LETTERS

Autophosphorylation at serine 1987 is dispensable for murine Atm activation *in vivo*

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The ATM (ataxia telangiectasia mutated) protein kinase is activated under physiological and pathological conditions that induce DNA double-strand breaks (DSBs). Loss of ATM or failure of its activation in humans and mice lead to defective cellular responses to DSBs, such as cell cycle checkpoints, radiation sensitivity, immune dysfunction, infertility and cancer predisposition. A widely used biological marker to identify the active form of ATM is the autophosphorylation of ATM at a single, conserved serine residue (Ser 1981 in humans; Ser 1987 in mouse)[1]. Here we show that Atm-dependent responses are functional at the organismal and cellular level in mice that express a mutant form of Atm (mutation of Ser to Ala at position 1987) as their sole Atm species. Moreover, the mutant protein does not exhibit dominant-negative interfering activity when expressed physiologically or overexpressed in the context of Atm heterozygous mice. These results suggest an alternative mode for stimulation of Atm by DSBs in which Atm autophosphorylation at Ser 1987, like trans-phosphorylation of downstream substrates, is a consequence rather than a cause of Atm activation.

It has been demonstrated that ATM is sequestered as an inactive dimer or higher order multimer in the unperturbed cell[1]. The generation of a DSB was proposed to induce a relaxation in the surrounding chromatin that promotes the intermolecular phosphorylation of ATM at Ser 1981 (ref. 1). This autophosphorylation releases inhibitory contacts between the catalytic domain in one ATM molecule and the region surrounding Ser 1981 in an interacting partner, resulting in the dissociation of ATM dimers into active monomers. After activation, phosphorylated ATM monomers are recruited to DNA breaks by binding to the carboxy terminus of the DSB sensor NBS1 (refs 2, 3), where they phosphorylate numerous substrates. This model predicts that a non-phosphorylatable mutant ATM protein would have dominant-negative interfering activity in cells expressing wild-type ATM, and would be incapable of reconstituting ATM-deficient cells. Both predictions have been confirmed in transformed human cell lines in which the mutant protein is ectopically expressed[1].

To determine the role of ATM autophosphorylation in a physiological context, we chose a bacterial artificial chromosome (BAC) reconstitution method for the generation of a mouse model. As a proof of principle, transgenic mice from two independent founder lines expressing a wild-type BAC (RP24-122F10) spanning the genomic mouse *Atm* locus (Atm^{TgWT}; Supplementary Fig. 1a) were backcrossed to $Atm^{-/-}$ mice. Expression of the wild-type mouse transgene in the Atm null background rescued the defects in growth, lymphocyte development and meiotic arrest of the homozygous mutant mice. By contrast, a BAC containing human ATM (RP11-455M10) failed to express in the mouse (not shown).

We then mutated the 1987 serine residue of Atm to alanine in the mouse BAC using an oligonucleotide-based recombination method[4]. The presence of S1987A was confirmed by sequence analysis (Supplementary Fig. 1b), and the mutated BAC was subsequently used to generate transgenic mice. Several founder lines were produced, two of which were analysed in detail. In the first (B1), mutant Atm-S1987A protein expression was similar to endogenous levels in $Atm^{+/-}$ mice irrespective of the tissue (splenocytes, Fig. 1a, b; thymocytes and testes, not shown), whereas the second transgenic founder (A8) produced 12-fold higher levels of Atm compared to $Atm^{+/-}$ mice

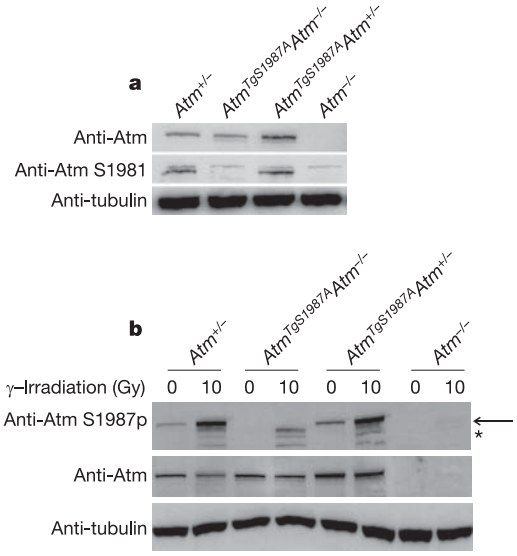

Figure 1 | Atm null and heterozygous mice express Atm-S1987A from a BAC transgene. a, Unphosphorylated Atm S1981 and total Atm protein in B cells from transgene (line B1) positive and negative littermates. **b**, B cells from $Atm^{+/-}$, $Atm^{-/-}$, $Atm^{TgS1987A}Atm^{+/-}$ and $Atm^{TgS1987A}Atm^{-/-}$ mice (B1) were harvested 30 min after no treatment (0 Gy) or after γ-irradiation (10 Gy), and phosphorylated Atm at S1987 was assessed by immunoblotting. Similar results were found for transgenic line A8 that expresses 12-fold higher levels of Atm-S1987A (see Supplementary Fig. 1c, d). The asterisk corresponds to a cross-reactive protein, subject to Atm- and damage-dependent phosphorylation, that migrates faster than authentic phosphorylated Atm at S1987 (indicated by arrow).

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(Supplementary Fig. 1c). Using an antibody that detects the unphosphorylated S1981 residue[1], we confirmed that $Atm^{TgS1987A}Atm^{-/-}$ mice do not express Atm containing serine at residue 1987 (Fig. 1a). Moreover, autophosphorylation at S1987 was undetectable in $Atm^{TgS1987A}Atm^{-/-}$ B cells exposed to irradiation, as assessed using phospho-specific monoclonal antibodies raised against mouse Atm S1987p (Fig. 1b and Supplementary Fig. 1d) or human ATM S1981p (Supplementary Fig. 2). We conclude that the mutant Atm-S1987A protein is the only potential source of Atm kinase activity in $Atm^{TgS1987A}Atm^{-/-}$ mice.

Antigen receptor rearrangements in lymphocytes generate DSBs that have the potential to phosphorylate Atm at S1987 *in vivo*[5]. Consistent with the activation of ATM during physiological V(D)J and immunoglobulin class-switch recombination, loss of ATM results in defective lymphocyte development and function, and accumulation of chromosomal aberrations involving antigen receptor loci. Expression of Atm-S1987A in the $Atm^{-/-}$ background rescued these defects, as demonstrated by the normal levels of mature single positive $CD4^{+}$T cells and expression of TCR-β in $Atm^{TgS1987A}Atm^{-/-}$ mice (Fig. 2a, b and Supplementary Fig. 3a). Similarly, B cells derived from $Atm^{TgS1987A}Atm^{-/-}$ mice and activated to undergo class-switch recombination produced normal levels of surface IgG1 (Fig. 2c and Supplementary Fig. 3b). Moreover, they did not accumulate a high frequency of chromosomal aberrations, in stark contrast to stimulated $Atm^{-/-}$ B cells[6] (Fig. 2d and Supplementary Fig. 3b). Thus, $Atm^{TgS1987A}Atm^{-/-}$ lymphocytes do not exhibit any of the hallmark defects found in $Atm^{-/-}$ mice.

Although it is formally possible that Atm (and the mutant Atm-S1987A protein) functions in a structural (rather than a catalytic) capacity to facilitate the processing of physiological V(D)J and class-switch recombination-induced DSBs, ATM kinase activity is known to be essential for cellular signalling in response to external damage. For example, ATM-dependent phosphorylation of Smc1, Chk2 and Chk1 contributes to the irradiation-induced intra-S-phase and G2/M checkpoints[7–11]. To determine whether mutant Atm-S1987A protein is capable of triggering DNA-damage-induced cell cycle checkpoints, we treated primary B cells with γ-irradiation. $Atm^{TgS1987A}Atm^{-/-}$ B cells assayed over a dose range of 0.5–10 Gy showed a reduction in the mitotic index and in the rate of DNA replication comparable to $Atm^{+/+}$ and $Atm^{+/-}$ controls, indicative of a normal G_2/M and intra-S-phase cell cycle checkpoint arrest, which contrasted with cells from $Atm^{-/-}$ littermates (Supplementary Figs 3c and 4a, b). Moreover, $Atm^{TgS1987A}Atm^{-/-}$ and $Atm^{TgS1987A}Atm^{+/-}$ ear fibroblasts were not hypersensitive to γ-irradiation (Supplementary Fig. 4c). To examine directly DNA damage signalling, we measured Atm substrate phosphorylation in primary B cells. γ-Irradiation-induced phosphorylation of Smc1, Chk2, Chk1 and p53 was barely detectable in $Atm^{-/-}$ B cells 30 min after treatment with 10 Gy γ-irradiation (Fig. 3a and Supplementary Fig. 1d). In contrast, $Atm^{TgS1987A}Atm^{-/-}$ B cells exhibited normal induction (Fig. 3a and Supplementary Fig. 1d) and kinetics (Supplementary Fig. 5a) of Atm substrate phosphorylation when compared to controls. Pre-treatment of $Atm^{TgS1987A}Atm^{-/-}$ cells with a small molecule inhibitor of Atm (KU55933; Supplementary Fig. 5b)[12], but not a DNA-PKcs inhibitor (NU7026; Supplementary Fig. 5c), abrogated γ-irradiation-induced phosphorylation of Chk2 and Smc1 and reduced γ-H2AX formation. In contrast, KU55933 did not affect Atr-mediated phosphorylation of Chk1 in response to hydroxyurea (Supplementary Fig. 5d). Thus, Atm and not related kinases such as Atr and DNA-PK mediate the γ-irradiation-induced phosphorylation events in $Atm^{TgS1987A}Atm^{-/-}$ mice. We therefore conclude that the mutant Atm-S1987A protein exhibits normal Atm

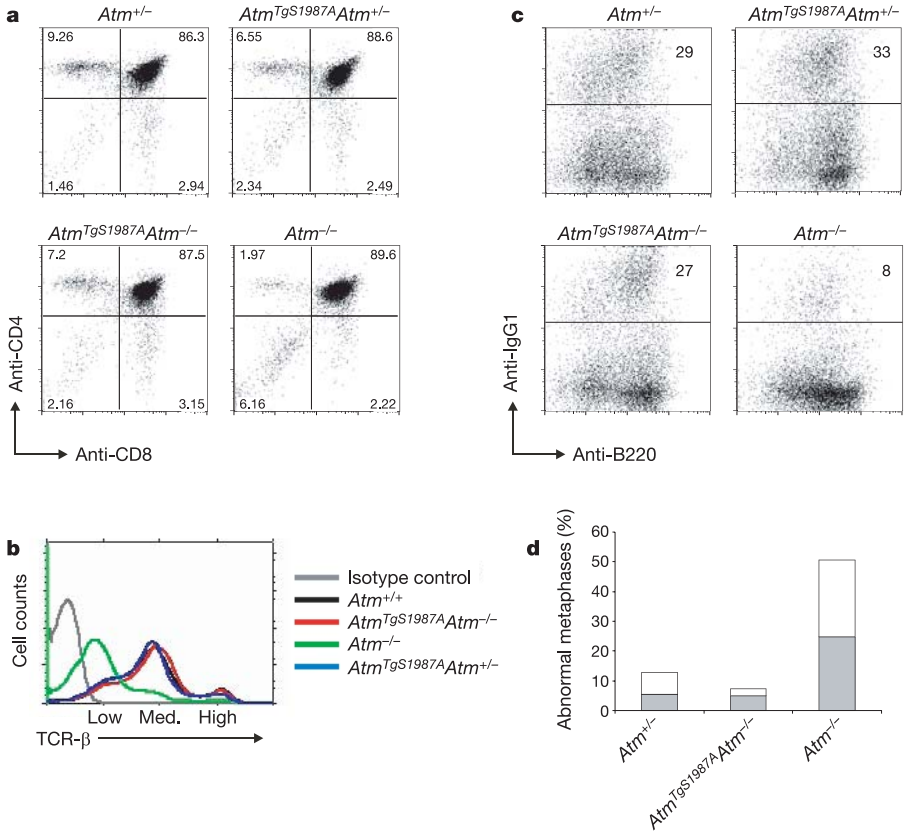
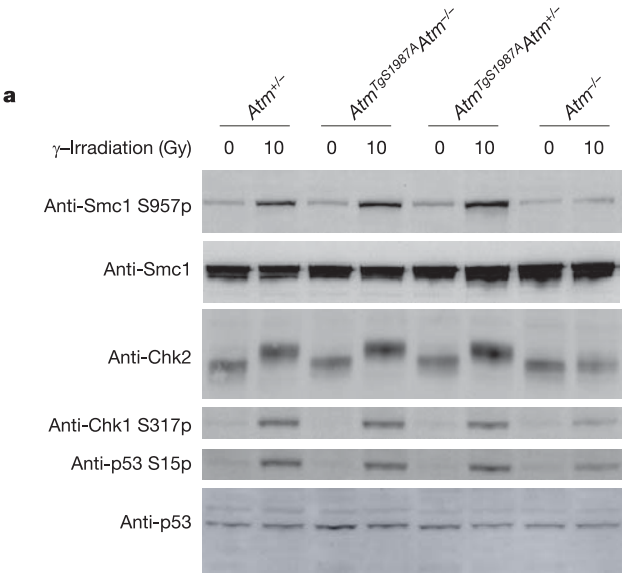


Figure 2 | Expression of Atm-S1987A reconstitutes lymphocyte development and restores genomic stability. **a**, Surface expression of CD4 versus CD8 in freshly isolated thymocytes. **b**, Surface expression of T-cell receptor-β (TCR-β). **c**, Efficiency of class switching to IgG1 in transgenic B cells. **d**, Percentage of metaphases with abnormalities specifically associated with chromosome 12 (filled bar) and with all other chromosomes (open bar) in B cells stimulated with LPS plus IL-4 for 72 h.

kinase activity, which is sufficient to trigger an efficient checkpoint response to γ-irradiation.

Many ATM substrates are phosphorylated at sites of DSBs[11]. To determine whether mutant Atm-S1987A is recruited to sites of DNA damage, we induced DSBs in primary fibroblasts and B cells using a laser microbeam, and the assembly of Atm coincident with γ-H2AX-marked sites of DSBs was tracked by fluorescence microscopy. We found that both wild-type and mutant Atm were rapidly recruited to breaks (Fig. 3b and Supplementary Fig. 6a). In addition, there was enhanced retention of Atm-S1987A at damaged sites in response to γ-irradiation, as demonstrated by the increase in protein that was resistant to detergent extraction in damaged $Atm^{TgS1987A}Atm^{-/-}$ B cells (Supplementary Fig. 6b). Thus, the dynamics of Atm-S1987A re-localization to DSBs seems to be similar to wild-type Atm.

Ectopic expression of ATM-S1981A protein has been reported to have dominant interfering activity in transformed cells[1]. However, our analysis of primary cells from $Atm^{TgS1987A}Atm^{+/-}$ mice that expressed physiological levels of transgenic Atm (line B1) revealed that Atm-S1987A does not interfere with endogenous Atm autophosphorylation (Fig. 1b and Supplementary Fig. 2), trans-phosphorylation of substrates (Fig. 3a), γ-irradiation-induced cell cycle checkpoints (Supplementary Fig. S4a, b) or chromatin binding (Supplementary Fig. 6b). Moreover, we excluded the possibility that overexpression of the Atm-S1987A protein contributes to a dominant interfering phenotype, as expression of 12-fold higher levels of Atm-S1987A in transgenic line A8 did not compromise endogenous Atm or downstream target phosphorylation (Supplementary Fig. 1d). Moreover, overexpressed Atm-S1987A was fully functional because A8-reconstituted $Atm^{-/-}$ mice rescued defects in damage signalling, lymphocyte development, cell cycle checkpoints, meiosis and radiation sensitivity (Supplementary Figs 1d, 3 and 7). We conclude that the S1987A non-phosphorylatable protein does not interfere with wild-type Atm.



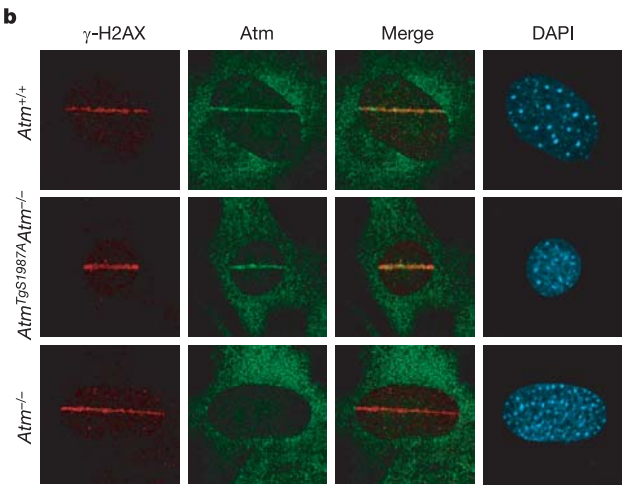


Figure 3 | S1987 phosphorylation is dispensable for Atm kinase activity and recruitment to DNA breaks. a, B cells were harvested for western blot analysis 30 min after no treatment (0 Gy) or after γ-irradiation (10 Gy). **b**, Distribution of Atm (green) in $Atm^{+/+}$, $Atm^{-/-}$ and $Atm^{TgS1987A}Atm^{-/-}$ fibroblasts 5 min after treatment with ultraviolet laser micro-irradiation. γ-H2AX (red) marks sites of DSBs.

Autophosphorylation of ATM has been interpreted as an indication of ATM activation[1,13]. Interference with autophosphorylation blocks ATM-mediated targeting of downstream substrates and the induction of cell cycle checkpoints. For example, inhibiting the activities of the MRE11–RAD50–NBS1 complex[14–19], 53BP1 (ref. 20), histone acetyltransferases[21,22] and protein phosphatase-5 (ref. 23) all decrease the formation of S1981-phosphorylated ATM and concomitantly suppress other readouts of ATM kinase activity. Despite this correlation, a causative role for S1981 phosphorylation in the activation of ATM remains controversial. First, the ATM-S1981A mutant protein does not block dimer dissociation or ATM kinase activity in *in vitro* assays[1,24]. Second, *Xenopus* ATM can be activated by DNA in the absence of autophosphorylation[25]. Third, ATM is constitutively phosphorylated in certain cell lines without rendering ATM active towards downstream targets[26]. Fourth, S1981 phosphorylation has no detectable effect on the oligomerization state of ATM: native ATM elutes as a single peak after gel filtration chromatography, and the position of this peak remains unchanged irrespective of irradiation in both human[27] and mouse (Supplementary Fig. 8) cells. Finally, pre-treatment of human cells with KU55933 (ref. 12), which blocks ATM autophosphorylation by competition with ATP, failed to inhibit the irradiation-induced increase in the kinase activity of immunoprecipitated ATM (Supplementary Fig. 9), further suggesting that autophosphorylation of ATM is not essential for its activation. Although we cannot exclude that the mechanism of ATM activation may operate differently in humans or that additional post-translational modifications may contribute to ATM activation[21,23], our findings demonstrate that phosphorylation of murine Atm at S1987 *in vivo* is not the signal that initially activates dormant Atm molecules, attracts Atm to sites of DNA damage, or facilitates its access to protein substrates.

Activation of ATM is dependent on MRE11–RAD50–NBS1-mediated DNA unwinding and recruitment of ATM to DNA ends[2,3,10,24,28]. In addition, recent evidence suggests that ATM S1981 phosphorylation occurs in the vicinity of a DSB[29] subsequent to its initial recruitment[3]. We speculate that after Atm is converted to a catalytically active conformation at a DSB, a large pool of Atm becomes accessible to rapid phosphorylation as a result of the high local concentration of available S1987 targets in Atm proximal to the lesion. Nevertheless, this post-translational modification is not required for the activation or activity of murine Atm *in vivo*.

METHODS

Generation of mice. BAC RP24-122F10, which consists of a 160-kilobase (kb) insert, including 48.3 kb of sequence upstream and 17.9 kb of sequence downstream of the *Atm* initiation and stop codons, respectively, was used to generate transgenic mice. Targeting of the BAC was performed as described[4]. The presence of the transgene was determined by screening tail DNA using the following PCR primer pairs: mAtmF 5′-AGCACAACCACACTGAATGC-3′ and SP6R 5′-GTTTTTTGCGATCTGCCGTTTC-3′; T7F 5′-TAATACGACTCACTATAGGG-3′ and mAtmR 5′-CTCAGGTGGAAATCTAACCTG-3′, which amplify a product of 600 base pairs (bp) and 300 bp at the 5′ and 3′ ends of the insert, respectively. Transgenic founders were crossed to $Atm^{+/-}$ mice.

Western blotting and immunofluorescence. For western blotting, primary antibodies were used at the following dilutions: anti-human ATM S1981p

(1:400, Rockland Immunochemicals), anti-human unphosphorylated ATM S1981 (1:2,500, Rockland Immunochemicals), anti-ATM 5C2 (1:500, Novus), anti-Smc1 S957p (1:500, Rockland Immunochemicals), anti-Smc1 (1:2,000, Novus), anti-p53 S15p (1:500, Cell Signaling), anti-p53 (1:1,000, Santa Cruz), anti-Chk1 317p (1:500, Bethyl Labs), anti-Chk2 (1:1,500, Upstate Biotechnology), anti-tubulin (1:10,000, Sigma) and anti-mouse Atm S1987p (1:500) was produced by immunization with the synthetic peptide SPTFEEGSpQGTTISS (Becton Dickinson). The association of Atm with chromatin after irradiation was determined using detergent extraction as described[30]. Primary B cells and ear fibroblasts were irradiated with a 364-nm ultraviolet laser, and processed for immunofluorescence analysis as described[29].

Cell cycle checkpoints and chromosomal stability. B cells were challenged with different doses of irradiation after stimulation with LPS and IL-4, and intra-S-phase and G2/M checkpoints were measured as described[19]. Mitotic chromosome spreads were prepared after B-cell stimulation for 72 h, and metaphases with abnormalities were quantified[6].

Additional materials and methods, including cell derivation, T-cell development, class switch recombination, immune complex assay, and histology are described in Supplementary Methods.

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