A Missense Mutation in Rev7 Disrupts Formation of Polζ, Impairing Mouse Development and Repair of Genotoxic Agent-induced DNA Lesions*

Received for publication, August 29, 2013, and in revised form, December 13, 2013. Published, JBC Papers in Press, December 19, 2013, DOI 10.1074/jbc.M113.514752

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**Background:** Rev7 encodes a subunit of Polζ for translesion DNA synthesis (TLS).
**Results:** We found a Rev7 mutation in mice that causes developmental defects and increases susceptibility for genotoxicity.
**Conclusion:** Rev7 is essential for mouse development through its function in cell proliferation.
**Significance:** These findings demonstrate a unique function of Polζ in development that is absent in other TLS polymerases.

Repro22 is a mutant mouse produced via N-ethyl-N-nitrosourea-induced mutagenesis that shows sterility with germ cell depletion caused by defective proliferation of primordial germ cells, decreased body weight, and partial lethality during embryonic development. Using a positional cloning strategy, we identified a missense mutation in Rev7/Mad2l2 (Rev7C70R) and confirmed that the mutation is the cause of the defects in repro22 mice through transgenic rescue with normal Rev7. Rev7/Mad2l2 encodes a subunit of DNA polymerase ζ (Polζ), 1 of 10 translesion DNA synthesis polymerases known in mammals. The mutant Rev7 did not interact with REV3, the catalytic subunit of Polζ. Rev7C70R/C70R cells showed decreased proliferation, increased apoptosis, and arrest in S phase with extensive γH2AX foci in nuclei that indicated accumulation of DNA damage after treatment with the genotoxic agent mitomycin C. The Rev7C70R mutation does not affect the mitotic spindle assembly checkpoint. These results demonstrated that Rev7 is essential in resolving the replication stalls caused by DNA damage during S phase. We concluded that Rev7 is required for primordial germ cell proliferation and embryonic viability and development through the translesion DNA synthesis activity of Polζ preserving DNA integrity during cell proliferation, which is required in highly proliferating embryonic cells.

The arrest of DNA replication after nucleotide damage disrupts the cell cycle and causes genome instability and cell death. Cells can relieve arrest through translesion DNA synthesis (TLS) that provides tolerance to DNA damage (1, 2). In processing damaged DNA through this mechanism, a nucleotide opposite the lesion is incorporated to bypass the damage but at the cost of increased rates of mutation (3, 4). Unlike classic DNA polymerases, many TLS DNA polymerases are present in most human cells (5, 6). One, DNA polymerase ζ (Polζ), is a member of the B family of DNA polymerases and consists of a catalytic and an accessory subunit encoded by Rev3 (official name, Rev3f) and Rev7 (official name, Mad2l2 for mitotic spindle assembly checkpoint protein MAD2B), respectively. Polζ has been documented to play a particularly important role in TLS, which is required for DNA repair, recombination, and chromosomal stability (1, 7–10).

In yeast, a strong epistatic relationship occurs between Rev3 and Rev7 mutations, enhancing susceptibility to genotoxic agents. Although these functions are conserved in vertebrates, studies using cultured cells with defects of these genes document greater complexity (11–13). In addition, a null Rev3 mutation in mouse results in severe early developmental lethality, leaving its function, especially its involvement in various cell lineages, largely unknown (14–16). A more restricted conditional null mutation of Rev3 in mouse embryonic fibroblast cells and adult B cells documents a need for Polζ, without which increased chromosomal aberrations lead to enhanced tumorigenesis (17, 18), reduced somatic mutations, and cell proliferation (3, 19). Although overexpression of Rev7 is associated

*This work was authored, in whole or in part, by National Institutes of Health staff. This work was supported in part by grants from the Japan Society for the Promotion of Science.

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The abbreviations used are: TLS, translesion DNA synthesis; APC, anaphase promoting complex; BAC, bacterial artificial chromosome; DSB, DNA double-strand break; FA, Fanconi anemia; ICL, interstrand cross-link; γH2AX, phosphorylated histone H2AX; MEF, mouse embryonic fibroblast; PGC, primordial germ cell; PN, pan-nuclear; Polζ, DNA polymerase ζ; RFLP, restriction fragment length polymorphism; PCNA, proliferating cell nuclear antigen.
A Mutation in Rev7 Impairs Mouse Development and DNA Repair

with high mortality in patients with colon cancer (20), relatively little is known about the role of REV7 and its regulation of Polζ function in TLS.

REV7/MAD2L2 is also homologous to the cell cycle checkpoint protein MAD2, and the two proteins share common HORMA domains. The MAD2 protein is a key component of the mitotic spindle assembly checkpoint as an inhibitor of anaphase promoting complex (APC), a surveillance mechanism that delays anaphase until all chromosomes are properly aligned on the metaphase plate (21, 22). Several reports have described the function of Mad2l2 in cell cycle regulation, and mammalian epithelial cells infected by Shigella show cell cycle arrest secondary to disruption of Mad2l2 with APC<sup>cdh1</sup> (23), which suggests that Rev7/Mad2l2 might regulate the cell cycle by interacting with APC.

Herein, we report a unique function of Rev7 in mouse development and DNA repair using a mouse model of infertility produced by a project known as ReproGenomics at The Jackson Laboratory (24). Repro22 mice are useful for studying the mechanism of mouse primordial germ cells (PGCs), as both males and females are sterile with a lack of PGCs. Interestingly, the mutant mice display severe embryonic lethality and reduced embryonic body weight. Using positional cloning, we identified a missense mutation in the N terminus of Polζ that disrupts Rev7 interaction with Rev3. In the absence of Polζ activity, cells become hypersensitive to interstrand cross-link (ICL) damaging agents and accumulate double-stranded breaks (DSBs) in their DNA. We next showed that Rev7 function is essential to resolving the replication stall in S phase of the cell cycle. Our results show that Rev7 is required for PGC proliferation and embryonic viability and development through the TLS activity of Polζ to preserve DNA integrity during cell proliferation, which is required in highly proliferating embryonic cells.

**EXPERIMENTAL PROCEDURES**

**Mice and Histological Preparations**—The heterozygous repro22/+ mice were obtained from The Jackson Laboratory, and the line was maintained through sib mating. The JF1/Ms mice were obtained from the National Institute of Genetics, Mishima, Japan. The repro22/+ heterozygous mice were crossed to JF1/Ms mice to generate F1 mice. The F1 mice carrying the mutant allele were intercrossed to generate F2 progeny. Congenic mouse lines were constructed by backcrossing heterozygous mice to C3H mice for 9 generations. Affected and normal mice were classified by the phenotype of the concepti. Affected and normal mice were rehydrated and stained with Mayer’s hematoxylin and eosin. After dehydration, tissues were embedded in paraffin wax and sectioned. After deparaffination in xylene, sections were rehydrated and stained with Mayer’s hematoxylin and eosin. After dehydration, tissues were embedded in paraffin wax and sectioned. After deparaffination in xylene, sections were rehydrated and stained with Mayer’s hematoxylin and eosin.

**Alkaline Phosphatase Staining**—The concepti used in this study were obtained by mating repro22/+ male and female mice. Noon on the day of vaginal plug identification was defined as embryonic day 0.5 (E0.5), and pregnant females were euthanized on days E7.5, E8.5, E9.5, E10.5, E11.5, E12.5, and E13.5. For whole-mount preparations, embryos were removed from decidua in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 2 h at 4 °C, rinsed in PBS, placed in 70% ethanol for 2–3 h at 4 °C, washed with distilled water, and processed as whole mounts for alkaline phosphatase staining (28, 29). The embryos were stained at room temperature for 10 min with Fast Red TR salt Sigma; F8764 and -naphthyl phosphate (Sigma; 71090) and α-naphthyl phosphate containing 5% borax Sigma; F8764 and 10% MgCl<sub>2</sub> in the staining solution. To obtain serial sections, we fixed the embryos in 4% paraformaldehyde and dehydrated them ethanol and xylene before embedding them in paraffin. The sections (7 μm) containing gonads were stained with NBT-BCIP (Boehringer DIG labeling detection kit).

**Linkage Mapping and Mutation Detection**—Genomic DNA was isolated from the tails of 1511 progeny with phenol/chloroform extraction, and fine mapping of the repro22 locus was performed using polymorphic microsatellite markers on mouse chromosome 4 (Table 1). These markers were genotyped as described previously (25).

For mutation detection, total RNA of the affected and normal animals was extracted from liver, testis, lung, spinal cord, and inner ear tissues using TRIzol reagent (Invitrogen) according to manufacturer instructions. After complementary DNA (cDNA) synthesis using random hexamers and Superscript III reverse transcriptase (Invitrogen), the entire coding regions of five candidate genes were amplified from the cDNAs of affected and normal mice. Primers designed to amplify the open reading frame of Rev7 messenger RNA are listed in Table 1. Purified polymerase chain reaction (PCR) products were cloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced using the dye-terminator method with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). The DNA sequences of normal and affected mice were compared. After identifying the missense mutation in Rev7, we designed primers to amplify a 239-bp fragment that contained it (see Table 1) to genotype the mutation with BstUI PCR-restriction fragment length polymorphism (RFLP).

**Transgenic Rescue Experiments**—To identify conserved genomic regions of Rev7 that may include potential regulatory regions of the gene, we performed a comparative analysis between mice and humans using VISTA (26). The BAC clone RP24–193K16 containing Rev7 and three other genes was digested using Clal restriction enzyme to isolate a 24.8-kb fragment containing the entire Rev7 gene including 5 kb of the potentially regulatory 5’ region and 3 kb of the 3’ untranslated region of the gene and part of the BAC vector. Transgenic mice were generated via intracytoplasmic sperm injection-mediated transgenesis (27). Oocytes were obtained through mating (C57BL/6j × C3H/HeJ) of F1 females and C3H/HeJ males and injected with the BAC fragment containing the intact copy of normal Rev7. Transgenic mice were identified with PCR using primers specific to the BAC vector sequence in the fragment (ATCCGGCGCGCCATTGTCATGCCC and CCTATATGTGAGTCGTATAGC) that amplified a 130-bp fragment. The transgenic founders with multiple copies of the normal Rev7 transgene (Rev7<sup>+/+</sup>) were crossed with repro22/+ females, and the resulting Rev7<sup>TG</sup>/<sup>+</sup>;repro22/+ offspring were crossed to...
establish Rev7\textsuperscript{TG/+}, repro22/repro22 mouse lines. The genotype of the endogenous Rev7\textsuperscript{+} was determined using genotypes of two flanking microsatellite markers (D4Mok8 and D4NiM1r1) as well as PCR-RFLP for the mutation site. The presence of Rev7\textsuperscript{TG} was determined with PCR of the BAC vector sequence and Southern hybridization.

Expression Analysis of Rev7 in Developing PGCs—We examined the expression of Rev7 in PGCs isolated from mouse embryos via fluorescence-activated cell sorting (FACS) of β-galactosidase-expressing cells (30). Total RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNase I. The first-strand cDNA was synthesized from the RNA template with reverse transcription using Superscript III reverse transcriptase with oligo-dT primers (Invitrogen). The expression of Rev7 was examined using semiquantitative reverse transcription-PCR.

In Vitro Interaction Assay—cDNA encoding human Rev7 and REV3 fragments (residues 1847–1898) was inserted into a pETDuet-1 vector (EMB Millipore, Billerica, MA). The result-ant plasmids encoded Rev7 with an N-terminal hexameric His tag and the REV3 fragment (31). The C70R substitution of Rev7 was introduced via the QuickChange protocol (Agilent, Santa Clara, CA). For the in vitro interaction assay between Rev7 and the REV3 fragments (32), His-tagged REV7 or Rev7\textsuperscript{C70R} mutant protein was co-expressed with the REV3 fragments in Escherichia coli BL21(DE3). The cell lysate was applied to Ni\textsuperscript{2+}-Sepharose resin (GE Healthcare) in buffer (50 mM HEPES-NaOH, pH 7.4, 500 mM NaCl, and 20 mM imidazole). After washing with buffer (50 mM HEPES-NaOH, pH 7.4, 1.5 mM NaCl, and 20 mM imidazole) bound proteins were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis with Coomasie Brilliant Blue staining.

Assay for DNA Damage Sensitivity—Mouse embryonic fibroblasts (MEFs) were isolated from interbred repro22/+ mice. After removal of the head and intestinal organs, each embryo was minced and digested with trypsin (PBS, 0.05% trypsin, 0.04% EDTA) at 37 °C. The cells were seeded in 100-mm culture dishes (passage 0). MEFs were cultured in 5% CO\textsubscript{2} at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), and experiments were performed with early passage MEFs. Cyto-toxic and cytostatic effects of DNA damage were assayed via toxic and cytostatic effects of DNA damage were assayed via flow cytometric analysis of mitomycin C-treated cells—Rev7\textsuperscript{C70R/C70R}, Rev7\textsuperscript{C70R/+}, and normal primary MEFs were treated with colcemid and harvested hourly for 8 h. The cells were fixed with 70% ethanol permeabilized with 1% BSA and 0.1% Triton X-100 in PBS containing 0.1% mg/ml RNase. The cells were then stained with Alexa Fluor 488 antibody recognizing phosphorylated histone H3 (Cell Signaling Technology, Beverly, MA) and stained with propidium iodide as described above. The ratio of phosphohistone H3 (Ser-10)-positive cells to total cells was recorded as the mitotic index.

Terminal Deoxyribonuclease-digoxigenin nick end labeling (TUNEL) and Proliferating Cell Nuclear Antigen (PCNA) Assay—Rev7\textsuperscript{C70R/C70R} cells were seeded in a 16-well glass chamber slide at 5 × 10\textsuperscript{5} cells/well. After 24 h of seeding, cells were treated with the indicated concentrations of mitomycin C for 18 h. Cells were washed with PBS and further cultured in fresh medium for 48 h. MEFs were then fixed with 3.7% formaldehyde in PBS for 5 min and permeabilized with 0.25% Triton-X in PBS for 5 min at room temperature. Apoptotic cells were detected with TUNEL assay using an In Situ Cell Death Detection kit, POD (Roche Diagnostics). After the TUNEL assay, preparations were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) in VECTASHIELD® Mounting Medium, H-1200 (Vector Laboratories, Burlingame, CA). A TUNEL assay for histological section was carried out using paraformaldehyde-fixed mouse tissues.

For the PCNA assay, tissue sections were incubated with PBS containing 0.3% H\textsubscript{2}O\textsubscript{2} for 30 min at room temperature. The sections were then incubated with biotin-conjugated anti-PCNA antibody (1:1000) with 3% BSA in PBS overnight at 4 °C. The sections were then incubated in series with streptavidin-HRP diluted with 3% BSA in PBS (1:1000) and 3,3’-diaminobenzidine tetrahydrochloride (DAB) choromogen (Dako, Kyoto, Japan). The sections were counterstained with Mayer’s hematoxylin and examined by light microscopy.

Statistical Analysis—Data are expressed as the means ± S.E. of the mean. Comparisons were made using the two-tailed Student's t

FEBRUARY 7, 2014 • VOLUME 289 • NUMBER 6

JOURNAL OF BIOLOGICAL CHEMISTRY 3813
dent’s test, \( t \) test, \( X^2 \), or analysis of variance and considered highly significant with \( p \) values of \( \leq 0.01 \) and significant with \( p \) values of \( \leq 0.05 \). The \( p \) values and significance levels are described in the figure legends.

RESULTS

Growth Retardation and Increased Embryonic Lethality of Homozygous repro22 Mice—During our initial analyses of homozygous repro22 mice, we found decreased size and body weight in adult mice of both genders compared with those of normal littermates. These differences were evident as early as E7.5 and E8.5 and persisted from birth into adulthood, suggesting that the genetic defect in repro22 mice causes severe growth retardation in prenatal development (Fig. 1A). We also found that a significant number of homozygous mutant animals died during embryogenesis. Analysis of 1511 F\(_2\) offspring from intercrosses of repro22/+ mice indicated a dramatically reduced number of affected mice. The distortion from Mendelian ratio with no sex ratio distortion suggested an autosomal single recessive mode of inheritance with partial lethality. The progeny data indicated that only \( \sim 40\% \) of affected animals survived after weaning (Fig. 1B), and both male and female mice were strikingly sterile.

Defective Development of PGCs in repro22 Mice—At 8 weeks of age, the male and female gonads of repro22 mice were \( \sim 10\% \) the size of those of normal mice (Fig. 1C). Examining gonadal histology, we observed complete absences of germ cells in male and female mice. Although testicular tubules were formed, they lacked germ cells, and neither follicles nor germ cells were present in adult ovaries (Fig. 1D). The documented absence of germ cells in adults of both sexes suggested a common defect in prenatal germ cell development. In mice, PGCs first appear in the developing gonad at E7.25 and are defined by their large size and strong alkaline phosphatase activity (28, 34). Therefore, we examined the developmental process of PGCs in affected and normal mouse embryos from E7.5 to E13.5 using alkaline phosphatase staining of gonadal sections and whole-mount embryos. The presence of pre-migratory PGCs in the hindgut pocket of affected and normal mice at E8.5 suggested that specification and initial formation of PGCs were not significantly

![FIGURE 1. Morphology and histology of repro22/repro22 gonads. A, comparison of body weights between repro22/+ and repro22/repro22 mice at E13.5. The embryos show significant reduction in body size. Values represent the means ± S.E. (\( n = 5 \)) and were statistically different at \( p < 0.03 \). B, the genotype of ~1500 progeny from repro22/+ heterozygous parents varied from expected Mendelian inheritance (\( p < 0.001 \)). C, repro22/+ and repro22/repro22 testes and female reproductive tracts from 8-week-old mice. D, section of testes and ovaries from 16-week-old repro22/+ and repro22/repro22 mice were stained with hematoxylin-eosin. The arrowhead points to the lumen in a normal seminiferous tubule. Scale bar, 100 \( \mu \)m. Ooc, oocyte.]
affected in repro22 mutant mice (Fig. 2A). In addition, PGCs were not significantly decreased in repro22 embryos at E7.25 compared with those in normal embryos (Fig. 2A), suggesting normal PGC specification in repro22 mice. However, the number of PGCs in the affected mice was approximately half that of normal mice at E8.5, and the differences became more pronounced in the hindgut mesentery at E9.5 and in the genital ridges at E11.5 (Fig. 2B). Notably, although fewer in number, PGCs in repro22 mice did successfully arrive and colonize the genital ridges at E11.5, suggesting that the defect in the mutant mouse is not in migration but in proliferation and survival of PGCs or both (Fig. 2A).

The widening difference in cell number persisted in E12.5 gonads, and at E13.5, when normal gonads were fully populated, few or no PGCs were detected in the mutant gonad either by alkaline phosphatase staining or PCAM1 immunostaining (Fig. 2C). We observed no differences in testicular cord formation, which was comparable with that of normal male gonads. Taken together, these observations during PGC development suggested that both defects in proliferation and survival of PGCs during PGCs development account for germ cell depletion in repro22 mice and an essential role in proliferation and survival of both somatic and germ cells during development.

**Fine Mapping and Positional Cloning of the repro22 Locus**—The repro22 mutant locus had been mapped initially to a 13-Mb region between D4Mit57 and D4Mit42 on the distal end of chromosome 4 (The Jackson Laboratory). To refine the location of the mutation, we determined the genotypes of microsatellite markers (Table 1) in this region in 160 affected mice (320 meiotic events) of 1511 F2 offspring from intercrosses of C57BL/6 and JF1/Ms mice as well as C57BL/6 and C3H/HeJ strains. No recombination between repro22 and D4Mok5 or D4Mok6 was observed, but one to four recombination events between repro22 and the other eight microsatellite markers were present. These data refined the critical region to a 70-kb interval between D4Mok3 and D4Mok7 markers (Fig. 3A), which included a cluster of three F-box genes (Fbox2, Fbox4, and Fbox6), one EST (2610109H07Rik), and Rev7 (Fig. 3B).

We determined the coding sequence of all of these genes in affected and normal mice by sequencing cDNA derived from various tissues. No functional mutation or nucleotide difference specific to the affected animals was observed in Fbox2, Fbox4, Fbox6, or 2610109H07Rik genes. However, in Rev7, we identified a single nucleotide substitution of T to C at nucleotide 208 in the coding region, which resulted in an amino acid substitution of Cys to Arg at codon 70 (C70R) in a highly conserved region of the HORMA domain, which is implicated in chromatin binding (Fig. 3, C and D). The complete association between the missense mutation and the repro22 phenotype in the F2 progeny was observed with PCR-RFLP analysis using Bstul endonuclease, and this allele was absent in various inbred strains (Fig. 3E). These findings indicated that the C70R muta-
Rev7 was responsible for germ cell depletion in repro22 mice. Transgenic Rescue—To confirm that the Rev7 C70R mutation was responsible for germ cell depletion in Rev7 C70R mice, we performed genetic complementation by establishing transgenic mice with a genomic fragment containing the normal Rev7 gene. A 24.8-kb genomic fragment containing the entire coding region and the predicted regulatory region of Rev7 was isolated from a BAC clone and injected into mouse oocytes via intracytoplasmic sperm injection-mediated transgenesis (Fig. 4, A and B). Mice with the Rev7 transgene were crossed with repro22/+ mice and subsequently intercrossed with Rev7 TG+/+;Rev7 C70R/− mice to produce Rev7 TG+/+;Rev7 C70R/C70R transgenic mice (Fig. 4C). Analysis of developing PGCs at E12.5 documented that the genital ridges of the Rev7 TG+/+;Rev7 C70R/C70R mice were as full of alkaline phosphatase-positive PGCs as those in normal control mice (Fig. 4D). Fertility was fully restored in both male and female Rev7 TG+/+;Rev7 C70R/C70R mice, and the size of their testes and ovaries were normal. On histological examination, the seminiferous tubules of the Rev7 TG+/+;Rev7 C70R/C70R mice were filled with germ cells including spermatogonia, spermatocytes, spermatozoa, and mature spermatids, and the ovaries contained multiple follicles with oocytes at various stages of development as well as corpora lutea (Fig. 4E). No histological difference was observed in}

**TABLE 1**

Sequences of PCR primers and expected size of amplified fragments

| Markers | Forward (5’-3’) | Reverse (5’-3’) | Size (bp) |
|---------|----------------|----------------|-----------|
| D4mit233 | CCCACCCCATCTATCATAAAA | AGGAGAGCCAGAGCTACACAA | 167 |
| D4mit285 | CTTAGGTAGACTTCCTGCTTT | GTGCGGAGGCAACCTTTCACCC | 100 |
| D4Nimr1 | AGGGAATCTGATGCCTTTGCA | TCAGTAAGTGTGCCTGCGTC | 220 |
| D4Mok3 | AAACTGGAAAGGGCAACATCTG | TCAGGCAAGGCTAGCCAGA | 183 |
| D4Mok4 | TTGATTGCTCTCTCTCTATTTACTTG | AACTTCCAGGACCCATTTGAG | 150 |
| D4Mok5 | TGAGTCTGGGCTAGCCACAC | CCTGCCGATAGCCATGCG | 184 |
| D4Mok6 | GCTCTCCTCTGGAACACAGT | AGCAGATCTCTCTGTTGTTG | 192 |
| D4Mok8 | GCCCGTGTAAACCTGACTTT | CCAACACCTCTGGCCCTCT | 226 |
| D4mit127 | GTGTCGCTGACGATCGA | GTACTGGGAGCAGCTGTGAGC | 145 |
| Mad212 | GTGTTGCTGGTATTGCTGTT | AGGCTTCTGGGCTTGAAG | 239 |

**FIGURE 3.** Molecular characterization of the repro22 mutation. A, segregation of haplotypes in 160 affected F2 mice. The number of mice for each haplotype is indicated at the bottom of the columns. B, localization of microsatellite markers, positions, and transcriptional orientations of three genes and an expressed sequence tag in the interval defined in A. An exon map of Rev7 is indicated at the bottom. C, nucleotide sequence of normal and repro22 alleles showing nucleotide substitution of T to C that results in missense mutation of Cys to Arg. D, alignment of the deduced amino acid sequences in the HORMA domain of vertebrate REV7 in which Cys-70 is well conserved. E, RFLP analysis of the specificity of the mutant allele using BstUI restriction enzyme in affected (A), parental heterozygous animals (S1, S2, D1, D2, and D3) and several inbred strains of mice.
between the testes or ovaries of normal and \( \text{Rev7}^{\text{C70R}} \) mice. Therefore, the \( \text{repro22} \) phenotypes were completely rescued by \( \text{Rev7}^{\text{TG}} \), demonstrating that the loss of function mutation of \( \text{Rev7}^{\text{C70R}} \) is responsible for germ cell depletion in \( \text{Rev7}^{\text{C70R}} \) mice.

**Expression Analysis of Rev7 in PGCs and Adult Tissues**—

Based on alkaline phosphatase staining in \( \text{Rev7}^{\text{C70R}} \) mutant mice, defects in PGCs were first apparent in the gonad at E8.5 and culminated with near complete loss of germ cells by E13.5. We, therefore, assessed the expression of the \( \text{Rev7} \) gene during the proliferative stage of PGC development using FACS sorting of \( \beta \)-galactosidase-tagged PGCs (30). \( \text{Rev7} \) expression in both male and female gonads was detected from E7.5 to E13.5 but not at E14.5 when the proliferative phase of PGCs was complete in male and female mice. Although a single transcript predominated during embryonic development, three additional isoforms of \( \text{Rev7} \) were present; particularly at E11.5, two variant transcripts specific to female PGCs were observed (Fig. 5 A).

We confirmed that these variants are alternatively spliced isoforms caused by skipping of exons 2 and/or 4 by sequencing of these transcripts.

We also examined expression of \( \text{Rev7} \) in various tissues of adult mouse. As shown in Fig. 5B, \( \text{Rev7} \) expressed ubiquitously in many tissues and highest expression was observed in testis.
It is to be noted that the expression of the Rev7 transcripts is also observed in tissues of Rev7<sup>C70R/C70R</sup> mice (Fig. 5B), indicating that the C70R mutation does not affect the level of Rev7 expression. The observed elimination of Rev7 expression in the mutant testis could be due to the absence of the germ cells in the mutant testis.

**Effects of the Rev7 Mutation on Somatic Cells**—The ubiquitous expression of Rev7 suggested that that Rev7 functions not only germ cells but also in various types of somatic cells, and the defects of REV7 could result in defective proliferation of somatic cells as observed in the germ cells. We, therefore, examined growth of MEFs isolated from normal, Rev7<sup>C70R/+</sup>, and Rev7<sup>C70R/C70R</sup> mouse embryos. As shown in Fig. 6A, the growth ratio C70R/C70R MEF was significantly lower than that of normal MEFs at 48 h of culture. These findings indicated that the defect of REV7 affects the proliferation of somatic cells.

Next, we examined *in vivo* effects of the Rev7 mutation on somatic cells. Because the effects of the Rev7 mutation are expected to be more apparent in rapidly proliferating cells, we assessed ratios of growth and apoptosis in intestinal epithelial cells. As shown in Fig. 6, B and C, the numbers of PCNA-positive S-phase cells were not significantly different between normal and Rev7<sup>C70R/C70R</sup> mice. However, the number of TUNEL-positive cells in Rev7<sup>C70R/C70R</sup> mice was significantly increased, and the number of epithelial cells in each villus was significantly reduced in Rev7<sup>C70R/C70R</sup> mice. These findings indicated that the Rev7 mutation results in growth defects of intestinal epithelial cells by an increased number of apoptotic cells.

**Defects in Spindle Assembly Checkpoint Function Do Not Account for the repro22 Phenotype**—REV7/MAD2L2 is homologous to the MAD2 protein, which is a key component of the mitotic spindle assembly checkpoint. Thus, Rev7<sup>/Mad2l2</sup> might also regulate cell cycle progression as an arbiter of the spindle assembly checkpoint and could contribute to the growth retardation and germ cell depression observed in Rev<sup>C70R</sup> mutant mice. To test this possibility, we treated Rev7<sup>C70R/C70R</sup> MEF with colcemid and harvested the cells hourly over 8 h. The cells were fixed and stained with phospho-histone H3 (Ser-10) antibody, a marker of mitosis, and the mitotic index was measured for each genotype (11, 35). We observed increased cell arrest at mitosis in the presence of colcemid in all genotypes (Fig. 7A) without significant difference. We conclude that that Rev7<sup>C70R</sup> is unlikely to be involved in the regulation of the spindle assembly checkpoint.

**Rev7<sup>C70R</sup> Disrupts Interaction with REV3**—Therefore, we focused on the possibility that the Rev7<sup>C70R</sup> mutation affects...
A Mutation in Rev7 Impairs Mouse Development and DNA Repair

interactions with Rev3, the catalytically active subunit of DNA Polζ, and affects TLS activity of the cell. The crystal structure of human Rev7 in complex with the Rev3 fragment documents that Cys-70 contacts Ile-163, Ile-166, and Trp-171 of Rev7, which is adjacent to Pro-1880 of Rev3 (Fig. 7B). Thus, the C70R mutation of Rev7 would significantly perturb the interaction between Trp-171 of Rev7 and Pro-1880 of Rev3, which is crucial for Rev7-Rev3 interaction, and the C70R mutation could disrupt the formation of DNA Polζ. To confirm whether C70R disrupts Rev7-Rev3 interactions, we examined the interaction of Rev7 with the Rev3 fragment using an in vitro binding assay (Fig. 7B). The mutant Rev7 protein with C70R bound little if at all to the Rev3\(^{1847–1898}\) fragment, whereas normal Rev7 protein showed a strong signal for binding to the Rev3\(^{1847–1898}\) fragment. Thus, we conclude that the Rev7\(^{C70R}\) mutation significantly impairs Rev3 interactions that form the DNA Polζ complex, resulting in defective TLS activity.

Rev7\(^{C70R}\) Mutation Sensitizes Embryonic Cells to ICL Agents—
The Rev family of proteins is uniquely associated with protection against cisplatin- and mitomycin C-induced DNA damage and repair of ICL in human cells as well as in chicken DT40 cell lines (11, 36). We, therefore, investigated whether the Rev7\(^{C70R}\) mutation sensitizes cells to a particular type of DNA damage similar to that observed in siRNA-directed knockdown of Rev7 in human (36) or nasopharyngeal carcinoma cells lines (37). Relative cell survival rates of normal, Rev7\(^{−/−}\), and Rev7\(^{C70R/C70R}\) MEFs were measured with microculture tetrazolium assays after treatment with various concentrations of mitomycin C. The cytotoxicity of mitomycin C is ascribed largely to its capability to generate ICLs in DNA (38). Unlike normal controls, Rev7\(^{C70R}\) mutant cells were hypersensitive to ICL-inducing mitomycin C (Fig. 7C). These findings indicate that the disruption of Rev7-Rev3 interaction caused by the Rev7\(^{C70R}\) mutation increases the susceptibility of cells to agents that cause ICL damage.

Analysis of γH2AX and 53BP1 Localization in Response to ICL Agents—Because Rev7\(^{C70R/C70R}\) cells showed significant sensitivity to DNA-damaging agents, we next investigated the efficiency of DNA repair systems in mutant cells under induced DNA damage. DNA damages, including DSB, can be visualized as subnuclear foci of γ-H2AX and/or 53BP1. The γH2AX foci were dramatically increased in Rev7\(^{C70R/C70R}\) cells. Cells were classified according to the number of γH2AX foci per nucleus, and when uniform staining of the entire nucleus without foci was observed, the cells were classified as displaying pan-nuclear (PN) staining (Fig. 8A). Although <20% of normal and Rev7\(^{C70R/+}\) cells had PN staining, >60% of Rev7\(^{C70R/C70R}\) cells had PN staining (Fig. 8B), indicating accumulated DNA damage in the mutant cells after mitomycin C treatment. As a result of increased ratio of PN staining, <20% of Rev7\(^{C70R/C70R}\) nuclei showed 0–30 γH2AX foci, whereas ~50% of normal and Rev7\(^{C70R/+}\) cells showed 0–30 γH2AX foci (Fig. 8B). Interestingly, when the cells were double-stained with antibodies to γH2AX and 53BP1, a significant portion of the γH2AX foci in nucleus with low density foci co-localized with 53BP1, but the PN-stained cells showed no 53BP1 co-localization (Fig. 8C). Because γH2AX is accumulated at the site DNA damages, including DSB and ICL, which cause stalling of replication fork, 53BP1 protein is mainly enriched at sites of DSB (39). Therefore, the PN staining of γH2AX

![Figure 7. Rev7\(^{C70R}\) mutation disrupts DNA polymerase/(Polζ) formation and sensitizes cells to ICL agents.](image-url)
without 53BP1 focus observed in the present study is suggested to represent replication fork stalling caused by ICL rather than DSB.

**Rev7 Is Required for S Phase Repair of ICL Damage**—To determine the cell cycle phase that requires DNA Pol H9256, we performed cytometry analysis in normal, Rev7 C70R / H11001, and Rev7 C70R / C70R cells. MEFs were stained with γH2AX antibody and counterstained with propidium iodide to determine the cell cycle stage 16 and 48 h after mitomycin C treatment (33). Control cells from each genotype without mitomycin C treatment had weak or negative signals. However, a striking difference was observed after treatment with mitomycin C. At 16 h, a large number of cells with strong positive signals for γH2AX corresponding to PN staining was observed in all genotypes. Forty-eight hours after mitomycin C treatment, the number of strongly positive cells decreased dramatically in normal and Rev7 C70R / H11001 cells, indicating DNA repair and cell cycle resumption. However, Rev7 C70R / C70R cells remained strongly positive for γH2AX staining, and the portion of cells in S phase was dramatically increased compared with that in normal cells (Fig. 9, A and B), indicating stalling of the cell cycle at S phase. An increased number of TUNEL-positive cells was also observed in Rev7 C70R / C70R MEFs after mitomycin C treatment (Fig. 9C). Thus, in the absence of functional DNA Pol H9256, Rev7 C70R / C70R cells do not undergo ICL repair, increasing apoptosis after mitomycin C treatment and suggesting hypersensitivity to ICL-inducing agents.

**DISCUSSION**

Homozygous repro22 mice display growth retardation, germ cell depletion, and sterility. Using positional cloning, we characterized repro22 as a C70R missense mutation in a highly conserved region of the HORMA (DNA binding) domain of the Rev7 gene. Mouse Rev7 has significant homology with yeast rev7, a member of the yeast rev family of genes including rev1, rev3, and rev7. The encoded REV7 protein interacts with REV3 to form DNA Pol H9256, a critical component of TLS (7, 40). The structure of human REV7 in complex with a REV3 fragment has been defined (32), and replacement of Cys-70 with Arg introduces an elongated and positively charged side chain predicted to interfere with normal interactions within the REV7 safety belt and prevent binding to REV3. This outcome was confirmed experimentally by the observation that REV3 bound to REV7, but not to Rev7 C70R, when co-expressed in bacteria. The REV7-REV3 interaction is indispensable for REV3 function (32). Thus, the Rev7 C70R mutation is predicted to affect Pol H9256 catalytic activity and prevent the TLS activity required to repair DNA damage and necessary to ensure cell cycle progression and proliferation during development. Beyond TLS activity, Rev7 is widely accepted to be a multifunctional protein acting as an adaptor in various biological pathways. Rev7 interacts with various proteins, including the Shigella effector IpaB in bacterial invasion of host cells, and is an activator of APC by interacting with Cdh1, Cdc20, or both (23). We examined whether Rev7 C70R mutation displays defects in spindle assembly checkpoint function by treating the MEFs with colcemid and observed no difference between normal and Rev7 C70R / C70R MEFs. Because colcemid is a microtubule-depolymerizing drug and could trigger the spindle assembly checkpoint, these data indicated no defect related to APC function of Rev7.
A significant number of DNA lesions occur daily in every cell, and the majority of them stall DNA polymerases, resulting in DNA replication stress. As a curative measure, canonical polymerases can be replaced by TLS polymerases that are adapted to correct replicative defects including those arising as interstrand adducts. This formulation is consistent with our experimental observation that reduction of Polε/H256 in Rev7/C70R cells leads to increased susceptibility to the DNA-damaging agent mitomycin C, which catalyzes ICL. Therefore, we conclude that the inability of Rev7/C70R cells to correct DNA lesions resulted in DNA replication stress.

The repair of ICL is not well understood in mammals but involves a recombination-independent pathway in the G1 phase and a replication- and recombination-dependent pathway in the S phase of the cell cycle (41, 42). The Fanconi anemia (FA) pathway plays a pivotal role in resolving ICL at S phase and acts upstream of TLS DNA polymerases (Fig. 8). The FA core complex contains an E3 ubiquitin ligase that is recruited to the DNA lesion. Mono-ubiquitination of FANCD2 and FANCI (FA II) coordinates endonucleolytic cleavage of the blocked replication fork followed by unhooking that leaves the cross-linked nucleotide tethered to the complementary strand that is then bypassed by TLS DNA polymerase (41–43). Polη, Polθ, and Polζ have been implicated in these pathways (42), and reconstitution of ICL repair in vitro has documented that depletion of Polζ impairs the process in Xenopus laevis egg extracts (44). We found that Rev7/C70R cells are hypersensitive to ICL agents, displaying increased DSBs and replication stalling in S phase. Involvement of Polζ in ICL repair has also been reported in HeLa cells using small interfering RNA technology (36). The processing of ICLs in G1 phase involves nucleotide excision repair and endonucleolytic cleavage of ICL. In addition to the FA core complex, PCNA becomes mono-ubiquitinated in response to DNA damage, which allows loading of y-family TLS polymerases and bypass of the lesion followed by nucleotide excision repair of the damaged DNA (Fig. 8A) (43, 45). Whether Rev7/C70R mutant cells bypass the G1 phase of ICL repair is unknown. However, because Polk is known as a primary participant in G1 phase ICL DNA repair (46), Rev7/C70R mutant cells are unlikely to have G1 phase ICL repair defects caused by a lack of Polζ function in replication-independent ICL repair.

Among genetically targeted mice with germ cell depletion, mutations affecting the FA complex proteins FANCL, FANCC, and FANCA show phenotypes similar to those of Rev7/C70R mice, with drastic reduction of germ cells proliferation and infertility (47–52). In particular, FancL mutant mice (51) and Usp1−/− Fancd2−/− double-mutant mice (52) show the most severe phenotypes similar to those observed in Rev7/C70R mutant mice. Unlike Rev7/C70R, mutations in other TLS polymerases do not affect PGC development (53–58). In addition,
similar to cells with the Rev\(^{7C70R}\) mutation, cells derived from FA and Usp1 null mutants are sensitive to ICL-inducing agents. Our data show that the lack of REV7 function results in accumulation of H2AX without 53BP1 colocalization at S phase, which could represent unresolved replication-fork stalling caused by DNA damages (39). These findings indicated that S phase ICL repair requires Rev7 and suggest that DNA Pol\(^\eta\) functions downstream of the FA pathway. The more severe phenotypes of Rev7\(^{C70R}\) mutant mice show that although some redundancy might occur in the FA and Usp1 families of genes, the TLS polymerases downstream of these pathways are restricted to control by Pol\(^\eta\). Interestingly, other TLS polymerases, including Polk, the primary participant in G\(_1\) phase ICL DNA repair, showed neither defects related to fertility nor developmental defects.

A recently reported targeted mutation of Rev7 (Rev7\(^{NA}\)) (59) shows phenotypes similar to those of Repro22 mice, with growth retardation and embryonic loss of germ cells. That report and our own show lost PGCs during development from E8.5 to E13.5, reduced body size, and embryonic lethality. Moving beyond the common phenotypes observed in both cases and taking advantage of a point mutation in Rev7, we performed additional functional analysis of Rev7, the findings of which are summarized as below.

We studied formation of PGCs at E7.5 and observed that it is unaffected by Rev7\(^{7C70R}\) mutation, which demonstrates that Rev7 is not involved in PGC formation. In addition, a previous study (59) could not show any clear expression of Rev7 in PGCs during development. Instead, we isolated PGCs from both male and female embryos during development from E7.5 to E14.5.
and clearly show that Rev7 is expressed during that period and that the expression was absent on E14.5 coincident with a well known PGC proliferation stage.

PGC development has three important steps, namely formation, migration, and proliferation. We found that the Rev7\textsuperscript{C70R} cells are proliferation-deficient through three pieces of evidence obtained in the present study. First, accumulated cells arrested at S phase of the cell cycle and strongly expressed γH2AX and displayed widespread apoptosis. Second, although Rev7 is expressed ubiquitously in various tissues, quiescent cells are not affected by the Rev7 mutation; it affects mainly proliferative cells during embryonic development. Third, the expression pattern in PGC development is consistent with the well known period of proliferation between E7.5 and E13.5. The expression ceased on E14.5, when the proliferation of PGCs stopped in both male and female mice. Rather than the proposed defect in germ cell maintenance (34), our results with Rev7\textsuperscript{C70R} mice indicate a defect in proliferation secondary to absent TLS activity. Consistent with our results, conditional targeting of Rev3 in skin epithelia (60) does not affect survival but is essential for responses requiring rapid proliferation of epithelial cells. Also notable is that the present findings indicated decreased growth ratio of the Rev7\textsuperscript{C70R} MEFs and increased apoptosis in Rev7\textsuperscript{C70R} intestinal epithelial cells, which are among the most rapidly proliferating cells. These findings also support our hypothesis that the rev7 mutation mainly affects rapidly proliferating cells and accounts for growth retardation and increased embryonic lethality observed in Rev7\textsuperscript{C70R/C70R} mice.

The point mutation of the Rev7\textsuperscript{C70R} disrupts its interaction with Rev3, therefore, impairing Polζ formation. We next excluded the possibility that this point mutation might affect another proposed function of Rev7 as an APC activator. We found that Rev7\textsuperscript{C70R} mutant cells are hypersensitive to ICL agents and that ICL repair is abrogated at S phase in Rev7\textsuperscript{C70R/C70R} cells, suggesting that ICL repair at this stage requires Rev7 and Polζ. Interestingly, the phenotypes in repro22 mice were similar to those of mice with mutations in the FA pathway. This pathway is well known for its function in replication-dependent ICL repair during S phase, which acts upstream of a TLS polymerase. We, therefore, show that the TLS polymerase required for replication-dependent ICL repair is Polζ.

Using the results of the present study, we proposed a model (Fig. 10) in which Polζ is required for ICL repair at S phase of the cell cycle and acts in concert and downstream of the FA pathway. Rev7\textsuperscript{C70R/C70R} mice will provide an excellent model for investigation of the function of Polζ in genome stability in both somatic and germ cells.

Acknowledgments—We thank Dr. J. Dean for critical discussions and editorial comments. We thank Drs. J. J. Eppig, M. A. Handel, and J. C. Schimenti for providing the repro22 mutant mice produced by the ReproGenomics Program at The Jackson Laboratory. We also thank Maho Maekawa for technical assistance.

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