the DNA end, as observed *in vitro*³¹, or whether MR in most cases first cleaves
49 DNA at greater distances from the DNA end, as observed in meiotic cells³⁰. The single-stranded

overhangs are then elongated by the long-range resection machinery including Exo1 or Dna2-Sgs1 to more than 1000 nt in length for homology-directed repair^{16,17,30,32}.

Upon DNA damage in eukaryotic cells, MR relocates from a homogeneous, nuclear distribution to discrete foci consisting of hundreds to thousands of MR molecules^{33–38}. This localized MR accumulation then recruits a cascade of DNA repair and signaling proteins, including ATM/Tel1^{35,36}. Given that MR foci formation at DSBs and the following array of protein interactions are conserved in eukaryotes, the capacity of MR to quickly accumulate at DSBs in a regulated manner appears to be an essential characteristic of efficient DSB repair³⁵. MR has been shown to dimerize to M₂R₂ complexes, whereby the Mre11 nuclease and Rad50 ATPase domains form a globular head domain from which two Rad50 coiled-coils protrude³⁹. Foci formation could be facilitated by higher-order oligomerization of MR dimers [(M₂R₂)_n] *via* globular head domain interactions or by zinc hook-mediated tethering of the protruding Rad50 coiled-coils^{40,41}. Although DNA binding occurs *via* the MR head domain³⁹ and hints of head domain oligomerization have been observed for various species *in vitro*, especially for eukaryotic MR^{37,40,42–44}, predominantly coiled-coil tethering was investigated and functionally associated with DNA movement, condensation and end fixation in repair^{37,40,41}. Also, while high resolution structural data from crystallography and cryo-electron microscopy on the MR dimer^{39,41,45–50} contributed majorly to the understanding of the diverse MR functions, they provided only limited information on more heterogeneous higher-order structures formed by MR dimers at DSBs. Thus, the relevance of MR head domain interactions for higher-order oligomerization and for the appearance of clearly detectable MR foci upon DNA damage remains unclear. Taken together, defining the molecular mechanisms of foci assembly and identification of the interaction interfaces underlying MR oligomerization are of great importance for understanding MR-mediated functions and mechanisms in DNA damage signaling and repair.

Here we visualized DNA end resection reactions by combining biochemical reconstitution and negative staining transmission electron microscopy (TEM), and thereby gained unprecedented insights into the function and architecture of active DNA end resection complexes at the single molecule level. We show that short-range DNA end resection is driven by MR oligomerization mediated by specific MR head domain interactions, which stabilize MR on DNA and enhance its endonuclease activity. Mutational analysis revealed that a small interface in the globular part of Rad50 modulates MR oligomerization, DNA end resection, as well as MR-dependent DNA damage signaling and foci formation in *S. cerevisiae*. Together, these results identify MR oligomerization as a physiologically relevant process that is pre-requisite for efficient DSB repair, and suggest a molecular mechanism for foci formation at DSBs.

84 **Results**

85 **MR forms oligomers in solution and cooperatively binds DNA**

86 To understand the role and regulation of MR oligomerization in DSB repair, we purified
87 recombinant *S. cerevisiae* MR from insect cells and visualized its different assemblies with negative
88 staining TEM (Fig. 1a; Supplementary Fig. 1a,b). In accord with previous studies of MR
89 homologs^{37,39–41,43}, we observed the formation of MR dimers (M_2R_2) with a globular head domain
90 of ~ 11 nm in diameter and protruding ~ 50 nm long Rad50 coiled-coils. The coiled-coils were either
91 in their open state (Fig. 1a-i, ii; Supplementary Fig. 1b-i) or closed to a clamp *via* zinc hooks at the
92 apex of the structure (Fig. 1a-iii; Supplementary Fig. 1b-ii). Additionally, M_2R_2 also displayed the
93 capacity to oligomerize *via* its head domain, allowing the formation of tetramers (M_2R_2)₂ and larger
94 assemblies (M_2R_2)_n with protruding coiled-coils that could then further interact *via* tethering (Fig.
95 1a-ii-iv; Supplementary Fig. 1b-i-iii). ATP binding stimulates MR association with DNA^{47,51}, and
96 MR endonuclease activity is magnesium- and manganese-dependent and requires ATP hydrolysis
97 by Rad50^{22,28,52,53}. Accordingly, under more physiological conditions as also used for *in vitro* MR
98 nuclease assays (30 °C with Mg²⁺, Mn²⁺, but ATPγS instead of ATP to prevent ATP hydrolysis and
99 stabilize Rad50 interactions), the MR oligomers were even more evident (Fig. 1a-v; Supplementary
100 Fig. 1b-iv). Higher temperature facilitated the formation of larger MR oligomers (compare 4 °C in
101 Supplementary Fig. 1b-i-iii with 30 °C in Supplementary Fig. 1b-iv and 42 °C in Supplementary
102 Fig. 1d-i). The MR oligomers were stable at physiological salt concentrations (150 mM KCl or
103 NaCl) and only dissociated into MR dimers (M_2R_2) at high salt (260 mM KCl) (Supplementary Fig.
104 1c). Oligomer size decreased linearly with increasing KCl concentration, suggesting a charge
105 screening effect of salt on electrostatic interactions between the MR head domains (100 nM MR,
106 Supplementary Fig. 1c). However, higher temperature (42 °C) and MR concentration (600 nM)
107 could overcome this charge screening effect at high salt conditions, restoring MR oligomerization
108 (compare 260 mM KCl in Supplementary Fig. 1c-i with Supplementary Fig. 1d-ii). Together, the
109 high local MR concentrations at DSBs and the growth temperature of yeast may thus counteract the
110 charge screening effect of chromatin DNA⁵⁴, and allow MR oligomerization under physiological
111 conditions.

112 To directly examine the effect of DNA on MR oligomerization, we investigated MR
113 assembly on linear and circular plasmid DNA. At lower MR:DNA ratios [50-300 nM MR, 10-30:1
114 with respect to DNA molecules], the MR oligomers loaded onto DNA and dissociated into dimers
115 that bound DNA ends (Fig. 1b-i, top) or internally (Fig. 1b-ii, iii; Supplementary Fig. 1e). Only few
116 isolated MR dimers were visible without another dimer bound adjacently on DNA (pink arrows
117 denote a few examples in Fig. 1b). The majority of MR dimers could be detected either in pairs

118 (orange arrows) or as “pearls-on-a-string” (red arrows), interacting laterally through the head
119 domains (Fig. 1b-ii, iii). This indicates a fast, yet ordered accumulation of MR molecules at certain
120 locations on DNA (Fig. 1b-ii, iii), while other DNA stretches were left almost protein-free (white
121 arrows, Fig. 1b). Such polymerization behavior, reminiscent of positive cooperativity, was observed
122 both in the presence and absence of DNA ends (Fig. 1b-ii, iii; Supplementary Fig. 1e). The
123 oligomerization was apparent not only in case of MR molecules bound next to each other on DNA,
124 but also at distant DNA sites. The observed DNA loops (Fig. 1b-i, bottom) and substrate compaction
125 (Fig. 1b-ii, iii; Supplementary Fig. 1e) suggest that the head domain interactions and subsequent
126 Rad50 tethering of MR clusters can bring distant parts of DNA into close proximity, as reported
127 previously for archaeal MR³⁷. The cooperative accumulation of multiple MR units on DNA was
128 confirmed by electrophoretic mobility shift assays (EMSAs), demonstrating a distinct banded
129 pattern of protein-DNA species and a rapid shift to the wells at higher MR concentrations (Fig. 1c-
130 i; Supplementary Fig. 1f). Cooperative MR binding to linear or circular DNA was comparable,
131 irrespective of free or protein-blocked ends or DNA supercoiling (Fig. 1c-ii; Supplementary Fig.
132 1f). The limited end preference of MR binding to DNA was observed previously⁵⁵; Xrs2, albeit not
133 essential for resection *in vivo*, facilitates end-binding of the MRX complex^{26,55}.

134 To ascertain that MR oligomers are active entities, we performed *in vitro* nuclease assays³¹
135 in the presence of phosphorylated Sae2 (pSae2) to trigger MR endonuclease activity (Fig. 1d-i).
136 Resection was monitored with a radio-labeled oligonucleotide annealing to the produced 3'-
137 overhang. Indeed, DNA end resection was stimulated with increasing MR concentrations that led to
138 binding of multiple MR entities on DNA in EMSA assays (Fig. 1c-i; Supplementary Fig. 1f).
139 Interestingly, negative staining TEM analysis of the MR:DNA ratio with the highest resection
140 activity in the nuclease assay (200 nM MR with 2.5 nM DNA, MR:DNA ratio 80:1; Fig. 1d-i)
141 revealed that at higher MR:DNA ratio the MR dimers formed larger oligomeric clusters on DNA,
142 in which the individual MR dimers could not be easily distinguished (Fig. 1d-ii), in contrast to the
143 “pearls-on-a-string”-like arrangement of distinguishable MR dimers on DNA observed at lower
144 MR:DNA ratios (Fig. 1b-ii, iii; Supplementary Fig. 1e). In MR foci with a high local concentration
145 of MR and hence a high MR:DNA ratio, MR thus likely assembles to larger oligomeric clusters on
146 DNA that could serve as nucleation sites for cooperative foci formation. The combination of
147 imaging and biochemical experiments demonstrates that the observed MR oligomers are not
148 aberrant aggregates, but functional units that may allow resection beyond the vicinity of DNA ends
149 as observed *in vivo*^{16,30}.

150 Additionally, a higher MR:DNA ratio (60:1; Supplementary Fig. 1g) also stabilized MR-
151 MR interactions on DNA against higher salt concentration, thus counteracting the expected salt
152 sensitivity of MR-DNA association⁴⁹. Moreover, MR preferentially binds longer DNA substrates,

153 as shown by the apparent dissociation constants (K_D) derived from EMSA experiments with
154 oligonucleotides (Fig. 1e; Supplementary Fig. 2a). EMSA experiments also revealed that longer
155 DNA molecules stabilized MR on DNA against high salt concentrations (Supplementary Fig. 2b-
156 d), similarly to higher MR:DNA ratios (Supplementary Fig. 1g). Mass photometry measurements
157 confirmed better MR binding to longer oligonucleotides, as seen by the increase of the MR-DNA
158 peak area between the two dashed lines (Fig. 1f; Supplementary Fig. 2e; molecular weight of M_2R_2
159 460 kDa). Consistent with a cooperative, sequential mechanism of MR binding to DNA, a higher
160 molecular weight peak of ~ 1000 kDa could be detected and properly fitted for longer
161 oligonucleotides (zoom insets for 106 bp and 197 bp), corresponding to two MR dimers bound per
162 DNA molecule. In addition, larger MR clusters were likely formed on longer DNA that are beyond
163 the detection limit of mass photometry⁵⁶, as indicated by the decrease in the observed MR-DNA
164 peak areas upon binding of 106 bp or 197 bp DNA compared to 73 bp (Fig. 1f). The formation of
165 larger MR-DNA assemblies with different fractions of MR molecules bound to plasmid substrate
166 as observed in negative staining TEM (Fig. 1b-ii, iii; Supplementary Fig. 1e) could only be detected
167 in mass photometry when MR and plasmid DNA were mixed in low salt conditions (25 mM KCl to
168 facilitate DNA binding), and immediately measured without incubation (see tail of counts at high
169 molecular weight > 2500 kDa; Supplementary Fig. 2f, 25 mM KCl). Incubation for 30 min at 30 °C
170 and low salt conditions (25 mM KCl), as required for MR binding to oligonucleotide DNA (Fig. 1f;
171 Supplementary Fig. 2e), however, led to much larger MR-plasmid assemblies that were barely
172 detectable by mass photometry (Supplementary Fig. 2f, 25 mM KCl, 30 min).

173 Taken together, these observations suggest that MR molecules form oligomers in solution,
174 which dissociate to bind DNA and cooperatively reassemble to larger MR clusters on DNA (Fig.
175 1g). MR oligomerization may thus allow rapid loading of multiple MR units onto DNA, thereby
176 facilitating short-range resection of DNA ends. Moreover, MR's physiological substrate, long
177 chromatin DNA, could promote cooperative and stable binding of high numbers of MR molecules,
178 allowing foci formation.

179 **MR clustering on DNA initiates end resection**

180 To be able to ultimately image active DNA end resection reactions, we first reconstituted
181 short-range (MR and pSae2) and long-range (Exo1) resection *in vitro* using recombinant
182 *S. cerevisiae* proteins and linear plasmid DNA with free or streptavidin-blocked ends, and employed
183 established gel-based assays. As expected, DNA with free ends was rapidly processed by Exo1
184 alone (Fig. 2a, lane 2; Supplementary Fig. 3a), as visualized by the characteristic pattern of resection
185 products, depending on the extent of resected DNA and whether resection was initiated at one or
186 both DNA ends (Fig. 2a, lane 2, see cartoons on the right). Adding MR-pSae2 to the Exo1 reaction

187 moderately enhanced resection (Fig. 2a, compare lane 2 with lanes 4, 6 and 8; Supplementary Fig.
188 3a), as observed previously^{31,57,58}. In contrast, MR-pSae2 alone resulted in slow resection that was
189 limited to the vicinity of DNA ends (Fig. 2a, lanes 3, 5 and 7; Supplementary Fig. 3a)^{16,31}. As
190 anticipated, on linear DNA with streptavidin-blocked ends, long-range resection by Exo1 alone was
191 reduced (Fig. 2b, lane 2; Supplementary Fig. 3a,b), but this activity could be restored by the addition
192 of MR-pSae2 (Fig. 2b, lanes 4, 6 and 8; Supplementary Fig. 3a). Thereby, MR-pSae2 was able to
193 overcome the protein block and resect blocked DNA ends similarly to free DNA (Fig. 2b, lanes 3,
194 5 and 7; Supplementary Fig. 3a), creating an entry site for Exo1-mediated long-range resection.
195 Using shorter, 3'-labeled oligonucleotide-based DNA with one streptavidin-block at the 5'-
196 terminated end revealed a gradual shortening of the 5'-strand by MR-pSae2 (Fig. 2c), indicating the
197 formation of several endonucleolytic incisions. MR oligomerization may promote such stepwise
198 DNA end resection³¹. Together, our reconstituted assays confirm that Exo1 requires MR-pSae2-
199 dependent short-range resection at protein-blocked DNA ends to initiate long-range resection.

200 We next set out to visualize the DNA end resection reactions, using the same recombinant
201 protein complexes, in negative staining TEM. As shown in Fig. 2d, we readily observed MR clusters
202 in conjunction with tetrameric pSae2 bound to DNA with free or streptavidin-blocked ends (Fig.
203 2d-i,II,III; Fig. 2d-iii,II; magenta arrows). While free ends of linear DNA were not clearly visible
204 and likely buried within MR-pSae2 clusters (Fig. 2d-i,II), streptavidin-blocked ends of linear DNA
205 could be easily distinguished (Fig. 2d-iii,II, dark blue arrows). MR-pSae2 clustering could occur
206 internally on DNA and harbor several DNA molecules, as apparent from the number of streptavidin-
207 blocked ends in particular protein-DNA assemblies (Fig. 2d-iii,II, left panel, dark blue arrows),
208 reminiscent of the assembly of repair foci involved in the repair of several broken DNA molecules
209 *in vivo*^{35,59}. Interestingly, resection of DNA ends was observed in TEM independently of protein
210 blocks, producing up to ~ 300 nt-long stretches of single-stranded DNA (Fig. 2d-i,III; Fig. 2d-iii,II),
211 consistent with *in vivo*^{16,30} and previous biochemical data³¹. These single-stranded tracts were
212 readily coated by RPA, thus appearing more electron-dense than double-stranded DNA (Fig. 2d-
213 i,III; Fig. 2d-iii,II, right panel; arrows in light blue). In accordance with the biochemical assay in
214 Fig. 2a, MR-pSae2 activity was not required to trigger Exo1-dependent long-range resection on
215 DNA templates with free ends, as visualized by the appearance of plasmid-length RPA-coated
216 single-stranded DNA with Exo1 alone (Fig. 2d-ii,II, arrows in light blue). In contrast, MR-pSae2-
217 dependent end processing was required for subsequent tail elongation by Exo1 when using DNA
218 substrates with blocked ends (Fig. 2d-iii,II-IV). Accordingly, no RPA-coated single-stranded DNA
219 overhangs were observed when imaging streptavidin-blocked DNA with Exo1 without prior short-
220 range resection by MR-pSae2 to remove the protein block (Fig. 2d-iii,I). Taken together, these single
221 molecule assays recapitulate that short-range resection by MR-pSae2 is essential for initial

222 processing of protein-blocked ends to allow handover to the Exo1 exonuclease to execute long-
223 range resection. By providing snapshots of the short- and long-range resection pathways, our TEM
224 analysis could unravel the underlying mechanisms, and visualize that MR clusters accumulating at
225 free and blocked DNA ends in conjunction with pSae2 are a prerequisite for short-range processing.

226 **A conserved Rad50 interface mediates MR oligomerization**

227 To define the functional relevance of cooperative DNA binding and cluster formation of MR
228 on DNA, we next set out to identify the interaction interface(s) between the MR head domains that
229 mediate oligomerization. A recent crystallographic structure of eukaryotic *C. thermophilum* (*Ct*)
230 Rad50 dimers on oligonucleotide DNA indicated that an anti-parallel, two-stranded beta-sheet (Fig.
231 3a, black rectangle, β) and its connecting loop (Fig. 3a, black rectangle, L) protrude from the head
232 domain of one Rad50 dimer in one asymmetric unit towards the identical structure of another Rad50
233 dimer in the adjacent asymmetric unit of the crystal (Fig. 3a, black rectangle and zoom in, RCSB
234 Protein Data Bank PDB identifier: 5DAC^{50,60}). The residues constituting this small beta-sheet and
235 its connecting loop, or their physicochemical characteristics, are conserved between
236 *C. thermophilum*, *S. cerevisiae* and *H. sapiens* Rad50 (Fig. 3b), which is intriguing given the limited
237 overall sequence similarity of MR between species⁶¹. Interestingly, mutations derived from cancer
238 predisposed individuals (* in Fig. 3b, from ClinVar/MedGen databases) cluster in this conserved
239 beta-sheet and could be relevant for Rad50 functions in DNA repair and cancer avoidance.

240 To investigate whether beta-sheet interactions are indeed involved in MR oligomerization,
241 we stained MR oligomers with Thioflavin T (ThT), a dye used to detect beta-sheet-rich structures⁶².
242 Indeed, a ThT signal was detected in the presence of MR oligomers already at 4 °C and enhanced
243 at higher temperature (42 °C), suggesting the presence of extended beta-sheet interactions between
244 MR molecules (Fig. 3c). In order to test the possible role of the identified protruding beta-sheet and
245 its connecting loop in MR oligomerization, we engineered two *S. cerevisiae* (*Sc*) Rad50 variants,
246 each with several point mutations in the beta-sheet to disrupt either electrostatic or hydrophobic
247 interactions between MR molecules. Addressing the electrostatic component of MR oligomerization
248 (Supplementary Fig. 1c), we substituted a polar residue in the potential interaction interface with an
249 uncharged, non-polar alanine (*Sc* N121A, in *Ct* N122, red in Fig. 3a zoom in and Fig. 3b, Rad50^{ho})
250 and removed a negative charge to alleviate a potentially strong repulsive force between the aspartate
251 residues D124 (*Ct* E125, red in Fig. 3a zoom in and Fig. 3b, Rad50^{ho}) of interacting *Sc*Rad50
252 molecules. In the second *Sc*Rad50 variant, four hydrophobic beta-sheet residues were mutated to
253 alanine to disrupt the beta-sheet structure⁶³ (*Sc* Rad50^{L116A/I119A/T127A/L128A}; *Ct* Rad50^{L118/L120/T127/I129};
254 blue in Fig. 3a zoom in and Fig. 3b, Rad50^{lo}). These two *Sc*Rad50 mutants were co-expressed and

co-purified with Mre11, showing that the physical interaction between Mre11 and Rad50 was not notably affected (Supplementary Fig. 4a).

We first characterized the oligomerization capacity of the MR variants in negative staining TEM in the absence of DNA. This analysis revealed that the quadruple mutant MR^{L116A/I119A/T127A/L128A} formed only dimers (thus henceforth denoted low oligomerization mutant, MR^{lo}), while the MR^{N121A/D124N} mutant (henceforth denoted high oligomerization mutant, MR^{ho}) assembled to even larger oligomers than wild-type MR (MR^{wt}) (Fig. 3d). Mass photometry confirmed that the MR^{ho} mutant formed larger oligomers than MR^{wt}, as in absence of DNA only a small dimeric fraction of MR^{ho} could be detected (- DNA, zoom inset ~ 460 kDa; Fig. 3e; Supplementary Fig. 4b). The MR^{ho} mutant was also more resistant towards high salt (240 mM KCl), as ~ 50 % more MR^{ho} oligomer assemblies could be harvested in a pelleting assay compared to MR^{wt} after incubation at 1.2 μM MR and higher temperature (42 °C) (ho:wt ratio ~ 1.5 in 42 °C pellet; Fig. 3f; Supplementary Fig. 4c). Consistently, mass photometry detected less MR^{ho} dimers at ~ 460 kDa after 30 min incubation at 250 mM KCl and 30 °C compared to MR^{wt}, suggesting the formation of larger MR^{ho} assemblies beyond the detection limit (Supplementary Fig. 4d). Upon addition of oligonucleotides with different lengths, MR^{ho} bound DNA with positive cooperativity and higher affinity to longer substrates, similarly to MR^{wt} (Fig. 3e; Fig. 1f). Interestingly, MR^{ho} formed larger oligomers on longer oligonucleotide DNA compared to MR^{wt}, as the peak at ~ 1000 kDa of two MR^{ho} dimers binding one DNA molecule could be detected and properly fitted already for 73 bp with a larger peak area than for MR^{wt} (compare 73 bp zoom insets in Fig. 3e and Fig. 1f). Moreover, the areas of the MR-DNA peaks decreased more with 106 bp and 197 bp DNA for MR^{ho} than MR^{wt}, indicative of the formation of larger MR^{ho} nucleoprotein assemblies beyond the detection limit (compare peak areas for 106 bp and 197 bp in Fig. 3e and Fig. 1f). For MR^{lo}, on the other hand, binding to oligonucleotide DNA could not be observed in mass photometry at the low MR and DNA concentrations required for optimal peak separation (197 bp in Supplementary Fig. 4b).

EMSA experiments revealed a moderate DNA binding impairment of the MR^{lo} mutant compared to MR^{wt} (100 bp in Fig. 3g, Supplementary Fig. 2a, Supplementary Fig. 4e). Nevertheless, MR^{lo} could be bound to linear plasmid-length DNA at a higher MR:DNA ratio (90 nM MR^{lo}, 35 mM KCl, MR^{lo}:DNA 36:1) in negative staining TEM, appearing as dispersed single dimers (Fig. 3h, top). This suggests that the targeted beta-sheet motif is indeed required in its intact form for MR oligomerization. Surprisingly, DNA binding of the MR^{ho} mutant was also reduced in EMSA experiments (Fig. 3g; Supplementary Fig. 2a; Supplementary Fig. 4e), indicating that higher oligomeric structures may interfere with disassembly to smaller MR^{ho} complexes including dimers and thus efficient loading onto DNA. Mass photometry experiments at low salt (25 mM KCl, zoom insets in Supplementary Fig. 4f) revealed that once MR^{ho} loaded onto plasmid DNA, it assembled

one or maximally two MR^{ho} dimers per DNA molecule (in 1500-2500 kDa range, zoom inset in Supplementary Fig. 4f) and then rapidly formed large nucleoprotein assemblies beyond the detection limit. Thus, the tail of events > 2500 kDa, corresponding to smaller nucleoprotein assemblies, was significantly reduced for MR^{ho} compared to MR^{wt} (25 mM KCl, zoom insets in Supplementary Fig. 4f), and even more so after incubation (25 mM KCl, 30 min, zoom insets in Supplementary Fig. 4f). Moreover, TEM analysis demonstrated that MR^{ho} assembled into defined, condensed “worm”-like structures once it bound plasmid DNA (90 nM MR^{ho}, 35 mM KCl, MR^{ho}:DNA 36:1; Fig. 3h-ii), at conditions when MR^{wt} formed much smaller clusters (100-300 nM MR^{wt}, 30 mM KCl, MR^{wt}:DNA 20-30:1; Fig. 1b-ii, iii; Supplementary Fig. 1e). Taken together, these results indicate that the beta-sheet interactions underlying MR oligomerization are more stable in the MR^{ho} mutant. It is likely that MR^{ho} dimers pack more closely and their oligomerization is less salt-sensitive due to the abolished charge repulsion of D124N.

MR oligomerization modulates its endonuclease activity

We next performed a clipping assay using oligonucleotide DNA with blocked ends to assess if MR oligomerization affects its endonuclease activity in conjunction with pSae2, which recapitulates the first step of short-range end resection. Interestingly, the MR^{lo} mutant was entirely endonuclease-deficient, even when used at a concentration (200 nM) that supports DNA binding (Fig. 4a-i; Fig. 3g; Supplementary Fig. 5a; Supplementary Fig. 4e-ii). Conversely, MR^{ho} was slightly more active than MR^{wt} (Fig. 4a-i, ii), which was particularly apparent upon short incubation times (Fig. 4b,c). Using plasmid-length DNA, the initial resection by MR^{ho} could be efficiently extended by Exo1 (Supplementary Fig. 5b), similarly to MR^{wt} (Fig. 2a,b). Yet, MR^{lo} and MR^{ho} were proficient as exonucleases and could be inhibited by ATPγS addition, implying that both are capable of ATP binding (Supplementary Fig. 5c). Moreover, both MR variants were able to bind pSae2 (Fig. 4d), as shown with a protein interaction assay using the conserved C-terminal part of Sae2 (pSae2 ΔN169)²⁸. These experiments demonstrate that although functionally deficient, the Rad50^{lo} variant is clearly distinct from the Rad50S mutants, which fail to physically and functionally interact with Sae2^{28,64}. Taken together, these data suggest that the conserved beta-sheet motif in Rad50 drives cooperative MR oligomerization, and that MR clustering is required for short-range resection to promote MR endonuclease activity.

MR oligomerization is required for foci formation and response to DNA damage

In order to probe the function of MR oligomerization *in vivo*, C-terminally yEVENUS-tagged wild-type and the two mutant Rad50 variants (*mr^{ho}: rad50^{N121A/D124N}-yEVENUS*, *mr^{lo}: rad50^{L116A/I119A/T127A/L128A}-yEVENUS*) were introduced into an *S. cerevisiae rad50Δ* strain. DNA damage sensitivity

assays with the topoisomerase inhibitor and DSB-inducer camptothecin (CPT) and the replication inhibitor hydroxyurea (HU) causing multiple types of DNA damage revealed that *mr^{ho}* cells coped with DNA lesions similarly to wild-type, while *mr^{lo}* cells displayed a phenotype comparable to *rad50Δ* cells (Fig. 4e). This defect was recapitulated by proliferation assays with 5 μM or 25 μM CPT: whereas the doubling times of wild-type and *mr^{ho}* cells only marginally increased upon CPT-treatment, *mr^{lo}* and *rad50Δ* cells were more sensitive to DNA damage, especially at a higher CPT concentration (25 μM; Fig. 4f). Western blot analysis showed that the expression levels of MR wild-type and the mutants remained rather unchanged upon DNA damage, with lower levels of the MR^{lo} mutant protein compared to MR^{ho} or wild-type controls (Fig. 4g).

Live-cell imaging of yEVENus-tagged wild-type, *mr^{ho}* and *mr^{lo}* strains before and after CPT treatment revealed the formation of small Rad50 foci in the nucleus at DNA damage sites (zoom insets and arrows, Fig. 4h-i). Interestingly, although we cannot exclude that the reduced protein levels contribute to this defect, the dimeric MR^{lo} mutant failed to assemble distinguishable Rad50 foci. Conversely, *mr^{ho}* cells formed Rad50 foci even in the absence of DNA damage (Fig. 4h-i, ii; Supplementary Fig. 5d), supporting the notion that MR oligomerization promotes cooperative and stable DNA binding required for the assembly of large repair structures *in vivo*.

Upon DNA damage, the checkpoint-kinase Tel1 is recruited by MR to repair foci at DNA lesions, which triggers phosphorylation of target proteins including Rad53, and delays progression into mitosis until the DNA damage is repaired⁶⁵. MR oligomerization at DNA lesions could thereby amplify the checkpoint signal. Indeed, western blot analysis of Rad53 in wild-type cells showed the appearance of an upshifted band corresponding to phosphorylated Rad53 after HU-induced DNA damage (Fig. 4i). As expected, Rad53 phosphorylation was impaired in *mr^{lo}* and *rad50Δ* cells. In contrast, higher Rad53 phosphorylation levels were reached earlier in *mr^{ho}* cells compared to wild-type, consistent with hyperactivation of the Tel1-mediated checkpoint pathway. Fluorescence activated cell sorting (FACS) analysis of cell cycle stages in untreated conditions confirmed that in contrast to wild-type and *mr^{ho}*, *mr^{lo}* and *rad50Δ* cells arrest in the G2/M phase, implying that these cells cannot pass the G2/M DNA damage checkpoint as they fail to efficiently repair even spontaneous DNA damage (Supplementary Fig. 5e). Taken together, these data suggest that cooperative MR oligomerization at DNA damage sites *via* a conserved beta-sheet motif is required to assemble repair foci, trigger Tel1-dependent checkpoint activation and ensure efficient DNA repair *in vivo*.

354 Discussion

355 Here we show that *S. cerevisiae* MR dimers oligomerize with positive cooperativity to form
356 nucleolytically active assemblies at DNA ends. Using TEM, we visualized that MR activity is
357 required to resect blocked ends and orchestrate handover to Exo1 to execute processive long-range
358 resection. Assembly of these higher-order MR complexes is mediated by a conserved beta-sheet
359 motif in the head domain of Rad50. Biochemical reconstitutions coupled with TEM imaging
360 revealed that MR oligomerization is required for pSae2-stimulated endonucleolytic DNA cleavage
361 and hence short-range resection, but is dispensable for MR exonuclease activity. Mutational analysis
362 of the beta-sheet motif identified specific mutations that preserve MR dimerization but decrease or
363 enhance MR head domain oligomerization *in vitro*. Interestingly, analysis of these Rad50 mutants
364 *in vivo* confirmed the functional importance of MR head domain oligomerization, which likely
365 cooperates with Rad50 coiled-coil tethering to form the MR foci observed at DSBs *in vivo*^{33–36,38}.

366 Visualization of short- and long-range DNA end resection by TEM-based single molecule 367 analysis

368 In cells, DSBs are likely bound by the highly abundant NHEJ-factor Ku that is rapidly
369 recruited to DNA ends but must be removed by the MR-pSae2 complex to initiate repair *via*
370 HR^{13,19,21,29,66,67}. MR also recruits and activates the checkpoint-kinase Tel1 to delay cell cycle
371 progression until the DNA damage is repaired^{14,18–21,35,65}. To this end, MR complexes accumulate
372 at the break sites and process the DNA ends by short-range resection, thereby removing Ku and
373 generating the overhangs needed for strand invasion in HR. For the processing of clean breaks,
374 however, MR-mediated short-range resection is dispensable in yeast, as it can be catalyzed by the
375 Exo1 nuclease¹⁵. Using a defined, recombinant system we could visualize the different molecular
376 steps governing this resection process *in vitro*. Our biochemical assays and single molecule TEM
377 analysis recapitulated MR-pSae2-dependent short-range processing and subsequent tail elongation
378 by Exo1 on streptavidin-blocked DNA substrates. TEM imaging further revealed that loading and
379 clustering of MR dimers and MR-pSae2 complexes to higher-order assemblies can occur internally
380 of DNA, but resection is restricted to DNA ends. This suggests that the MR-pSae2 assemblies slide
381 along the substrate⁶⁸ until they accumulate at a DNA end and activate the MR endonuclease. The
382 resulting short-range processing of blocked ends is a prerequisite for subsequent handover to Exo1
383 mediating long-range resection. After cleavage, MR-pSae2 clusters dissociate from RPA-coated
384 single-stranded DNA ends to allow RPA-replacement by Rad51 and the coordination of strand
385 invasion together with other DNA repair factors.

386 **MR oligomerization promotes short-range resection**

387 Previous work showed an initial endonucleolytic cut by MRX-pSae2 15-35 nt away from
388 the DNA end when using oligonucleotide-based DNA²². On longer DNA templates, however,
389 additional MRX-pSae2 incisions were found that led to the resection of several hundred nucleotides
390 in length³¹, consistent with observations from meiotic cells lacking the long-range resection
391 pathways^{16,30}. Accordingly, a stepwise DNA cleavage model for 5'-DNA end resection by MRX-
392 Sae2 was proposed³¹. Here, our experiments revealed that MR dimers assemble *via* a protruding
393 beta-sheet motif into oligomers, which are required for short-range resection at DNA breaks. MR
394 oligomerization is especially pronounced on longer DNA molecules, which should have a stronger
395 disruptive polyelectrolyte effect on MR oligomers yet allow binding of more MR molecules *via*
396 positive cooperativity, ultimately stabilizing the MR oligomers on DNA. Surprisingly, a Rad50
397 mutant with defective oligomerization (MR^{lo}) showed a complete lack of endonuclease activity *in*
398 *vitro*, although its complex formation with co-expressed Mre11, interaction with pSae2, ATP
399 binding and exonuclease activity were unaffected. This defect is unlikely explained by reduced
400 DNA binding, as increased MR^{lo} concentrations restore DNA binding but not enzymatic activity.
401 Moreover, a Rad50 mutant with increased oligomerization behavior (MR^{ho}) was more active as an
402 endonuclease compared to wild-type MR, despite being also impaired in DNA binding affinity. We
403 thus propose that MR oligomerization on DNA might facilitate the drastic conformational changes
404 required for nuclease activation^{45-48,69-71}, and thereby promote the activation of adjacent MR
405 molecules on the 5'-terminated DNA strand, creating a “domino-like” effect close to the DNA ends
406 that explains step-wise short-range resection.

407 **Human RAD50 germline mutations cluster in the beta-sheet required for MR oligomerization**

408 Our functional analysis of yeast Rad50 revealed that a conserved beta-sheet drives
409 oligomerization *in vitro* and *in vivo*. Interestingly, database (ClinVar/MedGen) and literature
410 reports^{10,72} suggest that human individuals with a hereditary pre-disposition for BRCA1/2- and
411 PALB2-associated cancers carry especially germline point mutations of hydrophobic residues in the
412 identified RAD50 beta-sheet motif. The tumor suppressor BRCA1 interacts with PALB2 and
413 BRCA2, and localizes to repair foci at DNA lesions similarly to MR⁷³⁻⁷⁵. Loss of BRCA1/2 function
414 leads to aberrant degradation of nascent DNA by MRE11 upon replication stress, and a dramatic
415 increase in the lifetime risk for ovarian and breast cancer^{6,8,74,75}. The abrogation of MRE11 activity
416 in BRCA1/2-deficient cells, on the other hand, was linked to PARP inhibitor resistance in cancer
417 chemotherapy⁵. It is intriguing that MR^{lo}, bearing beta-sheet-disrupting alanine mutations of four
418 hydrophobic residues also altered in cancer pre-disposed individuals, shows impaired endonuclease
419 activity *in vitro*. These loss-of-function RAD50 mutations could restrict MRE11-mediated

420 degradation of nascent DNA at challenged replication forks in PARP-resistant, BRCA1/2-PALB2-
421 mutated cells, and explain tumor chemoresistance. However, the importance of these RAD50
422 mutations for cancer risk and survival is not yet well understood and more extensive studies are
423 needed.

424 **Oligomerization may promote phase transition to form MR foci at DNA breaks**

425 Interestingly, several lines of evidence suggest that the MR complex is not only required for
426 short-range processing, but may also tether broken DNA ends to help HR- or NHEJ-mediated
427 repair^{40,41,69,76–78}. Moreover, repair foci with hundreds to thousands of MR molecules were found at
428 DNA breaks in yeast and mammalian cells^{33–38}. ChIP-seq data of human MRN shows an
429 accumulation of the complex across a DSB on both strands of broken DNA, covering ~ 1 kb of
430 DNA with strongest MRN localization at the DNA ends, indicating MRN focus formation around a
431 DSB and tethering of broken ends⁷⁹. In accord, our TEM analysis revealed that MR clusters can
432 hold several DNA molecules together, mediated on the one hand by oligomerization of the MR head
433 domains as well as interactions *via* the zinc hooks. We thus propose that MR head domain
434 oligomerization together with DNA end tethering may explain the coordinated resection observed
435 at two-ended DNA breaks⁸⁰. Consistent with this interpretation, no nuclear foci were detected upon
436 DNA damage when analyzing the yEVENus-tagged oligomerization-defective MR^{lo} mutant in *S.*
437 *cerevisiae*, while the hyper-oligomerizing MR^{ho} mutant formed nuclear foci even in the absence of
438 exogenous DNA lesions.

439 It remains to be elucidated whether MR assemblies in solution and in foci at DNA breaks
440 possess liquid-like biophysical characteristics such as described e.g. for the NHEJ-factor 53BP1,
441 the strand-exchange protein Rad52, the ATR checkpoint kinase-activator TopBP1 or the telomere-
442 associated TRF2, which self-assemble to liquid droplets that form phase-separated foci at nucleation
443 sites^{81–84}. Thereby, cooperative interactions can facilitate droplet assembly and foci formation, as
444 seen for TopBP1 or the meiotic DSB-binding Rec114-Mei4-Mer2 complex^{81,85}. Furthermore, the
445 fact that the higher-order MR assemblies could be pelleted in high-speed centrifugation similarly to
446 droplets formed by the NHEJ- and HR-factor FUS may hint towards the formation of MR
447 droplets^{86,87}. MR oligomerization and tethering *via* the protruding Rad50 coiled-coils could thereby
448 lead to a flexible, dynamic mesh that could engulf a DNA damage site. MR droplet fusion could
449 also bring the ends of two broken DNA molecules together and ensure coordinated resection on
450 both ends⁸⁰. However, ThT staining detected large beta-sheet-based structures, which could indicate
451 a more stable rather than a liquid-like structure. It is imaginable that MR, as one of the first proteins
452 binding DSBs, would assemble to a rather stable core inside the repair foci that may act as a scaffold
453 for down-stream repair and signaling components including pSae2/pCtIP and Tel1/ATM, which

transiently join the repair center in a more liquid-like outer shell with exchange to the surrounding nucleoplasm. Consistent with this notion, the extent of MR oligomerization at DNA lesions does not only drive end resection, but could also trigger the DNA damage checkpoint. Although even a single DNA break can activate this signaling response⁸⁸, MR oligomerization and simultaneous activation of multiple Tel1/ATM molecules may contribute to amplify the checkpoint signal.

Methods

Preparation of recombinant proteins

Yeast Mre11-Rad50 complexes (wild-type and variants) were co-expressed in *S. frugiperda* 9 (*Sf9*) cells with an optimal ratio of baculoviruses producing wild-type Mre11-6xHis and untagged wild-type or mutant Rad50^{ho/lo} that was codon-optimized for *Sf9* expression to achieve 1:1 MR subunit stoichiometry. MR complexes were purified by NiNTA affinity chromatography and ion-exchange chromatography²⁶. MR^{lo} was expressed at considerably lower amounts compared to MR^{wt/ho}, indicating that MR oligomerization may stabilize the complex. Recombinant Mre11 was purified by Amylose and NiNTA affinity chromatography, while Xrs2 and Rad50 were purified with FLAG affinity resin³¹. Exo1 was purified by FLAG affinity chromatography followed by ion-exchange chromatography⁵⁷. Recombinant phosphorylated Sae2 (pSae2) was purified from *Sf9* cells by affinity chromatography in the presence of phosphatase inhibitors²⁸. Yeast RPA was expressed from the p11d-scRPA vector (kind gift from M. Wold, University of Iowa) in *E. coli* (BL21 DE3 pLysS) and purified as described for human RPA²⁵.

DNA substrates

Oligonucleotides used in this study are listed in Supplementary Table S1. To prepare the DNA substrates (20, 31, 70, 100 and 197 bp) for DNA binding and endonuclease experiments, the respective oligonucleotides were labeled at the 3'-end with [α -32P]dCTP (Perkin Elmer) and terminal transferase (New England BioLabs) following manufacturer's recommendations. The enzyme was inactivated, and the reaction purified on Micro Bio-Spin P-30 Gel Columns (Bio-Rad). The labeled oligonucleotide was then annealed with two-fold excess of the related unlabeled oligonucleotide in 1x PNK buffer (New England BioLabs). The 5'-labeled 70 bp substrate for exonuclease assays was prepared similarly, except that the PC210 oligonucleotide was labeled at the 5'-end with [γ -32P]ATP (PerkinElmer) and T4 polynucleotide kinase (New England BioLabs). Oligonucleotides for hot annealing (5'_OligoA and 3'_OligoA) were labeled at the 5'-end with [γ -32P]ATP (PerkinElmer) and T4 polynucleotide kinase (New England BioLabs) following manufacturer's recommendations. For mass photometry, oligonucleotides of 20, 50, 73, 106 and 197 bp (Supplementary Table S1) and 2.7 kbp-long pUC19 were used. For TEM, circular 5.3 kbp-

487 long pFBDM (Addgene; Fig. 1b-iii; Supplementary Fig. 1e), linear 2.7 kbp-long pUC19 with free
488 4 nt-overhangs or a 2.8 kbp-long, pUC19-derived linear plasmid with streptavidin-blocked ends
489 were used. For EMSAs and nuclease assays (Fig. 1c,d; Fig. 2a,b; Supplementary Fig. 1f;
490 Supplementary Fig. 3a,b; Supplementary Fig. 5b) circular, relaxed or linear 2.7 kbp-long pUC19
491 with free 4 nt-overhangs, and a 2.8 kbp-long, pUC19-derived blunt linear plasmid with streptavidin-
492 blocked ends were used. The circular, supercoiled form of pUC19 was relaxed with Topoisomerase
493 I from *E. coli* (New England Biolabs) and purified using a MinElute PCR purification kit (Qiagen).
494 Linearized pUC19 was prepared with restriction digestion using KpnI-HF (New England BioLabs)
495 and 0.8 % agarose-gel purification. The 2.8 kbp-long, pUC19-derived linear plasmid substrate with
496 biotin at the ends was prepared using pAttP-S vector, annealed PC210 and PC211 oligonucleotides
497 and Φ C31 integrase⁸⁹. After incubation with monovalent streptavidin (kind gift from M. Howarth,
498 University of Oxford), streptavidin-blocked 2.8 kbp-long pUC19 was purified with Chromaspin TE-
499 200 columns (Takara).

500 **Negative staining transmission electron microscopy (TEM)**

501 Recombinant, purified proteins were mixed at the indicated concentrations with or without plasmid
502 substrates to 5, 10 or 15 μ l reactions on ice in a reaction buffer containing 25 mM Tris acetate pH 7.5
503 and 1 mM dithiothreitol (DTT) as well as the co-factors 5 mM magnesium acetate (Sigma-Aldrich,
504 M5661), 1 mM manganese chloride (Sigma-Aldrich, M3634) and 1 mM ATP γ S (Sigma-Aldrich,
505 A1388), unless indicated otherwise. 1 mM ATP (Sigma-Aldrich, A2383) was used instead of
506 ATP γ S in Supplementary Fig. 1e-ii and for resection reactions in Fig. 2d. KCl in phosphate buffer
507 (storage buffer of MR; 20 mM phosphate solution pH 7.4 [KH₂PO₄, K₂HPO₄], 10 % glycerol, 1 mM
508 phenylmethylsulfonyl fluoride [PMSF], 1 mM DTT) was added to adjust salt concentrations as
509 indicated. Assessment of oligomer size in Fig. 1a, Fig. 3d and Supplementary Fig. 1b was performed
510 at 120 mM KCl. For MR binding to plasmid DNA, 30 mM KCl was used (Fig. 1b; Supplementary
511 Fig. 1e), unless stated otherwise (Fig. 1d-ii; Fig. 2d; Fig. 3h; Supplementary Fig. 1g). The reactions
512 were then incubated at 30 °C for 30 min, unless indicated otherwise (Fig. 1a; Fig. 2d; Supplementary
513 Fig. 1b; Supplementary Fig. 1d).

514 For Fig. 2d-i,I and Fig. 2d-ii,I, 5 nM 2.7 kbp-long, linear plasmid DNA (in molecules) was
515 incubated for 2.5 h with 75 nM RPA at 30 °C and 40 mM KCl. For Fig. 2d-i,II,III, 50 nM MR and
516 200 nM pSae2 were added and incubated for 2.5 h. For Fig. 2d-ii,II, 5 nM 2.7 kbp-long, linear
517 plasmid DNA (in molecules) was incubated with 50 nM Exo1 and 200 nM RPA for 5 min at 30 °C
518 and 50 mM KCl. For Fig. 2d-iii,I, 5 nM 2.8 kbp-long, linear plasmid DNA (in molecules) carrying
519 two streptavidin blocks per end was incubated with 50 nM Exo1 and 75 nM RPA for 2.5 h at 30 °C
520 and 40 mM KCl. For Fig. 2d-iii,II, 50 nM MR and 200 nM pSae2 were incubated with 5 nM of the

521 substrate (in molecules) for 2.5 h at 30 °C and 40 mM KCl. For Fig. 2d-iii,III, IV, 50 nM Exo1 and
522 RPA to a final concentration of 670 nM were added to the reaction with MR-pSae2 (Fig. 2d-iii,II)
523 for 10 min at 30 °C and 50 mM KCl.

524 For TEM grid preparation, 5 µl of the reaction mixture was incubated for 1 min at room
525 temperature on a carbon film 300 mesh copper grid (CF300-CU from Electron Microscopy
526 Sciences) that was negatively glow discharged with the Emitech K100X glow discharge system for
527 45 s with 25 mA. After blotting away the excess sample with Whatman filter paper, the grid was
528 washed twice in EM buffer (20 mM HEPES pH 7.55, 130 mM NaCl, 2 % glycerol, 0.5 mM DTT),
529 stained in two droplets of 2 % uranyl acetate and air dried. TEM micrographs were acquired with a
530 FEI Morgagni 268 microscope at 100 kV using a CCD 1376 × 1032 pixel camera at different
531 magnifications. All samples were measured at least 3 times, and image analysis was performed
532 using ImageJ and GraphPad Prism.

533 **Pelleting assay**

534 60 µl reactions of 1.2 µM MR^{wt/ho} were incubated for 30 min at 4 °C or 42 °C in phosphate buffer
535 containing 20 mM phosphate solution pH 7.4 (KH₂PO₄, K₂HPO₄), 10 % glycerol, 1 mM PMSF,
536 1 mM DTT and 240 mM KCl. After incubation, the reactions were centrifuged at 21'000 g for
537 10 min at 4 °C. The resulting pellets were washed twice with phosphate buffer, resuspended in urea
538 sample buffer (116 mM Tris-HCl pH 6.8, 5 % glycerol, 8 M urea, 5 % SDS, 1 % beta-mercapto-
539 ethanol, 0.1 % bromophenol blue) and loaded likewise to the supernatant fractions onto a denaturing
540 4-12 % BisTris SDS-PAGE (ThermoFisher Scientific) that was subsequently stained with
541 Coomassie. MR^{wt/ho} bands were normalized in intensity to their input, statistical analysis was
542 performed using GraphPad Prism.

543 **ThT fluorescence**

544 20 µl reactions of 1.2 µM MR^{wt/ho} were incubated for 30 min at 4 °C or 42 °C in phosphate buffer
545 as described for the pelleting assay. Thioflavin T (ThT, Sigma-Aldrich, T3516) was dissolved in
546 water to a final concentration of 2.5 mM and filtered (0.2 µm, Millipore). ThT solution was then
547 added (1:10 dilution) to the samples, and fluorescence intensity was measured in a 384-well plate
548 (Corning Life Sciences) using a CLARIOstar plate reader (BMG Labtech). Excitation wavelength
549 was set at 450 nm, and emission was recorded at 490 nm. Statistical analysis was performed using
550 GraphPad Prism.

551 **Nuclease assays**

552 Endonuclease assays (15 µl volume, unless indicated otherwise) were performed with 1 nM (in
553 molecules) 70 bp-long DNA substrate labeled at the 3'-end in reaction buffer containing 25 mM Tris

554 acetate pH 7.5, 1 mM DTT, 80 U/ml pyruvate kinase (Sigma-Aldrich), 1 mM phosphoenolpyruvate
555 (PEP), 0.25 mg/ml bovine serum albumin (BSA) as well as the co-factors 5 mM magnesium acetate,
556 1 mM manganese acetate and 1 mM ATP. 30 nM monovalent streptavidin was added and incubated
557 for 5 min at room temperature. The indicated proteins were added and incubated for 30 min at 30 °C.
558 After incubation, the reaction was stopped with 0.5 µl of 14–22 mg/ml proteinase K (Roche), 0.5 µl
559 of 10 % (w/v) sodium dodecyl sulfate (SDS), and 0.5 µl of 0.5 M ethylenediaminetetraacetic acid
560 (EDTA) and incubated at 50 °C for 30 min. For time-course experiments, proteins were added to a
561 single master mix and incubated at 30 °C. 15 µl aliquots of the reaction were collected at the
562 indicated time and stopped as described above. Each sample was mixed with equal volume of
563 loading dye (95 % formamide, 20 mM EDTA, 1 mg/ml bromophenol blue) and boiled for 4 min at
564 95 °C. The products were separated by denaturing polyacrylamide electrophoresis using a 15 %
565 polyacrylamide gel with 7 M urea. The gels were fixed for 30 min in fixing solution (40 % methanol,
566 10 % acetic acid, 5 % glycerol) and then dried on 3MM paper (Whatman), exposed to phosphor
567 screen and imaged with a Typhoon imager (GE Healthcare). The resulting images were analyzed
568 with ImageJ and GraphPad Prism.

569 Exonuclease assays were performed with 1 nM (in molecules) 70 bp-long DNA substrate
570 labeled at the 5'-end in reaction buffer containing 25 mM Tris acetate pH 7.5, 1 mM DTT,
571 0.25 mg/ml BSA as well as the co-factors 5 mM manganese acetate and 1 mM ATP or ATP γ S where
572 indicated. After protein addition, the reactions were incubated for 30 min at 30 °C, and subsequently
573 stopped and processed as described above for the endonuclease assays.

574 Nuclease assays on plasmid-length substrate were performed with 1 nM (in molecules)
575 2.8 kbp-long, pUC19-derived substrate obtained with Φ C31 integrase (as described in the DNA
576 substrates section) in reaction buffer as used for endonuclease assays containing 25 mM Tris acetate
577 pH 7.5, 1 mM DTT, 80 U/ml pyruvate kinase (Sigma-Aldrich), 1 mM PEP, 0.25 mg/ml BSA as
578 well as the co-factors 5 mM magnesium acetate, 1 mM manganese acetate and 1 mM ATP. The
579 substrate was incubated, where indicated, for 5 min at room temperature with 30 nM monovalent
580 streptavidin. The indicated proteins were added and incubated at 30 °C. For Fig. 1d-i, the salt
581 concentration was adjusted with KCl as indicated to match the TEM imaging conditions. At
582 different time points (0, 15 and 30 min), 14 µl of the reaction was collected and transferred into
583 tubes containing 1 µl of Exo1 or equal amount of storage buffer and further incubated for 10 min at
584 30 °C. The samples without MR-pSae2 were collected from the master mix before the addition of
585 MR and pSae2 and processed with or without Exo1 as described above. After the additional 10 min,
586 the reactions were stopped by the addition of 5 µl of 2 % STOP solution (30 mM EDTA, 2 % SDS,
587 30 % glycerol and 1 mg/ml bromophenol blue) and 1 µl Proteinase K (14-22 mg/ml). The reactions
588 were deproteinated at 37 °C for 1 h, then 15 µl of each sample was mixed with a three-fold excess

of the indicated 22 nt-long radio-labeled probe compared to the plasmid-length substrate (in molecules). For Fig. 1d, Fig. 2a,b and Supplementary Fig. 5b-i, ii, the radio-labeled probe anneals 160 nt away from the 5'-DNA end where the 5'-terminated strand was resected. For Supplementary Fig. 3b and Supplementary Fig. 5b-iii, the radio-labeled probe anneals 160 nt away from the 3'-DNA end where the complementary strand was resected. The mix was incubated for 3 min at 60 °C in a water bath and subsequently cooled down overnight to room temperature. The annealing reactions were loaded on a 1 % agarose gel. The gels were then dried on DE81 chromatography paper (Whatman) and processed as described above.

Electrophoretic mobility shift assays (EMSAs)

DNA binding experiments with oligonucleotide-based DNA (Fig. 1e; Fig. 3g; Supplementary Fig. 2a-d; Supplementary Fig. 4e) were carried out in buffer containing 25 mM Tris acetate pH 7.5, 1 mM DTT and 0.25 mg/ml BSA with co-factors 5 mM magnesium acetate and 1 mM ATP γ S. To keep the total DNA amount constant, 5 nM 20 bp, 3.22 nM 31 bp, 1.43 nM 70 bp, 1 nM 100 bp and 0.51 nM 197 bp DNA were used, such that 100 nM (in base pairs) of 3'-labeled DNA substrate was incubated with the indicated amount of protein at 30 °C for 15 min in 15 μ l reaction volume at 30 mM NaCl. For Supplementary Fig. 2b-d, the indicated final salt concentrations were reached by NaCl addition. After incubation, the samples were supplemented with 5 μ l of EMSA loading dye (50 % glycerol with bromophenol blue) and loaded on a 6 % polyacrylamide gel (TAE). The gel was run for 90 min at 80 V (2.7 V/cm) on ice (75 min for the 20 bp substrate) in a Mini-PROTEAN® Tetra Cell system (BioRad). After separation, the gels were dried and exposed to a phosphor screen. The screen was then imaged with a Typhoon imager (GE Healthcare), quantitated with ImageJ and statistically analyzed with GraphPad Prism.

For DNA binding experiments with plasmid-based DNA in Fig. 1c and Supplementary Fig. 1f, 2.5 nM (in molecules) circular 2.7 kbp-long Topoisomerase I-relaxed or KpnI-linearized pUC19 plasmid was used, as well as 2.5 nM (in molecules) streptavidin-blocked pUC19-derived 2.8 kbp substrate. Reactions of 20 μ l were mixed in buffer containing 25 mM Tris acetate pH 7.5, 1 mM DTT and 80 mM KCl, as well as the co-factors 5 mM magnesium acetate, 1 mM manganese chloride and 1 mM ATP γ S. After incubation for 1 h at 30 °C, the reactions were mixed with TriTrack DNA loading dye 6x (Thermo Scientific™) to 1x and loaded on a 0.5 % agarose gel (TB). The gel was run for 1 h at 140 V (11.7 V/cm) on ice. After running, the gels were stained with GelRed Nucleic Acid Gel Stain (Biotium), imaged with a Typhoon imager (GE Healthcare) and quantified using ImageJ and GraphPad Prism.

621 Mass photometry

622 Mass photometry measurements were carried out on a OneMP device (Refeyn Ltd). Glass coverslips
623 (No. 1.5 H thickness, 24 x 50 mm, VWR) were cleaned by sonication, first in isopropanol and then
624 in deionized water for 15 min. After cleaning, the coverslips were dried under a clean stream of
625 nitrogen. For measurements with the 2.7 kbp-long pUC19 plasmid, coverslips were coated with a
626 1 % poly-lysine solution. For each measurement, a clean coverslip was placed onto the objective.
627 For sample delivery, a silicone gasket (CultureWell™ Reusable Gasket, Grace Bio-Labs) with 4
628 wells was fixed on the surface of the coverslip. Before measurements, samples were incubated in
629 buffer containing 25 mM Tris acetate pH 7.5 and 1 mM DTT with co-factors 0.1 mM ATPγS, 5 mM
630 magnesium acetate and 1 mM manganese chloride at 30 °C for 30 min.

631 Mass photometry experiments with oligonucleotides in Fig. 1f and Fig. 3e were performed
632 at 25 mM KCl with 5 nM MR^{wt/ho} and 27.7 nM 20 bp, 9.8 nM 50 bp, 6.7 nM 73 bp, 4.6 nM 106 bp
633 and 2.5 nM 197 bp DNA to keep the total DNA amount constant (100 nM base pairs). In Fig. 1f,
634 free MR dimer was observed at 452 ± 40 kDa ($\pm$ SD) (- DNA, theoretically 460 kDa). Peaks of one
635 (452 ± 34 kDa to 550 ± 65 kDa) or two MR dimers (1035 ± 80 kDa and 1050 ± 160 kDa, zoom
636 insets for 106 bp and 197 bp) bound to a DNA molecule were detected and fitted where possible. In
637 Fig. 3e, a peak was observed for free MR dimer at 460 ± 18 kDa (- DNA). Moreover, peaks
638 corresponding to one (458 ± 50 kDa to 540 ± 80 kDa) or two MR dimers (1000 ± 80 kDa to $1050 \pm$
639 75 kDa, zoom insets 73-197 bp) bound to a DNA molecule were detected and fitted with Gaussian
640 functions where possible. For Supplementary Fig. 2e and Supplementary Fig. 4b, 5 nM Mre11,
641 Rad50 and MR^{wt/ho/lo} were measured with or without 2.5 nM 197 bp-long oligonucleotide DNA at
642 25 mM KCl. In Supplementary Fig. 2e, a peak was observed for pure Mre11 at 75 ± 15 kDa
643 (theoretically 78 kDa). For pure Rad50, a monomer peak at 150 ± 45 kDa (theoretically 153 kDa)
644 and a dimer peak at 306 ± 36 kDa (theoretically 305 kDa) were detected. In the MR^{wt} sample, free
645 Mre11 was observed at 82 ± 16 kDa in addition to the MR dimer peak, as well as free monomeric
646 Rad50 at 130 ± 20 kDa and dimeric Rad50 at 305 ± 45 kDa. The MR^{wt} complex peak without DNA
647 bound was detected at 452 ± 40 kDa (theoretically 460 kDa) and the DNA-bound MR^{wt} fraction at
648 550 ± 65 kDa. In Supplementary Fig. 4b, Mre11 peaks were distinguishable at 80 ± 15 kDa and
649 62 ± 19 kDa in addition to the MR^{wt/ho} dimer peak, respectively. Also monomeric and dimeric Rad50
650 peaks were observable for the MR^{wt/ho} samples at 130 ± 20 kDa and 305 ± 45 kDa, and 134 ± 27 kDa
651 and 291 ± 25 kDa, respectively. MR^{wt/ho/lo} dimer peaks without DNA bound were at 452 ± 40 kDa,
652 460 ± 18 kDa and 438 ± 60 kDa, respectively, while the DNA-bound fractions were detected at
653 550 ± 65 kDa for MR^{wt} and 530 ± 80 kDa for MR^{ho}. For mass photometry experiments in
654 Supplementary Fig. 4d without DNA, 10 nM MR^{wt/ho} was incubated at 250 mM KCl or 25 mM KCl
655 and 30 °C for 30 min with co-factors as afore-mentioned. Binding was monitored for 40 s.

656 For Supplementary Fig. 2f and Supplementary Fig. 4f with 2.7 kbp plasmid DNA, 20 nM
657 MR^{wt/ho} was first incubated with 1 nM substrate (in molecules) for 30 min at 250 mM KCl and 30 °C
658 and subsequently measured (labeled as 250 mM KCl, 30 min). MR^{wt} (~ 460 kDa) and plasmid DNA
659 (~ 1300 kDa) appeared mostly unbound, while MR^{ho} formed oligomers without DNA beyond the
660 detection limit such that less free MR^{ho} dimers could be detected at ~ 460 kDa. Secondly, the salt in
661 sample was diluted to 25 mM KCl, while keeping the protein and DNA concentrations constant,
662 and the sample was immediately analyzed (labeled 25 mM KCl). Much less unbound MR^{wt/ho} and
663 plasmid DNA could be detected. For MR^{wt}, a tail of counts at high molecular weight > 2500 kDa,
664 corresponding to higher-order MR-DNA assemblies, could be detected. MR^{ho}, on the other hand,
665 formed much larger nucleoprotein assemblies beyond the detection limit (smaller tail of counts
666 > 2500 kDa). Third, the sample with 25 mM KCl was incubated for 30 min at 30 °C and then
667 measured (labeled 25 mM KCl, 30 min). For both MR^{wt} and MR^{ho}, even less higher-order
668 assemblies on DNA could be detected > 2500 kDa, as they surpassed the detection limit. Binding
669 was monitored for 100 s.

670 For data acquisition, the gasket well was filled with 9 µl measuring buffer for adjustment of
671 surface focusing. Afterwards, 1 µl of sample was added to the buffer in the well resulting in the final
672 sample concentration. Protein and complex binding to the coverslip surface was monitored using
673 AcquireMP (Refeyn Ltd, Version 2.3.0). Data analysis was performed by DiscoverMP (Refeyn Ltd,
674 version 2.3.0), OriginPro 2017 and a custom-written Python program. For contrast to mass
675 conversion, a known protein mass standard calibrant (NativeMarkTM Unstained Protein Standard,
676 Invitrogen) was measured on the same day. Due to calibration with respect to protein mass, the
677 pUC19 peak in plasmid measurements appeared at ~ 1300 kDa. All samples were measured at least
678 3 times.

679 **pSae2-Rad50 protein interaction assay**

680 50 µl of soluble extract from *Sf9* cells expressing the C-terminal part of pSae2 (pSae2 ΔN169) with
681 MBP-tag²⁸, prepared with phosphatase inhibitors, was incubated with 50 µl Amylose resin (New
682 England Biolabs) in 450 µl lysis buffer (50 mM Tris-HCl pH 7.5, 2 mM beta-mercaptoethanol,
683 1 mM EDTA, 15.6 % glycerol, 305 mM NaCl, 30 µg/ml leupeptin, 1 mM PMSF and protease
684 inhibitor cocktail [1:400, Sigma-Aldrich, P8340]) for 1 h at 4 °C with agitation. The resin was then
685 centrifuged at 2'000 g for 2 min at 4 °C and washed 5 times with wash buffer containing 50 mM
686 Tris-HCl pH 7.5, 150 mM NaCl, 0.2 % NP40, 2 mM EDTA and 1 mM PMSF in a total volume of
687 1.3 ml. After the addition of 1.5 µg purified MR^{wt/ho/lo} and 250 µl wash buffer, the mix was further
688 incubated for 1 h at 4 °C with agitation. MR^{wt/ho/lo} controls were also incubated with amylose resin
689 without pSae2 ΔN169. The resin was then centrifuged and washed 5 times as described above.

690 MBP-tagged pSae2 ΔN169 bound to pulled-down MR^{wt/ho/lo} via Rad50 interactions was eluted with
 691 100 μl wash buffer containing 20 mM maltose (Carl Roth). 20 μl of the eluate was then loaded with
 692 1x NuPAGE™ LDS Sample buffer on a denaturing 4-12 % Bis-Tris SDS-PAGE (both
 693 ThermoFisher Scientific). Western blots were performed with monoclonal mouse anti-MBP
 694 antibody (1:1'000, Abcam, ab49923), rabbit polyclonal anti-Rad50 antibody (1:1'000,
 695 ThermoScientific, PA5-32176) and goat anti-rabbit or anti-mouse HRP-conjugated secondary
 696 antibody (1:2'000, BioRad, 170-6515 or 170-6516, respectively).

697 **Yeast plasmids and strains construction, media and growth conditions**

698 Construction of yeast plasmids and strains was performed according to standard molecular biology
 699 methods. Plasmids used in this study for yeast strain construction are listed in Supplementary Table
 700 S2. Yeast strains used in this study are derivatives of BY4741 and are listed in Supplementary Table
 701 S3. Cells were grown at 30 °C in yeast extract pentose dextrose, YPD (2 % glucose, 2 % peptone,
 702 1 % yeast extract) or synthetic SD media (2 % glucose, 0.17 % yeast nitrogen base, 0.5 % NH₄
 703 sulfate and amino acids).

704 **DNA damage sensitivity spotting and growth assays**

705 Serial dilutions of exponentially growing cells were spotted on YPD agar plates without or with
 706 DNA damaging agents: Camptothecin, CPT 25 μM (Sigma-Aldrich, C9911) and Hydroxyurea, HU
 707 25 mM (Sigma-Aldrich, H8627). Agar plates were incubated at 30 °C and imaged after 2 days. For
 708 growth rates, OD₆₀₀ of exponentially growing cells was measured in absence or presence of CPT
 709 (5 μM or 25 μM) in a 24-well plate using a plate reader (CLARIOStar BMG Labtech). Doubling
 710 times for exponential growth were calculated using the following formula:

$$711 \quad \text{Doubling time} = \frac{\text{Duration} \times \log(2)}{\log(\text{Final concentration}) - \log(\text{Initial concentration})}$$

714 Results from independent replicates (n = 3) were averaged and displayed using GraphPad Prism.

715 **Rad50 and Rad53 Western blots**

716 Total proteins extracts were obtained upon lysis with 1.85 M NaOH and 7.4 % beta-mercaptoethanol
 717 and precipitation with 50 % trichloroacetic acid (TCA) according to standard procedures. Proteins
 718 extracts were resuspended in NuPAGE™ LDS sample buffer (ThermoFisher Scientific) and loaded
 719 on a 7.5 % acrylamide gel. The following antibodies were used for immunoblotting: mouse
 720 monoclonal anti-Rad53 antibody (1:100, clone 11G3G6, kind gift of S. Gasser, Friedrich Miescher
 721 Institute for Biomedical Research⁹⁰), rabbit polyclonal anti-Rad50 antibody (1:1'000,

722 ThermoScientific, PA5-32176), mouse monoclonal anti-Tubulin antibody (1: 20'000, Sigma-
723 Aldrich T5168) and goat anti-rabbit or anti-mouse HRP-conjugated secondary antibody (1:2'000,
724 BioRad, 170-6515 or 170-6516, respectively).

725 **Live-cell fluorescence microscopy and image analysis**

726 Yeast cells were grown overnight in SD media and then diluted into fresh growth medium to resume
727 exponential phase growth. Before imaging, cells were left untreated or treated with 200 μ M CPT
728 for 2 h. Live-cell microscopy was performed using a spinning disk confocal fluorescence
729 microscope (Visitron system) equipped with an inverted microscope (Nikon Eclipse TiE), a EM-
730 CCD camera (Andor iXon Ultra, Andor), a motorized XY stage and a piezo Z drive. The system is
731 controlled by VisiVIEW software (Visitron system). Cells in 96-well plates were imaged at 30 °C
732 in a temperature-controlled incubator with a 100x objective lens (N.A.=1.4, Nikon CFI Plan Apo).
733 For image analysis, the percentage of cells containing Rad50 foci in the nucleus, based on histone
734 Hta2-mCherry signal, was quantified using the Cell Counter plugin in ImageJ software. Results of
735 independent biological replicates (n = 3) were averaged and displayed with GraphPad Prism.
736 Furthermore, automated single cell image analysis was performed using YeastQuant software⁹¹ on
737 raw images running in Matlab. Cells were segmented based on brightfield channel images and nuclei
738 were segmented based on Hta2-mCherry. Relocalization and foci formation of Rad50-yEVENUS
739 were quantified as normalized standard deviation of yEVENUS average intensity signal. At least 50
740 cells per condition were analyzed and quantified.

741 **Fluorescence activated cell sorting (FACS)**

742 Exponentially growing cells were fixed in 70 % ethanol. After a wash in 50 mM Tris-HCl pH 7.9,
743 cells were resuspended in the same buffer containing 10 μ g/ μ l RNase A (Sigma-Aldrich,
744 10109169001) and incubated at 37 °C over night. After centrifugation, pellets were resuspended in
745 55 mM HCl containing 5 mg/ml pepsin (Applichem, A4289) and incubated at 37 °C for 30 min.
746 Pellets were washed in 200 mM Tris-HCl pH 7.5, 211 mM NaCl and 78 mM MgCl₂, and
747 resuspended in the same buffer containing 0.1 mg/ml propidium iodide (Sigma-Aldrich, 81845).
748 After brief sonication (10-12 sec at medium intensity, Diagenode Bioruptor Standard), the cell cycle
749 stages of cells were analyzed using a FACS Canto (BD Biosciences) and FlowJo software.

750 **Statistical analysis**

751 All data shown in this study are representative of at least three independent experiments if not stated
752 otherwise in the figure legends. See respective figure legends and methods sections for details to
753 plotted data points, error bars and specific data analyses. Statistical unpaired, two-tailed significance
754 t-tests were performed using GraphPad Prism. For Fig. 1e, the t-value was 12.63 with 5 degrees of

freedom. For Fig. 3c, the t-value was 22.51 with 6 degrees of freedom. For Fig. 3f, the t-values were 3.37 (Pellet) and 3.05 (Sup.), each with 4 degrees of freedom. For Fig. 4a, the t-values were 2.95 (12.5 nM), 4.18 (25 nM) and 6.04 (50 nM), each with 4 degrees of freedom. For Fig. 4h, the t-value was 3.03 with 4 degrees of freedom. For the non-significant t-test in Supplementary Fig. 1c, the t-value was 1.47 with 198 degrees of freedom. For Supplementary Fig. 3a, the t-values were 4.28 (**), 6.6 (***) and 22.86 (****) with 6 degrees of freedom each.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data supporting this study are included in the article, its Supplementary materials or are available from the corresponding authors upon reasonable request. Source data are provided with this paper including uncropped images of gels, TEM images and blots.

Code availability

Custom code used in this study is available from the authors upon reasonable request.

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985 **Author information**

986 **Contributions**

987 V.M.K designed the MR mutants, purified proteins, developed the *in vitro* pathway
988 reconstitution technique in negative staining TEM, prepared grids, performed transmission electron
989 microscopy and executed biochemical and statistical analyses. G.R. purified proteins, performed
990 radioactive DNA binding and activity assays, and analyzed the results. E.B. designed the yeast
991 experiments, engineered yeast strains, performed the FACS analysis, carried out the quantitative
992 live-cell imaging analysis together with S.S.L., conducted immunoblotting and growth
993 measurements with the help of J.T., and analyzed the data. J.T. engineered the MR mutant yeast
994 strains, and performed spotting assays and data analysis. K.K. and R.S. are responsible for the mass
995 photometry analysis, G.C. performed the ThT fluorescence measurements and pelleting assays, and
996 R.G. helped with the structural analysis of the MR mutants. M.P. and P.C. designed the study, and
997 V.M.K., P.C. and M.P. wrote the paper with critical input from all authors.

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1001 **Ethics declaration**

1002 **Competing interests**

1003 The authors declare no competing interests.

Fig. 1: MR head domain interactions promote positive cooperative binding and MR oligomerization on DNA.

Fig. 1: MR head domain interactions promote positive cooperative binding and MR oligomerization on DNA.

a TEM of recombinant *S. cerevisiae* Mre11-Rad50 dimers (M_2R_2) and higher-order complexes (M_2R_2)_n with open (**i, ii**) or closed (**iii**) Rad50 coiled-coils. MR dimers oligomerize *via* head domains (arrows in **ii, iii**; grey cartoon lines in **iii, iv**) and can further interact through coiled-coil tethering (arrows in **iv**). **v**: At 30 °C with co-factors ATP γ S, Mg²⁺ and Mn²⁺, M_2R_2 forms even larger oligomers *via* head domains. n = 3. Scale bar: 100 nm.

b TEM of MR binding to plasmid DNA at 30 mM KCl and conditions as in Fig. 1a-v. **i**: M_2R_2 bound to DNA end (**Top**) or forming DNA loops (**Bottom**). **ii, iii**: MR oligomers dissociate into dimers polymerizing on some DNA stretches, but not others, irrespective of DNA ends. n = 3. Scale bar: 100 nm.

c Representative EMSA of MR binding to linear plasmid DNA at 80 mM KCl (**i**), and quantification (**ii**, Supplementary Fig. 1f). Mean $\pm$ SEM, n = 3, sigmoidal fit.

d **i**: MR oligomers are non-aberrant entities able to catalyze resection with pSae2 and ATP at 80 mM KCl. Resection detection with probe (green box in cartoon) binding to the 3'-overhang produced. n = 2. **ii**: TEM of **i** without pSae2 but with ATP γ S to stabilize MR on DNA. n = 3. Scale bar: 100 nm.

e Quantification of EMSAs as in Supplementary Fig. 2a with oligonucleotides of different lengths (100 nM base pairs). K_D as MR concentration at 50 % DNA binding $\pm$ SEM of n_{20, 31 bp} = 4, n_{70, 100, 197 bp} = 3, unpaired two-tailed t-test.

f Molecular mass distributions from mass photometry show cooperative binding of MR^{wt} to oligonucleotides of different lengths (100 nM base pairs). Cartoons illustrate the respective species in the peaks. Measured molecular weight (MW, kDa) $\pm$ SD given in grey. Zoom insets show MW range where a peak of two MR dimers bound to one DNA molecule would be expected and was fitted where possible. n = 3.

g Model illustrating that in presence of DNA, MR oligomers dissociate to dimers which polymerize with positive cooperativity at DNA ends or internally *via* head domain interactions, forming large MR clusters and DNA loops. DNA compaction suggests additional coiled-coil tethering.

Fig. 2: MR oligomerization on DNA is the first step in short-range and handover to long-range end resection.

1033 **Fig. 2: MR oligomerization on DNA is the first step in short-range and handover to long-range**
1034 **end resection.**

1035 **a** Nuclease assay recapitulating short- and long-range end resection of MR^{wt} on linear plasmid
1036 DNA with free ends and ATP. After incubation with MR-pSae2, Exo1 was added for 10 min where
1037 indicated (+). Resection detection with probe (green box see cartoons) binding to the 3'-overhang
1038 produced. n = 4.

1039 **b** As **a**, but with linear plasmid DNA with streptavidin-blocked ends. n = 4.

1040 **c** Nuclease assay demonstrating stepwise resection by MR-pSae2 (small black arrows) on the
1041 5'-terminated DNA strand with ATP. The red star marks the radio-label position. n = 2.

1042 **d** Representative collection of TEM images showing short- and long-range end resection by
1043 MR^{wt} on linear plasmid DNA with free (**i**, **ii**) and streptavidin-blocked ends (**iii**) under conditions
1044 comparable to **a** and **b** in the presence of ATP, n = 3. Scale bars: 100 nm. The legend shows cartoons,
1045 indicative arrows and corresponding micrographs of the reaction components. **i**, **I**: Linear plasmid
1046 substrate shows no significant stretches of RPA-coated, single-stranded DNA in absence of MR-
1047 pSae2 or Exo1. **ii**, **II**: Upon addition of MR and pSae2, large clusters of pSae2-MR are formed on
1048 DNA, in agreement with cooperative binding. **iii**, **I**: After 2.5 h, resected RPA-coated, single-stranded
1049 DNA overhangs are visible with pSae2-MR still bound, as well as not yet resected double-stranded
1050 DNA. **IV**: pSae2-MR complexes dissociate from DNA and form their respective oligomers. **ii**, **I**: as
1051 in **i**, **I**. **ii**, **II**: Fully resected and RPA-coated linear plasmid substrates are visible after 5 min
1052 incubation with Exo1, with Exo1 molecules still bound. **iii**, **I**: No resection of Exo1 can be detected
1053 on linear plasmid DNA with two streptavidin blocks per end. **II**: After incubation for 2.5 h with MR
1054 and pSae2, pSae2-MR clusters are formed that can harbor several streptavidin-blocked DNA
1055 substrates, and short-range resection is observed on one DNA end. **III**, **IV**: Fully resected plasmids
1056 are produced by Exo1 within 10 min after MR-pSae2 resection, and free MR oligomers with
1057 protruding Rad50 coiled-coils, pSae2 oligomers and Exo1 can be detected.

Fig. 3: Conserved beta-sheet motif in Rad50 head domain mediates MR oligomerization and cooperative DNA binding.

Fig. 3: Conserved beta-sheet motif in Rad50 head domain mediates MR oligomerization and cooperative DNA binding.

a Crystal structure of eukaryotic *C. thermophilum* (*Ct*) Rad50 dimers on oligonucleotide DNA (PDB: 5DAC^{50,60}), indicating that adjacent Rad50 dimers could interact *via* a small beta-sheet (β) and its connecting loop (L) protruding from the Rad50 head domain. Zoom inset shows red and blue residues mutated in the *S. cerevisiae* MR^{ho/lo} mutants. Repulsive E125 residues of adjacent *Ct* Rad50 dimers are spaced by 4.8 Å within the crystal but could come closer in solution.

b Sequence alignment of the *C. thermophilum*, *S. cerevisiae* and *H. sapiens* Rad50 beta-sheet motif. Color code as in **a**. Germline point mutations listed on ClinVar/MedGen for humans with cancer pre-disposition are indicated (*). Cartoons show multiple point mutations introduced in *S. cerevisiae* Rad50 to disrupt electrostatic (Rad50^{ho} mutant) or hydrophobic (Rad50^{lo}) interactions.

c Thioflavin T (ThT) fluorescence measurement of MR^{wt} at 240 mM KCl. Detected signal indicates formation of beta-sheet rich, higher-order structures. Mean $\pm$ SEM, n = 4, unpaired two-tailed t-test.

d **i:** Representative TEM of *S. cerevisiae* MR^{wt/ho/lo} as in Fig. 1a-v. **ii:** Quantification of mean head domain diameters $\pm$ SEM of MR^{wt/ho/lo} oligomers from **i** along their longest axis. n_{MR-wt} = 22 molecules, n_{MR-ho} = 72 and n_{MR-lo} = 46. Grey area indicates diameter range of ~ 8-14 nm measured for MR^{wt} dimers. Scale bar: 100 nm.

e Molecular mass distributions from mass photometry show cooperative binding of MR^{ho} to oligonucleotides of different lengths (100 nM base pairs). Cartoons illustrate the respective species in the peaks. Measured molecular weight (MW, kDa) $\pm$ SD given in grey. Zoom insets show MW range where a peak of two MR dimers bound to one DNA molecule would be expected and was fitted where possible. n = 3.

f Quantification of MR^{wt/ho} pelleting assays at 240 mM KCl (see Supplementary Fig. 4c). MR^{ho} forms ~ 1.5-fold more higher-order oligomers than MR^{wt} at higher temperature (Pellet, 42 °C). Mean $\pm$ SEM, n = 3, unpaired two-tailed t-tests.

g Quantification of EMSAs as in Supplementary Fig. 2a and 4e of MR^{wt/ho/lo} binding to 100 bp-long DNA with Mg²⁺ and ATP γ S. Mean $\pm$ SEM, n = 3, sigmoidal fit.

h TEM of MR^{ho/lo} binding to linear plasmid DNA. n = 3. **Top:** MR^{lo} binds dispersedly on DNA at 35 mM KCl without start of polymerization by two MR dimers compared to MR^{wt} (Fig. 1b-ii, iii). **Bottom, i:** Many MR^{ho} dimers condense DNA to a “worm”-like structure at 50 mM KCl. **ii:** At conditions as in **Top** panel, MR^{ho} forms more condensed, “worm”-like structures compared to MR^{wt} (Fig. 1b-ii, iii). Scale bar: 100 nm.

Fig. 4: MR oligomerization modulates endonuclease activity, foci formation and response to DNA damage.

1091 **Fig. 4: MR oligomerization modulates endonuclease activity, foci formation and response to**
1092 **DNA damage.**

- 1093 **a** Representative endonuclease assays of MR^{wt/ho/lo} with pSae2 and ATP (**i**) and quantification
1094 (**ii**). Mean ± SEM, n = 3, unpaired two-tailed t-tests. The red star marks the radio-label position.
- 1095 **b, c** Representative assay showing endonuclease activity kinetics of MR^{wt/ho} with pSae2 and ATP
1096 (**b**), and quantification (**c**). Mean ± SEM, n = 3. The red star marks the radio-label position.
- 1097 **d** Pulldown of MR^{wt/ho/lo} via Rad50 interactions with the C-terminal part of pSae2 (pSae2
1098 ΔN169). Ponceau and anti-MBP western blot indicate elution of MBP-tagged pSae2 ΔN169. Anti-
1099 Rad50 western blot shows interaction of pSae2 ΔN169 with Rad50 in MR^{wt/ho/lo}. n = 2.
- 1100 **e** Serial dilutions of exponentially growing *S. cerevisiae rad50Δ* cells expressing Rad50^{wt/ho/lo}-
1101 yEVENUS spotted on agar plates without (YPD) and with DNA damage-inducing camptothecin
1102 (CPT) or hydroxyurea (HU), incubated at 30 °C and imaged after 2 days. n = 3.
- 1103 **f** Quantification of doubling times of exponentially growing *rad50Δ* cells and *rad50Δ* strains
1104 expressing Rad50^{wt/ho/lo}-yEVENUS with and without CPT treatment to induce DSBs. Mean and
1105 minimum to maximum values are shown, n = 3.
- 1106 **g** Anti-Rad50 western blot showing expression levels of Rad50^{wt/ho/lo}-yEVENUS in *rad50Δ* cells
1107 before and after CPT treatment for 1 h. α-Tubulin as loading control. n = 3.
- 1108 **h** Representative live-cell imaging of *S. cerevisiae rad50Δ* cells expressing Rad50^{wt/ho/lo}-
1109 yEVENUS with histone Hta2-mCherry as nuclear marker (**i**), and quantification (**ii**). The percentage
1110 of cells with nuclear Rad50 foci (zoom insets and arrows in **i**) is shown for wild-type, *mr^{ho}* and *mr^{lo}*
1111 cells before and after CPT treatment. Mean ± SD, n = 3, unpaired two-tailed t-test. Scale bars: 5 μm.
- 1112 **i** Anti-Rad53 western blot⁹⁰ demonstrating MR^{wt/ho/lo}-mediated Tel1-DNA damage checkpoint
1113 activation and subsequent Rad53 phosphorylation (*) upon DNA damage induction with HU in
1114 *rad50Δ* cells. n = 3.

Suppl. Fig. 1: MR head domain interactions allow MR oligomerization on DNA.

1113 **Suppl. Fig. 1: MR head domain interactions allow MR oligomerization on DNA.**

1114 **a** Representative Coomassie-stained SDS-PAGE of recombinant *S. cerevisiae* wild-type
1115 Mre11-Rad50 (MR^{wt}) used in this study. Mre11 was 6xHis-tagged, while Rad50 was untagged but
1116 codon-optimized for *Sf9* expression.

1117 **b** Uncropped TEM images showing the heterogeneity of MR assemblies. *n* = 3. Areas in dashed
1118 rectangles were selected for Fig. 1a to highlight specific MR species. Scale bar: 100 nm.

1119 **c** Salt-sensitivity of MR oligomers. *n* = 3. Scale bars: 100 nm. **i:** TEM images with cartoons
1120 indicating the predominant oligomer species. **ii:** Mean head domain diameters of MR oligomers
1121 measured at their longest axis (see cartoon) ± SEM as a function of KCl concentration, fitted with a
1122 linear regression. *n*_{40 mM KCl} = 91 molecules, *n*_{60 mM KCl} = 33, *n*_{120 mM KCl} = 30, *n*_{150 mM KCl} = 104, *n*_{200 mM}
1123 *KCl* = 38 and *n*_{260 mM KCl} = 10. Grey area indicates the diameter range of ~ 8-14 nm measured for MR
1124 dimers. **iii:** As **i** but with 150 mM NaCl. **iv:** Mean head domain diameters of MR oligomers at their
1125 longest axis ± SD are comparable at 150 mM NaCl and KCl (non-significant unpaired two-tailed t-
1126 test, *n*_{150 mM NaCl} = 96 molecules, *n*_{150 mM KCl} = 104).

1127 **d** TEM of MR at 42 °C. *n* = 3. **i:** MR oligomers are larger at 42 °C than 30 °C at otherwise equal
1128 conditions (Supplementary Fig. 1b-iv) or at 4 °C without co-factors (Supplementary Fig. 1b-i-iii).
1129 **ii:** Higher MR concentration (600 nM) and higher temperature (42 °C) stabilize MR oligomers at
1130 high salt (240 mM KCl). Scale bar: 100 nm.

1131 **e** TEM of MR binding to circular plasmid DNA at 30 mM KCl. *n* = 3. **i:** Co-factors with ATP_γS.
1132 MR polymerization is similar as on linear DNA in Fig. 1b-ii. **ii:** Co-factors with ATP. MR
1133 polymerization on DNA is comparable with ATP and ATP_γS (Fig. 1b-iii). Scale bar: 100 nm.

1134 **f** Representative EMSAs of MR with topoisomerase-relaxed circular, or linearized plasmid
1135 DNA with free or streptavidin-blocked ends at 80 mM KCl. Left image is also shown in Fig. 1c-i.
1136 *n* = 3.

1137 **g** **i:** TEM showing salt-sensitivity of MR binding to linear plasmid DNA at higher KCl
1138 concentrations (compare with Fig. 1b-ii at 30 mM KCl). **ii:** Higher MR concentration (300 nM) can
1139 stabilize MR on plasmid DNA even at 150 mM KCl. *n* = 3. Scale bar: 100 nm.

Suppl. Fig. 2: MR oligomerization and positive cooperativity stabilize MR binding on longer DNA.

Suppl. Fig. 2: MR oligomerization and positive cooperativity stabilize MR binding on longer DNA.

a Representative EMSAs showing MR binding to oligonucleotides of different lengths (100 nM base pairs) with co-factors ATP γ S and Mg²⁺ at 30 mM NaCl (**i**), and quantification (**ii**). Mean $\pm$ SEM, n_{20, 31 bp} = 4, n_{70, 100, 197 bp} = 3, sigmoidal fit. Dashed line indicates K_D as MR concentration at 50 % DNA binding; K_D per oligonucleotide plotted in Fig. 1e. Red stars mark the radio-label position.

b EMSAs with MR and (**i**) 20 bp or (**ii**) 100 bp-long oligonucleotides (100 nM base pairs) with co-factors ATP γ S and Mg²⁺ and various NaCl concentrations. n = 3. Red stars mark the radio-label position.

c, d Representative EMSAs with MR and (**i**) 20 bp or (**ii**) 100 bp-long oligonucleotides (100 nM base pairs) at 75 mM or 250 mM NaCl with co-factors ATP γ S and Mg²⁺ (**c**) and quantification (**d**). Red stars mark the radio-label position. Mean $\pm$ SEM, n_{20 bp, 75 mM KCl} = 4, n_{20 bp, 250 mM KCl} = 3, n_{100 bp, 75 mM KCl} = 5 and n_{100 bp, 250 mM KCl} = 4, sigmoidal fit.

e Molecular mass distributions from mass photometry of Mre11, Rad50 and MR^{wt} $\pm$ 197 bp-long DNA. n = 3. Cartoons illustrate the respective species in the observed peak. Note that the individual complex subunits do not bind DNA, in contrast to the full MR dimers. See Methods for detailed assignment of the observed peaks.

f Molecular mass distributions from mass photometry show the formation of MR^{wt} oligomers on plasmid DNA at 25 mM KCl (tail of events > 2500 kDa, zoom inset). n = 3. Cartoons show the respective species in the observed peak. After 30 min at 30 °C and 25 mM KCl, large nucleoprotein assemblies beyond the detection limit are formed (smaller tail of events detected > 2500 kDa). At 250 mM KCl, even after 30 min at 30 °C, MR^{wt} (~ 460 kDa) and plasmid DNA (~ 1300 kDa) are predominantly unbound.

Suppl. Fig. 3: Controls of end resection pathway reconstitutions in nuclease assays.

1164 **Suppl. Fig. 3: Controls of end resection pathway reconstitutions in nuclease assays.**

1165 **a** Quantification of nuclease assays as shown in Fig. 2a,b. Mean $\pm$ SEM, n = 4, unpaired two-
1166 tailed t-tests. Resection activity (%) was normalized such that the activity of MR-pSae2 together
1167 with Exo1 on DNA substrate with free ends at 40 min was 100 %.

1168 **b** Representative nuclease assay with Exo1 on streptavidin-blocked plasmid DNA. Faint signal
1169 of resection on the complementary strand was detected with a probe (red box in cartoon) annealing
1170 160 nt from the 3'-DNA end, suggesting that not all substrate molecules were blocked at all ends
1171 with streptavidin (see also Fig. 2b). n = 2.

Suppl. Fig. 4: Characterization of MR mutants *in vitro*.

1172 **Suppl. Fig. 4: Characterization of MR mutants *in vitro*.**

1173 **a** Representative Coomassie-stained SDS-PAGE showing the purified *S. cerevisiae* MR^{ho/lo}
1174 mutants.

1175 **b** Molecular mass distributions from mass photometry of MR^{wt/ho/lo} ± 197 bp-long DNA. n = 3.
1176 Cartoons show the respective species in the observed peak. Note that for MR^{lo} no DNA binding was
1177 apparent.

1178 **c** Representative pelleting assays of MR^{wt/ho} at 240 mM KCl (see Fig. 3f). After centrifugation,
1179 the supernatant (Sup.) and pellet of MR^{wt/ho} were analyzed by SDS-PAGE. MR^{ho} forms more and/or
1180 larger assemblies than MR^{wt} that can be pelleted more efficiently, especially at higher temperature.
1181 n = 3.

1182 **d** Molecular mass distributions from mass photometry show the formation of MR^{wt/ho} oligomers
1183 without DNA at 250 mM or 25 mM KCl. MR^{ho} higher-order assemblies beyond the detection limit
1184 resist higher salt concentration, thus less MR^{ho} dimers could be detected compared to MR^{wt} (peak
1185 with dimer cartoon). n = 3.

1186 **e** Representative EMSAs of (i) MR^{ho} and (ii) MR^{lo} with 100 bp-long DNA and co-factors
1187 ATP_γS and Mg²⁺ as in Supplementary Fig. 2a for MR^{wt}. n = 3. The red star marks the radio-label
1188 position.

1189 **f** Mass photometry experiments show the formation of MR^{wt/ho} oligomers on plasmid DNA at
1190 25 mM KCl. Cartoons indicate the respective species in the observed peak. For MR^{wt}, a tail of counts
1191 at high molecular weight > 2500 kDa, corresponding to higher-order MR-DNA assemblies, could
1192 be detected, while MR^{ho} formed much larger nucleoprotein assemblies beyond the detection limit
1193 (smaller tail of counts > 2500 kDa, 25 mM KCl). After 30 min at 30 °C, also MR^{wt} formed larger
1194 assemblies on DNA beyond the detection limit (smaller tail of counts > 2500 kDa, 25 mM KCl,
1195 30 min), however, MR^{ho} still assembled larger clusters per DNA molecule compared to MR^{wt}, thus
1196 less unbound plasmid DNA was detected at ~ 1300 kDa with MR^{ho} (25 mM KCl, 30 min). At
1197 250 mM KCl, even after 30 min at 30 °C, MR^{wt/ho} (~ 460 kDa) and plasmid DNA (~ 1300 kDa) are
1198 predominantly unbound.

Suppl. Fig. 5: Characterization of MR mutants *in vitro* and *in vivo*.

1199 **Suppl. Fig. 5: Characterization of MR mutants *in vitro* and *in vivo*.**

1200 **a** Representative assay demonstrating the lack of MR^{lo} endonuclease activity on streptavidin-
1201 blocked DNA with pSae2 even at higher MR^{lo} concentration (200 nM). n = 2. The red star marks
1202 the radio-label position.

1203 **b** Representative nuclease assays recapitulating short- and long-range end resection of MR^{ho} on
1204 linear plasmid DNA with free (i) or streptavidin-blocked ends (ii) and ATP. After incubation with
1205 MR-pSae2, Exo1 was added for 10 min where indicated (+). Signal detection with a probe (green
1206 box in cartoons) binding where the 5'-terminated strand was resected. n = 2. **iii:** Representative
1207 nuclease assay with Exo1 on streptavidin-blocked plasmid DNA. A faint signal of resection on the
1208 complementary strand was detected with a probe (red box in cartoon) annealing 160 nt from the 3'-
1209 DNA end, suggesting that not all substrate molecules were blocked at all ends with streptavidin.
1210 n = 2.

1211 **c** Representative assays demonstrating exonuclease activity of MR^{wt/ho/lo} on 70 bp-long DNA
1212 with co-factors (ATPγS where indicated). Bands of exonuclease products are visible for MR^{wt/ho/lo}.
1213 As shown in lanes 3, 5 and 7, the exonuclease of Mre11 was inhibited upon ATPγS addition,
1214 demonstrating that all Rad50 variants bind ATP. Rad50^{wt} was supplemented to account for free
1215 Mre11 subunits not in complex with Rad50 variants, which could mediate exonuclease activity
1216 insensitive to inhibition by ATP(γS) (ii). n = 2. The red star marks the radio-label position.

1217 **d** Nuclear relocalization of MR^{wt/ho/lo}-yEVENus in untreated conditions and upon DSB-induction
1218 with 2 h of 200 μM camptothecin (CPT) calculated as normalized standard deviation of nuclear
1219 Rad50-yEVENus intensity from images as shown in Fig. 4h. Values were normalized to untreated
1220 conditions = 1. Median of n = 3 is shown. MR^{wt} relocalizes more upon DNA damage than MR^{ho}, as
1221 the latter formed foci already before DSB induction and did not accumulate many more foci upon
1222 DNA damage.

1223 **e** FACS-based cell cycle analysis of exponentially growing, MR^{wt/ho/lo}-yEVENus-expressing
1224 cells in untreated conditions. Gating is indicated. n = 3.

1225 **Suppl. Table S1: Oligonucleotides used in this study**

Name	Sequence (5' to 3')	Notes
PC216_20 nt	CGTACTCCACCTCATGCATC	Labeled oligonucleotide for 20 bp substrate
PC216_C_20 nt	GATGCATGAGGTGGAGTACG	Unlabeled oligonucleotide for 20 bp substrate; bold T indicates biotin position
X12-3SC	TTGCTAGGACATGCTGTCTAGAGACTATCGC	Labeled oligonucleotide for 31 bp substrate
X12-4SC	GCGATAGTCTCTAGACAGCATGTCCTAGCAA	Unlabeled oligonucleotide for 31 bp substrate
PC210	GTAAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGC TCCCCGGGCGCGTACTCCACCTCATGCATC	Labeled oligonucleotide for 70 bp substrate; bold T indicate biotin positions
PC211	GATGCATGAGGTGGAGTACGCGCCCGGGGAGCCCAAGGGC ACGCCCTGGCACCCGCACCGCGGCACTTAC	Unlabeled oligonucleotide for 70 bp substrate; bold T indicate biotin positions
BIO100C	GATGCAGGAGGCTGCTACGACCATGGCAGAAGATTATGAG GTGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGC ACCCGCACCGCGGCACTTAC	Labeled oligonucleotide for 100 bp substrate
BIO100	GTAAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGC TCCCCGGGCGCGTACTCCACCTCATAATCTTCTGCCATGGT CGTAGCAGCCTCCTGCATC	Unlabeled oligonucleotide for 100 bp substrate; bold T indicates biotin position
197mer_top	GCTTCGTATGTTTCTCCTGCTTATCACCTTCTTGAAGGCTTC CCATTCATTTCAGGAACCGCCTTCTGGTGATTGTGCAAGAACG CGTACTTATTCGCCACCATGATTATGACCAGTGTTTCCAGT CCGTTCAAGTTGTTGCAGTGGAATAGTCAGGTAAATTTAAT GTGACCGTTTATCGCAATCTGCCGACCACTCG	Labeled oligonucleotide for 197 bp substrate; bold G indicates biotin position; used for mass photometry
197mer_bottom	GCGAGTGGTCGGCAGATTGCGATAAACGGTCACATTAAAT TTAACCTGACTATTCCACTGCAACAACTGAACGGACTGGAA ACACTGGTCATAATCATGGTGCGAATAAGTACGCGTTCTT GCAAATCACCAGAAGGCGGTTCTGAATGAATGGGAAGCC TTCAAGAAGGTGATAAGCAGGAGAAACATACGAAG	Unlabeled oligonucleotide for 197 bp substrate; used for mass photometry
5'_OligoA	GGGACTGGGAAAACCCTGGCGT	Probe to detect resection of the 5' end; the

		underlined sequence is complementary to plasmid DNA
3'_OligoA	<u>GGACGCCAGGGTTTTCCCAGTC</u>	Probe to detect resection of the 3' end; the underlined sequence is complementary to plasmid DNA
20 bp top	GACCGAGATAGGGTTGAGTG	Used for mass photometry
20 bp bottom	CACTCAACCCTATCTCGGTC	
50 bp top	GGACCACGCATAATATACCTATATCAATGGCCTCCCACGCA TAAGCAGTG	
50 bp bottom	CACTGCTTATGCGTGGGAGGCCATTGATATAGGTATATTAT GCGTGGTCC	
73 bp top	GACCACCCTTTTTGATATAATATACCTATATCAATGGGC TCCCACGCAATTTCGGCAGATACGTTCTGAGGGAA	
73 bp bottom	GCCCATTGATATAGGTATATTATATCAAAAAGGGTGGTC TTCCCTCAGAACGTATCTGCCGAATTGCGTGGGA	
106 bp top	TTAGCTCCTACGCGGAACGAGACCTACCAATATACCTATAT CAATGGCCTCCCACGCATAAGCCTCGGGAGCTCGACCATCT GGTCTCATCGGACACTTAGGACCA	
106 bp bottom	TGGTCCTAAGTGTCCGATGAGACCAGATGGTCGAGCTCCCG AGGCTTATGCGTGGGAGGCCATTGATATAGGTATATTGGTA GGTCTCGTTCCGCGTAGGAGCTAA	
oJT11	ATCACAAATTGAGTGGGTCGATATTAACAGAGTCACTTATG GTGACGGTGCTGGTTTA	To amplify yEVENUS from pKT090
oJT12	CAATCAAAGTCTATCCCTTCGTAGATATTATGGGGTCTTTT CGATGAATTCGAGCTCG	
EB181	ATCAACTAGTCTACAATACAAAAAATAGACAATAGTATA TTTTTCTTTTGCTTGAAATT	To amplify --end promoter--Rad50-Venus-ADH1 terminator-- with restriction overhangs for cloning in pSIVu (URA) plasmid
EB182	ATTAGGTACCCCGGTAGAGGTGTGGTCAATAAGAG	

1226 **Suppl. Table S2: Plasmids used for yeast strain construction in this study**

Plasmid name	Plasmid number	Description	Source
	pKT090	pFA6-link-yEVENUS-Sp.His5	Euroscarf
	pSIV URA	pSIV URA empty vector	Ref ⁹²
	pGT LEU2 mCherry	pGT LEU2 mCherry	Ref ⁹²
MR ^{wt}	pJT12	pSIVu-SpeI-prom-rad50-yEVENUS-ADH1 term-KpnI	this paper
MR ^{ho}	pJT13	pSIVu-SpeI-prom-rad50 ^{N121A/D124N} -yEVENUS-ADH1 term-KpnI	this paper
MR ^{lo}	pJT14	pSIVu-SpeI-prom-rad50 ^{L116A/I119A/T127A/L128A} -yEVENUS-ADH1 term-KpnI	this paper

1227 **Suppl. Table S3: Yeast strains used in this study**

Strain name	Strain number	Genotype	Source
	BY4741	<i>Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	OpenBiosystems
<i>rad50Δ</i>	yJT305	BY4741, <i>rad50::kanMX</i>	dissected from diploid collection
<i>mr^{ho}</i>	yJT308	BY4741, <i>rad50::kanMX</i> , <i>rad50^{N121A/D124N}-yEVENUS::URA3</i>	this paper
<i>mr^{lo}</i>	yJT311	BY4741, <i>rad50::kanMX</i> , <i>rad50^{L116A/I119A/T127A/L128A}-yEVENUS::URA3</i>	this paper
MR ^{wt}	yJT322	BY4741, <i>rad50::kanMX</i> , <i>rad50-yEVENUS::URA3</i>	this paper
Histone-tagged MR ^{wt}	yEB371	BY4741, <i>rad50::kanMX</i> , <i>rad50-yEVENUS::URA3</i> , <i>hta2::Hta2-mCherry::LEU2</i>	this paper
Histone-tagged <i>mr^{ho}</i>	yEB373	BY4741, <i>rad50::kanMX</i> , <i>rad50^{N121A/D124N}-yEVENUS::URA3</i> , <i>hta2::Hta2-mCherry::LEU2</i>	this paper
Histone-tagged <i>mr^{lo}</i>	yEB415a	BY4741, <i>rad50::kanMX</i> , <i>rad50^{L116A/I119A/T127A/L128A}-yEVENUS::URA3</i> , <i>hta2::Hta2-mCherry::LEU2</i>	this paper