The conserved Mre11-Rad50 complex is crucial for the detection, signaling, end tethering and processing of DNA double-strand breaks. While it is known that Mre11-Rad50 foci formation at DNA lesions accompanies repair, the underlying molecular assembly mechanisms and functional implications remained unclear. Combining pathway reconstitution in electron microscopy, biochemical assays and genetic studies, we show that *S. cerevisiae* Mre11-Rad50 with or without Xrs2 forms higher-order assemblies in solution and on DNA. Rad50 mediates such oligomerization, and mutations in a conserved Rad50 beta-sheet enhance or disrupt oligomerization. We demonstrate that Mre11-Rad50-Xrs2 oligomerization facilitates foci formation, DNA damage signaling, repair, and telomere maintenance in vivo. Mre11-Rad50 oligomerization does not affect its exonuclease activity but drives endonucleolytic cleavage at multiple sites on the 5′-DNA strand near double-strand breaks. Interestingly, mutations in the human RAD50 beta-sheet are linked to hereditary cancer predisposition and our findings might provide insights into their potential role in chemoresistance.
misfunction of the MRE11-RAD50-NBS1 (MRN) complex is associated with several pathologies, including various hereditary and sporadic cancers. Aberrant degradation of nascent DNA by MRE11 was observed in tumor suppressor BRCA1/2-deficient cells, and abrogation of MRE11 activity is linked to PARP inhibitor resistance in cancer chemotherapy. At the molecular level, human MRN and yeast Mre11-Rad50-Xrs2 (MRX) promote DNA double-strand break (DSB) repair via non-homologous end joining (NHEJ) and homologous recombination (HR) by means of DNA tethering, activation of downstream pathway components, and ATP-dependent resection of the 5′-terminated strands at DSBs. The latter function is particularly relevant for HR and a variant of the end-joining pathway termed alternative end-joining (A-EJ). The MRN/X complex also activates the DNA damage checkpoint kinase ATM/Tel1, which in turn phosphorylates targets to orchestrate cell cycle arrest and DNA repair. Moreover, MRN/X and ATM/Tel1 contribute to the maintenance of normal telomere length.

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

In budding yeast S. cerevisiae, Mre11 nuclease activity is essential for processing protein-blocked DNA ends. This activity can be bypassed in case of free DNA ends by long-range nucleases essential for processing protein-blocked DNA ends. This activity is mediated by the Rad50 coiled-coils. The MRN/X complex also activates the DNA damage checkpoint kinase ATM/Tel1, which in turn phosphorylates targets to orchestrate cell cycle arrest and DNA repair. Moreover, MRN/X and ATM/Tel1 contribute to the maintenance of normal telomere length.

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In budding yeast S. cerevisiae, Mre11 nuclease activity is essential for processing protein-blocked DNA ends. This activity can be bypassed in case of free DNA ends by long-range nucleases normally acting downstream, including Exo1 and Dna2. NBS1/Xrs2 is essential for resection in humans, but largely dispensable in yeast. It is, however, needed for Tel1 activation and nuclear import of the MRX complex. Due to the requirement of a sister chromatid as homologous template for repair via HR, resection is limited to the S/G2 phase of the cell cycle, which is achieved through cell cycle-regulated stimulation of Mre11 endonuclease activity by phosphorylated Sae2 (pSae2). In vitro, MR first incises the 5′-terminated strand ~15–35 bp away from the DSB end with its endonuclease function, and then exonucleolytically degrades DNA backwards to the end in 3′–5′ direction. In cells, Mre11-dependent resection can generate 3′-overhangs of up to ~300 nt in length. It is unclear whether the short-range resection tracts are generated by DNA cleavage that proceeds stepwise away from the DNA end, as observed in vitro, or whether MR in most cases first cleaves DNA at greater distances from the DNA end, as observed in meiotic cells. The single-stranded overhangs are then elongated to more than 1000 nt by the long-range resection machinery including Exo1 or Dna2-Sgs1 for homology-directed repair.

Upon DNA damage in eukaryotic cells, MR relays from a homogeneous, nuclear distribution to discrete foci consisting of hundreds to thousands of MR molecules. This localized MR accumulation then recruits a cascade of DNA repair and signaling proteins, including ATM/Tel1. Given that MR foci formation at DSBs and the resultant 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 repair.

MR was shown to dimerize to M2R2 complexes with one or two subunits of NBS1/Xrs2, whereby the Mre11 nuclease and Rad50 ATPase domains form a globular head from which two Rad50 coiled-coils protrude, culminating at a zinc hook at the apex of the structure. DNA binding occurs mainly via the head domain of Mre11 and Rad50, but Xrs2 and the Rad50 coiled-coils were also shown to bind DNA. Foci formation could be facilitated by higher-order oligomerization of MR dimers via globular head domain interactions or by zinc hook-mediated tethering of the protruding Rad50 coiled-coils. Although hints of head domain oligomerization have been observed for various species in vitro, especially for eukaryotic MR, coiled-coil tethering was predominantly investigated and functionally associated with DNA movement, condensation, and end fixation in repair. High-resolution structures of the MR dimer from crystallography and cryo-electron microscopy contributed significantly 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, the molecular mechanisms of foci assembly and identifying 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 examine DNA end resection reactions by combining biochemical reconstitution and negative staining transmission electron microscopy (TEM), and thereby provide insights into the function and architecture of active DNA end resection complexes at the single molecule level. We demonstrate that short-range DNA end resection is driven by MR oligomerization mediated by MR head domain interactions, which stabilize MR on DNA and enhance its endonuclease activity at multiple sites along the 5′-terminated DNA strand. Mutational analysis shows that a small interface in the globular part of Rad50 modulates MR oligomerization and DNA end resection, as well as MR-dependent foci formation, DNA damage signaling, and telomere maintenance 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.

**Results**

MR oligomerizes via head domain and forms nucleolytically active assemblies on DNA. To understand the role and regulation of MR(X) oligomerization in DSB repair, we purified recombinant S. cerevisiae MRX from insect cells and visualized it with negative staining TEM (Fig. 1a and Supplementary Fig. 1a, b). Since Xrs2 is largely dispensable for DNA end resection and integrated with variable copy numbers into purified MRX complexes, we mainly analyzed MR to reduce complexity. In accord with previous studies of MR homologs, we observed the formation of MR dimers (M2R2) with a globular head domain of ~11 nm in diameter and protruding ~50 nm long Rad50 coiled-coils. The coiled-coils were either open (Fig. 1a-i, ii and Supplementary Fig. 1a-i) or closed to a clamp (~15 nm in diameter and protruding ~50 nm long Rad50 coiled-coils) with protruding coiled-coils that could interact with protruding coiled-coils that could then further interact via tethering (Fig. 1a-i–ii; Supplementary Fig. 1b-i–iii). ATP binding stimulates MR association with DNA, and MR endonuclease activity is magnesium- and manganese-dependent and requires ATP hydrolysis by Rad50. Additionally, M2R2 also displayed the capacity to oligomerize via its head domain, allowing the formation of tetramers (M4R4) and larger assemblies (M2R2) with protruding coiled-coils that could then further interact via tethering (Fig. 1a-ii–iii; Supplementary Fig. 1b-i–iii). ATP binding stimulates MR association with DNA, and MR endonuclease activity is magnesium- and manganese-dependent and requires ATP hydrolysis by Rad50. Accordingly, under more physiological conditions as used for in vitro MR nuclease assays (~30 °C with Mg2+, Mn2+, but ATP is instead of ATP to prevent ATP hydrolysis and stabilize Rad50 interactions), the MR oligomers were even more evident (Fig. 1a-v and Supplementary Fig. 1b-iv). Higher temperature
Fig. 1 MR oligomerizes via head domain and forms nucleolytically active assemblies on DNA. a TEM of S. cerevisiae Mre11-Rad50 dimers (M2R2) and oligomers (M2R2)n with open (i, ii) or closed (iii) Rad50 coiled-coils. MR oligomerization via head domains (arrows in ii, iii; gray cartoon lines in iii, iv) and further interaction through coiled-coil tethering (arrows in iv). (v) Larger MR oligomers at 30 °C with co-factors ATPγS, Mg2+, and Mn2+ (Supplementary Fig. 1a-c). Images representative of n = 3. b TEM of MR binding to plasmid DNA at 30 °C with co-factors as above. (i) M2R2 binding the DNA end. (ii, iii) Linear plasmid DNA tetramerization via head domain and forms nucleolytically active assemblies on DNA. (iv) Higher oligomerization via head domain and forms nucleolytically active assemblies on DNA. n = 3 independent experiments. c EMSA of MR binding to plasmid DNA (i) and quantification (ii; Supplementary Fig. 1g). Mean ± SEM, n = 3 or n = 4 independent experiments, sigmoidal fit. d (i) Resection assay with probe (green box in cartoon) binding to the 3′-overhang produced. n = 2. (ii) TEM of (i) without pSae2 but with ATPγS to stabilize MR on DNA. n = 3. Large MR oligomers still catalyze resection with pSae2 and ATP. e Quantification of EMSAs as in Supplementary Fig. 2a with oligonucleotides of different lengths (100 nM base pairs). Kd as MR concentration at 50% DNA binding ± SEM of p20,31bp = 4, p70,100,197bp = 3, unpaired two-tailed t-test. f Molecular mass distributions from mass photometry show better MRwt binding to longer oligonucleotides (100 nM base pairs). Cartoons illustrate species in peaks. Measured molecular weight (MW, kDa) ± SD in gray. 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 (Supplementary Fig. 3c). n = 3 independent experiments. g Model illustrating that in the presence of DNA, MR oligomers dissociate to dimers which then oligomerize again via head domain interactions at DNA ends or internally to "pearls-on-a-string"-like assemblies and larger clusters. Substrate compaction suggests DNA loop formation and coiled-coil tethering. Scale bars: 100 nm (a, b, d).

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facilitated the formation of larger MR oligomers (compare 4°C in Supplementary Fig. 1b-i–iii, 30°C in Supplementary Fig. 1b-iv and 42°C in Supplementary Fig. 1d-i). These MR oligomers were stable at physiological salt concentrations (150 mM KCl or NaCl) and only dissociated into MR dimers (M₃R₂) at high salt (260 mM KCl) (Supplementary Fig. 1c). Oligomer size decreased linearly with increasing KCl concentration, suggesting a charge screening effect of salt on electrostatic interactions between the MR head domains (100 nM MR, Supplementary Fig. 1c). However, higher temperature (42°C) and increased MR concentration (600 nM) could overcome this charge screening effect at high salt conditions, restoring MR oligomerization (compare 260 mM KCl in Supplementary Fig. 1c-i with Supplementary Fig. 1d-i). Together, the high local MR concentrations at DSBs and the growth temperature of yeast may thus counteract the charge screening effect of chromatin DNA, allowing MR oligomerization under physiological conditions.

To examine the effect of DNA on MR oligomerization, we investigated MR assembly on linear and circular plasmid DNA. At lower MR:DNA ratios (50–100 nM MR, 10–20:1 with respect to DNA moles), the MR oligomers bound onto DNA and dissociated into dimers that bound DNA ends (Fig. 1b-i) or internally (Fig. 1b-ii, iii and Supplementary Fig. 1e, f). A few isolated MR dimers or pairs of MR dimers bound adjacent on DNA were visible (Fig. 1b, pink and orange arrows denote examples). The majority of MR molecules, however, were non-randomly distributed and oligomerized on DNA (Fig. 1b-ii, iii; red arrows). Some of these MR molecules appeared to form “pearls-on-a-string”-like assemblies on DNA, seemingly interacting with each other through the head domains, oftentimes with DNA loops in between (Fig. 1b-ii, iii zoom-insets and cartoons). The dense accumulation of MR units at some DNA regions contrasted with other DNA stretches that were left almost protein-free, as observed both in the presence and absence of DNA ends with ATP or ATPγS (Fig. 1b-ii, iii and Supplementary Fig. 1e, f). The detected DNA loops and substrate compaction suggest that the head domain interactions and subsequent Rad50 tethering of MR oligomers can bring distant parts of DNA into close proximity, as reported previously for archaeal MR. In electrophoretic mobility shift assays (EMSAs) we observed a distinct banded pattern at low MR concentrations (up to 100 nM), followed by a rapid shift of the protein-DNA complexes to the wells in a narrow MR concentration range (from 200 to 400 nM) (Fig. 1c-i and Supplementary Fig. 1g). This switch-like binding behavior of MR was comparable on linear and circular DNA, irrespective of free or protein-blocked ends or DNA supercoiling (Fig. 1c-ii and Supplementary Fig. 1g), and was suggestive of a stabilization of MR units bound to DNA as a protein cluster. Limited binding preference of MR to DNA ends was observed previously, as Xrs2, albeit not essential for resection in vivo, facilitates end-binding of the MRX complex. To ascertain that MR oligomers are active entities, we performed in vitro nuclease assays in the presence of phosphorylated Sae2 (pSae2) to trigger MR endonuclease activity (Fig. 1d-i). Resection was monitored with a radio-labeled oligonucleotide annealing to the produced 3’-overhang. Efficient DNA end resection was observed at the MR concentrations that led to the binding of multiple MR units on DNA in EMSA assays (Fig. 1c-i and Supplementary Fig. 1g). Interestingly, negative staining TEM analysis of the MR:DNA ratio with the highest resection activity in the nuclease assay (200 nM MR with 2.5 nM DNA, MR:DNA ratio 80:1; Fig. 1d-i) revealed that the MR dimers formed large oligomers on DNA, in which the individual MR dimers could no longer be easily distinguished (Fig. 1d-ii), in contrast to the assemblies with distinguishable MR dimers on DNA observed at lower MR:DNA ratios (Fig. 1b-ii, iii and Supplementary Fig. 1e). The combination of imaging and biochemical experiments demonstrates that the observed MR oligomers are not inactive assemblies, but rather functional units that allow resection to proceed through multiple endonucleolytic cuts beyond the vicinity of DNA ends, as observed in vivo.

A high MR:DNA ratio (60:1; Supplementary Fig. 1h) also stabilized MR on DNA against higher salt concentration, thus counteracting the expected salt sensitivity of MR-DNA association. Moreover, MR preferentially bound longer DNA substrates, as shown by the trend of apparent dissociation constants (K_D) derived from EMSA experiments with oligonucleotides, which decreased with increasing DNA length (Fig. 1e and Supplementary Fig. 2a). EMSA experiments also revealed that longer DNA molecules stabilized MR on DNA against high salt concentrations (Supplementary Fig. 2b-d), similarly to higher MR:DNA ratios (Supplementary Fig. 1h). As MR-DNA species remaining in the wells may bias the K_D values, we performed mass photometry, in which the large MR-DNA assemblies stay in solution. These measurements confirmed better MR binding to longer oligonucleotides, as the peak area between the two dashed lines, corresponding to one MR dimer binding to one DNA molecule, grew with increasing DNA length (Fig. 1f; theoretical molecular weight of M₃R₂ is 460 kDa). Moreover, a higher molecular weight peak at ~1000 kDa could be detected and properly fitted for longer oligonucleotides, corresponding to two MR dimers bound per DNA molecule (zoom-insets for 106 and 197 bp). In addition, larger MR oligomers were likely formed on longer DNA that were beyond the detection limit of the instrument, as indicated by the sudden decrease in the observed MR-DNA peak areas with 106 or 197 bp, compared to 73 bp-long DNA (Fig. 1f). Taken together, these observations suggest that MR dimers form oligomers in solution, which dissociate to bind DNA and reassemble in a switch-like manner to larger MR assemblies on DNA (Fig. 1g). As expected, purified MRX behaved comparably in TEM, EMSAs, and mass photometry experiments (Supplementary Fig. 3a–e), demonstrating that Xrs2 binding does not diminish MR oligomerization. MR(X) oligomerization may allow a rapid loading of multiple MR(X) units onto DNA, thereby facilitating short-range resection of DNA ends and foci formation.

**MR oligomerization on DNA accompanies end resection.** Next, we reconstituted short-range (MR and pSae2) and long-range (Exo1) resection in vitro using recombinant S. cerevisiae proteins and linear plasmid DNA with free or streptavidin-blocked ends. Gel-based assays confirmed that DNA with free ends could be rapidly processed by Exo1 alone, as visualized by the characteristic pattern of resection products, depending on the extent of resected DNA and whether resection was initiated at one or both DNA ends (Fig. 2a, lane 2, see cartoons on the right; Supplementary Fig. 4a). Adding MR-pSae2 to the Exo1 reaction moderately enhanced resection (Fig. 2a, compare lane 2 with lanes 4, 6 and 8 and Supplementary Fig. 4a), as observed previously. In contrast, MR-pSae2 alone resulted in slow resection that was limited to the vicinity of DNA ends (Fig. 2a, lanes 3, 5 and 7 and Supplementary Fig. 4a). As expected, long-range resection by Exo1 alone was strongly reduced on linear DNA with streptavidin-blocked ends (Fig. 2b, lane 2 and Supplementary Fig. 4a, b), but this activity could be restored by the addition of MR-pSae2 (Fig. 2b, lanes 4, 6 and 8 and Supplementary Fig. 4a). Thereby, MR-pSae2 was able to overcome the protein block and resect blocked DNA ends similarly to free DNA (Fig. 2b, lanes 3, 5 and 7 and Supplementary Fig. 4a), creating an entry site for Exo1-mediated long-range resection. Using shorter, 3’-labelled oligonucleotide-based DNA with one streptavidin-block at the 5’-terminated end revealed a gradual shortening of the 5’-strand by
MR-pSae2 and the generation of DNA fragments of similar length (Fig. 2c). This indicates the sequential formation of several endonucleolytic incisions and the stepwise DNA end resection by many MR-pSae2 molecules that together produce the single-stranded overhang observed21. MR oligomerization may promote such joint DNA end resection 21. Together, our reconstituted assays demonstrate that Exo1 requires MR-pSae2-dependent short-range resection at protein-blocked DNA ends to initiate long-range resection.

We next set out to visualize the architecture of DNA end resection complexes, using the same recombinant proteins and reaction conditions, in negative staining TEM. As shown in...
Fig. 2 MR oligomerization on DNA accompanies end resection. a Short-range end resection of MR-pSae2 and handover to Exo1 for long-range resection (10 min, +) on plasmid DNA with free ends. Resection detection with probe (green box see cartoons) binding to the 3′-overhang produced. Image is representative of 4 experiments. b As a, but plasmid DNA with streptavidin-blocked ends (Supplementary Fig. 4a, b). n = 4. c Stepwise resection by MR-pSae2 (small black arrows) on the 5′-terminated DNA strand. Red star marks the position of the radio-label. n = 2. d Representative TEM images visualizing short- and long-range end resection by MR-pSae2 and Exo1 on plasmid DNA with free (i, ii) and streptavidin-blocked ends (iii) under conditions comparable to a and b. n = 3. Scale bars: 100 nm. Legend shows cartoons, indicative arrows, and corresponding micrographs of the reaction components. Schematic cartoons in (i–iii) illustrate adjacent micrographs of the different reactions. Due to the resolution of the negative staining images, the number of molecules or their orientation drawn in the cartoons may not exactly correspond to the assembly in the micrographs. (i, i) Linear plasmid substrate shows no significant stretches of RPA-coated, single-stranded DNA in the absence of MR-pSae2 or Exo1. (ii–IV) MR-pSae2 oligomerize on DNA (magenta arrows and labels; Supplementary Fig. 4c) and produce short resected, RPA-coated single-stranded DNA overhangs in 2.5 h, before dissociating from DNA and likely forming their respective oligomers. (ii) Exo1 can resect entire plasmid substrates with free ends in 5 min. (iii) Plasmid DNA with two streptavidin blocks per end can only be resected by Exo1 if pSae2–MR oligomers process these DNA substrates beforehand. pSae2–MR clusters (magenta arrows and labels) can harbor several streptavidin-blocked DNA substrates (II, left) and execute short-range resection on one DNA end within 2.5 h, resulting in short resected, RPA-coated single-stranded DNA overhangs (II, right). Exo1 can then fully resect the DNA substrates within 10 min (III–IV).

Mutagenesis of conserved Rad50 interface regulates MR oligomerization. To define the functional relevance of MR oligomerization on DNA, we next set out to identify the interaction interface(s) between the MR head domains. As a basis for mutational analysis, we employed a recent crystallographic structure showing a eukaryotic C. thermophillum (Ct) Rad50 dimer bound to a DNA molecule that forms a "quasi-continuous" structure with another Rad50 dimer-bound DNA molecule adjacent in the crystal, indicating a possible arrangement of two Rad50 dimers on crystal, indicating a possible arrangement of another Rad50 dimer-bound DNA molecule adjacent in the crystal. As a basis for mutational analysis, we employed a recent crystallographic structure showing a eukaryotic C. thermophillum (Ct) Rad50 dimer bound to a DNA molecule that forms a "quasi-continuous" structure with another Rad50 dimer-bound DNA molecule adjacent in the crystal, indicating a possible arrangement of two Rad50 dimers on DNA (Fig. 3a; RCSB Protein Data Bank PDB identifier: 5DAC48, https://doi.org/10.2210/pdb5dac/pdb). We hypothesized that an anti-parallel, two-stranded beta-sheet (black rectangle, β) and its connecting loop (black rectangle, L) protruding from the head domain of one Rad50 dimer in one asymmetric unit towards the identical structure of another Rad50 dimer in the adjacent asymmetric unit of the crystal could represent a possible interaction site, especially on a continuous DNA molecule and in solution when MR molecules could come even closer on DNA than in the crystal structure shown. The residues constituting this small beta-sheet and its connecting loop, or their physicochemical characteristics, are conserved between C. thermophillum, S. cerevisiae, and H. sapiens Rad50 (Fig. 3b), which is intriguing given the limited overall sequence similarity of MR between species49. Interestingly, mutations derived from cancer predisposed individuals (β in Fig. 3b, from ClinVar/MedGen databases) cluster in this conserved beta-sheet and could be relevant for Rad50 functions in DNA repair and cancer avoidance.

To investigate whether beta-sheet interactions are indeed involved in MR oligomerization, we stained S. cerevisiae (Sc) MR oligomers with Thioflavin T (ThT), a dye used to detect beta-sheet-rich structures50. Indeed, a ThT signal was detected in the presence of ScMR oligomers at 4°C and enhanced at higher temperature (42°C), suggesting the presence of extended beta-sheet interactions between MR molecules (Fig. 3c). In order to test the possible role of the identified protruding beta-sheet and its connecting loop in MR oligomerization, we engineered two ScRad50 variants, each with several point mutations in the beta-sheet to disrupt either electrostatic or hydrophobic interactions between the Rad50 subunits of MR dimers. Addressing the electrostatic component of MR oligomerization (Supplementary Fig. 1c), we substituted a polar residue in the potential interaction interface with an uncharged, non-polar alanine (Sc N121A, Ct N122) and removed a negative charge (Sc D124, Ct E125) to disrupt an attractive salt bridge between the D124 and R125 residues (Ct E125, R126) or to alleviate a potentially strong repulsive force between the aspartate residues D124 (Ct E125) of interacting ScRad50 molecules (red residues mutated in Fig. 3a zoom-inset and Fig. 3b; Rad50β). In the second ScRad50 variant, four hydrophobic beta-sheet residues were mutated to alanine to disrupt or destabilize the beta-sheet structure51 (ScRad50L116A/I119A/T127A/L128A; CtRad50I118/L120/T127/I129, blue residues mutated in Fig. 3a zoom-inset and Fig. 3b; Rad50β). These two ScRad50 mutants were co-expressed and co-purified with Mre11, showing that the physical interaction between Mre11 and Rad50 was not notably affected (Supplementary Fig. 5a).

We first characterized the oligomerization capacity of the ScMR variants in negative staining TEM in the absence of DNA. This analysis revealed that the quadruple mutant MRL116A/I119A/T127A/L128A formed only dimers (thus...
Fig. 3 Mutagenesis of conserved Rad50 interface regulates MR oligomerization. a Crystal structure of two *C. thermophila* (CT) Rad50 dimers bound to one oligonucleotide molecule each (PDB: 5DAC48, 10.2210/pdb5dac/pdb) used to generate a plausible interaction model as guide for validation by mutagenesis. Possible interactions of adjacent Rad50 dimers on DNA via a small beta-sheet (p) and its connecting loop (L) protruding from the Rad50 head domain (rectangle), especially if bound DNA was continuous. Zoom-inset shows red/blue residues mutated in the *S. cerevisiae* (Sc) Mre11-Rad50ho/lo variants. Gray dotted line: salt bridge between E125 and R126 residues of adjacent Mre11 dimers. Red dotted line: repulsive electrostatic interaction between MRwt MRho MRlo dimers spaced by 4.8 Å within the crystal, but could come closer on continuous DNA and in solution. b Sequence alignment of the CT, Sc, and *H. sapiens* Rad50 beta-sheet motifs. Color code as in a. * indicates germline point mutations listed on ClinVar/MedGen for cancer-predisposed patients. Cartoons show multiple point mutations introduced in ScRad50 to disrupt electrostatic (Rad50ho) or hydrophobic (Rad50lo) interactions (Supplementary Fig. 5a). c Thioflavin T (ThT) fluorescence of ScMRwt indicates beta-sheet rich higher-order structures (Supplementary Fig. 5e). Mean ± SEM, n = 3 independent experiments, unpaired two-tailed t-test, a.u. arbitrary units. d (i) Representative TEM of ScMRwt/ho/lo as in Fig. 1a-v. (ii) Quantification of mean head domain diameters ± SEM of ScMRwt/ho/lo oligomers from (i) along their longest axis. Mean ± SEM, n = 30 molecules, nMR- ho = 72, and nMR-lo = 46. Gray area indicates diameter range of ~8-14 nm measured for ScMRwt dimers. Scale bar: 100 nm. e Molecular mass distributions from size-exclusion chromatography show better ScMRho binding to longer oligonucleotides (100 nM base pairs). Cartoons illustrate species in peaks. Measured molecular weight (kDa) ± 5D in gray. 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 (Supplementary Fig. 3b). n = 3 independent experiments. f Quantification of ScMRwt/ho pelleting assays at 4 °C or 42 °C (see Supplementary Fig. 5c). Sup. supernatant. Higher temperature promotes oligomerization. Mean ± SEM, n = 3, unpaired two-tailed t-tests.

henceforth denoted low oligomerization mutant, MRlo), while the MRN121A/D124N mutant (henceforth denoted high oligomerization mutant, MRho) assembled to even larger oligomers than wild-type MR (MRwt) (Fig. 3d). Mass photometry confirmed that the MRho mutant formed larger oligomers than MRwt, as in the absence of DNA only a small dimeric fraction of MRho could be detected (~DNA, zoom-inset ~460 kDa; Fig. 3e and Supplementary Fig. 5b). The MRho mutant was also more resistant towards high salt (240 mM KCl), as significantly more MRho oligomer assemblies could be harvested in a pelleting assay compared to MRwt after incubation at 1.2 µM MR and higher temperature (42 °C) (42 °C pellet; Fig. 3f and Supplementary Fig. 5c). Consistently, mass photometry detected less MRho dimers at ~460 kDa after 30 min incubation at 250 mM KCl and 30 °C compared to MRwt, suggesting the formation of larger MRho assemblies beyond the detection limit (Supplementary Fig. 5d). Similar to MRwt, MRho was stabilized on longer oligonucleotide substrates (Figs. 1f and 3e). However,
**Fig. 4 MR oligomerization influences DNA binding behavior and endonuclease activity.**

a) Quantification of EMSAs of MR<sup>wt</sup>/ho/lo binding to 100 bp-long DNA as in Supplementary Figs. 2a and 5f. Mean ± SEM, n = 3 independent experiments, sigmoidal fit. b) TEM of MR<sup>ho</sup>/lo binding to plasmid DNA. Images representative of n = 3 experiments. MR<sup>lo</sup> binds dispersedly on DNA without oligomerization, while MR<sup>ho</sup> dimers oligomerize on DNA to a denser "worm-like" structure compared to the "pears-on-a-string" assembly of MR<sup>wt</sup> (Fig. 1b-i) at similar conditions (see "Methods"). c) TEM of Mre11<sup>wt</sup> and Rad50<sup>wt</sup>/ho/lo binding to plasmid DNA, n = 3. In contrast to purified Mre11<sup>wt</sup>, Rad50<sup>wt</sup> forms "pears-on-a-string"-like structures on linear plasmid DNA. Rad50<sup>ho</sup> fails to oligomerize, thus binds dispersedly on DNA, while Rad50<sup>ho</sup> clusters into large oligomers on DNA, similarly to MR<sup>ho</sup>. d) Rad50<sup>ho</sup> clusters into large oligomers on DNA, similarly to MR<sup>ho</sup>. e) Endonuclease assays of MR<sup>wt</sup>/ho/lo/pSae2 (d, Supplementary Fig. 6a) with quantification (e). Images representative of n = 3 experiments. Mean ± SEM, unpaired two-tailed t-tests. Red star marks the position of the radio-label. f) Representative endonuclease activity kinetics of MR<sup>wt</sup>/ho/pSae2 (f) with quantification (g). Mean ± SEM, n = 3 independent experiments. Red star marks the position of the radio-label. h) Representative resection assay of MR<sup>ho</sup> with probe (green box in cartoon) binding to the 3'-overhang produced, n = 2. Note that the large MR<sup>ho</sup> oligomers in Fig. 4b are active in resection. i) Interaction assay of MR<sup>ho</sup>/lo with the C-terminal part of pSae2 (pSae2 ∆N169). Ponceau and anti-MBP western blot indicate elution of MBP-tagged pSae2 ∆N169. The anti-Rad50 western blot shows interaction of pSae2 ∆N169 with Rad50 in MR<sup>wt</sup>/ho/lo. One out of two independent experiments is shown. Scale bars: 100 nm (b, c).

MR<sup>ho</sup> formed larger oligomers on longer oligonucleotide DNA compared to MR<sup>wt</sup>, as the peak at ~1000 kDa of two MR<sup>ho</sup> dimers binding to one DNA molecule could be detected and properly fitted already for 73 bp with a larger peak area than for MR<sup>wt</sup> (compare 73 bp zoom-insets in Figs. 1f and 3c). Moreover, the areas of the MR-DNA peaks decreased more with 106 and 197 bp DNA for MR<sup>ho</sup> than for MR<sup>wt</sup>, indicating the formation of larger MR<sup>ho</sup> nucleoprotein assemblies beyond the detection limit (compare peak areas for 106 and 197 bp in Figs. 1f and 3c). For MR<sup>lo</sup>, on the other hand, binding to oligonucleotide DNA could not be observed in mass photometry at the low MR and DNA concentrations required for measuring (197 bp in Supplementary Fig. 5b).

MR oligomerization influences DNA binding behavior and endonuclease activity. EMSA experiments revealed a moderate DNA binding impairment of the MR<sup>lo</sup> mutant compared to MR<sup>wt</sup> (Fig. 4a and Supplementary Figs. 2a and 5f); however, at ~90 nM, MR<sup>lo</sup> efficiently bound the 100 bp-long DNA substrate. Similarly, 90 nM MR<sup>ho</sup> was also bound to linear plasmid-length DNA (35 mM KCl, MR<sup>lo</sup>/DNA 36:1) in negative staining TEM, appearing as dispersed single dimers (Fig. 4b). Surprisingly, the DNA binding of the MR<sup>ho</sup> mutant was also reduced in EMSA experiments (Fig. 4a and Supplementary Figs. 2a and 5f), indicating that higher oligomeric structures may interfere with disassembly to smaller MR<sup>ho</sup> complexes including dimers and thus efficient loading onto DNA. Mass photometry measurements at low salt (25 mM KCl, zoom-insets in Supplementary Fig. 5g) revealed that once MR<sup>ho</sup> loaded onto plasmid DNA, it assembled as one or maximally two MR<sup>ho</sup> dimers per DNA molecule (in 1500–2500 kDa range, zoom-inset in Supplementary Fig. 5g) 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<sup>ho</sup> compared to MR<sup>wt</sup> (25 mM KCl, zoom-insets in Supplementary Fig. 5g), and even more so after incubation (25 mM KCl, 30 min, zoom-insets in Supplementary Fig. 5g). Moreover, TEM analysis demonstrated that MR<sup>ho</sup> assembled into defined, condensed “worm”-like structures once it bound plasmid DNA (90 nM MR<sup>ho</sup>, 35 mM KCl, MR<sup>ho</sup>:DNA 36:1; Fig. 4b), while MR<sup>wt</sup> formed much smaller oligomers at comparable conditions (100 nM MR<sup>wt</sup>; Fig. 1b–ii and Supplementary Fig. 1e). We next expressed the Mre11<sup>wt</sup> and Rad50<sup>wt/ho/lo</sup> subunits individually from yeast, and observed that Mre11<sup>wt</sup> alone did not oligomerize on DNA (Fig. 4c). In contrast, oligomerization of the Rad50<sup>wt/ho/lo</sup> subunits recapitulated the behavior of the respective MR heterodimers (Figs. 1b–ii and 4b, c); Rad50<sup>ho</sup> formed larger oligomeric assemblies on DNA compared to Rad50<sup>wt</sup>, while Rad50<sup>lo</sup> bound DNA dispersely. Taken together, these results suggest that Rad50 is necessary and sufficient to drive oligomerization of the MR complex, most likely through a conserved beta-sheet motif.

We next performed clamping assays using oligonucleotide-based DNA with blocked ends to assess if MR oligomerization affects its endonuclease activity in conjunction with pSae2. Interestingly, the MR<sup>lo</sup> mutant was entirely endonuclease-deficient, even when used at a concentration (200 nM) that supports DNA binding (Fig. 4d, e and Supplementary Fig. 6a). Conversely, MR<sup>ho</sup> was slightly more active than MR<sup>wt</sup> (Fig. 4d, e), which was particularly apparent upon short incubation times during kinetic experiments with oligonucleotide-based DNA (Fig. 4f, g). Using plasmid-length substrates, we observed that conditions resulting in large “worm”-like assemblies support DNA end resection (90–100 nM MR<sup>ho</sup>, Fig. 4b, h). A further increase of the MR<sup>ho</sup> concentration to 200 nM additionally enhanced resection activity (Fig. 4b), suggesting that the assemblies are sites of efficient DNA end resection. The initial resection by MR<sup>ho</sup> could efficiently be extended by Exo1 (Supplementary Fig. 6b–d), similarly to MR<sup>wt</sup> (Fig. 2a, b), indicating that the large protein assembly does not interfere with downstream long-range resection.

Notably, both MR<sup>ho</sup> and MR<sup>lo</sup> were proficient as exonucleases and functionally interacted with ATP (Supplementary Fig. 6e). Moreover, both MR variants were able to bind pSae2 (Fig. 4i), as shown in a protein interaction assay using the conserved C-terminal part of Sae2 (pSae2 ΔN169)<sup>19</sup>. These experiments demonstrate that although endonuclease-deficient, the Rad50<sup>lo</sup> variant is clearly distinct from the Rad50<sup>wt</sup> mutants, which fail to physically and functionally interact with Sae2<sup>19,52</sup>. Taken together, these data suggest that the conserved beta-sheet motif in Rad50 regulates MR oligomerization, and that oligomerization in turn promotes MR resection activity.

**Foci formation and DNA repair require MRX oligomerization in vivo.** In order to probe the function of MRX oligomerization in vivo, we introduced Rad50<sup>wt</sup> or the Rad50<sup>ho/lo</sup> mutants in an S. cerevisiae rad50 deletion (rad50Δ) strain together with a C-terminal yEVenus- tag (rad50<sup>wt</sup>-yEVenus, rad50<sup>ho</sup>; rad50<sup>N121A/D124N-yEVenus, rad50<sup>ho</sup>; rad50<sup>116A/119A/T127A/L128A-yEVenus</sup>, rad50<sup>ho</sup>), while Mre11 and Xrs2 were unaltered. DNA damage sensitivity similarly to wild-type, while cells with Rad50<sup>ho</sup> displayed a phenotype comparable to rad50Δ (Fig. 5a). This defect was recapitulated by proliferation assays with 5 or 25 μM CPT; whereas the doubling times of wild-type and Rad50<sup>ho</sup>, expressing cells only marginally increased upon CPT-treatment, cells with Rad50<sup>ho</sup> or rad50Δ were more sensitive to DNA damage, especially at higher CPT concentrations (Fig. 5b). We then engineered wild-type yeast strains overexpressing untagged Rad50<sup>ho/lo</sup> from the galactose-inducible GAL1 promoter (Fig. 5c, d). We found that overexpression of Rad50<sup>ho</sup> on top of endogenous Rad50<sup>wt</sup> results in a pronounced growth defect and DNA damage sensitivity (Fig. 5d). Although we cannot exclude that the introduced mutations affect the endonuclease activity by altering the local structure, these results suggest that Rad50<sup>ho</sup> retains the capacity to act in a dominant-negative manner, most likely by inhibiting oligomerization of the wild-type MRX pool.

Live-cell imaging of yEVenus-tagged Rad50 variants revealed the formation of small Rad50<sup>ho/lo</sup> foci at potential DNA damage sites in the nucleus of CPT-treated cells (arrows, Fig. 5e). Interestingly, the Rad50<sup>ho</sup> mutant instead failed to assemble distinguishable foci (Fig. 5e), although we cannot exclude that its slightly reduced protein levels contribute to this defect (Fig. 5f). Conversely, Rad50<sup>lo</sup> formed foci even in the absence of CPT-induced DNA damage (Fig. 5e, g and Supplementary Fig. 7a). These data support the notion that MRX oligomerization promotes stable DNA binding required for the assembly of large repair structures detectable as foci in vivo.

**DNA damage checkpoint activation and telomere maintenance.** Upon DNA damage, the checkpoint-kinase Tel1 is recruited by MRX to repair foci at DNA lesions and phosphorylates target proteins including Rad53, which delays cell cycle progression until the DNA damage is repaired<sup>5</sup>. A single unrepaired DSB is sufficient to trigger the DNA damage checkpoint in yeast<sup>53</sup>. We hypothesized that MRX oligomerization at DNA lesions could have a function in amplifying the checkpoint signal. Indeed, the phosphorylation-mediated mobility shift of Rad53 upon DNA damage was reduced in cells expressing untagged Rad50<sup>ho</sup> compared to Rad50<sup>wt</sup> or Rad50<sup>lo</sup> in western blotting; however, the limited Rad53 phosphorylation was still sufficient to allow full arrest of Rad50<sup>ho</sup>-expressing cells after HU treatment, as demonstrated by flow cytometry (Fig. 6a and Supplementary Fig. 7b). MRX and Tel1 are, however, also required for the maintenance of telomere length — a function that does not require the nuclease activity of the MRX complex<sup>41</sup>. A characteristic feature of rad50Δ cells are thus shortened telomeres (Fig. 6b). Telomeric PCR experiments revealed that yeast cells carrying untagged Rad50<sup>ho</sup> suffered from short telomeres similarly to rad50Δ, while cells with Rad50<sup>ho</sup> maintained normal telomere length (Fig. 6b, c and Supplementary Fig. 7c). Taken together, these data suggest that MRX oligomerization at DNA damage sites, governed by the conserved Rad50 beta-sheet motif, facilitates the assembly of protein foci, which is required for the DNA repair, signaling, and telomere maintenance functions of MRX in vivo.

**Discussion**

Here, we show that S. cerevisiae MR(X) dimers oligomerize via the head domain of Rad50 to nucleolytically active assemblies at DNA ends. The formation of these higher-order MR complexes is governed by a conserved beta-sheet motif in the Rad50 head domain. Mutational analysis of the beta-sheet motif identified specific mutations that preserve MR dimerization but decrease or enhance MR head domain oligomerization in vitro. Biochemical reconstitutions coupled with TEM imaging revealed that MR oligomerization is required for pSae2-stimulated endonucleolytic DNA cleavage and hence short-range resection but is dispensable for MR exonuclease activity. Subsequent analysis of the Rad50 mutants in vivo confirmed the importance of MRX oligomerization for its cellular functions in DNA repair, signaling, and telomere maintenance.
Fig. 5 Foci formation and DNA repair require MRX oligomerization in vivo. a Serial dilution of exponentially growing S. cerevisiae rad50Δ cells expressing Rad50wt/ho/lo-yEVenus, spotted on agar plates (YPD) +/- DNA damage-inducing camptothecin (CPT) or hydroxyurea (HU), and incubated for 2 days at 30 °C. n = 3. b Doubling times of exponentially growing rad50Δ cells and rad50Δ strains expressing Rad50wt/ho/lo-yEVenus +/- CPT treatment. Mean and minimum to maximum values shown, n = 3 independent experiments. c Anti-Rad50 western blot showing expression levels of Rad50wt/ho/lo-yEVenus in wild-type cells (–Gal-OE). Center panel is contrast-enhanced to show endogenous Rad50wt levels (wild-type, α-tubulin) and pSae2 complexes to higher-order assemblies can occur internally of DNA, but resection is restricted to DNA ends. By sliding along DNA as previously shown55, MR could accumulate at the DNA end, where the MR endonuclease is locally activated by pSae2. The resulting short-range processing of blocked ends is prerequisite for subsequent handover to Exo1-mediated long-range resection. After cleavage, MR-pSae2

Using reconstituted biochemical reactions, we could visualize intermediates of DNA end resection in vitro. Our biochemical assays coupled with TEM analysis recapitulated MR-pSae2-dependent short-range processing and subsequent tail elongation by Exo1. TEM imaging revealed that oligomerization of MR and pSae2 complexes to higher-order assemblies can occur

\[ a \text{ YPD} \]

\[ 25 \mu M \text{ CPT} \]

\[ 25 \text{ mM HU} \]

do similarly resistant to DNA damage as wild-type cells due to endogenous Rad50wt (and minimum to maximum values shown, untreated or CPT-treated α-tubulin controls equal loading. n = 3. d Serial dilution of exponentially growing wild-type cells expressing galactose-inducible Rad50wt/ho/lo-yEVenus, spotted on SD-URA agar plates with glucose (–) or galactose (+ variant overexpression), and incubated at 30 °C for 2 days. Before overexpression, cells carrying Rad50wt/ho/lo were similarly resistant to DNA damage as wild-type cells due to endogenous Rad50wt (–, top). n = 3. e Representative live-cell images of untreated or CPT-treated rad50Δ cells expressing Rad50wt/ho/lo-yEVenus with histone Hta2-mCherry as nuclear marker. Arrows indicate nuclear Rad50foci (Supplementary Fig. 7a). f Anti-Rad50 western blot showing expression levels of Rad50wt/ho/lo-yEVenus in rad50Δ cells before (–) and after (+) CPT treatment. α-Tubulin controls equal loading. n = 3. g Quantification of live-cell imaging in e. The percentage of cells with nuclear Rad50-yEVenus foci (arrows in e) is shown for untreated and CPT-treated rad50Δ cells expressing Rad50wt/ho/lo-yEVenus. Mean ± SD, n = 3 independent experiments, unpaired two-tailed t-test. Scale bars: 5 μm.
assemblies dissociate from RPA-coated single-stranded DNA ends to allow subsequent steps in the DSB repair pathway.

Previous work showed that an initial endonucleolytic cut by MRX-pSae2 is positioned 15–35 nt away from the DNA end when using oligonucleotide-based DNA\(^\text{12}\). On longer DNA templates, however, additional MRX-pSae2 incisions were found that led to the resection of several hundred nucleotides in length\(^\text{21}\), consistent with observations from meiotic cells lacking the long-range resection pathways\(^\text{5,20}\). Accordingly, a stepwise DNA cleavage model for 5′-DNA end resection by MRX-Sae2 was proposed\(^\text{21}\). Here, our experiments revealed that MR molecules form large assemblies, which provide a structural basis to explain the multiple endonucleolytic cuts along the 5′-terminated DNA strand during short-range resection.

Mutations in a protruding beta-sheet in the Rad50 subunit allowed us to construct two MR mutants, one with enhanced (MR\(^\text{ho}\)) and one with reduced oligomerization (MR\(^\text{lo}\)). Although available results demonstrate that MR oligomerization is dependent on the Rad50 head domain and beta-sheet interactions, the resolution of the TEM images does not allow to prove that the beta-sheet motif directly mediates this effect. Nevertheless, MR\(^\text{ho}\) shows slightly increased resection activity compared to MR\(^\text{wt}\), and enhanced foci formation in living cells. As MR\(^\text{ho}\) displays enhanced oligomerization despite the loss of an attractive salt bridge between Sc D124 and R125 (Ct E125 and R126) due to the D124N mutation, we propose that the abolished charge repulsion of D124N residues between MR\(^\text{ho}\) molecules could drive this behavior. Although in the crystal structure model with two separate and only “quasi-continuous” DNA molecules (Fig. 3a) the Ct E125 (Sc D124) residues of adjacent Rad50 dimers are ~5 Å apart, they may be closer on a continuous DNA molecule in solution. Moreover, the hydrophobic residues in the beta-sheet could come closer when charge interactions are alleviated, which could explain the lower salt-sensitivity of MR\(^\text{ho}\) oligomers and the more pronounced beta-sheet signal in ThT staining. Yet, other head domain interactions are possible and high-resolution structures of MR dimers adjacent bound on DNA will ultimately be needed to demonstrate the exact residues mediating beta-sheet-driven MR oligomerization.

In contrast to MR\(^\text{wt}\) and MR\(^\text{ho}\), MR\(^\text{lo}\) was endonuclease-deficient in vitro and failed to form DNA damage-induced nuclear foci in vivo, resulting in cellular sensitivity to DNA damage, an altered checkpoint response and short telomeres. Moreover, Rad50\(^\text{lo}\) acts as a dominant-negative factor on endogenous Rad50\(^\text{wt}\) in overexpression experiments in yeast cells. Although we cannot exclude that the mutations in MR\(^\text{lo}\) negatively affect MR\(^\text{lo}\) activity beyond disrupting oligomerization, we note that oligomerization accompanies resection in MR\(^\text{wt}\), and that the Rad50\(^\text{lo}\) mutant still binds pSae2, Mre11, and Xrs2 that mediates the observed nuclear localization in cells\(^\text{17}\). Moreover, MR\(^\text{lo}\) shows normal levels of exonuclease activity, which is inhibited by ATPyS, as observed for wild-type MR\(^\text{21}\), indicating that the nuclease active site of MR\(^\text{lo}\) is sufficiently intact, given that the endo- and exonuclease activities of MR are mediated through the same active site\(^\text{56}\). The defects of MR\(^\text{lo}\) are unlikely explained with reduced DNA binding, as increased MR\(^\text{lo}\) concentrations restored DNA binding but not its enzymatic activity. In addition, MR\(^\text{ho}\) was more active as endonuclease than MR\(^\text{wt}\), despite being impaired in DNA binding as well. We thus propose that MR oligomerization on DNA might facilitate the drastic conformational changes required for nuclease activation\(^\text{38,57}\), and promote activation of adjacent MR molecules on the 5′-terminated DNA strand, thereby creating a “domino-like” effect close to the DNA ends that explains step-wise short-range resection.

Repair foci with hundreds to thousands of MRX/N molecules were found at DNA breaks in yeast and mammalian cells\(^\text{23–28}\). ChIP-seq data of human MRN show an accumulation of the complex across a DSB on both sides of broken DNA, covering ~1 kb of DNA, with the strongest MRN localization at the DNA ends, indicating MRN focus formation around a DSB and tethering of broken ends\(^\text{58}\). In accord, our TEM analysis revealed that MR clusters can hold several DNA molecules together, mediated by oligomerization of the MR head domains and...
interactions via the zinc hooks. We thus propose that MR head domain oligomerization together with DNA end tethering may explain the coordinated resection observed at two-ended DNA breaks. Consistent with this interpretation, no nuclear foci were detected upon DNA damage for γEVENus-tagged oligomerization-defective MRΔX in S. cerevisiae, while the hyper-oligomerizing MRΔ50X mutant formed nuclear foci even in the absence of exogenous DNA lesions. Moreover, pulldown and ThT assays detected stable MR assemblies, which could build a rather rigid core inside potentially liquid-like repair foci, as formed, e.g., by the NHEJ-factor 53BP1 or the strand-exchange protein Rad52Δ51, and act as scaffold for transiently joining downstream repair and signaling components including pSae2/pCIP and Teli/ATM.

Interestingly, database (ClinVar/MedGen) and literature reports suggest that human individuals with a hereditary predisposition for BRCA1-2- and PALB2-associated cancers carry germline point mutations of predominantly hydrophobic residues in the human RAD50 beta-sheet motif. The tumor suppressor BRCA1 interacts with PALB2 and BRCA2, and localizes to repair foci at DNA lesions similarly to MRΔ50. Loss of BRCA1/2 function leads to aberrant degradation of nascent DNA by MRE11 upon replication stress, and a dramatic increase in the lifetime risk for ovarian and breast cancer.65–67 The abrogation of MRE11 activity in BRCA1-2-deficient cells, on the other hand, was linked to PARP inhibitor resistance in breast chemotherapy.68 It is intriguing that MRΔ90, bearing beta-sheet-disturbing alanine mutations of four hydrophobic residues altered in cancer predisposed individuals, shows impaired endonuclease activity in vitro. These loss-of-function RAD50 mutations could restrict MRE11-mediated degradation of nascent DNA at challenged replication forks in PARP-resistant, BRCA1/2-PALB2-mutated cells, and may help to explain tumor chemoresistance. However, the importance of these RAD50 mutations for cancer risk and survival is not well understood and thus more extensive studies are needed.

Methods

Preparation of recombinant proteins. Yeast Mre11-Rad50 complexes were co-expressed in S. cerevisiae in high yields from high-copy (9) cells with an optimal ratio of baculovirus-produced Mre11wt-6xHis and untagged Rad50wt, Rad50ho, or Rad50lo that was optimized for S9 expression to achieve 1:1 MR subunit stoichiometry. MR complexes were expressed using vectors for His-tagged Mre11, untagged Rad50, and FLAG-tagged Xrs2 and purified via NINTA and FLAG affinity chromatography45. Recombinant phosphorylated Sae2 (pSae2) was purified from S9 cells by amylase and NINTA affinity chromatography in the presence of phosphatase inhibitors. Yeast RPA was expressed from the p11d-scRPA vector (kind gift from M. Wold, University of Iowa) in E. coli (BL21 DE3 pLYS) and purified as described for human RPA.13

DNA substrates. Oligonucleotides used in this study are listed in Supplementary Table 1. 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 5'-end with [γ-32P]ATP (PerkinElmer) and terminal transferase (New England Biolabs) following the manufacturer’s recommendations. For mass photometry, oligonucleotides of 20, 50, 73, 106, and 197 bp (Supplementary Table 1) and 2.7 kb-long pUC19 were used. For TEM, circular 5.3 kb-long pFBDM (Addgene; Fig. 1b-iii and Supplementary Fig. 1), linear 2.7 kb-long pUC19 with free 4 nt-overhangs or a 2.8 kb-long, pUC19-derived linear plasmid with 50 nM MR subunit stoichiometry were used. Where indicated, circular, relaxed or linear 2.7 kb-long pUC19 with free 4 nt-overhangs, and a 2.8 kb-long, pUC19-derived blunt linear plasmid with streptavidin-blocked ends were used for EMSAs and nucleases assays. The circular, supercoiled form of pUC19 was relaxed with Topoisomerase I from E. coli (New England Biolabs) and purified using a MiniElute PCR purification kit (Qiagen). Linearized pUC19 was prepared with restriction digestion using KpnI-HF (New England Biolabs) and 0.8% agarose-gel purification. The 2.8 kb-long, pUC19-derived linear plasmid substrate with biotin at the ends was prepared using pAtgP5-vector, annealed PC210 and PC211 oligonucleotides for hot annealing (5′-end), and 5′-C31 integrase. After incubation with monovalent streptavidin (kind gift from M. Howarth, University of Oxford), streptavidin-blocked 2.8 kb-long pUC19 was purified with Chromaspin TE-200 columns (Takara). For Telo-meric PCR, the 100 µM stocks of oligonucleotides listed in Supplementary Table 1 were diluted in sterile water to a working concentration of 10 µM. OBL359 was purchased from Microsynth; OBL358, OBL360, and OBL361 from Merck Millipore.

Negative staining TEM. Purified proteins were mixed at the indicated concentration with or without plasmid substrate (5, 10, and 15 µg/ml) in a reaction buffer containing 25 mM Tris acetate pH 7.5, 1 mM dithiothreitol (DTT) and the co-factors 5 mM magnesium acetate (Sigma-Aldrich, M5661), 1 mM manganese chloride (Sigma-Aldrich, M3634) and 1 mM ATPγS (Sigma-Aldrich, A1388), unless indicated otherwise. In Supplementary Fig. 1f-ii and for resection in Fig. 2d-iii, III, IV, 50 nM Exo1 and RPA to a 2.8 kb-long, pUC19-derived linear plasmid (in molecules) was incubated for 2.5 h at 30 °C and 50 mM KCl. For Fig. 2d-i, II, III, 50 nM MRwt and 200 nM pSae2 were added and incubated for 2.5 h at 30 °C with 40 mM KCl. For Fig. 2d-ii, IV, 5 nM 2.8 kb-long, linear plasmid DNA (in molecules) was incubated with 50 nM Exo1 and 200 nM RPA for 5 min at 30 °C and 40 mM KCl. For Fig. 2d-ii, IV, 5 nM 2.8 kb-long, linear plasmid DNA (in molecules) carrying two streptavidin blocks per end was incubated with 50 nM Exo1 and 75 mM RPA for 2.5 h at 30 °C and 40 mM KCl. For Fig. 2d-ii, IV, 50 nM MRwt and 200 nM pSae2 were incubated with 5 nM of the substrate (in molecules) for 2.5 h at 30 °C and 40 mM KCl. For Fig. 2d-ii, IV, 50 nM MRwt and 200 nM pSae2 were incubated with 5 nM of the substrate (in molecules) for 2.5 h at 30 °C and 40 mM KCl. For Fig. 2d-ii, III, IV, 50 nM Exo1 and RPA to a final concentration of 670 nM were added to the reaction with MRwt-pSae2 (Fig. 2d-iii, II) for 10 min at 30 °C and 50 mM KCl.

For TEM grid preparation, 5 µl of the reaction mixture were incubated for 1 min at room temperature on a carbon film 300 mesh copper grid (CF300-CU from Electron Microscopy Sciences) that was negatively glow discharged with the Emsco Group® glow discharge system for 45 s with 25 mA. After blotting away the excess sample with Whatman filter paper, the grid was washed twice in EM buffer (20 mM HEPES pH 7.55, 130 mM NaCl, 2% glycerol, 0.5 mM DTT), stained in two droplets of 2% uranyl acetate, and air dried. TEM micrographs were acquired with a FEI Morgagni 268 microscope at 100 kV using the Morgagni User Interface 3.0, TemCam® software and a CCD camera at 80,000× magnification. All samples were imaged at least three times, and image analysis was performed using Fiji/ImageJ 1.52t, Excel 2016, and GraphPad Prism 9.

EMSAs. DNA binding experiments with oligonucleotide-based DNA were carried out in buffer containing 25 mM Tris acetate pH 7.5, 1 mM DTT, 0.25 mg/ml bovine serum albumin (BSA), 5 mM magnesium acetate, and 1 mM ATPγS. To keep the total DNA amount constant, 5 nM 20 bp, 3.22 nM 31 bp, 1.43 nM 70 bp, 0.814 nM 197 bp, and 0.51 nM 317 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 µl reaction volume at 30 mM NaCl. Other indicated final salt concentrations were reached by NaCl addition. After incubation, the samples were supplemented with 5 µl loading dye (50% glycerol with bromophenol blue) and loaded onto a 6% polyacrylamide gel. 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 using a Typhoon FLA 9000 imager with Typhoon FLA 9500 software (GE Healthcare), quantitated with Fiji/ImageJ 1.52t and Excel 2016, and statistically analyzed with GraphPad Prism 9.

For DNA binding experiments with plasmid-based DNA 2.5 nM (in molecules) circular 2.7 kb-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 kb plasmid substrate. Reactions of 20 µl were mixed in buffer containing 25 mM Tris acetate pH 7.5, 1 mM DTT, 0.25 mg/ml bovine serum albumin, 0.5 mM magnesium chloride, and 1 mM ATPγS. After incubation for 1 h at 30 °C, the reactions were mixed with TriTrack DNA loading dye 6x (Thermo Scientific®) to

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Nuclease assays. Endonuclease assays (15 µl volume, unless indicated otherwise) were performed with 1 nM (in molecules) 70 bp-long DNA substrate labeled at the 5′-end in reaction buffer containing 25 mM Tris acetate pH 7.5, 1 mM DTT, 80 µl pyruvate kinase, 1 mM PEP, 0.25 mg/ml BSA, 5 mM magnesium acetate, 1 mM ATP and ATPγS where indicated. After protein addition, the reactions were incubated for 30 min, 30 °C, and subsequently stopped and processed as described above for the endonuclease assays.

Nuclease assays on plasmid-length substrate were performed with 1 nM (in molecules) 2.8 kb-long, pUC19-derived substrate obtained with ΦC31 integrase (as described in the “DNA substrates” section) in reaction buffer as used for endonuclease assays but in the addition of 10% glycerol. The reactions were stopped by addition of 0.5 µl of 4-12% agarose gel. The gels were then dried on DE81 chromatography paper. 1% agarose gel. The gels were then dried on DE81 chromatography paper. 1% agarose gel. The gels were then dried on DE81 chromatography paper.

For sample delivery, a silicone gasket (CultureWell™ Reusable 1% poly-lysine solution. For each measurement, a clean coverslip was placed onto a 1% agarose gel (Whatman) and processed as described above.

DNA to keep the total DNA amount constant (100 nM base pairs). Peaks were detected and fitted with Gaussian functions where possible. For Supplementary Figures 3c and 5b, 5 nM Mre11/Rad50/Xrs2/Mrc1/toothless/MRX were measured with or without 2.5 mM 197 bp-long oligonucleotide DNA at 25 mM KCl. For mass spectrometry experiments, Supplementary Figure 6d without DNA, 10 nM Mrc1 was incubated at 250 mM KCl or 25 mM KCl and 30 °C for 30 min with co-factors as afore-mentioned. Binding was monitored for 40s.

For Supplementary Figs. 3e and 5g with 2.7 kbp plasmid DNA, 20 nM MRXwt MRX was first incubated with 1 nM substrate (in molecules) for 30 min at 250 mM KCl and subsequently measured (labeled as 250 mM KCl, 30 min). MRXwt (~460 kDa) and plasmid DNA (~1300 kDa) appeared mostly unbound, while MRX and MRXroformed oligomers without DNA beyond the detection limit such that free MRX or MRXrodimers could be detected at ~550 or ~460 kDa, respectively. Secondly, the salt in the sample was diluted to 25 mM KCL while keeping the protein and DNA concentrations constant, and the sample was immediately analyzed (labeled 25 mM KCl). Much less unbound MRX, MRXro, and plasmid DNA could be detected. For MRX and MRXro, however, the decrease in unbound plasmid DNA was smaller than for MRX, indicating that they form larger oligomers in the absence of DNA molecule. For MRXro, a tail of counts at higher molecular weight >2500 kDa, corresponding to higher-order MR-DNA assemblies, could be detected. MRX and MRXro, on the other hand, formed much larger nucleoprotein assemblies beyond the detection limit (smaller tail of counts >2500 kDa). Third, the sample with 25 mM KCl was incubated for 30 min at 30 °C and then measured (labeled 25 mM KCl, 30 min). For both MRX and MRXro, even fewer higher-order assemblies on DNA could be detected >2500 kDa, as they surpassed the detection limit, while for MRX the tail of counts >2500 kDa remained similar. Binding was monitored for 100 s.

For data acquisition, the gasket well was filled with 9 µl measuring buffer for adjustment of surface focusing. Afterwards, 1 µl of sample was added to the buffer in the well resulting in the final sample concentration. Protein and complex binding to the coverslip surface was monitored using AcquireMFP (Reflyn Ltd., version 2.3.0). Data analysis was performed by DiscoverMP (Reflyn Ltd, version 2.3.0). OriginPro 2017 and a custom-written Python program (Supplemental Software). For contrast to mass conversion, a known protein mass standard (NativeMark™ TM Unstained Protein Standard; Invitrogen) was measured on the same day. Due to calibration with respect to protein mass, the pUC19 peak in plasmid mass measurements appeared ~1300 kDa. All samples were measured at least three times.

Thioflavin T (ThT) fluorescence. For ThT measurements, 20 µl reactions of 1.2 µM MRXwt or MRXro were incubated for 30 min at 4 or 42 °C in phosphate buffer containing 20 mM phosphate solution pH 7.4 (KH2PO4, K2HPO4), 10% glycerol, 1 mM PMSF, 1 mM DTT, and 240 mM KCl. Thioflavin T (ThT; Sigma-Aldrich, T3516) was dissolved in water to a final concentration of 2.5 mM and filtered (0.2 µm; Millipore). ThT solution was added (1:10 dilution) to the samples, and fluorescence intensity measured in a 384-well plate (Corning Life Sciences) using a ClARIOstar plate reader (BMG Labtech, 5.40 R3 software). Excitation wavelength was set at 450 nm and emission recorded at 490 nm. Statistical analysis was performed with GraphPad Prism 9.

Pelleting assay. For pelleting assays, 60 µl reactions of 1.2 µM MRXwt or MRXro were incubated for 30 min at 4 or 42 °C in phosphate buffer as for ThT assays. After incubation, the reactions were centrifuged at 21,000g for 10 min at 4 °C. The resulting pellets were washed once with phosphate buffer, resuspended in sample buffer (116 mM Tris-HCl pH 6.8, 5% glycerol, 8 µl 5%, SDS, 1% beta-mercaptoethanol, 0.1% bromophenol blue) and loaded likewise to the supernatant sample buffer (116 mM Tris-HCl pH 6.8, 5% glycerol, 8 µl 5%, SDS, 1% beta-mercaptoethanol, 0.1% bromophenol blue) and loaded likewise to the supernatant fractions onto a denaturing 4–12% Bis-Tris SDS-PAGE (Thermo Fisher Scientific) that was then Coomassie-stained. MRXwt and MRXro were normalized in intensity to their input using Fiji/ImageJ (5.2t) and Excel 2016; statistical analysis was performed with GraphPad Prism 9.

pSae2-Rad50 protein interaction assay. For interaction assays, 50 µl of soluble extract from S. cerevisiae, expressing the MBP-tagged C-terminal part of pSae2 (pSae2 ΔN169) with phosphate inhibitors, were incubated with 50 µl Amlyase resin (New England Biolabs) in 450 µl lysis buffer (50 mM Tris-HCl pH 7.5, 2 mM beta-mercaptoethanol, 1 mM EDTA, 15.6 % glycerol, 305 mM NaCl, 30 µg/ml leupeptin, 1 mM PMSF, and protease inhibitor cocktail (1:400, Sigma-Aldrich, P8340)) for 1 h at 4 °C with agitation. The resin was then centrifuged at 2000g for 2 min at 4 °C and washed five times with wash buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% NP40, 2 mM EDTA, and 1 mM PMSF in a total volume of 1.3 ml. After the addition of 1.5 µg purified histone H2A.1 and 250 mM KCl to the wash buffer, the mix was further incubated for 1 h at 4 °C with agitation. MRXwt and MRXro controls were also incubated with amylase resin without pSae2 ΔN169. The resin was then centrifuged and washed five times as described above. MBP-tagged pSae2 ΔN169 bound to pulled-down MRXwt or MRXro interactions was eluted with 100 µl wash buffer containing 20 mM KCl (CaCl2 and MgCl2 elution buffer) overnight and then loaded with 1× NuPAGE™ LDS Sample buffer on a denaturing 4–12% Bis-Tris SDS-PAGE (both Thermo Fisher Scientific). Western blots were probed with mouse a-MBP antibody (1:1000, Abcam, ab49923), rabbit a-Rad50 antibody (1:1000, ThermoScientific, PAS-31276), and goat a-rabbit or a-mouse HRP-conjugated secondary antibody (1:2000, BioRad, 170-6519 or 170-6516, respectively). The blots were imaged using a Vilber Fx6 system and FusionCapt Adv FX7 software.

Yeast plasmids and strains. Construction of yeast plasmids (Supplementary Table 2) and strains (Supplementary Table 3) was performed according to standard
molecular biology methods. Yeast strains used in this study are derivatives of BY4741. Cells were grown at 30 °C in yeast extract peptone dextrose, YPD (2% glucose, 1% yeast extract) or synthetic SD media (2% glucose, 0.17% yeast nitrogen base, 0.5% NH₄ sulfate, and amino acids). For galactose-induced overexpression experiments, overnight grown cells in SD media (2% raffinose) were diluted in SD 2% galactose or SD 2% glucose for 5 h before spotting assays or harvesting protein samples.

DNA damage sensitivity spotting and growth assays. Serial dilutions of exponentially growing cells were spotted on YPD or SD-Ura (with 2% glucose or 2% galactose) agar plates without or with DNA damaging agents: Camptothecin, CPT 25 µM (Sigma-Aldrich, C9911) and Hydroxyurea, HU 25 mM (Sigma-Aldrich, H8627). Agar plates were incubated at 30 °C and image after 2 days using a Vhz 350 confocal microscope (Vilber Lourmat, France) and FP condensed Axv7 software. For growth rates, OD₆₀₀ of exponentially growing cells was measured in the absence or presence of CPT (5 or 25 µM) in a 24-well plate using a plate reader (CLARIOStar BMG Labtech, 5.40 R3 software). Doubling times for exponential growth were calculated using the following Eq. (1):

\[ \text{Doubling time (hours)} = \frac{\text{duration (hours)} \times \log(2)}{\log(\text{final OD}_{600}) - \log(\text{initial OD}_{600})} \]  

Results from independent replicates (n = 3) were averaged and displayed using GraphPad Prism 9.

Live-cell fluorescence microscopy and image analysis. Yeast cells were grown overnight in SD media (2% glucose) and then diluted into fresh growth medium to resume exponential phase growth. Before imaging, cells were left untreated or treated with 250 µM CPT for 2 h. Live-cell microscopy was performed using a spinning disk confocal fluorescence microscope (Vistanorm system) equipped with an inverted microscope (Nikon Eclipse TiE, an EM-CCD camera (Andor iXon Ultra, Andor), a motorized XY stage, and a piezo Z drive. The system is controlled with the Fiji/IMAGEJ 1.52t software. For flow rates, OD₆₀₀ of exponentially growing cells was measured in the presence or absence of CPT (5 or 25 µM) in a 24-well plate using a plate reader (CLARIOStar BMG Labtech, 5.40 R3 software). Doubling times for exponential growth were calculated using the following Eq. (1):

\[ \text{Doubling time (hours)} = \frac{\text{duration (hours)} \times \log(2)}{\log(\text{final OD}_{600}) - \log(\text{initial OD}_{600})} \]  

Statistical analysis. All data shown in this study are representative of at least three independent experiments if not stated otherwise in the figure legends. See respective figure legends and “Methods” section for details to plotted data points, error bars, and specific data analyses. Statistical unpaired, two-tailed significance tests were performed using GraphPad Prism 9. 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 (Pelel) and 3.05 (Sup), each with 4 degrees of freedom. For Fig. 4e, 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. 5g, the t-value was 3.03 with 4 degrees of freedom. For the non-significant t-test in Supplementary Fig. 4a, the t-value was 1.47 with 198 degrees of freedom. For Supplementary Fig. 4a, the t-values were 4.28 (**), 6.6 (***) and 22.86 (****) with 6 degrees of freedom each. For Supplementary Fig. 5e, the t-values were 3.56 (**), 6.25 (***) and 22.51 (****) with 6 degrees of freedom each.

Code availability
An exemplary custom-written Python code for the analysis of mass photometer data is provided with this study as a Supplementary Software file.

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**Author contributions**

V.M.K. purified proteins, designed the Rad50 mutants with structural support from R.G., developed the in vitro pathway reconstitution technique in negative staining TEM, and performed experimental design, TEM analysis, biochemical assays, statistical analysis, conceptualization and visualization. G.R. purified proteins, and conducted, analyzed, and conceptualized radioactive DNA binding and activity assays. E.B. designed the yeast experiments, carried out the quantitative live-cell imaging, and performed image analysis with the help of S.S.L.; E.B. and J.T. engineered the yeast strains, and conducted immunoblotting, growth measurements, spotting assays, and data analysis. K.K. and R.S. are responsible for the mass photometry analysis; G.C. carried out the ThT fluorescence measurements and pelleting assays; N.S. and B.L. conducted the telomeric PCR, Rad53 immunoblotting, and flow cytometry analysis. M.P. and P.C. designed, supervised, and conceptualized the study; V.M.K., P.C., and M.P. wrote the paper with critical input from all authors. K.K. and J.T. contributed equally.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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