High-frequency developmental abnormalities in *p53*-deficient mice

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Background: Several strains of mice carrying null mutations of the tumour suppressor gene *p53* have been developed. It has been reported that homozygous mice from all of these strains develop normally to birth, but then succumb rapidly to neoplasia.

Results: Here, we report that a significant proportion of female $p53^{-/-}$ mice die during embryogenesis or in the period between birth and weaning, being subject to a spectrum of abnormalities. In a significant proportion (23 %) of $p53^{-/-}$ female embryos, the normal process of neural tube closure failed, leading to exencephaly and subsequent anencephaly. Although this phenomenon was predominantly associated with females, we observed one affected male embryo. In addition to a spectrum of neural tube defects, many of these embryos exhibited a range of craniofacial malformations, including ocular abnormalities and defects in upper incisor tooth formation. We observed a significant reduction in the number of $p53^{-/-}$ female progeny of $p53^{+/-} \times p53^{+/-}$ matings, and also in an *in utero* analysis of the $p53^{+/-}$ female progeny of $p53^{-/-} \times p53^{+/+}$ matings. When male mice were exposed to irradiation prior to mating, a significant increase in the rate of abnormality was seen in the progeny, which was specifically associated with *p53* deficiency.

Conclusions: We have identified a high rate of developmental abnormalities associated with *p53* deficiency. This manifests itself as a spectrum of lesions, predominantly female-associated defects in neural tube closure. These defects may arise either because p53 plays a physiological role at the time of neural tube closure, or because of an abnormally high frequency of mutation within the haploid gametes of *p53*-null parents.

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Background

Loss of *p53* function has been strongly linked to the development of malignancy [1]. In an attempt to define the mechanisms underlying this association, several mouse strains have recently been developed that carry an inactivated version of *p53* [2–5]. The reported phenotypes of these strains are all remarkably similar. Surprisingly, given the proposed role of *p53* in controlling cell-cycle progression, homozygous mice were found to survive to birth, but then to succumb rapidly to tumours, predominantly of the thymic lymphoid lineages [2,6,7]. Analysis of a variety of cell types from homozygotes, both *in vivo* and *in vitro*, has shown normal *p53* function to be essential for preventing the propagation of DNA damage to daughter cells, whether by causing cell-cycle arrest or by inducing apoptosis [4,8–11].

Although the survival of $p53^{-/-}$ mice has led to the conclusion that *p53* function is not essential for normal embryonic development, we have observed in our breeding stock a deficiency in the number of female homozygotes at the time of weaning. Jacks *et al.* [6] reported a reduction in the number of $p53^{-/-}$ homozygous animals from heterozygous ($p53^{+/-} \times p53^{+/-}$) crosses, and have suggested that a proportion of homozygotes may be lost during embryogenesis, or in the period between birth and weaning. One possible explanation for these observations is that p53 is involved in the normal process of spermatogenesis. Evidence in support of this idea comes from two sources. First, histological analysis of the testes of $p53^{-/-}$ mice [12] has shown an increased level of multinucleate giant cells, spreading from the periphery into the lumen of the seminiferous tubules. These were interpreted to arise as a consequence of failed repair during spermatogenesis, leading to aberrant division. Second, a role for *p53* during meiosis has been suggested by the pachytene stage-specific expression of *p53* in primary spermatocytes [13].

In order to investigate these observations further, a study of the embryonic development of *p53*-null mice has been undertaken. A significant percentage of female $p53^{-/-}$ mice were found to die during embryogenesis or in the period between birth and weaning. A spectrum of developmental abnormalities was observed, with failure of the anterior neural tube to close being the principal defect. In order to investigate whether any of the developmental abnormalities were attributable to mutation, rather than as a result of a defect in a p53-dependent physiological pathway, we carried out further experiments in which DNA damage was induced in gametes of homozygous animals by γ irradiation.

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Results

Analysis of progeny from *p53*-deficient matings

Breeding data from several stocks of *p53*-mutant mice, derived from a strain carrying a deletion in *p53* of exons 2–6 [3], are shown in Table 1. All show a clear deficiency of female $p53^{-/-}$ progeny. This was, however, most prominent in the inbred 129/Ola stock, which parallels the report of a high incidence of testicular abnormality within *p53*-null animals of this strain [12]. Hence, although this phenomenon is influenced by genetic background, it is not completely determined by it. All subsequent analyses of this phenomenon were carried out using the outbred stock 'cross 1', which segregates for BALB/c, SWR and 129/Ola genomes, and shows a 50 % reduction in female homozygotes at weaning.

We analyzed embryos from $p53^{-/-} \times p53^{-/-}$ matings, isolated between gestation day 11.5 and birth (Table 1). No resorptions were observed. Analysis of the total number of embryos revealed a small reduction (17 %) in the number of female homozygotes. Although not statistically significant, this suggests that some loss may be occurring either during the development of X chromosome-bearing gametes, or during the pre- or very early post-implantation period. Of the female embryos analyzed, a significant proportion (23 %) were observed at mid-gestation to exhibit exencephaly, as a consequence of failure of the cephalic neural tube to close, which resulted in anencephaly at birth (Fig. 1).

These combined losses predict a reduction in the number of females at weaning of 33 %. This figure is, however, less than the observed reduction for this cross (50 %), implying that further losses may be occurring between birth and weaning. That the abnormalities were variable in their severity is supported by the finding of two adult animals that had skull malformations (Fig. 1g,h); these are presumed to represent the mildest observable form of this phenotype.

Progeny from matings between $p53^{-/-}$ and $p53^{+/+}$ animals were also examined, with only one exencephalic embryo being observed (Table 2). There was, however, a

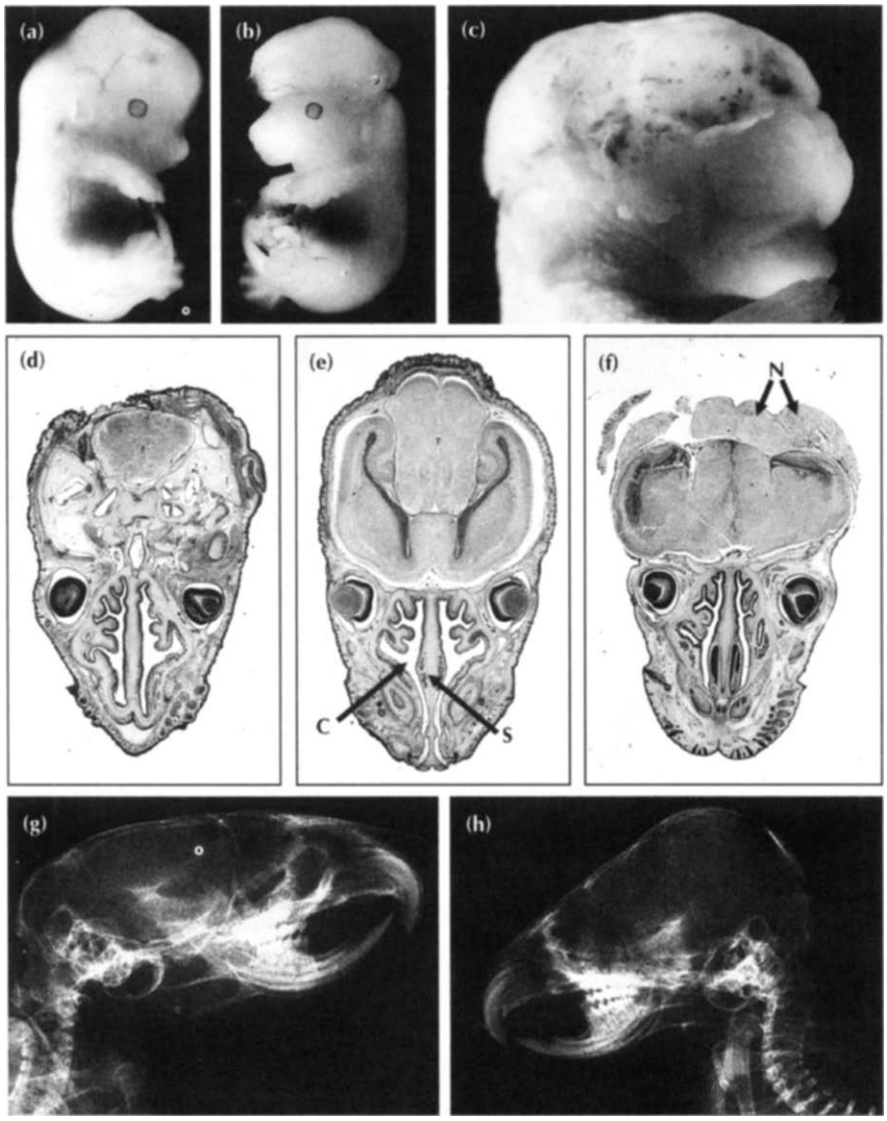

Fig. 1. Abnormalities of the central nervous system in $p53^{-/-}$ mice. **(a,b)** External morphologies of 13.5 day (a) unaffected and (b) exencephalic embryos. **(c)** 16.5 day embryo showing the eversion and exposure of neural tissue characteristic of exencephaly. **(d–f)** Haematoxylin and eosin stained transverse sections through the head region of (d) a 19.5 day exencephalic embryo, (e) an unaffected $p53^{-/-}$ 19.5 day embryo, and (f) a 14.5 day exencephalic embryo. Note that there is a considerable volume of brain tissue present in the 14.5 day exencephalic embryo, whereas relatively little is observed by 19.5 days. This is a characteristic feature in the $p53^{-/-}$ exencephalic mice. C, nasal cavity; S, nasal septum; N, exposed neural tissue. **(g,h)** Lateral radiographs of adult mouse heads (48 days). (g) The $p53^{+/+}$ skull displays normal morphology, whereas (h) the $p53^{-/-}$ (non-exencephalic) skull exhibits a much enlarged cranial vault.

Table 1. Number of *p53*-mutant progeny, either at weaning or *in utero*, isolated between gestation day 11.5 and birth.

	Progeny of +/– x +/– matings from cross 1 (at weaning)			Progeny of –/– x –/– matings (at weaning)				Progeny of –/– x –/– matings from cross 1 (*in utero*)		
	–/–	+/–	+/+	129/Ola (inbred)	Cross 1 (outbred)	Cross 2 (outbred)	Cross 3 (outbred)	Total number	Normal embryos	Exencephalic embryos
No. males / no. females	87/43	175/188	82/94	37/6	84/42	74/32	59/35	128/106	127/86	1/20
Relative deficiency of females	– 50%	+9.3%	+9.3%	–84%	–50%	–57%	–40.7%	–17%	–33%	
	$p < 0.01$	NS	NS	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.05$	NS	$p < 0.01$	

Percentage figures represent the relative deficiency (–) or excess (+) of females, expressed as a percentage of the number of males assuming a Mendelian distribution. Probability values are calculated according to the binomial distribution. Crosses 1 and 2 segregate for BALB/c, SWR and 129/Ola genomes, but represent separate cohorts; cross 3 segregates for SWR, 129/Ola and C57BL/6. NS, not significant.

statistically significant reduction in the number of female progeny sired by male homozygotes (the proportion of males and females were tested by binomial distribution, $p < 0.05$).

Karyotypic analysis was carried out on fibroblast cultures derived from two $p53^{-/-}$ exencephalic embryos. G-band analysis of both cultures showed them to have a modal chromosomal number of 40, suggesting that the genetic damage, if any, carried by these animals was relatively subtle (data not shown).

Developmental abnormalities associated with *p53* deficiency

Histological examination of 19 $p53^{-/-}$ embryos with exencephaly (including 4 new-born $p53^{-/-}$ mice which had progressed to anencephaly) showed that all regions of the brain were affected (Fig. 1a–f). The extent to which neural tube closure had failed to occur normally varied. Although in most embryos this resulted in exencephaly, leading to anencephaly, one embryo was found to have a persistently open neural tube in the lumbosacral region. Amongst exencephalic embryos, there was a marked female prevalence, although a single male embryo was identified (Table 1). This reflects a similar sex bias to that seen in human embryos with neural tube defects, which is assumed to arise as a consequence of sex-determined differences in the development of early embryos [14]. The transition from exencephaly to anencephaly by the time of birth is necessarily associated with massive cell loss. Electron microscopic and histological analysis of the exencephalic embryos confirmed the presence of apoptosis in neural tissue from *p53*-deficient mice (Fig. 2g,h). This apoptosis must be *p53*-independent.

In addition to exencephaly, we observed a spectrum of associated abnormalities (Fig. 2). In 5 of the 19 exencephalic embryos examined, fusion of the upper incisors was observed, giving rise to a single midline tooth (Fig. 2a,b). In 4 of these 5 cases of fusion, there was evidence of an additional median dental element, which in 3 cases was incorporated into the fused tooth (termed *dens-in-dente* [15]). Ocular abnormalities were also found, involving both the neural retina and the lens. Retinal dysplasia with multiple folding of the neural retina was present in 5 of the 19 embryos (Fig. 2c). Adhesion of the lens to the cornea was observed in 13 of these embryos,

Table 2. Numbers of *p53*-mutant progeny derived from matings where the male has previously been subject to whole body irradiation.

Mating type (M x F)	Progeny type	Radiation dose (Gy)	No. exencephalic embryos / total no. embryos (M)	(F)	Mean litter size (no. litters)
–/– x +/+	+/–	0	0/83	0/56	6.6 (21)
–/– x +/+	+/–	2 or 5*	0/40	0/44	5.2 (16)
+/+ x –/–	+/–	0	0/23	1/27	7.1 (7)
+/+ x +/+	+/+	0	0/42	0/45	7.9 (11)
+/+ x +/+	+/+	5	0/12	0/12	3.4 (7)
–/– x +/–	–/–	2 or 5*	0/14 (1 dead)	6/10	5.5 (12)
–/– x +/–	+/–	2 or 5*	0/21	0/21	5.5 (12)

The numbers given in this table are summarized from day 11 of gestation to birth. *Combined data from males exposed to either 2 Gy or 5 Gy prior to mating.

and also in many of the unaffected $p53^{-/-}$ litter mates, showing that this feature is not exclusively associated with exencephaly. A single case of bilateral preaxial polydactyly of the hindlimbs was seen (Fig. 2d).

Analysis of progeny from irradiated animals

An increased incidence of developmental abnormalities following γ-irradiation of parental animals has been reported previously [16]. This must arise as a consequence of increased genetic damage within the gametes of parental animals. This fact, together with observations that imply a role for *p53* in normal spermatogenesis [12,13], prompted us to examine the frequency of developmental abnormalities following γ-irradiation of *p53*-mutant parental animals. Prior to mating, males were subjected to whole body exposure to either 2 or 5 Gray (Gy); the results are shown in Table 2. No abnormal embryos were observed amongst the progeny of control wild-type matings, $p53^{-/-} \times p53^{+/+}$ matings, or in the $p53^{+/-}$ progeny of $p53^{-/-} \times p53^{+/-}$ matings, although litter sizes were reduced. There was, however, a significantly increased level of exencephaly amongst female $p53^{-/-}$ progeny derived from $p53^{-/-} \times p53^{+/-}$ matings (6/10, corrected $\chi^2 = 6.68$, $p < 0.01$).

Discussion

Taken together, these results show clearly an increased level of developmental abnormalities in *p53*-null animals, primarily defects associated with neural tube formation. These defects were strongly, but not exclusively, associated with female animals. Similar sex distortion associated with exencephaly has been reported for both humans and mice [14]. We therefore interpret our find-

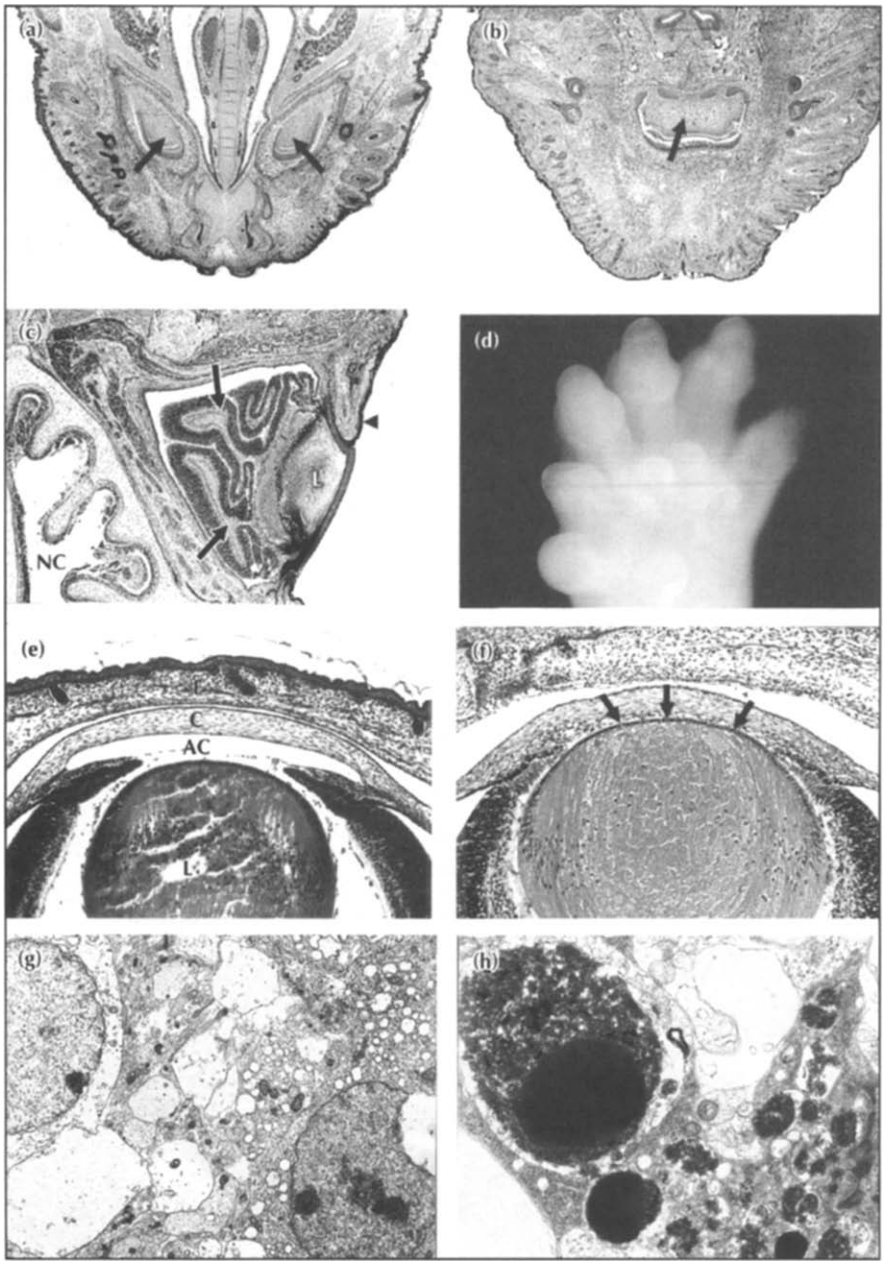

Fig. 2. Morphological abnormalities associated with *p53* deficiency. **(a,b)** Transverse sections through comparable areas of the upper jaw of (a) an unaffected 19.5 day embryo, and (b) a new-born anencephalic mouse. The unaffected embryo has two upper incisor teeth present (arrows), whereas the anencephalic mouse has a single midline tooth formed by the fusion of the two upper incisor teeth (arrow). **(c)** Transverse section through the eye of a new-born anencephalic mouse. The neural retina is extensively folded (arrows), the lens (L) is abnormal and the eyelids are unfused (arrowhead, eyelid; NC, nasal cavity). **(d)** Hindlimb with an extra digit seen in a 15.5 day exencephalic embryo. **(e)** Transverse section through the eye of a 17.5 day $p53^{+/+}$ embryo. AC, anterior chamber; C, cornea; L, lens; E, fused eyelids. **(f)** Transverse section through the eye of a 19.5 day unaffected $p53^{-/-}$ embryo, showing adhesion (arrows) between the posterior surface of the cornea and the anterior surface of the lens. (a–c,e,f) Haematoxylin and eosin stained samples. **(g)** Electron micrograph from the posterior column of upper spinal cord in a 16.5 day $p53^{-/-}$ embryo showing normal nuclear morphology. **(h)** Sections from the same level of spinal cord in a $p53^{-/-}$ embryo with exencephaly, showing classical features of apoptosis: cell shrinkage, organelle aggregation, nuclear chromatin condensation and nuclear fragmentation.

ings as an exaggeration, by virtue of *p53* deficiency, of the normal sex bias for this phenomenon.

There are two possible explanations for the observed increase in developmental abnormalities. First, *p53* may have a physiological role at the time of neural tube closure. Evidence in support of this comes from studies of neural tube closure, which suggest that the neural folds may develop as a consequence of local changes in cell turnover [14]. These changes include alterations to the rates of cell-cycle progression, cell division and programmed cell death, each of which, individually or synergistically, may alter the course and outcome of neural tube formation and closure. In view of the importance of *p53* in the direct and indirect regulation of all of these processes, and the modulation of *p53*-dependent pathways by other mechanisms such as growth factors [17], it is possible that the process of neural tube closure is adversely affected in an otherwise normal, *p53*-null environment.

The second possible explanation for the defects is that they may arise as a consequence of a higher mutation rate in gametes from homozygous parents. A role for *p53* in preventing the propagation of mutations, either by the initiation of G1 arrest or by the induction of apoptosis, has been established in many other cell types [4,8–11]. Several predictions arise from this hypothesis. The first is that one would expect to observe a range of phenotypes in the affected animals as a consequence of mutations in different genes. As we have shown, this was found to be the case.

The second prediction is that there might be an increase in the frequency of chromosomal abnormalities within the exencephalic embryos, although one might also argue that embryos which have acquired sufficient genetic damage to result in chromosomal abnormalities may be efficiently deleted by the known *p53*-independent damage responsive pathways [18]. We have been unable to demonstrate any gross chromosomal damage, but this does not rule out the existence of more subtle changes.

The third prediction is that abnormalities might be observed in progeny from matings between $p53^{-/-}$ and $p53^{+/+}$ animals. Although only one such abnormality was observed, there was a statistically significant reduction in the number of female progeny sired by male $p53^{-/-}$ mice. A similar sex-ratio distortion was not seen amongst progeny from irradiated animals, although litter size was reduced, nor was it seen in heterozygous progeny from unirradiated female homozygotes. These results are consistent with an increased mutation rate in male-derived gametes, but one which does not result in exencephaly by virtue of *p53*-dependent loss of affected heterozygote embryos.

A fourth prediction follows, namely that the rate of developmental abnormality should increase following γ-irradiation of the parental animals. No abnormalities were observed in the progeny of wild-type matings, $p53^{-/-} \times p53^{+/+}$ matings, or in the $p53^{+/-}$ progeny from $p53^{-/-} \times p53^{+/-}$ matings, although litter sizes were reduced. There was, however, a significantly increased level of exencephaly amongst female $p53^{-/-}$ progeny derived from $p53^{-/-} \times p53^{+/-}$ matings. We can make no comment with regard to a similar phenomenon within animals derived from female *p53*-null gametes, as the reciprocal irradiation experiment — the irradiation of female $p53^{-/-}$ animals prior to mating — was not performed.

Conclusions

These results demonstrate a clear link between *p53* deficiency and developmental abnormalities. We observed a broad spectrum of craniofacial defects and ocular abnormalities in *p53*-deficient mice. This may reflect the importance of *p53* in normal physiological development, possibly by altering the frequency of cell-cycle arrest or apoptosis. However, the incidence of abnormalities is markedly elevated by irradiation of parental gametes, raising the intriguing additional possibility that increased mutation in a *p53*-null environment may also be important.

Materials and methods

Analysis of embryos

Embryos were dissected from the yolk sac and developmentally staged [19]. They were then fixed in buffered formalin, embedded in paraffin wax, serially sectioned at 3–5 μm and stained with haematoxylin and eosin. Embryos were genotyped from yolk sac DNA by the polymerase chain reaction (D.J. Harrison, R.D.G. Malcomson, A.R. Clarke, S. Coutts, A. Peter and S.E.M. Howie, unpublished data), and sexed either morphologically or by the polymerase chain reaction [20].

Electron microscopy

1 mm cubes of tissue were fixed for 16 h in ice-cold 1 % gluteraldehyde, and post-fixed in osmium tetroxide. Blocks were embedded in Araldite resin, sectioned with a glass knife and stained with uranyl acetate. Sections were examined on nickel grids using a Philips 301 transmission electron microscope.

Irradiation

Adult male mice were either mock-irradiated or exposed to γ-irradiation from a ^{137}Cs source (0.3 Gy min^{-1}). Appropriate exposure doses were as stated in the text. Where male animals were irradiated and then mated to untreated females, analysis of progeny was restricted to 4 weeks post-irradiation.

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