

Supporting Information

REV7 Safeguards Spermatogonial Stem Cell Genome Integrity through Non-Homologous End Joining

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Supplementary Tables

Table S1: Baseline clinical characteristics of the recruited patients.

Clinical Characteristics	NOA Group (n = 3)	OA Group (n = 3)	P Value
Age (years)	29.00 ± 6.93	42.33 ± 12.01	0.189
Testosterone (nmol/L)	7.29 ± 1.60	13.30 ± 2.87	0.709
Luteinizing Hormone, LH (U/L)	11.89 ± 7.75	4.63 ± 3.17	0.242
Follicle-Stimulating Hormone, FSH (IU/L)	24.27 ± 21.26	8.25 ± 8.21	0.323
Estradiol (nmol/L)	0.14 ± 0.08	0.14 ± 0.05	0.942
Prolactin, PRL (ug/L)	10.75 ± 1.28	9.36 ± 4.41	0.647
Left Testis Volume (mL)	12.37 ± 5.29	16.43 ± 9.74	0.569
Right Testis Volume (mL)	12.97 ± 5.69	16.23 ± 8.96	0.627
Average Testis Volume (mL)	12.67 ± 5.47	16.33 ± 9.29	0.594

Note: Continuous variables are presented as mean ± SD. Group comparisons were performed using the Mann-Whitney U test. The FSH levels in the OA group were generally within the normal range, consistent with the endocrine profile of obstructive azoospermia. In contrast, FSH levels were broadly elevated in the NOA group, reflecting compensatory pituitary secretion secondary to impaired inhibin B feedback from the damaged seminiferous epithelium. Notably, one NOA patient (NOA-2) exhibited severely elevated FSH (48.46 IU/L) and LH (20.83 IU/L) alongside a Y chromosome AZFc microdeletion, indicating a dual etiology for the severe spermatogenic failure. All patients underwent micro-TESE, and the collected testicular tissues were evaluated pathologically prior to downstream immunohistochemistry and immunofluorescence analyses.

Table S2: Primers used for mouse genotyping

Primer	Sequence (5'→3')	Use / expected product
Rev7 F1	CCCGGTTTAACTTTGGACACAC	F1/R1 allele PCR; F1/R2 recombination PCR
Rev7 R1	CTTAACATCTCACACACAAGGTGC	F1/R1: WT 125 bp; floxed 200 bp
Rev7 F2	TAGCCTCACTGTGTAGATGGAGA	F2/R2 allele PCR
Rev7 R2	CATAGCTGAGTGGCTACAAGAGA	F2/R2: WT 134 bp; floxed 201 bp; F1/R2 recombinant 200 bp
Vasa-Cre forward	CACGTGCAGCCGTTTAAGCCGCG	240 bp
Vasa-Cre reverse	TTCCCATTCTAAACAACACCCTGAA	240 bp
Stra8-Cre forward	GATGGATTTCGCTCTCTGGTGTAG	443 bp
Stra8-Cre reverse	CCCATTTAATCTCCTCTTCTCCG	443 bp

WT, wild type. Primer pair F1/R2 detects the Cre-recombined allele.

Table S3: RT-PCR primers used to validate primary mouse SSC identity

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Product (bp)
Actb	TGCTGTCCCTGTATGCCTCT	GTGATGTCACGCACGATTTC	222
Klf4	GAAGGGAGAAGACACTGCGT	GTCTAGGTCCAGGAGGTCGT	625
Mycn	GCGGTCACTAGTGTGTCTGT	AGTGGTTACCGCCTTGTGT	393
Pou5f1 (Oct4)	TCTTTCCACCAGGCCCGG	CTCTGCGGGCGGACATGGGGAGATCC	837
Sox2	AGTGGTACGTTAGGCGCTTC	CCCAGCAAGAACCCTTTCCT	547
Nanog	CTGATTCTTCTACCAGTCCC	AAACAGATGCGTTCACCAGATAGCC	380
Ddx4 (Vasa)	AGCATTCCCATTGTATTAGC	AGGCACTTGCCCAACAGCGACA	573
Zbtb16 (Plzf)	ACCCATACTGGCACGGACAT	TGTGAACCCTGTAGTGCGTCTC	346
Dazl	GTTAGGATGGATGAAACCGA	AATCAGATTAAAGCACTGCCCCGAC	739
Stra8	AGGCAACCAACCCAGTGATG	TCCTGTTCTGAATATGAATCTTTGT	156

Gene aliases used in the figures are provided in parentheses. SSC, spermatogonial stem cell.

Table S4: Primers used for ChIP-qPCR

Locus	Forward primer (5'→3')	Reverse primer (5'→3')
AsiSI-flanking DSB locus	CCAGGTCCATCCATTTGGCT	GTCTGGCGGTTCTCAGAAA
ACTB negative-control locus	TGTCCATTCAATCCAGGCC	GTACAGGAATAGCCTCCGCC

The primer pair for the AsiSI-flanking DSB locus is located upstream of the AsiSI cleavage site on chromosome 14. The ACTB locus served as a negative-control region for AsiSI-site enrichment.

Supplementary Figures

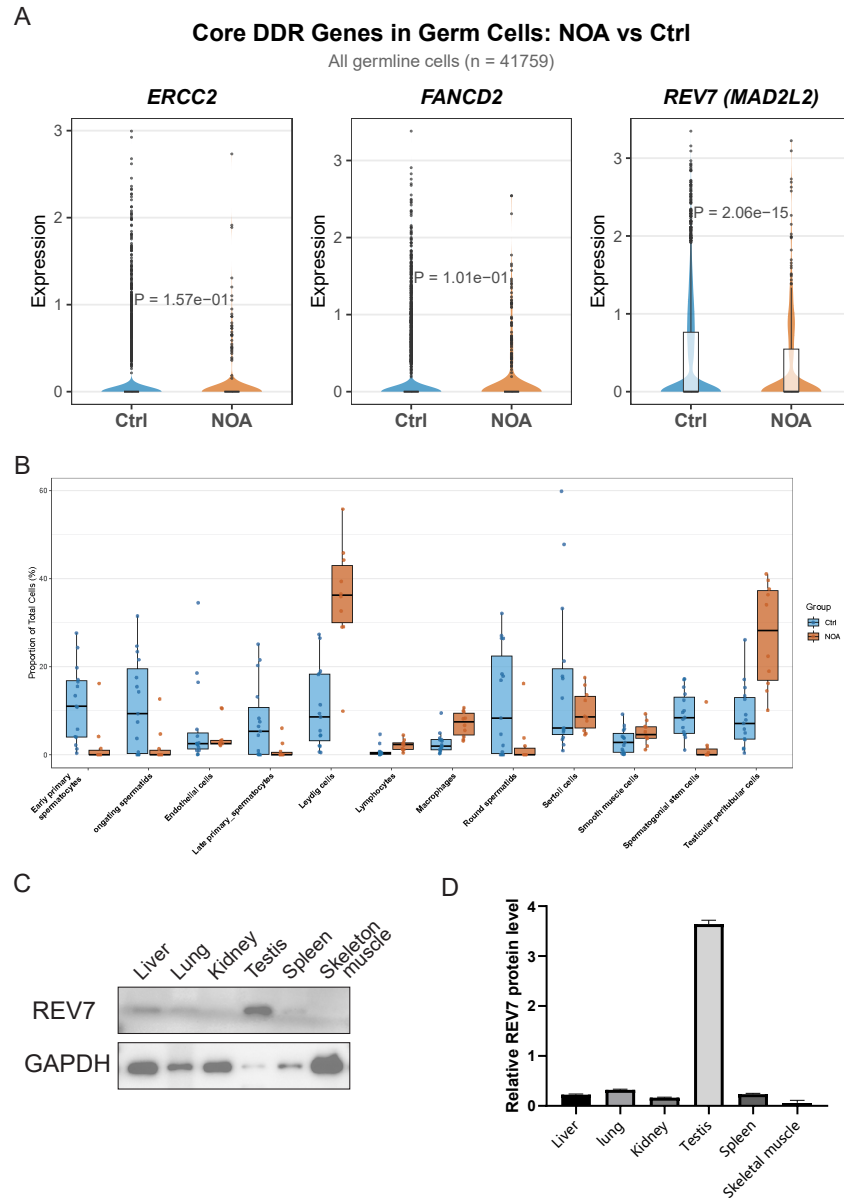


Figure S1: Bioinformatics analysis and tissue-specific expression validation of testis-enriched DDR genes. (A) Violin plots comparing the expression levels of ERCC2, FANCD2, and REV7 (MAD2L2) in germ cells between the NOA and Ctrl groups. The analysis included a total of 129,511 cells (Ctrl: 76,249; NOA: 53,262), from which 41,759 were identified as germ cells (Ctrl: 37,248; NOA: 4,511). The y-axis represents the normalized expression value $\log(\text{Expression} + 1)$. (B) Quantitative analysis of cell proportion shifts. The boxplot compares the absolute proportion of spermatogonial stem cells (SSCs) between CTR and NOA donors, with each dot representing an individual clinical sample. (C) Western blot analysis of REV7 protein levels across various tissues (liver, lung, kidney, testis, spleen, and skeletal muscle) from adult wild-type C57BL/6 mice. GAPDH served as the internal loading control. (D) Semi-quantitative analysis of relative REV7 protein levels in the indicated tissues, confirming the highest expression in the testis. (Data in D are presented as mean $\pm$ SD. Statistical significance was determined by the Wilcoxon rank-sum test for A and B. *** $P < 0.001$).

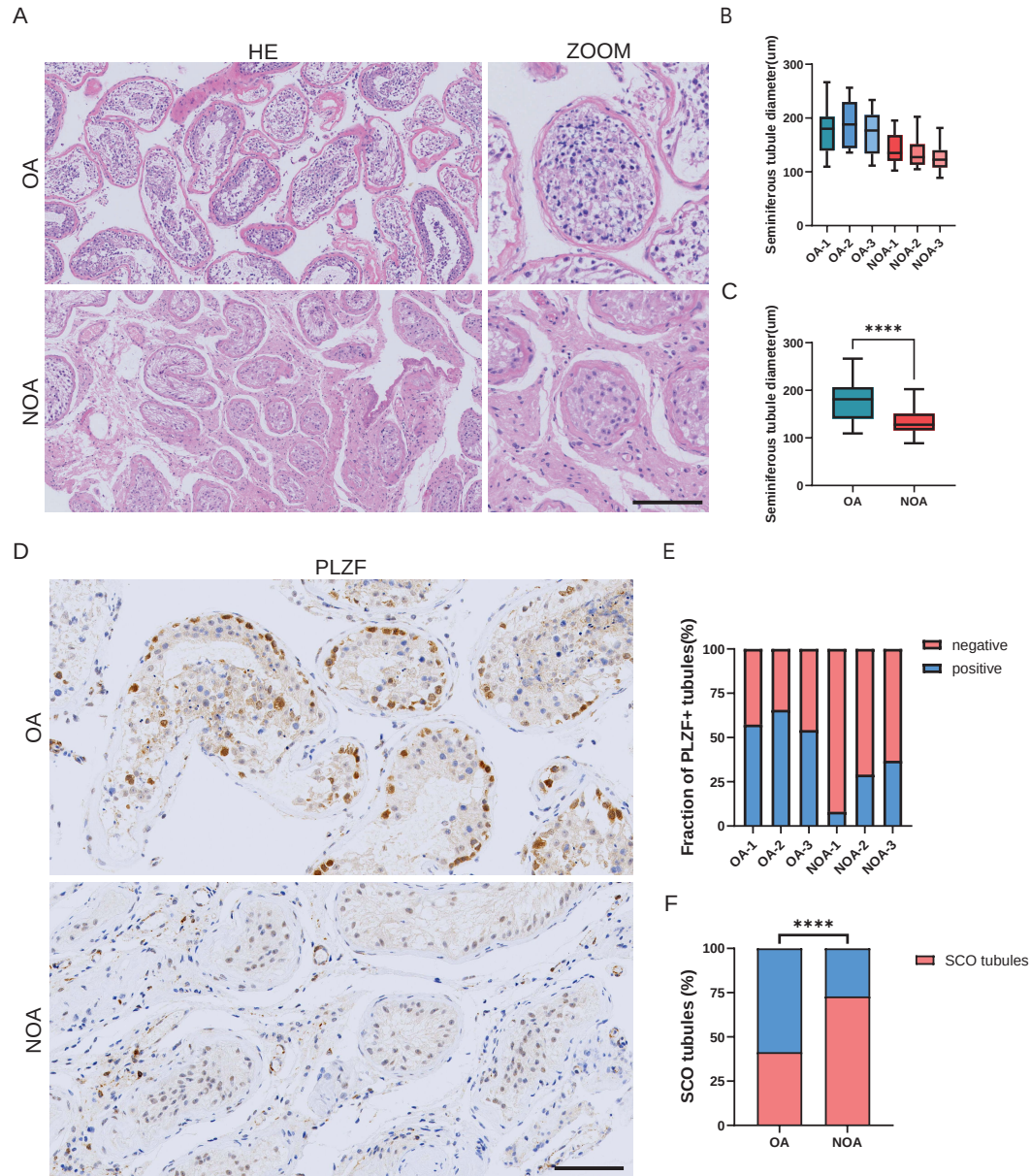


Figure S2: Histopathological evaluation and SSC pool quantification in human testicular biopsies. (A) Representative panoramic hematoxylin and eosin (H&E) staining images (left, scale bar = 100 μ m) and high-magnification views of individual seminiferous tubules (right) from OA ($n = 3$) and NOA ($n = 3$) patients. The NOA group displays a thinned seminiferous epithelium, massive germ cell loss, and an absence of mature spermatozoa. (B) Boxplot depicting individual seminiferous tubule diameters across the six samples ($n = 10$ –20 tubules per sample). (C) Combined quantitative comparison of seminiferous tubule diameters between the OA group ($n = 48$ tubules; $179.9 \pm 41.5 \mu$ m) and the NOA group ($n = 48$ tubules; $134.0 \pm 26.0 \mu$ m). (D) Representative immunohistochemical (IHC) staining images of PLZF in OA and NOA testicular tissues (scale bar = 100 μ m). PLZF⁺ SSCs (brown nuclear staining) are localized at the basement membrane. (E) Stacked bar charts illustrating the individual quantitative analysis of the fraction of PLZF⁺ (positive) and PLZF⁻ (negative) tubules across the three OA and three NOA samples. (F) Combined stacked bar chart comparing the percentage of PLZF⁺ tubules versus Sertoli cell-only (SCO) tubules between the OA (44.2%, 250/565 tubules) and NOA (27.1%, 70/258 tubules) groups. (Boxplots in B and C show the median line, IQR box, and 1.5xIQR whiskers. **** $P < 0.0001$. Statistical significance was determined using the Mann-Whitney U test for C and the Chi-square test for E and F).

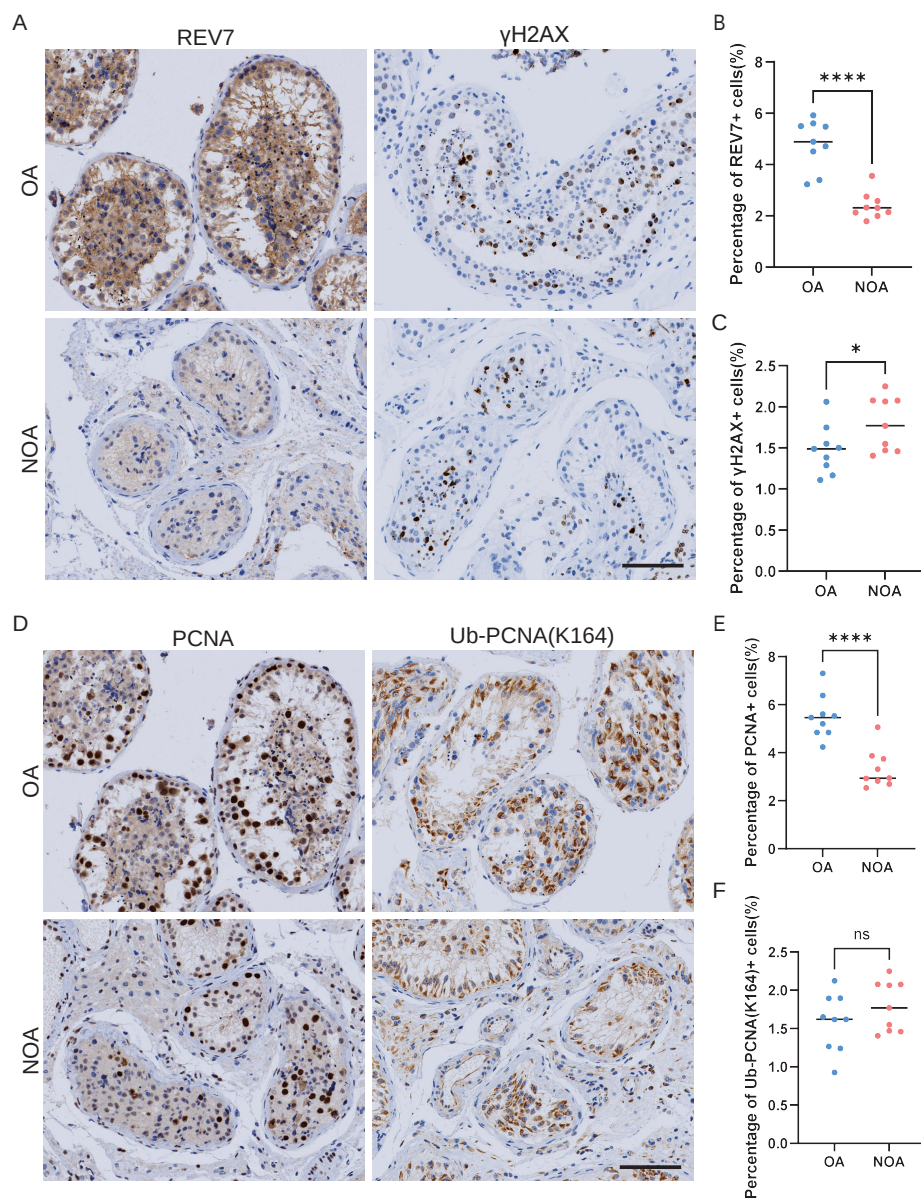


Figure S3: Immunohistochemical evaluation of REV7, DNA damage, and proliferation markers in human testes. (A) Representative IHC staining images of REV7 (left) and γH2AX (right) in testicular tissues from OA and NOA patients (scale bar = 100 μm). (B, C) Quantitative statistical analysis of the positive area percentage (PAP) for REV7 and γH2AX, respectively. (D) Representative IHC staining images for PCNA (left, a proliferation marker) and Ub-PCNA (K164) (right, a TLS pathway marker) in OA and NOA testicular tissues (scale bar = 100 μm). (E, F) Quantitative statistical analysis of the PAP for PCNA and Ub-PCNA (K164), respectively. (Data are presented as mean ± SD. $P < 0.01$, *** $P < 0.001$, ns: not significant. Statistical significance was determined using the Mann-Whitney U test).

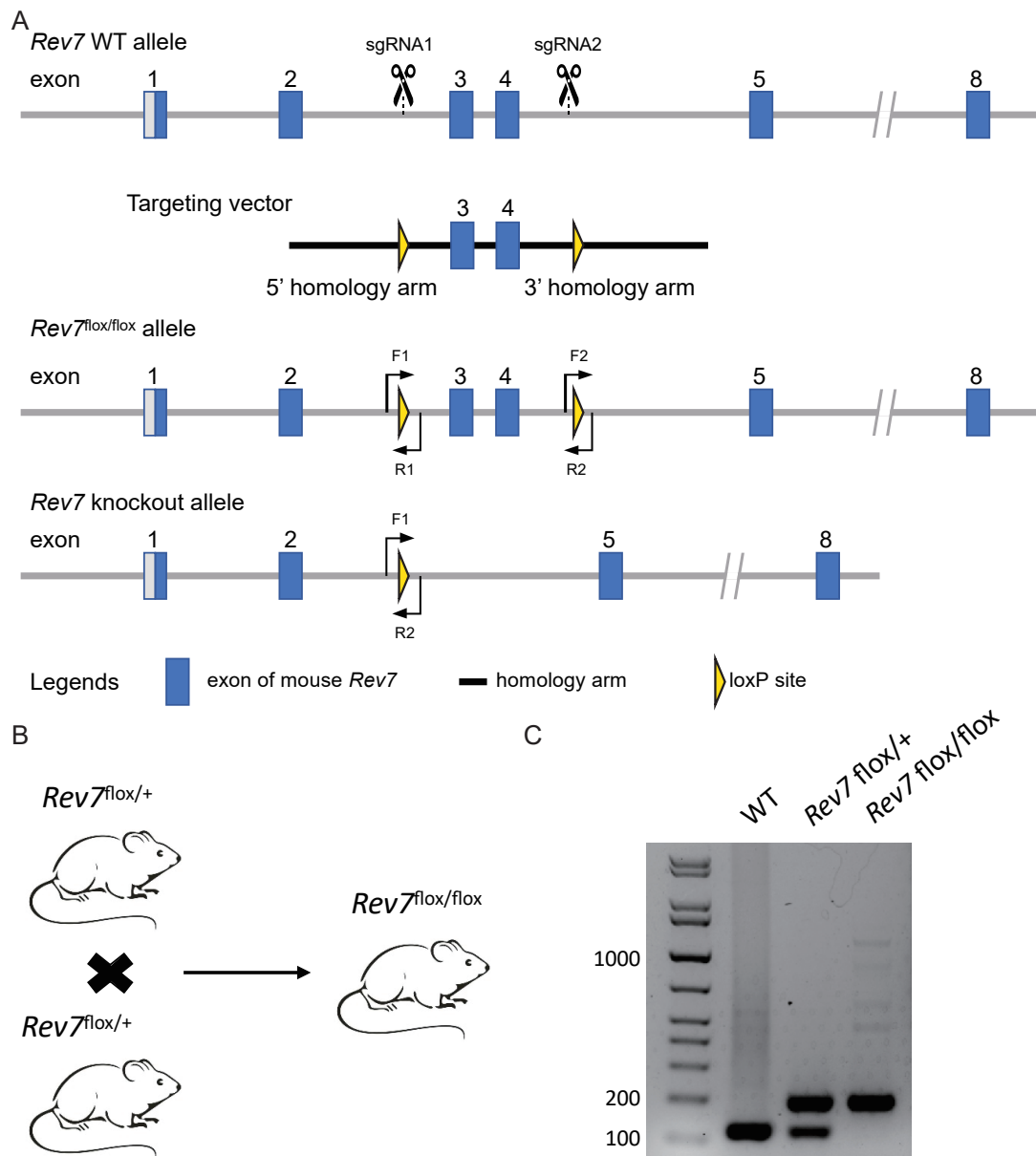


Figure S4: Generation and genotyping of the *Rev7* conditional knockout mouse model. (A) Schematic diagram of the gene targeting strategy for the *Rev7* floxed allele. LoxP sites were inserted flanking the critical exons (Exon 3 and 4) of the *Rev7* gene using CRISPR/Cas9. (B) Schematic illustration of the breeding strategy utilizing *Rev7^{flox/+}* heterozygous intercrosses to generate homozygous *Rev7^{flox/flox}* mice. (C) Representative agarose gel electrophoresis for PCR genotyping, demonstrating the size differences among the WT band, the flox band, and the heterozygous double bands.

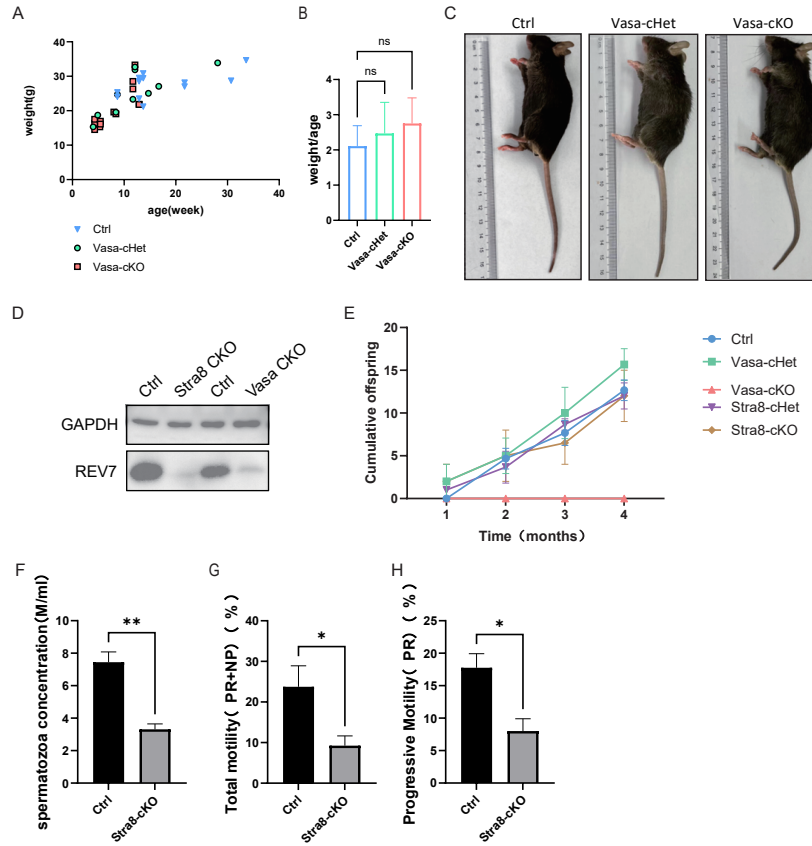


Figure S5: Growth, development, and reproductive phenotypes of Vasa-Cre and Stra8-Cre mediated Rev7 conditional knockout mice. (A) Body weight growth curves of Ctrl (representing the *Rev7^{f/f}* genotype, serving as the control; $n = 12$), heterozygous knockout (Vasa-Het, $n = 10$), and homozygous knockout (Vasa-cKO, $n = 13$) male mice monitored from 4 weeks of age. (B) Statistical comparison of the weight growth rate (g/week) among the three genotypes. (C) Representative macroscopic appearance of age-matched adult male mice across the three genotypes. (D) Western blot validation of REV7 protein deletion in the testicular tissues of Ctrl, Stra8-cKO, and Vasa-cKO mice, with GAPDH as the internal control. (E) Line graph showing the cumulative number of offspring from Ctrl, Vasa-cHet, Vasa-cKO, Stra8-cHet, and Stra8-cKO adult male mice ($n = 3$ per group) continuously mated with wild-type (WT) females for 4 months. (F–H) Quantitative evaluation of epididymal sperm parameters comparing Ctrl ($n = 4$) and Stra8-cKO ($n = 4$) adult males, including sperm concentration (F), total motility (G), and progressive motility (PR) (H). (Data are presented as mean $\pm$ SD. * $P < 0.05$, $P < 0.01$, ns: not significant. Statistical significance was determined using Student's t-test).

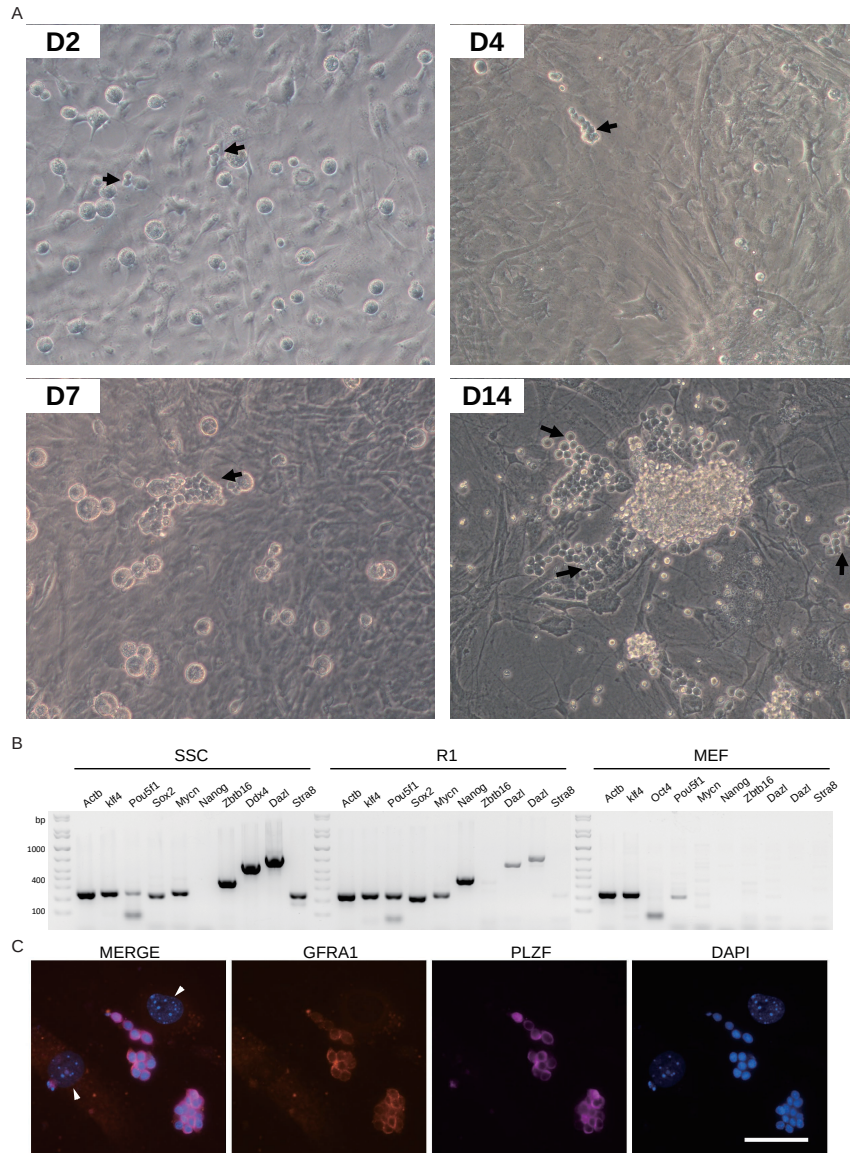


Figure S6: Isolation, culture, and molecular characterization of primary mouse spermatogonial stem cells (mSSCs). (A) Representative bright-field morphological images of primary mSSCs cultured in vitro at day 2 (D2), day 4 (D4), day 7 (D7), and day 14 (D14). The number and size of the colonies gradually increased over the extended culture period (200 $\times$). (B) RT-PCR validation of mSSC-specific gene transcription. The mRNA expression levels of co-expressed genes (*Actb*, *Klf4*), stemness markers (*Oct4*, *Sox2*, *Mycn*, *Nanog*), and germ cell-specific markers (*Plzf*, *Vasa*, *Dazl*, *Stra8*) were analyzed in mSSCs, utilizing mouse embryonic stem cells (R1) and mouse embryonic fibroblasts (MEFs) as controls. (C) Immunofluorescence identification of mSSC-specific protein expression. Representative images demonstrate that the colony-forming round mSSCs highly express the membrane receptor GFR α 1 and the nuclear transcription factor PLZF. MEFs in the background (indicated by white arrows) serve as negative controls (scale bar = 50 μ m).

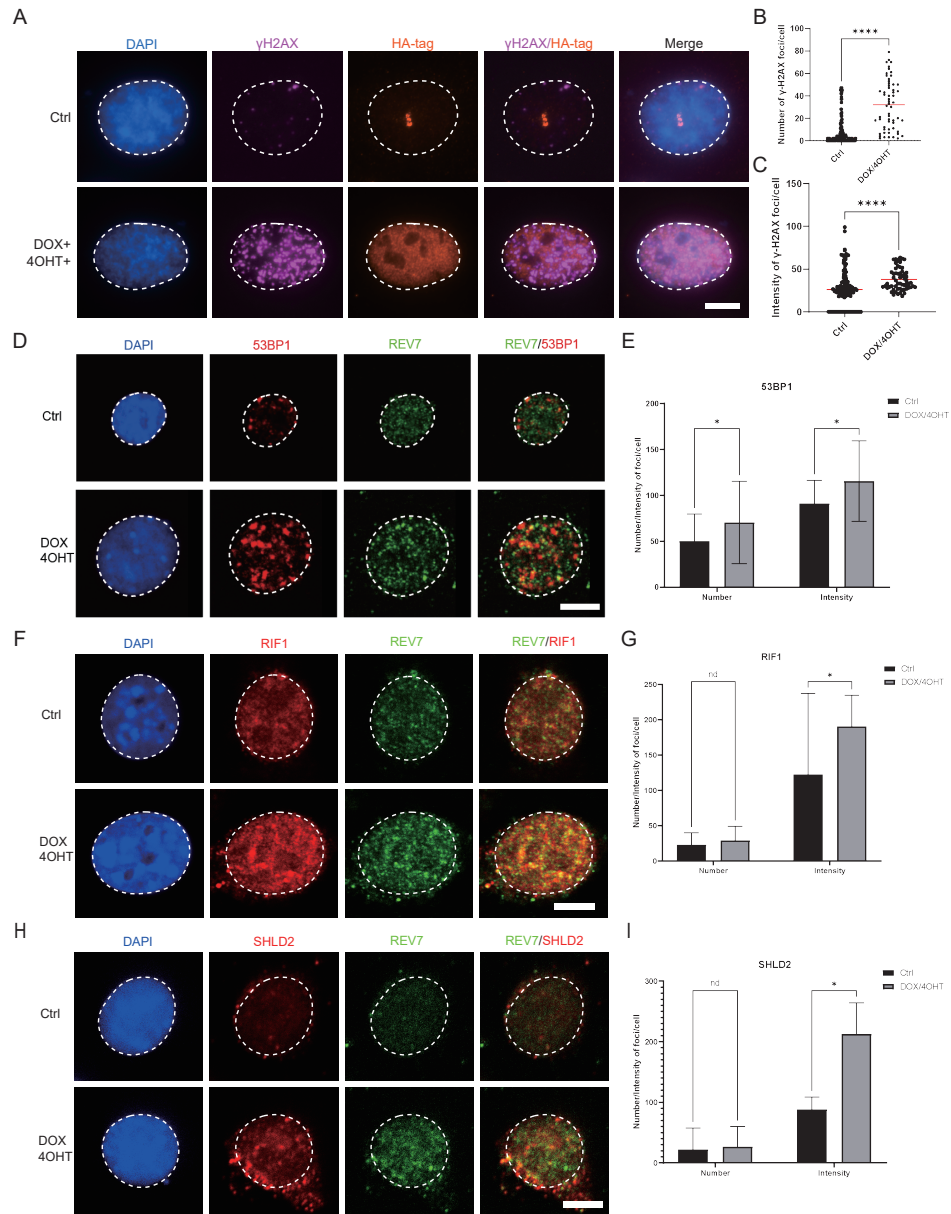


Figure S7: Co-localization of REV7 with the 53BP1-RIF1-Shieldin axis at targeted DNA double-strand breaks. (A) Representative high-magnification immunofluorescence images of individual C18-4 cells displaying the co-staining of HA-tag (marking ER-AsiSI, red) and γ H2AX (purple) following DOX and 4-OHT treatment, compared to a DMSO control (scale bar = 10 μ m). (B, C) Quantitative statistics of γ H2AX foci number and average fluorescence intensity in HA⁺ versus HA⁻ (control) cells, verifying the efficiency of the ER-AsiSI targeted DSB induction system. (D) Representative immunofluorescence images of REV7 (green) and 53BP1 (red) following DSB induction (scale bar = 10 μ m). (E) Quantitative analysis of 53BP1 foci number and fluorescence intensity. (F) Representative images of REV7 (green) and RIF1 (red) co-staining following DSB induction (scale bar = 10 μ m). (G) Quantitative analysis of RIF1 foci number and fluorescence intensity. (H) Representative images of REV7 (green) and SHLD2 (red) co-staining following DSB induction. (I) Quantitative analysis of SHLD2 foci number (left) and average fluorescence intensity (right). (Data are presented as mean $\pm$ SD. * $P < 0.05$. Statistical significance was determined using the Mann-Whitney U test).