Disruption of the developmentally regulated *Rev3l* gene causes embryonic lethality

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The *REV3* gene encodes the catalytic subunit of DNA polymerase (pol) ζ, which can replicate past certain types of DNA lesions [1]. *Saccharomyces cerevisiae rev3* mutants are viable and have lower rates of spontaneous and DNA-damage-induced mutagenesis [2]. Reduction in the level of Rev31, the presumed catalytic subunit of mammalian pol ζ, decreased damage-induced mutagenesis in human cell lines [3]. To study the function of mammalian Rev31, we inactivated the gene in mice. Two exons containing conserved DNA polymerase motifs were replaced by a cassette encoding G418 resistance and β-galactosidase, under the control of the *Rev3l* promoter. Surprisingly, disruption of *Rev3l* caused mid-gestation embryonic lethality, with the frequency of *Rev3l*$^{-/-}$ embryos declining markedly between 9.5 and 12.5 days *post coitum* (dpc). *Rev3l*$^{-/-}$ embryos were smaller than their heterozygous littermates and showed retarded development. Tissues in many areas were disorganised, with significantly reduced cell density. *Rev3l* expression, traced by β-galactosidase staining, was first detected during early somitogenesis and gradually expanded to other tissues of mesodermal origin, including extraembryonic membranes. Embryonic death coincided with the period of more widely distributed *Rev3l* expression. The data demonstrate an essential function for murine Rev31 and suggest that bypass of specific types of DNA lesions by pol ζ is essential for cell viability during embryonic development in mammals.

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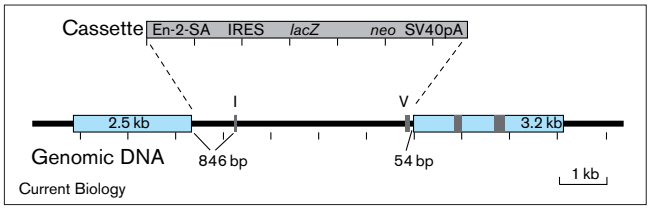
Results and discussion

The targeting vector was designed to inactivate the predicted polymerase activity of the Rev3l protein by eliminating two exons encoding conserved polymerase motif V and part of motif I [4]. These were replaced with a dicistronic cassette containing *lacZ* fused to a neomycin-resistance gene (Figure 1). Heterozygous intercrosses yielded only *Rev3l*$^{+/-}$ and *Rev3l*$^{+/+}$ progeny in a 2.3:1 ratio, demonstrating that disuption of the Rev3l polymerase domain results in embryonic lethality (Table 1). Morphology, fertility and viability of *Rev3l*$^{+/-}$ animals appeared normal, indicating no influence of gene dosage and no dominant-negative effect of any truncated protein that might be produced. The non-viability of *Rev3l*$^{-/-}$ embryos was unexpected as yeast with a deletion of *REV3* show no growth defects, are modestly sensitive to DNA damaging agents, and have lower rates of induced and spontaneous mutations [2]. Furthermore, humans with a defect in pol η, another DNA polymerase that bypasses damage, can live for decades and show relatively mild symptoms [5,6].

Figure 1



Targeted disruption of the *Rev3l* gene. A targeting vector was constructed by subcloning genomic sequences (blue) into pBluescript II KS$^+$. A promoterless cassette containing the splice acceptor from the *En-2* gene (En-2-SA), an internal ribosome entry site (IRES), *lacZ–neo*^{R} selection marker and a polyadenylation sequence (SV40pA) was placed between the arms, replacing the indicated region including two exons encoding amino acids 2776–2860 of Rev3l.

To determine the developmental stage at which the polymerase disruption causes lethality, embryos from *Rev3l*$^{+/-}$ intercrosses were analysed. Beginning at 10.5 dpc, the fraction of embryos that were homozygous for the disruption became progressively lower (Table 1). At 7.5 dpc, *Rev3l*$^{-/-}$ and *Rev3l*$^{+/-}$ embryos appeared equivalent in size and developmental stage. From 8.5 dpc, *Rev3l*$^{-/-}$ embryos were smaller than their heterozygous and wild-type littermates. Between 9.5 dpc and 12.5 dpc, mutants were about 60% of the expected length and there was a developmental delay of up to 2 days, accompanied by defective morphogenesis (Figure 2 and data not shown). Although *Rev3l*$^{-/-}$ embryos had not turned by 9.5 dpc (Figure 2d), some *Rev3l*$^{-/-}$ embryos did complete axial rotation by

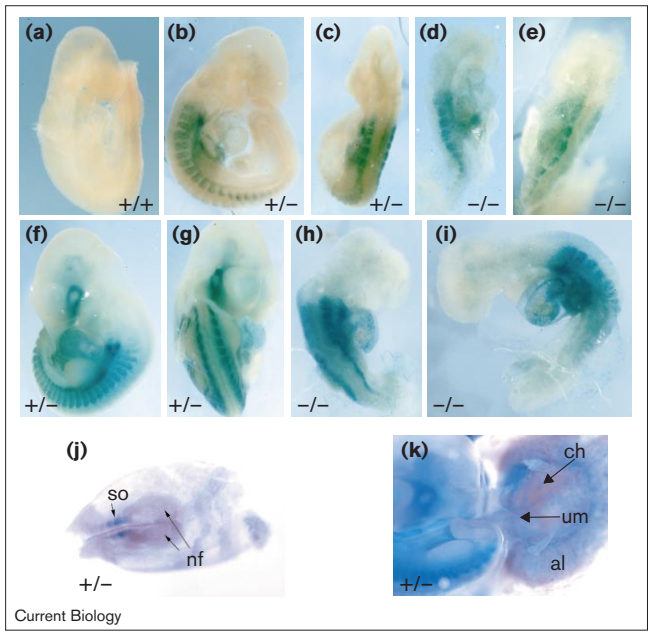
Table 1

Embryos and progeny from Rev31$^{+/-}$ intercrosses.

Age		Number per genotype		
(dpc)	Total number	+/+	+/–	–/–
7.5	7	1	4	2
8.5	9	3	4	2
9.5	3	0	2	1
9.5	11	3	5	3
9.5	12	2	4	6
Total 9.5	**26**	**5**	**11**	**10**
10.5	5	3	2	0
10.5	6	0	4	2
10.5	5	3	1	1
10.5	10	3	7	0
10.5	8	2	5	1
10.5	14	5	5	4
Total 10.5	**48**	**16**	**24**	**8**
11.5	10	5	4	1
11.5	9	2	5	2
Total 11.5	**19**	**7**	**9**	**3**
12.5	6	2	4	0
12.5	5	4	1	0
12.5	6	3	3	0
12.5	7	2	4	1
Total 12.5	**24**	**11**	**12**	**1**
Mice born	**164**	**50**	**114**	**0**

Timed matings were set up and the day on which the copulation plug was found was designated as 0.5 dpc. Embryos were explanted and dissected away from fetal membranes. Yolk sacs were washed in phosphate-buffered saline and frozen on dry ice. DNA was extracted from the yolk sacs of embryos or tail snips of 14-day-old mice using a Qiagen DNeasy Tissue Kit. PCR genotyping is described in the Supplementary materials and methods.

Figure 2



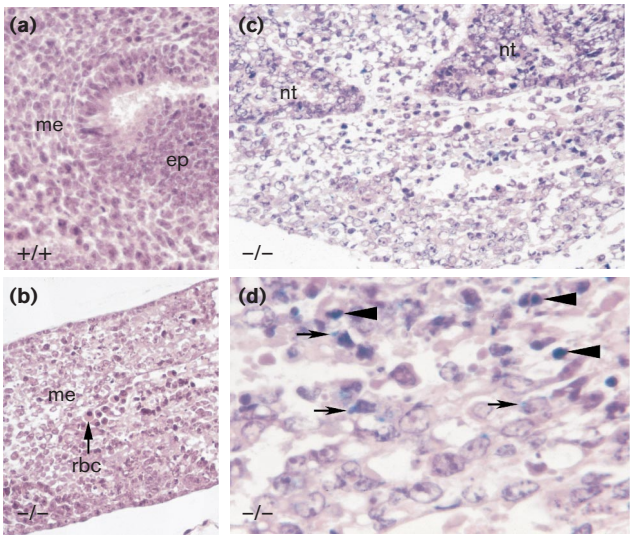
Rev3l expression in mouse embryos at **(a–e)** 9.5 dpc and **(f–i,k)** 10.5 dpc and **(j)** 7.5 dpc. (j) At 7.5–8.0 dpc, expression was seen in the somites (so). The neural folds (nf) are indicated. The 10.5 dpc embryo and its implantation site are shown in (k); al, allantoic mesenchyme; ch, chorion; um, umbilicus. *Rev3l*$^{+/-}$ mice were mated and pregnant females sacrificed. Embryos were dissected from their yolk sacs, washed in phosphate-buffered saline, fixed and stained with X-gal. Length of embryos from head to tail: *Rev3l*$^{+/-}$ 9.5 dpc, 2.5 mm; *Rev3l*$^{-/-}$ 9.5 dpc, 1.5 mm; *Rev3l*$^{+/-}$ 10.5 dpc, 3.5 mm; *Rev3l*$^{-/-}$ 10.5 dpc, 2–2.5 mm.

10.5 dpc (Figure 2i). Head, abdominal and tail regions formed, as did the neural tube, somite pairs, branchial arches and limb buds (Figure 2). *Rev3l*$^{-/-}$ embryos could be distinguished readily by their craniofacial and abdominal abnormalities. For example, at 9.5 and 10.5 dpc, the somites in *Rev3l*$^{-/-}$ embryos had a diffuse, irregular structure and were fewer in number than in heterozygous littermates (Figure 2).

Rev3l$^{-/-}$ embryos with beating heart structures containing blood were visible until the latest time of viability, but pericardial sac edema was commonly observed at later stages (Figure 2i). Blood within yolk sac vasculature was also observed in *Rev3l*$^{-/-}$ embryos, although the vessels were less branched than in their heterozygous and wild-type littermates. The yolk sacs of *Rev3l*$^{-/-}$ embryos were often fragile, with loose attachment of the embryo to the decidual implantation site, possibly because of delayed and/or suboptimal chorioallantoic fusion. Abnormalities in the development and maintenance of embryonic mesoderm were major features associated with disruption of *Rev3l*. Mesenchyme from *Rev3l*$^{-/-}$ embryos was often discohesive and degenerate, with pyknotic bodies and debris visible throughout the tissue, although occasional mitotic figures were still seen (Figure 3). Nucleated red blood cells indicative of embryonic hemopoeisis were abundant in the *Rev3l*$^{-/-}$ embryos (Figure 3b). Abnormalities were not confined to tissues of mesodermal origin. Epithelial and neuroepithelial cell pleomorphism was also commonly seen, possibly a consequence of discohesion and degeneration of the surrounding mesenchyme (Figure 3c). Attempts to generate embryonic fibroblasts from such *Rev3l*$^{-/-}$ mid-gestation embryos have been unsuccessful.

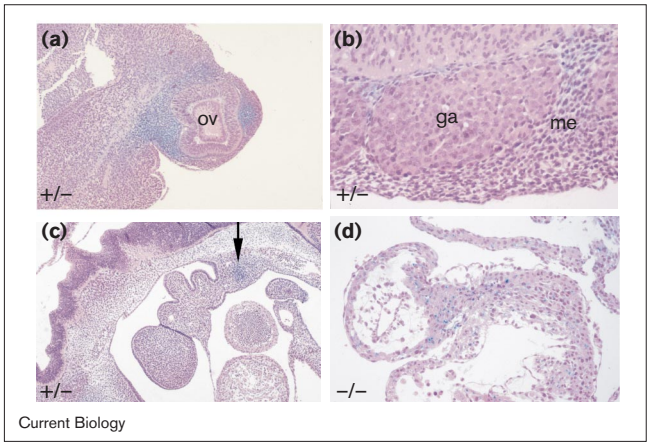
The targeting strategy enabled us to monitor expression from the *Rev3l* promoter by staining embryos for β-galactosidase activity. Expression of *Rev3l* was first detectable within the earliest somite pairs formed at 7.5–8.0 dpc (Figure 2j) and continued during further somitogenesis. From 9.5 dpc, the *lacZ* transgene was expressed within the developing myocytes of the heart and in the mesenchyme close to the aorta (Figure 4d). At 10.5 dpc, strong expression of *Rev3l* was found in the peri-optic mesenchyme surrounding the optic vesicle (Figure 4a). This striking increase in expression

Figure 3



Mesenchymal degeneration in *Rev3l*$^{-/-}$ embryos. Embryos (10.5 dpc) were stained for β-galactosidase activity, fixed and embedded. Sections shown were stained with haematoxylin and eosin. **(a)** Cephalic region of *Rev3*$^{+/+}$ embryo showing normal tissue density in mesenchyme (me) and epithelium (ep) and absence of dying cells. **(b)** Degenerated gut mesenchyme in *Rev3*$^{-/-}$ embryos. The large cells seen in the centre are nucleated red blood cells (rbc). **(c)** Highly disorganised and degenerated mesenchyme in a *Rev3l*$^{-/-}$ embryo. Degenerated neural tube tissue (nt) is also indicated. **(d)** Higher power oil immersion view of a lower right section of (c). Small arrows, examples of staining with X-gal; arrowheads, examples of pyknotic nuclei.

Figure 4



Differential expression of *Rev3l* in tissues from embryos at **(a,b,d)** 11.5 dpc and **(c)** 10.5 dpc. The embryos were processed as in Figures 2 and 3. Only *Rev3l*$^{+/-}$ and *Rev3l*$^{-/-}$ embryos exhibited β-galactosidase staining. (a) Polar mesenchymal expression in the region around the optic vesicle (ov) of a *Rev3l*$^{+/-}$ embryo. (b,c) Staining in the mesenchyme (me) around (b) a posterior root ganglion (ga) and (c) the fourth branchial arch (arrow) adjacent to the dorsal aorta in *Rev3l*$^{+/-}$ embryos. (d) Expression in myocytes in the developing heart of a *Rev3l*$^{-/-}$ embryo, showing some disorder of mesenchymal tissue.

in the peri-optic region occurred between 9.5 and 10.5 dpc (compare Figure 2b with Figure 2f,g). Some expression was also detected in retroperitoneal mesenchyme and in mesenchyme surrounding dorsal root ganglia (Figure 4b and data not shown). Dark mid-ventral staining was detected in whole mounts (Figure 2b,d,f,i). Some of this was accounted for by staining in and near the heart, but additional strong expression was found near the fourth branchial arch and in thyroid precursor mesenchyme near the lung bud (Figure 4c). The staining of tissues in *Rev3l*$^{-/-}$ embryos was generally more diffuse (Figure 2d,e,h,i) and is likely to be the result of the marked disorganisation and cell loss in tissues often found in the homozygous mutants. Areas of β-galactosidase staining could be found within such degenerated tissue (Figure 3c,d).

In adult tissues, *Rev3l* expression has been examined by northern blotting in mouse [7] and by reverse transcription coupled to PCR (RT–PCR) of human DNA [8]. Some expression was detected in all tissues examined, including those predominantly derived from ectoderm (brain), endoderm (lung) and mesoderm (muscle). Our results indicate that high-level expression of *Rev3l* is developmentally regulated during embryogenesis, occurring first in early somitogenesis and then in other mesodermal tissues up to at least 11.5 dpc. This differential expression seems likely to account for the predominant disorder and lack of integrity found mainly in mesenchymal tissues. Lack of proper development of the heart and large circulatory vessels might in itself be the immediate cause of death.

To examine whether *Rev3l* is expressed within extraembryonic membranes, decidua were partially dissected to observe implanted embryos. In heterozygous and homozygous mutants, β-galactosidase activity was observed within the amnion, allantoic mesenchyme and major blood vessels of the umbilical cord (Figure 2k). These tissues are partially or wholly derived from mesoderm. Nevertheless, *Rev3l* expression was not detectable within all extraembryonic tissues, as the chorion beneath the allantoic mesoderm appeared unstained. As *Rev3l* is normally expressed within extraembryonic membranes, the absence of functional Rev3l in *Rev3l*$^{-/-}$ embryos could be the cause of the pericardial sac edema, yolk sac fragility and weak attachment to the decidual implantation site. Yolk-sac malfunction can induce osmotic imbalance, leading to edema, whereas delayed and/or suboptimal chorioallantoic fusion could result in an implantation defect. Defects of the chorioallantoic placenta or yolk sac are a common cause of murine lethality *in utero* [9] and could contribute to the embryonic lethality of the *Rev3l* disruption.

Unexpectedly, disruption of the polymerase domain of Rev3l causes loss of embryonic viability between 9.5 and

12.5 dpc. The expression pattern of Rev3l suggests a crucial role of this protein in mesodermal tissues of embryonic and/or extraembryonic origin. If this role is not fulfilled, there is degeneration and cell loss throughout the embryo. How does Rev3l prevent cell death? There are several hints from the phenotype of yeast *rev3* mutants and the activity of the gene product. First, yeast *rev3* mutants are defective in damage-induced mutagenesis [2]. This appears to be a direct consequence of the fact that DNA pol ζ has the ability to bypass certain types of DNA damage, and in the process often inserts a non-complementary nucleotide. Lack of pol ζ causes yeast cells to be only slightly sensitive to killing by ultraviolet light. This probably indicates that other DNA repair and lesion bypass processes in yeast can combat most of the mutagen toxicity in the absence of pol ζ, but that pol ζ is still the main enzyme used for bypass in some situations. A second role for pol ζ is indicated by experiments which show that one pathway of double-strand break repair in *S. cerevisiae* normally makes use of pol ζ, as indicated by a Rev3-dependent pattern of mutations introduced in the vicinity of the break site during repair [10].

The Rev3l-defective mouse embryos studied here arose from zygotes that were able to propagate and differentiate through many cell divisions before embryonic death. During this time, the rapidly proliferating cells in the embryo might gradually accumulate DNA damage that in normal organisms is bypassed by pol ζ. Numerous DNA lesions caused by unavoidable oxidative and hydrolytic processes are constantly formed in genomes [11]. Similarly, double-strand breaks can form when DNA replication forks encounter nicked templates, and these stalled replication forks must be reactivated by replication or repair. Cells in adult tissues or in culture have mechanisms to cease division or DNA replication temporarily in order to allow time for accurate and specific DNA repair enzymes to act before proceeding through the cell cycle. However, embryonic development adheres to a strict temporal programme that requires rapid cell division. Under such conditions, enzymes that can rapidly bypass DNA lesions may be expected to be particularly important. We have been unable to isolate homozygous *Rev3l*$^{-/-}$ ES cells by culturing *Rev3l*$^{+/-}$ ES cells in high G418 concentrations or by retargeting *Rev3l*$^{+/-}$ cells with a disruption construct conferring hygromycin resistance. ES cells may be a special case of a cell type primed to undergo apoptosis after accumulating low levels of DNA damage [12]. We have, however, been unable so far to obtain live homozygous progeny by crossing the Rev3l defect onto a p53-defective background. The mesodermal tissues in which *Rev3l* is most highly expressed may have a special need for the polymerase activity of pol ζ if certain types of DNA adducts or strand breaks accumulate in them. An intolerable load of damaged DNA in critical embryonic or extra-embryonic cells would then lead to death. It is also possible that Rev3l has an additional, unknown function.

Supplementary material

A figure showing more details of the targeting construct, additional discussion and methodological detail are available at http://current-biology.com/supmat/supmatin.htm.

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