The role of the Mre11–Rad50–Nbs1 complex in double-strand break repair—facts and myths

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Received January 19, 2016; Revised February 16, 2016; Accepted February 23, 2016

ABSTRACT

Homologous recombination (HR) initiates double-strand break (DSB) repair by digesting 5′-termini at DSBs, the biochemical reaction called DSB resection, during which DSBs are processed by nucleases to generate 3′ single-strand DNA. Rad51 recombinase polymerizes along resected DNA, and the resulting Rad51–DNA complex undergoes homology search. Although DSB resection by the Mre11 nuclease plays a critical role in HR in Saccharomyces cerevisiae, it remains elusive whether DSB resection by Mre11 significantly contributes to HR-dependent DSB repair in mammalian cells. Depletion of Mre11 decreases the efficiency of DSB resection only by 2- to 3-fold in mammalian cells. We show that although Mre11 is required for efficient HR-dependent repair of ionizing-radiation-induced DSBs, Mre11 is largely dispensable for DSB resection in both chicken DT40 and human TK6 B cell lines. Moreover, a 2- to 3-fold decrease in DSB resection has virtually no impact on the efficiency of HR. Thus, although a large number of researchers have reported the vital role of Mre11-mediated DSB resection in HR, the role may not explain the very severe defect in HR in Mre11-deficient cells, including their lethality. We here show experimental evidence for the additional roles of Mre11 in (i) elimination of chemical adducts from DSB ends for subsequent DSB repair, and (ii) maintaining HR intermediates for their proper resolution.

KEYWORDS: double-strand break repair, double-strand break resection, etoposide, homologous recombination, ionizing radiation, Mre11, non-homologous end joining, topoisomerase

THE CHOICE OF THE TWO MAJOR DSB REPAIR PATHWAYS: HOMOLOGOUS RECOMBINATION AND NONHOMOLOGOUS END JOINING

DNA double-strand breaks (DSBs) are the most dangerous type of DNA damage, as a single unrepaired DSB can trigger apoptosis. DSBs are generated during physiological replication, and are induced by radiotherapy and chemotherapeutic reagents such as the topoisomerase poison. There are two major DSB repair pathways in eukaryotic cells: homologous recombination (HR) and non-homologous end joining (NHEJ).

The choice of the two pathways in Saccharomyces cerevisiae is alternative so that DSB resection can inhibit NHEJ, because DSBs containing 3′ single-strand DNA cannot be repaired by canonical NHEJ (Fig. 1A). It has been widely believed that the alternative choice model is also relevant to mammalian cells [1], though the relevance has not yet been demonstrated. The method of examining the precise structure of resected DSB sites is currently available only for meiotic HR in S. cerevisiae, but not for mammalian cells [2, 3]. Although DSB resection would strongly inhibit canonical NHEJ, quick initiation of DSB resection does not reduce the overall efficiency of DSB repair in S. cerevisiae due to the very small contribution of NHEJ to DSB repair [3]. By contrast, DSB resection from DSB ends (Fig. 1A) would result in a considerable decrease in the overall efficiency of DSB repair in mammalian cells due to the major role of NHEJ in DSB repair.

A structural study of resected DSBs during meiotic HR in S. cerevisiae revealed that DSB resection is initiated by a single-strand break (SSB) on the strand to be resected up to 300 bases from the 5′-terminus of the DSB [2] (Fig. 1B). This SSB is subjected to subsequent bidirectional resection, both in the 5′→3′ direction away from the DSB and in the 3′→5′ direction towards the DSB end. The Mre11 nuclease forms a complex with Rad50 and Xrs2 (the yeast ortholog of mammalian Nbs1) [4]. The resulting MRX complex is responsible for the formation of the SSB, followed by the 5′→3′ direction resection in the meiotic HR of S. cerevisiae. This model agrees with the in vitro nuclease activity of purified Mre11. It
remains elusive whether this bidirectional resection also plays a role in mitotic HR in mammalian cells as well as in yeast [5].

Based on findings in chicken DT40 cells (Fig. 1C) [6], we here propose another model for DSB resection (Fig. 1D). If resection from the SSB is carried out only in the 5′–3′ direction away from the DSB, but not in the 3′–5′ direction, DSB ends would be maintained as duplex DNA (Fig. 1D). The absence of homologous single-stranded tails at DSB ends does not significantly interfere with homology search in S. cerevisiae [7], while the presence of duplex DNA at DSB ends would ensure efficient repair by canonical NHEJ. The new model shown in Fig. 1D predicts that HR and NHEJ are able to work in parallel, without interfering with each other. In other words, while HR undergoes homology search with the Rad51 recombinase polymerized on resected DSBs, NHEJ is able to efficiently ligate DSB ends.

The new model shown in Fig. 1D is supported by the ionizing radiation sensitivity of DT40 cells deficient in both HR and NHEJ [6] (Fig. 1C). In this study, we completely inactivated canonical NHEJ by disrupting the KU70 gene, and partially inactivated HR by disrupting the RAD54 gene. Note that the complete inactivation of HR by disrupting the RAD51 gene causes cellular lethality associated with severe genome instability, whereas disrupting the RAD54 gene allows for normal mouse development. Nonetheless, the loss of Rad54 completely inhibits HR-dependent repair of ionizing radiation–induced DSBs, as evidenced by data indicating that the loss of Rad54 reduces cellular tolerance to ionizing radiation in the S/G2 phases to the tolerance seen in the G1 phase [6]. Rad54 does not affect DSB resection, but facilitates homology search by resected DSBs associated with polymerized Rad51 (Fig. 1A) [8]. HR is preferentially used over canonical NHEJ for DSB repair in the G2 phase in DT40 cells, as shown by comparable radiosensitivity between wild-type and canonical-NHEJ–deficient KU70−/− cells (Fig. 1C). The alternative choice model (Fig. 1A) predicts that canonical NHEJ could not substitute for abortive HR in RAD54−/− cells because the precedent formation of the 3′ single-strand tail would inhibit canonical NHEJ. However, the radiotolerance of RAD54−/− DT40 cells is considerably higher than that of KU70−/−/RAD54−/− DT40 cells (Fig. 1C), indicating that canonical NHEJ can efficiently ligate
aborted HR intermediates generated in RAD54−/− cells. We, therefore, propose that the molecular mechanism for DSB resection in S. cerevisiae distinctly differs from that in metazoan cells (compare Fig. 1A and D). The new model agrees with the phenotype of RAD54−/− and KU70−/−/RAD54−/− DT40 cells, where canonical NHEJ is able to efficiently repair DSBs, even after polymerization of Rad51 at the DSB sites in RAD54−/− cells.

**LOSS OF MRE11 ONLY CAUSES UP TO 2- TO 3-FOLD DECREASE IN DSB RESECTION IN MITOTIC HR IN VERTEBRATE CELLS**

The current model of DSB resection in mammalian cells is based on the findings about HR in S. cerevisiae, since its molecular mechanism for DSB resection is most precisely defined [2] (Figs 1B and 2A). Mre11 is essential for DSB resection in the meiotic HR of S. cerevisiae. Mre11 also plays an important role in DSB resection when HR repairs ionizing radiation–induced DSBs, particularly in the G2 phase [3]. Extrapolating these findings of S. cerevisiae, the vast majority of the manuscripts and reviews have suggested that Mre11 also plays the major role in DSB resection in mammalian cells [9].

It should be noted that the essential role of Mre11 in meiotic HR is irrelevant to some of the mitotic HR in S. cerevisiae. For example, the selective inactivation of nuclease activity in Mre11 causes only a modest delay in DSB resection at the DSB site formed by the HO restriction enzyme, which generates ‘clean’ DSBs containing no abnormal chemical modification [3, 10]. Thus, other nucleases can effectively substitute for the defective nuclease activity of Mre11 in the resection at ‘clean’ DSBs in S. cerevisiae. Moreover, the importance of the nuclease activity in mammalian Mre11 has been indicated by Buis et al. [11], who conditionally generated nuclease-deficient MRE11ΔHIS200 and MRE11-null mutant (MRE11Δ−−) mice. Both mutant mice exhibited a very similar phenotype, including the mortality of cells associated with dramatic genomic instability, hypersensitivity to ionizing radiation, and 2- to 3-fold reduction in the efficiency of DSB resection. The data have been interpreted as compelling evidence for the critical role of Mre11-nuclease-dependent DSB resection in HR. However, this interpretation raises two questions. First, is only 2- to 3-fold reduction in DSB resection solely responsible for a severe defect in HR? Second, does a defect in HR fully explain the dramatic genomic instability? To define the role of Mre11 nuclease activity in DSB resection and genome stability, we conditionally generated MRE11ΔHIS200 and MRE11Δ−− cells from the chicken DT40 and human TK6 B cell lines [12]. Note that the TK6 cell line is widely used for evaluating the genotoxicity of industrial chemicals by regulators due to its stable karyotype and phenotype [13]. These MRE11 mutants retain the capability of performing DSB resection in nearly normal kinetics in both DT40 (Fig. 3A and B) and TK6 (Fig. 3C) cells. The data agree with a recent study directly measuring the length of resected 3′ single-strand overhangs; that study shows only about a 50 to 70% decrease in DSB resection upon depletion of Mre11 [14]. Collectively, the loss of Mre11 causes only up to 2- to 3-fold reduction in the efficiency of DSB resection in vertebrate cells. If DSB resection initiates from a SSB away from a DSB end, the very poor 3′ to 5′ exonuclease activity of Mre11 would allow DSB ends to be maintained as duplex DNA (Fig. 2B).

**DSB RESECTION BY MRE11 IS DISPENSABLE FOR EFFICIENT HR-DEPENDENT DSB REPAIR**

The prominent question is whether only 2- to 3-fold reduction in DSB resection has an impact on the efficiency of HR-dependent DSB repair. A previous study of our laboratory, as well as a genetic study of yeast, consistently indicate that such a small reduction has virtually no impact on the efficiency of HR-dependent DSB repair. Although the selective loss of the Mre11 nuclease activity reduces the resection kinetics of ‘clean’ DSBs, subsequent HR-dependent DSB repair occurs efficiently in S. cerevisiae [3, 10]. We generated the TK6 mutant cells (CIPW/NW cells), where the amount of CtIP was reduced by five times and the DSB resection efficiency decreased by two times. The resulting mutant is capable of completing HR-dependent DSB repair with normal kinetics (Fig. 3C) and is fully proficient in HR between homologous chromosomes induced by 1-Scel restriction enzyme–mediated DSBs [12]. Thus, 2- to 3-fold reduction in DSB resection does not have a negative impact on the overall efficiency of HR-dependent DSB repair. In summary, DSB resection by Mre11 does not account for the critical role of Mre11 in HR-dependent repair of ionizing radiation–induced DSBs.

If DSB resection by Mre11 has a minor role in DSB resection, an important question is which nuclease plays a critical role in DSB
resection and HR? Four nucleases (CtIP, Dna2, Exo1 and Mre11) contribute to DSB resection in both *S. cerevisiae* and mammalian cells. Short-range DSB resection, removing up to a few hundred nucleotides from the 5′ strand by Mre11 and Sae2 (the CtIP ortholog), is followed by long-range DSB resection, more than 10 kb in length, by Dna2 and Exo1 in *S. cerevisiae* [15, 16]. The short-range resection is sufficient for efficient HR. To measure the relative contribution of the four nucleases to DSB resection, we generated isogenic DT40 mutants deficient in individual nucleases. We assessed DSB resection by measuring Rad51 focus formation at 1 h after ionizing radiation (Fig. 3A). Only CtIP and Dna2, but not Exo1 or Mre11, are required for efficient DSB resection (Fig. 3B) [12, 17]. Nuclease-deficient DNA2<sup>−/−</sup>/D245A as well as null-mutant DNA2<sup>−/−</sup> cells show a significant defect in DSB resection, indicating Dna2 contributes to DSB resection as a nuclease. In contrast, mammalian CtIP has the non-catalytic role in DSB resection, and does not work as a nuclease in HR [18]. We showed that the non-catalytic role played by CtIP is essential for DSB resection by recruiting Dna2 to DSB sites [17].

The modest contribution of Exo1 to DSB resection agrees with the normal development of Exo1-deficient mice, despite the embryonic mortality of various HR-deficient mice [19]. Collectively, Dna2 plays the dominant role in DSB resection required for efficient HR among the four nucleases in the chicken DT40 cell line [17] (Fig. 2B). Given the very minor contribution of Dna2 to DSB resection for efficient HR in *S. cerevisiae*, the relative contribution of the four nucleases to DSB resection and HR is very different between *S. cerevisiae* and the DT40 cells.

**THE ROLE OF MRE11 NUCLEASE ACTIVITY IN ELIMINATION OF CHEMICAL ADDUCTS FROM DSB ENDS**

Although human MRE11<sup>−/−</sup>/H129N as well as MRE11<sup>−/−</sup> TK6 cells retain the capability of efficiently performing DSB resection, these mutants show delayed resolution of Rad51 focus formation after ionizing irradiation (Fig. 3C). Similarly, although chicken MRE11<sup>−/−</sup> DT40 cells efficiently perform DSB resection, the cells show a severe
defect in repairing ionizing radiation–induced DSBs by HR [20, 21]. These observations indicate that Mre11 plays important roles in DSB repair other than DSB resection. We hereafter discuss two roles played by Mre11: (i) removal of chemical adducts from DSB ends, and (ii) maintenance of Holliday junction HR intermediates.

Although Mre11-nuclease–deficient S. cerevisiae shows only a modest delay in DSB resection at the HO restriction enzyme–induced DSB sites in asynchronous populations, the mutant yeast is hypersensitive to ionizing radiation [3, 22]. Likewise, in the fission yeast, Schizosaccharomyces pombe (S. pombe), the Mre11 nuclease plays an important role in the repair of ionizing radiation–induced DSBs, but is not required for resection at an HO-induced DSB [23]. The data suggests that Mre11 may eliminate chemical modification induced by ionizing radiation before DSB repair by HR as well as NHEJ. This idea is further supported by the data that Mre11 nuclelease activity of S. pombe is involved in the removal of both Topoisomerase I (Top1) and Top2 from 3′ and 5′ DNA ends, respectively, in vivo [24]. Top2 resolves DNA catenates by catalyzing the transient formation of double-strand DNA breakage, enabling intact DNA to pass through the DSB and re-ligating the DSB [25]. During such transient DSB formation, Top2 becomes covalently bound to the 5′-DNA end of the breakage, forming Top2-DNA cleavage complex (Top2cc) intermediates. A chemotherapeutic agent, etoposide, strongly stabilizes Top2cc, leading to the formation of DSBs [26]. Mre11-nuclease–deficient S. pombe shows a more significant accumulation of Top2cc than wild-type cells, following treatment with etoposide. Moreover, Mre11 promotes NHEJ of etoposide-induced DSBs in G1 in mammalian cells [27]. These observations indicate that the incision activity of Mre11 eliminates Top2 cc adducts from DSB ends (Fig. 2C) prior to the repair of the DSB by NHEJ. Note that the incision activity is likely to be independent of the role of Mre11 in DSB resection (Fig. 2B), since the incision can occur in the G1 phase, when DSB resection is inhibited.

To directly test whether the nuclease activity of Mre11 is involved in elimination of Top2 cc from DSB ends, we measured the amount of Top2cc (Top2 cc covalently associated with genomic DNA), following exposure of cells to etoposide (Fig. 4A). We separated Top2cc from free Top2 by subjecting the cellular extract to cesium-chloride-gradient ultracentrifugation, followed by fractionation and western blot analysis with anti-Top2 antibody. Top2cc is seen only in cells treated with etoposide (Fig. 4B). The heterozygous mutation of the nuclease activity (MRE111+/H129N) significantly increased the amount of Top2cc. Moreover, overexpression of tyrosyl-DNA-phosphodiesterase 2 (TDP2), an enzyme eliminating covalently bound Top2, normalized the amount of Top2cc [28]. The prominent phenotype of the heterozygous mutant (MRE111+/H129N) cells suggests the important role of the Mre11 nuclease activity in elimination of the Top2 cc adduct, as yeast Mre11 is essential for removing the Spo11 topoisomerase from DSB ends (Fig. 1B). The dependency of DSB resection on Mre11 is significantly higher at ‘dirty’ DSBs induced by ionizing radiation than at ‘clean’ DSBs generated by restriction enzymes [3]. These observations support the notion that the nuclease activity of mammalian Mre11 is required for processing various forms of blocked DSB ends, including those containing damaged nucleotides and topoisomerase adducts, for subsequent DSB repair by NHEJ as well as HR. We propose that Mre11 contributes to cellular tolerance to ionizing radiation through processing of DSB ends (Fig. 2C) rather than DSB resection (Fig. 2B).

THE ROLE OF MRE11 IN HR AT A STEP AFTER THE FORMATION OF HOLLIDAY JUNCTION HR INTERMEDIATES

Nuclease-dead Mre11 mutants have a significantly milder phenotype during mitosis than do null-Mre11 mutants in S. cerevisiae [3, 29]. Likewise, nuclease-deficient Mre11 mutant DT40 cells are able to slowly proliferate, although the null-Mre11 mutant DT40 cells are lethal [12]. However, the non-catalytic functioning of Mre11 in HR has not yet been defined. This functioning might depend on the capability of the MRN complex to tether two DNA strands, since the complex has a structure similar to that of cohesion and condensin [30]. Although human MRE11−/− as well as MRE11−/−H129N–TK6 cells initiate DSB

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Fig. 4. Accumulation of topoisomerase II adduct (Top2cc) in human TK6 cells heterozygous for nuclease-deficient Mre11 (MRE111+/H129N). (A) Schematic of the in vivo Top2cc measurement by immunodetection with anti-Top2 antibody. A 200 μg genomic DNA sample was subjected to sedimentation by CsCl-gradient ultracentrifugation. Genomic DNA of wild-type TK6 cells that had been treated with 10 μM etoposide for 2 h was included as a control in every ultracentrifugation. The treatment reduced the survival of wild-type cells by ~3% relative to untreated cells. Individual fractions were blotted to nylon filters, followed by western blot using anti-Top2 antibody. The remaining two fractions include free Top2 (yellow circle), while the other fractions include Top2 covalently associated with genomic DNA. (B) Western blot analyses of Top2 in the indicated TK6 cells that had been treated with 10 μM etoposide (+) or DMSO (−) for 2 h. ‘+TDP2’ indicates overexpression of a TDP2 transgene. The Tdp2 enzyme eliminates Top2 cc adducts from DSB ends.
Fig. 5. (A) Model for generation of isochromatid-type breaks following ionizing irradiation at the G2 phase. The irradiation generates DSBs in one of two sister chromatids. Defective initiation of HR leads to chromatid-type breaks in mitotic chromosome spreads. Formation of HR intermediates such as Holliday junctions followed by failure of their resolution could generate isochromatid-type breaks, where two sister chromatids are broken at the same sites due to a defect in local chromosome condensation at HR intermediates. (B) The numbers of the indicated chromosome aberrations in the mitotic chromosome spreads after ionizing irradiation at the G2 phase. DT40 cells carrying the indicated genotypes were exposed to 0.3 Gy γ-rays and harvested at 3 h for chromosome analysis. The numbers shown are per 100 mitotic cells. (C) The spatial distance between two sister chromatids is much bigger in mammalian cells than in yeast. Following DNA replication, dynamic chromosome condensation significantly separates two sister chromatids. Accordingly, HR intermediates are constantly pulled in opposite directions, as shown by the arrows. The MRN complex may stabilize HR intermediates by tethering DNA strands. (D) Model for the repair of ionizing radiation–induced DSBs. Ionizing radiation causes ‘dirty DSBs’ to associate with chemical modifications at DSB ends. The nuclease activity of Mre11 plays the major role in eliminating chemical modifications, particularly from 5’ ends. *Saccharomyces cerevisiae* Mre11 coordinates initiation of DSB resection at the two ends of individual DSBs [3]. We propose that Mre11 maintains HR intermediates for their proper resolution.
resection with nearly normal kinetics, these mutants showed a significant delay in resolution of ionizing radiation–induced Rad51 foci (Fig. 3C). The data suggest a role for Mre11 in a late step of HR as well as in processing the ends of ‘dirty’ DSBs.

There is no reliable phenotypic assay for analyzing HR intermediates in mammalian cells or mitotic yeast. However, phenotypic analyses of meiotic HR in S. cerevisiae most significantly contribute to our understanding of the precise molecular mechanism for resolution of HR intermediates [31]. DT40 cells provide a unique phenotypic analysis for evaluating resolution of HR intermediates. HR plays the dominant role in repairing ionizing radiation–induced DSBs during the G2 phase in DT40 cells. Thus, we can assess the efficiency of HR-dependent DSB repair by exposing G2-phase cells to γ-irradiation and measuring the number of chromosome aberrations in the subsequent M phase [32]. Moreover, morphological observation of mitotic chromatids for their proper resolution. A critical difference in HR between yeast cells and vertebrate cells is a spatial distance between donor sequences in intact sister chromatids and recipient homologous sequences in broken sister chromatids. The distance is much smaller in yeast in comparison with mammalian cells (Fig. 5C) [33, 34]. Moreover, dynamic condensation of sister chromatids may constantly increase their distance after DNA replication in mammalian cells. Accordingly, HR intermediates containing two sisters may be pulled in opposite directions, leading to dissociation of the intermediates. The following two mechanisms may prevent the dissociation of the HR intermediates. Extensive strand exchange between two sister chromatids leads to the formation of stable structure such as the double Holliday junction [31]. Moreover, the tethering by MRN may further stabilize HR intermediates and promote the completion of DSB repair by HR. This MRN-dependent mechanism might be relevant to mitotic HR in S. cerevisiae, since yeast Mre11 also has a prominent non-catalytic role in genome maintenance [3, 29].

CONCLUSION

The genetic study of yeast species provides a framework for examining molecular mechanisms underlying HR in mammalian cells. The genetic study of yeast, but not mammalian cells, allows for examining the structure of various HR intermediates. In addition, since HR is the most complex DNA repair reaction, involving more than 100 molecules (including histone-modifying enzymes [4]), neither the biochemical study nor genetic study of mammalian cells allows for dissecting the role of individual factors in various HR reactions. Accordingly, the functions of individual mammalian HR factors have been postulated by the function of their yeast ortholog proteins. A large number of researchers of mammalian Mre11 seem to have interpreted their own data by extrapolating known functions of S. cerevisiae Mre11. However, although mammalian Mre11 does contribute to DSB resection, its limited contribution does not necessarily account for the very severe defect in HR-dependent repair of ionizing radiation–induced DSBs [11, 14]. The present data suggest that the severe defect reflects the important role of Mre11 in DSB repair aside from DSB resection: (i) processing of DSB ends and (ii) maintenance of HR intermediates. This conclusion is also supported by genetic studies of S. cerevisiae indicating (i) the greater contribution of Mre11 to repair of ‘dirty’ DSBs than ‘clean’ DSBs, and (ii) a severer phenotype of the null-Mre11 mutant compared with that of the nuclease-deficient Mre11 mutant [3]. Future studies will clarify the roles of mammalian Mre11 in the processing of DSB ends and the maintaining of HR intermediates (Fig. 5D).

ACKNOWLEDGEMENTS

We thank A. Noguchi and A. Kobayashi for their technical assistance and the lab members for their stimulating discussion. Immunocytochemical analyses were performed at the Medical Research Support Center, Graduate School of Medicine, Kyoto University.

FUNDING

This work was supported by the Japan Society for the Promotion of Science (JSPS) Core-to-Core Program and a Grant-in-Aid for Scientific Research (S) (to S.T.) and Grants-in-Aid from the Ministry of Education, Science, Sport and Culture (to H.S. [KAKENHI 26740018] and S.T. [KAKENHI 25650006 and 23221005]). Funding to pay the Open Access publication charges for this article was provided by KAKENHI 23221005 and KAKENHI 26740018.

REFERENCES


