The *Rad50<sup>S</sup>* allele promotes ATM-dependent DNA damage responses and suppresses ATM deficiency: implications for the Mre11 complex as a DNA damage sensor

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Genetic and cytologic data from *Saccharomyces cerevisiae* and mammals implicate the Mre11 complex, consisting of Mre11, Rad50, and Nbs1, as a sensor of DNA damage, and indicate that the complex influences the activity of ataxia-telangiectasia mutated (ATM) in the DNA damage response. *Rad50<sup>S/S</sup>* mice exhibit precipitous apoptotic attrition of hematopoietic cells. We generated ATM- and Chk2-deficient *Rad50<sup>S/S</sup>* mice and found that *Rad50<sup>S/S</sup>*, cellular attrition was strongly ATM and Chk2 dependent. The hypomorphic *Mre11⁴<sup>ATLD1</sup>* and *Nbs1²<sup>H9004</sup>* alleles conferred similar rescue of *Rad50<sup>S/S</sup>*-dependent hematopoietic failure. These data indicate that the Mre11 complex activates an ATM–Chk2-dependent apoptotic pathway. We find that apoptosis and cell cycle checkpoint activation are parallel outcomes of the Mre11 complex–ATM pathway. Conversely, the *Rad50<sup>S</sup>* mutation mitigated several phenotypic features of ATM deficiency. We propose that the *Rad50<sup>S</sup>* allele is hypermorphic for DNA damage signaling, and that the resulting constitutive low-level activation of the DNA damage response accounts for the partial suppression of ATM deficiency in *Rad50<sup>S/S</sup>* *Atm<sup>−/−</sup>* mice.

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The cellular response to DNA damage includes activation of DNA damage-dependent cell cycle checkpoints, DNA repair, and in some contexts, apoptosis. The Mre11 complex, consisting of the highly conserved proteins Mre11, Rad50, and Nbs1, has been implicated in DNA damage recognition and the subsequent activation of cell cycle checkpoints, as well as in DNA repair. Human and mouse hypomorphic *Nbs1* and *Mre11* mutations [Carney et al. 1998; Stewart et al. 1999; Kang et al. 2002; Williams et al. 2002; Theunissen et al. 2003; Difilippantonio et al. 2005] in Nijmegen breakage syndrome [NBS] and the ataxia-telangiectasia-like disorder [A-TLD], respectively, are associated with ionizing radiation [IR] sensitivity, checkpoint deficiency, and chromosome instability. Despite these defects in the DNA damage response pathway, IR-dependent apoptotic induction does not appear to be compromised in *Nbs1* or *Mre11* mutant mice [Theunissen et al. 2003].

Several lines of evidence demonstrate that the Mre11 complex functions with the ataxia-telangiectasia mutated [ATM] kinase to affect aspects of the response to DNA double-strand breaks [DSBs]. This conclusion is supported by molecular observations in addition to the phenotypic similarities between NBS, A-TLD, and ataxia-telangiectasia [A-T] [Petrini 2000]. Nbs1 is phosphorylated by ATM in response to DNA damage, and this event is required for activation of the intra-S-phase checkpoint [Gatei et al. 2000; Lim et al. 2000; Wu et al. 2000, Zhao et al. 2000]. The complex also functions upstream of ATM and possibly the ataxia-telangiectasia and Rad3-related [ATR] transducing kinase by recognizing DNA damage and potentiating their activation and activity. The complex associates with DNA damage in-

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Genetic determinants of Rad50<sup>S/S</sup> pathology

In order to determine the mechanism of Rad50<sup>S/S</sup>-dependent cellular attrition, we defined the genetic dependencies of this Rad50<sup>S/S</sup> phenotypic outcome. First, to determine whether Rad50<sup>S</sup> is a hypermorphic allele, we crossed Rad50<sup>S/S</sup> with Rad50<sup>S/A</sup> mice, reasoning that reduced Rad50<sup>S</sup> dosage would increase phenotypic severity if it were hypomorphic, or decrease severity if it were hypermorphic (Luo et al. 1999). Although most Rad50<sup>S/S</sup> mice died from hematopoietic failure, the mean survival was significantly higher than that of Rad50<sup>S/S</sup>: 58% of Rad50<sup>S/S</sup> mice survived to 5 mo [Fig. 1A]. The increased survival of Rad50<sup>S/A</sup> was associated with malignancy as three out of 29 mice died with lymphoma [Fig. 1A]. Hence, reduced Rad50<sup>S</sup> dosage in Rad50<sup>S/S</sup> mice enhanced survival, indicating that Rad50<sup>S</sup> is a hypermorphic allele.

Since the Mre11 complex and ATM function in the same DNA damage response pathway [D’Amours and Jackson 2002, Petrini and Stracker 2003], we established Rad50<sup>S/S</sup> Atm<sup>−/−</sup> double-mutant mice to determine whether the hypermorphic character of the Rad50<sup>S</sup> allele was ATM dependent. Whereas Rad50<sup>S/S</sup> mice are recovered at 50% of the expected Mendelian frequency [Bender et al. 2002], Rad50<sup>S/S</sup> Atm<sup>−/−</sup> mice were recovered at normal Mendelian ratios.

Phenotypic rescue was also observed in mature animals. Peripheral blood cell numbers in Rad50<sup>S/S</sup> Atm<sup>−/−</sup> mice were comparable to wild-type levels (red blood cell percentages: wild type, 41%; Rad50<sup>S/S</sup>, 32%; Rad50<sup>S/S</sup> Atm<sup>−/−</sup>, 39%; platelet numbers: wild type, 575 ± 10<sup>4</sup> cells/mL; Rad50<sup>S/S</sup>, 252 ± 10<sup>4</sup> cells/mL; Rad50<sup>S/S</sup> Atm<sup>−/−</sup>, 500 ± 10<sup>4</sup> cells/mL). Flow cytometry on 4-wk-old mice indicated that hematopoietic cells in both Rad50<sup>S/S</sup> Atm<sup>−/−</sup> and Rad50<sup>S/S</sup> Atm<sup>−/−</sup> mice were comparable to those in Atm<sup>−/−</sup> mice [Fig. 2, Supplementary Table 1]. The rescue of Rad50<sup>S/S</sup> hematopoietic attrition was associated with significantly increased survival of Rad50<sup>S/S</sup> Atm<sup>−/−</sup> and Rad50<sup>S/S</sup> Atm<sup>−/−</sup> mice compared with Rad50<sup>S/S</sup> [Fig. 1B]. Whereas most (>90%) Rad50<sup>S/S</sup> mice died during the first 5 mo of age, 85% of Rad50<sup>S/S</sup> Atm<sup>−/−</sup> mice and 42% of Rad50<sup>S/S</sup> Atm<sup>−/−</sup> mice lived longer than 5 mo. The increase in survival relative to Rad50<sup>S/S</sup> alone was significant at 5, 10, and 15 mo of age (P values < 0.001). Rad50<sup>S/S</sup> Atm<sup>−/−</sup> died of malignancy and Rad50<sup>S/S</sup> Atm<sup>−/−</sup> predominantly succumbed to anemia, albeit significantly later than most Rad50<sup>S/S</sup> mice. The data indicated that the attrition of hematopoietic cells in Rad50<sup>S/S</sup> mice was dependent on ATM.
Genetic interactions with Mre11 complex mutants

As with cells established from A-TLD patients, cells from mice expressing the Mre11<sup>ATLD1</sup> allele (Mre11<sup>ATLD1/ATLD1</sup> mice) exhibit indices of attenuated ATM activity (Theunissen et al. 2003). We established Rad50<sup>S/S</sup> Mre11<sup>ATLD1/ATLD1</sup> double mutants to determine whether reduced ATM activation by the Mre11 complex would also rescue Rad50<sup>S/S</sup> cellular attrition. As with the Atm-null allele, Mre11<sup>ATLD1</sup> increased Rad50<sup>S/S</sup> survival in a dose-dependent manner [Fig. 1C]. Cohorts of 29 Rad50<sup>S/S</sup> Mre11<sup>ATLD1/ATLD1</sup> and 49 Rad50<sup>S/S</sup> Mre11<sup>+/ATLD1</sup> mice were aged and monitored for indications of Rad50<sup>S/S</sup>-dependent pathology. Twenty-four out of 29 Rad50<sup>S/S</sup> Mre11<sup>ATLD1/ATLD1</sup> mice were alive and older than 5 mo with no overt signs of anemia at the time of this writing, and four double mutants developed lymphoma.

A similar, but less pronounced rescue was observed in Rad50<sup>S/S</sup> Mre11<sup>+/ATLD1</sup> mice. Seventeen Rad50<sup>S/S</sup> Mre11<sup>ATLD1/+</sup> and 24 Rad50<sup>S/S</sup> Mre11<sup>ATLD1/+</sup> mice were aged and monitored for indications of Rad50<sup>S/S</sup>-dependent pathology. Ten out of 17 Rad50<sup>S/S</sup> Mre11<sup>ATLD1/+</sup> mice were alive and older than 5 mo with no overt signs of anemia at the time of this writing, and three double mutants developed lymphoma.

Figure 1. Rescue of Rad50<sup>S/S</sup> survival by Rad50-null, Atm-null, Mre11<sup>ATLD1</sup>, and Nbs1<sup>ΔA/B</sup> alleles. [A] Kaplan-Meier survival curves of Rad50<sup>S/S</sup> and Rad50<sup>S/S</sup> mice. [B] Kaplan-Meier survival curves of Rad50<sup>S/S</sup>, Atm<sup>−/−</sup>, Rad50<sup>S/S</sup> Atm<sup>−/−</sup>, and Rad50<sup>S/S</sup> Atm<sup>+/−</sup> mice. [C] Kaplan-Meier survival curves of Rad50<sup>S/S</sup>, Rad50<sup>S/S</sup> Mre11<sup>+/ATLD1</sup>, Rad50<sup>S/S</sup> Mre11<sup>ATLD1/ATLD1</sup>, Rad50<sup>S/S</sup> Nbs1<sup>ΔA/B</sup>, and Rad50<sup>S/S</sup> Nbs1<sup>ΔA/B</sup> mice. [A–C] In each table below the survival curves, for each cohort, the number of mice that were found dead without overt signs of anemia and malignancy is equal to the number of mice minus the number of alive mice, the number of dead mice with anemia, and the number of dead mice with lymphoma. [D] Hematoxylin and eosin staining (upper pictures) or cleaved Caspase-3 detected by immunohistochemistry (lower pictures) in bone marrow sections from 4-wk-old Rad50<sup>S/S</sup>, Rad50<sup>S/S</sup> Atm<sup>−/−</sup>, Rad50<sup>S/S</sup> Mre11<sup>+/ATLD1</sup>, and Rad50<sup>S/S</sup> Mre11<sup>ATLD1/ATLD1</sup> mice. Magnification, 100×. Bar, 100 µM.
Mice were alive and older than 5 mo. Rad50 mice were rescued by Eµ-Bcl2 expression (genotype dosage (Fig. 1C). Rad50 mice with Mre11 and Nbs1 alleles were within two-fold of wild-type levels, showing that Rad50 hematopoietic attrition was affected by a single copy of Mre11 (Fig. 2, Supplementary Table 1).

Mre11/SATLD1 mice were alive and older than 5 mo. Out of the 32 Rad50/S S Mre11/SATLD1 mice that died, 19 exhibited hematopoietic attrition, 10 succumbed to lymphoma, and three died without signs of anemia and malignancy (Fig. 1C). Flow-cytometric analysis of bone marrow from 6-wk-old animals confirmed that increased survival in Rad50/S Mre11/SATLD1 and Rad50/S Mre11/SATLD1 was associated with reduced hematopoietic attrition. The numbers of double-negative (DN) T cells, pro-B cells, and macrophages in both Rad50/S Mre11/SATLD1 and Rad50/S Mre11/SATLD1 were within 2.5-fold of wild-type levels, showing that Rad50/S hematopoietic attrition was affected by a single copy of Mre11 (Fig. 2, Supplementary Table 1).

Nbs1AB/AB mice also exhibit reduced indices of ATM activity (Williams et al. 2002). Similar to the hematopoietic rescue of Rad50/S by Mre11/SATLD1, the numbers of DN T cells, pro-B cells, and macrophages in both Rad50/S Nbs1AB/AB and Rad50/S Nbs1AB/AB were within fivefold of wild-type levels (Fig. 2, Supplementary Table 1). As with ATM and MRE11, the increase in Rad50/S survival was dependent on NBS1 gene dosage (Fig. 1C).

Rad50/S cellular attrition is due to apoptosis

Attrition of Rad50/S hematopoietic cells was attributable to apoptosis. Immunohistochemical analysis of Rad50/S bone marrow revealed markedly increased levels of cleaved Caspase-3, whereas wild type did not show appreciable staining (Fig. 1D). The staining of cleaved Caspase-3 was reduced to wild-type levels in Rad50/S ATM−/−, Rad50/S Mre11/SATLD1/SATLD1, and Rad50/S Mre11/SATLD1 bone marrow (Fig. 1D). Similar results were obtained in thymus and testes sections (data not shown). This interpretation was further supported by genetic evidence. We crossed Rad50/S with Ep-Bcl2 mice, which carry a transgene of the anti-apoptotic BCL2 gene (Strasser et al. 1990). This transgene increases resistance to apoptosis in B and T lymphocytes [Strasser et al. 1991a,b]. The depletion of hematopoietic stem cells in Rad50/S mice appears to be partially attributable to the attrition of committed precursors [Bender et al. 2002]. Therefore, we reasoned that reduction in the loss of B and T cells in Rad50/S Ep-Bcl2 would confer increased survival. This was indeed the case, as Rad50/S Ep-Bcl2 mice succumbed to anemia significantly later than Rad50/S [Fig. 3A]. These observations confirm that apoptosis underlies the precipitous Rad50/S hematopoietic failure, consistent with the previously established p53 dependency for Rad50/S cellular attrition [Bender et al. 2002]. Collectively, the experiments in which reduced ATM activity rescues Rad50/S hematopoiesis demonstrate that Rad50/S, and by extension, the Mre11 complex, promotes ATM-dependent apoptosis.

Genetic interactions with downstream ATM targets

To define the ATM targets that are activated in this context, we crossed Rad50/S with Chk2−/− and Smc1S957A/S966A (Smc1S2SA/S2SA) mutant mice [Hiroo et al. 2002, Kitagawa et al. 2004]. Rad50/S Chk2−/− hematopoietic cell numbers were within twofold of wild type (Fig. 2, Supplementary Table 1). In our cohort of Rad50/S Chk2−/− mice, 52 out of 65 were alive and older than 5 mo at the time of this writing (Fig. 3B). Although less pronounced than with ATM, MRE11, or NBS1, the rescue of Rad50/S survival by Chk2 deficiency was partially dependent on gene dosage, with 10 of 41 Rad50/S Chk2−/− mice alive at 5 mo-of-age [Fig. 3B].

In contrast, Rad50/S Smc1S2SA/S2SA mice were indistinguishable from Rad50/S with respect to hematopoietic cell numbers and survival (Figs. 2, 3B). These data demonstrate that Rad50/S activates Mre11 complex ATM-dependent apoptosis through the Chk2 kinase. Given that phosphorylation of SMC1 by ATM is necessary for activation of the intra-S-phase checkpoint [Kim et al. 2002, Yazdi et al. 2002, Kitagawa et al. 2004], the data further indicate that apoptotic induction and intra-S-phase checkpoint activation are parallel endpoints of the Mre11 complex–ATM pathway.
ATM-dependent signaling was examined at the molecular level to identify the targets responsible for the Rad50S/S apoptotic phenotype. Irradiation induces auto-phosphorylation of ATM Ser1981, and this phosphorylation event correlates with ATM activation (Bakkenist and Kastan 2003). In asynchronous primary ear fibroblast cultures, phosphorylation of ATM Ser1981 was increased in unirradiated Rad50S/S cells compared with wild type [Fig. 4A]. This outcome was associated with phosphorylation in untreated cells of the ATM targets H2AX [Fig. 5D], 53BP1 [Fig. 4B], and SMC1 [Fig. 4C] on Ser139, Ser25, and Ser957, respectively. Constitutive ATM auto-phosphorylation [Fig. 4A] and SMC1 phosphorylation were reduced in the genetic contexts that led to enhanced Rad50S/S survival, i.e., Rad50S/S Mre11A/ATLD1/ATLD1, Rad50S/S Atm+/−, and moderate reduction in Rad50S/S Mre11A/ATLD1 [Fig. 4C], consistent with the interpretation that ATM-dependent signaling accounts for cellular attrition in Rad50S/S.

Rad50S/S suppresses ATM deficiency

Remarkably, the phenotypic rescue in Rad50S/S Atm+/− was bidirectional. Whereas the data clearly demonstrated that the Mre11 complex activates ATM-dependent apoptotic pathways, Rad50S/S Atm+/− exhibited an additional striking feature. The latency of lymphomagenesis associated with ATM deficiency was profoundly increased in Rad50S/S Atm+/− double mutants. The majority of Atm+/− mice in our colony died with thymic lymphomas by 5 mo of age, 2.9% lived to 10 mo, and none lived beyond 15 mo [Fig. 1B]. In contrast, Rad50S/S Atm+/− mice showed increased survival with Atm+/− mice: 20.5% of Rad50S/S Atm+/− mice survived to 10 mo [P < 0.005] and 18% survived to 15 mo [P < 0.001]. It is conceivable that the increased latency of lymphomagenesis is due to a qualitative difference between the lymphomas arising in Atm+/− versus Rad50S/S Atm+/− mice. Unlike Atm+/− mice, T-cell development in Rad50S/S Atm+/− mice was essentially the same as in wild-type mice (data not shown).

To analyze the mechanism(s) by which the Rad50S/S allele suppressed ATM deficiency, we assessed DNA damage signaling in primary Rad50S/S Atm+/− cells. Substrates of the three ATM-related kinases, ATM, DNA-PKcs, and ATR have SQ/TQ motifs at the site of phosphorylation [Kim et al. 1999]. We reasoned that a general assessment of DNA damage signaling in Rad50S/S and Rad50S/S Atm+/− cells could be obtained by immunofluorescence experiments using phospho-SQ/TQ antiserum. This approach has recently been applied to the analysis of Mlh1-dependent checkpoint signaling [Stojic et al. 2004]. Consistent with the previous data indicating constitutive DNA damage signaling, SQ/TQ foci were observed in unirradiated Rad50S/S cells at a significantly higher frequency than in wild-type cells [Fig. 5A,B]. After γ-irradiation, >90% of wild-type and Rad50S/S cells formed foci, whereas only 16% of Atm+/− fibroblasts had foci. Rad50S/S Atm+/− fibroblasts exhibited SQ/TQ foci in 36% of the cells [Fig. 5A]. Focus formation was reduced in caffeine (20 mM)-treated Rad50S/S Atm+/− cells [Fig. 5C], and partial colocalization with 53BP1 foci was observed in all genotypes studied (data not shown). Although we cannot rule out the possibility that Rad50S/S cells exhibit a small amount of DNA damage, these data suggest that Rad50S/S activates a compensating DNA damage response pathway that leads to the rescue of ATM deficiency.

H2AX phosphorylation to create γ-H2AX is another general indicator of DNA damage signaling. This event can be effected by ATM, ATR, or DNA-PKcs according to the experimental setting [Burma et al. 2001; Ward and Chen 2001]. Consistent with previous data [Bender et al. 2002], γ-H2AX was present in unirradiated Rad50S/S mouse embryonic fibroblasts [MEFs] [Fig. 5D]. γ-H2AX formation was observed in response to camptothecin [CPT], hydroxyurea [HU], ultraviolet light [UV], and IR treatment in wild-type and Rad50S/S MEFs. Atm+/− cells ex-

| Genotype | Number (#) | # alive mice | # dead mice with anemia with lymphomas |
|----------|------------|--------------|----------------------------------------|
| Rad50S/S | 83 | 0 | 82 |
| Rad50S/S Ep-Bcl2 | 15 | 7 (3) | 0 |

Figure 3. Rescue of Rad50S/S survival by Chk2-null allele and the Ep-Bcl2 transgene. (A) Kaplan-Meier survival curves of Rad50S/S and Rad50S/S Ep-Bcl2 mice. (B) Kaplan-Meier survival curves of Rad50S/S, Rad50S/S Chk2+/−, Rad50S/S Chk2−−, and Rad50S/S Smc1S/S mice. Each table below the survival curves is as in Figure 1.
Figure 4. Constitutive ATM activity in Rad50<sup>S/S</sup> is rescued by ATM deficiency and Mre11<sup>ATLD1</sup>. (A) Analysis of ATM Ser1981 phosphorylation. Extracts from wild-type [WT], Rad50<sup>S/S</sup>, Rad50<sup>S/S</sup> Mre11<sup>ATLD1</sup>/ATLD1, and ATM<sup>−/−</sup> p4 ear fibroblasts were prepared after mock treatment and 1 h after 10 Gy of IR. For the ATM Ser1981 phosphorylation Western blot (ATM-Ser1981-P), the lower reactive species represents ATM, as indicated by the ATM Western blot (ATM), and the upper band is nonspecific. SMC1 is included as a loading control. (B) Analysis of 53BP1 phosphorylation in primary ear fibroblasts. Ser25-phosphorylated 53BP1 was immunoprecipitated from untreated or irradiated (10 min after 4 Gy) primary ear fibroblast extracts, and detected with a 53BP1 antibody. A 53BP1 Western blot of the same extracts is shown as a loading control. (C) Analysis of SMC1 Ser957 phosphorylation in the indicated mutants. Extracts from p4 ear fibroblasts were prepared after mock treatment and 1 h after 10 Gy of IR. The extracts were sequentially immunoblotted with SMC1-Ser957-P and SMC1 (loading control) antisera. [WT] Mock-treated wild-type lane underloaded for SMC1. As untreated Mre11<sup>−/−</sup> cells were also significantly inhibited weak γ-H2AX signal following camptothecin, HU, and UV, whereas signal was absent following IR. In contrast, substantial γ-H2AX formation was seen in Rad50<sup>S/S</sup> ATM<sup>−/−</sup> cells following all treatments, including IR (Fig. 5D).

These molecular indices were associated with the mitigation of cellular phenotypic features in Rad50<sup>S/S</sup> ATM<sup>−/−</sup> cells. Cultured ATM<sup>−/−</sup> MEFs exhibit senescence-like proliferative failure (Barlow et al. 1996; Xu and Baltimore 1996). In contrast, the growth of Rad50<sup>S/S</sup> ATM<sup>−/−</sup> MEFs was comparable to wild type and Rad50<sup>S/S</sup> (Fig. 6A). Rad50<sup>S/S</sup> ATM<sup>−/−</sup> cells were also significantly more radiosensitive than ATM<sup>−/−</sup> cells, with the double mutants approximating wild-type and Rad50<sup>S/S</sup> survival at low IR doses (Fig. 6B). The enhanced radiosensitivity, as well as the increase in SQ/TQ foci formation, is not an effect of cell cycle distribution [data not shown]. Rad50<sup>S/S</sup> did not reduce Mre11<sup>ATLD1</sup>/ATLD1 IR sensitivity, as both Mre11<sup>ATLD1</sup>/ATLD1 and Rad50<sup>S/S</sup> Mre11<sup>ATLD1</sup>/ATLD1 MEFs exhibited the same degree of IR sensitivity (Fig. 6C).

In addition, Rad50<sup>S/S</sup> ATM<sup>−/−</sup> cells had a slight reduction in chromosome instability relative to ATM<sup>−/−</sup>; however, Rad50<sup>S/S</sup> had no effect on instability in Mre11<sup>ATLD1</sup>/ATLD1 [Supplementary Tables 2, 3]. Although certain aspects of ATM deficiency were substantially mitigated in Rad50<sup>S/S</sup> ATM<sup>−/−</sup> mice, this was relatively circumscribed. Rad50<sup>S/S</sup> did not rescue the IR-induced G1/S, intra-S-phase, and G2/M checkpoint defects in ATM<sup>−/−</sup> or Mre11<sup>ATLD1</sup>/ATLD1 [Supplementary Fig. 1A–C]. Nor did Rad50<sup>S/S</sup> rescue the IR-dependent ATM<sup>−/−</sup> phosphorylation defects on 53BP1 Ser25 [Fig. 4B], SMC1 Ser957, Chk2, and p53 Ser18 [Fig. 4C; Supplementary Fig. 1D], or the IR-dependent Mre11<sup>ATLD1</sup>/ATLD1 and Nbs1<sup>ΔAR</sup> phosphorylation defects on SMC1 Ser957 and Chk2 [Supplementary Fig. 1D]. This suggests that the alternative pathway activated by Rad50<sup>S</sup> has a relatively limited overlap with the canonical ATM pathway.

Discussion

In this study, we exploit the behavior of the Rad50<sup>S</sup> allele to examine the Mre11 complex’s role in promoting apoptosis. We show that the precipitous apoptotic attrition of hematopoietic cells in Rad50<sup>S/S</sup> mice is abrogated by ATM deficiency. Consistent with the ATM dependence of this rescue, hypomorphic mutations in the Mre11 complex [Mre11<sup>ATLD1</sup> and Nbs1<sup>ΔAR</sup> alleles] that are associated with reduced ATM activity also abrogate Rad50<sup>S/S</sup> cellular attrition. Similar rescue is effected by Chk2, and p53 deficiency. These data indicate that the Mre11 complex governs an ATM-Chk2-p53-dependent apoptotic pathway. Our results further indicate that in addition to the aforementioned ATM-dependent apoptotic pathway, Rad50<sup>S</sup> also partially suppresses the lymphomagenesis, senescence, and radiosensitivity associated with ATM deficiency by activating a compensating pathway.

Parallel endpoints of the Mre11 complex–ATM DNA damage response pathway

Whereas it is well-established that the Mre11 complex and ATM collaborate in the activation of cell cycle checkpoints, [D’Amours and Jackson 2002; Stracker et al. 2004], these data provide the first indication that the Mre11 complex has an effect on ATM-dependent apoptosis. This finding was somewhat unexpected as neither Nbs1<sup>ΔAR</sup> nor Mre11<sup>ATLD1</sup>/ATLD1 mice exhibit defects in IR-induced thymocyte apoptosis [Theunissen et al. 2003]. Hematopoietic attrition in Rad50<sup>S/S</sup> mice is rescued by reduced ATM dosage, and by two Mre11 complex mutations that attenuate ATM activity, the Mre11<sup>ATLD1</sup> allele [Theunissen et al. 2003] and the
Nbs1R allele [Williams et al. 2002]. Accordingly, levels of cleaved Caspase-3 are reduced in the thymus and bone marrow of these mice. Rad50S/S,E-bcl-2 mice also exhibit significantly increased survival compared with Rad50S/S mice, confirming that the Rad50S allele activates apoptosis. Chk2 and p53 are the downstream regulators of the apoptotic pathway governed by the Mre11 complex, since deficiency of those proteins abrogates the rapid apoptotic attrition seen in Rad50S/S mice (Bender et al. 2002; this study).

The Mre11 complex’s influence on apoptosis was separable from its influence on checkpoint activation. SMC1 is constitutively phosphorylated in Rad50S/S mice. This outcome is abrogated by ATM deficiency and Mre11ATLDD. We reasoned that if apoptosis were a downstream consequence of SMC1 phosphorylation, Smc12SA/2SA would rescue Rad50S/S cellular attrition. However, Smc12SA/2SA had no effect on the Rad50S/S phenotype, indicating that SMC1 is not in the apoptotic pathway governed by the Mre11 complex. Thus, SMC1 defines a branch in the Mre11 complex–ATM pathway, and apoptosis and checkpoint activation are parallel outcomes of the pathway. Collectively, these data reveal that the Mre11 complex is required for ATM to initiate parallel DNA damage responses, leading to the regulation of chromosome stability and cell cycle checkpoints on one hand, and to the induction of apoptosis on the other.

Figure 5. DNA damage signaling is enhanced in Rad50S/S Atm−/− cells compared with Atm−/− cells. (A) SQ/TQ foci formation in wild-type (WT), Atm−/−, Rad50S/S, and Rad50S/S Atm−/− primary ear fibroblasts was assessed by immunofluorescence. Primary ear fibroblasts were harvested 30 min after mock treatment (white bars) or 4 Gy of IR (gray bars). Two-hundred-fifty cells were counted for presence or absence of SQ/TQ foci. A cell is considered positive when it shows ≥10 foci. Data are from three experiments. (B) SQ/TQ foci from mock-treated or 4 Gy-irradiated primary ear fibroblasts. (C) Primary ear fibroblasts were pretreated for 1 h with caffeine (20 mM) or vehicle and harvested 30 min after treatment with 4 Gy IR as indicated. For each treatment, 250 cells were counted for presence or absence of SQ/TQ foci. Data are from two experiments run in duplicate. (D) Extracts from primary MEF cultures prepared after mock treatment (−); 1 h after 1 µM CPT, 2 mM HU, or 20 J/m² UV; or 10 min after 5 Gy of IR treatment were sequentially immunoblotted with γ-H2AX and Actin [loading control] antisera. “Short” and “Long” represent short and long exposure, respectively.
Several lines of evidence from this study support this hypothesis. Reducing Rad50<sup>+</sup> dosage (in Rad50<sup>−/−</sup> mice) enhances survival. If Rad50<sup>+</sup> were a hypomorphic allele that affected Mre11 complex-mediated DNA repair, Rad50<sup>−/−</sup> would likely exhibit a more severe phenotype than Rad50<sup>+</sup>/<sup>+</sup>. The fact that neither Nbs1<sup>A17/A17</sup> nor Mre11<sup>ATTLD/ATTLD</sup>, both of which are bona fide hypomorphic mutants, enhance the severity of Rad50<sup>−/−</sup> is also inconsistent with the idea that impaired DNA damage metabolism underlies the Rad50<sup>+</sup>/<sup>+</sup> phenotype. The Mre11 complex’s cytologic behavior is normal in Rad50<sup>+</sup>/<sup>+</sup> cells [Bender et al. 2002], and we have not observed a significant increase in Rad51 spontaneous foci [data not shown]. These observations, together with the fact that Rad50<sup>+</sup>/<sup>+</sup> cells do not exhibit sensitivity to a broad range of clastogens [Bender et al. 2002], support the interpretation that the enhanced signaling in Rad50<sup>−/−</sup> is not due to a defect in DSB repair. Finally, the Rad50<sup>+</sup> allele’s extremely mild effect on chromosome stability would appear to be insufficient to account for the severity of its effect on apoptosis [Bender et al. 2002, de Jager and Kanaar 2002]. For comparison, nothing resembling the Rad50<sup>+</sup>/<sup>+</sup> phenotype is seen in murine mutants associated with chromosome instability or DNA repair defects. Some examples include Mre11<sup>ATTLD/ATTLD</sup> mice [Theunissen et al. 2003], Ku [Difilippantonio et al. 2000], scid [Bosma et al. 1983], Rad54 [Essers et al. 1997], Mus81 [McPherson et al. 2004], Brca1 [Ludwig et al. 2001], and H2AX [Bassing et al. 2003, Celeste et al. 2003]. Notably, the level of chromosome breakage, both spontaneous and induced, appears to be substantially higher in Mre11<sup>ATTLD/ATTLD</sup> mice than in the strains noted above, yet bears no phenotypic resemblance to Rad50<sup>−/−</sup>.

An alternative, nonexclusive hypothesis is also supported by the data. The promotion of apoptosis by the Rad50<sup>+</sup> allele may reflect a gain of function that leads to the induction or stabilization of a unique lesion. This lesion would necessarily be one that is not a major product of the clastogenic agents we have used [IR, mitomycin C, or HU], as no sensitivity of Rad50<sup>+</sup>/<sup>+</sup> cells to those agents is evident [Bender et al. 2002, data not shown]. Supporting this possibility is the fact that Rad50<sup>−/−</sup>/Rad50<sup>−/−</sup> double mutants in which cellular attrition is attenuated are frequently associated with lymphomagenesis. Rad50<sup>−/−</sup> Chk2<sup>−/−</sup> is particularly affected, 10 of the 12 mice died with lymphoma [Fig. 3B]. It is important to consider that the lesion hypothesis does not exclude the possibility that Rad50<sup>−/−</sup> is hypermorphic for signaling, and that the Rad50<sup>−/−</sup> phenotype may be a composite outcome of the two.

An intriguing possibility for the Rad50<sup>−/−</sup>/Rad50<sup>−/−</sup> lesion is a covalent DNA–protein complex. The Mre11 complex is implicated in the nucleolytic removal of covalently attached topoisomerase II or topoisomerase II-like proteins in bacteriophage T4 and S. cerevisiae [Keeney and Kleckner 1995; Nairz and Klein 1997; Tsubouchi and Ogawa 1998; Stohr and Kreuzer 2001]. For example, the removal of Spo11 from meiotic DSBs is completely blocked in S. cerevisiae rad50 mutants [Keeney and Kleckner 1995]. Recent data demonstrate that Spo11 is

Possible mechanisms of Rad50<sup>+</sup>-dependent pathology

The molecular basis of chronic DNA damage signaling in Rad50<sup>−/−</sup> is not clear. We have proposed that in S. cerevisiae and mammals, the Rad50-containing Mre11 complex is an up-mutant for DNA damage signaling, and in this regard, may analogize a receptor mutant in which a signaling takes place in the absence of ligand—in this speculative scenario, the ligand in question would be DNA damage [Petrini and Stracker 2003]. This hypothesis is based in part on the observation that, despite indices of chronic genotoxic stress, no evidence for the accumulation of DNA damage in either S. cerevisiae or murine Rad50<sup>+</sup> mutants has been obtained, and neither organism exhibits overt DNA repair defects.

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removed from DSB ends by endonucleolytic cleavage adjacent to the bound protein in both *S. cerevisiae* and mice, and further, that this mode of repair may be generally relevant to the removal of covalent topoisomerase II cleavage complexes (Neale et al. 2005). The involvement of the Mre11 complex in this repair process remains to be addressed.

**Activation of an alternative DNA damage response pathway in Rad50<sup>S/S</sup> mice**

An equally striking aspect of Rad50<sup>S/S</sup> is its effect on ATM deficiency. Rad50<sup>S/S</sup> rescues the lymphomagenesis, senescence, and radiosensitivity of ATM-deficient mice and cells. This rescue is associated with enhancement of several generic readouts of ATM/ATR signaling in Rad50<sup>S/S</sup> Atm<sup>−/−</sup> cells. For example, SQ/TQ foci form in response to IR treatment at a higher frequency in Rad50<sup>S/S</sup> Atm<sup>−/−</sup> compared with Atm<sup>−/−</sup> fibroblasts. These results support the hypothesis that activation of a compensating DNA damage response pathway in Rad50<sup>S/S</sup> is the underlying basis for suppression of ATM deficiency in Rad50<sup>S/S</sup> Atm<sup>−/−</sup> mice. This situation is reminiscent of the observation that a *S. cerevisiae* rad50S mutation is associated with partial suppression of the checkpoint defects in Mec1-deficient cells. These outcomes are dependent upon the Mec1 paralog, Tel1, and the Mre11 complex (Usui et al. 2001).

Rad50<sup>S/S</sup> did not mitigate phenotypic outcomes in *Mre11<sup>ATLD1/ATLD1</sup>*. This may reflect that *Mre11<sup>ATLD1/ATLD1</sup>* impairs the activity of both the alternative and the ATM-dependent pathway. Supporting this interpretation, *Mre11<sup>ATLD1/ATLD1</sup>* is synthetically lethal with Atm<sup>−/−</sup> (Table 1), as is *Nbs1<sup>AB/AB</sup>* [Williams et al. 2002].

Since neither DNA-PKcs nor SMG1 have been implicated in the suppression of lymphomas, the alternative pathway is most likely to be ATR dependent. Consistent with this view, recent data suggest that Nbs1 influences the activity of ATR on certain targets [Stiff et al. 2004]. However, the data in hand do not rigorously exclude a role for DNA-PKcs or SMG1 in the Rad50<sup>S/S</sup>-dependent suppression of ATM deficiency. Irrespective of the kinase involved, the data clearly support the view that the influence of the Mre11 complex on the DNA damage response is not limited to ATM-dependent functions.

Finally, the suggestion that Rad50<sup>S/S</sup> activates both ATM-dependent and ATM-independent responses may have therapeutic implications. On one hand, the fact that a pathway that mitigates ATM deficiency exists and can be activated may provide a basis for the development of treatments for ataxia-telangiectasia. On the other hand, the potent pro-apoptotic behavior of Rad50<sup>S</sup> raises the possibility that compounds mimicking this activity could be developed as anti-tumor agents that act with reduced mutagenic effects.

**Materials and methods**

**Mice derivation and genotyping**

Rad50<sup>S/S</sup>, Rad50<sup>+/−</sup>, Atm<sup>−/−</sup>, *Mre11<sup>ATLD1/ATLD1</sup>*, *Nbs1<sup>AB/AB</sup>* Chk2<sup>−/−</sup>, Smc1<sup>2DA/2DA</sup>, Smc1<sup>S957A/S966A/S957A/S966A</sup>, and *ErbB2* mice, survival, and genotyping have been described (Strasser et al. 1990; Barlow et al. 1996; Luo et al. 1999; Bender et al. 2002; Hirao et al. 2002; Williams et al. 2002; Thunissen et al. 2003; Kitagawa et al. 2004). All mice maintained on mixed 129/SvEv and C57BL6 background. Rad50<sup>S/S</sup>, Atm<sup>−/−</sup>, Rad50<sup>S/S</sup> Atm<sup>−/+</sup>, and Rad50<sup>S/S</sup> Atm<sup>−+</sup> mice were derived at the University of Wisconsin Medical School and at Memorial Sloan-Kettering Cancer Center, and Figure 1B is a compilation of mice in both colonies. All other survival curves include mice born only at Memorial Sloan Kettering Cancer Center.

**Cell derivation and culture**

Murine embryonic fibroblasts (MEFs) and car fibroblasts were generated and cultured as described (Bender et al. 2002). Immunofluorescence was achieved by pX-8 transfection, an SV40 plasmid containing an inactivated origin of replication [Fromm and Berg 1982]. For cumulative growth, the fold increase in cell number ([R]) was calculated from passage number 2 to passage number 6. Between each passage number, 100,000 cells were plated on six-well plates, and R = X/100,000 with X = cell number after 3 d. The cumulative cell number Y at each passage number [p] was obtained by solving Y<sub>p</sub> = Y<sub>p−1</sub> × R with Y<sub>3</sub> = 100,000 and p = 3, 4, 5, and 6.

**Survival analyses**

Statistical significance determined by Wilcoxon Rank Sum Test or Fisher’s exact test using Mstat software [Norman Drink...
water, Mc Ardle Laboratory for Cancer Research). Kaplan Meier survival curves made with Prism 4 (GraphPad Software).

Hematopoietic cell preparation and analysis

Single-cell suspensions from the thymus and bone marrow prepared and analyzed as described [Bender et al. 2002]. Briefly, the number of DN T cells was equal to the number of CD4 CD8 double-negative cells in the thymus, the number of macrophages was equal to the number of the B220-negative and CD43-positive bone marrow cells, and the number of pro-B cells was equal to the number of B220 CD43 double-positive bone marrow cells.

Cellular assays

Sensitivity to IR, intra-S-phase, and G2/M checkpoint functions assessed as described [Williams et al. 2002]. Cells were irradiated with a Mark I 137Cs source at 220 cGy/min. G1/S checkpoint assays carried out as described [Xu et al. 1996]. Chromosomal analyses were prepared as described previously [Bender et al. 2002] and carried out on blinded samples. Splenocytes were stimulated with lipopolysaccharide for 48 h prior to addition of colcemid for 3 h. Ear fibroblasts were incubated for 4 h with colcemid.

Immunohistochemistry

Four percent paraformaldehyde-fixed tissues were processed at the Research Animal Resource Center of the Cornell University Medical College. Paraffin-embedded 5–10-μm sections were stained with hematoxylin and eosin, and cleaved Caspase-3 (Cell Signalling; #9661) immunohistochemistry was performed at the Molecular Cytology Core Facility of Memorial Sloan-Kettering Cancer Center. Color images were captured using a Nikon digital camera and processed using Adobe Photoshop.

Immunoprecipitations and Western blotting

For 53BP1 IP, whole-cell extracts of primary ear fibroblasts were obtained in lysis buffer (300 mM NaCl, 20 mM Hepes at pH 7.9 and 0.5% NP-40 with protease and phosphatase inhibitors). One microgram of 53BP1-Ser25-P antibody (gift from P. Carpenter, Harvard Medical School, Boston, MA) was added to the extracts incubated for 30 min in 0.1 M HCl, and the resulting supernatant was used for immunoprecipitation. Chk2 (Upstate Biotechnology; 05-649) antiserum used at a 1:500 dilution in 1% BSA/PBS. For 53BP1 IP, whole-cell extracts of primary ear fibroblasts were obtained in lysis buffer (200–400 mM NaCl, 50 mM Tris-HCl at pH 7.5, 1% NP-40 with protease and phosphatase inhibitors). To prepare extracts for γ-H2AX detection, the chromatin pellet from regular lysates was further incubated for 30 min in 0.1 M HCl, and the resulting supernatant was used for immunoblotting. Chk2 [Upstate Biotechnology; 05-649] antiserum used at 1:400 dilution in 2% BSA/PBS.

Immunofluorescence assays

Immunofluorescence experiments were done as previously described [Maser et al. 1997]. SQ/TQ antibody (Cell Signaling; #2851) was used at a 1:500 dilution in 1% BSA/PBS.

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