Loss of ATM kinase activity leads to embryonic lethality in mice

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The ataxia telangiectasia (A-T) mutated (ATM) kinase is a key deoxyribonucleic acid (DNA) damage signaling kinase that regulates DNA repair, cell cycle checkpoints, and apoptosis. The majority of patients with A-T, a cancer-prone neurodegenerative disease, present with null mutations in Atm. To determine whether the functions of ATM are mediated solely by its kinase activity, we generated two mouse models containing single, catalytically inactivating point mutations in Atm. In this paper, we show that, in contrast to Atm-null mice, both D2899A and Q2740P mutations cause early embryonic lethality in mice, without displaying dominant-negative interfering activity. Using conditional deletion, we find that the D2899A mutation in adult mice behaves largely similar to Atm-null cells but shows greater deficiency in homologous recombination (HR) as measured by hypersensitivity to poly (adenosine diphosphate-ribose) polymerase inhibition and increased genomic instability. These results may explain why missense mutations with no detectable kinase activity are rarely found in patients with classical A-T. We propose that ATM kinase-inactive missense mutations, unless otherwise compensated for, interfere with HR during embryogenesis.

Introduction

The ataxia telangiectasia (A-T) mutated (ATM) kinase plays a key role in the DNA damage response by phosphorylating numerous substrates that signal for DNA repair, cell cycle checkpoint activation, and apoptosis (Banin et al., 1995; Canman et al., 1998; Matsuoka et al., 2007; Bensimon et al., 2010; Olsen et al., 2010; Beli et al., 2012). The critical function of ATM is underscored by its high conservation in eukaryotes and its deficiency in the recessive, cancer-prone A-T disease (Savitsky et al., 1995). The exact mechanism by which ATM is activated in vivo still remains unclear (Pellegrini et al., 2006; Daniel et al., 2008), but one current model suggests that autophosphorylation triggers its activation (Bakkenist and Kastan, 2003; Kozlov et al., 2006, 2011; So et al., 2009). A prediction is that loss of ATM kinase activity would be equivalent to loss of the ATM protein. Indeed, patient cells reconstituted with kinase-inactive ATM (Bakkenist and Kastan, 2003) and treatment of cells with a pharmacological ATM kinase inhibitor (Hickson et al., 2004; Bredemeyer et al., 2006; Callén et al., 2007; Rainey et al., 2008; White et al., 2008) support the notion that inhibition of cellular ATM kinase activity mirrors complete ATM deficiency.

The classical form of A-T is most frequently caused by compound heterozygote-truncating mutations that result in a total loss of destabilized ATM protein (~90% of cases; Gilad et al., 1996; Lakin et al., 1996; Li and Swift, 2000; Micol et al., 2011).
Mouse models deficient in ATM have been invaluable for the study of A-T (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996; Difilippantonio and Nussenzweig, 2007; Lavin, 2008; Stracker and Petrini, 2011). In stark contrast to Atm-null mice (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996), we show here that single point mutations disrupting the kinase activity of ATM lead to early embryonic lethality in mice. These results reveal a previously unknown essential role for ATM kinase activity during embryonic development and provide a potential explanation for why kinase-inactive missense mutations do not constitute a significant portion of the molecular lesions in childhood A-T. Furthermore, our finding that inhibition of ATM kinase activity in vivo does not always equate to complete loss of ATM protein suggests that treatments using ATM inhibitors (ATMi’s) could be more toxic than previously anticipated.

Results and discussion

Mutations in the ATM kinase domain display severely impaired kinase activity in vitro and cause embryonic lethality in mice

To determine whether the diverse in vivo functions of ATM are mediated solely by its kinase domain, we used bacterial artificial chromosome (BAC) recombineering to generate two kinase-inactive mouse models of ATM. To identify a single residue for mutagenesis without any high-resolution structural information for ATM, we took two approaches. In a structural modeling approach, we identified the p110 phosphoinositide 3-kinase domain as having a statistically significant protein sequence alignment with the ATM kinase domain and found that an essential catalytic aspartic acid residue in the active site of the p110 kinase structure aligns with D2889 of human ATM. Moreover, a p110 knockin mouse substituting this residue with alanine, p110D2899A/D2910A, demonstrated that this mutation abolishes kinase activity in vitro and in vivo without affecting protein stability (Okkenhaug et al., 2002); therefore, we chose to generate the conserved D2899A mutation in the mouse (Fig. 1 A). For the second approach, we decided to model a rare missense variant from a patient with classical A-T, c.8189A>C (p.Gln2730Pro), whose cells displayed ATM protein expression without detectable kinase activity (Taylor and Byrd, 2005; Barone et al., 2009).

Before generating BAC transgenic mouse models, we tested whether the D2889A and Q2730P mutations in human ATM are required for ATM kinase activity in a completely reconstituted system. Kinase assays with wild-type (WT), D2889A, and Q2730P mutant dimeric human ATM complexes were performed using p53 as a substrate (Lee and Paull, 2005). Although WT ATM dimers displayed p53 S15 phosphorylation in an MRN (MRE11-RAD50-NBS1)- and DNA-dependent manner, kinase activity of D2889A and Q2730P mutant dimers was completely abolished (Fig. 1 B). Thus, the D2889A and Q2730P mutations are required for human ATM kinase activity.

As both D2889 and Q2730 residues in human ATM are conserved in the mouse (Fig. 1 A), we recombineered the equivalent mutations in a WT BAC spanning the genomic mouse Atm locus to generate BAC transgenic mice as previously described (Pellegrini et al., 2006; Daniel et al., 2008). Both mutation sites were confirmed by sequencing (Fig. 1 C). Founder lines expressing mutant murine ATM from the BAC transgene were identified and bred to Atm<sup>−/−</sup>. Founder lines G1 and H7 of Atm<sup>TgD2899A</sup> mice expressed transgenic murine ATM near the level observed in Atm<sup>−/−</sup> mice, whereas founder lines F5, D4, and I2 overexpressed murine ATM (Fig. 1 D). For Atm<sup>TgQ2740P</sup> mice, founder line G4 expressed transgenic murine ATM near the level observed in Atm<sup>−/−</sup> mice, whereas the C8 and A3 founder lines overexpressed ATM (Fig. 1 E). Similar ATM protein levels were observed from splenocytes and thymocytes of transgenic animals (Fig. 1, D and E; and not depicted).

We crossed Atm<sup>TgD2899A</sup>Atm<sup>−/−</sup> and Atm<sup>TgQ2740P</sup>Atm<sup>−/−</sup> mice with Atm<sup>−/−</sup> but, to our surprise, failed to generate transgenic animals on the Atm<sup>−/−</sup> background (Fig. 1, F and G). This failure to generate Atm<sup>Tg</sup>Atm<sup>−/−</sup> live-born pups was observed across all founder lines of both mutations (Fig. 1, F and G) and was not the result of transgene integration on the chromosome containing endogenous Atm, as shown by FISH (Fig. S1). To begin to elucidate the stage of embryonic development that is impaired with deficient ATM kinase activity, we prepared embryos at embryonic day 9.5 (E9.5) from D2899A mutant mice. We found that Atm<sup>TgD2899A</sup>Atm<sup>−/−</sup> embryos were produced at a dramatically lower frequency than expected, indicating that D2899A mutant embryos die before E9.5 (Table 1). Transgenic animals with one or both WT copies of Atm were found at the expected frequency, indicating an absence of dominant-negative interfering activity. We conclude that the kinase-inactive ATM mutation leads to early embryonic lethality in mice.

We speculated that the severe defect during embryogenesis is the result of ATM kinase recruitment at DNA breaks, which may impair the function of other proteins by occluding their access to DNA damage. To support this, we found that a kinase-inactive human ATM D2870A mutant protein, expressed in cells that do not express endogenous ATM, is recruited to sites of laser-induced DNA damage (Fig. S2). Moreover, WT human ATM was similarly recruited to DNA damage sites in cells treated with the KU55933 ATMi (Fig. S2). These results are consistent with other studies in human cells showing that ATM kinase activity is dispensable for recruitment of epitope-tagged ATM to sites of DNA breaks after laser- or ionizing radiation (IR)–induced DNA damage (Barone et al., 2009; Davis et al., 2010). They are also consistent with results from Xenopus laevis egg extracts showing an increase in ATM association to damaged chromatin in the presence of caffeine or the KU55933 ATMi (You et al., 2007, 2009).

Double-stranded break (DSB)–induced activation and recruitment of ATM to chromatin is dependent on Nbs1 (Uziel et al., 2003; Difilippantonio et al., 2005). If recruitment of kinase-dead ATM to DNA breaks is toxic, we reasoned that we might be able to rescue viability by breeding with Nbs1<sup>−/−</sup> mice, a hypomorphic Nbs1 mutant mouse that exhibits a mild defect in ATM activation (Williams et al., 2002). However, no Nbs1<sup>−/−</sup>Atm<sup>TgD2899A</sup>Atm<sup>−/−</sup> mice were born (Table 2). Thus, a mutation that is predicted to reduce ATM activation and association with chromatin cannot rescue viability of ATM kinase-inactive mice.
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297ATM kinase-dead mice are embryonically lethal • Daniel et al. (Table 2), suggesting that disruption of DNA-PKcs recruitment to DNA breaks does not rescue ATM kinase-inactive toxicity. Similarly, loss of 53BP1, which reduces nonhomologous end joining (NHEJ) and promotes resection of DSBs (Ward et al., 2004; Xie et al., 2007; Difilippantonio et al., 2008; Dimitrova et al., 2008; Bunting et al., 2010), also did not rescue the viability of $\text{Atm}^{-/-}$ mice (Table 2).

Conditional ATM D2899A B lymphocytes display defects in development, genome stability, and sensitivity to poly (ADP-ribose) polymerase (PARP) inhibitor To overcome the embryonic lethality of $\text{Atm}^{\text{TgD2899A}}^{-/-}$ mice, we crossed a previously described conditional $\text{Atm}$ knockout

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**Figure 1.** ATM D2899A and Q2740P mutations display severely impaired kinase activity in vitro and cause embryonic lethality in mice. (A) Pairwise local alignments of human ($H. sapiens$) and mouse ($M. musculus$) ATM protein sequences surrounding sites of mutagenesis. (B) Kinase assay with 0.2 nM WT, D2899A, or Q2730P mutant dimeric human ATM, 3.6 nM MRN, 50 nM GST-p53 substrate, and linear DNA probed with an antibody to p53 S15 phosphorylation. A titration of ATM is shown for each sample. (C) Sequence chromatograms showing the amino acid mutations generated in mouse BAC RP24-122F10. (D and E) Thymocytes were harvested for lysate preparation and Western blot analysis. Molecular mass markers are in kilodaltons. (F and G) Tables showing the expected and observed genotypes of 3-wk-old pups from breeding $\text{Atm}^{\text{TgD2899A}}^{-/-} \times \text{Atm}^{-/-}$ mice. The boxes highlight the comparison between the total number of ATM kinase-inactive and Atm-null live-born pups.

Recently, a DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) mutant mouse model with three phosphorylation site substitutions revealed that the DNA-PKcs mutant protein causes a more severe phenotype than $\text{Dnapkcs}$-null mice (Zhang et al., 2011). Because DNA-PKcs has overlapping functions with ATM (Gurley and Kemp, 2001; Sekiguchi et al., 2001; Callén et al., 2009; Gapud and Sleckman, 2011), we reasoned that if ATM and DNA-PKcs are being recruited to DNA breaks and occluding access of other factors, disruption of the DNA-dependent protein kinase holoenzyme may partially alleviate the toxic effect of kinase-inactive ATM. To test this hypothesis, we crossed $\text{Ku80}$-null mice (Nussenzeig et al., 1996) with our kinase-inactive ATM mice. However, $\text{Atm}^{\text{TgD2899A}}^{-/-}$ live-born pups were not recovered in the $\text{Ku80}^{-/-}$ background (Table 2), suggesting that disruption of DNA-PKcs recruitment to DNA breaks does not rescue ATM kinase-inactive toxicity. Similarly, loss of 53BP1, which reduces nonhomologous end joining (NHEJ) and promotes resection of DSBs (Ward et al., 2004; Xie et al., 2007; Difilippantonio et al., 2008; Dimitrova et al., 2008; Bunting et al., 2010), also did not rescue the viability of $\text{Atm}^{\text{TgD2899A}}^{-/-}$ mice (Table 2).
Table 1. Embryonic lethality of ATM D2899A mice occurs before E9.5

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<th>Founder line</th>
<th>Atm&lt;sup&gt;TgD2899A&lt;/sup&gt;Atm&lt;sup&gt;+/-&lt;/sup&gt;</th>
<th>Atm&lt;sup&gt;+/-&lt;/sup&gt;</th>
<th>Atm&lt;sup&gt;TgD2899A&lt;/sup&gt;Atm&lt;sup&gt;+&lt;/sup&gt;/-</th>
<th>Atm&lt;sup&gt;+/-&lt;/sup&gt;</th>
<th>Atm&lt;sup&gt;TgD2899A&lt;/sup&gt;Atm&lt;sup&gt;-/-&lt;/sup&gt;</th>
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Shown are the expected and observed genotypes from breeding Atm<sup>TgD2899A</sup>Atm<sup>-/-</sup> with Atm<sup>+/-</sup> mice. Note the comparison between the total number of ATM kinase-inactive and Atm-null embryos.

Table 2. Embryonic lethality of ATM D2899A [G1 founder line] mice is not rescued by disruption of Nbs1, Ku80, or 53bp1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Atm&lt;sup&gt;TgD2899A&lt;/sup&gt;Atm&lt;sup&gt;+/-&lt;/sup&gt;</th>
<th>Atm&lt;sup&gt;+/-&lt;/sup&gt;</th>
<th>Atm&lt;sup&gt;TgD2899A&lt;/sup&gt;Atm&lt;sup&gt;+&lt;/sup&gt;/-</th>
<th>Atm&lt;sup&gt;+/-&lt;/sup&gt;</th>
<th>Atm&lt;sup&gt;TgD2899A&lt;/sup&gt;Atm&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Atm&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<td>11</td>
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<tr>
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Shown are the expected and observed genotypes of 3-week-old pups from breeding Nbs1<sup>+-/-</sup>Atm<sup>TgD2899A</sup>Atm<sup>-/-</sup> with Nbs1<sup>+-/-</sup>Atm<sup>-/-</sup> mice (n = 238), intercrossing Ku80<sup>-/-</sup>Atm<sup>TgD2899A</sup>Atm<sup>-/-</sup> mice (n = 207), or intercrossing 53bp1<sup>-/-</sup>Atm<sup>TgD2899A</sup>Atm<sup>-/-</sup> mice (n = 102). Note the numbers of ATM kinase-inactive live-born pups in the different Ku80 backgrounds, the comparison between the number of ATM kinase-inactive and Atm-null live-born pups in the 53bp1<sup>-/-</sup> background, and the numbers of ATM kinase-inactive live-born pups in the different Nbs1 backgrounds.
causes defects in the overall fitness of stimulated cells, we activated splenic B cells in culture with lipopolysaccharide (LPS) and IL4 and monitored YFP frequency of B220+ cells over time by flow cytometry. Although YFP frequency in live ATMlox/+ B cells remained constant over 6 d in culture, ATMlox/+ B cells displayed a reproducible decrease in YFP frequency, with ATMlox/+ B cells showing an intermediate phenotype (Fig. 2 D). ATMlox/+ B cells expressing WT ATM showed similar results as ATMlox/+ B cells (Fig. 2 D), supporting an absence of dominant-negative interfering activity in the D2899A mutant. Thus, although the D2899A point mutation in mice leads to severe defects in embryonic development, defects in B lymphocyte development and activation are only mildly more severe than ATM-deficient controls.

Chromosomal instability is a hallmark of both human and murine ATM-deficient cells (Xu et al., 1996; Shiloh, 2003; Callén et al., 2007) and is also a rapid consequence of treatment of WT cells with ATM kinase inhibitors (Hickson et al., 2004; Bredemeyer et al., 2006; Callén et al., 2007; Rainey et al., 2008; White et al., 2008). To test whether genetic ablation of the murine ATM kinase causes defects in genome stability, metaphase chromosome spreads of YFP+ cells sorted from ATMlox/+ and control B cell cultures were analyzed for chromosome abnormalities. As expected, ATM-deficient cells...
displayed a higher number of aberrations per cell compared with controls (Fig. 2 E). Atm$^{TgD2899A Atmflox^-}$ cells showed a 1.7-fold increase in aberrations per cell compared with ATM-deficient cells, marked by chromosome breaks and chromatid breaks (Fig. 2 E). We conclude that ATM D2899A mutant murine B cells exhibit more genomic instability than ATM-deficient control cells.

The increase in chromatid breaks observed in D2899A mutant cells suggested that these cells may have more severely impaired homologous recombination (HR). To test this, we used flow cytometry to assess the YFP frequency of live B220$^+$ cells cultured with PARP inhibitor. Relative to the YFP frequency measured with no drug treatment, this assay recapitulated the reported sensitivity of ATM-deficient human cells to PARP inhibitor (Fig. 2 F; Bryant and Helleday, 2006; McCabe et al., 2006). Using YFP as a marker for Cre-expressing cells in competition with nondeleted cells, our analyses indicated that Atm$^{TgD2899A Atmflox^-}$ cells were mildly more sensitive to PARP inhibitor treatment compared with ATM-deficient cells (Fig. 2 F), suggesting a more significant defect in HR. We conclude that D2899A mutant murine B cells display more severe defects in genome stability and PARP inhibitor sensitivity compared with ATM-deficient cells.

**Conditional ATM D2899A B lymphocytes display defects in variable, diversity, and joining (V(D)J) recombination and immunoglobulin class switching**

Deficiency in murine ATM leads to impaired DNA end joining in lymphocytes undergoing V(D)J recombination (Bredemeyer et al., 2006). To test whether the D2899A mutation causes similar V(D)J recombination defects, we generated v-Abl (viral Abl) kinase-transformed pre-B cell lines (herein referred to as abl pre-B cells) from bone marrow of Atm$^{TgD2899A Atmflox^-}$ mice expressing an Eμ-Bcl2 transgene. These cells were transiently transfected with Cre recombinase to generate Atm$^{TgD2899A Abl^-}$ abl pre-B cells and retrovirally infected with the pMX-INV abl pre-B cells to generate Atm$^{TgD2899A Abl^v}$ abl pre-B cells. To induce recombination activating gene (RAG)–mediated DSBs, Atm$^{Abl^-}$INV, Atm$^{TgD2899A Abl^v}$-INV, and Atm$^{Abl^-}$ INV abl pre-B cells were treated with the Abl kinase inhibitor STI571. Southern blot analysis of pMX-INV rearrangement revealed that the defects in V(D)J recombination in Atm$^{TgD2899A Abl^v}$-INV abl pre-B cells were similar to those observed in Atm$^{Abl^-}$-INV abl pre-B cells. Specifically, there was a decrease in normal coding joint formation with an accumulation of unrepaird coding ends and an increase in hybrid joint formation (Fig. S3). We conclude that ATM D2899A mutant murine lymphocytes display V(D)J recombination defects similar to Atm-null lymphocytes.

During an immune response, physiological DSBs at the IgH locus occur in B lymphocytes during class switch recombination (CSR; Stavnezer et al., 2008). ATM is required for efficient CSR and has been proposed to play a role as a DNA damage response factor in the synopsis of two broken switch regions (Pan et al., 2002; Lumsdon et al., 2004; Reina-San-Martin et al., 2004). To test whether the D2899A mutation affects CSR, splenic B cells from Atm$^{TgD2899A Atmflox^-}$ and control mice were stimulated with LPS and IL-4, and the frequency of YFP$^+$ cells positive for surface IgG1 was assessed by flow cytometry. Consistent with previous studies using germ-line Atm$^-$/mice (Lumsden et al., 2004; Reina-San-Martin et al., 2004), B cells from Atm$^{flox^-}$ mice with CD19-cre led to a 2.5-fold decrease in IgG1 CSR, relative to the IgG1$^+$ frequency in YFP$^+$ cells from Atm$^{flox^-}$ mice (Fig. 3, A and B). B cells from Atm$^{TgD2899A Atmflox^-}$ mice displayed a 1.9-fold defect in IgG1 CSR, albeit less severe than ATM-deficient controls (Fig. 3, A and B). We conclude that ATM D2899A mutant B cells display defects in CSR that are mildly less severe than ATM-deficient controls.

**DNA-PKcs and A-T and Rad3 related (ATR) are functional in conditional ATM D2899A B cells**

Because DNA-PKcs and ATM have redundant roles in development and CSR (Gurley and Kemp, 2001; Sekiguchi et al., 2001; Callén et al., 2009), we directly tested whether DNA-PKcs kinase activity is functional during CSR in D2899A mutant B cells. To this end, we stimulated Atm$^{TgD2899A Atmflox^-}$ and control cells to undergo CSR in the presence or absence of the DNA-PKcs inhibitor NU7026 (Callén et al., 2009). In contrast to Atm$^{flox^-}$ cells, inhibiting DNA-PKcs kinase activity in either Atm$^{TgD2899A Atmflox^-}$ or Atm$^{flox^-}$ B cells resulted in a nearly twofold reduction in IgG1 CSR (Fig. 3 C), a decrease consistent with PKcs inhibitor treatment of germ-line Atm$^-$/ B cells (Callén et al., 2009). We conclude that DNA-PKcs is functional in conditional ATM D2899A mutant B cells.

A different phosphoinositide 3-kinase–like kinase, ATR, is required for cell cycle progression and genome stability during replicative stress (Brown and Baltimore, 2003; López-Contreras and Fernandez-Capetillo, 2010), and there is evidence that ATM can regulate ATR function. For example, ATM is required for ATR activation and CHK1 phosphorylation in response to IR; however, ATR-dependent CHK1 phosphorylation in response to replication-associated DNA damage occurs independently of ATM (Adams et al., 2006; Cuadrado et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006). To test whether ATR is functional in ATM D2899A mutant cells, we examined CHK1 S317 phosphorylation in YFP$^+$ sorted Atm$^{TgD2899A Atmflox^-}$ and control cultured B cells treated with hydroxyurea. We found that hydroxyurea-induced CHK1 S317 phosphorylation was indistinguishable between mutant and control cells (Fig. 3 D). Double deficiency in the human ATM and ATR kinases leads to extensive chromosome fragmentation in the presence of the DNA replication inhibitor aphidicolin (Ozeri-Galai et al., 2008); however, Atm$^{TgD2899A Atmflox^-}$ cells were also indistinguishable from controls in response to aphidicolin in our previously described flow cytometric YFP frequency assay (unpublished data). Altogether, our results indicate that expression of kinase-inactive murine ATM does not abrogate DNA-PKcs or ATR function in B cells.

Although an explanation for the embryonic lethality in kinase-inactive ATM mice is yet to be determined, Atm-null mutations clearly can become embryonically lethal when combined
In dividing embryonic cells, an HR defect in Nbs1<sup>1ΔNΔB</sup> mice may synergize with ATM deficiency to cause an accumulation of DNA damage above a tolerable threshold. Similarly, we found that D2899A mutant B cells were mildly more sensitive than ATM-deficient controls to PARP inhibition, and we hypothesize that this may be caused by a more severe decrease in resection. Although D2899A mutant B cells show a decrease in HR, they also show a mild increase in the frequency of class switching compared with ATM-deficient cells, suggestive of increased NHEJ activity. Based on these findings, we suggest that the mild imbalances in DNA repair pathways in Atm<sup>TgD2899A</sup>/Atm<sup>−/−</sup> cells above those governed by ATM could result in very severe developmental defects, while having less dramatic outcomes in adult tissues.

with deficiencies in various DNA damage response pathways. For instance, Atm-null mice show embryonic lethality when disrupted in combination with Fangc<sup>−/−</sup> (Kennedy et al., 2007), Parp1<sup>−/−</sup> (E8.0; Ménisser-de Murcia et al., 2001), Parp2<sup>−/−</sup> (between E9.5 and newborn; Huber et al., 2004), Ku70<sup>−/−</sup> and Ku80<sup>−/−</sup> (earlier than E11.5), Dnapkcs<sup>−/−</sup> (E7.5–8.5; Gurley and Kemp, 2001; Sekiguchi et al., 2001; Gladdy et al., 2006), H2ax<sup>−/−</sup> (E11.5–12.5; Zha et al., 2008), and Nbs1<sup>1ΔNΔB</sup> (E10; Williams et al., 2002). Some of these synthetic lethal interactions may arise from additive defects in DNA repair. For example, it is possible that Nbs1<sup>1ΔNΔB</sup> mice are synthetically lethal with Atm<sup>−/−</sup> (Williams et al., 2002) because of the essential role of the MRN complex in DSB resection that is independent of its function in murine ATM activation (Buis et al., 2008).
Only rarely do A-T patients with the classical phenotype display relatively normal ATM protein levels without detectable kinase activity (Barone et al., 2009; Demuth et al., 2011; Jacquemin et al., 2012). We hypothesize that similar kinase-inactive mutations also cause embryonic lethality in humans and expect that patients with a similar genotype as the one modeled here must have additional mutations to bypass the embryogenesis defect. Indeed, there is clear precedence for secondary mutations that rebalance DSB repair pathways and alleviate early embryonically lethal phenotypes. For example, loss of the NHEJ factor 53BP1 rescues the early embryonic lethality observed in Bcr1-null mice (Cao et al., 2009; Bouwman et al., 2010; Bunting et al., 2012). Although loss of 53BP1 and Ku deficiency both increase HR (Pierce et al., 2001; Bunting et al., 2010, 2012), 53bp1+/−/AtmΔD2899A/Atm+/− mice and Ku80+/−/AtmΔD2899A/Atm−/− mice were not obtained (Table 2), suggesting that other mutations might compensate for kinase-inactive ATM protein. In sum, our results contribute a novel perspective to the role of ATM kinase activity during embryogenesis and raise caution regarding the consequences of ATM1 mutation in cancer patients and in experimental systems.

Materials and methods

Generation of mice

The D2899A and Q2740P mutations were targeted using BAC recombineering as previously described (Yang and Sharan, 2003; Pellegrini et al., 2006; Daniel et al., 2008). In brief, mutations were introduced into the murine ATM BAC RP24-122F10, which consists of a 160-kb insert including 17.9 kb of sequence downstream of the Atm initiation and stop codons, respectively, along with an engineered EcoRI site between exons 35 and 36 for a PCR-based method to distinguish between AtmΔATM−/− and AtmΔATM+/− genotypes as described previously (Pellegrini et al., 2006; Daniel et al., 2008). Both mutant BACs were used to generate transgenic mice. The presence of the transgene was determined by PCR as previously described (Pellegrini et al., 2006) using mouse ATM forward, 5′-AGCGACA-ACCACACGAAATGCGC-3′; and reverse, 5′-TTGGTTTGGCGATCTGCCGTTTC-3′, primers. To distinguish between AtmΔATM+/− and AtmΔATM+/+ genotypes, EcoRI digestion of the PCR purified product from amplification using mouse ATM 5′, 5′-GGCGATCTGCTACATCTAGTGGACCT-3′, and Atm WT reverse, 5′-CGAAATTCGACGAGTTGCTGAG-3′, primers. To perform co-transgenic mice yielded the predicted digest products running at 600, 400, and 200 bp, whereas AtmΔATM−/− mice yielded the predicted products at 400 and 200 bp. Transgenic founders were crossed to Atm+/− mice. All experiments were performed in compliance with the Animal Welfare Act regulations and other federal statutes relating to animals and adhered to the principles set forth in the Guide for Care and Use of Laboratory Animals (National Research Council, 1996). J. Petrini (Memorial Sloan-Kettering Cancer Center, New York, NY) provided Nbs1−/− mice. F. Alt (Harvard Medical School, Boston, MA) provided Atm conditional knockout mice.

Lymphocyte cultures, flow cytometry, and genome stability

Single-cell suspensions of ACK-treated bone marrow and spleenocytes from 6–12-wk-old mice were stained with a-B220–FITC, a-IgM–R-phycocerythrin, and a-CD43–Biotin followed by streptavidin–allophycocyanin. Cultured B cells were isolated from spleens of 6–12-wk-old mice by immunomagnetic depletion with anti-CD43 beads (Miltenyi Biotec) and stimulated with either 25 mg/ml LPS (Sigma-Aldrich), 5 µg/ml interleukin 4 (Sigma-Aldrich), and/or 2.5 µg/ml R1015 (BD) as indicated. For assessing class switching, cultured B cells were harvested and stained in single-cell suspensions with a-IgG1–bantin followed by streptavidin–R-phycocerythrin. Cells were acquired through a propidium iodide–negative live-lymphocyte gate with either a FACSCalibur (BD) or an LSR II (BD) flow cytometer. Live YFP+ cells were sorted on a cell sorter (FACSAria II; BD). Data were analyzed using FlowJo software (Tree Star). All statistical significance analyses were determined by a two-tailed t test assuming unequal variance. For genome stability analyses, cultured B cells were arrested at mitosis with 0.1 µg/ml colcemid (Roche) treatment for 1 h, live YFP+ cells were sorted by FACS, and metaphase chromosome spreads were prepared following standard procedures. FISH was performed on slides with a probe for telomere repeat–specific peptide nucleic acid conjugated to Cy3 fluorochrome (Panagene) and counterstained with DAPI. Metaphase images were acquired with an upright microscope (Axioplan 2; Carl Zeiss) equipped with a 63x, NA 1.4 objective lens (Plan-Apochromat, Nikon) and a monochrome charge-coupled device camera (ORCA-ER; Hamamatsu Photonics) using MetaMorph software (Molecular Devices). Abl cell lines were generated, and V(DJ) recombination was assayed as described including the AtmΔATM−/−INV (A70.3INV3) and AtmΔATM−/−INV (AtmΔATM−/−INV14) lines (Bredemeyer et al., 2006). The pMXINVI retroviral recombination substrate contains GFP cDNA and human CD4 cDNA downstream of an internal ribosomal entry site. For Cre deletion in AtmΔATMATM+/− abl cells, cells were transfected with mouse stem cell virus–thymidine kinase (TK) plasmid DNA using a transfection system (1,500 V/15 ms/4 pulses; Neon; Invitrogen). 24 h after transfection, cells were sorted using biotin anti-Thy1.1 antibodies (BD) and antibiotin microbeads according to the company’s protocol (Miltenyi Biotec). The sorted cells were subcloned by limiting dilution and identified using a 3′ ATM southern probe from a 635-bp fragment generated by PCR using 5′-GGGATCCGTGCTACTGCTGACATATTACGCGGGG3′ and 5′-GGGGGATCATCGCATGGATGCAGCGAGG3′ primers with standard procedures as described previously (Zha et al., 2008).

Western blotting

Whole-cell lysates from single-cell suspensions of thymocytes or B lymphocytes were prepared as previously described (Dillipantipato et al., 2005), and Western blotting was performed as previously described (Daniel et al., 2008). In brief, 40 µg protein was loaded on 4–12% Bis-Tris gels (NuPAGE; Life Technologies) and probed with the following antibodies using HRP-conjugated secondary antibodies and ECL Western blotting detection reagents (GE Healthcare): ATM (clone SC2; 1:1000; Novus Biologicals), α-Tubulin (clone B512; 1:30,000; Sigma-Aldrich), KAP1 S824p (1:700; Bethyl Laboratories, Inc.), CHK1 S317p (1:500, Bethyl Laboratories, Inc.), and SMC1 (1:8,000; Novus Biologicals). Human ATM complexes were purified from 293T cells by sequential anti-Flag and anti-HA immunoprecipitation, and kinase assays were performed as previously described (Lee and Paul, 2005). In brief, kinase assays were performed in kinase buffer composed of 50 mM Hepes, pH 7.5, 50 mM KCl, 5 mM MgCl2, 10% glycerol, 1 mM ATP, and 1 mM DTT for 90 min at 30°C.

Online supplemental material

Fig. S1 shows that integration of the Atm D2899A mutant transgene in each founder occurs on a different chromosome than endogenous ATM using FISH. Fig. S2 shows that ATM kinase activity is dispensable for ATM recruitment to DNA breaks using laser microirradiation and immunofluorescence microscopy. Fig. S3 shows FACS histograms of YFP expression in bone marrow and spleen subsets and Southern blot analysis of digested genomic DNA assaying for rearrangement of the pMX-INV–integrated substrate within ν-Abi1-transformed pre-B cell lines, indicative of RAG-dependent DNA recombination. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201204035/DC1.

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