Disease-associated MRE11 mutants impact ATM/ATR DNA damage signaling by distinct mechanisms

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DNA double-strand breaks (DSBs) can lead to instability of the genome if not repaired correctly. The MRE11/RAD50/NBS1 (MRN) complex binds DSBs and initiates damage-induced signaling cascades via activation of the ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia- and rad3-related (ATR) kinases. Mutations throughout MRE11 cause ataxia-telangiectasia-like disorder (ATLD) featuring cerebellar degeneration, and cancer-predisposition in certain kindreds. Here, we have examined the impact on DNA damage signaling of several disease-associated MRE11A alleles to gain greater understanding of the mechanisms underlying the diverse disease sequelae of ATLD. To this end, we have designed a system whereby endogenous wild-type Mre11a is conditionally deleted and disease-associated MRE11 mutants are stably expressed at physiologic levels. We find that mutations in the highly conserved N-terminal domain impact ATM signaling by perturbing both MRE11 interaction with NBS1 and MRE11 homodimerization. In contrast, an inherited allele in the MRE11 C-terminus maintains MRN interactions and ATM/ATR kinase activation. These findings reveal that ATLD patients have reduced ATM activation resulting from at least two distinct mechanisms: (i) N-terminal mutations destabilize MRN interactions, and (ii) mutation of the extreme C-terminus maintains interactions but leads to low levels of the complex. The N-terminal mutations were found in ATLD patients with childhood cancer; thus, our studies suggest a clinically relevant dichotomy in MRE11A alleles. More broadly, these studies underscore the importance of understanding specific effects of hypomorphic disease-associated mutations to achieve accurate prognosis and appropriate long-term medical surveillance.

INTRODUCTION

DNA damage is commonplace and results from a variety of endogenous and exogenous sources (1). DNA double-strand breaks (DSBs) are a highly toxic form of damage and arise from replication fork collapse, ionizing radiation (IR) or as intermediates in programmed rearrangements during meiosis and lymphocyte development. If not properly repaired, these lesions may result in deletion, duplication or translocation of genomic material leading to cellular dysfunction and potentially neoplastic transformation. Hence, maintenance of genomic integrity is essential for cellular and organismal viability. To deal with the constant threat of DNA damage, organisms have evolved elaborate DNA damage response (DDR) machinery to attempt DNA repair, and if unsuccessful, remove cells from the proliferative pool.

The MRE11/RAD50/NBS1 (MRN) complex is central to the detection and repair of DSBs, as well as the restart of stalled replication forks (2). This complex serves as a hub for regulation of kinases involved in damage responses. When DSBs arise, MRN directly binds DNA ends and facilitates activation of ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia- and rad3-related (ATR). These are the apical kinases of the DDR that are responsible for cell cycle arrest, initiation of DNA repair and, if necessary, apoptosis. DNA ends are bound directly and stabilized in proximity by a heterotetramer composed of two MRE11 and two RAD50 protomers. When MRN is engaged at DSBs, it recruits and activates ATM through direct interaction with the NBS1 subunit as well as other possible contacts (3–5). MRE11 possesses endo- and exo-nuclease activities that initiate resection of DNA ends (6,7). This end processing facilitates generation of single-stranded DNA upon which ATR can be subsequently loaded and activated (3,8). MRN-dependent resection is promoted by ATM; thus, DSB-induced ATR activation is also downstream of ATM activity (9,10).

Inherited deficiencies in DNA damage signaling are responsible for a spectrum of disorders with diverse sequelae involving the central nervous system, the immune system and
musculoskeletal development (1). Biallelic mutations in ATM or NBN (NBS1) result in ataxia-telangiectasia (AT; MIM 208900) or Nijmegen breakage syndrome (NBS; MIM 251260), respectively (11–13). These syndromes are marked by neurologic phenotypes (cerebellar ataxia and microcephaly, respectively), immunodeficiency, cellular genomic instability and IR hypersensitivity (14,15). As anticipated from the important role of DNA repair in maintaining genomic stability, AT and NBS patients display strong predisposition to malignancy. In fact, a single hypomorphic ATM or NBS1 allele can be sufficient to increase cancer risk (16–24). This cancer predisposition is also apparent in mouse models. Though Nbs1-null mice are not viable (25), mice with Nbs1 heterozygosity or hypomorphism are predisposed to oncogenesis (26,27). Furthermore, Atm-null mice are predisposed to lymphoma (28), and mice carrying one hypomorphic Atm allele are cancer predisposed (29). Other components of the DSB repair machinery are also tumor suppressors—for example, BRCA1 and BRCA2 (30,31).

Given that MRE11 functions in a complex with NBS1, it would be anticipated that inherited MRE11 deficiencies would phenocopy NBS. However, this is not commonly observed. Germline biallelic MRE11A (MRE11) mutation results in a syndrome more similar to AT than NBS and has thus been termed ataxia-telangiectasia-like disorder (ATLD; MIM 604391) (32–37). Like AT, ATLD is characterized by cerebellar ataxia which develops after birth through neurodegeneration rather than the microcephaly at or shortly after birth associated with NBS. In addition, the earliest identified ATLD patients appear to differ from both AT and NBS in their cancer predisposition. ATLD patients homozygous for the first identified ATLD allele, MRE11A\textsuperscript{ATLD\textsubscript{1}}, have not been reported to develop early cancer, nor are Mre11a\textsuperscript{ATLD\textsubscript{1}/ATLD\textsubscript{1}} mice predisposed to the development of lymphoma—the malignancy to which AT and NBS patients are most markedly predisposed (32,38). However, MRE11A\textsuperscript{ATLD\textsubscript{1}} has been associated with a familial cancer syndrome inherited in an autosomal dominant manner (39). Also, more recently identified ATLD patients harboring novel MRE11A mutations do seem cancer prone. Two ATLD-afflicted brothers both died of lung cancer (pulmonary adenocarcinoma) at the remarkably young ages of 9 and 16 (35). These two individuals had cerebellar degeneration but additionally had developmental defects suggestive of NBS.

Comparison of these DDR disorders makes clear that while there are some common features, there are also critical differences among specific patient groups. Complete absence of any MRN component is early embryonic lethal in mice; thus, the disease alleles are partial loss-of-function mutations that must preserve essential functions of the complex (25,40,41). It is likely that the differing impact of each of the mutations is largely responsible for the varied spectrum of sequelae in the resulting disorders. Understanding and predicting these differences has important implications for prognosis and treatment for these disorders. Given that mutations in AT and MRN components can affect the wider population through haplo-insufficiency and somatic mutation, greater understanding of these factors has important health implications.

We endeavored to define the specific defects in DNA damage signaling caused by several MRE11A alleles associated with diverse clinical outcomes. To this end, we designed a structure–function system to express mutant alleles at physiologic levels in murine cells harboring an endogenous Mre11a conditional Cre/LoxP allele (Mre11a\textsuperscript{cond}) engineered in our laboratory (41). We find that the MRE11 mutants have distinct consequences on stability of the MRN complex and DNA damage signaling, which appear to correlate with differences in cancer predisposition. Furthermore, these studies provide evidence that MRE11 dimer formation and MRE11–NBS1 interaction are mechanistically linked in mammals thus providing insight into how certain MRE11A mutations abrogate DNA damage signaling.

RESULTS
Selection of four distinct disease-associated MRE11A mutant alleles

We sought to determine if disease-associated MRE11A mutations impact DNA damage signaling in differing ways and if unique functional defects relate to variability in disease sequelae (35,39,42). Unfortunately, most ATLD-associated MRE11A mutations cause low levels of the entire MRN complex, preventing structure–function analyses through study of patient cell lines (32,33,35–37). To circumvent this limitation, we engineered cell lines to express MRE11A mutants of interest at approximately physiologic levels and in which endogenous wild-type Mre11a could be inactivated through Cre/LoxP-mediated deletion (41).

Inherited alleles of interest were chosen based on maximum differences in patient outcome (Fig. 1A). To this end, we compared the impact of the first reported ATLD allele, MRE11A\textsuperscript{ATLD\textsubscript{1}}, to that of recently identified alleles in compound heterozygotes, MRE11A\textsuperscript{ATLD\textsubscript{17}} and MRE11A\textsuperscript{ASM} (Fig. 1B). MRE11A\textsuperscript{ATLD\textsubscript{1}} contains a nonsense mutation that truncates 76 amino acid residues from the MRE11 C-terminus (32). Although MRE11A\textsuperscript{ATLD\textsubscript{17}/ATLD\textsubscript{17}} patients and Mre11a\textsuperscript{ATLD\textsubscript{17}/ATLD\textsubscript{17}} mice exhibited cellular hallmarks of cancer, they do not appear to be cancer predisposed (32,38). In contrast, two brothers harboring both MRE11A\textsuperscript{ATLD\textsubscript{17}} and MRE11A\textsuperscript{ASM} died of pulmonary adenocarcinoma at the ages of 9 and 16 years (35). While this patient cohort is small, the early age of lung cancer is striking given that it is normally a disease of old age (43). The brothers' wild-type MRE11A heterozygous parents and sibling did not have ATLD or lung cancer at last report (44).

The MRE11A\textsuperscript{ATLD\textsubscript{17}} mutation (c.727T>C) results in substitution at a highly conserved residue (p.W243R) near motif IV of the N-terminal phosphodiesterase domain (Fig. 1B). MRE11A\textsuperscript{ASM} possesses an intronic mutation, c.1098+5G>A, near a splice donor site associated with skipping of the 81-nucleotide exon 10. The resulting mRNA maintains the open reading frame, thus encoding a predicted protein lacking 27 amino acid residues (p.Δ340–366). The deleted residues reside in the capping domain, a structural feature unique to MRE11 compared with other known phosphoesterases (45,46).

In addition to inherited mutations causing ATLD, MRE11A has been found mutated in the context of familial and sporadic cancers. Mutational analysis of MRE11A in unselected primary tumors revealed a heterozygous mutation (c.1715G>A) encoding MRE11 R572Q in a lymphoma (42). While it is not known
whether this allele was inherited or if it played a causal role in this lymphoma, the mutation nonetheless resides in an interesting region of mammalian MRE11. R572 is in a glycine-arginine-rich (GAR) motif, portions of which are conserved among metazoans. Methylation of GAR motif arginines has been shown to be important for MRE11 exonuclease activity and activation of the ATR kinase (47–50). On the organismal level, alteration of the GAR motif arginines caused radiation hypersensitivity, a trait common among cancer-prone genomic instability syndromes. However, similar to most studied ATLD alleles, the murine MRE11 protein lacking GAR motif arginines was present at low levels relative to wild-type MRE11 (50). Thus, we engineered the lymphoma-associated MRE11 GAR motif mutant (MRE11GRM) into our expression system to facilitate study of this mutant with the protein at physiologic levels.

Figure 1. Disease-associated MRE11 mutants in this study. (A) Summary of the name, nickname (alias), amino acid change and clinical context for each mutant. (B) A HsMRE11 stick diagram with pertinent domains and motifs. Note that the phosphodiesterase domain includes both the nuclease (catalytic) domain and the capping domain. Amino acid changes are labeled along with mutant alleles and associated human diseases (top). Alignments of the residues surrounding the mutant sites are shown (bottom).

Generation of cell lines expressing Mre11a mutant alleles

Immortalized murine embryonic fibroblasts (MEFs) harboring one Mre11a allele rendered null in the germline (Mre11a−/) and one floxed conditional allele (Mre11acond) (41) were stably transfected with pEF6-Mre11a constructs expressing the mutants of interest from cDNA. Clones were isolated and exposed to adenovirus expressing Cre recombinase to convert the endogenous Mre11acond allele to null (Mre11aΔ). Clones expressing at least physiologic levels of mutant MRE11 protein were used for experimentation, and protein levels were confirmed before and after each experimental time period (Fig. 2A). Untagged proteins were expressed to avoid disruption of MRE11 interactions as previously reported (51). For MRE11ATLD1 and MRE11ASM, gel mobility distinguished these from wild-type MRE11 (Fig. 2B). For
the missense mutants MRE11\textsuperscript{ATLD17} and MRE11\textsuperscript{GRM}, expression was assessed by an RT-PCR-sequencing strategy (Fig. 2C and Supplementary Material, Fig. S1) combined with confirmation of endogenous allele deletion by genomic PCR (data not shown) and by comparison with Mre11\textsuperscript{a}\textsuperscript{cond} cells treated in parallel during each experiment.

**Activation of ATM and ATR kinases**

MRN recruits ATM to DSBs and facilitates kinase activation through direct interaction of ATM with NBS1 (3–5). ATM activation and activity can be monitored by measuring ATM autophosphorylation at S1987 (52) and ATM phosphorylation of KRAB associated protein (KAP1, also called TIF1\textbeta, TRIM28 and KRIP-1) (53). Also, ATM kinase and MRE11 nucleolytic activities together mediate DNA resection (9,10,41). The resulting replication protein A-coated ssDNA can be loaded with ATRIP-ATR at which time ATR phosphorylates substrates such as the CHK1 kinase (3,8,10). To determine the ability of each MRE11 mutant to activate ATM and ATR in the presence of DSBs, pATM S1987, pKAP1 S824 and pCHK1 S345 induction was measured.

We used IR to experimentally induce DSBs, and observed robust KAP1 and CHK1 phosphorylation in the presence of wild-type MRE11, but only weak induction in the absence of MRE11 or ATM (Fig. 3A and B). We next assessed the impact of MRE11\textsuperscript{a}\textsuperscript{cond} alleles found in the ATLD siblings who succumbed to pulmonary adenocarcinoma. Cells expressing MRE11\textsuperscript{ATLD17} showed deficiencies in pATM and pKAP1 induction but not pCHK1 induction (Fig. 3C and D). Decreased pKAP1 induction was observed following IR doses ranging from 0.5 to 10 Gy and following recovery times from 6 min to 8 h (Supplementary Material, Fig. S2A–C). Induction of pKAP1 was also substantially abrogated in cells expressing MRE11\textsuperscript{ASM} (Fig. 3D and Supplementary Material, Fig. S2D). In addition, MRE11\textsuperscript{ASM}-expressing cells showed reduced levels of IR-induced pCHK1, suggesting this mutant was defective in facilitating ATR activity. Hence, whereas both alleles in the ATLD patients with pulmonary adenocarcinoma had a negative impact on ATM activity, only MRE11\textsuperscript{ASM}-expressing cells had deficient ATR activity.

The MRE11\textsuperscript{GRM} mutant identified in a lymphoma supported approximately wild-type levels of ATM activation (Fig. 3E and F). A modest 10% reduction in KAP1 phosphorylation was noted (Fig. 3G) but is likely not biologically significant. In contrast, pCHK1 induction was reduced to 50% of wild-type (Fig. 3E and F, and Supplementary Material, Fig. S2E), indicating reduced ATR activation. This implies that even a subtle disruption of the MRE11 GAR motif is sufficient to compromise ATR activity.

Finally, we analyzed cells expressing MRE11\textsuperscript{ATLD1}, a mutant from ATLD patients who do not appear to be cancer prone. In stark contrast to the mutants described above, MRE11\textsuperscript{ATLD1} expression was able to complement pATM, pKAP1 and pCHK1 induction (Fig. 3F and (51)). Therefore, this disease-associated mutant retains the ability to activate both ATM and ATR kinases. Quantitation of pKAP1 and pCHK1 induction is shown in Figure 3G and H. ATM/ATR activation results are summarized in Table 1.

**Assessment of the G2/M cell cycle checkpoint**

Upon DNA damage, ATM triggers cell cycle arrest at the G2/M transition (54,55). We therefore assessed the competency of the early G2/M checkpoint in our mutant MRE11-expressing lines. Cells were mock-treated or treated with 10 Gy IR, and the mitotic index was calculated based on the percentage of cells positive for the mitosis-specific phospho-histone H3 S10 marker (56). Activation of the G2/M checkpoint is reflected by a significant drop in mitotic index 60 min post-IR. A defective checkpoint is identified by a significantly higher mitotic index post-IR compared with wild-type control. Consistent with previous reports, we found that cells lacking MRN or ATM had a higher mitotic index post-IR compared with wild-type, reflecting the defective G2/M checkpoint (Fig. 4 and Table 1) (38,54).
MRE11\textsuperscript{ASM}, which conferred defects in both ATM and ATR kinase activation, caused a defective G\textsubscript{2}/M checkpoint (Fig. 4 and Table 1). In fact, the impact was similar to that in ATM-deficient cells. MRE11\textsuperscript{ATLD17} did not have a measurable impact on the G\textsubscript{2}/M checkpoint, despite the reduced activation of ATM. This mutant did permit greater ATM activity than MRE11\textsuperscript{ASM}; thus it is likely that the observed difference in checkpoint proficiencies is due to differences in ATM activation. MRE11\textsuperscript{GRM}, which supported normal ATM activation, both appeared capable of maintaining the G\textsubscript{2}/M checkpoint (summarized in Table 1).

**MRE11/RAD50/NBS1 complex stability**

The findings above support the notion that unique MRE11\textsubscript{A} mutations can impact ATM and ATR functions in differing ways. Kinase activation by MRN involves conformational

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**Figure 3.** Assessment of DNA damage-induced ATM/ATR kinase activation. (A – F) Western blot analyses for representative substrates of the ATM and ATR kinases. The indicated cell lines (top) were either untreated or treated with 10 Gy IR and allowed to recover for 30 min. Whole cell lysates were immunoblotted for the proteins indicated (left). GAPDH is a loading control. Samples were loaded in various combinations to allow accurate comparison of phospho-protein levels across multiple gels. Immunoblots are representative of at least three independent experiments. EV, empty vector controls. (G, H) Quantitation of pKAP S824 (G) and pCHK S345 (H) induction from immunoblots. After accounting for protein loading, induction was normalized to the weighted mean induction of the wild-type lines. Each bar represents at least three independent experiments. Error bars represent standard error of the mean.
Previous studies have demonstrated that disrupted MRN complex formation can cause abnormal ratios of the complex components; patients with MRE11A mutations that compromise MRE11–NBS1 interaction have reduced NBS1:MRE11 molar ratios (34,35,37). We assessed cellular RAD50:MRE11 and NBS1:MRE11 ratios to determine if they suggested defects in MRN complex stability.

Mre11a deletion resulted in proportionally decreased RAD50 levels (Fig. 5A and B). Expression of wild-type MRE11, or each of the four MRE11 mutants, resulted in a proportional increase in RAD50 levels. A least-squares best fit for all Mre11a alleles yielded a line with an $R^2 = 0.93$ (Fig. 5B). RAD50:MRE11ATLD1 and RAD50:MRE11GRM ratios were consistent with those found in ATLD17 and ATLD1 patient cells (32,35). Thus, each mutant can maintain RAD50 levels to a similar extent as wild-type MRE11.

Depletion of MRE11 also resulted in decreased NBS1 levels (Fig. 5A and C). In contrast to what was seen for RAD50: MRE11, cells expressing either MRE11ATLD1 or MRE11ASM had reduced NBS1:MRE11 ratios suggestive of compromised interaction with NBS1. The least squares fit for wild-type cell lines had a slope appreciably different from the best fit for MRE11ATLD1 or MRE11ASM-expressing lines (Fig. 5C). Cells expressing MRE11ATLD1 or MRE11GRM had wild-type-like NBS1:MRE11 ratios, implying normal interactions. In support of our observations, ATLD17/18 patient cells were found to have reduced NBS1:MRE11 ratios (35), whereas ATLD1/2 patient cells had normal NBS1:MRE11 ratios (32) despite both lines having reduced overall levels of MRN. These findings are summarized in Table 2.

Figure 4. Assessment of the DNA damage-induced G2/M checkpoint. Cells were either mock treated or treated with 10 Gy IR, allowed to recover for 60 min and then stained for the mitosis-specific p-histone H3S10 modification, p-H3S10 was detected by flow cytometry, and mitotic index was determined by the percentage of p-H3S10-positive cells. Relative mitotic index (Y-axis) reflects the ratio of the mitotic index of IR-treated cells to untreated cells for a given line. MRE11 wild-type cells (WT) show a significant reduction in mitotic index after IR, reflecting activation of the G2/M checkpoint. Absence of MRE11 (EV, empty vector) or ATM ($\Delta$ATM) shows significantly higher relative mitotic indices compared with WT controls, reflecting a defective G2/M checkpoint. A minimum of three independent experiments were performed for each cell line. Error bars represent standard error of the mean.

Table 1. Comparison of ATM/ATR activation among the four disease-associated Mre11 alleles in this study

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MRE11–NBS1 interaction and MRE11 homodimerization

To directly determine whether the four MRE11 mutants in our study impact NBS1 binding, we utilized the yeast two-hybrid system. Wild-type and mutant genes were cloned into pGBK (bait) and pGAD (prey) plasmids, and binding was quantitatively assessed using $\alpha$-galactosidase expression and qualitatively using colony growth in double selection (-His, -adenine). Both MRE11ATLD1 and MRE11ASM displayed substantial reductions in NBS1 binding (Fig. 7A, B, and Supplementary Material, Fig. S4A). However, MRE11ATLD1 and MRE11GRM interacted with NBS1 to similar extents as wild-type MRE11.

Within the MRN complex, MRE11 forms a dimer which bridges DNA ends in close proximity and positions DNA termini within the nuclease domain (46,62). Using the yeast two-hybrid approach, we determined whether the four mutants in our
study impact the ability of MRE11 to homodimerize. Compared with wild-type MRE11, MRE11ATLD17 homodimerization was reduced (Fig. 7C, D, and Supplementary Material, Fig. S4B). Strikingly, homodimerization was dramatically reduced for MRE11ASM and MRE11 ASM with MRE11 ATLD17, appearing similar to the results obtained using empty pGBK and pGAD vectors. The MRE11ASM fusion proteins were confirmed to be present in the yeast strains through immunoblotting (Supplementary Material, Fig. S4C and E). No defect in MRE11ATLD or MRE11GRM homodimerization was apparent. See Table 2 for summary.

### A mammalian MRE11 homodimerization mutant

Structural studies of proteins from the single-celled eukaryote *Schizosaccharomyces pombe* have suggested a mechanistic...
The findings from examination of the four mutants in this study suggest that this relationship may hold true in mammals and represent an important cause of variable disease sequelae for syndromes associated with MRN dysfunction. The two mutants that impacted MRE11–NBS1 interaction, MRE11ATLD17 and MRE11ASM, also reduced MRE11 dimer stability, whereas the two remaining mutants, MRE11GRM and MRE11ATLD1, preserved both functions. We therefore wished to determine the extent to which mammalian MRE11 dimer stability and NBS1 interaction are functionally linked. To this end, we sought to design an Mre11a mutation that would specifically disrupt dimer formation, using structural and biochemical studies from the archael species Pyrococcus furiosus as a guide (62). Archea lack an NBS1 homologue as well as eukaryotic-specific structural motifs of MRE11 involved in NBS1 binding (46,60). Therefore, residues in mammalian MRE11 that are conserved with those required for dimerization in P. furiosus Mre11 likely represent the core features for this function.

We identified murine and human L72 as being homologous to P. furiosus Mre11 L61, the latter of which is necessary for Mre11 dimer stability (Fig. 8A) (62). L72 is an aliphatic hydrophobic residue that participates in formation of a hydrophobic pocket at the homodimer interface (Fig. 8B). We mutated this residue to aspartate and utilized the yeast two-hybrid system to assess MRE11 L72D homodimerization and direct interaction with NBS1. We observed that homodimeration of MRE11 L72D was impaired, using both α-galactosidase levels and colony growth (Fig. 8C and D). Thus, the function of this portion of mammalian MRE11 is conserved despite significant divergence in nearby structures involved in interaction with eukaryote-
specific NBS1 (46,60). Importantly, direct interaction with NBS1 was also abrogated by the MRE11 L72D dimerization mutant (Fig. 8E and F).

A key impact on MRE11–NBS1 interaction would be predicted to affect ATM activation. We tested this by stably expressing MRE11 L72D from cDNA in Mre11a/D cells (data not shown). ATM activation was measured by autophosphorylation 30 min after exposure to IR. Indeed, ATM activation was significantly reduced compared with that in cells expressing wild-type MRE11 (Fig. 8G). Thus, our studies have revealed an important functional link between MRE11 homodimerization, MRE11–NBS1 interaction and ATM signaling in mammals.

**DISCUSSION**

Here, we have shown that unique inherited alleles of MRE11A can have varying impact on control of cellular signaling upon DNA damage. A common theme among the ATLD alleles is reduced activation of the ATM kinase, likely accounting for similar disease sequelae such as cerebellar degeneration. However, the mechanisms differ greatly which may explain clinical variation, such as cancer predisposition. Reduced signaling in MRE11A<sup>ATLD1</sup>/ATLD1 individuals likely results primarily from low levels of the MRN complex because we have shown that expression of MRE11<sup>ATLD1</sup> to normal levels supports ATM activity. On the contrary, in MRE11A<sup>ATLD17/ASM</sup> patients, both mutant alleles impact crucial N-terminal structural features required for MRE11–NBS1 interaction and, therefore—ATM activation. The specific defects caused by these two N-terminal mutations suggested a mechanistic link between MRE11 homodimer formation and interaction with NBS1, which we demonstrated through generation of a single-amino acid substitution that abrogates both functions.

The N-terminal half of MRE11 is highly conserved throughout nature. Recent co-crystallization of Mre11 and Nbs1 fragments from the single-celled eukaryote Schizosaccharomyces pombe shows that SpMre11 forms a dimer that interacts with two SpNbs1 fragments (60). Nbs1 binds in a highly asymmetric manner. Although each Nbs1 fragment binds one Mre11 protomer on a peripheral surface, only one Nbs1 fragment binds across the Mre11 dimer interface and makes contact with both Mre11 protomers. This contact occurs at a eukaryote-specific portion of Mre11 recently dubbed ‘latching loops’ (60). The mutant residue in MRE11<sup>ATLD17</sup> (HsMRE11 W243, SpMre11 W248) does not lie within the latching loops. Instead, it forms the hydrophobic core of the structural region encompassing the peripheral
Figure 8. Engineered MRE11 homodimerization mutant. (A) MRE11 stick diagram and alignment indicating amino acid residue L72. (B) Crystal structure of the HsMRE11 N-terminal domain homodimer determined by Park et al. (46) with mutation sites indicated as follows: L72D (dimerization mutant, pink), W243R (MRE11ATLD17, violet) and Δ340–366 (MRE11ASM, mocha). (C) Homodimerization assessed by Y2H colorimetric assay as described in legend to Figure 7A. MRE11 L72D homodimerization was similar to background. (D) MRE11 homodimerization assessed by Y2H colony growth assay as described in legend to Figure 7B. (E) MRE11 L72D interaction with NBS1 assessed by Y2H colorimetric assay as described in legend to Figure 7A. MRE11 L72D–NBS1 interaction was reduced by 59% compared with that of wild-type MRE11–NBS1. (F) MRE11 L72D–NBS1 interaction assessed by Y2H colony growth assay as described in legend to Figure 7B. (G) Cells stably expressing either wild-type MRE11 or MRE11 L72D were treated with 10 Gy IR, allowed to recover for 30 min and lysed. MRE11 L72D failed to complement ATM activation as measured by ATM autophosphorylation. Tubulin was used as a loading control.
binding sites. Hence, its substitution could disrupt folding within the N-terminal domain, thereby effecting binding by Nbs1 at the peripheral sites as well as stability of the Mre11 dimer.

Our studies on the MRE11 L72D mutant have illuminated the mechanistic connection between the homodimer interface helices and NBS1 binding. These helices show high conservation, and studies of Mre11 from prokaryotes and *S. pombe* implicate them in dimer stability independent of the eukaryote-specific latch loops (45,60,63,64). MRE11 L72 lies within these helices, and the impact of MRE11 L72D on homodimerization provides strong evidence that they function in dimer stability in mammals. The influence of this mutant on NBS1 binding indicates that during evolution eukaryotes have built upon the core dimer structure to provide scaffolding for NBS1-dependent functions such as initiation of DNA damage signaling.

The severe impact of the *MRE11A*<sub>ATLD17</sub> mutation could in part explain unique aspects of the disease features of the affected patients. *MRE11A*<sub>ATLD17</sub> was an allele in the two ATLD17/18 children who succumbed to lung cancer, an outcome not reported in other ATLD patients (32,34–37,65). However, the second allele in these individuals must also be considered. *MRE11A<sub>ASM</sub>* represents a loss of 27-amino acid residues from a feature unique to MRE11, the capping domain (45). In unicellular organism MRE11 orthologues, the capping domain has DNA- and RAD50-binding sites (58,62,64,66). Therefore, the capping domain is likely to be crucial for folding of the greater N-terminal phosphodiesterase domain and thus required for stability of the MRE11 protein as a whole. In support of this notion, although *MRE11A<sub>ASM</sub>* mRNA was found in ATLD17/18 patient cells, no evidence of ASM protein was apparent (35,44). Collectively, the available evidence suggests that *MRE11A<sub>ASM</sub>* may functionally mimic a null allele in the patient cells, leaving the MRE11<sup>ATLD17</sup> protein as the disease-causing entity.

In contrast to the well-conserved N-terminus, the C-terminus of MRE11 is divergent in several respects. Prokaryotic Mre11 has little sequence beyond the capping domain, whereas eukaryotes have ~300 additional residues. The primary sequence of these residues is poorly conserved when comparing different evolutionary groups of eukaryotes, and no structural information is yet available. The Mre11<sup>ATLD17</sup> nonsense mutation lies near the 3-prime end of the Mre11 coding sequence and encodes a protein lacking the final 76-amino acid residues (32,38). This truncated protein maintains interactions among MRN components and supports ATM activation. We conclude from this that ATLD1/2 cells have reduced ATM activity and patients have cerebellar degeneration due to low levels of the MRN complex rather than a specific functional defect in the MRE11<sup>ATLD17</sup> protein *per se*. This could provide a rational explanation for the differences in disease course of ATLD1/2 patients compared with syndromes resulting from mutations that cause significant structural alterations in the MRN complex. Examples of the latter would include ATLD17/18—as demonstrated in this study—and NBS resulting from early termination and alternative translation initiation of mutant NBS1 (67). Some ATLD mutations are likely to have intermediate effects compared with those in this study. For example, ATLD3/4 patients harbor a mutation in the N-terminus of MRE11 (N117S), but have not been reported to develop cancer. Interestingly, the ratio of NBS1 to Mre11 proteins in ATLD3/4 is impacted far less than in ATLD17/18, suggesting a lesser impact on MRN structural integrity in ATLD3/4 (32,35). Therefore, we postulate that cancer predisposition may arise in the context of major structural abnormalities in the MRN complex and be less probable when mutations have subtle effects on complex stability or primarily effect MRN protein levels.

How the *MRE11A*<sub>ATLD1</sub> nonsense mutation causes low MRE11 levels is not understood. The mammalian MRE11 C-terminus has recently been shown to interact with cyclin-dependent kinase 2 (CDK2) and to be required for CDK2-dependent phosphorylation of the DNA repair factor CTIP (RBBP8) (51). In principle, loss of this interaction could potentially destabilize MRE11 in a manner similar to disrupted interactions with NBS1. However, CDK2 levels are not reduced in MRE11 deficiency, and MRE11 is present at normal levels in CDK2 knockout mouse fibroblasts (data not shown and (51)). It is possible that additional protein interactions occur at the eukaryote-specific extended MRE11 C-terminus that would explain why levels are low in ATLD1/2. In addition, because mutation of the GAR motif in mice resulted in reduced MRE11 levels, it is formally possible that loss of the C-terminal 76-amino acid residues causes low MRE11 levels by impacting the GAR motif at a distance. Understanding this portion of mammalian MRE11 represents an important future goal for insight into DDRs as well as disease mechanisms of MRN deficiencies.

Although the N-terminal domain of MRE11 is highly conserved, there may be important differences in downstream events under its control. For example, the *S. pombe* mutation analogous to MRE11<sup>ATLD17</sup> (*Sp*Mre11 W248R) was not defective in activation of Tel1 (the *S. pombe* ATM orthologue) despite causing a significant disruption of MRN complex stability (68). Our studies support the notion that mammalian MRE11 dimer formation and NBS1 interaction are mechanistically linked and that this linkage plays an important role in activation of ATM. While a similar linkage may occur between *S. pombe* Mre11 and Nbs1, communication to the respective DNA damage kinases (ATM versus Tel1) might differ. This makes telo-elogic sense since simple organisms need only activate checkpoints to provide sufficient time for repair, whereas metazoan cells must decide whether to risk attempting repair, versus initiating senescent or apoptotic programs.

Our studies highlight the notion that crucial differences in disease sequelae among related disorders can result from subtle differences in multi-protein complex stability. In the case of MRN, all inherited disease alleles are hypomorphic owing to embryonic lethality of null alleles. Therefore, this spectrum of disorders provides a superb platform for our greater understanding of inherited syndromes of this sort. Defining the relationship between disease outcomes and specific protein structures and functions has important diagnostic and prognostic implications. With sufficient knowledge, informed decisions can be made regarding long-term medical surveillance and care catered to the specific mutations present. This type of information will also aid in the emerging strategy of developing therapeutics that manipulate protein–protein interactions rather than directly targeting a catalytic site. In the near future, it may be possible to develop compounds that compensate for structural mutations, restoring interactions and catalytic activity.
MATERIALS AND METHODS

DNA construct creation

Select Mre11a mutations were introduced into pEF6-MmMre11a using site-directed mutagenesis (Stratagene) or—for the alternative splice mutant—PCR amplification followed by ligation. These mutants were shuttled into pGBKKT7 and pGADT7 (Clontech). MmNbn (GeneCopoeia) were TOPO PCR subcloned (Life Technologies) and shuttled into the Y2H vectors.

MEF engineering and culture

Mre11a<sup>condΔ</sup> murine embryonic cell lines (41) were maintained using standard culture conditions. To create clones, cells were transfected (Lipofectamine 2000, Life Technologies) with empty vectors or wild-type MRE11- or mutant MRE11-expressing constructs and clones were isolated and grown under blasticidin (Life Technologies) selection. Prior to each experiment, cells were grown under blasticidin selection for 3 days, split, allowed to recover for a day, treated with replication-defective adeno-cre (University of Michigan Vector Core), allowed to recover for 2 days, split and allowed to recover for 2–3 days after which time experiments were performed. ATM<sup>−/−</sup> MEFs were as described previously (51). Where IR treatment is indicated, cells were exposed to a 137Cs source.

Mre11a RNA typing

5e5 cells per sample were pelleted, RNA was isolated (AllPrep DNA/RNA Mini Kit, Qiagen), RT-PCR was performed (forward primer: GCAATCTCAACATTTCCATTCC, reverse primer: GTTTCTTCTTGGGCAACTACTG) and amplicons were subjected to Sanger sequencing (sequencing primers: forward primer: GCAATCTCAACATTTCCATTCC, reverse primer: GTTTCTTCTTGGGCAACTACTG). Primer and amplicon sequences are listed in Supplementary Material (available online).

Immunoprecipitation

Ten to twenty million cells were lysed with 50 mM Tris–Cl, 300 mM NaCl, 10% glycerol and 1% NP-40. Lysates were pre-cleared with protein A agarose beads (Roche); lysate protein concentrations were measured by BCA assay (Thermo Scientific); 20 μg/μl protein solutions were made; beads were incubated with either anti-MRE11 antibody (Cell Signaling), anti-RAD50 antibody (Bethyl) or anti-NBS1 antibody (Novus); and 0.5 mg protein was added along with phosphatase and protease inhibitors (Roche). After an overnight incubation, beads were washed four times. Proteins were eluted from the beads with Laemmli buffer (BioRad), and extracts were heated at 95°C for 10 min.

Yeast two-hybrid

Y2HGold (Clontech) were cotransformed using the Yeastmaker Yeast Transformation system (Clontech) per the manufacturer’s protocol. To select for cotransformed cells, transformation reactions were plated onto SD-L-W agar (Clontech) plates, and colonies were picked and streaked onto SD-L-W agar plates. To test for interaction by colorimetric assay, the restreaked yeast were picked, grown in SD-L-W (Clontech) overnight, assessed for their density (by OD<sub>600nm</sub>) and briefly centrifuged. α-Galactosidase activity was visualized as conversion of p-nitrophenyl-α-D-galactopyranoside (colorless) to p-nitrophenoxy (yellow, λ<sub>max</sub> = 410 nm). Sixteen microliters of supernatant was aliquoted per reaction, 48 µl assay buffer (2 volumes 0.5 M NaOAc, pH 4.5 (aq) and 1 volume 100 mM p-nitrophenyl-α-D-galactopyranoside (Sigma-Aldrich) (aq)) was added and the reactions were incubated at least overnight. Each reaction was quenched with 136 µl 1 Na<sub>2</sub>CO<sub>3</sub> (aq), and OD<sub>410nm</sub> readings were taken. Colony growth was assessed by culturing yeast overnight, normalizing yeast density by OD<sub>600nm</sub> and plating five 10X serial dilutions onto SD-L-W (loading control) and SD-L-W-H-ade (interaction test) agar plates.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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