Targeted disruption of the cell-cycle checkpoint gene *ATR* leads to early embryonic lethality in mice

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Checkpoints of DNA integrity are conserved throughout evolution, as are the kinases ATM (Ataxia Telangiectasia mutated) and ATR (Ataxia- and Rad-related), which are related to phosphatidylinositol (PI) 3-kinase [1–3]. The *ATM* gene is not essential, but mutations lead to ataxia telangiectasia (AT), a pleiotropic disorder characterised by radiation sensitivity and cellular checkpoint defects in response to ionising radiation [4–6]. The *ATR* gene has not been associated with human syndromes and, structurally, is more closely related to the canonical yeast checkpoint genes *rad3*^{Sp} and *MEC1*^{Sc} [7,8]. ATR has been implicated in the response to ultraviolet (UV) radiation and blocks to DNA synthesis [8–11], and may phosphorylate p53 [12,13], suggesting that ATM and ATR may have similar and, perhaps, complementary roles in cell-cycle control after DNA damage. Here, we report that targeted inactivation of ATR in mice by disruption of the kinase domain leads to early embryonic lethality before embryonic day 8.5 (E8.5). Heterozygous mice were fertile and had no aberrant phenotype, despite a lower *ATR* mRNA level. No agents. Attempts to target the remaining wild-type *ATR* allele in heterozygous *ATR*$^{+/-}$ ES cells failed, supporting the idea that loss of both alleles of the *ATR* gene, even at the ES-cell level, is lethal. Thus, in contrast to the closely related checkpoint gene *ATM*, *ATR* has an essential function in early mammalian development.

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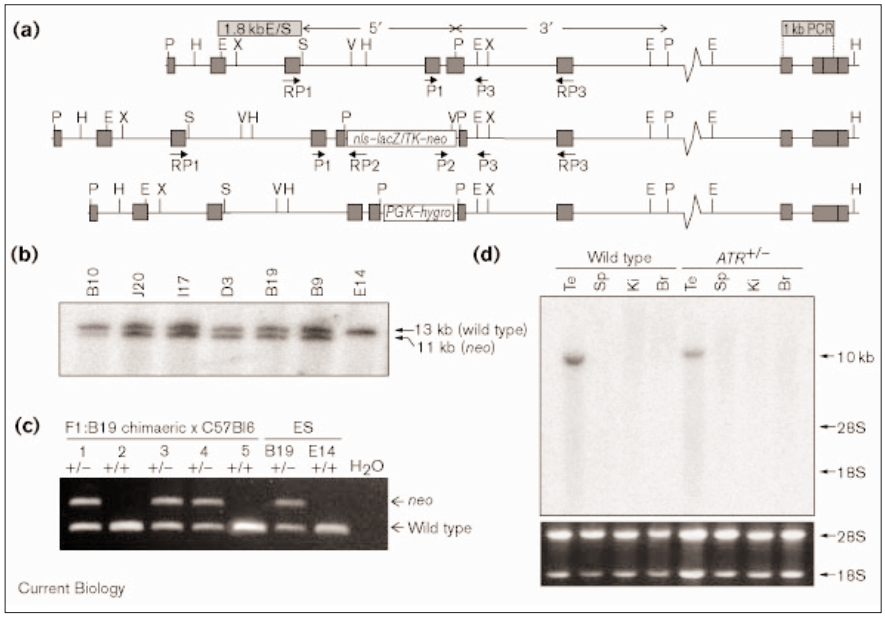
Results and discussion

The yeast *ATR* homologues *MEC1*^{Sc} and *rad3*^{Sp} play pivotal roles in arresting the cell cycle following DNA damage caused by a wide range of genotoxic agents [1,2]. Hitherto, no human disorder has been associated with *ATR*. To assess the impact of *ATR* mutations in mice, we isolated genomic clones representing the 3′ end of the mouse *ATR* locus (as the 3′ end encodes the critical kinase domain) and constructed a vector for gene targeting in mouse ES cells. The positions of exons corresponding to the coding region around the conserved PI 3-kinase domain [8] were determined, and this domain was found to be distributed over the last seven exons (Figure 1a). The corresponding kinase domain of the human *ATM* gene is spread over eight exons that do not coincide [14], indicating that the intron/exon structures are not strongly conserved between these closely related genes.

Fluorescence *in situ* hybridisation (FISH) localised the mouse *ATR* gene to chromosome 9, band E4/F (Figure 2a), which is close to the mouse *ATM* locus. To exclude the possibility of cross-hybridisation, *ATR* and *ATM* genomic probes were used in a dual-colour FISH. This demonstrated (Figure 2b) that the *ATM* gene was indeed located slightly more proximal on mouse chromosome 9 in band C, confirmed the presence of several human regions syntenic to mouse chromosome 9, including human chromosome 11q23 (the position of the *ATM* gene) [16] and human chromosome 3q (where human *ATR* is located) [7,8].

To inactivate ATR, an *nls–lacZ/TK–neo* cassette was inserted in-frame into the *ATR* gene in the *Pst*I site located just 5′ to the sequence encoding the most highly conserved part of the kinase domain (Figure 1a), thus disrupting this important portion of the encoded protein. The linearised plasmid was electroporated into E14 129/Ola ES cells, and 205 neomycin-resistant clones were isolated and screened for homologous integration. Six clones (Figure 1b) were found in which *nls–lacZ/TK–neo* cassette had correctly replaced the segment encoding the PI 3-kinase domain of ATR. Four independent ES cell clones (designated B9, B19, J20 and D3) were injected into mouse 129/Sv blastocysts, yielding 39 chimaeras in total. Two of the chimaeric mice (derived from different ES cell transformants) gave offspring with germ-line transmission (Figure 1c), and mice heterozygous for the targeted *ATR* gene were established. Both male and female *ATR*$^{+/-}$ mice were fertile but, when *ATR*$^{+/-}$ mice were interbred, homozygous mutants for the targeted *ATR* allele were absent from the large number of offspring analysed (Table 1). The observed ratio of *ATR*$^{+/-}$ to wild-type mice was close to 2:1, indicating embryonic

Figure 1



Targeted disruption of the murine *ATR* locus. **(a)** Genomic structure of the 3′ end of the *ATR* locus (top), and the *ATR* loci targeted with *nls–lacZ/TK–neo* (middle) or *PGK–hygro* (bottom). Dark grey boxes, exons; unshaded boxes, the inserted *nls–lacZ/TK–neo* [10] and *PGK–hygro* cassettes; light grey boxes, DNA probes; arrows above the wild-type locus, the 5′ and 3′ flanking regions of the targeting constructs; small arrows, PCR primers (see the Supplementary material); E, *Eco*RI; V, *Eco*RV; H, *Hin*dIII; P, *Pst*I; S, *Sph*I; X, *Xba*I. **(b)** Neomycin-resistant ES cell clones (targeted with *nls–lacZ/TK–neo*) were digested with *Hin*dIII and *Eco*RV, blotted and probed with the 1 kb 3′ PCR probe. ES cell clones B10, J20, I17, D3, B19 and B9 were correctly recombined and contained the 11 kb *nls–lacZ/TK–neo*-targeted (*neo*) allele. The E14 parental ES cell clone showed only the 13 kb wild-type allele. **(c)** Germ-line transmission of the B19 chimaeric mouse. Offspring were genotyped using the P1, P2 and P3 primers. The position of the 375 bp wild-type *ATR* and the 511 bp *ATR–neo* (*neo*) fragment are indicated. **(d)** Expression of the *ATR* gene in mouse tissues. Total RNA (20 μg) extracted from the indicated tissues of a wild-type and $ATR^{+/-}$ mouse was electrophoresed, blotted and hybridised with a ^{32}P-labelled 0.8 kb *ATR* cDNA probe. The ethidium-bromide-stained gel before blotting (bottom) was used as a control for loading. The 28S and 18S ribosomal RNA bands and the 10 kb *ATR* mRNA transcript are indicated. Te, testis; Sp, spleen; Ki, kidney; Br, brain.

lethality of the *ATR*-deficient phenotype. Heterozygous matings were analysed at E10.5 and E8.5, but again no $ATR^{-/-}$ embryos were found. At both time-points, we observed a significantly high proportion of residual placentas containing no embryos. Unfortunately, because of the presence of maternal tissue, unequivocal genotyping was not possible, but the residual placentas indicate post-implantation lethality, that is, later than E4.5. We are currently establishing ES cell lines from blastocysts of matings between $ATR^{+/-}$ females and $ATR^{+/-}$ males.

As a complementary approach to generate an ATR-deficient cell line, we attempted to inactivate the remaining wild-type *ATR* allele in the neomycin-resistant $ATR^{+/-}$ ES cells by using an *ATR* construct carrying a marker for hygromycin resistance (*PGK–hygro*) (Figure 1a). In wild-type ES cells, homologous recombination yielded 7 (out of 250) correctly targeted ES cell clones, which is comparable to the frequency observed before with the *nls–lacZ/TK–neo* cassette. Extensive efforts to inactivate the remaining wild-type allele in the neomycin-resistant $ATR^{+/-}$ ES cells failed, however, although retargeting of the *ATR/nls–lacZ/TK–neo* allele was observed. Among the 639 tested hygromycin-resistant ES cell transformants, at least eight $ATR^{-/-}$ clones would have been expected. The absence of $ATR^{-/-}$ transformants was, statistically, highly significant ($p = < 0.001$, Student's *t*-test), suggesting that ATR is essential for the viability of ES cells.

Analysis of heterozygous *ATM* mice and mouse embryonic fibroblasts (MEFs) suggests that *ATM* haplo-insufficiency results in increased sensitivity to ionising radiation [5,6,17] The *ATR* gene is expressed at very low levels and its 10 kb transcript cannot be detected by northern blot analysis in most tissues, with the notable exception of the testis [18]. Therefore, the effect of targeting one of the *ATR* alleles at the molecular level was investigated using this tissue. As shown by the northern blot in Figure 1d, the amount of *ATR* mRNA was decreased in the testes of $ATR^{+/-}$ mice compared with wild-type mice. If, as in the case of ATM, this was also reflected at the protein level, we could infer that a 50% reduction of the ATR product does not result in any obvious phenotype at the level of the intact organism. Both male and female $ATR^{+/-}$ mice were fertile, developed normally (body weight) and produced $ATR^{+/-}$ offspring in a Mendelian fashion. The survival of the $ATR^{+/-}$ mice after one year (> 95%) was not significantly different from wild-type mice, nor did we see any tumours developing in the $ATR^{+/-}$ mice during this period. We have not excluded the possibility that differences in cancer predisposition become apparent later in life or after chronic or acute treatment with DNA-damaging agents.

The *ATR–lacZ* fusion transcript, expected to be 3 kb larger than the wild-type transcript, was not visible by northern analysis with an *ATR* probe (Figure 1d) or with an *nls–lacZ/TK–neo* probe (data not shown). Using reverse

Table 1

Genotyping of offspring and embryos from matings of heterozygous *ATR* mice.

	Number of litters	Total number	Genotype			No result
			+/+	+/–	–/–	
Offspring	46	301	99 (75.3)	202 (150.5)	0 (75.3)	–
E10.5	4	30*	10 (7)	18 (14)	0 (7)	2
E8.5	3	20†	5 (4.5)	13 (9)	0 (4.5)	2

These numbers exclude the 7* or 6† residual placentas that were not genotyped. The numbers within parentheses indicate the number of offspring expected on the basis of Mendelian inheritance.

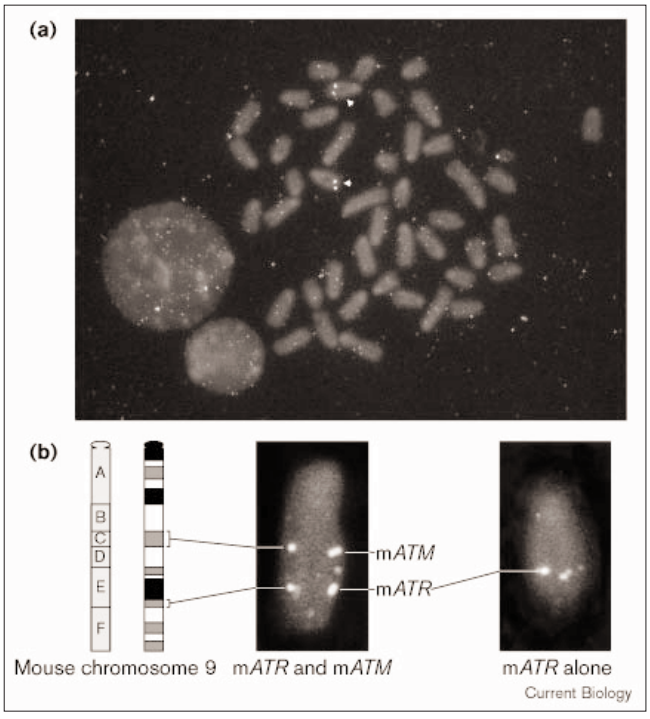
transcription (RT)–PCR, *ATR–lacZ* fusion mRNAs were detected in testes of $ATR^{+/-}$ mice and in $ATR^{+/-}$ ES cells. Alternatively spliced *ATR* mRNAs that skipped the *nls–lacZ/TK–neo* exon were not observed. The *ATR–lacZ* fusion transcript was, however, unstable or expressed at very low levels as, unlike previous positive results with this *nls–lacZ/TK–neo* cassette [19], we did not observe any β-galactosidase activity in the testes of $ATR^{+/-}$ mice or ES cells.

In view of the increased sensitivity of ATM heterozygotes, we examined whether the heterozygous ES cell clones displayed altered sensitivity to DNA-damaging agents. Exposure to UV radiation, to the DNA crosslinking agent mitomycin C, or to γ-irradiation, failed to cause any significant differences in survival for any of the tested neomycin- (Figure 3) or hygromycin-resistant ES cell clones.

In contrast to *ATR*, the closely related checkpoint gene *ATM* is not essential during early embryogenesis [4–6]. Mutants of the budding yeast and fission yeast *ATM* orthologues $TEL1^{Sc}$ and $tel1^{Sp}$, respectively, display a much less severe phenotype than mutants with loss of the *ATR* orthologues $MEC1^{Sc}$ or $rad3^{Sp}$ [1,2,20]. ATM-deficient mice are viable and display many aspects of the pleiotropic phenotype of AT patients, including infertility, sensitivity to ionising radiation and cancer susceptibility [5,6]. Premature senescence of ATM-deficient mouse embryonic fibroblasts is rescued in a p53-deficient background [21]. Although the viability of cells with a dominant-negative ATR mutation was not dependent on the presence of functional p53 [10], absence of p53 might have an effect on the lethality of $ATR^{-/-}$ embryos.

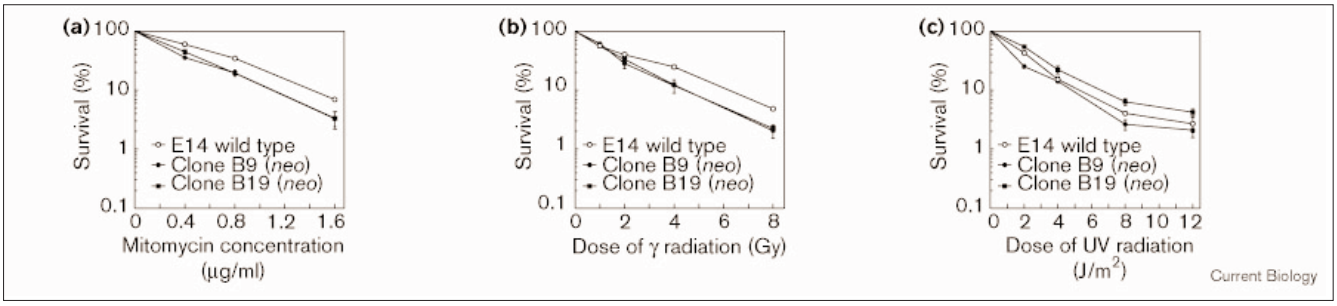
Our work demonstrates that *ATR* is an essential gene, required for embryogenesis and probably general cell proliferation. This is consistent with the observations that high-level dominant-negative ATR expression causes cell death [10,11] and that modest overexpression of wild-type ATR inhibits MyoD-induced differentiation and cell-cycle abnormalities [9]. At present, it is not clear whether embryonic lethality and the effect on cell differentiation and proliferation are intrinsic to its checkpoint function. Perhaps, as for the $MEC1^{Sc}$ gene [1,22], a more subtle mutation or Cre–*lox*-mediated conditional mutation of *ATR* in mouse tissues will enable the different functions of ATR to be segregated and studied. An embryonic lethal phenotype has also been observed in an independent ATR knockout [23].

Figure 2



Localisation of *ATR*, and colocalisation of *ATR* and *ATM*, on mouse chromosome 9. **(a)** Metaphase spreads of the mouse erythroid Red8 cell line were hybridised with a biotinylated murine *ATR* cosmid clone as described [24]. A specific hybridisation signal (arrowheads) was detected on mouse chromosome 9. Counterstaining (not shown) with 4,6-diamidino-2-phenylindole (DAPI) revealed the chromosome banding pattern, and the identity of chromosome 9 was confirmed using control chromosome 9 probes. **(b)** Specific FISH signals on chromosome 9 using a biotinylated murine *ATR* cosmid probe alone (right), or biotinylated murine *ATR* and *ATM* cosmid probes used simultaneously (left). The signals of the *ATR* and *ATM* genes are indicated on the chromosomes and in the diagrams of the chromosome 9 banding pattern.

Figure 3



Effect of DNA-damaging agents on wild-type and *nls–lacZ/TK–neo*-targeted ES cells. Clonogenic survival assay after treatment with **(a)** increasing concentrations of the DNA crosslinking agent mitomycin C, or increasing doses of **(b)** ^{137}Cs γ-irradiation and **(c)** 254 nm UV-C light. The percentage of surviving cells, as measured by their colony-forming ability, was plotted as a function of the indicated concentration of mitomycin C , or dose of γ- or UV-radiation. All measurements were performed in triplicate as described [25] and the standard error of the mean are indicated.

Supplementary material

Supplementary material including additional methodological detail, a figure showing expression of the *ATR* gene and *ATR–lacZ* fusion transcripts in different mouse tissues and ES cells, and a figure showing the murine *ATR* cDNA sequence is available at http://current-biology.com/supmat/supmatin.htm.

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