Mre11-Rad50-Nbs complex is required to cap telomeres during *Drosophila* embryogenesis

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Edited by Mary-Lou Pardue, Massachusetts Institute of Technology, Cambridge, MA, and approved April 22, 2009 (received for review March 12, 2009)

Using *Drosophila* as a model system, we identified here a stringent requirement for Mre11-Rad50-Nbs (MRN) function in telomere protection during early embryonic development. Animals homozygous for hypomorphic mutations in either *mre11* or *nbs* develop normally with minimal telomere dysfunction. However, they produce inviable embryos that succumb to failure of mitosis caused by covalent fusion of telomeric DNA. Interestingly, the molecular defect is not the absence of MRN interaction or of Mre11 nuclease activities, but the depletion of the maternal pool of Nbs protein in these embryos. Because of Nbs depletion, Mre11 and Rad50 (MR) are excluded from chromatin. This maternal effect lethality in *Drosophila* is similar to that seen in mice carrying hypomorphic *mrn* mutations found in human patients, suggesting a common defect in telomere maintenance because of the loss of MRN integrity.

maternal lethality | telomere capping | targeted mutagenesis | ALTD

The Mre11-Rad50-Nbs (MRN) complex is multifunctional in genome maintenance (1). In DNA double strand break (DSB) repair and damage checkpoint activation, MRN resects DNA ends in cooperation with other proteins (2, 3), and activates the ataxia telangiectasia-mutated (ATM) checkpoint kinase (4). Biochemical studies suggest an additional structural role of MRN in genome maintenance through tethering of DNA ends (5). The Nbs subunit serves to recruit an Mre11 and Rad50 (MR) complex to the nucleus (6–8).

The mechanism of MRN function at telomeres is poorly understood. MRN is partially responsible for maintaining a 3′ DNA overhang at telomeres (9, 10), and this structure is important for loading telomerase activities and the CDC13 capping protein in yeast (11–13). More recently, a direct role in capping was proposed for yeast MRX (14). In *Drosophila* where telomeres are not elongated by a telomerase, loss of MRN leads to telomere fusion (15–19). At *Drosophila* telomeres, MRN may function to maintain a chromatin structure appropriate for loading of the capping machinery (20).

The regulation of MRN function in development is also poorly understood. Mammalian *mrn*-null mutations lead to cell lethality (21). Human hypomorphic mutations in *mre11* and *nbs* cause AT-like disorder (LD) and Nijmegen breakage syndrome (NBS), respectively. Female mice that are homozygous for such mutations produce embryos that die within a few cell divisions after fertilization, suggesting that MRN is important for early animal development (8, 22, 23). To our knowledge, the reason for this requirement remains undetermined.

We used the more amenable *Drosophila* model to dissect the requirements for MRN during early embryo development. We discovered that animals with hypomorphic mutations in either *mre11* or *nbs* develop normally. However, these females produced inviable embryos that suffered gross chromosome segregation defects during the early cell cycles. We developed new molecular and cytological methods that identified the cause of this mitotic catastrophe as telomere uncapping leading to telomere association. We show that this association is accompanied by covalent linkage of telomeric DNA. In the developing mutant embryos, MR proteins are excluded from chromatin because of the depletion of Nbs protein. We suggest that the evolutionarily conserved requirement for MRN during early development is to prevent telomere fusion.

Results

Hypomorphic *mre11* and *nbs* Mutations Cause Maternal Effect Lethality. The cell divisions that occur before the activation of zygotic expression during *Drosophila* embryogenesis have many features that are common in early development of other animals, such as being rapid and having no Gap phases (24). We hypothesized that these unique features might impose a stringent requirement for MRN function. MRN-null embryos from heterozygous crosses develop normally owing to the maternal contribution of wild type protein. Germ-line clones that are null for *rad50* cannot be generated efficiently (25). Therefore, the study of MRN in embryogenesis would be facilitated by hypomorphic alleles that allow the survival of homozygous mutant females.

We chose to study $mre11^{58S}$, the most thoroughly studied *mre11* hypomorphic mutation. It is caused by a single histidine to tyrosine substitution at an invariant residue that is essential for the nuclease activity of Mre11 in both yeast and humans (26, 27). Although $mre11^{58S}$ is the strongest point mutation in various in vitro assays, many of the cellular defects that it causes in yeast are intermediate to those of the null mutation (28). We identified his^{230} in *Drosophila* Mre11 as equivalent to his^{213} in yeast and his^{217} in human Mre11 (Fig. S1), and generated the his to tyr change at the endogenous *mre11* locus by ends-in gene targeting (*SI Materials and Methods*). For *nbs*, we isolated a hypomorphic allele during our development of the site-specific integrase mediated repeated targeting (SIRT) method (29). The nbs^{2K} allele is predicted to encode an N-terminally truncated Nbs protein (*SI Materials and Methods*).

Adult flies homozygous for $mre11^{58S}$ or transheterozygous for $mre11^{58S}$ and the $mre11^{\Delta 35K1}$-null allele (hemizygous for $mre11^{58S}$) are viable, and recovered at a Mendelian ratio. Similar results were obtained for animals homozygous or hemizygous for nbs^{2K}. This is in contrast to *mre11* or *nbs*-null mutants, which die late in development as pupae because of telomere uncapping with an average of 2.5 telomere associations per nucleus (15, 16). We observed that both $mre11^{58S}$ and nbs^{2K} animals display mild telomere-capping defects: 0.2 telomere associations per $mre11^{58S}$ nucleus ($n = 118$), and 0.3 associations per nbs^{2K} nucleus ($n = 122$), compared with the wild-type level of 0.04 (15). Also, both $mre11^{58S}$ and nbs^{2K} males are fertile.

Author contributions: G.G., X.B., and Y.S.R. designed research; G.G., X.B., J.C., D.S., and Y.S.R. performed research; G.G., X.B., and Y.S.R. analyzed data; and G.G. and Y.S.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0902707106/DCSupplemental.

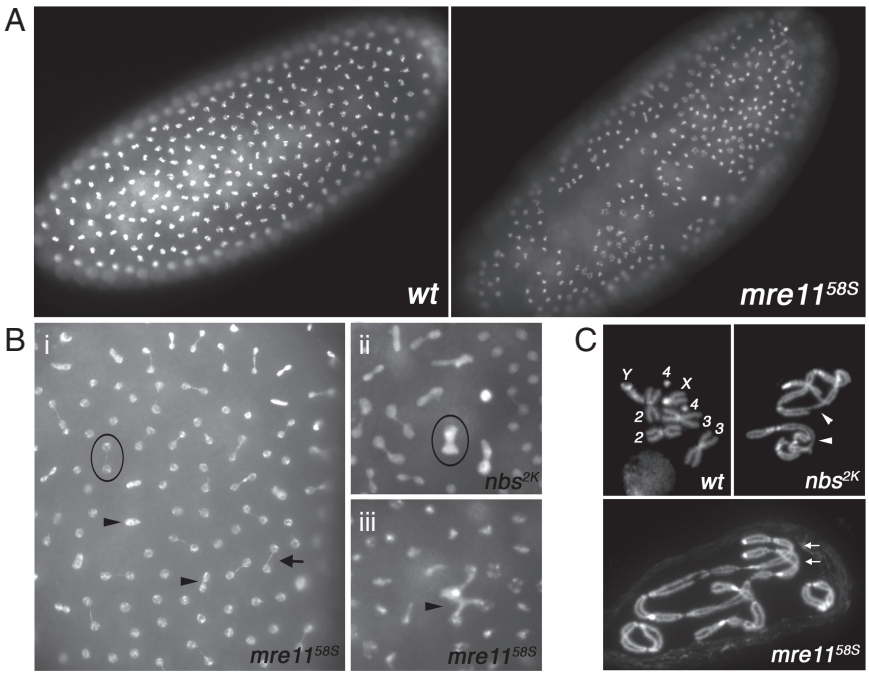

Fig. 1. Chromosome segregation defects in *mre11*^{58S} and *nbs*^{2K} embryos. All panels are pictures of DAPI staining. (*A*) Low magnification pictures showing 2 similarly staged embryos from wild-type (*wt*) and *mre11*^{58S} mothers. In the mutant, nuclei are missing from large areas of the embryo cortex. (*B*) Close-up pictures of *mre11*^{58S} and *nbs*^{2K} embryonic nuclei. (*i*) The arrow points to one of many *mre11*^{58S} nuclei connected by chromosome bridge(s). Arrowheads point to 2 of many polyploid nuclei that probably resulted from failed mitoses. The nucleus in the black circle has lagging chromosome(s). (*ii*) A polyploid *nbs*^{2K} nucleus is circled. (*iii*) The arrowhead points to a multilobed *mre11*^{58S} nucleus possibly having had multiple spindles. (*C*) Mitotic chromosome spreads from embryonic nuclei. In the *wt* nucleus, all chromosomes are labeled. In the *nbs*^{2K} nucleus, arrowheads point to single telomere fusions. In the *mre11*^{58S} nucleus, arrows point to 2 double fusions each between 2 pairs of sister telomeres. This nucleus was polyploid.

Despite the normal appearance of homozygous or hemizygous *mre11*^{58S} or *nbs*^{2K} females, they laid embryos that did not hatch (>10,000 embryos counted), even when they were mated to wild-type males. Therefore, both *mre11*^{58S} and *nbs*^{2K} mutations cause maternal effect lethality. For simplicity, we refer to these embryos as *mre11*^{58S} or *nbs*^{2K} embryos, but note that they have a wild-type copy of the paternal *mre11* and *nbs* genes. The lethality of these embryos is caused by a defect in the maternal contribution of MRN.

Maternal Effect Lethality Is Caused by Nuclear Division Defects. Analyses of DAPI stained mutant embryos suggest that lethality is caused by failure of chromosome segregation (Fig. 1). Early embryos (those examined before cycle 7) appeared to be mostly normal, with occasional nuclei connected by chromosomal bridges (8%, $n = 454$) (Fig. 1*B*). As the embryos developed, more nuclei appeared connected by bridges, and nuclei with abnormal DNA content became abundant. Sister nuclei separation failed in 69 out of 228 mitoses; likely, the result of unresolved chromosome bridges. Some of these polyploid nuclei apparently attempted to divide in the next mitosis, creating multiple-lobed nuclei (Fig. 1*B*). To improve understanding of the chromosome segregation defect, we performed live imaging analyses of *mre11*^{58S} embryos, using GFP-tagged histones to visualize chromosomes. A movie showing nuclear divisions of such an embryo is presented in Movie S1 and Movie S2. Two hundred nuclei were monitored for 2 or more divisions. Mitotic bridges were observed in 38% of anaphases and telophases.

Drosophila embryos can clear abnormal nuclei by "sinking" them interiorly (30, 31). Most late stage *mre11*^{58S} or *nbs*^{2K} embryos had large nuclei-free areas (Fig. 1*A*), and their interior was filled with abnormally large and highly condensed nuclei. They rarely developed signs of gastrulation. We concluded that abundant chromosome bridging led to excessive loss of nuclei and the death of those embryos.

Telomere Fusions Cause Mitotic Catastrophe. We offer unresolved telomere associations as the most likely mechanism leading to chromosome bridging. Previously, telomere associations in *mrn*-null mutants were visualized in mitotic chromosome spreads from larval neuroblasts. To provide evidence for telomere associations in embryos, we developed a mitotic squashing technique, and observed mitotic configurations indicative of frequent telomere associations in mutant nuclei (Fig. 1*C*). Telomere associations were unequivocally identified in 95.3% of *mre11*^{58S} nuclei ($n = 257$), and 93.4% of *nbs*^{2K} nuclei ($n = 91$).

The occurrence of unresolved mitotic bridges suggests that these telomere associations probably involve covalent DNA linkage. To provide direct evidence, we developed a specialized PCR protocol to isolate telomere fusion junctions, taking advantage of the fact that telomere associations in *mre11* or *nbs* mutants are not associated with significant loss of telomeric DNA (15, 16).

The ends of *Drosophila* chromosomes are populated by 3 terminal specific retro-transposons (32). They attach to chromosome ends by the 3′ end of the transcript, resulting in tandem arrays of retro-elements in directed repeats. A telomere fusion can result in a pair of telomere-pointing primers facing each other, leading to a PCR product that would not be produced from wild-type samples (Fig. 2*A*). Using a series of telomere-pointing primers from the conserved GAG region of the most abundant telomere element, *HeT-A*, we recovered PCR products from 0- to 2-h *mre11*^{58S} or *nbs*^{2K} embryos, but such products were rarely recovered from the wild-type controls (Fig. 2*B*). Sequencing of 9 independent clones from *mre11*^{58S} and 10 from *nbs*^{2K} samples revealed that all represented "head-to-head" attach-

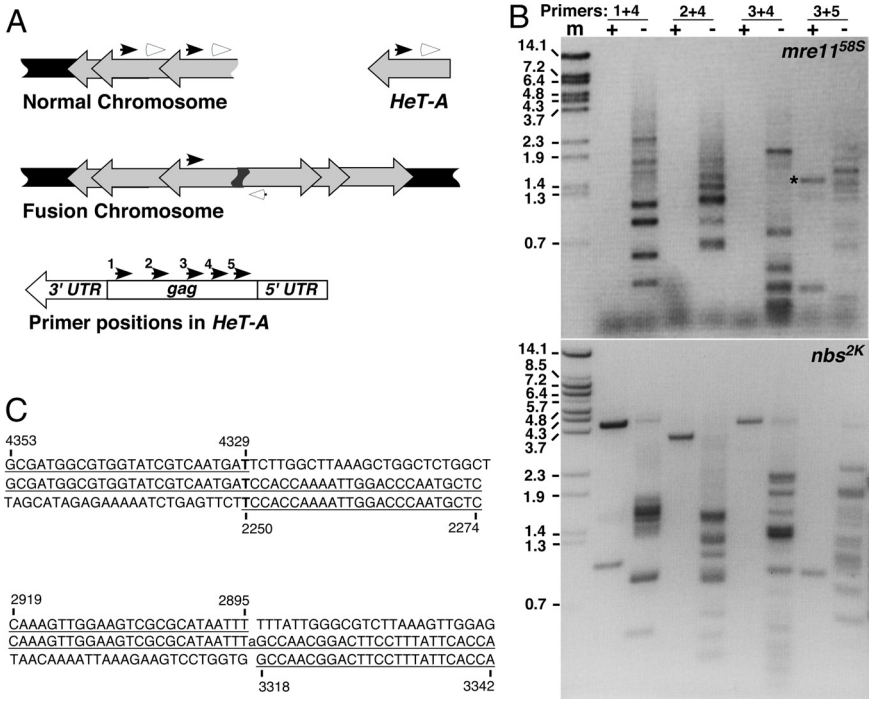

Fig. 2. Telomeres in *mre11*^{58S} and *nbs*^{2K} embryos engage in covalent fusions. (*A*) Schematic representation for isolating telomere fusion junctions. *HeT-A* retro-transposons are depicted as block arrows in gray. They attach to chromosomal DNA (black) unidirectionally. Black and white arrowheads denote a pair of end-facing primers. They can anneal to multiple positions along the *HeT-A* arrays. (*Top*) Wild-type situation in which the PCRs are not expected to be productive. (*Middle*) Telomere fusion in which some of the primer pairs can lead to the formation of PCR products. (*Bottom*) Several features of a *HeT-A* element: 3′ UTR, gag ORF, and 5′ UTR. The approximate positions are indicated for the 5 end-facing primers (arrowheads). (*B*) Agarose gel pictures show PCR products obtained using wild-type (+), *mre11*^{58S} (−; *Upper*), or *nbs*^{2K} (−; *Lower*) DNA templates from embryos. The primer combinations are listed at the top. m, marker DNA with sizes in kilobase. The band marked by * might correspond to nucleotides 2081–3496 in GenBank entry U06920.2 for *HeT-A*, with 3496 corresponding to the first nucleotide in primer 3. (*C*) Sequences of fusion junctions from *mre11*^{58S} (*Upper*) and *nbs*^{2K} (*Lower*). The numbers are nucleotide numbers from U06920.2. Three strands (in the 5′ to 3′ direction) are shown for each junction, with the actual sequences connected by the fusion underlined. The rest of the sequences are those predicted from U06920.2. In each fusion, the top strand came from one telomere that fused with the other telomere shown in the bottom strand, giving rise to the fusion product denoted in the middle strand. The top fusion used the overlapping T (in bold) for repair. The bottom fusion had an a (in small case) as a filler base for repair.

ment of *HeT-A* elements, as predicted for telomere fusions, and appeared to be the products of nonhomologous end joining repair of DSBs (Fig. 2*C*). Therefore, covalent telomere fusions were abundant in mutant embryos. The 6 clones sequenced from wild-type samples were all linear amplifications of a *HeT-A* region primed from a single *HeT-A* primer. These products showed no evidence of telomere fusion, although the mechanism leading to the formation of these products is unknown.

MR Are Excluded from Chromatin in the Mutant Embryos. To gain insight into the molecular defect of telomere dysfunction, we studied the cellular distribution of MRN in syncytial embryos using antibodies against each subunit. Immunostaining analyses using MR antibodies produced very similar, if not identical, distribution patterns (Fig. 3*A*; Fig. S2). In wild-type embryos, MR showed strong staining indicative of abundant maternal contributions of these proteins. We observed MR foci throughout the cell cycle, but they were especially prominent on condensed chromosomes. MR foci did not appear to be localized to telomeres in wild-type cells (Fig. 3*A*). For example, MR foci are not seen frequently on the dot-like chromosome 4. Also, MR foci are often absent from lagging telomeres in anaphase or telophase. A nontelomeric localization of Rad50 was observed in larval nuclei (17). In wild-type cells, Nbs does not colocalize extensively with Mre11 or Rad50 (Fig. 3 *B* and *C*). Instead, Nbs is evenly distributed throughout the interphase nucleus, and is underrepresented on metaphase chromatin, whereas Rad50 forms bright foci.

MR was absent from interphase chromatin in both *mre11*^{58S} and *nbs*^{2K} embryos, although cytoplasmic staining remained (Fig. 3*D*; Fig. S2). MR was also absent from chromatin during metaphase (Fig. 3*D*; Fig. S2), suggesting that nuclear membrane breakdown before mitosis was insufficient to restore MR binding to chromatin in the mutants. Thus, mutations in 2 different genes (*mre11* and *nbs*) both led to the exclusion of MR from chromatin.

Maternal Nbs Is Depleted in *nbs*^{2K} and *mre11*^{58S} Mutants. The Nbs subunit regulates nuclear localization of MR in mammalian cell. We examined whether *Drosophila* cells employ a similar mechanism. We did not detect Nbs in *mre11*^{58S} or *nbs*^{2K} nuclei by immunostaining, and used Western blot analyses to document the extent of this Nbs reduction. There was a greatly reduced level of Nbs, but normal amounts of MR in extracts from *mre11*^{58S} or *nbs*^{2K} adult females, suggesting that the initial maternal store of Nbs is probably less than the wild-type level. In both *mre11*^{58S} and *nbs*^{2K} embryos, the amount of Nbs was below the level of detection, even after enrichment by IP. Although MR proteins were present at near normal levels in the extracts, they were absent from anti-Nbs immuno-precipitates (Fig. 4*A*).

The *nbs*^{2K} mutation encodes an N-terminally truncated protein (Fig. 4*B Center*; Fig. S1), and similarly truncated proteins are unstable in yeast and mammals (7, 33). Thus, the absence of Nbs in extracts from *nbs*^{2K} embryos was expected. However, Nbs depletion was not expected for *mre11*^{58S} embryos, because these embryos were from females with both wild-type copies of the *nbs*

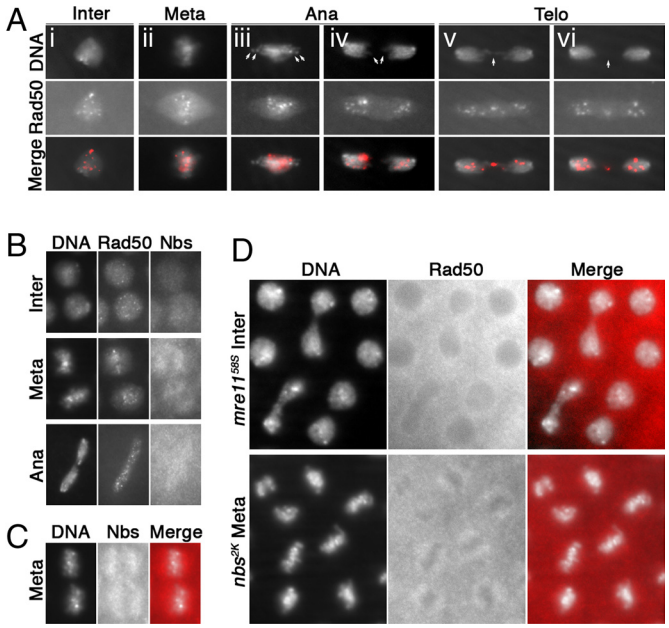

Fig. 3. Rad50 forms large nuclear foci in wild-type, but not mutant embryos. DNA (DAPI) and antibody signals are shown as grayscale pictures in separate panels. In merged pictures, antibody signals are shown in red. (*A*) Rad50 distribution in wild-type cells. (*iii*) Arrows indicate the dot-like chromosome 4s without Rad50 signals. (*iv*) Arrows indicate 2 lagging chromosome ends with no Rad50 signals. (*v*) The arrow points to 2 juxtaposing chromosome ends with strong Rad50 signals. (*vi*) The arrow points to an area with strong Rad50 signals that appear not to be associated with chromatin. (*B*) Nbs does not colocalize with Rad50 in wild type cells. (*C*) Nbs is excluded from mitotic chromatin in wild-type cells. (*D*) Rad50 is excluded from chromatin in an *mre11*^{58S} cell in interphase (*Upper*) and an *nbs*^{2K} cell in metaphase (*Lower*). Cell-cycle phases are indicated as follows: Inter, interphase; Meta, metaphase; Ana, anaphase; and Telo, telophase.

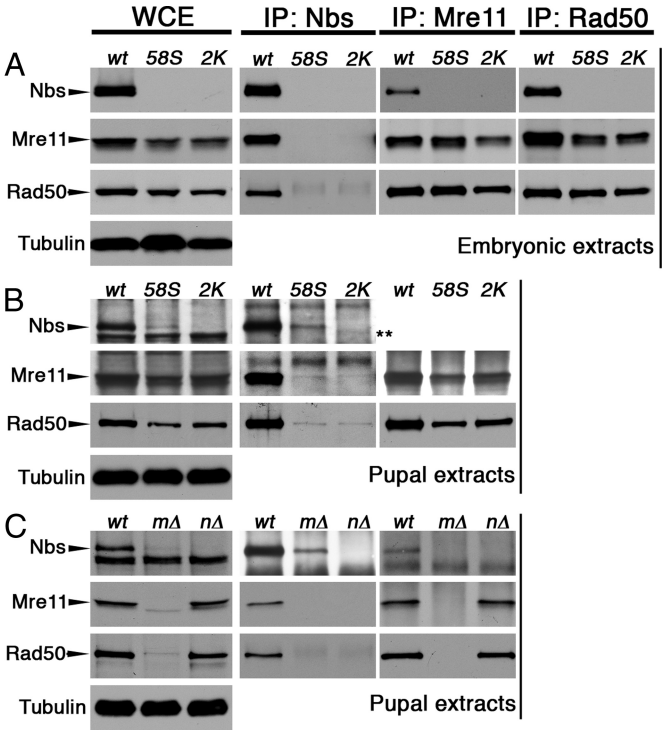

Fig. 4. Biochemical characterization of MRN during development. Western blot analysis results using extracts from syncytial embryos produced from *wt*, *mre11*^{58S} (*58S*), or *nbs*^{2K} (*2K*) mothers (*A*), extracts from pupae of wild-type, *mre11*^{58S} homozygotes, or *nbs*^{2K} homozygotes from heterozygous crosses (*B*), or extracts from pupae of wild-type, *mre11*$^{\Delta}$ homozygotes (*mΔ*), or *nbs*$^{\Delta}$ homozygotes (*nΔ*) (*C*). The antibodies used are indicated at the left. Arrowheads indicate the positions of the protein of interest except for Tubulin. Other bands are recognized by the antibodies nonspecifically. **, Presumed truncated Nbs protein from *nbs*^{2K} homozygous extracts; WCE, whole-cell extract. IP with the antibody indicated.

gene on a chromosome separate from the *mre11* locus. It is possible that defective MRN interactions in *mre11*^{58S} might have led to Nbs depletion, because the mutation disrupts such interactions in yeast (26). However, results from our IP experiments did not support this hypothesis (Fig. 4*A*). In *mre11*^{58S} extracts, interactions between Mre11^{58S} and Rad50 were about as efficient as those between normal MR proteins in wild-type extracts. In *nbs*^{2K} extracts, interactions between MR were also proficient. Therefore, defective MR interaction was not observed in the mutant extracts. Nbs depletion has prevented us from assaying the efficiency of Mre11-Nbs interaction in the mutant extracts.

Residual MRN Function in Postembryonic Tissues of *mre11* and *nbs* Mutants. Contrary to the severe capping defect in the embryos, *mre11*^{58S} and *nbs*^{2K} postembryonic animals developed mild telomere dysfunction, and were viable. We investigated possible mechanisms for this difference by Western blot and IP analyses. As already indicated in Figs. 3 and 4*A*, there is abundant maternal deposition of MRN in wild-type syncytial embryos. This maternal supply probably runs out during the third instar larval stage. Two observations support this hypothesis. First, telomere uncapping in *mre11*$^{\Delta 35K1}$-null mutants first occurs during larval development (*SI Materials and Methods*). Second, maternal Mre11 and Nbs proteins are absent in pupal extracts from respective deletion mutants (Fig. 4*C*). In *mre11*^{58S} pupal extracts, the levels of Rad50 and the mutant Mre11^{58S} proteins were slightly reduced, whereas Nbs level was reduced to a much greater extent (Fig. 4*B Left*). Similar to the situation observed in embryo extracts, the interactions between Mre11^{58S} and Rad50 were efficient in pupal extracts (Fig. 4*B Right*). Interestingly, the residual Nbs proteins seemed to able to interact with both Mre11^{58S} and Rad50, because we detected a very small amount of MR in anti-Nbs IPs (Fig. 4*B Center*). We surmise that this residual amount of Nbs, possibly in complex with MR, might be sufficient to provide telomere protection and to support viability.

We detected a protein that migrates faster than wild-type Nbs in *nbs*^{2K} extracts (marked with 2 asterisks in Fig. 4*B*), which might correspond to the N-terminally truncated protein as our antibody recognized C-terminal epitopes in Nbs. This protein was much less abundant than Nbs in wild-type cells, consistent with our hypothesis that the truncation destabilized the mutant protein. Similar to the situation in *mre11*^{58S}, this residual level of Nbs in *nbs*^{2K} might be sufficient to maintain telomere integrity. We tested whether the remaining Nbs function in *nbs*^{2K} could be reduced further by the *mre11*^{58S} mutation, and found that *mre11*^{58S} and *nbs*^{2K} double mutants were lethal as pupae. They suffered a more severe telomere dysfunction than either single mutant (1.4 fusions per nucleus, $n = 81$).

Discussion

We have used *Drosophila* to study the functions of MRN in a developmental context. We discovered that *mre11* and *nbs* hypomorphic mutations yielded maternal effect lethality caused by telomere fusions resulting in failure of mitosis. This loss of telomere protection is probably caused by the depletion of the maternal Nbs pool and the subsequent exclusion of MR from chromatin.

GENETICS

Regulation of MR Function by Nbs. The abnormal localization of MR in nbs^{2K} embryos can be explained by the depletion of maternal Nbs due to a destabilizing *nbs* mutation. Based on this result, we suggest that the presence of Nbs is essential for MR loading to chromatin, and that this loading is unlikely to occur during mitosis, because Nbs is excluded from mitotic chromatin in normal cells (Fig. 3*C*). The situation is less clear for $mre11^{58S}$ embryos. The H230Y mutation might have led to the exclusion of MR from chromatin and a separate defect that caused Nbs destabilization. An alternative hypothesis is that the exclusion of MR from chromatin is secondary to Nbs depletion, similar to the situation in nbs^{2K}. We cannot distinguish between these 2 possibilities.

The $mre11^{58S}$ mutation in this study was modeled after the yeast mutation, but the 2 have different effects on MRN integrity. The yeast $Mre11^{58}$ protein does not interact with either Rad50 or Xrs2 (the yeast Nbs equivalent), but those defective interactions do not lead to Xrs2 depletion in vivo (26). However, we observed Nbs depletion in $mre11^{58S}$ animals despite efficient interactions of $Mre11^{58S}$ with Rad50 in embryonic and pupal extracts (Fig. 4). This difference may be organism specific, and an explanation of how $mre11^{58S}$ compromises MRN integrity in *Drosophila* will require further investigation.

In contrast to syncytial embryos from $mre11^{58S}$ or nbs^{2K} homozygous mothers, $mre11^{58S}$ or nbs^{2K} mutant embryos from heterozygous parents have sufficient maternal contributions to support proliferation passed the critical point of zygotic activation. Subsequently, zygotic *nbs* expression may be sufficient for maintaining a low level of Nbs in postembryonic cells to prevent telomere uncapping in these mutant animals. However, other developmental factors could have also contributed to the different degrees of telomere dysfunction in $mre11^{58S}$ and nbs^{2K} animals during embryonic versus postembryonic development. For example, although Nbs is essential for MR binding to chromatin in embryonic cell cycles, other proteins might, in late stages of development, partly fulfill the function of recruiting MR. This proposition would be consistent with previous results showing that nbs^{1} mutants have a partial defect in chromatin retention of Rad50 during metaphase in larval neuroblasts (18).

Other Possible Defects in $mre11^{58S}$ and nbs^{2K} Embryos. Our results from mitotic chromosome spreads and telomere fusion PCR analyses suggest that telomere fusion is abundant in $mre11^{58S}$ and nbs^{2K} embryonic nuclei, and is probably the major cause for mitotic failures. However, there may be other possibilities, because we showed nuclear MR foci that were not at the telomere (Fig. 3). Consistently, we did not detect interactions between MRN and the HOAP capping protein (34, 35) in coIP experiments. Future work decoding the chromosomal addresses for these MR foci may implicate other defects in chromatin architecture. It is conceivable that other types of chromosome fusions occur such as those between DSBs that persist because of MRN dysfunction, or between DSBs and uncapped telomeres.

MRN is also required for meiotic DSB repair and *mrn* mutations disrupt meiotic processes in animals (8, 25, 36, 37). In *Drosophila* females, defects in meiotic DSB repair lead to the activation of a checkpoint that disrupts axis formation in oocytes, giving rise to embryos with a "spindle" phenotype (38). Female $mre11^{58S}$ and nbs^{2K} mutants lay abundant embryos, and these embryos display no spindle phenotype. Also, meiotic progression in $mre11^{58S}$ females is normal with a delay in the repair of some DSBs (K. McKim, personal communication). Therefore, we have no evidence to suggest that mitotic catastrophes in $mre11^{58S}$ and nbs^{2K} embryos are a manifestation of a gross defect in meiotic DSB repair.

MRN controls various checkpoints that arrest the cell cycle in response to DNA damage (21). The rapid embryonic cycles in *Drosophila* are atypical, and lack conventional checkpoints, because replication inhibitors or X-ray irradiation are unable to arrest cell-cycle progression (30, 39). However, loss of MRN could activate other specialized cell-cycle control mechanisms operating during the early cell divisions. For example, mitotic failure in $mre11^{58S}$ and nbs^{2K} embryos could activate CHK2 in subsequent cell cycle(s) leading to nuclei displaced from the embryo cortex (31). Also, the mitotic spindle checkpoint might also be activated because of abnormal chromosome behaviors in mutant embryos. Silva et al. (40) identified mitotic defects in embryos that were devoid of maternal ATM. Because of the intimate functional relationship between ATM and MRN, it is possible that telomere uncapping was also responsible for those defects.

Critical Requirement for MRN in Early Development Is Conserved in Animals. The maternal effect lethality uncovered in our study is similar to that described for $mre11^{ATLD}$ and $nbs^{\Delta B}$ mice, which suggests that the requirement for MRN in early development is conserved in animals. Theunissen et al. (23) speculated that a synergistic effect of defective checkpoint and recombination functions was responsible for the rapid loss of cell proliferation in those mice. We suggest that telomere dysfunction, because of the instability of the maternal Nbs supply, might be the major contributing cause for maternal lethality.

In *mrn* and other telomere uncapping mutants in *Drosophila*, one of the most striking consequences of chromosome segregation failure is the generation of polyploid cells (Fig. 1). This observation suggests that the mitotic apparatus might not exert enough force to overcome multiple covalent linkages between DNA molecules (41). Interestingly, many chicken DT40 cells and mouse B-lymphocytes become polyploid after a gradual loss of MRN (42, 43), suggesting that these polyploid cells might have occurred as a result of telomere fusion. Although telomere elongation in *Drosophila* is independent of the telomerase function common in most other animals, the telomere capping function of *Drosophila* MRN is probably a conserved feature.

Materials and Methods

Primer sequences are listed in Table S1. Information about primers used for sequencing is available on request. Fly stocks and antibody information, as well as a detailed protocol for extract preparations, are provided in *SI Materials and Methods*.

Mitotic Spreads of Larval and Embryonic Nuclei. Mitotic spreads from larval neuroblasts were performed as described (44). For embryonic mitotic spreads, the larval protocol and a protocol for metabolic labeling of embryos (45) were combined and modified as follows. Embryos (0–1 h) were collected onto a Netwell insert with 74-μm mesh (Costar, Corning). Embryos were dechorionated with 50% bleach for 90 s, and washed extensively with embryo wash buffer (0.7% NaCl/0.05% Triton X-100). Embryos were dried with blotting paper, then submerged in a small amount of octane that just covered the embryo layer. All subsequent treatments were performed on a rotating platform, and embryos were agitated periodically using a pipette. Embryos were treated for 5 min in octane, dried, and rehydrated in embryo wash for 1 min with agitation. Embryos were treated with 0.05 mM colchicine in 0.7% NaCl for 20 min, followed by 5 min in a hypotonic solution of 0.5% sodium citrate. The hypotonic solution was substituted with embryo wash, the embryos were transferred to V-vials (Wheaton), and the aqueous solution was removed as much as possible; 1 mL of a methanol, acetic acid, and water mix (11:11:2) was added to the embryos, and they were fixed for at least 5 min on a rotator, and stored at 4 °C until used for chromosome spreading. A drop of the fixative carrying embryos was placed on a slide. To avoid overcrowding of mitotic figures, $<$10 embryos were loaded per slide. A clean siliconized coverslip was placed on the embryos. The embryos were squashed first gently, then hard. All remaining steps were performed according to the protocol for larval neuroblasts (44).

Isolation of Telomere Fusion Junctions. As templates, we used DNA from 0- to 2-h embryos from $mre11^{58S}$ or nbs^{2K} females that had been mated with their heterozygous male siblings. Similarly staged embryos from matings between heterozygous male and female siblings were used as controls. Embryos were homogenized in a buffer (100 mM Tris, pH 8.8/200 mM NaCl/5 mM EDTA/0.2% of

SDS) with a final concentration of 5 ng/μL of proteinase K freshly added, and left at room temperature for 2 h or at 4 °C overnight. DNA was phenol extracted, and iso-propanol precipitated. Between 0.2 and 1 μg of genomic DNA was used in a 50 to 100 μL PCR using Extaq (Takara) with the following PCR program: 98 °C 30 s, 68 °C 5–8 min for 40 cycles followed by a single 72 °C 10-min step. Primers were HeT-A453rev (primer 1), HeT-A1196rev (primer 2), HeT-A1751rev (primer 3), HeT-A1997rev (primer 4), and HeT-A2615rev (primer 5). The combinations 1 + 4, 2 + 4, 3 + 4, and 3 + 5 were used for junction isolation (Fig. 2). PCR products were cloned by TOPO TA cloning and sequenced.

Whole-Mount DAPI and Antibody Staining of Embryos. DAPI and Immunostaining of whole-mount embryos were performed essentially as described using boiling as the initial fixation method (46). Anti-Rad50 sera from either rabbit or guinea pig were used at a 1:200 dilution. Anti-Mre11 and anti-Nbs sera from guinea pigs were used at a 1:100 dilution. Secondary antibodies (Invitrogen) were used at a 1:500 dilution.

ACKNOWLEDGMENTS. We thank Dr. Paul Goldsmith's group at National Cancer Institute (NCI) for purifying anti-Rad50 antibodies; Drs. Kami Ahmad (Harvard University, Cambridge, MA) and Maurizio Gatti (University of Rome, Rome, Italy) for reagents; members of the Rong lab for comments on the manuscript; and Drs. Maxine Singer, Michael Lichten, and Dhruba Chattoraj at NCI for their efforts in improving this manuscript. This work was supported by the NCI Intramural Program.

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