Synthetic lethality between mutation in Atm and DNA-PK$_{cs}$ during murine embryogenesis

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The gene product mutated in ataxia telangiectasia, ATM, is a ubiquitously expressed 370 kDa protein kinase that is a key mediator of the cellular response to DNA damage [1]. ATM-deficient cells are radiosensitive and show impaired cell cycle arrest and increased chromosome breaks in response to ionizing radiation. ATM is a member of the phosphatidylinositol-3-kinase (PI3K)-related protein kinase superfamily, which includes the catalytic subunit of DNA-dependent protein kinase (DNA-PK$_{cs}$) and ATR [2]. DNA-PK is a 470 kDa protein kinase that is required for proper end-to-end rejoining of DNA double-strand breaks [3]. *Prkdc*$^{scid/scid}$ mice have a homozygous mutation in the gene encoding DNA-PK$_{cs}$ and, like *Atm*$^{-/-}$ mice, are viable and radiosensitive [4–8]. To determine if Atm and DNA-PK$_{cs}$ show genetic interaction, we attempted to generate mice deficient in both gene products. However, no *scid/scid Atm*$^{-/-}$ pups were recovered from *scid/scid Atm*$^{+/-}$ intercrosses. Developmental arrest of *scid/scid Atm*$^{-/-}$ embryos occurred around E7.5, a developmental stage when embryonic cells are hypersensitive to DNA damage [9]. This reveals synthetic lethality between mutations in Atm and DNA-PK and suggests that Atm and DNA-PK have complementary functions that are essential for development.

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Results and discussion

Atm$^{-/-}$ mice are viable, although slightly runted [6–8], and have a partial defect in development of T cells [10], Purkinje cells [11], and in gametogenesis. *Scid/scid* mice have a homozygous nonsense mutation at amino acid 4046 of the gene encoding DNA-PK$_{cs}$, *Prkdc* [4, 5], resulting in loss of the last 82 amino acids of the protein, which includes the conserved carboxy-terminal region. Scid cells have reduced DNA-PK$_{cs}$ protein levels, greatly reduced protein kinase activity, and are defective in DNA double-strand break repair. *Scid/scid* mice lack mature T and B cells due to a failure to complete V(D)J antigen receptor rejoining but are otherwise developmentally normal. As DNA-PK$_{cs}$ and ATM are related proteins that have different but partially overlapping functions, we attempted to generate mice deficient in both gene products to study their interaction. We crossed *Atm*-deficient mice to *Prkdc* mutant *scid/scid* mice and then bred these progeny to obtain *scid/scid Atm*$^{+/-}$ mice that were then intercrossed. Out of a total of 278 weaned mice derived from intercrosses of *scid/scid Atm*$^{+/-}$ mice, 101 were *scid/scid Atm*$^{+/+}$, 177 were *scid/scid Atm*$^{+/-}$, and none were *scid/scid Atm*$^{-/-}$ (Table 1). This differed significantly from the Mendelian expectation of 70 mice of *Atm*$^{-/-}$ genotype. In contrast, intercrosses of *Atm*$^{+/-}$ mice or *scid*/+ mice generated *Atm*$^{-/-}$ and *scid/scid* newborns, respectively, at close to Mendelian frequencies. As the *scid/scid Atm*$^{+/-}$ intercross was a segregating cross between BALB/c and 129 strains (see Materials and Methods), there was a possibility that the lethality of *Atm*$^{-/-}$ embryos was due to genetic background effects and not to synthetic lethality between mutations in *Prkdc* and *Atm*. To check for this possibility, we also crossed *scid/scid Atm*$^{+/-}$ mice to *Atm*$^{+/-}$ mice such that all progeny were *scid*/+ and of the same segregating BALB/c 129 genetic background as the above cross. In this case, *scid*/+ *Atm*$^{-/-}$ pups were recovered at close to Mendelian frequencies (23 *Atm*$^{+/-}$ 32 *Atm*$^{+/+}$ and 15 *Atm*$^{-/-}$), indicating that genetic background was not the cause of *Atm*$^{-/-}$ embryonic lethality. As a final test to rule out genetic background effects, we obtained the *scid* mutation on a different genetic background, e.g., C3HSmn.C *Prkdc*^{scid}/J mice, and crossed these to 129 *Atm*$^{+/-}$ mice. Intercrosses of C3H 129 *scid/scid Atm*$^{+/-}$ mice again failed to yield *Atm*$^{-/-}$ offspring (25 *Atm*$^{+/+}$ 56 *Atm*$^{+/-}$ and 0 *Atm*$^{-/-}$), confirming the specificity of the *Prkdc Atm* interaction.

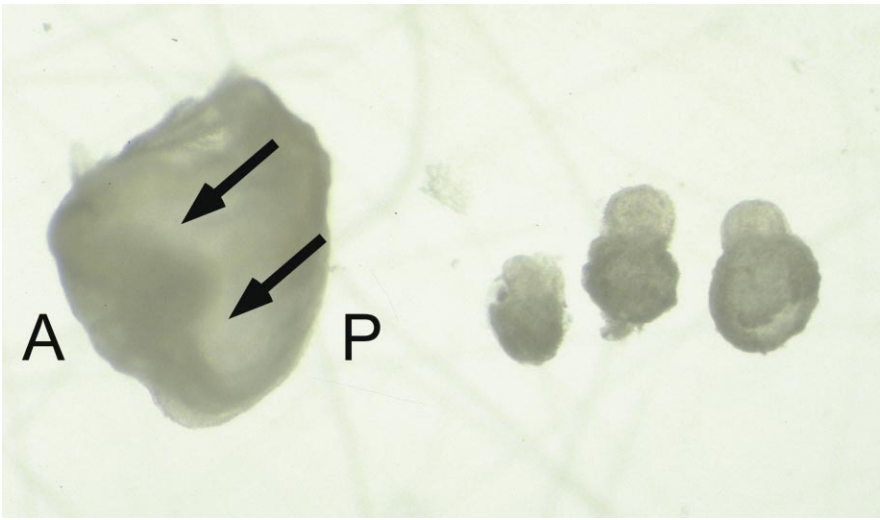
Table 1

Embryo and offspring analysis from ***scid/scid Atm*$^{+/-}$ intercrosses**

Age	*Atm*$^{+/+}$	*Atm*$^{+/-}$	*Atm*$^{-/-}$	Resorbed	Total
E3.5	3	14	3	na	20
E7.5	2	12	2	0	16
E8.5	4	12	4 (small)	0	20
E9.5	12	16	3 (small)	3	34
E11.5–13.5	13	12	0	19	44
Neonatal (<24 h)	6	11	0	na	17
Weaned	101	177	0	na	278

scid/scid Atm$^{+/-}$ mice were intercrossed and embryos collected on the indicated days and genotyped by PCR. All embryos were homozygous for the mutation in DNA-PK$_{cs}$, e.g., *Prkdc scid/scid*. na, not applicable.

Figure 1



Simultaneous loss of DNA-PK$_{cs}$ and Atm results in early embryonic lethality. Shown are embryos within yolk sacs isolated at E8.5. On the left is a *Prkdc*$^{scid/scid}$ *Atm*$^{+/+}$ conceptus showing a normal appearing embryo (arrows) within the yolk sac (A, anterior; P, posterior). The three much smaller concepti on the right are *Prkdc*$^{scid/scid}$ *Atm*$^{-/-}$ and show arrested development.

To determine when lethality of developing *scid/scid Atm*$^{-/-}$ embryos occurred, timed pregnancies of *scid/scid Atm*$^{+/-}$ intercross breeders were set up and embryos examined at several time points postcoitum. Out of 25 normal appearing embryos examined between E11.5 to E13.5, none were genotyped as *Atm*$^{-/-}$ (Table 1). Six of these litters had an average of 42% reabsorbing embryos. Yolk sacs were recovered from four of these reabsorbing embryos at E12.5 and genotyped. Two were *Atm*$^{+/-}$ and two were *Atm*$^{-/-}$. This indicates that *Prkdc* and *Atm* compound mutant embryos failed to thrive at some point prior to E11.5. Next, we examined preimplantation concepti. Out of 20 blastocysts recovered and genotyped at E3.5, three were *Atm*$^{-/-}$, which is close to the expected number of five, indicating that lethality occurred after implantation. Out of 16 normal appearing embryos recovered at E7.5, after the start of gastrulation, two were *Atm*$^{-/-}$. At E8.5, four out of twenty embryos were *Atm*$^{-/-}$. However, these embryos, dissected free of yolk sac, were significantly smaller and less developed then wild type embryos of the same age (Figure 1). By E9.5, out of 28 normal embryos recovered, none were *Atm*$^{-/-}$ (Table 1). Six out of 34 embryos at this age were reabsorbing with noticeably smaller deciduae. Residual embryonic material was recovered from three of these and all were genotyped as *Atm*$^{-/-}$. Together, these results indicate that the development of *scid/scid Atm*$^{-/-}$ embryos was arresting around E7.5, at the mid primitive streak stage.

The observation that both *scid/scid Atm*$^{+/-}$ and *scid*/+ *Atm*$^{-/-}$ mice develop to term, whereas *scid/scid Atm*$^{-/-}$ embryos die very early in embryogenesis, indicates a potent synergistic interaction between mutations in these two PI3K-related protein kinases. What is the basis of this interaction?

ATM mutant cells show several cell cycle defects (reviewed in [1]). In response to ionizing radiation, ATM-deficient cells fail to arrest in G1, in part due to impaired activation of p53 and upregulation of the Cdk inhibitor p21. ATM mutant cells in S phase exhibit radio-resistant DNA synthesis and continue to synthesize DNA following exposure to ionizing radiation. ATM mutant cells fail to arrest in G2, inappropriately enter mitosis following DNA damage, and show increased chromosome gaps and breaks. Finally, some cell types from ATM mutant mice fail to undergo p53- dependent apoptosis in response to ionizing radiation [7, 12]. These results indicate that the radiosensitivity of ATM mutant cells is not due to enhanced apoptosis but is more likely due to mitotic failure and/or lethal levels of unrejoined chromosome breaks [1, 13].

Unlike ATM, DNA-PK-deficient cells have not shown defects in cell cycle checkpoints or apoptotic responses to ionizing radiation [14–16]. However, these cells show a clear defect in end-to-end rejoining of DNA breaks, which is a major repair pathway for double-strand breaks in mammalian cells [17]. Thus, the proximal cause of radiosensitivity of DNAPK mutant cells is likely due to lack of DNA repair while that of ATM mutant cells may be due lack of cell cycle arrest and resultant chromosome breaks.

Unlike ATM, DNA-PK$_{cs}$ requires regulatory subunits, termed Ku for activity. Following the generation of DNA breaks, Ku70 and Ku 80 subunits bind to the free DNA ends and recruit DNA-PK$_{cs}$. DNA-PK$_{cs}$ is subsequently activated when bound to DNA and catalyses the end-to-end rejoining of DNA breaks. Although there are many in vitro substrates for DNA-PK$_{cs}$, the relevant in vivo

substrates remain largely undefined [3]. Substrates identified for ATM include those involved in cell cycle arrest, including Mdm2, p53, Chk2, and, indirectly, Chk1 [1]. The kinase domain of DNA-PK$_{cs}$ is 28% identical and 51% similar to that of ATM [2]. Thus, although structurally related, these proteins have distinct functions in response to DNA damage.

Previous results have shown that *Atm*$^{-/-}$ embryos irradiated with 0.5 Gy at E6.5 fail to develop to term whereas *Atm*$^{+/-}$ and wild type littermates do [9]. Thus, the combination of DNA damage and Atm deficiency is lethal during embryogenesis. Mutation in DNA-PK$_{cs}$ as occurs in the *scid/scid* mutant is likely to result in persistence of unrepaired breaks that arise spontaneously during the rapid cell cycles that occur during embryogenesis. These unrepaired breaks would normally activate Atm-dependent events including cell cycle checkpoints. In the absence of Atm, these cells might continue to progress through the cell cycle, resulting in fixation of damage or conversion of DNA breaks to chromosomal breaks. As Atm is involved in multiple checkpoints, we asked whether loss of p53, which is critical for the DNA damage induced G1 checkpoint, also interacted with DNA-PK. Intercrosses of *scid/scid p53*$^{+/-}$ mice [18] yielded mice of the expected p53 genotypes (56 *scid/scid p53*$^{+/+}$, 91 *scid/scid p53*$^{+/-}$, and 39 *scid/scid p53*$^{-/-}$), indicating that loss of p53 and the associated G1 checkpoint is insufficient to result in lethality in a DNA-PK$_{cs}$ mutant background. Furthermore, intercrosses of *p53*$^{-/-}$ *Atm*$^{+/-}$ mice also yielded mice of the expected Atm genotypes (20 *p53*$^{-/-}$ *Atm*$^{+/+}$, 51 *p53*$^{-/-}$ *Atm*$^{+/-}$, and 17 *p53*$^{-/-}$ *Atm*$^{-/-}$), indicating no measurable interaction of Atm and p53 during normal embryogenesis. These results clearly highlight the importance of other functions of Atm, besides regulation of p53 and G1 arrest, in cellular survival and interaction with DNA-PK. These functions could be those involved in controlling G2 arrest or in maintaining chromosomal integrity in the face of DNA breaks. It has been suggested that in ATM mutant cells, DNA breaks are more efficiently converted into chromosome breaks due to lack of cell cycle arrest and/or due to alterations in chromatin structure [1]. This could be the mechanistic basis for synergy between mutations in Atm and DNAPK. It is also possible that Atm and DNA-PK have other, yet to be described, overlapping functions that are essential for viability.

It has long been known that certain stages of embryonic development are more sensitive to DNA damage than others. During gastrulation of the mouse, at E7.5, embryos are by far the most sensitive to irradiation, showing a very large increase in apoptosis in response to 0.5 Gy and substantial cell death after doses as low as 0.05 Gy [9]. Earlier or later stages of development do not show this high degree of radiosensitivity. Cells of the embryo at this stage of development have an extreme rate of proliferation, with a cell generation time of as little as 2–3 hr [19]. Thus, defects in DNA repair and cellular responses are likely to have the greatest impact during this developmental window and this could explain the demise of *scid/scid Atm*$^{-/-}$ embryos at this stage. Indeed, mouse concepti deficient in other DNA repair or response genes such as *Atr* [20, 21], *Rad 50* [22], *Rad 51* [23], *AP endonuclease* [24], *Brca1*, and *Brca2* [25] also die at about the same stage of development. ATR, another PI3K-related family member, is more highly related to ATM than is DNA-PK$_{cs}$, sharing 31% identity at the PI3K domain [2]. ATR, like ATM, is a protein kinase that is activated by DNA damage and initiates signaling important in cell cycle checkpoints [26, 27]. ATR-deficient cells are sensitive to DNA damage, and lethality of *Atr*$^{-/-}$ embryos prior to E7.5 is reportedly due to extensive chromosomal fragmentation [20, 21]. These data support the idea that defects in DNA repair or response pathways are critical just prior to or during gastrulation.

Genetic interaction among PI3K-related protein kinase members has also been reported in yeast. *TEL1* and *MEC1* from *S. cerevisiae* share significant sequence similarity to Atm, Atr, and DNAPK$_{cs}$ [2] and are involved in telomere maintenance and in response to DNA damage [28]. Simultaneous mutations in both *TEL1* and *MEC1* resulted in synergistically increased sensitivity to DNA damaging agents [28] and a senescent phenotype [29]. Just one copy of *TEL1* was sufficient to suppress the lethality and radiation sensitivity of a truncation allele of *MEC1* [28]. Thus, *TEL1* and *MEC1* are functionally related, and it was suggested that functions of the *ATM* gene are divided between these two *S. cerevisiae* homologs [28].

A complete picture of the signaling pathways and functions of these PI3K-related family members has yet to emerge. The strong genetic interactions observed in both yeast and mammalian homologs suggest they have overlapping or complementary functions, which provides important clues to their function in the context of the intact organism.

Materials and methods

129/SvEv *Atm*$^{(ins5790neo)}$ knockout mice were obtained from A. Wynshaw-Boris [6] and genotyped by PCR of toe-derived DNA using the following primers: Atm forward, 5′GACTTCTGTCAGATGTTGCTGCC3′; Atm reverse, 5′CGAATTTGCAGGAGTTGCTGAG3′; and Atm neo, 5′GGG TGGGATTAGATAAATGCCTG3′ The product sizes are WT, ~170 bp, and Mutant, ~410 bp. PCR conditions were 1× PCR buffer, 2.7 mM $MgCl_2$, 0.5 mM dNTPs, 0.1 mM primers, 1 unit of Taq, and 200 ng DNA in 25 μl water. Cycling conditions were 95° for 5 min followed by 25 cycles of 93° for 45 s, 55° for 45 s, and 72° for 90 s.

BALB/cByJSmn-*Prkdc*^{scid}/J (BALB/c *scid*) and C3HSmn.C*Prkdc*^{scid}/J mice were purchased from Jackson Laboratory and genotyped as described [4]. To generate compound mutant mice, 129/Sv *Atm*$^{+/-}$ mice were crossed to BALB/c *scid/scid* mice, yielding 129×BALB/c F1 *Atm*$^{+/-}$ *scid*/+ mice. These were backcrossed to BALB/c *scid/scid* mice to generate *scid/scid Atm*$^{+/-}$ mice. These were intercrossed in an attempt to generate *scid/scid Atm*$^{-/-}$ mice. No mice of this genotype were recov-

ered, so pregnancies were set up and embryos removed at defined time points postcoitum.

Blastocysts and embryos were genotyped using standard Atm primers and Qiagen HotStar Taq DNA Polymerase. Blasts, and embryos were collected in Qiagen 1× buffer with 1 μg/μl of Proteinase K (Sigma). These were incubated for 2 hr at 57°C, and 1 μl of resulting DNA was used per 10 μl PCR reaction. PCR conditions were those suggested by Qiagen with 1.5 μl of 25 mM $MgCl_2$ added. 40 cycles were used for blasts and 25 cycles for embryos.

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