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Loss of DNA polymerase zeta enhances spontaneous tumorigenesis

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Abstract

Mammalian genomes encode at least 15 distinct DNA polymerases, functioning as specialists in DNA replication, DNA repair, recombination, or bypass of DNA damage. Although the DNA pol zeta(ζ) catalytic subunit REV3L is important in defense against genotoxins, little is known of its biological function. This is because REV3L is essential during embryogenesis, unlike other translesion DNA polymerases. Outstanding questions include whether any adult cells are viable in the absence of Polζ, and whether Polζ status influences tumorigenesis. REV3L-deficient cells have properties that could influence the development of neoplasia in opposing ways: markedly reduced damage- induced point mutagenesis, and extensive chromosome instability. To answer these questions, *Rev3L* was conditionally deleted from tissues of adult mice using *MMTV-Cre*. Loss of REV3L was tolerated in epithelial tissues, but not in the hematopoietic lineage. Thymic lymphomas in $Tp53^{-/-}$ *Rev3L* conditional mice occurred with decreased latency and higher incidence. The lymphomas were populated predominantly by *Rev3L* null T-cells, demonstrating that loss of *Rev3L* can promote tumorigenesis. Remarkably, the tumors were frequently oligoclonal, consistent with accelerated genetic changes in the absence of *Rev3L*. Mammary tumors could also arise from *Rev3L*-deleted cells, in both $Tp53^{+/+}$ and $Tp53^{+/-}$ backgrounds. Mammary tumors in $Tp53^{+/-}$ mice deleting *Rev3L* formed months earlier than mammary tumors in $Tp53^{+/-}$ control mice. Prominent preneoplastic changes in glandular tissue adjacent to these tumors occurred only in mice deleting *Rev3L* and were associated with increased tumor multiplicity. Polζ is the only specialized DNA polymerase yet identified that inhibits spontaneous tumor development.

Keywords

DNA damage; DNA polymerase; conditional mouse gene disruption; REV3L; mammary tumor; thymic lymphoma

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Introduction

Despite the cell's many DNA repair mechanisms, genomic replication and cell division frequently take place with unrepaired DNA lesions still present. Bypass of such lesions can be performed by enzymes known as translesion synthesis DNA polymerases, which have activities on differing substrates (1,2). Included in this group of enzymes in mammals are polζ, polη, pol*ι*, polκ, polθ, polν, and Rev1 (1,2). The best characterized is pol η, which inserts bases across from UV radiation-induced cyclobutane pyrimidine dimers in DNA. A deficiency of the human enzyme causes the hereditary skin cancer- prone disorder xeroderma pigmentosum variant (XP-V) (1).

Polζ has been difficult to study because unlike other specialized DNA polymerases, its catalytic subunit REV3L is essential for development. This indicates that polζ has a non-redundant function, perhaps bypass of endogenous DNA damage in the rapidly growing embryo (3–6). The mammalian *REV3L* gene (7,8) encodes a polypeptide of ~350 kDa, twice as large as the catalytic subunit of yeast polζ, Rev3. Reduced REV3L function in human and mouse fibroblasts decreases base change mutagenesis induced by UV light and other mutagens (9–11). REV3L therefore differs markedly from the other DNA polymerases, because interfering with its function reduces rather than increases point mutagenesis.

Polζ-defective cell lines have only been isolated in p53-compromised backgrounds. *Rev3L* null mouse embryonic fibroblasts exhibit spontaneous chromosomal instability, with an increase of ~10-fold in the frequency of chromosome translocations (12); other *Rev3L* null cells also have increased frequencies of chromosome breaks and translocations (13–16). This is consistent with a role for DNA polζ in bypass of adducts that would otherwise block DNA replication and lead to replication fork collapse and breakage. Alternatively, REV3L might function in the repair synthesis that occurs during some forms of DNA double strand break repair (16–19). We undertook a study to answer two basic questions. The first is if Rev3L loss can be tolerated in normal adult cells. A second is whether the loss of REV3L in adult cells affects the development of spontaneous neoplasia.

Materials and Methods

Conditional Rev3L allele targeting construct

The conditional targeting construct (Fig. 1A) was based on that used to replace two exons containing the 3′ end of motif I and all of motif V within the REV3L polymerase domain active site (5). The plasmid backbone (pBluescript II KS+) and the *Rev3L* genomic homology arms were retained and loxP sites were added at the internal edges of the homology arms. At the 3′ end of the 5′ homology arm (*Bsp*E1 site), a neomycin resistance gene surrounded by loxP sites was added. This cassette was excised from plasmid pL2-neo (created by Hua Gu), contained a 1.1 kb linker regions and is expressed by the HSV *tk* promoter. A loxP site formed by annealing oligonucleotides was ligated to the 5′ end of the 3′ homology arm (*Sal*I site). Finally the 4.6 kb of genomic sequence missing between the homology arm sequences and containing the essential polymerase exons was amplified from 129/Ola genomic DNA using Expand High Fidelity polymerase (Roche). This PCR product was cloned between the 5′ neo cassette flanked by loxP sites and the single 3′ loxP site. The final targeting construct contained 3 loxP sites and 4 exons (2 within the original 3′ homology arm and the 2 essential pol exons now flanked by loxP sites). The sequence of the exons and *loxP* sites was confirmed by DNA sequencing. It is not currently known whether any truncated REV3L protein is produced by this conditional construct.

Targeted ES cell identification

The 3loxP targeting construct was linearized and electroporated into 129/Ola ES cells. Two ES cell clones were found to be correctly targeted (both 5′ and 3′ arms recombined) by nested PCR using decreasing temperature annealing steps. Southern blotting was performed to confirm this and to ensure that a second non-homologous integration had not occurred. The *neo* selection cassette flanked by loxP sites was removed by transient expression of a Cre recombinase plasmid within the ES cells. Individual colonies were then replica plated with one plate grown in the presence of G418 to identify those cells likely to have deleted the neo cassette. PCR screening was then employed to distinguish between colonies that had deleted the neo cassette only (to create the lox construct) versus those that also deleted the *Rev3L* exons because the outermost loxP sites had been utilized by Cre (creating a null allele). Southern blotting confirmed results and eliminated mixed ES cell colonies.

Mouse strains and breeding

Two ES cell clones with the two essential DNA polymerase exons flanked by loxP sites were injected into C57BL6/J blastocysts. One clone generated pups with a transgenic germline. The *Rev3L* and *Tp53* (20) strains were mixed B6;129. Mouse strains Tg(*MMTV-Cre)1mam* and Tg(*MMTV-Cre)4mam*, originally obtained from the Jackson laboratory on a FVB background, were bred to the $Rev3L^{+/-}$ strain (5). Mice were maintained as outbred by non-sibling matings. All experimental crosses were $Rev3L^{lox/lox}$ to $Rev3L^{+/-}$ *MMTV-Cre*, including those examining the effects of a *Tp53* null background. Details of animal monitoring, necropsy and histology are in Supplementary Information.

Rev3L conditional allele deletion assay

A PCR assay was developed to determine the relative levels of conditional allele deletion in tissues and tumors of $Rev3L^{+/lox}$ *Cre* and $Rev3L^{-/lox}$ *Cre* mice. Genomic DNA was prepared from tissues and tumors using DNeasy columns (Qiagen). Keratinocyte culture and bone marrow lineage separation methods are in Supplementary Information. Triplex PCR used a forward primer common to both the *lox* and *Δlox* alleles and reverse primers unique to each allele. PCR reaction mixtures (25 μl) contained 200 μM each dNTP, 0.6 μM common primer, 0.3 μM of each allele-specific primer, 25–100 ng genomic DNA, and 0.13 μl Qiagen HotStar TaqPol (0.63 units). The annealing temperature (57 °C) was optimized to reflect defined input ratios of *lox* and *Δlox* genomic DNAs obtained from pure ES cell clones. After 30 cycles PCR, the 647 bp (*lox*) and 511 bp (*Δlox*) PCR products were separated on a 1.2% agarose gel.

Clonality analysis of thymic tumors

Genomic DNA from thymic lymphomas was analyzed to survey DJ rearrangements within *Tcrb*, essentially as described (21,22). Details are in Supplementary Information.

Results

Polζ deletion is tolerated in epithelial but not hematopoietic tissues

Because of the embryonic lethality of *Rev3L* deletion, we developed a conditional knock-out mouse to determine the consequence of Polζ deletion for viability of different adult cell types and spontaneous tumorigenesis. Two essential exons in the DNA polymerase domain of the *Rev3L* gene were flanked by loxP sites so that they could be deleted by Cre recombinase (Fig. 1A). No differences in phenotype were apparent among $Rev3L^{+/+}$, $Rev3L^{+/-}$, $Rev3L^{+/lox}$ and $Rev3L^{-/lox}$ mice. To conditionally inactivate *Rev3L*, the lines Tg(*MMTV-Cre)1mam* and Tg(*MMTV-Cre)4mam* were used in crosses (23). In adults, both show highest levels of Cre expression in secretory tissues, the hematopoietic system

(including B and T cells, megakaryocytes, and the erythroid lineage), and epithelia. Some MMTV-Cre expression may occur in embryos after 11.5 days of development (23). The life spans and weights of adult mice deleting the conditional *lox* allele to generate either *Rev3L* heterozygous (*+ /Δlox*) or Rev3L null (*−/Δlox*) cells were not statistically different from non-Cre controls (Fig. S1A).

A PCR assay was developed to quantify deletion in genomic DNA from Cre-expressing tissues (Fig. 1B). Significantly, deletion of *Rev3L* to null status was tolerated in epithelial tissues, including samples from the tail, ear and salivary gland, though probably with some loss of viability (Fig. 1B). *Rev3L* null cells were still present in these tissues in two year old *Rev3L*$^{-/lox}$*Cre* mice. We confirmed that *Rev3L* null keratinocytes are viable and can divide *ex vivo* (Fig. S2A).

The consequence of *Rev3L* deletion was quite different in hematopoietic tissues. In *Rev3L*$^{-/lox}$*Cre* mice, the deleted form of the conditional *Rev3L* allele was not detected or was present in only trace amounts in bone marrow, spleen, thymus and lymph nodes (Fig. 1C). The conditional *Rev3L* allele was consistently disrupted at a level of ~50% in the hematopoeitic tissues of all *Rev3L*$^{+/lox}$*Cre* control mice tested (Fig. 1C). Poor viability of *Rev3L*-deleted lymphoid cells was not a consequence of the introduction of double-strand breaks mediated by V(D)J recombination during the maturation of lymphocytes (Fig. S2B). This demonstrates an essential requirement for pol ζ in maintaining viability in adult hematopoietic lineages.

Formation of Rev3L-null lymphoma

A lack of *Rev3L* could accelerate the formation of oncogenic rearrangements or inactivation of tumor suppressors. Lymphomas were the most common tumor in *Tp53*$^{+/+}$ *Rev3L*$^{-/lox}$*Cre* mice and occurred with frequencies similar to the control genotypes. Most did not contain *Rev3L* null cells (Fig. S3A). Nevertheless, among the hematopoietic masses examined from 8 *Rev3L*$^{-/lox}$*Cre* mice, one T-lymphoblastic lymphoma/leukemia occurred very early (3.5 months) and was dominated by *Rev3L* null cells (Fig. 2A, S3B). Thus complete loss of *Rev3L* was an early event in this tumor and the final change(s) required for uncontrolled growth occurred in a cell lacking *Rev3L* function.

Loss of Rev3L enhances incidence and accelerates the development of lymphoma

Mice were placed in a *Tp53*-null background, where the influence of *Rev3L* disruption on the frequency and latency of spotaneous thymic lymphoma could be measured. Cellular lethality upon *Rev3L* deletion is partially due to p53-imposed controls (12,14,15,24). Deletion of *Tp53* did not globally allow blood cells to proliferate without Rev3L function, as there was near or total absence of cells with complete disruption in the bone marrow, spleen and mesenteric lymph node of *Rev3L*$^{-/lox}$*Tp53*$^{-/-}$*Cre* mice (Figs. S4A and B). However, examination of *Rev3L* deletion in the thymus of two *Rev3L*$^{-/lox}$*Tp53*$^{-/-}$*Cre4mam* mice without obvious lymphoma revealed that absence of *Tp53* did promote the ability of *Rev3L* null thymocytes to divide *in vivo* (Fig. S4A and B). In one case (Fig. S4B), *Rev3L* deleted cells were found only in a single thymic lobe, suggesting that absence of *Tp53* is necessary but not sufficient for bypass of the genomic defect(s) induced by *Rev3L* loss.

Twenty-one of the 22 mice in the *Rev3L*$^{-/lox}$*Tp53*$^{-/-}$*Cre* group succumbed to thymic lymphoma by 142 days (Table 1). The one that did not was euthanized at six weeks due to exencephaly known to occur in *Tp53* null mice (25). The incidence of thymic lymphomas in the *Rev3L*$^{-/lox}$*Tp53*$^{-/-}$*Cre1mam* group was thus 100%, revealing complete penetrance in mice capable of forming *Rev3L* null lymphocytes. This is significantly greater than the 70 – 77% frequency in control genotypes ($p<0.03$, Table 1), a frequency consistent with other

studies of *Tp53*$^{-/-}$ mice (20,26). Eleven tumors from *Rev3L*$^{-/lox}$ *Tp53*$^{-/-}$*Cre1mam* mice were examined histologically and all were precursor T-lymphoblastic leukemia/lymphomas, based on CD3^{+}TdT^{+} immunostaining and morphology. This is the predominant form of lymphoma arising in *Tp53* null mice (27).

Mice from the *Rev3L*$^{-/lox}$*Tp53*$^{-/-}$*Cre* group forming *Rev3L* null lymphomas have significantly decreased survival compared to the three control genotypes retaining at least one functional *Rev3L* allele (Fig. 3; $p<0.0002$, Table 1). Similar differences between the deleting and non-deleting genotypes were found by comparing only the survival of mice with lymphoma (Table 1). Genotyping multiple samples from 14 of the lymphomas from the *Rev3L*$^{-/lox}$*Tp53*$^{-/-}$*Cre1mam* group showed that 10 were predominantly constituted by *Rev3L* null cells (representative results, Fig. 2B). One lymphoma had a mixed population that was mostly *Rev3L* null, but had one thymus sample also containing parental, non-deleted *Rev3L* cells (Fig. 2C). Three lymphomas contained only cells with the intact conditional allele. Thus, loss of *Rev3L* enhances development of thymic lymphoma in *Tp53* null mice both by increasing incidence and by shortening the latency of tumors.

Rev3L null tumors are frequently oligoclonal

To test whether *Rev3L*$^{-}$ cells have an increased probability of neoplastic conversion, we asked whether thymic lymphomas arising from them are multiclonal. T-cell lymphomas from *Tp53* null mice have clonal *Tcrb* rearrangements (28). Clonality in thymic lymphomas was assessed by determining the number, distribution and sizes of D-J rearrangements within the *Tcrb* (*TCR*-β) gene (22) in 10 *Rev3L*$^{+}$ and 10 *Rev3L*$^{-}$ thymic lymphomas. To assess whether cell populations differed across the tumor, multiple genomic DNA samples from non-adjacent sites of each tumor were assayed simultaneously. D1-J1, D2-J2 and D1-J2 joined segments were amplified in separate PCR reactions (Fig. 4A) that paired *Rev3L*$^{-}$ tumors with *Rev3L*$^{+}$ tumors (Fig. 4B, C, D). Non-tumor thymus DNA from a *Tp53* null mouse served as a control to identify the sizes of normal rearrangements, all of which can be found in a normal thymus, including those involving a J2 pseudosegment (Fig. 4C, D, right).

From the ten *Rev3L*$^{+}$ tumors, 48 total independent samples were analyzed; only one instance was found with different clonal proliferations at different locations within the tumor (Fig. 4B). Analysis of 39 samples from different sites of the ten *Rev3L*$^{-}$ tumors revealed that five *Rev3L*$^{-}$ tumors had different clonal populations in separate samples from the same tumor (Fig. 4B, C, D).

D-J recombination can occur on both alleles of the *Tcrb* locus (29). To classify a tumor sample as biclonal based on number of rearrangements, more than two J1 or J2 rearrangements must be present in an individual sample. No *Rev3L*$^{+}$ tumor samples had so many products, whereas three *Rev3L*$^{-}$ tumors had more than two D2-J2 rearrangements in an individual sample. One of these was not among the five having different clonal populations at non-adjacent sites within the same tumor. The analyses together identify a significantly increased number of *Rev3L* null tumors that are at least biclonal (Fig. 4B).

The *Rev3L*$^{-}$ lymphoma group also had five D-J rearrangement products with sizes distinctly different than the standard rearrangements (Fig. 4B, C, D); only one such rearrangement occurred in the *Rev3L*$^{+}$ group (Fig. 4B). The occurrence of aberrantly migrating D-J rearrangements suggests that processing of VDJ intermediates might be altered in *Rev3L* null T-cells. Rev3L may also be involved in the repair of strand breaks occurring during class switch recombination (16).

Loss of Rev3L in mammary tumors

Mammary tumors were also observed in *Rev3L*-deleting females in the $Tp53^{+/+}$ cohort (Table S2). Notably, 5 of the 6 mammary tumors in $Rev3L^{-/lox}Cre$ females were composed largely of *Rev3L* null cells (Fig. S5A). The single mammary tumor (a solid carcinoma) from the $Rev3L^{+/lox}Cre$ genotype had no *Rev3L* deletion (Fig. S5B).

To further examine the effect of *Rev3L* deletion on tumor incidence and latency, we generated cohorts of *Rev3L*-deleting and control genotypes in a *Tp53*-sensitized background. On the $Tp53^{+/-}$ background, significantly more mammary tumors occurred in mice deleting *Rev3L* than those retaining *Rev3L* function (Table S3, p=0.044). Eighteen mammary tumors were found in 11 $Rev3L^{-/lox}Tp53^{+/-}Cre1mam$ mice, with 4 of these in males. The numbers of females with mammary tumors from the $Tp53^{+/-}$ and $Tp53^{+/+}$ cohorts were combined for each *Rev3L* genotype (although tumor numbers were smaller in the $Tp53^{+/+}$ cohort). The number of *Rev3L*-deleting females that developed mammary tumors was significantly greater than the combined $Rev3L^{+/lox}Cre$ control group, which had the next highest incidence (14 vs. 5 females, 41 total females in each group, p=0.035, Fisher's exact test).

Four $Rev3L^{-/lox}Tp53^{+/-}Cre1mam$ mice but only a single control mouse developed multiple mammary tumors. As in the $Tp53^{+/+}$ cohort, most mammary tumors (12/17 analyzed) in *Rev3L*-deleting mice were comprised largely of *Rev3L* null cells; samples from 2 other tumors contained 25–50% deletion. The increased number of mammary tumors populated by *Rev3L* null cells in *Rev3L*-deleting females strongly suggests that loss of REV3L promotes mammary tumorigenesis.

Mammary tumors in *Rev3L* deleting mice had a shorter latency than in control genotypes in the $Tp53^{+/-}$ background (median 295 vs. 448 days, p=0.02, log-rank). Loss of *Rev3L* thus reduced tumor latency in addition to increasing tumor incidence. Survival of $Rev3L^{-/lox}Tp53^{+/-}Cre1mam$ mice as a group was not significantly different from controls (Fig. S1B). Similar numbers of lymphomas developed in all 4 $Tp53^{+/-}$ genotypes (22–34%), comparable to previously reported incidences (26).

Increased formation of mammary preneoplastic lesions in mice deleting Rev3L

Mice on the $Tp53^{+/-}$ background, regardless of *Rev3L* status, developed a similar spectrum of mammary tumors, including carcinosarcomas, adenosquamous carcinomas, solid carcinomas, and sarcomas (Table S3). Half of the mammary tumors in $Rev3L^{-/lox}Tp53^{+/-}Cre1mam$ mice were solid carcinomas (compared to 1 of 7 mammary tumors in the 3 control genotypes); these tumors generally had the highest proportion of *Rev3L* null cells among the mammary tumors from *Rev3L* deleting mice.

Glandular tissue adjacent to the mammary tumors from *Rev3L*-deleting mice often had mammary intraepithelial neoplasia (MIN) and marked atypical hyperplasia (Fig. 5A). In these mice, MIN was observed adjacent to at least 7 tumors of different types. The 6 control genotype tumors with adjacent glandular tissue had no MIN and only the single control mouse with multiple mammary tumors had mild to moderate hyperplasia (Fig. 5A). In the $Tp53^{+/+}$ cohort, MIN was found adjacent only to one *Rev3L* null adenosquamous carcinoma ($Rev3L^{-/lox}Cre1mam$ female). Atypical hyperplasia and MIN would clearly predispose *Rev3L*-deleting mice to the development of multiple mammary tumors. Moreover, some mammary tumors in *Rev3L*-deleting mice may have been formed by the confluence of multiple neoplastic foci, as 3 of the 4 $Rev3L^{-/lox}$ $Tp53^{+/-}Cre1mam$ mice with multiple tumors (Fig. 5B) also had multiple MIN adjacent to the tumors. Thus loss of *Rev3L* in a $Tp53^{+/-}$ background causes an increase in the number of both preneoplastic and neoplastic lesions.

Discussion

The conditional *Rev3L* knockout mouse permitted a test of the viability of tissues lacking polζ function. Epithelial cells from the tail, ear and salivary gland tolerated deletion of *Rev3L*, and null keratinocytes underwent at least 3–4 cell divisions in culture. In contrast, *Rev3L* null hematopoietic cells did not proliferate, consistent with the severe early hematopoiesis defect previously found in *Rev3L* null embryos (6). Hematopoietic tissues may be especially dependent on *Rev3L* for their survival, due to high basal levels of p53 (30), a higher load of reactive oxygen species (31), or a greater dependence on polζ-dependent DNA replication and repair. Removal of p53 did not enable viability of all blood cells, but promoted survival of *Rev3L* null thymocytes. This suggests that a *Tp53*-independent response is present, but that it can be bypassed or inactivated. Both *Tp53*-dependent and *Tp53*-independent pathways exist to induce cell cycle arrest, senescence, stasis, and apoptosis (32–34).

Remarkably, REV3L-negative tumors grew aggressively, although *Rev3L* is essential for embryonic development and viability of MEFs and blood cells. In $Tp53^{-/-}$ mice, deleting the conditional *Rev3L* allele reduced lifespan and increased thymic lymphoma incidence to 100%. Most lymphomas consisted primarily of *Rev3L* null cells, indicating that REV3L loss accelerates lymphoma development. Loss of *Rev3L* reduced mammary tumor latency in $Tp53^{+/-}$ mice, increased tumor multiplicity, and promoted the occurrence of mammary tumors in males. For both T-cell and mammary tumorigenesis, *Rev3L* loss apparently increases the number of cells at risk for neoplastic conversion. More $Rev3L^{-}$ tumors were oligoclonal, and mammary intraepithelial neoplasia was a distinct feature of adjacent glandular tissue in *Rev3L* null mammary tumors.

Genomic instability due to *Rev3L* loss has been previously documented in chicken, mouse and human cells, with increased formation of DNA breaks and translocations (12–16). This provides a likely explanation for enhanced tumorigenesis in *Tp53*-deficient mice deleting *Rev3L*. Increased genetic instability is known to accelerate tumorigenesis, although the generation of DNA breaks may require that *Tp53*-mediated controls be removed or attenuated.

The precise cellular function of REV3L in limiting genome instability remains to be determined. One possibility is that DNA polζ has a role in bypass of some endogenously formed adducts that would otherwise block DNA replication and lead to replication fork collapse and breakage. Alternatively, REV3L might function in the repair synthesis that occurs during DNA double strand break repair. The mid-gestation lethality of *Rev3L* deletion and severe proliferation defect of null embryonic fibroblasts somewhat resembles knockouts of genes involved in homologous recombination (HR), such as *Rad51*, *Rad51d*, *Xrcc2*, and *Brca* (3). *Rev3L* and HR null knockouts also share features of chromosome instability and *Tp53* absence enables cells lacking these genome maintenance genes to overcome blocked cell division (35). Deletion of *Brca1* or *Brca2* in $Tp53^{+/+}$ mice by *MMTV-Cre* results in mammary tumors with average latencies similar to that caused by *Rev3L* deletion (36,37). *Tp53* heterozygosity cooperates with the loss of all 3 of these genes to reduce the average latency of mammary tumor formation to between 8.5 months (*Brca1*) and 10.5 months (*Rev3L*). Deletion of *Rev3L* by MMTV-Cre in $Tp53^{+/-}$ mice induced multiple mammary tumors, as it did for the (more penetrant) *Brca1* (36). Deletion of the *Brca* alleles in $Tp53^{-/-}$ mice using the T-cell lineage specific *Lck-Cre* (38,39) has effects on incidence and latency of thymic lymphoma similar to the acceleration of T-cell lymphoma caused by *Rev3L* deletion.

The present results with *Rev3L* identify a specialized DNA polymerase that uniquely inhibits spontaneous tumor development in mammals The finding that epithelial cells can survive in the absence of pol zeta opens the way to further investigations of the specific roles of this enzyme in epithelial neoplasia, including UV-induced skin cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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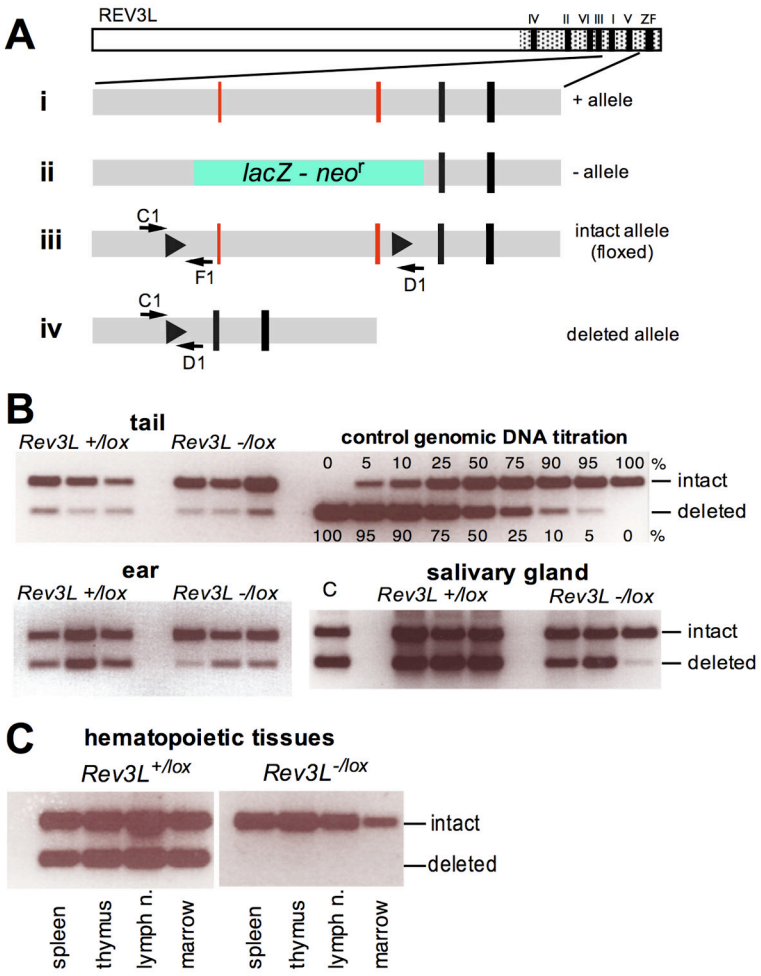


Figure 1. Conditional inactivation of Rev3L

A. *Rev3L* cDNA encodes a 3122 amino acid protein, with conserved DNA polymerase motifs (I - VI) and a zinc finger (ZF) motif near the C-terminus. **(i).** A segment of *Rev3L* genomic DNA (+ allele) with exons (vertical bars). The two exons in red contain part of motif I and all of V. **(ii)**. These two conserved exons encoding amino acids 2776–2860 were replaced by a *lacZ-neo*^{r} cassette to disrupt the gene (− allele) as described (5). **(iii).** For the functional "floxed allele" two LoxP sites (black triangles) are incorporated into introns flanking these exons. **(iv).** Cre recombinase deletes these exons and intervening sequence to give the *Δlox* allele. The *lox* allele is detected with PCR primers C1 and F1 (arrows) and the *Δlox* allele with PCR primers C1 and D1.

B. Epithelial cells tolerate deletion of *Rev3L*. Genomic DNA samples (35 ng) from tail, ear, and salivary gland of *Rev3L* conditional knockout mice contain *Rev3L* null cells (*−/Δlox*) as well as cells with an intact allele (*lox*). For each genotype, DNA was analyzed from tissues of three different mice possessing the *MMTV-Cre4mam* transgene at 7 months (tail), 13–16 months (ear) and 16 months (salivary gland). The right side of the top panel shows a control PCR titration with the indicated ratios of *lox* and *Δlox* genomic DNA (35 ng total per lane). Lanes with a 1:1 mixture of *lox* and *Δlox* DNA are indicated by C.

C. Adult hematopoietic tissues do not normally tolerate *Rev3L* deletion. Samples (35 ng genomic DNA) from spleen, thymus, lymph node, and bone marrow of *Rev3L* conditional knockout mice do not contain detectable *Rev3L* null cells (*−/Δlox*). If one wild-type allele of *Rev3L* is present, deletion produces *Rev3L*$^{+/\Delta lox}$ cells. The tissues shown at bottom are from

single 14 month old mice of each genotype, both possessing the *MMTV-Cre4mam* transgene.

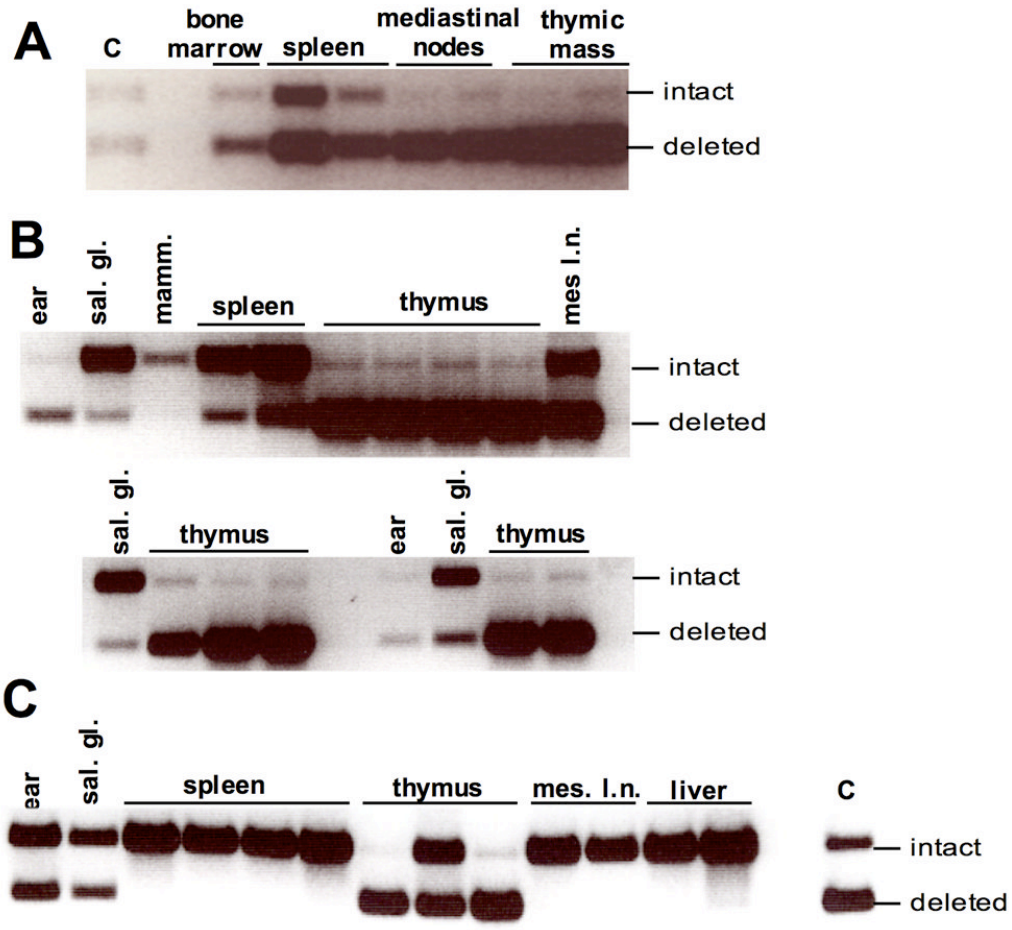


Figure 2. Rev3L null T-cells can form tumors in mice

A. Samples of tumor tissue, spleen and bone marrow from a 3.5 month-old *Rev3L*$^{-/lox}$ *Tp53*$^{+/+}$ *Cre1mam* mouse show major populations of *Rev3L* null cells, identified as precursor T-lymphoblastic leukemia/ lymphoma (Fig. S3B). Control sample (C) is a 1:1 mixture of *lox* and *Δlox* DNA.

B. Thymic lymphomas in *Rev3L*$^{-/lox}$ *Tp53*$^{-/-}$ *Cre1mam* mice are usually populated by *Rev3L* null cells. Tissues from three different mice are displayed. All animals were dead or moribund at three months of age. Multiple samples from thymus show that *Rev3L* null cells dominate the thymic tumor in all three mice. Abbreviations: mesenteric lymph node “mes l.n.”, salivary gland, “sal. gl.”, mammary gland “mamm.”.

C. *Rev3L* null cells dominated two lobes of this thymic lymphoma from a *Rev3L*$^{-/lox}$ *Tp53*$^{-/-}$ *Cre1mam* mouse, while another lobe showed a mixed population. Control samples (C) as in part A, other abbreviations as in part B.

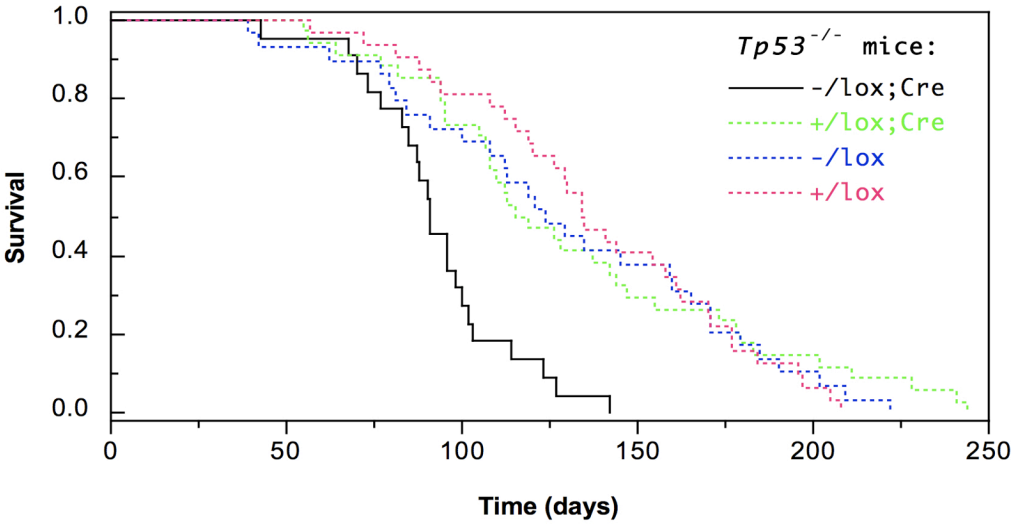
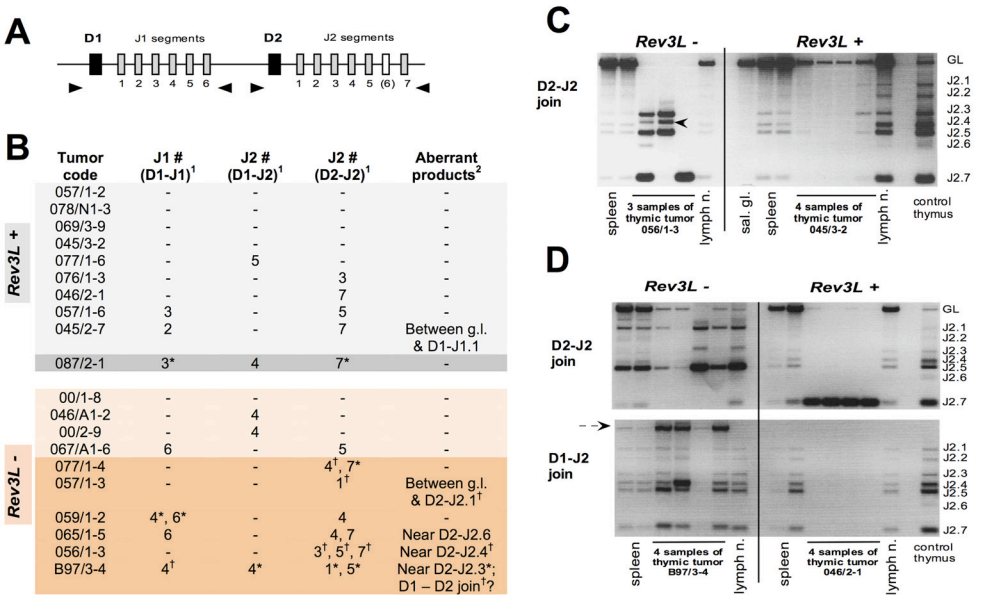


Figure 3. Accelerated tumorigenesis in Tp53-null mice deleting Rev3L

Kaplan-Meier plot of mouse survival with time. Sample sizes, mean and median values in Table 1. The difference in survival of *Rev3L*$^{-/lox}$ *Tp53*$^{-/-}$ *Cre1mam* to the other 3 genotypes was highly significant by two-sided log-rank analysis: *Rev3L*$^{+/lox}$ *Tp53*$^{-/-}$ *Cre* (P<0.0001), *Rev3L*$^{-/lox}$ *Tp53*$^{-/-}$ (p=0.0002) and *Rev3L*$^{+/lox}$ *Tp53*$^{-/-}$ (p<0.0001). *Rev3L*$^{-/lox}$ *Tp53*$^{-/-}$ *Cre1mam* solid black; *Rev3L*$^{+/lox}$ *Tp53*$^{-/-}$ *Cre1mam* dotted green; *Rev3L*$^{-/lox}$*Tp53*$^{-/-}$ dotted blue; *Rev3L*$^{+/lox}$ *Tp53*$^{-/-}$ dotted magenta.



	Tumor code	J1 # (D1-J1)[1]	J2 # (D1-J2)[1]	J2 # (D2-J2)[1]	Aberrant products[2]
Rev3L +	057/1-2	-	-	-	-
	078/N1-3	-	-	-	-
	069/3-9	-	-	-	-
	045/3-2	-	-	-	-
	077/1-6	-	5	-	-
	076/1-3	-	-	3	-
	046/2-1	-	-	7	-
	057/1-6	3	-	5	-
	045/2-7	2	-	7	Between g.l. & D1-J1.1
	087/2-1	3*	4	7*	-
Rev3L -	00/1-8	-	-	-	-
	046/A1-2	-	4	-	-
	00/2-9	-	4	-	-
	067/A1-6	6	-	5	-
	077/1-4	-	-	4†, 7*	-
	057/1-3	-	-	1†	Between g.l. & D2-J2.1†
	059/1-2	4*, 6*	-	4	-
	065/1-5	6	-	4, 7	Near D2-J2.6
	056/1-3	-	-	3†, 5†, 7†	Near D2-J2.4†
	B97/3-4	4†	4*	1*, 5*	Near D2-J2.3*; D1 – D2 join†?

Figure 4. Increased oligoclonality within Rev3L null tumors

A. Schematic of D-J segments within the *Tcrb* gene. Locations of primers are indicated by arrowheads. J2.6 is a pseudosegment in the mouse, not yielding productive rearrangements. Segment D2 rearrangement is possible with any one of 6 different J2 gene coding segments. The D1 segment generally joins with 6 J1 coding segments and to a lesser extent, the 6 J2 segments.

B. D–J rearrangements of the *Tcrb* gene in murine $Tp53^{-/-}$ thymic lymphomas. "g.l" is germline. Darker shaded rows indicate tumors that were at least biclonal. The increased number of biclonal tumors in the $Rev3L^{-}$ group relative to the $Rev3L^{+}$ group (6 versus 1) is statistically significant (Fisher's exact test, one-tailed, p=0.03).

[1] "-", no rearrangement of this type was detected. [2] verified by independent PCR. * product found in only one sample from a tumor, and verified by independent PCR. † product found in two or more, but not all samples from a tumor

C. Non-adjacent samples of lymphomas from $Rev3L^{-}$ and $Rev3L^{+}$ samples were surveyed for D2-J2 rearrangements. In this example, two $Rev3L^{-}$ lymphoma samples show distinct populations of *Tcrb* rearrangements and one sample contains both populations. The arrow shows an aberrant product not corresponding to the set of normal outcomes of recombination in 2 samples. The full distribution of rearrangements is found in the non-tumor thymus sample at the right.

D. Non-adjacent samples of a $Rev3L^{-}$ and a $Rev3L^{+}$ lymphoma were surveyed for D2-J2 and D1-J2 rearrangements. More than one clone of rearranged cells is present within the $Rev3L^{-}$ tumor. The arrow shows an aberrant event, as D1-J2 PCR will not amplify a product of germline size. A product with this size would arise if a rare D1 to D2 joining event had occurred. The $Rev3L^{+}$ lymphoma is monoclonal, harboring a D2-J2.7 rearrangement in the four tumor samples.

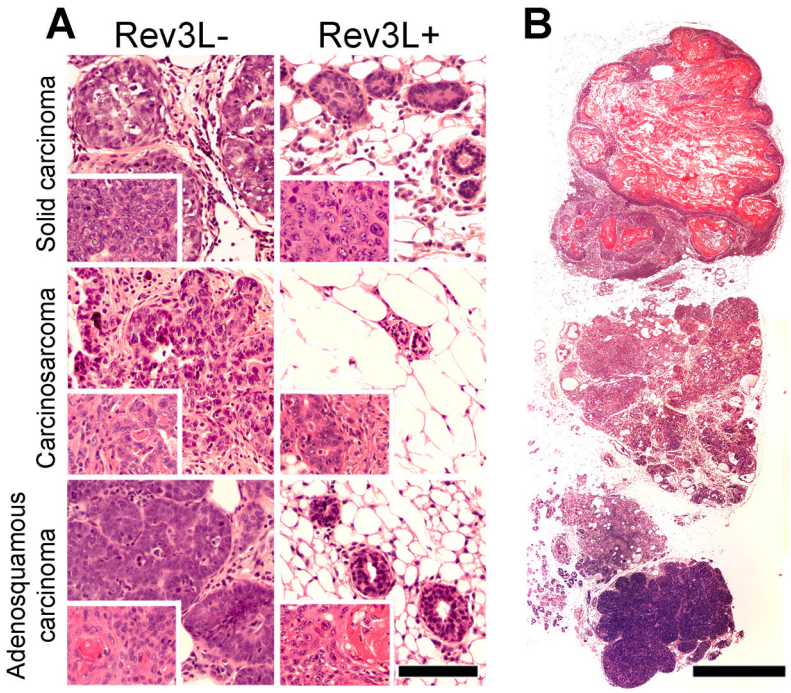


Figure 5. Neoplasia and preneoplasia in Tp53$^{+/-}$ mice deleting Rev3L

A. Multifocal atypical hyperplasia and mammary intraepithelial neoplasia (MIN), characterized by increased cellularity, cellular atypia, and filling of alveoli with solid, papillary, or multilayered proliferations of epithelium, were seen only in mice deleting *Rev3L*, although mammary tumor morphology was similar for both Rev3L^{-} and Rev 3L^{+} tumors in Tp53$^{+/-}$ mice. Atypical hyperplasia and MIN was seen in 5 of the 9 *Rev3L*$^{-/lox}$ *Tp53*$^{+/-}$*Cre1mam* mice for which glandular tissue adjacent to tumors could be examined. All tissues shown at the same magnification. (Scale bar: 100 μm).

B. Multiple adjacent preneoplastic and hyperplastic mammary nodules were observed in Tp53$^{+/-}$ mice deleting *Rev3L*, as shown in this panel, but not in mice that retained *Rev3L*. (Scale bar: 1 mm).

Table 1

Thymic lymphoma incidence and survival of *Tp53*$^{-/-}$ mice deleting *Rev3L with Cre1mam*

Genotype(all *Tp53*$^{-/-}$)	n	n for lymphoma incidence[1]	# with lymphoma in thymus	% with lymphoma in thymus	*P* value[2]	median survival[3] (days)	oldest mouse (days)	*P* value[4]
Rev3L$^{+/lox}$	32	30	21	70%	0.0068	134 (120–161)	208	<0.0001
Rev3L$^{-/lox}$	29	26	20	77%	0.0265	124 (108–160)	222	0.0002
Rev3L$^{+/lox}$ ***Cre***	34	31	22	71%	0.0073	117 (108–144)	244	<0.0001
Rev3L$^{-/lox}$ ***Cre***	22	21	21	100%	–	91 (85–100)[5]	142	–

[1] Mice excluded from the sample population to determine thymic lymphoma incidence were those where definitive necropsy was not possible after death, and those surviving < 2 months as no mice of any genotype in our colony developed thymic lymphoma within that period.

[2] Fisher's Exact Test (two-tailed) compared to *Rev3L*$^{-/lox}$ *Tp53*$^{-/-}$ *Cre1mam*

[3] median survival time, with 95% confidence interval below.

[4] Log rank *P* (two-sided) compared to *Rev3L*$^{-/lox}$ *Tp53*$^{-/-}$*Cre1mam.*

[5] Comparing only mice with thymic lymphoma, the median survival of the *Rev3L*$^{-/lox}$ *Tp53*$^{-/-}$ *Cre1mam* genotype (91 days, n = 21) is also decreased by more than 3 weeks relative to the median survival of mice with lymphoma from the other groups: *Rev3L*$^{-/lox}$ *Tp53*$^{-/-}$ (140 days, n = 20), *Rev3L*$^{+/lox}$ *Tp53*$^{-/-}$ (135 days, n = 21) and *Rev3L*$^{+/lox}$ *Tp53*$^{-/-}$ *Cre* (122 days, n = 22).