

DNA polymerase zeta is required for proliferation of normal mammalian cells

Sabine S. Lange¹, John P. Wittschieben² and Richard D. Wood^{1,*}

¹Department of Molecular Carcinogenesis, The University of Texas MD Anderson Cancer Center Science Park, and the Graduate School of Biomedical Sciences at Houston, Smithville Texas, P.O. Box 389, Smithville, TX 78957, USA and ²Novo Nordisk Foundation, Tuborg Havnevej 19, 2900 Hellerup, Denmark

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ABSTRACT

Unique among translesion synthesis (TLS) DNA polymerases, pol ζ is essential during embryogenesis. To determine whether pol ζ is necessary for proliferation of normal cells, primary mouse fibroblasts were established in which *Rev3L* could be conditionally inactivated by Cre recombinase. Cells were grown in 2% O₂ to prevent oxidative stress-induced senescence. Cells rapidly became senescent or apoptotic and ceased growth within 3–4 population doublings. Within one population doubling following *Rev3L* deletion, DNA double-strand breaks and chromatid aberrations were found in 30–50% of cells. These breaks were replication dependent, and found in G1 and G2 phase cells. Double-strand breaks were reduced when cells were treated with the reactive oxygen species scavenger N-acetylcysteine, but this did not rescue the cell proliferation defect, indicating that several classes of endogenously formed DNA lesions require *Rev3L* for tolerance or repair. T-antigen immortalization of cells allowed cell growth. In summary, even in the absence of external challenges to DNA, pol ζ is essential for preventing replication-dependent DNA breaks in every division of normal mammalian cells. Loss of pol ζ in slowly proliferating mouse cells *in vivo* may allow accumulation of chromosomal aberrations that could lead to tumorigenesis. Pol ζ is unique amongst TLS polymerases for its essential role in cell proliferation.

INTRODUCTION

Genomic DNA lesions are formed continually by environmental agents and toxic intermediates of metabolism. Some of this damage escapes removal by DNA repair

and is encountered by the DNA replication apparatus, which can block replication fork progression. Mechanisms are available that allow cells to temporarily tolerate such DNA damage (1). One major tolerance mechanism uses specialized DNA polymerases to incorporate a nucleotide opposite a damaged template base in a process called translesion synthesis (TLS). TLS permits continued replication, but may cause mutations. At least 7 of the 15 DNA polymerases in mammalian cells have a capacity for TLS (1).

It is important to understand the cellular function of each DNA polymerase. DNA polymerase ζ (pol ζ) is unique among TLS polymerases in mammalian cells, because inactivation of the gene encoding its catalytic subunit (*Rev3L*) leads to embryonic lethality in the mouse. This was not anticipated from extensive studies of budding yeast, as *Saccharomyces cerevisiae* containing a deletion of the homologous *REV3* gene are viable.

The inviability of *Rev3L*-defective mice suggests that pol ζ performs one or more irreplaceable functions in proliferation of at least some cell types. Mouse models for conditional deletion of pol ζ show that cells from the hematopoietic lineage normally do not proliferate, while some tissues of epithelial origin remain viable in mosaic form (2,3).

Experiments to reduce *Rev3L* function in normal cells with antisense RNA or siRNA have had inconsistent and variable outcomes. Antisense inhibition of *Rev3L* expression in human fibroblasts resulted in viable cells that were less susceptible to induced point mutations (4). A recent study reported that administration of siRNA impaired the growth of tumor cell lines, but not normal cell lines (5). However, only modest suppression of *Rev3L* expression was achieved in the latter experiments, leaving open the possibility that mammalian cells can survive with low levels of *Rev3L* and that complete ablation is incompatible with growth.

On the other hand, several viable *Rev3L*-defective cell lines have been derived from cells compromised for p53

*To whom correspondence should be addressed. Tel: +1 512 237 9431; Fax: +1 512 237 6532; Email: rwood@mdanderson.org

function (6–9). These cells show signs of ongoing genomic stress and they exhibit increased chromosomal abnormalities. Thus, it remains possible that the severe loss of viability during mouse development might be ascribed to a specialized function for REV3L in embryogenesis, rather than a role in normal cell proliferation.

A definitive answer to whether pol ζ is necessary for growth of normal mammalian cells requires a tightly controlled system for efficient elimination of DNA polymerase function, together with isogenic controls. We report here on experiments where such an approach was developed and used to inactivate *Rev3L* function from primary cell lines by defined genetic deletion.

MATERIALS AND METHODS

Cell culture

The primary mouse embryonic fibroblasts (MEFs) were cultured in medium containing high glucose, glutamax-DMEM (Invitrogen), 15% Hyclone FBS (Thermoscientific), non-essential amino acids, Na pyruvate, MEM vitamin solution, penicillin/streptomycin (Invitrogen) and if indicated, 1 mM N-acetylcysteine (Sigma). The SV40 TAG-immortalized MEFs were cultured in medium containing high glucose, glutamax-DMEM (Invitrogen), 10% FBS (Atlanta Biologics) and penicillin/streptomycin. All culture, unless otherwise noted, was conducted in air-tight containers

(Supplementary Figure S1A), based on Wright & Shay (10). These chambers were filled with a gas mixture containing 93% N₂, 5% CO₂ and 2% O₂ (Praxair) and incubated at 37°C. The low oxygen environment was monitored using an oxygen analyzer and monitor (Teledyne Analytical Instruments). Primary MEFs were derived from e13.5 embryos with genotypes *Rev3L*^{-/lox}, *Rev3L*^{+/lox}, mT/mG^{+/-} *Rev3L*^{-/lox} and mT/mG^{+/-} *Rev3L*^{+/lox}, as per Pines & Backendorf (11). The mT/mG Cre-reporter transgene is described in Figure 1 (12). At least two independently derived primary MEF cell lines were used for each of the following experiments.

Deletion of a floxed copy of *Rev3L* used adenovirus Cre (University of Iowa Gene Transfer Vector Core) and the adfection protocol provided. Viral particles and 25 μ M CaCl₂ were added to serum-free DMEM, incubated for 20 min at room temperature and then with cells for 1 h. Mock-treated cells were adfected without virus. Cell number was monitored at every passage (Countess, Invitrogen), and green fluorescent protein (GFP)-positive cells were observed and counted using a Nikon TS-100 fluorescent microscope equipped with a DS-L2 camera.

Assessment of genomic *Rev3L* loxP deletion by PCR analysis was completed using the following primers: Common forward: 5'-ATA AGA GCC TGC CTG ATG AGC CAG-3' (0.8 μ M), 2lox reverse: 5'-AGG AGG AGG GCA CAC ACA AAA AGT TAG G-3' (0.4 μ M), and 1lox reverse: 5'-GAA TTC CCA CAA TTC ACG CTT

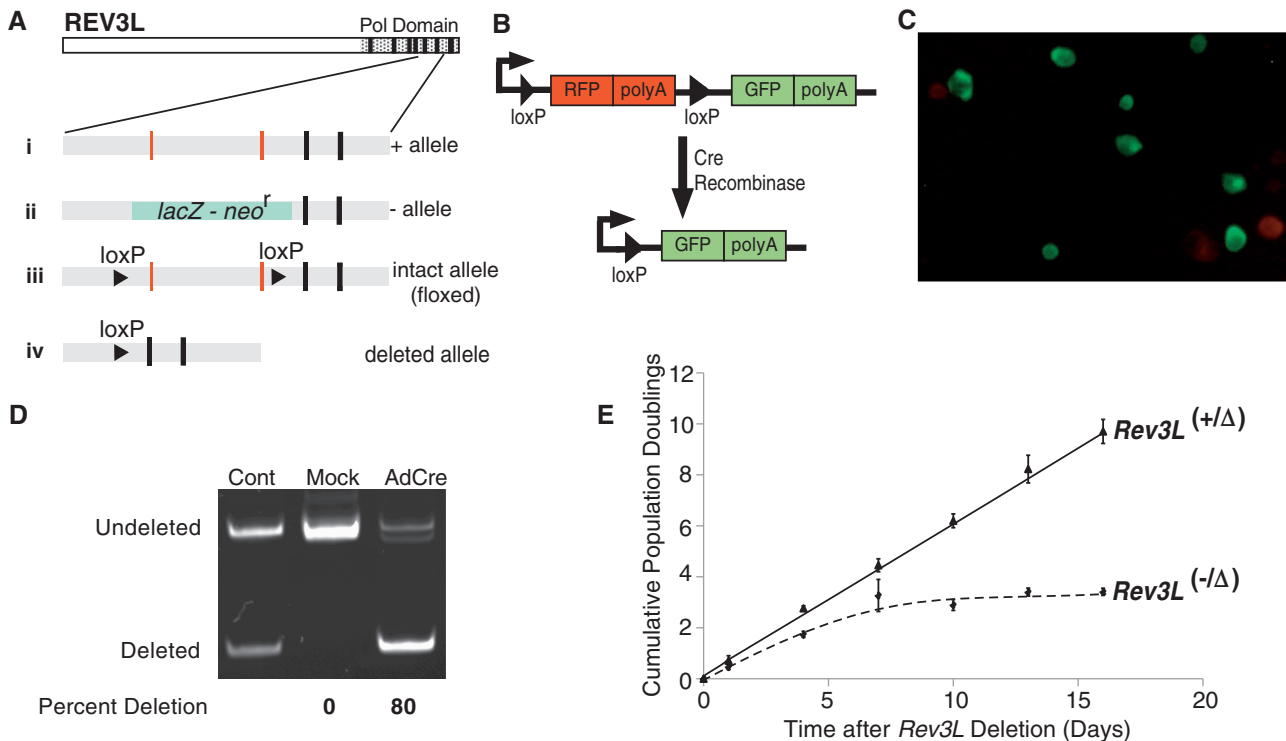


Figure 1. Disruption of *Rev3L* function prevents cell proliferation. (A) Schematic of (i) wild-type, (ii) knockout, (iii) floxed intact and (iv) floxed deleted alleles of the murine *Rev3L* gene (2); Vertical bars represent exons (red bars contain part of DNA polymerase motif I and all of motif V), triangles are loxP sites. (B) Action of an mT/mG Cre reporter gene present in the MEFs. The transgene constitutively expresses membrane-tagged tomato-RFP (mT). With Cre, the RFP is removed, and membrane-tagged GFP is expressed (mG) (12). (C) mT/mG primary MEFs expressing RFP (no Cre activity) or GFP (Cre activity) after introduction of AdCre at an MOI of 25. (D) PCR of undeleted and deleted (by AdCre at MOI 25) floxed *Rev3L* alleles, Cont is control PCR. (E) Growth of primary *Rev3L*^{-/ Δ} (dashed line) and *Rev3L*^{+/ Δ} (solid line) MEFs after treatment with MOI 25 AdCre in 2% O₂ (n = 4). Data points indicate average \pm SEM.

CTC C-3' (0.7 μ M). At an annealing temperature of 62°C, the 2loxP (undeleted allele) produces a 423 bp product, and the 1loxP (deleted allele) produces a 213 bp product.

Senescence assays

For senescence-associated β -galactosidase activity (SA- β -gal) assays, primary MEFs were plated in 8-well chamber slides at 1 or 7 days post-AdCre. At 3 or 9 days, respectively, the cells were fixed, and incubated overnight with X-gal, as per manufacturer's instructions (Senescence detection kit, Abcam). The cells were washed, permeabilized for 5 min with 0.5% Triton X-100 in PBS, and mounted with DAPI. At least four independent experiments were conducted with each cell line at each time point, with total cell number assessed by DAPI fluorescence (at least 650 cells were counted per genotype and treatment group), and β -gal-positive cells assessed under bright field illumination. For IL-6 secretion, conditioned media (>48 h) was removed from primary MEFs at 9 days post-AdCre and IL-6 measured using a Quantikine ELISA Kit (R&D Systems) and a Biotek Synergy 2 plate reader (Winooski, VT), using Gen5 software. The signal was normalized to cell number.

Apoptosis assays

Primary MEFs (not carrying the mT/mG transgene) were plated in 8-well chamber slides at 1 or 7 days post-AdCre, and at 3 or 9 days post-AdCre, respectively, the cells were fixed with 1% paraformaldehyde. They were incubated with TUNEL substrate (*in situ* cell death detection kit, fluorescein, Roche) for 1 h at 37°C, and then were mounted with DAPI. At least four independent experiments were conducted with each cell line at each time point, with total cell number assessed by blue DAPI fluorescence (at least 1600 cells were counted per genotype and treatment group), and TUNEL positive cells counted by green fluorescence.

Chromosome analysis

At 3 days post-AdCre, primary MEFs (containing the mT/mG transgene) were treated with 0.03 μ g/ml demecolcine solution (Sigma) for 4 h. The cells were then trypsinized and fluorescence-associated cell sorting (FACS)-sorted, and GFP-only and RFP-only cells were then exposed to 0.075 M KCl for 15 min at 37°C, and were fixed in 3:1 MeOH:glacial acetic acid. The cells were spread on glass slides, Giemsa stained and metaphases were analyzed using a BX41 Olympus microscope, with 60X or 100X oil objectives. Photographs were taken with the 60X oil objective on a Spot Idea 5 color digital camera. Four independent experiments were conducted with each cell line, counting 100 metaphases per experiment.

Immunofluorescence

Primary MEFs were plated in 8-well chamber slides at 1 day or 7 days post-AdCre. At 3 days or 9 days, respectively, the cells were fixed with MeOH and permeabilized with Triton X-100. MeOH fixation destroys the mT/mG-induced fluorescence, allowing visualization of

the immunofluorescence. Cells incubated with BrdU were also treated with 2 M HCl to denature their DNA. The cells were incubated overnight in blocking buffer (0.1 M lysine, 1% BSA in PBS) with primary antibodies against 53BP1 (rabbit polyclonal, 1:400, Bethyl Labs), γ -H2AX (mouse monoclonal, 1:400, JBW301, Millipore), or BrdU (mouse monoclonal, 1:500, B44, BD Biosciences). The preparation was incubated with AlexaFluor-488 goat-anti-mouse or AlexaFluor-594 goat-anti-rabbit secondary antibodies (1:400, Invitrogen) and mounted with DAPI. At least four independent experiments were conducted with each cell line and time point, assessing cell number and 53BP1 foci-positive cells by red fluorescence (>550 cells counted per genotype and treatment group), and γ -H2AX or BrdU positive cells by green fluorescence. The brightness of the entire microscope field was increased to better display the fluorescence for publication, using Adobe Photoshop C3.

Flow cytometry

For cell cycle analysis, primary MEFs (containing the mT/mG transgene) were harvested 3 days post-AdCre infection. The cells were fixed in 2% paraformaldehyde for 1 h at 4°C, and incubated overnight at -20°C in 70% EtOH. The cells were resuspended in permeabilization solution (1% BSA, 0.1% Triton X-100 in PBS-A), and FxCycle Far Red DNA stain (200 nM; Invitrogen) and RNase A (0.1 mg/ml) were added. The cells were analyzed by FACS on an LSRFortessa (BD Biosciences) for DNA content (Far Red stain, 670/30 filter set) and GFP expression (indicative of Cre activity, and therefore of *Rev3L* deletion; 530/30 filter set). Cell cycle analysis was done on FlowJo software (Tree Star). Analysis of cell cycle and γ -H2AX staining was done using primary MEFs (lacking the mT/mG transgene), as per Huang & Darzynkiewicz (13), using 1 μ g of γ -H2AX antibody (JBW301, Millipore) per 10⁶ cells, and 0.5 μ g of AlexaFluor-488 goat-anti-mouse secondary antibody (Invitrogen) per 10⁶ cells. Cells were labeled with Far Red for DNA content.

Statistical analysis

Cell cycle and statistical analysis for FACS experiments were done using FloJo software. To assess the statistical difference between distributions of γ -H2AX fluorescence, population binning was used, which provides a χ^2 statistic (14). Analysis of apoptosis, senescence, chromosomal aberrations and immunofluorescence used a two-sample T-test for independent samples with equal variances (two-tailed, $P < 0.05$).

RESULTS

Rev3L is necessary for normal cell proliferation

To develop a system that would allow a direct test of pol ζ function in primary cells, we used mice harboring one inactivated allele of *Rev3L* and the other allele with essential exons flanked by loxP sites (2). These 'floxed' *Rev3L*^{-/lox} mice were crossed with the mT/mG mouse strain, which carry an inducible GFP transgene to track

Cre expression (Figure 1B) (12). Primary fibroblasts were derived from embryos of the progeny, so that introduction of Cre into these cells would inactivate pol ζ by site-specific recombination (Figure 1A, B). Control MEFs were derived from $Rev3L^{+/lox}$ embryos carrying the mT/mG reporter (2,12). Upon introduction of an adenovirus expressing Cre (AdCre), $\sim 70\%$ of the cells deleted the floxed copy of $Rev3L$, as demonstrated by PCR (Figure 1D) and by GFP transgene expression (Figure 1C), thus generating $Rev3L^{+/\Delta}$ and $Rev3L^{-/\Delta}$ cells. A low multiplicity of infection (MOI) with AdCre was used to limit secondary Cre-induced breaks in genomic DNA (15). The cells were incubated in a 2% O₂ atmosphere (Supplementary Figure S1A) to minimize reactive oxygen species (ROS)-induced stress and senescence (16).

Cell counts and GFP expression were monitored following inactivation of the floxed $Rev3L$ allele. The $Rev3L^{+/\Delta}$ cells grew exponentially in culture with a doubling time of ~ 43 h (Figure 1E). In contrast, deletion of pol ζ impaired growth of $Rev3L^{-/\Delta}$ cultures within a few days, and the cultures completely ceased growth before 3 population doublings (Figure 1E).

Rev3L deletion increases senescence and apoptosis

The adverse effects of $Rev3L$ deletion required time for cells to attempt proliferation. At day 3, when all cells had undergone approximately one population doubling, $Rev3L^{-/\Delta}$ and $Rev3L^{+/\Delta}$ cells had similar cell cycle stage distributions (data not shown), and low levels of senescence or apoptosis (Figure 2). However, 9 days after

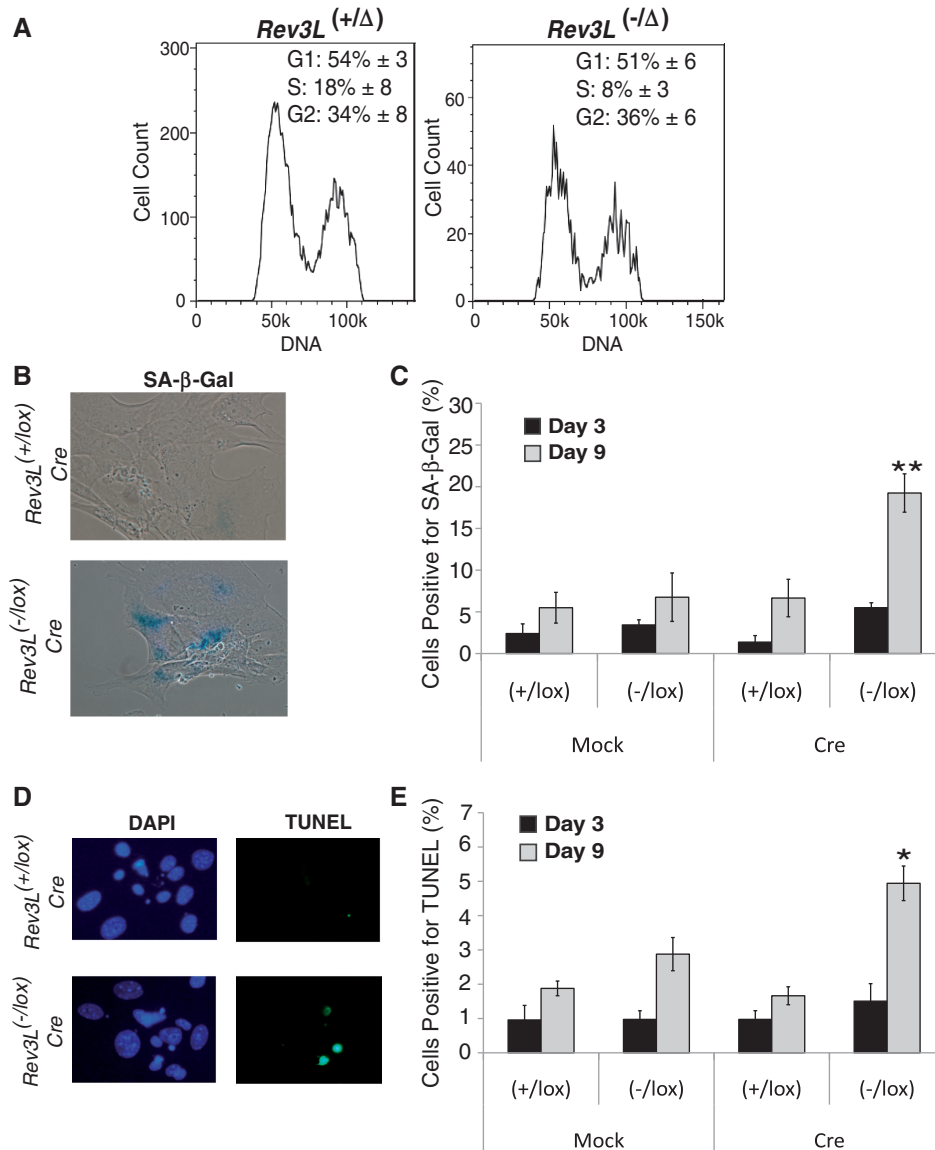


Figure 2. Rapid senescence and apoptosis after deletion of $Rev3L$. (A) Cell cycle analysis of $Rev3L^{-/\Delta}$ and $Rev3L^{+/\Delta}$ primary MEFs at day 9 post- $Rev3L$ deletion, with proportion of cells in each cell cycle phase. (B) Staining of 9 day $Rev3L^{-/lox}$ Cre and $Rev3L^{+/\lox}$ Cre cells for SA- β -gal. (C) Quantification of SA- β -gal staining in $Rev3L^{-/lox}$ Cre and $Rev3L^{+/\lox}$ Cre MEFs. (D) DAPI and TUNEL-stained $Rev3L^{-/lox}$ Cre and $Rev3L^{+/\lox}$ Cre MEFs at 9 days. (E) Quantification of TUNEL staining of $Rev3L^{-/lox}$ Cre and $Rev3L^{+/\lox}$ MEFs; * $P < 0.05$; ** $P < 0.01$ ($n = 4$). Data points indicate average \pm SEM.

Rev3L deletion, the proportion of *Rev3L*^{-Δ} cells in S phase was greatly diminished as determined by flow cytometry (Figure 2A) and incorporation of BrdU (data not shown). At this time there was at least a 3-fold increase in senescence-associated β-gal in *Rev3L*^{-Δ} cells compared to control cells (Figure 2B, C), and an increase in IL-6 secretion (Figure 5C). These characteristics show that induced senescence was a primary consequence of *Rev3L* deletion. More apoptotic cells were also present in *Rev3L*^{-Δ} cultures compared to controls (Figure 2D, E). Therefore, elimination of pol ζ from primary mouse cells leads to rapid activation of pathways leading to cell death and senescence.

Rev3L inactivation leads to chromatid gaps and breaks in each cell division

To dissect the reasons for inhibition of proliferation, we analyzed the integrity of mitotic chromosomes following inactivation of *Rev3L* in primary MEFs (Figure 3A, B). Chromosomal abnormalities accumulated rapidly. After 3 days, the number of metaphases containing chromatid-type aberrations (gaps and breaks) was increased 5-fold, and the number per metaphase increased 10-fold, relative to controls (Table 1). Chromosome-type aberrations (double-minutes and acentric chromosomes) did not increase in frequency. Metaphases with radial chromosomes (<5%) also appeared uniquely in *Rev3L*-deleted cells. Mitotic chromosome spreads with ploidy of 2N, 4N and occasionally greater were found in all groups, typical of the polyploidization that occurs early during culture of MEFs (17). There was no evidence of increased aneuploidy in *Rev3L*-deleted cells, and bivalents and centromeres in *Rev3L*-deleted cells otherwise appeared normal. This indicates that pol ζ does not directly influence chromosome segregation.

Rapid formation of double-strand breaks in the absence of pol ζ function

The appearance of chromatid gaps and breaks within one population doubling of REV3L loss suggested that strand breaks were forming rapidly in the DNA. To investigate this, fixed cells were stained with antibodies recognizing 53BP1 and γ-H2AX (Figure 3C). When foci of these two proteins are found in the same nuclear domains, they reliably mark sites of DNA double-strand breaks (DSBs) in cells. Primary MEFs harboring γ-H2AX foci almost always contained 53BP1 foci, with <3% of cells displaying foci for only one marker. The majority of cells containing foci for both 53BP1 and γ-H2AX exhibited colocalization of these markers. Less than 1% of the MEFs displayed pan-nuclear γ-H2AX staining, and these typically had an abnormal or degraded nuclear appearance, consistent with one hypothesis suggesting that these represent pre-apoptotic cells (18). Many of the 53BP1 foci were large, consistent with nuclear domains that sequester broken DNA in G1 phase (19,20). Therefore most of the foci-positive MEFs that we observed did contain DSBs, rather than other structures that have been hypothesized to activate H2AX phosphorylation (18). DSBs as assessed with these markers increased 2-fold over the control by

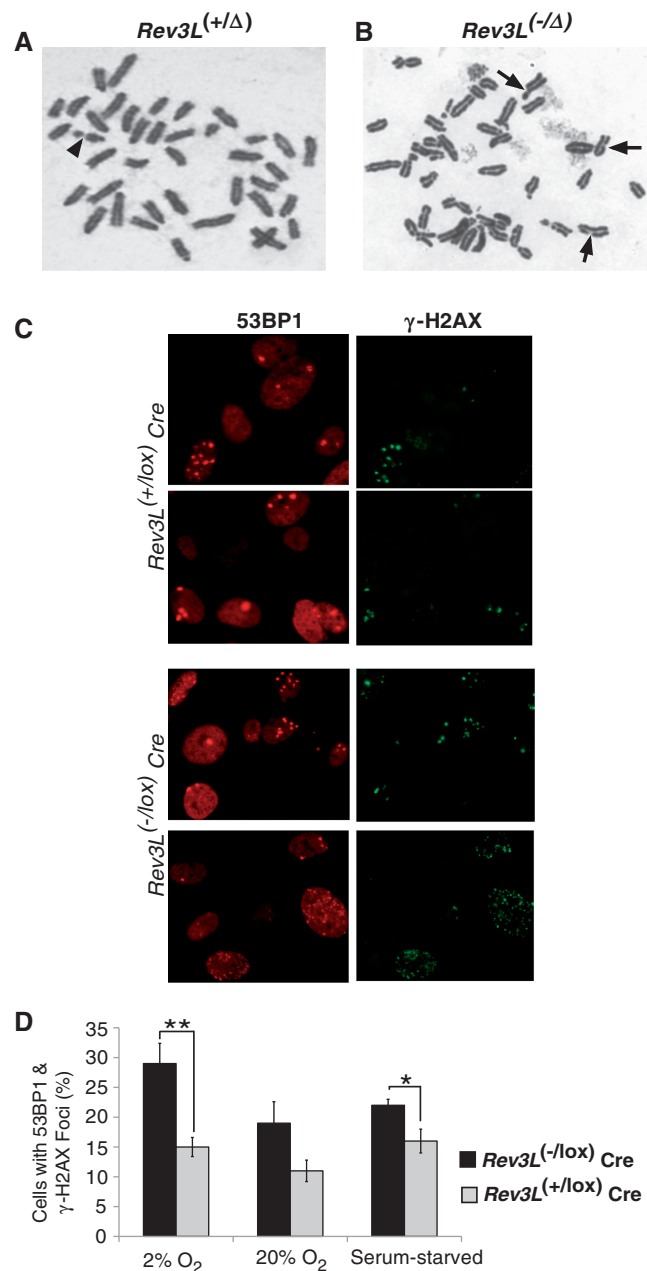


Figure 3. Chromatid aberrations and double-strand breaks form rapidly in *Rev3L*-deleted primary fibroblasts. Metaphase spreads from 3 day *Rev3L*^{+Δ} (A) and *Rev3L*^{-Δ} (B) primary MEFs; arrows point to gaps and breaks in the chromosomes; the arrowhead points to an acentric chromosome. (C) Immunofluorescence of 53BP1 and γ-H2AX in 3 day *Rev3L*^{-lox} Cre and *Rev3L*^{+lox} Cre MEFs. (D) Quantification of *Rev3L*^{-lox} Cre (black bars) and *Rev3L*^{+lox} Cre (grey bars) MEFs positive for both >2 53BP1 foci and γ-H2AX foci, at day 3 post-deletion in 2% oxygen, in 20% oxygen, or in 2% oxygen with serum starvation; **P* < 0.05; ***P* < 0.01 (*n* = 4). Data points indicate average ± SEM.

3 days (one population doubling) after *Rev3L* inactivation (Figure 3D).

The proportion of *Rev3L*-deleted cells with DSBs was reduced by cultivation between days 2 and 3 in medium lacking serum, which diminishes replication (Figure 3D). This suggests that DNA replication is required for the

Table 1. Chromosomal aberrations 3 days after *Rev3L* deletion

Genotype	Total metaphases counted	Acentrics and double-minutes per metaphase	Metaphases containing acentrics and double-minutes (%)	Gaps and breaks per metaphase	Metaphases containing gaps and breaks (%)
<i>Rev3L</i> ^{+lox} Mock	412	0.34 ± 0.17	7.2 ± 2.6	0.11 ± 0.04	10.4 ± 1.4
<i>Rev3L</i> ^{-lox} Mock	408	0.18 ± 0.05	6.8 ± 1.3	0.15 ± 0.05	9.4 ± 2.1
<i>Rev3L</i> ^{+lox} Cre	400	0.22 ± 0.08	9.3 ± 1.1	0.11 ± 0.01	7.5 ± 1.6
<i>Rev3L</i> ^{-lox} Cre	404	0.14 ± 0.02	9.2 ± 1.0	1.71 ± 0.34 ^a	50 ± 6.7 ^b

Numbers indicate average ± SEM ($n = 4$).

^a $P < 0.05$ compared to other groups.

^b $P < 0.01$ compared to other groups.

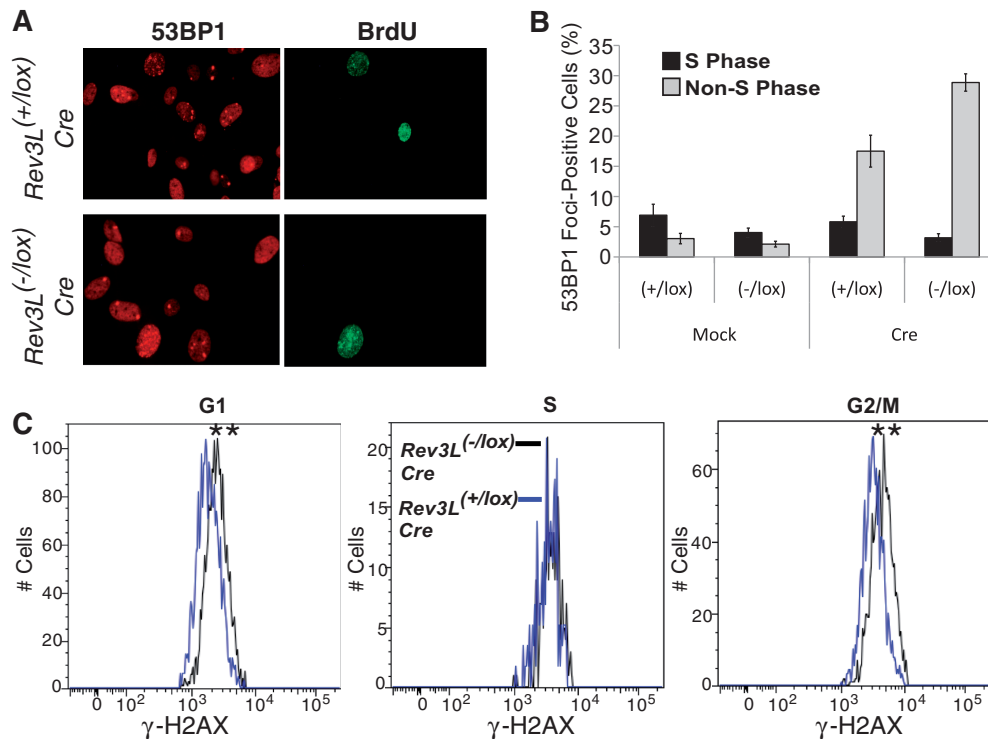


Figure 4. Following *Rev3L*-deletion, most double-strand breaks are not in S phase cells. (A) 53BP1 immunofluorescence of *Rev3L*^{-lox} Cre and *Rev3L*^{+lox} Cre primary MEFs in cells labeled with BrdU for 2 h prior to fixation. (B) Quantification of percent of 53BP1-foci positive *Rev3L*^{+lox} and *Rev3L*^{-lox} MEFs that were positive (S phase, black bars) or negative (non-S phase, grey bars) for BrdU incorporation. (C) *Rev3L*^{+lox} Cre (blue line) and *Rev3L*^{-lox} Cre (black line) MEFs were flow-sorted for γ -H2AX and DNA content (to determine cell cycle stage). The γ -H2AX signal is plotted relative to cell number for each cell cycle stage. ****** $P < 0.01$ ($n = 4$); Data points indicate average ± SEM.

DSBs formed in the absence of REV3L. Growth at 20% O₂ also decreased formation of DSBs (Figure 3D), likely a consequence of the inhibition of proliferation in 20% O₂ (16) (Supplementary Figure S1B).

To determine the cell cycle stage where most of the DSBs were present, cells were labeled with BrdU (marking ongoing DNA replication) or phospho-histone H3 (marking M phase cells), and analyzed by immunofluorescence. Only 10% of the DSBs in the *Rev3L*^{-Δ} MEFs were present in cells with ongoing DNA replication (Figure 4A, B), and only ~5% of the *Rev3L*^{-Δ} cells containing DSBs were in M phase (data not shown). To further examine cell cycle stage, the MEFs were fixed at day 3 and analyzed by flow-sorting for γ -H2AX and DNA content. *Rev3L*^{-lox} Cre cells had an increased γ -

H2AX content in both G1 and G2/M phases, compared to *Rev3L*^{+lox} Cre cells, but there was no difference in γ -H2AX signal between the cell lines during S phase (Figure 4C). These data suggest that while replication is involved in the formation of the DSBs, most of the observed breaks were in cells that were not undergoing DNA replication.

Reduction of ROS diminishes DSBs but does not rescue growth of *Rev3L*-deleting cells

The DNA breaks in the primary MEFs in these experiments arise in a DNA replication-dependent process, and are of endogenous origin. As reactive oxygen species are a ubiquitous source of DNA damage, we treated cells with the ROS scavenger N-acetyl cysteine (NAC) and

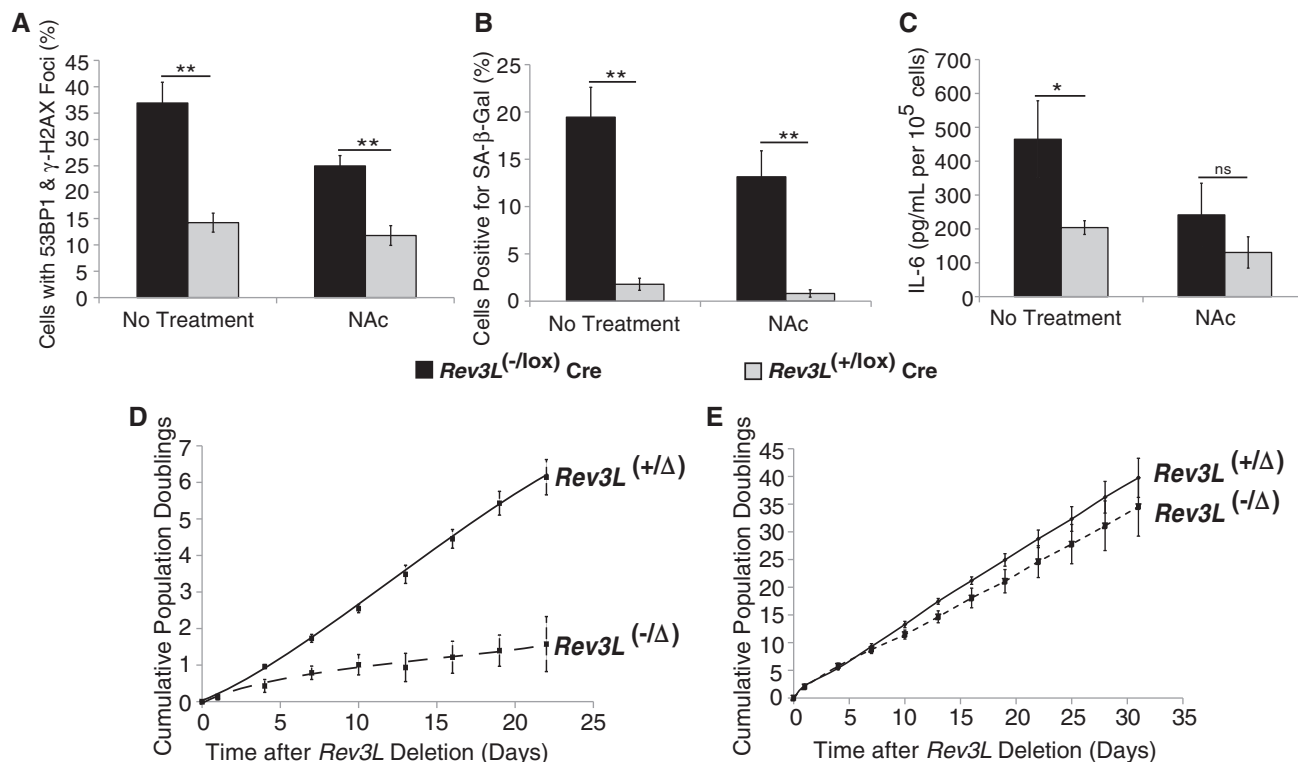


Figure 5. Some phenotypes of $Rev3L$ deletion are partially ameliorated by anti-oxidants at day 9 post-deletion. (A) Quantification of $Rev3L^{-/lox}$ Cre (black bars) and $Rev3L^{+/lox}$ Cre (grey bars) MEFs positive for both >2 53BP1 foci and γ -H2AX foci, with or without treatment with 1 mM N-acetyl cysteine (NAC). (B) Quantification of SA- β -gal staining in $Rev3L^{-/lox}$ Cre and $Rev3L^{+/lox}$ Cre MEFs with or without 1 mM NAc. (C) Quantification of IL-6 secretion into the media of $Rev3L^{-/lox}$ Cre and $Rev3L^{+/lox}$ Cre MEFs with or without treatment with 1 mM NAc. (D) Growth curve of primary $Rev3L^{-/\Delta}$ (dashed line) and $Rev3L^{+/\Delta}$ (solid line) MEFs over time after treatment with adenovirus Cre at 2% O_2 in the presence of 1 mM NAc. (E) Growth of TAG-immortalized $Rev3L^{-/\Delta}$ (dashed line) and $Rev3L^{+/\Delta}$ (solid line) MEFs after treatment with MOI 50 AdCre at 2% O_2 ($n = 4$).; * $P < 0.05$; ** $P < 0.01$ ($n = 4$). Data points indicate average \pm SEM.

assessed DSBs, cell growth and senescence. This treatment produced a statistically significant reduction in DSB formation after deletion of $Rev3L$, but not in the control cells (Figure 5A). The NAc treatment also demonstrated a trend towards reduction of senescence, as indicated by senescence-associated β -gal staining (Figure 5B) and IL-6 secretion (Figure 5C). Given the variability between assays, the difference between NAc and untreated $Rev3L^{-/lox}$ Cre cells did not attain significance. This suggests that part of the toxic effect of $Rev3L$ deletion may be mediated by ROS. However, NAc did not rescue the cell growth defect of $Rev3L$ -deleting cells with NAc treatment (Figure 5D). Further, combining growth in a 20% O_2 atmosphere with $Rev3L$ deletion did not synergistically inhibit proliferation (Supplementary Figure S1B). These results indicate that the endogenous sources of DNA damage leading to toxicity in $Rev3L$ -deleting cells are not limited to ROS.

Rev3L deletion is tolerated in T-antigen immortalized cells

Several $Rev3L$ -defective cell lines have been isolated from parental cells or embryos with compromised p53 function (6–9). A major effect of T-antigen immortalization is to inactivate p53 function, which controls checkpoint and cell death pathways. We tested whether T-antigen immortalized cells could tolerate deletion of $Rev3L$.

$Rev3L^{-/lox}$ and $Rev3L^{+/lox}$ MEFs were immortalized with a T-antigen expression vector, and then $Rev3L$ was deleted with AdCre. The T-antigen immortalization allowed cell growth and proliferation in the absence of $Rev3L$ (Figure 5E).

DISCUSSION

Function for DNA pol ζ in normal cell proliferation

Several potential outcomes could have been envisioned for the experiments described here with normal primary cells. One possibility would have been that potentially toxic events that require pol ζ for tolerance occur only occasionally, but would accumulate gradually over many cell divisions and result in abnormalities that would prevent sustainable proliferation. However, the results show that complete deletion of $Rev3L$ is not tolerated in p53-proficient cells and is needed in essentially every cell cycle. Unchallenged cells rapidly accumulate DNA damage leading to senescence and apoptosis. Thus DNA pol ζ is necessary not only for development, but for normal cellular viability. This is a unique phenotype for pol ζ , standing in contrast to the other mammalian DNA polymerases with TLS activity.

These results explain why viable $Rev3L^{-/-}$ embryonic stem cells, blastocysts or p53-proficient MEFs have not

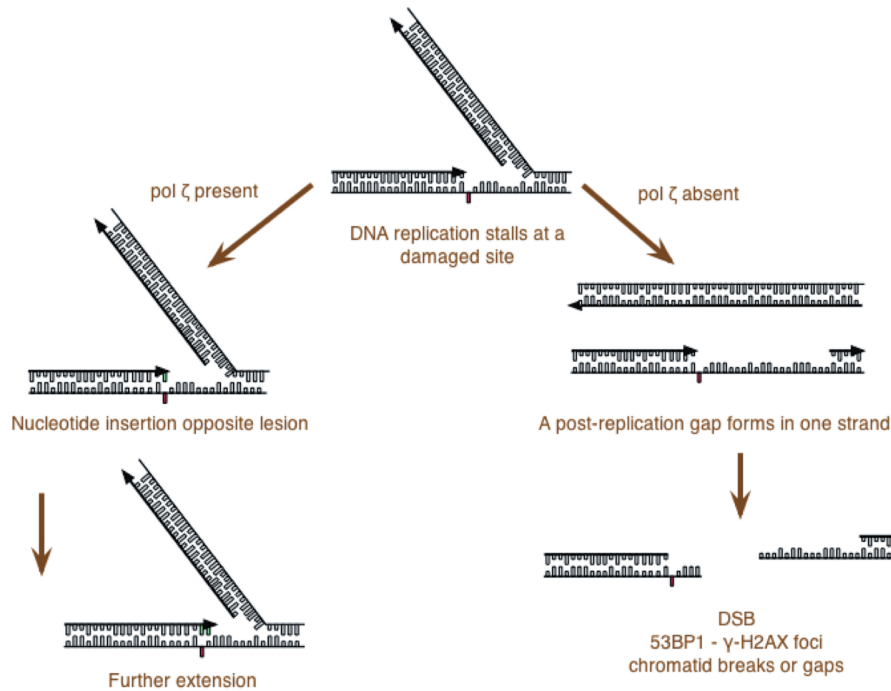


Figure 6. Model of DNA polymerase ζ action in mammalian cells.

been isolated (21). We note that a recent study came to a contrasting conclusion, suggesting that siRNA suppression of human *Rev3L* suppresses cell growth in cancer cell lines, but not in normal cell lines (5). However, the extent of *Rev3L* suppression in those experiments (50% or at most 25% of initial levels) was not sufficient to enable definitive conclusions. It seems likely that toxicity to the cell lines originated from other experimental factors.

These observations suggest a model as follows (Figure 6). In all cells, even with proficient DNA repair, template damage is frequently encountered during DNA replication. This includes DNA damage mediated by ROS (shown here to contribute to increased DSBs in the absence of REV3L), as well as other endogenously formed lesions (e.g. abasic sites, lipid peroxidation-induced DNA damage) that may require pol ζ function for bypass. In S phase, there are several options when the replication fork encounters a DNA lesion: a TLS process involving REV3L can bypass the site (potentially causing mutations); a homologous recombination (HR) mediated pathway can perform a template switch (an error-free pathway); or a gap could be formed by re-initiation of replication downstream (22) or by convergence of an adjacent replicon. The gap would remain in the DNA, which would still need to be resolved in late S or G2 phases. This model is supported by work showing that while gaps are formed during S phase after UV irradiation of *Rev3L*^{-/-} p53^{-/-} MEFs, there is no immediate replication defect (23). Damaged *Rev3L*-defective cells move into G2 phase but are unable to fill in the replication-induced gaps.

If TLS-mediated bypass of the lesion during G2 is unsuccessful, an unrepaired gap will be prone to DNA

breakage. There is evidence that DNA breaks can persist through mitosis and are sequestered during G1 phase into domains containing 53BP1 and γ -H2AX (19,20). Eventually, the breaks can trigger responses leading to apoptosis or senescence. This model explains not only why replication is required for generation of DNA breaks, but also why the breaks are mostly observed in G1 and G2 phases, and are not associated with replicating DNA. We note that the release of IL-6 observed in *Rev3L*-deleted MEFs is consistent with a secretory phenotype observed in murine cells with senescence caused by DNA breaks, but not with senescence caused by culture in 20% O₂ (24).

In the absence of REV3L, DSB avoidance would be expected to be more dependent on HR-mediated template switching. Indeed, deletion of both *Rev3L* and *Rad54* is synthetically lethal in DT40 chicken cells, and yields extensive chromosomal aberrations (8). In this DT40 system, REV3L is not epistatic with HR. In mammalian cells, pol zeta may contribute more directly to DSB repair (25).

REV3L interacts with the MAD2 protein (26,27) raising the possibility that REV3L plays a role in normal chromosome segregation. However, the present results show that disruption of *Rev3L* gives cellular phenotypes quite distinct from a *Mad2* disruption in the mouse. *Mad2* knockout cells have obvious chromosome segregation defects, and no increased γ -H2AX formation (28).

Implications for *Rev3L* knockout models

The activity of pol ζ in preventing DNA breaks arising from endogenous DNA lesions helps clarify why *Rev3L* is

critical for embryogenesis. This also accounts for the observation that rapidly proliferating hematopoietic tissues in adult mice cannot survive in the absence of REV3L (2). Tissues with a slow rate of replication may be able to better tolerate the loss of REV3L. During gradual accumulation of genetic changes, cells may bypass blocks to proliferation. Detailed studies await regarding the tolerance of normal epithelial tissues for complete *Rev3L* deletion.

The approach used here demonstrates that *Rev3L* deletion in normal cells rapidly leads to chromosome breaks and cell death. The chromosomal instability present in previously established *Rev3L*-null cell lines provides challenges for interpretation of data from these lines. These cell lines usually have slower growth rates, are refractory to spontaneous and DNA damage-induced mutagenesis (29,30), and show a persistent accumulation of chromosomal breaks and translocations (3). With every cell division, more aberrations accumulate that could lead to phenotypes arising from secondary genetic changes. This genetic instability, when allowed to persist in a background of compromised checkpoints *in vivo*, provides a fundamental explanation for the increased selection for *Rev3L* null tumors when mice conditionally delete the *Rev3L* gene in a mosaic background (2).

The results presented here represent a significant shift in our knowledge of the activity of DNA pol ζ . In yeast, deletion of *REV3* reduces TLS in cells damaged by radiation or chemical mutagens, but does not affect cell proliferation (31). In contrast, mammalian pol ζ has a role in preventing DSBs and chromosome aberrations that arise from ongoing spontaneous DNA damage in every cell division.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figure S1.

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