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Making the Best of the Loose Ends: Mre11/Rad50 complexes and Sae2 promote DNA double-strand break resection

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Abstract

Double-strand breaks in chromosomal DNA are repaired efficiently in eukaryotic cells through pathways that involve direct religation of broken ends, or through pathways that utilize an unbroken, homologous DNA molecule as a template for replication. Pathways of repair that require homology initiate with the resection of the 5' strand at the break site, to uncover the 3' single-stranded DNA that becomes a critical intermediate in single-strand annealing and in homologous strand exchange. Resection of the 5' strand is regulated to occur most efficiently in S and G₂ phases of the cell cycle when sister chromatids are present as recombination templates. The mechanisms governing resection in eukaryotes have been elusive for many years, but recent work has identified the major players in short-range processing of DNA ends as well as the extensive resection of breaks that has been observed *in vivo*. This review focuses on the Mre11/Rad50/Xrs2(Nbs1) complex and the Sae2(CtIP) protein and their roles in initiating both short-range and long-range resection, the effects of topoisomerase-DNA conjugates on resection *in vivo*, and the relationship between these factors and NHEJ proteins in regulating 5' strand resection in eukaryotic cells.

1. Introduction

The repair of DNA double-strand breaks (DSBs) through mechanisms of homologous recombination initiates with the processing of the broken DNA strands, primarily removal of the 5' strand [1,2]. This process of 5' strand resection is essential for the eventual establishment of a Rad51 filament on the 3' strand, which performs the homology search for a target to use as a template for replication [3]. In eukaryotes, the 3' single-stranded DNA (ssDNA) generated during resection is first coated by the RPA complex before exchange for Rad51 by mediator proteins [4], and plays an important biological role in recruitment and activation of Mec1(ATR) in the replication checkpoint [5]. In budding yeast, both resection and checkpoint activation occur much more efficiently in S and G₂ phases of the cell cycle when sister chromatids are present [6,7]. 5' strand resection is thus a critical event in the initiation of homologous recombination as well as in S phase cell cycle checkpoint control in eukaryotic cells. Resection enzymes and their roles *in vivo* have recently been reviewed in DNA Repair [8], thus this review focuses primarily on the mechanisms by which the Mre11/Rad50/Xrs2(Nbs1) (MRX(N)) complex and Sae2(CtIP) facilitate DNA end processing.

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2. DSB resection in budding yeast

DSB resection was observed decades ago [1] but only in the last few years have the molecular details of this process become more apparent [8-10]. Most of these details have been elucidated in *S. cerevisiae*, where resection of DSBs has primarily been measured during meiotic recombination, or at sites of inducible endonuclease-generated breaks in vegetatively growing cells. The extent of resection can vary depending on the presence of functional strand invasion machinery, as well as the availability and location of a homologous template, which also determines the kinetics of repair [11,12]. Under circumstances where strand invasion is blocked by the absence of recombination factors or a homologous template, tens of thousands of nucleotides can be removed from the 5' strand at the break site [11,13]. When a homologous template is available and strand invasion can occur, the extent of resection is significantly less (~2 to 4 kb in the case of allelic recombination and ~3 to 6 kb in the case of ectopic recombination), which correlates well with the faster kinetics of gene conversion between homologs relative to recombination between different chromosomes [11]. The rate of resection has been reported to be approximately 4 kb/hr in vivo at different chromosomal locations [11-14], although the initiation of resection is not necessarily concerted [2].

3. Mechanisms of MRX/Sae2 stimulation of resection

3.1. MRX and Sae2 introduction

Mre11/Rad50 complexes are found in all organisms and are important for efficient DSB repair, as well as for signaling of DSBs that occurs through the Tel1(ATM) protein kinase in eukaryotes [15,16]. The Mre11 protein is in the lambda phosphatase family of phosphoesterases, and recombinant Mre11 proteins from several divergent species exhibit exo- and endonuclease activity in vitro [17-24]. Exonuclease activity in manganese occurs in the 3' to 5' direction, while endonuclease activity is observed in manganese on single-strand/double-strand transitions and on hairpin loops. In magnesium, Mre11 does not act as an exonuclease but rather shows weak endonucleolytic activity on the 5' strand of linear DNA ends [25-27]. Mre11 binds to the Rad50 protein, which is in the Structural Maintenance of Chromosomes (SMC) family of large ATPases containing long coiled-coils that separate Walker A and Walker B ATP-binding domains. Rad50 proteins exhibit ATPase and adenylate kinase activities in vitro [28-30], and the human MRN complex was also shown to catalyze opening of duplex DNA ends in a manner that is stimulated by ATP [31]. In eukaryotic cells, Mre11 and Rad50 also associate with a third protein, Nbs1(NBN, Nibrin, Xrs2 in budding yeast), which regulates the activities of the other components and is essential for the functional interactions between the MRX(N) complex and Tel1(ATM) [31-35].

In organisms that undergo sexual reproduction, MRX(N) complexes are essential for the processing of DSBs that are created by Spo11, a topoisomerase-like enzyme related to TOP6A from archaea [36] that forms a covalent attachment with DNA at a catalytic tyrosine residue [37,38]. In many organisms, MRN also regulates telomere maintenance through telomerase activity and also through telomerase-independent recombination [8], and in some organisms MRN is important for efficient nonhomologous end joining (NHEJ)[39]. All of these scenarios involve DNA ends, which are bound by MRN and in some cases also processed through the activity of Mre11 [25-27]. MRX complexes are required for the association of DSB ends in vivo in budding yeast [40,41], and have been observed tethering DNA ends together in vitro [42]. Mre11 dimers from *P. furiosus* were crystallized with two molecules of DNA associating end to end [43], suggesting that Mre11 may hold the ends of DNA molecules together in the context of Mre11/Rad50 complexes.

The Sae2 protein in budding yeast does not associate physically with MRX but deletion strains share many phenotypes with strains expressing hypomorphic alleles of the MRX complex, most notably Mre11 nuclease-deficient alleles and *rad50S* alleles that block meiotic DSB processing [44-46]. Sae2-deficient yeast strains were also found to exhibit defects in mitotic DSB repair, specifically those involving hairpin structures as intermediates [47-49]. Rattray et al observed that large palindromic duplications resulted from misrepair of a DSB within an inverted repeat in *sae2Δ* cells, which was postulated to result from fold-back and replication of 3' single-stranded DNA intermediates [47,48]. Lobachev et al also found that the processing of spontaneous DNA breaks at sites of inverted repeats was altered in *sae2Δ*, MRX-deficient, Mre11 nuclease-deficient, and *rad50S* strains, which was also suggested to arise because of a failure to process hairpin DNA structures [49]. Recombinant Sae2 binds linear DNA independently of MRX and exhibits endonuclease activity on 5' flap structures as well as on hairpin DNA in vitro [50]. Hairpin structures were cleaved within single-stranded regions adjacent to the hairpin rather than at the hairpin tip, thus it was suggested that Mre11 nuclease activity might help to create single-stranded DNA which could then be cleaved by Sae2.

The MRX complex and the Sae2/Com1 protein have been associated with DSB resection for many years because mutations in the genes encoding these factors generate a complete block to 5' strand removal in meiosis. *rad50S* and *mre11S* hypomorphic mutations, Mre11 nuclease-deficient alleles, and null mutations in Sae2 yield covalent complexes of Spo11 on the 5' strand of DNA at the break site [24,44-46,51,52]. The strict requirement for Mre11 nuclease activity and for the Sae2 protein is not as pronounced in vegetatively growing yeast cells, however, where resection of endonuclease-induced breaks is only delayed in the absence of MRX [53], and nearly unaffected by mutations that inactivate Mre11 nuclease activity [52]. Deletion of Sae2 also causes a delay of resection, similar to that seen with *rad50S* strains [54]. The primary difference between meiotic and mitotic DSBs is the presence of covalent Spo11-DNA intermediates in meiosis, which presumably are removed by a combination of MRX nuclease activity and the activity of Sae2.

3.2. Long-range resection of DSBs in yeast

Long-range resection of endonuclease-induced breaks in budding yeast was shown to be dependent on two redundant pathways: the 5' to 3' exo- and endonuclease Exo1, or an alternative pathway consisting of the 5' flap endonuclease Dna2 in combination with the helicase Sgs1 and its partners Rmi1 and Top3 [13,55-57]. Although inactivation of either of these pathways alone has little effect on DSB resection, removal of both blocks all long-range (0.5 to 30 kb) resection. In cells lacking Exo1 and either Sgs1 or Dna2, the broken DNA ends were processed very slowly on the 5' strand but only 50 to 300 nt was removed [13,55]. This short-range processing was found to be dependent on the MRX complex and the Sae2 protein.

MRX deficient yeast cells with functional Exo1 and Dna2/Sgs1/Rmi1/Top3 pathways show deficiencies in resection, primarily at early time points and at sites close to the site of the break [13,55]. The study by Zhu et al examined the kinetics of resection in wild-type vs. *rad50Δ* cells, showing that 5' strand degradation very close to the break site is significantly delayed in cells lacking MRX [13]. The rate of resection far from the break (28 kb) was not as significantly altered in the absence of MRX, but ~20% of the breaks were never resected in *rad50Δ* cells. These observations clearly point to a role for MRX in the initiation of resection; not that MRX is essential for the start of resection at every break site, but that it accelerates the rate of initiation. This also suggests that initiation of resection is a rate-limiting step, consistent with a recent study showing that initiation of resection likely occurs with a stochastic, slower rate compared to the rate measured for processive resection far from the break site, based on quantitative measurement of ssDNA intermediates in vivo [2].

The observation that overexpression of Exo1 can at least partially suppress the sensitivity of MRX-deficient strains to DNA damaging agents [58-62] is also consistent with the idea that one of the primary functions of MRX in DSB resection is to help load Exo1 (or Dna2/Sgs1/Rmi1/Top3) onto DNA ends.

It is also clear that the stimulatory effect of MRX on the extensive resection of 5' strands can occur independently through both redundant pathways: Exo1 and Dna2/Sgs1/Rmi1/Top3 [13]. Evidence for MRX stimulation of Exo1 comes from the comparison of *sgs1Δ* and *sgs1Δrad50Δ* cells [13], where it is clear that the *sgs1Δrad50Δ* cells show a marked decrease in resection at sites close to the break point. Similarly, MRX also appears to be important for the Sgs1 pathway since an *exo1Δrad50Δ* strain exhibits a population of unresected DSBs, which differs dramatically from *exo1Δ* cells that are essentially wild-type in resection close to the DSB [13].

3.3. Recruitment of Exo1 and Dna2

Recombinant yeast Exo1 was used to reconstitute resection in vitro and to determine the effects of MRX and Sae2 on its activity [25]. These experiments showed that there are at least 3 distinct mechanisms that contribute to the effects of MRX and Sae2 on yeast Exo1 (Fig. 1). The first of these occurs through recruitment of the Exo1 protein, which is a relatively inefficient, distributive 5' to 3' exonuclease as well as 5' flap endonuclease in vitro [63]. Exo1 showed little activity on linear DNA when present at a molar excess over ends of 3-5:1, yet under these conditions MRX and Sae2 dramatically stimulated Exo1 nuclease activity [25]. At higher ratios of Exo1 to DNA, Exo1 activity was observed in the absence of MRX and Sae2, but MRX and Sae2 still markedly increased the extent of resection. Most of this effect was attributed to an increase in Exo1 binding to DSB ends in the presence of MRX and Sae2. This effect was not dependent on the nuclease activity of Mre11, as a nuclease-deficient MRX complex exhibited ~80% of the activity of wild-type MRX. Protein-protein interactions were not observed between Exo1 and either MRX or Sae2, so it is possible that a change in DNA structure, created by MRX and Sae2, may increase the affinity of Exo1 for DNA. Previous experiments with the human MRN complex suggest that this is likely a branched structure [31,35], consistent with the observation that Exo1 preferentially binds to branched DNA in comparison to fully complementary ends [25]. In addition, MRX and Sae2 stimulated the endonucleolytic products generated by Exo1 on the 5' strand of DNA, also consistent with the presence of an opened structure at the DNA end.

The recruitment of Exo1 by MRX has also been observed in vivo, where loading of Exo1 at an HO-induced break was observed to be significantly decreased in *mre11Δ* or *rad50Δ* strains [64]. A similar effect was also observed with Dna2 in MRX-deficient strains [64], also suggesting that the stimulatory effect of MRX is substantially attributable to recruitment of the enzymes responsible for long-range DNA end processing.

3.4. DNA end processing by MRX/Sae2

In addition to Exo1 recruitment in the presence of MRX/Sae2, a less efficient mechanism of Exo1 stimulation was also observed which acts at the step of DSB processing [25]. In previous studies, Mre11/Rad50 complexes from *P. furiosus*, *D. radiodurans*, *E. coli*, *S. cerevisiae*, and humans were all found to exhibit 3' to 5' exonuclease activity and endonuclease activity in manganese in vitro [17-24]. However, in more physiological conditions with magnesium, the *P. furiosus*, *S. cerevisiae*, and humans complexes showed very weak, if any, 3' to 5' exonuclease activity, but did show endonuclease activity on the 5' strand of linear DNA ends [25-27]. The predominant cleavage sites for the 5' endonuclease activity were between 10 and 50 nt from the DSB. In resection assays with either *P. furiosus* MR or yeast MRX, incubation of the linear DNA substrate with MR was found to increase

subsequent resection by downstream enzymes (the helicase/nuclease complex HerA/NurA in the case of *P. furiosus* and Exo1 with the *S. cerevisiae* proteins), even when the MR(X) was removed prior to this incubation [25,27]. Limited 5' strand resection by an unrelated 5' to 3' exonuclease also promoted Exo1 activity [25]. Thus, removal of the 5' strand to generate a short 3' overhang has a stimulatory effect on the downstream enzymes that act together with Mre11/Rad50 complexes, which also affects binding of the downstream enzymes to the processed (3' overhang) DNA ends [27]. In vivo, MRX/Sae2-dependent processing at endonuclease-induced break sites can only be observed directly when Exo1 and either Sgs1 or Dna2 is inactivated [13,55]. Under these conditions, about 100 nt [55] (up to 200 or 300 nt in a minority of cells [13]) were found to be removed from the 5' strand. The 5' endonucleolytic cleavage of DSBs observed in vitro is likely the source of this inefficient processing seen in *exo1Δsgs1Δ* cells in vivo. One question that is difficult to answer in vivo is whether MRX and Sae2 actually cleave the 5' strand at a break under circumstances where the Exo1 and Dna2 pathways are functional. In vitro experiments with MRX and Exo1 suggest that the Exo1 endonuclease activity on the 5' strands at a break is much more robust than MRX/Sae2 activity [25], thus it is likely that in a wild-type cell, Exo1 (or Dna2/Sgs1/Rmi1/Top3) is recruited to DNA ends and performs the majority of the processing of the 5' strand.

While Mre11 nuclease activity appears to play a relatively minor role in the resection of DSBs induced by endonucleases in budding yeast, nuclease-deficient MRX complexes show only partial complementation of *mre11Δ* strains when assayed for survival of ionizing radiation (IR) [52]. Since MRX is recruited to DSB sites generated by either an endonuclease or by IR [65], it is possible that the requirement for MRX in the repair of IR-induced damage is due to the fact that IR can generate base damage, protein-DNA adducts, or other altered structures at the end of DSBs that require Mre11 nuclease activity for removal. These complicated structures would likely not be present on all the ends, thus one might expect a hypomorphic phenotype with the nuclease-deficient mutant. MRX-dependent generation of oligonucleotides from linear DNA was also observed in *Xenopus* egg extracts, where the oligonucleotide products of 5' strand resection were shown to regulate ATM protein kinase activity [66]. These oligonucleotides may thus constitute an additional link between DNA end processing and checkpoint activation in eukaryotic cells.

Interestingly, budding yeast cells expressing an Mre11 nuclease-deficient allele are extremely sensitive to IR when also deleted for Dna2 and the Pif1 helicase, despite the presence of wild-type Exo1 in these cells [56]. The Dna2 nuclease function, rather than the helicase function, was found to be responsible for this effect. Thus the function of Dna2 is partially redundant with MRX with respect to processing of IR-induced damage, and this function is distinct from that of Exo1. Expression of a helicase-proficient, nuclease-deficient form of Dna2 was also shown to be lethal in cells expressing an Mre11 nuclease-deficient allele, suggesting that structures generated by Dna2 (presumably at replication forks) specifically require Mre11-dependent processing in the presence of a helicase+/nuclease-form of Dna2 [56].

3.5. Rad50S mutations and DSB processing

rad50S mutations in MRX completely block meiotic DSB resection [46] but only delay resection in vegetatively growing yeast cells, similar to strains lacking Sae2 [25,54]. The *rad50S* mutations were identified by mutagenesis of the N-terminal ATPase domain and genetic screening for alleles that abolish Rad50 function in meiosis but show nearly wild-type function in vegetatively growing cells [46]. Despite the location of these mutations in the ATPase domain, *rad50S* mutations do not block the ATPase activity of MRX [67]. Functionally equivalent mutations were also identified in Mre11 [24,51,52]. Overexpression of Sae2 can suppress the delay of resection observed in *rad50S* strains [54], suggesting that

these mutations may alter the relationship between MRX and Sae2 on DNA ends. The MRX complex remains at DSB sites longer in *rad50S* strains [68], also similar to that seen in *sae2Δ* strains, thus the unknown event(s) that precipitate loss of MRX from DNA ends involve both MRX and Sae2 interactions and are abrogated or delayed when Rad50 proteins contain the *rad50S* mutation. Sae2 still localizes to DSBs in *rad50S* cells, however, consistent with the ability of Sae2 to bind to DNA independently of MRX, thus the defect is not simply in Sae2 binding [68]. The molecular basis of the *rad50S* defect is not understood, but reconstitution of Exo1-mediated resection in vitro also showed a defect in Exo1 activity in the presence of Rad50S MRX in comparison to the wild-type complex [25], thus it may be possible to dissect the properties of this mutant with purified components.

rad50S forms of the mammalian MRN complex have also been studied extensively in the mouse, where it is clear that Rad50S MRN generates constitutive checkpoint signaling by ATM [69-71]. *rad50S* mice exhibit increased apoptosis in hematopoietic cells and spermatogonia, leading to early death due to anemia, and these outcomes can be suppressed by loss of even one allele of ATM, Chk2, or p53 [69,72]. Increased signaling in *rad50S* yeast cells is also observed in a *mec1* background (in which DNA damage-induced checkpoint signaling is Tel1-dependent), leading to constitutive formation of activated Rad53/Rad9 complexes [73]. Rad50S MRX(N) complexes behave genetically as hypermorphic alleles relative to the function of MRN in ATM signaling, in that their phenotypes can be abrogated by mutations that disrupt the MRN complex or ATM [69,73].

Usui et al demonstrated that *rad50S* or a *sae2Δ* deletion in yeast can rescue MMS sensitivity and checkpoint functions in a *mec1Δ* deletion strain [73]. This result suggests a relationship between 5' strand processing and Tel1(ATM) activation, such that stabilization of the end of the 5' strand promotes Tel1(ATM) kinase activity, while removal of the 5' strand inhibits activity. Evidence in support of this model was also obtained in human cell extracts, where ATM activation was found to decrease proportionally with 5' strand processing, opposite to that of ATR activation which depends on extensive, RPA-coated ssDNA [74]. ATM activation in vitro through the human MRN complex was shown to be dependent on opening of linear duplexes to expose ssDNA ends [35], also consistent with the idea that the single strands, particularly the 5' strand, are an essential component of the ATM activation mechanism. It is possible that Rad50S MRX(N) complexes may hold the 5' strand of DNA at a break in a configuration that is not as suitable for endonucleolytic cleavage by Mre11 or Sae2, and in this way may activate Tel1(ATM) longer than would be the case in wild-type cells. This would conceivably yield novel phenotypes associated with ATM hyperactivation that would be very different from cells lacking MRX(N).

The defect in Rad50S MRX(N) complexes may be related to processing of topoisomerase lesions, since cells from *rad50S* mice are hypersensitive to camptothecin, a topoisomerase I poison, yet show little sensitivity or chromosomal aberrations in the context of IR or UV irradiation, or other DNA damaging agents [70]. There is not necessarily a perfect correspondence between camptothecin sensitivity and hypersignaling through Tel1, however, since Mre11 nuclease-deficient mutants are camptothecin sensitive yet do not rescue signaling in *mec1* strains [73].

3.6. MRX/Sae2 and processing of topoisomerase covalent conjugates

In contrast to vegetatively growing cells, budding yeast strains expressing nuclease-deficient Mre11 alleles show a complete block in meiotic DSB resection [52], because the putative type II topoisomerase Spo11 is covalently attached to the 5' strands at every break [37]. Spo11 was subsequently found to be removed from chromosomes with a short oligonucleotide (~12 nt or 21-37 nt) covalently attached [75], showing that DSB processing during meiosis initiates with an endonucleolytic incision of the DNA rather than direct

reversal of the tyrosyl-DNA bond. Removal of Spo11 by this mechanism was also shown to be dependent on MRX and Sae2 [75], consistent with their activity as 5' endonucleases in vitro [25,50]. Rec12(Spo11) removal from DSBs in fission yeast is similarly dependent on MRN and Ctp1 and requires Mre11 nuclease activity, although in this case only one size of oligonucleotide (13 to 29 nt) was observed attached to Rec12 [76-78].

The strict requirement for Mre11 nuclease activity in Spo11 removal also suggests the possibility that MRX may be involved in removing topoisomerase II covalent complexes in vegetatively growing cells. This hypothesis was tested in budding yeast using the same immunoprecipitation/Tdt labeling technique used to assay Spo11-DNA processing, but generation of the topoisomerase II-oligonucleotide product was not altered in *rad50S* or *sae2Δ* cells [75]. The idea that MRX(N) complexes may cleave covalent topoisomerase conjugates is still of interest, however, because other studies have found that MRX and Sae2 do contribute to the survival of budding yeast cells during exposure to camptothecin, a topoisomerase I poison, in a parallel pathway to that of Tdp1, a 3' tyrosyl-DNA phosphodiesterase [79-81]. A study in fission yeast also found that *rad50S* and nuclease-deficient forms of the MRN complex exhibit hypersensitivity to both topoisomerase I and II poisons (camptothecin and TOP-53, respectively) relative to methylmethanesulfonate and IR sensitivity [82]. In addition, TOP-53-induced topoisomerase II covalent complexes were observed at elevated levels in *rad50Δ*, Mre11 nuclease-deficient, and *ctp1Δ* (functional equivalent of Sae2 in fission yeast) strains. Studies with the E. coli Mre11/Rad50 complex, SbcCD, also showed that the complex can cleave DNA on the 5' strand with a biotin-avidin complex attached to that strand, suggesting that removal of large protein complexes from DNA ends through endonucleolytic cleavage of the DNA is a conserved activity of this complex [83].

In contrast to the situation in yeast, Mre11 nuclease activity appears to be critically important in mammalian cells, as a nuclease-deficient mutation in Mre11 causes early embryonic lethality in the mouse and prevents proliferation of mouse embryonic fibroblasts [84], similar to genetic knockout of the Mre11 gene [85]. The reasons for this striking phenotype are not clear, although it was shown using a conditional allele that a failure in DNA repair (as measured by IR sensitivity, genomic instability, RPA and Rad51 foci after IR, and ISceI-induced homology-directed repair), rather than a defect in ATM activation, are responsible [84]. These findings show that Mre11 nuclease activity plays an important role in DNA repair at non-meiotic DSBs, to the extent that Mre11 null cells and cells expressing the Mre11 nuclease-deficient allele appeared identical for all of the repair phenotypes. It is not clear if removal of topoisomerase II covalent complexes constitutes a large part or any part of the role of Mre11 nuclease activity in human cells, as a novel phosphodiesterase (Tdp2/TTRAP) has been identified in human cells that directly removes 5' tyrosyl linkages [86], similar to the 3' tyrosyl removal catalyzed by Tdp1 [87], and this was shown to be the major source of this activity in human cells. It is certainly possible that MRN could provide an alternative pathway of endonucleolytic removal of topoisomerase II, however, in parallel to the direct removal by Tdp2. It will be important to determine what proportion of topoisomerase I and II covalent complexes are removed via endonucleolytic cleavage of the DNA adjacent to the attachment site, and if the MRN complex has a role in these cleavage events in mammalian cells.

3.7. MRX/Sae2 and competition with Ku

In addition to recruitment of downstream resection enzymes and processing of DNA ends to facilitate 5' strand resection, there is at least one other mechanism by which MRX promotes DSB resection in eukaryotic cells which involves factors important for NHEJ. NHEJ is often considered to be a pathway that competes with homologous recombination, particularly in the G₁ phase of the cell cycle. In mammalian cells, NHEJ operates throughout the cell cycle

[88], however, and it is still not clear what controls pathway choice at DSBs that occur during S and G₂. A number of studies have shown that DSB resection is increased in efficiency in the absence of Ku, and to a lesser extent in the absence of other core NHEJ factors Dnl4 (ligase IV) and Lif1 (Xrcc4) [2,89-91]. This effect is primarily observed in G₁ and is dependent on the MRX complex and Exo1, although resection is limited to the region proximal to the break site [2,64,90]. Resection is not increased by deletion of Ku in G₂ cells, but can be delayed by overexpression of Ku [90]. These results suggest that Ku inhibits MRX(N) binding to DNA, which has also been observed in vitro with recombinant human Ku and MRN complexes [31].

While Ku inhibits MRX-dependent resection (at least in G₁), it appears that the converse is also true: MRX inhibits Ku activity at DNA ends. MRX deletion strains show a dramatic increase in the binding of Ku to DSBs [64,89]. The *mre11-3* nuclease deficient allele of Mre11 can suppress this effect [89], showing that the presence of MRX, rather than its DNA processing activity, is important in inhibiting Ku binding. Inhibiting Ku is not the only role of MRX in the processing of DNA breaks, however, as deletion of Ku in a strain lacking MRX does not restore resection rates to wild-type levels and only partially restores radiation resistance [64,90,92]. In vitro experiments have demonstrated that Ku strongly inhibits Exo1-mediated resection and that MRX/Sae2 can at least partially recover Exo1 activity under these conditions [64], consistent with the idea that MRX and Ku compete for DNA ends.

The role of NHEJ factors in regulating DSB resection is intimately linked to cell cycle control of resection, since NHEJ is used as an alternative to homologous recombination in G₁, but not in G₂ [6,7,90,93]. Cyclin-dependent kinase (CDK) activity is essential for long-range resection, which favors homologous recombination during S and G₂, a period of the cell cycle when sister chromatids are present. Limited DSB resection can occur in G₁ [65] which increases in the absence of Ku, but even this is limited to DNA regions close to the break site [2]. The difference between G₁ and G₂ cells may be explained by differential binding of Ku, because Ku was reported to be associated with DSBs more efficiently in G₂ relative to G₁, in a manner that is dependent on CDK activity [93]. However, another study reported that levels of Ku at break sites were similar in G₁ vs G₂, thus it is unclear at this point how and if Ku loading onto DNA ends is regulated during the cell cycle phase [90]. Ku does not appear to be a direct target of CDK [93], so the mechanism by which CDK inhibits NHEJ during G₂ is unknown.

3.8. Sae2/CtIP and regulation of resection by CDK

Identification of CDK targets that regulate resection has been a major interest in the field. One of the only CDK phosphorylation events that has been shown to be important for resection in vivo is the single CDK site on the Sae2 protein, the endonuclease that cooperates with MRX in recruitment of Exo1 and Dna2/Sgs1 and DNA end processing. Mutation of S267 in Sae2 to alanine blocks DSB resection in vivo [94], although we do not yet know what the phosphorylation does to activate the protein. Monomeric Sae2 (both wild-type and S267A) produced in *E. coli* is active in DNA binding and endonuclease activity while dimeric Sae2 is relatively inactive [50,95], thus it is possible that the CDK phosphorylation in yeast helps to activate the inactive dimer, perhaps in conjunction with Tel1/Mec1 (ATM/ATR) phosphorylation events that occur in S and G₂ [96]. Sae2 and MRX can bind independently to DNA breaks, shown in vivo [68] and in vitro [50], and Sae2 was found to form foci at time points when MRX foci were dissociating, almost always before the appearance of Rad52 foci [68]. It is attractive to speculate that post-translational modification of Sae2 may regulate the kinetics of these events, but whether this is the case is not known. Mutation of five Tel1/Mec1 sites in Sae2 to alanine generated MMS sensitivity that approached the sensitivity of a *sae2Δ* strain [96], suggesting that these sites are

important for Sae2 function in vivo. However, mutation of the same sites in vitro caused a deficiency in nuclease activity, even though the *E. coli*-expressed protein is not phosphorylated [50]. Thus these mutations in SQ/TQ sites cause functional problems independently of phosphorylation and make the investigation of the roles of these modifications difficult in vivo.

The functional ortholog of Sae2 in other eukaryotes is the CtIP/Ctp1 protein, which shows almost no sequence similarity to Sae2 apart from a short stretch of amino acids in the C-terminus of the proteins, yet has been shown to be remarkably similar in its functions in DSB resection [97-102]. Ctp1 in fission yeast was shown to be required for efficient resection of DSBs and is regulated at the level of transcription to be present at high levels in S and G₂ phases of the cell cycle [103]. In chicken DT40 cells and in human cells in culture, CtIP also is important for resection in S and G₂ and is phosphorylated by CDK at two sites that control the efficiency of resection [101,102]. One of these sites (S327) also regulates association of CtIP with the Brca1 tumor suppressor [104], a factor which has been known to be critical for homologous recombination for some time [105] but the exact function of which remains unknown. CtIP in DT40 cells was also shown to act in G₁ phase, in addition to its known roles in S and G₂, although its function in G₁ cells appears to be in the microhomology-mediated end-joining (MMEJ) pathway [101]. A function for CtIP in MMEJ was not unexpected because Sae2 also is an important component of Ku-independent MMEJ in budding yeast [106].

There are a few differences between CtIP and Sae2, most notably the fact that Ctp1 in *S. pombe* and CtIP in mammalian cells require MRN for localization to DSBs [103] whereas Sae2 localizes independently [68,99]. Mammalian CtIP binds directly to the MRN complex in a manner that involves both the N-terminus and the C-terminus of CtIP, with the N-terminus being particularly important for DNA damage localization [99,107]. CtIP in mammals is significantly larger than yeast Sae2 (897 a.a. vs. 345 a.a.) and also is lacking in recognizable domains apart from a coiled-coil region identified in the N-terminus that mediates homodimerization [108,109].

CtIP was also shown to be required for DSB resection in *Xenopus* egg extracts, where the MRN complex and ATM were found to be essential for CtIP loading onto DNA ends [100]. The kinase activity of ATM was required for CtIP localization on damaged DNA, but the identity of the target of ATM is currently unknown. You et al. also identified a DNA-binding motif within the central region of CtIP that is necessary and sufficient for localization to DNA damage sites, although binding of a small fragment containing this motif was independent of ATM activity [100]. The authors suggest that modification of full-length CtIP (by ATM or other enzymes) after DNA damage might uncover this DNA-binding motif to promote damage localization.

4. Summary and future questions

We now understand much more about the mechanisms of DSB resection than we did a few years ago. The enzymes responsible for long-range resection in *S. cerevisiae* have been identified as Exo1 and Dna2/Sgs1/Rmi1/Top3 [13,55-57], and it appears that at least Exo1 function in resection has been conserved in human cells [110]. The RecQ helicase BLM also has been shown to stimulate the activity of human Exo1 in vitro [111], and to affect DSB resection in human cells [57], suggesting that it may be an important component of the system in higher organisms. The WRN helicase and Dna2 have also been implicated in DSB resection in *Xenopus* egg extracts [112], so it is possible that both the Exo1 and Dna2 pathways may operate in higher eukaryotes.

It is now clear that the MRX complex does not catalyze long-range resection directly, but rather is involved in a number of activities that together promote long-range resection: recruitment of downstream enzymes, processing of DNA ends to promote recruitment and remove covalent adducts, and blocking the binding of NHEJ factors to DSB ends (summarized in Fig. 1). We also know that CDK phosphorylation regulates Sae2/CtIP activity at DNA breaks [94], but relatively little detail has emerged about how this affects the activities of these proteins, or how CDK activity might block the inhibitory effects of Ku on DSB resection. Finally, there is evidence that ATM activity is important for CtIP loading on DNA but the target and mechanism of this effect is still under investigation.

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Figure 1.

Schematic diagram of proposed mechanisms by which the MRX complex and Sae2 facilitate the activity of the downstream enzymes, shown as “Nuc”, to represent either Exo1 or Dna2(Sgs1/Rmi1/Top3) nucleases. See text for details. Recruitment (left panel): MRX and Sae2 promote nuclease binding to DNA by creating a higher-affinity binding site for the nuclease (here shown as a branched DNA structure). Nuclease bound to the MRX/Sae2-bound DNA makes an endonucleolytic cut (scissors) on the 5' strand. Further resection occurs by nucleases independently of MRX/Sae2. The event that triggers MRX and Sae2 dissociation is not known. End processing on unmodified ends (center panel, left side): MRX and Sae2 cleave the 5' strand of the DNA end through endonucleolytic activity (scissors); in the case of Exo1, this short 3' overhang facilitates nuclease binding and digestion. End processing on modified ends (center panel, right side): MRX and Sae2 cleave an adduct from the DNA (shown here as the 5' strand but in theory could be the 3' strand), allowing access of nucleases to the ends. Alternatively, Dna2 may also perform adduct cleavage [56]. Ku inhibition (right panel): the Ku heterodimer binds avidly to DNA ends; this occurs in competition with MRX binding. Some of the time MRX wins this competition and blocks Ku from binding; this allows loading of Exo1 and resection. Dna2 binding to DNA ends in vivo is relatively unaffected by Ku, while Exo1 binding is strongly affected [64], thus the nuclease in this case is likely to be Exo1. The exact configuration of the proteins on the DNA is not known, although Exo1 has been shown to crosslink to a DNA end on the 5' strand, while Sae2 crosslinks to the DNA on the 3' strand in strand-specific crosslinking experiments [25].