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

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Abstract

Spermatogonial stem cells (SSCs) sustain lifelong sperm production, yet the mechanisms that protect their genomes remain incompletely understood. Integrated human testicular transcriptomic and proteomic analyses identify REV7, encoded by *MAD2L2*, as a testis-enriched DNA damage response factor. REV7 expression is reduced in residual SSCs from men with non-obstructive azoospermia. Germline-specific *Rev7* deletion in mice depletes the SSC pool, disrupts spermatogenesis, and causes infertility. Following DNA double-strand break induction, REV7 rapidly accumulates at damage sites. REV7 deficiency impairs SSC proliferation and stemness, delays DDR signal resolution, increases DNA damage, disrupts cell-cycle progression, and promotes apoptosis. Mechanistically, REV7 supports SHLD2 of Shieldin recruitment and favors non-homologous end joining over homologous recombination. Drug induced *Rev7* deletion in adult SSCs suppresses spermatogenesis-related transcriptional programs before overt stem-cell loss. These findings establish REV7 as a genome-protective factor that links DNA double-strand break repair to SSC homeostasis, spermatogenic capacity, and male fertility.

Keywords: Spermatogonial stem cell | REV7 | Genome stability | Non-homologous end joining | Male infertility

1 Introduction

Male infertility is frequently caused by impaired spermatogenesis, with non-obstructive azoospermia (NOA) representing its most severe clinical manifestation [1, 2, 3]. Although microdissection testicular sperm extraction (micro-TESE) combined with intracytoplasmic sperm injection (ICSI) enables some patients with NOA to achieve biological paternity, these procedures recover residual sperm rather than restore defective spermatogenesis [4, 5]. Moreover, the molecular causes remain unidentified in many patients [6, 7]. Elucidating the mechanisms responsible for maintaining spermatogonial stem cells (SSCs) is therefore important for improving the diagnosis and treatment of male infertility [8, 9].

SSCs sustain lifelong sperm production through coordinated self-renewal and differentiation [10]. DNA damage caused by oxidative stress [11], environmental toxicants [12], aging [13], ionizing radiation, and chemotherapy [14, 15] may impair SSC survival and function. Persistent damage can deplete the SSC pool and lead to oligozoospermia or azoospermia, whereas inaccurate repair in surviving SSCs may transmit genetic alterations to spermatozoa and subsequent generations [16, 14, 17]. Our previous studies showed that mouse SSCs exhibit a distinctive DNA damage response characterized by limited homologous recombination and preferential utilization of 53BP1-dependent non-homologous end joining (NHEJ) [18, 19]. We further identified the NHEJ-mediated pathway may contribute genome maintenance in SSCs [20]. However, the downstream machinery connecting 53BP1 signaling to DNA-end protection remains incompletely understood.

REV7, encoded by *MAD2L2*, is a multifunctional genome-maintenance protein [21]. It participates in translesion DNA synthesis as a component of DNA polymerase ζ , protects stalled replication forks, and forms the Shieldin complex with SHLD1, SHLD2, and SHLD3 [22, 23]. Downstream of 53BP1–RIF1, REV7–Shieldin restricts DNA-end resection and promotes NHEJ over homologous recombination [23, 24, 25].

More importantly, previous studies have relied predominantly on constitutive knockout models or models targeting embryonic primordial germ cells (PGCs). Since *Rev7* deficiency results in PGC depletion before the establishment of the SSC pool, these models may not adequately determine whether REV7 exerts a cell-autonomous genome-protective function in adult SSCs, nor can they fully delineate the mechanisms by which REV7 preserves genome stability in SSCs within the adult testis [26, 27]. Previous studies have further demonstrated that DNA damage responses in SSCs exhibit distinct cell type-, differentiation stage-, and tissue context-dependent characteristics, involving coordinated regulation of DNA repair, cell-cycle checkpoints, and apoptosis [17]. Nevertheless, the molecular mechanisms through which REV7 safeguards SSC genome stability remain poorly understood.

At the human level, nuclear REV7 expression has been detected in germ cells within

non-neoplastic seminiferous tubules [28]; however, existing human studies have been largely descriptive or conducted in the context of testicular germ cell tumors and chemoresistance [29, 30, 31]. Systematic investigations of REV7 expression and function in testicular tissues from infertile men, primary human SSCs, and different forms of spermatogenic failure are still lacking. Therefore, systematic investigation of the molecular and biological functions of REV7 in human male germline stem cells is warranted to clarify its potential contribution to spermatogenic failure and male infertility.

2 Results

2.1 REV7 Is Involved in Male Spermatogenesis and Is Essential for SSC Homeostasis

To identify DNA damage response (DDR) factors with a potential role in spermatogenesis, protein and transcript expression data from three independent datasets (HPA, GTEx, and the Mouse Expression Atlas) were integrated (Figure 1A, B). Venn diagram intersection identified only *ERCC2*, *FANCD2*, and *REV7* (*MAD2L2*) as shared testis-enriched core candidates (Figure 1A). Specifically, *REV7* mRNA exhibited overwhelmingly high expression in the human testis, which was experimentally validated at the protein level in adult wild-type mice (Figure S1C, D).

To elucidate its cell-type-specific role, single-cell RNA-sequencing data from control testes with normal spermatogenesis and testes from patients with NOA were analyzed (Figure 1C, E). Compared with controls, NOA testes showed a marked contraction of the germ-cell compartment and a pronounced shift in cellular composition, including a significant depletion of the SSC/undifferentiated spermatogonia population alongside the loss of later germ-cell populations (Figure 1C, E, F; Figure S1B).

Among the three conserved candidates, *REV7* was selectively downregulated in NOA germ cells, whereas *ERCC2* and *FANCD2* did not differ significantly (Figure 1D; Figure S1A). Cell-stage analysis confirmed reduced *REV7* expression particularly in SSCs and round spermatids (Figure 1I). Consistent with extensive germ-cell loss, NOA testes ex-

hibited broad suppression of programs associated with germ-cell development, spermatid differentiation, and DSB repair (Figure 1G, H, J, K). Together, these data linked reduced *REV7* expression to disruption of the SSC compartment and to a broader failure of genome-maintenance programs in NOA.

2.2 *REV7* Expression in Spermatogonial Stem Cells Is Associated with Male Infertility

To validate the transcriptomic findings, we examined clinically defined testicular specimens from patients with obstructive azoospermia (OA, serving as controls) and NOA (Table S1, Figure S2A–C). Co-immunofluorescence staining showed robust nuclear *REV7* signal in PLZF-positive cells from OA testes, which was markedly diminished in the residual PLZF-positive cells of NOA testes (Figure 2A, C). This overall reduction in *REV7* protein abundance was independently confirmed by broad immunohistochemical (IHC) staining (Figure S3A, B).

This change was not accompanied by a generalized decrease in the upstream DSB-response factor 53BP1, indicating that *REV7* loss represented a selective alteration within the DDR network (Figure 2B, D). In parallel, NOA patients exhibited a significantly contracted SSC pool, elevated global DNA damage (γ H2AX), and decreased proliferation (PCNA) (Figure 2E; Figure S2D–F; Figure S3A, C–E). Notably, the expression of Ub-PCNA (K164), a marker of TLS pathway remained unchanged (Figure S3D, F). Therefore, reduced *REV7* abundance within individual SSCs coincided with substantial contraction of the SSC pool and severe spermatogenic impairment in human NOA.

2.3 Testis-Specific Conditional Deletion of *Rev7* Leads to Infertility through SSC reduction

To determine whether *REV7* is functionally required in the male germ line, we generated two stage-resolved conditional knockout models utilizing *Vasa-Cre* and *Stra8-Cre* drivers (Figure 3A; Figure S4A–C; Figure S5D). Continuous mating assays revealed a striking

functional divergence: while adult *Vasa-Cre; Rev7^{fl/fl}* (Vasa-cKO) males were completely infertile, *Stra8-Cre; Rev7^{fl/fl}* (Stra8-cKO) males retained normal fertility (Figure S5E).

Vasa-cKO mice developed striking testicular and epididymal atrophy with significantly reduced organ indices, whereas the fertile Stra8-cKO mice showed a much milder reduction (Figure 3B–E). Histological analysis confirmed severe spermatogenic failure in Vasa-cKO mice, characterized by atrophied seminiferous tubules, thinned epithelium, and collapsed epididymal lumens devoid of mature spermatozoa (Figure 3F–I). While Stra8-cKO testes retained more intact tubules, quantitative evaluation revealed that their epididymal sperm parameters were still significantly compromised compared to controls (Figure S5F–H).

Crucially, PLZF-positive spermatogonia were significantly depleted in Vasa-cKO testes, accompanied by an increased proportion of Sertoli cell-only tubules (Figure 3J–L). These findings established that early germ-cell deletion of *Rev7* produces profound SSC depletion, providing a cellular basis for the resulting male infertility.

2.4 REV7 Is Involved in the DNA Double-Strand Break Repair Response in SSCs

Because REV7 is a component of DSB repair machinery, we tested whether it responds dynamically to DNA damage in SSCs. Following ionizing radiation *in vivo*, REV7 rapidly accumulated into discrete nuclear foci within GFR α 1-positive SSCs, remaining prominent at 2 h and partially declining by 24 h (Figure 4A–C). To facilitate mechanistic studies, we isolated and validated primary mouse SSCs *in vitro* (Figure S6A–C). The dynamic REV7 response was faithfully reproduced in these cultured cells, where radiation-induced REV7 foci prominently co-localized with the established DSB marker 53BP1 (Figure 4D–F). This demonstrated that REV7 is reversibly mobilized to sites of DNA damage, consistent with its direct participation in the SSC DSB repair response.

2.5 REV7 Regulates Spermatogonial Stem Cell Proliferation and Cell Cycle

To define the cellular consequences of REV7 loss, we depleted *Rev7* in the C18-4 spermatogonial cell line using shRNA (Figure 5A). CCK-8 assays showed progressively impaired expansion of REV7-depleted cells. Notably, when subjected to ionizing radiation, *Rev7*-depleted cells exhibited a profound flattening of the growth curve, indicating significantly enhanced radiosensitivity and susceptibility to growth arrest (Figure 5B). Annexin V/propidium iodide flow cytometry revealed a marked increase in apoptosis after *Rev7* knockdown, alongside a redistribution of the cell-cycle profile characterized by increased G1/S fractions and a reduced G2/M fraction (Figure 5C–F). These findings strongly suggest that REV7 depletion triggers genomic instability and aberrant DDR signaling.

2.6 REV7 Promotes DNA Damage Response and Maintains Genome Stability in SSCs

Under basal conditions, *Rev7* knockdown increased the accumulation of γ H2AX, whereas total H2AX and Ub-PCNA (K164) remained broadly unchanged (Figure 6A, B). By contrast, K164-monoubiquitinated PCNA, a marker associated with translesion synthesis, was not consistently induced. Re-expression of Flag-tagged wild-type REV7 substantially reduced γ H2AX toward the control level, demonstrating the specificity of the damage phenotype (Figure 6C, D).

Following irradiation, control cells mounted a transient γ H2AX response concurrent with significant REV7 protein accumulation (Figure 6E, G). In sharp contrast, REV7-depleted cells displayed a premature γ H2AX increase and sustained signal elevation through 24 h, indicating substantially delayed repair (Figure 6E, F, H). RNA sequencing further revealed broad transcriptional consequences of *Rev7* depletion (Figure 6I, M), highlighting altered programs related to cell-cycle control, DNA replication, chromosome segregation, and p53-associated apoptosis (Figure 6J–P). Together, these data demonstrated that REV7 limits persistent DNA damage and preserves genome-stability

programs in spermatogonial cells.

2.7 REV7 Regulates NHEJ Repair Efficiency in SSCs

Alkaline comet assays confirmed that REV7-depleted cells exhibited markedly longer comet tails and significantly increased tail moments, verifying DNA lesion accumulation (Figure 7A, B). Evaluating repair pathway components showed that *Rev7* knockdown reduced the Shieldin subunits SHLD1 and SHLD2, whereas the upstream factors 53BP1 and RIF1 remained unchanged (Figure 7C, E). After irradiation, core NHEJ factors were reduced in REV7-depleted cells, while BRCA1 and phosphorylated RPA2—associated with DNA-end resection and HR—were increased (Figure 7D, F).

We directly quantified repair pathway usage with dual-reporter assays (Figure 7G). REV7 deficiency significantly reduced normalized NHEJ efficiency, while concurrently increasing HR efficiency (Figure 7H–K). Therefore, REV7 is a critical determinant that shifts DSB repair toward NHEJ in spermatogonial cells.

2.8 REV7 Promotes NHEJ Repair by Facilitating SHLD2 Recruitment to DNA Double-Strand Break Sites

Endogenous co-immunoprecipitation placed REV7 in a complex containing the upstream 53BP1 scaffold and the downstream Shieldin effector SHLD2 (Figure 8A). Using a doxycycline-inducible ER-AsiSI system to generate defined genomic DSBs (Figure 8B; Figure S7A–C), we observed that REV7 rapidly accumulated into distinct nuclear foci that significantly co-localized with γ H2AX, 53BP1, RIF1, and SHLD2 upon targeted cleavage (Figure 8C–F; Figure S7D–I), which provided compelling visual evidence that REV7 operates coordinately within the 53BP1-RIF1-Shieldin axis at actual sites of DNA damage.

While upstream 53BP1 efficiently accumulated at DSBs independently of REV7 expression (Figure 8G, H), targeted ChIP-qPCR demonstrated that robust enrichment of the downstream Shieldin subunit SHLD2 was nearly abolished upon *Rev7* knockdown (Figure 8I). REV7 ChIP-sequencing further confirmed pronounced REV7 enrichment centered on induced AsiSI cleavage sites (Figure 8J). These findings support a mechanism in which

lesion-bound REV7 stabilizes and facilitates the essential engagement of the downstream Shieldin complex at broken DNA ends.

2.9 REV7 Maintains the Spermatogenic Function of SSCs

To distinguish an ongoing requirement for REV7 in adult SSCs from embryonic effects, we acutely induced *Rev7* excision in adulthood before isolating the SSC-enriched THY1-positive population (Figure 9A, B). At the acute analysis time point, the proportion of sorted SSCs did not differ significantly between groups, allowing transcriptional changes to be evaluated before overt population loss (Figure 9C, D).

Gene Ontology and Gene Set Enrichment Analyses on differentially expressed genes revealed a striking and coordinated negative enrichment of programs specifically associated with spermatid differentiation, cilium movement, and overall sperm motility in the knockout group (Figure 9E, F). Thus, acute *Rev7* excision triggers a coordinated collapse of the sperm structural and motility program in adult SSCs, demonstrating a continuous requirement for REV7 beyond early development.

3 Discussion

Disruption of spermatogonial stem cell (SSC) homeostasis is closely associated with male infertility, yet the underlying mechanisms remain incompletely understood [10]. Previous studies have established genome stability as a key requirement for maintaining SSC homeostasis [16]; however, the molecular pathways that preserve genomic integrity in SSCs still require further clarification. In particular, how DNA double-strand breaks (DSBs) are repaired in SSCs and how repair pathway choice influences the stem-cell pool have not been fully resolved. REV7 participates in both translesion synthesis (TLS) and DSB repair pathway selection [36, 21]. In somatic cells, REV7 functions as the regulatory subunit of DNA polymerase ζ and cooperates with additional factors to mediate TLS, allowing replication forks to bypass damaged templates and sustain replication continuity [37]. REV7 also acts downstream of the 53BP1-RIF1 axis as a core component of the Shieldin complex,

where it limits DNA end resection, suppresses homologous recombination (HR) bias, and promotes non-homologous end joining (NHEJ) [23, 24]. These studies have established REV7 as an important regulator of genome stability and therapeutic response in proliferating non-germline cells. Nevertheless, whether REV7 exerts analogous or SSC-specific functions has remained largely undefined. Because SSCs constitute a small fraction of the testicular cell population and are responsible for both self-renewal and differentiation into downstream spermatogenic lineages, defining the relationship among REV7, SSC homeostasis, DSB repair pathway choice, and male infertility has substantial biological and clinical significance.

Previous studies reported that systemic *Mad2l2/Rev7* deficiency leads to a marked reduction in primordial germ cell (PGC) number, germ-cell depletion, and infertility, with proposed mechanisms involving increased apoptosis during PGC migration and cell-cycle abnormalities [26, 27]. However, several important questions remained unresolved. First, those studies primarily focused on embryonic PGC fate determination, maintenance, cell-cycle regulation, and chromatin reprogramming, rather than adult SSC function during postnatal spermatogenesis. Second, PGC-based models did not examine human infertile testicular specimens or determine whether REV7 is altered in the residual SSC population of patients with male infertility. Third, those studies did not clarify how REV7 participates in DSB repair pathway choice in germline stem cells, particularly whether REV7 acts through Shieldin-dependent NHEJ. Fourth, PGCs and adult SSCs differ substantially in developmental timing, niche environment, chromatin state, cell-cycle status, and biological function; therefore, PGC phenotypes alone cannot explain SSC exhaustion, seminiferous tubule collapse, or NOA-associated infertility. In the present study, we addressed these unresolved issues using stage-specific conditional knockout models, adult inducible deletion, SSC functional assays, and DNA repair analyses. More importantly, we directly linked REV7-dependent NHEJ repair with SSC genome stability, stem-cell maintenance, and male fertility, providing a new mechanistic basis for understanding how dysregulated DNA repair contributes to male infertility.

This study was designed as a stepwise translational and mechanistic investigation.

We first used cross-platform human and mouse expression databases to identify REV7 as a conserved testis-enriched DNA damage response (DDR) gene, suggesting that REV7 may participate in mammalian spermatogenesis. We then analyzed human NOA single-cell RNA-sequencing data and connected reduced REV7 expression with germ-cell loss, SSC depletion, and suppression of DNA repair programs. This association was further validated in clinical testicular tissues, where REV7 protein was reduced in PLZF-positive human SSCs from patients with NOA, consistent with our bioinformatic analysis. Functional causality was then examined using testis-specific conditional knockout models. Early germ-cell deletion of *Rev7* mediated by Vasa-Cre caused severe testicular atrophy, depletion of PLZF-positive SSCs, and loss of epididymal sperm, whereas later-stage deletion mediated by Stra8-Cre produced a milder phenotype. This comparison indicates that REV7 is particularly important at or before the SSC stage. We next established *in vivo* and *in vitro* DDR models and demonstrated that REV7 is recruited to nuclear foci in SSCs after DNA damage. We further examined the effects of REV7 on spermatogonial cell proliferation, apoptosis, and cell-cycle progression. Finally, we advanced from phenotype to mechanism through protein expression analyses, comet assays, NHEJ/HR reporter assays, REV7-53BP1-SHLD2 co-immunoprecipitation, and AsiSI-induced site-specific DSB recruitment assays. Together, this evidence chain, spanning database discovery, clinical validation, animal genetics, DDR biology, cellular function, repair pathway selection, and SHLD2-associated NHEJ mechanism, supports the conclusion that REV7 is not only associated with male infertility but is functionally required for SSC genome maintenance.

The experimental design of this study also considered the clinical heterogeneity of male infertility. Male infertility can be classified into three histopathological patterns, namely Sertoli cell-only syndrome, maturation arrest, and focal spermatogenesis [38]. For the NOA group, we selected residual testicular tissues from patients who underwent microdissection testicular sperm extraction (micro-TESE) and were clinically confirmed to have sperm retrieval, representing a focal spermatogenesis pattern. For the control group, we selected residual testicular biopsy tissues from patients with obstructive azoospermia (OA) who had a previous history of fertility and underwent testicular sperm retrieval for

assisted reproduction by intracytoplasmic sperm injection (ICSI). This sampling strategy allowed us to compare residual SSCs in clinically relevant infertile and spermatogenically preserved contexts. In the cellular and animal modeling experiments, the choice of ionizing radiation (IR) dose was guided by previously published studies of testicular DNA damage models [18, 16, 20]. We therefore designed appropriate time points for detecting DNA damage, enabling induction of measurable DSBs while also allowing observation of the early recruitment dynamics of repair proteins.

At the mechanistic level, this study demonstrates for the first time in germline stem cells that REV7 participates in 53BP1-dependent repair pathway choice and promotes NHEJ by regulating the downstream Shieldin subunit SHLD2. This provides an experimental extension of previous findings in non-germline systems [23, 25, 39]. REV7 knock-down reduced SHLD2 engagement at DSB sites in SSCs, thereby impairing NHEJ repair efficiency and leading to increased SSC apoptosis and cell-cycle arrest. By integrating clinical male infertility with the mechanism by which REV7 maintains SSC genome stability through NHEJ repair efficiency, our study reveals that REV7 plays an essential role in preserving the SSC pool. These findings support a model in which REV7 transmits upstream 53BP1-mediated damage recognition to the SHLD2-containing Shieldin complex at broken DNA ends. This process protects DNA ends, restrains BRCA1-associated end resection, and facilitates efficient completion of NHEJ. This mechanism also helps explain why SSCs, many of which reside in G0/G1 or early proliferative states, may be particularly dependent on NHEJ to maintain genome stability.

Several limitations should be acknowledged and addressed in future work. First, studies in somatic cells have shown that REV7 participates in both TLS and DDR processes [36, 21]. In the present study, Ub-PCNA levels were not markedly changed, whereas γ H2AX accumulation and DNA breaks were significantly increased, suggesting that defective DSB repair is the predominant phenotype. Nevertheless, we cannot exclude a role for REV7 in TLS, replication fork protection, or alternative end joining, especially under specific types of DNA damage or at particular cell-cycle stages. Although we focused on the function of REV7 in the SSC NHEJ pathway, the role of REV7 in regulating

TLS in SSCs remains to be experimentally explored. Second, unlike HR-mediated repair during meiotic progression [40, 41], our study demonstrates that SSCs can repair DNA damage through a REV7-mediated NHEJ pathway. However, NHEJ is intrinsically more prone to mutagenic repair. Because SSCs have not yet entered meiosis and cannot rely on homologous recombination for high-fidelity repair, whether this error-prone NHEJ mechanism introduces mutations into germline stem cells and thereby creates heritable genetic risk remains an important question for future studies. Third, Vasa-Cre-mediated *Rev7* deletion caused a sharp reduction in SSC number, yet a small number of PLZF-positive cells remained detectable, while the mice were nevertheless azoospermic. This observation suggests that REV7 likely functions not only in SSC maintenance but also in mitotic amplification of differentiating spermatogonia, meiotic progression, and later spermatogenic stages. The precise mechanisms by which REV7 acts during these stages remain to be clarified.

In summary, based on these experimental results, we conclude that REV7 maintains SSC genome stability by regulating NHEJ repair efficiency. This study reveals a critical role for REV7 in preserving genome integrity in SSCs and, for the first time, directly links REV7-dependent NHEJ repair with SSC homeostasis, spermatogenesis, and male infertility. Loss of REV7 decreases NHEJ repair efficiency, causes persistent accumulation of DNA damage, impairs SSC proliferation and stemness maintenance, and ultimately leads to SSC exhaustion and male infertility. These findings not only expand our understanding of SSC-specific DNA damage repair mechanisms but also provide a new theoretical basis for explaining the molecular etiology of a subset of idiopathic male infertility. They further offer new directions for future clinical exploration of biomarkers and therapeutic targets for male infertility.

4 Materials and methods

4.1 Cross-Database Identification of Testis-Enriched DNA Damage Response Genes

Expression profiles for 35 core DNA damage response (DDR) genes were compared across the Human Protein Atlas (HPA) [28], GTEx v8 [42], and the EBI Expression Atlas [43]. For HPA normal-tissue immunohistochemistry, staining categories were converted to an ordinal score (not detected, 0; low, 1; medium, 2; high, 3), and the highest reported score was retained when more than one measurement was available for a gene–tissue pair. Human transcript abundance (nTPM) was obtained for 36 GTEx tissues, standardized by gene as a Z score, and truncated at ± 3 for visualization. A human gene was classified as testis enriched when testis ranked first in nTPM among the evaluated tissues. Mouse baseline transcript abundance (TPM) was obtained for 104 tissues from the EBI Expression Atlas, averaged across compatible studies, and mapped to human orthologs. Mouse genes were classified as testis enriched when testis ranked first across the evaluated tissues. The intersection of HPA-high proteins, GTEx testis-rank-1 transcripts, and mouse testis-rank-1 transcripts was used to nominate conserved testis-enriched DDR candidates for subsequent single-cell analysis.

4.2 Human Testicular Single-Cell RNA-Sequencing Analysis

Processed UMI count matrices from human testes were obtained from Gene Expression Omnibus datasets GSE149512 (17 donors) [44], GSE254315 (25 donors) [45], and GSE202647 (5 donors) [46], yielding 47 clinical samples (37 controls and 10 patients with non-obstructive azoospermia [NOA]). Analyses were performed in R 4.5.1 with Seurat 5.3.0 [47]. Cells were retained when they contained at least 40 detected genes, 1,000–6,000 total UMIs, and less than 20% mitochondrial transcripts; genes detected in fewer than three cells were removed. Counts were normalized with LogNormalize (scale factor, 10,000), and 2,000 highly variable genes were selected. Dataset-associated effects were integrated with Harmony 1.2.4 [48]. UMAP [49] embedding and Louvain clustering

(resolution, 0.5) were based on the first 20 Harmony-corrected components.

After quality control, 129,511 cells were retained (76,249 control and 53,262 NOA cells). Clusters were annotated from canonical markers and cluster-enriched genes as SSCs, early primary spermatocytes, late primary spermatocytes, round spermatids, elongating spermatids, Sertoli cells, Leydig cells, peritubular cells, smooth muscle cells, endothelial cells, macrophages, or lymphocytes. Low-quality or uncharacterized clusters were excluded. Global differential expression between NOA and control cells was defined by $|\log_2 \text{fold change}|$ greater than 0.25 and Benjamini–Hochberg-adjusted P less than 0.05. Gene Ontology (GO) [50] and Kyoto Encyclopedia of Genes and Genomes (KEGG) [51] enrichment was performed with clusterProfiler [47] using a hypergeometric test and Benjamini–Hochberg correction. Gene set enrichment analysis (GSEA) was used to evaluate coordinated DDR pathway changes.

For the prespecified candidates *ERCC2*, *FANCD2*, and *MAD2L2* (*REV7*), expression in NOA and control cells was compared within predefined cell populations with Seurat FindMarkers using the Wilcoxon rank-sum test ($\logfc.threshold = 0$; $min.pct = 0$). Average $\log_2$ fold change was calculated after applying `expm1` to log-normalized values, followed by averaging and log transformation. Because only three candidates selected independently by the cross-database screen were tested, nominal two-sided P values are reported for this targeted analysis.

4.3 Human Testicular Samples and Ethics

Archived formalin-fixed, paraffin-embedded testicular tissues were obtained from men evaluated for azoospermia at Tongji Hospital, Tongji University. Azoospermia was established based on at least two semen analyses performed at least 2 weeks apart, with no sperm detected after centrifugation. The cohort consisted of two specific groups: men with non-obstructive azoospermia (NOA; $n = 3$) who underwent microdissection testicular sperm extraction (micro-TESE) and were ultimately histologically confirmed to have focal spermatogenesis; and patients with obstructive azoospermia (OA; $n = 3$) who had a prior history of vasectomy and subsequently presented to the Department of Repro-

ductive Medicine at Tongji Hospital with restored fertility desires. The archived residual tissues from these OA patients, obtained following conventional testicular sperm extraction (TESE) procedures, were utilized as controls representing normal spermatogenesis. Age, semen findings, serum hormone profiles (testosterone, luteinizing hormone, follicle-stimulating hormone, estradiol, prolactin), ultrasonographic testis volume, and available genetic findings were abstracted from the medical records. Cohort characteristics are reported in Table Table S1.

The retrospective study was approved by the Ethics Committee of Tongji Hospital, Tongji University (Approval No. SBKT-2026-065). The institutional ethics committee waived the requirement for individual informed consent strictly due to the retrospective nature of the study, as the research exclusively utilized archived residual tissues and de-identified clinical data.

4.4 Animals, Housing, and Ethical Approval

Male mice were maintained in a specific pathogen-free facility at the Animal Center of Shanghai Tongji Hospital at 22 ± 2 °C and $50\% \pm 10\%$ relative humidity under a 12 h light/12 h dark cycle, with food and water available ad libitum. C57BL/6 mice were used for tissue expression profiling and irradiation studies, and DBA/2 mice were used to establish primary mSSC cultures. Conditional *Rev7* mice and Cre-driver lines were obtained or generated through Cyagen Biosciences (Suzhou, China), as documented in the source records. All procedures were approved by the animal ethics committee of Shanghai Tongji Hospital, Tongji University (protocol no. 2026-DWSB-036) and were conducted in accordance with institutional animal-welfare requirements. Only male animals were included because the study addressed spermatogenesis and male fertility.

4.5 Generation of Conditional *Rev7* Mouse Models and Genotyping

A conditional *Rev7* allele was generated by CRISPR/Cas9-mediated insertion of loxP sites into introns 2 and 4, thereby flanking exons 3 and 4. *Rev7* floxed mice were

crossed with *Vasa-Cre* or *Stra8-Cre* mice to delete *Rev7* in early germ cells or differentiating spermatogonia, respectively. Cre-negative *Rev7*^{fl/fl} littermates served as controls. *Vasa-Cre;Rev7*^{fl/+} and *Stra8-Cre;Rev7*^{fl/+} males were designated Vasa-cHet and Stra8-cHet, whereas *Vasa-Cre;Rev7*^{fl/fl} and *Stra8-Cre;Rev7*^{fl/fl} males were designated Vasa-cKO and Stra8-cKO. For acute inducible deletion, *Rev7*^{fl/fl} mice were crossed with H11-CAG-MerCreMer mice.

Genomic DNA from tail or tissue was analyzed by PCR. Primer pair F1/R1 generated a 125 bp wild-type product and a 200 bp floxed product; primer pair F2/R2 generated 134 bp wild-type and 201 bp floxed products. *Vasa-Cre* and *Stra8-Cre* transgenes produced 240 and 443 bp amplicons, respectively. Cre-mediated recombination was detected with F1/R2 as a 200 bp product. Primer sequences are listed in Table Table S2.

For the THY1⁺-cell RNA-sequencing cohort, tamoxifen was dissolved in corn oil and administered to adult H11-CAG-MerCreMer;*Rev7*^{fl/fl} mice and control littermates on days 0, 2, 4, and 6; testes were collected on day 8, as shown in Figure 9A.

4.6 Fertility Testing and Epididymal Sperm Analysis

Eight-week-old males were housed continuously with proven-fertile wild-type females at a ratio of one male to two females for 4 months. Three males per genotype were evaluated, and the cumulative number of live offspring attributable to each male was recorded. For sperm analysis, the cauda epididymides of adult males were minced in 1 mL of prewarmed low-glucose DMEM and incubated at 38 °C for 40 min to permit sperm dispersal [52, 53, 54]. Sperm concentration, total motility (progressive plus nonprogressive), and progressive motility were quantified with a computer-assisted sperm analysis system. Four control and four Stra8-cKO males were analyzed.

4.7 Tissue Collection, Organ Indices, and Histology

Mice were euthanized at the ages or post-treatment intervals indicated in the figures. Testes, epididymides, and seminal vesicles were dissected and weighed. Organ indices were calculated as organ weight divided by body weight and multiplied by 100%. Testes and

epididymides were fixed overnight in 4% paraformaldehyde or Bouin solution, dehydrated, paraffin embedded, and sectioned at 5 μ m. Sections were stained with hematoxylin and eosin (H&E) using standard procedures.

For seminiferous tubule morphometry, circular or near-circular transverse profiles were selected throughout the section. The long and short axes were measured in ImageJ, and their mean was used as the tubule diameter. Sertoli-cell-only (SCO) profiles were scored from the absence of recognizable germ cells within the seminiferous epithelium. Human and mouse group comparisons were based on the numbers of patients, mice, and tubules stated in the corresponding legends.

4.8 Immunohistochemistry, Immunofluorescence, and Image Quantification

Paraffin sections were deparaffinized, rehydrated, and subjected to heat-mediated antigen retrieval. For immunohistochemistry (IHC), endogenous peroxidase was quenched, nonspecific binding was blocked, and sections were incubated with primary antibodies overnight at 4 °C. Bound antibody was detected with an HRP-conjugated secondary reagent and 3,3'-diaminobenzidine, followed by hematoxylin counterstaining. The percentage of immunopositive cells or immunopositive area was measured from fields acquired under matched settings in ImageJ, as specified for each figure.

For immunofluorescence, fixed cells or tissue sections were permeabilized with 0.3% Triton X-100, blocked with 5% bovine serum albumin or normal serum, and incubated overnight at 4 °C with primary antibodies. Alexa Fluor-conjugated secondary antibodies were applied for 1 h at room temperature, and nuclei were counterstained with DAPI. Primary targets included PLZF (Santa Cruz Biotechnology), REV7 (Invitrogen), 53BP1 (Cell Signaling Technology), GFR α 1 (Santa Cruz Biotechnology), γ H2AX (Cell Signaling Technology), PCNA (Santa Cruz Biotechnology), Ub-PCNA (K164) (Cell Signaling Technology), RIF1 (Abcam), SHLD2 (Invitrogen), or HA-tag (Abcam). Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific) were applied for 1 h at room temperature. Nuclei were counterstained with DAPI. Images were acquired with a Leica

STELLARIS 5 confocal microscope or a Nikon ECLIPSE Ni-E fluorescence microscope. Acquisition settings were held constant within each comparison. Nuclear foci were quantified per cell and fluorescence intensity was measured after background subtraction using a common threshold. Only global adjustments applied uniformly to the compared images were used for display.

4.9 Cell Line Culture and Primary mSSC Isolation

The mouse spermatogonial stem cell line C18-4 was cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin at 37 °C in a 5% CO₂ humidified incubator. For primary cultures, mSSCs were isolated from the testes of DBA/2 mice using a two-step enzymatic digestion process and maintained on mitomycin C-treated mouse embryonic fibroblast (MEF) feeder layers, following established protocols [55, 56]. The complete mSSC culture medium was formulated based on StemPro-34 Serum-Free Medium (SFM, Thermo Fisher Scientific) supplemented with Glial Cell Line-Derived Neurotrophic Factor (GDNF, 10 ng/mL), basic Fibroblast Growth Factor (bFGF, 10 ng/mL), and Leukemia Inhibitory Factor (LIF, 10³ U/mL) to robustly maintain self-renewal and an undifferentiated state. Mouse embryonic stem cells (R1) and MEFs were cultured in parallel as controls for marker validation.

4.10 RNA Extraction and RT-PCR Validation of mSSC Identity

Total RNA was isolated from primary mSSCs, R1 embryonic stem cells, and MEFs with TRIzol reagent. RNA was reverse transcribed, and endpoint RT-PCR was performed for *Actb* and *Klf4*, the pluripotency-associated genes *Pou5f1* (*Oct4*), *Sox2*, *Mycn*, and *Nanog*, and the germ-cell-associated genes *Zbtb16* (*Plzf*), *Ddx4* (*Vasa*), *Dazl*, and *Stra8*. PCR products were resolved by agarose-gel electrophoresis. Primer sequences and expected amplicon sizes are listed in Table Table S3. MSSC identity was further supported by colony morphology and coexpression of GFR α 1 and PLZF by immunofluorescence.

4.11 Lentiviral Knockdown and REV7 Rescue

Lentiviral vectors encoding three independent short hairpin RNAs against mouse *Rev7* (sh*Rev7* -1, sh*Rev7* -2, and sh*Rev7* -3) or a scrambled control (shCtrl) were produced by Genomeditech (Shanghai, China). Cells were transduced in the presence of polybrene and selected with puromycin. Knockdown was confirmed by immunoblotting before functional analyses. For rescue experiments, Flag-tagged wild-type REV7 was expressed from pInducer20 and introduced with Lipofectamine 2000. Rescue cultures were analyzed in parallel with matched vector controls.

4.12 Ionizing Radiation

Localized testicular irradiation was delivered to adult males at 2 Gy with an RS2000pro biological irradiator while the remainder of the body was shielded. C18-4 cells and primary mSSCs were also exposed to 2 Gy. Samples were collected at the post-irradiation intervals indicated in the figures (typically 0 or untreated, 30 min, 1 h, 2 h, 6 h, and 24 h) for immunofluorescence, immunoblotting, or transcriptomic analysis. Untreated or sham-irradiated samples collected in parallel served as controls.

4.13 Cell Proliferation, Apoptosis, and Cell-Cycle Analysis

For the CCK-8 assay, cells were seeded in 96-well plates in 100 μ L medium per well. At 24, 48, 72, and 96 h, 10 μ L CCK-8 reagent was added and plates were incubated for 1–4 h in the dark. Absorbance at 450 nm was measured with a microplate reader after confirming the absence of bubbles.

For apoptosis analysis, culture supernatants and adherent cells were collected together. After washing, 5×10^4 – 1×10^5 cells were suspended in 195 μ L binding buffer, stained with 5 μ L Annexin V-FITC and 10 μ L propidium iodide for 15 min at room temperature in the dark, and analyzed with a CytoFLEX flow cytometer. For cell-cycle analysis, cells were fixed in ice-cold 70% ethanol for 2–24 h at 4 °C. After washing, cells were suspended in 500 μ L staining buffer containing 10 μ L RNase A and 25 μ L propidium iodide, incubated for 30 min at 37 °C in the dark, and analyzed with a URIT BF-730 flow

cytometer. Cell-cycle distributions were calculated in FlowJo 7.6.1 software (Ashland, OR, USA) using the same gating model across groups.

4.14 Alkaline Comet Assay

DNA strand-break accumulation was measured with an alkaline comet assay kit. Cell suspensions were mixed with 0.7% low-melting-point agarose and layered onto slides pre-coated with 1% normal-melting-point agarose. After gelling at 4 °C, slides were lysed for 2 h at 4 °C in chilled lysis buffer containing 10% dimethyl sulfoxide. DNA was unwound in alkaline buffer for 20–60 min and electrophoresed for 20 min under chilled, light-protected conditions. Slides were neutralized and stained with propidium iodide for 10–20 min. Images were acquired with a Nikon ECLIPSE Ni microscope equipped with a DS-Ri2 camera. Tail length, percentage of DNA in the tail, and tail moment were calculated with CASP software [57].

4.15 Protein Extraction, Immunoblotting, and Co-Immunoprecipitation

Cells or homogenized tissues were lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein concentration was determined by BCA assay. Equal protein amounts were separated by SDS-PAGE, transferred to PVDF membranes, blocked in 5% nonfat milk, and incubated with primary antibodies overnight at 4 °C. HRP-conjugated secondary antibodies and enhanced chemiluminescence were used for detection. Targets included REV7 (Invitrogen/Thermo Fisher Scientific), Ub-PCNA (K164) (Cell Signaling Technology), γ H2AX (Cell Signaling Technology), 53BP1 (Cell Signaling Technology), RIF1 (Abcam), SHLD1 (Abcam), SHLD2 (Invitrogen/Thermo Fisher Scientific), Ku70 (Santa Cruz Biotechnology), Ku80 (Thermo Fisher Scientific), BRCA1 (Santa Cruz Biotechnology), pRPA2 (Cell Signaling Technology), and GAPDH (LABLEAD). Band intensity was quantified in ImageJ and normalized to GAPDH or the indicated loading control.

For endogenous co-immunoprecipitation, precleared C18-4 lysates were incubated overnight at 4 °C with anti-REV7 or species-matched control IgG. Protein A/G magnetic beads

(ABclonal Technology) were added, and immune complexes were washed, eluted, and analyzed by immunoblotting for REV7, 53BP1, and SHLD2. Input lysate was included to document target abundance.

4.16 NHEJ and Homologous Recombination Reporter Assays

NHEJ-GFP and HR-GFP reporters were provided by Zhiyong Mao (Tongji University) [58]. Reporter cleavage was induced with I-SceI. ShCtrl and shRev7 C18-4 cells were transfected with 0.6 μ g NHEJ-GFP or 1.0 μ g HR-GFP together with 0.2 μ g pDsRed-N1 as an internal control, using Lipofectamine 2000 (Invitrogen). After 72 h, GFP-positive and dsRed-positive cells were quantified by flow cytometry. Repair activity was expressed as the GFP/dsRed ratio to normalize for transfection efficiency. Each reporter experiment was performed in three independent replicates and analyzed with a common gating strategy in FlowJo.

4.17 Inducible ER-AsiSI DSB System

The HA-ER-NLS-AsiSI coding sequence was PCR-amplified from D1vA cell genomic DNA and cloned into a doxycycline-inducible (Tet-On) lentiviral vector [18, 59, 60]. A parallel control construct lacking the ER domain was generated to rigorously verify 4-OHT dependency. C18-4 cells were then transduced with this HA-tagged ER-AsiSI construct. This system employs a stringent dual-regulation mechanism: doxycycline induces the expression of the fusion protein, which is actively sequestered in the cytoplasm via its ER domain; subsequent treatment with 4-hydroxytamoxifen (4-OHT) triggers rapid nuclear translocation, leading to AsiSI-mediated, sequence-specific DSB generation genome-wide.

Experimentally, ER-AsiSI expression was induced with 200 ng/mL doxycycline for 24 h, followed by the addition of 500 nM 4-OHT for 4 h to trigger nuclear endonuclease translocation. Vehicle-treated cells were maintained in parallel as negative controls. Successful targeted DSB induction was strictly validated by assessing γ H2AX focus formation specifically within HA-positive nuclei in both transduced U2OS and C18-4 cells. Following targeted activation, these cells were immediately processed for subsequent im-

munofluorescence, ChIP-qPCR, and REV7 ChIP-sequencing analyses.

4.18 Chromatin Immunoprecipitation and qPCR

Control and *Rev7*-depleted ER-AsiSI cells were treated with doxycycline and 4-hydroxytamoxifen as described above. Proteins and DNA were crosslinked with 1% formaldehyde, and the reaction was quenched with . Chromatin was sheared by sonication to 200–500 bp and immunoprecipitated overnight at 4 °C with antibodies against γH2AX (EMD Millipore), REV7 (Abcam), 53BP1 (Invitrogen), and SHLD2 (Invitrogen). Following stringent washing and elution, crosslinks were reversed overnight at 65 °C and DNA was purified.

Recovered DNA was analyzed with SYBR Green chemistry on a CFX384 Touch system. The AsiSI-flanking locus and *ACTB* negative-control locus were amplified with the primers in Table Table S4. Enrichment was expressed as percentage of input: % input = $100 \times 2^{(\Delta Ct - Ct[input])}$, where $\Delta Ct = Ct[input] - \log_2(\text{input dilution factor})$.

4.19 Bulk RNA-Sequencing

Total RNA was isolated with TRIzol from the cell or sorted-cell populations indicated in the figures. Concentration and purity were assessed by NanoDrop and Qubit, and integrity was evaluated with an Agilent 2100 Bioanalyzer. Polyadenylated RNA was enriched with oligo (dT) beads, fragmented, reverse transcribed, end repaired, A-tailed, adapter ligated, and PCR amplified. Paired-end 150 bp libraries were sequenced on an Illumina NovaSeq 6000.

Adapters and low-quality bases were removed with fastp. Clean reads were aligned to the mouse mm10/GRCm38 reference genome with STAR using Ensembl release 102 gene models, and gene-level counts were generated with featureCounts. Differential expression was analyzed with DESeq2; genes with $|\log_2 \text{fold change}|$ greater than 1 and Benjamini–Hochberg-adjusted *P* less than 0.05 were considered differentially expressed. GO and KEGG enrichment and preranked GSEA against MSigDB gene sets were performed with clusterProfiler. For inducible *Rev7* deletion, approximately 12,000 THY1⁺ cells were collected from each mouse (three control and three knockout mice) for library preparation.

4.20 REV7 ChIP-Sequencing

Chromatin from vehicle-treated and activated ER-AsiSI C18-4 cells was immunoprecipitated with anti-REV7. Sequencing reads were quality filtered, aligned to mm10, and compared with the corresponding input chromatin. Peaks were called with MACS2 2.2.7.1 using $-\text{nomodel}$ and a P -value threshold of 1×10^{-2} to capture transient REV7 occupancy. Signal was aggregated around annotated AsiSI cleavage sites to generate target-centered enrichment profiles.

4.21 Isolation of THY1⁺ Spermatogonia by Fluorescence-Activated Cell Sorting

Testes were decapsulated and digested first with 4 mg mL⁻¹ collagenase IV for 15 min at 37 °C and then with 0.05% trypsin plus 1 mg mL⁻¹ DNase I for 10 min at 37 °C. Digestion was stopped with fetal bovine serum, and cells were passed through a 40 μ m strainer. The suspension was incubated for 20 min at 4 °C in the dark with PE/Cyanine7-conjugated anti-mouse CD90.2 (THY1.2; BioLegend). After washing in PBS containing BSA and 2% fetal bovine serum, THY1⁺ cells were purified on a BD FACSAria III. Gates were defined from the 0.125 m glycine corresponding unstained/negative controls and applied consistently across control and knockout samples.

4.22 Statistical Analysis

Statistical analyses were performed with GraphPad Prism 9.5.0 or R 4.5.1. Continuous data are presented as mean $\pm$ standard deviation (SD) unless otherwise stated. For two independent groups, a two-sided unpaired Student's t -test was used for analyses designated as parametric in the figure legends, and a two-sided Mann–Whitney U test was used for nonparametric or rank-based comparisons. More than two groups were compared by one-way analysis of variance followed by Tukey's multiple-comparisons test. Tubule-frequency data were compared with the chi-square test. Single-cell differential expression was evaluated with the Wilcoxon rank-sum test. Transcriptome-wide P values

were adjusted by the Benjamini–Hochberg procedure; nominal P values were used only for the prespecified three-gene analysis described above. All tests were two-sided. $P < 0.05$ was considered statistically significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. The exact experimental unit and sample size are given in the corresponding figure legends.

Author Contributions

Wei Le and Denglong Wu conceptualized and designed the overall study. Jiamin Zhang led the design and construction of the CCER system, and provided supervision and critical validation for the cellular experiments. Mingxiang Fan performed the core experiments, analyzed the data, and drafted the manuscript. Wei Le additionally performed the confocal microscopy experiments. Chang Lu assisted with the *in vivo* mouse experiments. Guoyao Du contributed to the data analysis. Wei Le, Denglong Wu, and Jiamin Zhang rigorously reviewed and edited the manuscript. All authors read and approved the final version to be published.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability

The publicly available single-cell datasets analyzed in this study are deposited in GEO under GSE149512, GSE254315, and GSE202647. Newly generated RNA-sequencing data will be made available through a public repository upon publication; accession number(s): GSE344716. Other data supporting the findings are available from the corresponding author upon reasonable request.

References

- [1] Agarwal, A. *et al.* Male infertility. *Lancet* **397**, 319–333 (2021).
- [2] Krausz, C. & Riera-Escamilla, A. Genetics of male infertility. *Nature Reviews. Urology* **15**, 369–384 (2018).
- [3] Piechka, A., Sparanese, S., Witherspoon, L., Hach, F. & Flannigan, R. Molecular mechanisms of cellular dysfunction in testes from men with non-obstructive azoospermia. *Nature Reviews. Urology* **21**, 67–90 (2024).
- [4] Flannigan, R. K. & Schlegel, P. N. Microdissection testicular sperm extraction: Preoperative patient optimization, surgical technique, and tissue processing. *Fertility and Sterility* **111**, 420–426 (2019).
- [5] Corona, G. *et al.* Sperm recovery and icsi outcomes in men with non-obstructive azoospermia: A systematic review and meta-analysis. *Human Reproduction Update* **25**, 733–757 (2019).
- [6] Yatsenko, A. N. *et al.* X-linked tex11 mutations, meiotic arrest, and azoospermia in infertile men. *The New England Journal of Medicine* **372**, 2097–2107 (2015).

- [7] Fakhro, K. A. *et al.* Point-of-care whole-exome sequencing of idiopathic male infertility. *Genetics in Medicine: Official Journal of the American College of Medical Genetics* **20**, 1365–1373 (2018).
- [8] Singh, S. R., Burnicka-Turek, O., Chauhan, C. & Hou, S. X. Spermatogonial stem cells, infertility and testicular cancer. *Journal of Cellular and Molecular Medicine* **15**, 468–483 (2011).
- [9] Diao, L., Turek, P. J., John, C. M., Fang, F. & Reijo Pera, R. A. Roles of spermatogonial stem cells in spermatogenesis and fertility restoration. *Frontiers in Endocrinology* **13**, 895528 (2022).
- [10] Kubota, H. & Brinster, R. L. Spermatogonial stem cells. *Biology of Reproduction* **99**, 52–74 (2018).
- [11] Bisht, S., Faiq, M., Tolahunase, M. & Dada, R. Oxidative stress and male infertility. *Nature Reviews. Urology* **14**, 470–485 (2017).
- [12] Skakkebaek, N. E. *et al.* Male reproductive disorders and fertility trends: Influences of environment and genetic susceptibility. *Physiological Reviews* **96**, 55–97 (2016).
- [13] Kong, A. *et al.* Rate of de novo mutations and the importance of father's age to disease risk. *Nature* **488**, 471–475 (2012).
- [14] Grewenig, A., Schuler, N. & Rübe, C. E. Persistent dna damage in spermatogonial stem cells after fractionated low-dose irradiation of testicular tissue. *International Journal of Radiation Oncology, Biology, Physics* **92**, 1123–1131 (2015).
- [15] Meistrich, M. L. Effects of chemotherapy and radiotherapy on spermatogenesis. *European Urology* **23**, 136–141; discussion 142 (1993).
- [16] Rübe, C. E., Zhang, S., Miebach, N., Fricke, A. & Rübe, C. Protecting the heritable genome: Dna damage response mechanisms in spermatogonial stem cells. *DNA repair* **10**, 159–168 (2011).

- [17] Ishii, K. *et al.* The trp53-trp53inp1-tnfrsf10b pathway regulates the radiation response of mouse spermatogonial stem cells. *Stem Cell Reports* **3**, 676–689 (2014).
- [18] Le, W. *et al.* Preliminary study of the homologous recombination repair pathway in mouse spermatogonial stem cells. *Andrology* **6**, 488–497 (2018).
- [19] Le, W. *et al.* Preliminary study on 53bp1-mediated dna double-strand break response in spermatogonial stem cells. *Reproductive Sciences* **30**, 1572–1584 (2023).
- [20] Zhou, F. *et al.* Sirt1 mediates ku70 to maintain genomic stability in spermatogonial stem cells via the nhej repair pathway. *Cell Death & Disease* **17**, 490 (2026).
- [21] Clairmont, C. S. & D'Andrea, A. D. Rev7 directs dna repair pathway choice. *Trends in Cell Biology* **31**, 965–978 (2021).
- [22] Paniagua, I. *et al.* Mad2l2 promotes replication fork protection and recovery in a shieldin-independent and rev3l-dependent manner. *Nature Communications* **13**, 5167 (2022).
- [23] Noordermeer, S. M. *et al.* The shieldin complex mediates 53bp1-dependent dna repair. *Nature* **560**, 117–121 (2018).
- [24] Ghezraoui, H. *et al.* 53bp1 cooperation with the rev7-shieldin complex underpins dna structure-specific nhej. *Nature* **560**, 122–127 (2018).
- [25] Boersma, V. *et al.* Mad2l2 controls dna repair at telomeres and dna breaks by inhibiting 5' end resection. *Nature* **521**, 537–540 (2015).
- [26] Watanabe, N. *et al.* The rev7 subunit of dna polymerase ζ is essential for primordial germ cell maintenance in the mouse. *The Journal of Biological Chemistry* **288**, 10459–10471 (2013).
- [27] Pirouz, M., Pilarski, S. & Kessel, M. A critical function of mad2l2 in primordial germ cell development of mice. *PLoS genetics* **9**, e1003712 (2013).

- [28] Uhlén, M. *et al.* Proteomics. tissue-based map of the human proteome. *Science* **347**, 1260419 (2015).
- [29] Sakurai, Y. *et al.* Inactivation of rev7 enhances chemosensitivity and overcomes acquired chemoresistance in testicular germ cell tumors. *Cancer Letters* **489**, 100–110 (2020).
- [30] Spinos, T. *et al.* Predictive tissue markers in testicular germ cell tumors: Immunohistochemical expression of mlh1 and rev-7 proteins. *International Urology and Nephrology* **56**, 1887–1898 (2024).
- [31] Murakumo, Y., Sakurai, Y., Kato, T., Hashimoto, H. & Ichinoe, M. Rev7 in cancer biology and management. *Cancers* **15**, 1721 (2023).
- [32] Hickford, D. E., Frankenberg, S., Pask, A. J., Shaw, G. & Renfree, M. B. Ddx4 (vasa) is conserved in germ cell development in marsupials and monotremes. *Biology of Reproduction* **85**, 733–743 (2011).
- [33] Gassei, K. *et al.* Ddx4-egfp transgenic rat model for the study of germline development and spermatogenesis. *Biology of Reproduction* **96**, 707–719 (2017).
- [34] Sadate-Ngatchou, P. I., Payne, C. J., Dearth, A. T. & Braun, R. E. Cre recombinase activity specific to postnatal, premeiotic male germ cells in transgenic mice. *Genesis* **46**, 738–742 (2008).
- [35] Endo, T. *et al.* Periodic retinoic acid-stra8 signaling intersects with periodic germ-cell competencies to regulate spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **112**, E2347–2356 (2015).
- [36] de Krijger, I., Boersma, V. & Jacobs, J. J. L. Rev7: Jack of many trades. *Trends in Cell Biology* **31**, 686–701 (2021).
- [37] Tomida, J. *et al.* Rev7 is essential for dna damage tolerance via two rev3l binding sites in mammalian dna polymerase ζ . *Nucleic Acids Research* **43**, 1000–1011 (2015).

- [38] Mostafa, T., Bocu, K. & Malhotra, V. A review of testicular histopathology in nonobstructive azoospermia. *Asian Journal of Andrology* **27**, 370–374 (2025).
- [39] Xu, G. *et al.* Rev7 counteracts dna double-strand break resection and affects parp inhibition. *Nature* **521**, 541–544 (2015).
- [40] Baudat, F., Imai, Y. & de Massy, B. Meiotic recombination in mammals: Localization and regulation. *Nature Reviews. Genetics* **14**, 794–806 (2013).
- [41] Gray, S. & Cohen, P. E. Control of meiotic crossovers: From double-strand break formation to designation. *Annual Review of Genetics* **50**, 175–210 (2016).
- [42] GTEx Consortium. The gtex consortium atlas of genetic regulatory effects across human tissues. *Science* **369**, 1318–1330 (2020).
- [43] Madrigal, P. *et al.* Expression atlas in 2026: Enabling fair and open expression data through community collaboration and integration. *Nucleic Acids Research* **54**, D147–D157 (2026).
- [44] Zhao, L. *et al.* Single-cell analysis of developing and azoospermia human testicles reveals central role of sertoli cells. *Nature Communications* **11**, 5683 (2020).
- [45] Cui, L. *et al.* Single-cell transcriptomic atlas of the human testis across the reproductive lifespan. *Nature Aging* **5**, 658–674 (2025).
- [46] Wang, S. *et al.* scrna-seq and scatac-seq reveal that sertoli cell mediates spermatogenesis disorders through stage-specific communications in non-obstructive azoospermia. *eLife* **13**, RP97958 (2025).
- [47] Wu, T. *et al.* clusterprofiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation (Cambridge (Mass.))* **2**, 100141 (2021).
- [48] Korsunsky, I. *et al.* Fast, sensitive and accurate integration of single-cell data with harmony. *Nature Methods* **16**, 1289–1296 (2019).

- [49] McInnes, L., Healy, J., Saul, N. & Großberger, L. Umap: Uniform manifold approximation and projection. *Journal of Open Source Software* **3**, 861 (2018).
- [50] Ashburner, M. *et al.* Gene ontology: Tool for the unification of biology. the gene ontology consortium. *Nature Genetics* **25**, 25–29 (2000).
- [51] Kanehisa, M. & Goto, S. Kegg: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research* **28**, 27–30 (2000).
- [52] Oliveira, H. *et al.* Evaluation of in vivo reproductive toxicity of potassium chromate in male mice. *Experimental and Toxicologic Pathology: Official Journal of the Gesellschaft Fur Toxikologische Pathologie* **62**, 391–404 (2010).
- [53] Wang, Y. Epididymal sperm count. *Current Protocols in Toxicology* **Chapter 16**, Unit16.6 (2003).
- [54] Tayama, K. *et al.* Measuring mouse sperm parameters using a particle counter and sperm quality analyzer: A simple and inexpensive method. *Reproductive Toxicology (Elmsford, N.Y.)* **22**, 92–101 (2006).
- [55] Kanatsu-Shinohara, M. *et al.* Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. *Biology of Reproduction* **72**, 985–991 (2005).
- [56] Guan, K. *et al.* Isolation and cultivation of stem cells from adult mouse testes. *Nature Protocols* **4**, 143–154 (2009).
- [57] Końca, K. *et al.* A cross-platform public domain pc image-analysis program for the comet assay. *Mutation Research* **534**, 15–20 (2003).
- [58] Mao, Z., Seluanov, A., Jiang, Y. & Gorbunova, V. Trf2 is required for repair of nontelomeric dna double-strand breaks by homologous recombination. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 13068–13073 (2007).

- [59] Massip, L., Caron, P., Iacovoni, J. S., Trouche, D. & Legube, G. Deciphering the chromatin landscape induced around dna double strand breaks. *Cell Cycle* **9**, 2963–2972 (2010).
- [60] Iacovoni, J. S. *et al.* High-resolution profiling of gammah2ax around dna double strand breaks in the mammalian genome. *The EMBO journal* **29**, 1446–1457 (2010).
- [61] Team, R. C. *R: A Language and Environment for Statistical Computing* (R Foundation for Statistical Computing, Vienna, Austria, 2025).
- [62] Shah, P. *et al.* Primordial germ cell dna demethylation and development require dna translesion synthesis. *Nature Communications* **15**, 3734 (2024).
- [63] The gtex consortium atlas of genetic regulatory effects across human tissues. *Science* .

Supporting Information

Additional supporting information can be found online in the Supporting Information section at the end of the article. This material includes the baseline clinical characteristics of the recruited patients (Table S1), primer sequences used for mouse genotyping, RT-PCR, and ChIP-qPCR (Tables S2–S4), as well as supplementary figures detailing bioinformatic analyses, human testicular histopathological evaluations, generation and phenotyping of the *Rev7* conditional knockout mouse models, and *in vitro* assessments of the DNA damage response in primary spermatogonial stem cells (Figures S1–S7).

Figures legends

Figure 1: Expression of testis-enriched DDR genes in NOA patients.

(A, B) Three independent data sources, including HPA protein levels (IHC = "High", 18 genes), GTEx human mRNA levels (Testis Rank-1, 9 genes), and Mouse Expression Atlas mRNA levels (Testis Rank-1, 5 genes), were utilized to extract testis-enriched DNA damage response (DDR) genes. Venn diagram analysis was subsequently performed to intersect these genes. Three overlapping genes, *ERCC2*, *FANCD2*, and *REV7* (*MAD2L2*), were identified as highly expressed in the testis. The tissue-specific expression of these three core genes was further evaluated across various tissues using the aforementioned HPA, GTEx, and Mouse Expression Atlas data. All three genes exhibited the highest expression (Rank-1) in the testis across the three datasets.

(C) Single-cell RNA sequencing (scRNA-seq) data of testicular cells from controls with normal spermatogenesis (Ctrl, n = 76,249 cells) and non-obstructive azoospermia patients (NOA, n = 53,262 cells) were analyzed via UMAP dimensionality reduction.

(D) The expression changes of the three candidate genes in germ cells were statistically analyzed. *REV7* was significantly downregulated in NOA germ cells, whereas *ERCC2* and *FANCD2* showed no statistical difference.

(E) The UMAP visualization was utilized to classify cells into 12 specific cell types, including spermatogonial stem cells (SSC) and other germ or somatic cells, to illustrate the cellular composition of Ctrl and NOA samples.

(F) The percentages of each cell type were calculated and compared between the two groups.

(G, H) Gene Ontology (GO) biological process enrichment analysis was performed for globally downregulated differentially expressed genes (DEGs) in NOA patients. Pathways related to spermatid differentiation and targeted core DDR pathways, including double-strand break (DSB) repair, were significantly suppressed in the testes of NOA patients.

(I) The expression differences of *REV7* across 5 germ cell subpopulations (NOA vs. Ctrl) were statistically analyzed.

(J, K) Gene Set Enrichment Analysis (GSEA) was utilized to assess the "DNA repair" and "Double-strand break repair" gene sets.

(Exact $\log_2FC$ values are annotated in the figures. *** $P < 0.001$, ns: no statistical significance. NES: normalized enrichment score).

Figure 2: REV7 and 53BP1 protein levels in SSCs of OA and NOA patients.

(A) Double immunofluorescence staining coupled with confocal microscopy was used to detect the expression of REV7 and 53BP1 proteins in PLZF-positive (PLZF⁺) spermatogonial stem cells (SSCs) within testicular tissue sections from obstructive azoospermia (OA) and non-obstructive azoospermia (NOA) patients. High REV7 fluorescence signals were observed in the PLZF⁺ nuclei of OA patients, whereas the REV7 signal was markedly diminished in the residual PLZF⁺ cells of NOA patients (scale bar = 25 μ m).

(B) In contrast, the 53BP1 signal within PLZF⁺ nuclei exhibited no significant difference between OA and NOA patients (scale bar = 25 μ m).

(C) The fluorescence intensity of REV7 in PLZF⁺ nuclei was quantitatively analyzed, demonstrating a significant decrease in the NOA group.

(D) This was further confirmed by the quantitative analysis of 53BP1 fluorescence intensity.

(E) Additionally, the overall SSC pool was evaluated by calculating the percentage of PLZF⁺ SSCs relative to the total counted cells in the sections. The quantitative analysis indicated a significant depletion of the SSC pool in NOA patients compared to OA controls.

*(Nuclei were counterstained with DAPI. Data are presented as mean $\pm$ SD, n = 3 patients per group. ** P < 0.01 while compared with OA group, Student's t-test).*

Figure 3: Reproductive phenotypes of germ cell-specific *Rev7* knockout mice.

(A) Schematic diagrams were used to illustrate the gene targeting strategy for the *Rev7* floxed allele and the temporal expression windows of *Vasa-Cre* and *Stra8-Cre* during mouse spermatogenesis. *Vasa-Cre* expression initiates at embryonic day 7.5 (E7.5), covering all germ cell stages including primordial germ cells (PGCs) and postnatal spermatogonial stem cells (SSCs), whereas *Stra8-Cre* initiates at postnatal days 3-4 (P3-4). The breeding strategy and resulting genotypes (+/+, fl/+, fl/fl) for *Vasa-Cre*-mediated *Rev7* conditional knockout mice are shown.

(B, D) Macroscopic observation and organ weight quantification were performed to evaluate gonadal development. Representative macroscopic images of testes and epididymides showed marked gonadal atrophy in both *Vasa*-cKO and *Stra8*-cKO adult male mice compared to Ctrl mice.

(C, E) Quantitative analysis of the testis index and epididymis index (percentage of organ weight to body weight) revealed a highly significant decrease in both knockout models compared to the Ctrl group.

(F, G) Hematoxylin and eosin (H&E) staining was utilized to assess the pathological changes in testis and epididymis tissues. Panoramic(top) and high-magnification(bottom) images demonstrated that *Vasa*-cKO mice exhibited severe spermatogenic failure, characterized by atrophied seminiferous tubules, thinned tubular walls, and collapsed epididymal lumens devoid of mature spermatozoa. In contrast, *Stra8*-cKO mice presented milder pathological changes with some mature spermatozoa still visible in the lumen (scale bar = 100 μ m).

(H, I) The seminiferous tubule diameters and epididymal tubular inner diameters were quantitatively analyzed, confirming a significant reduction in the *Vasa*-cKO group, whereas the *Vasa*-cHet group showed no statistical difference compared to the Ctrl.

(J) Immunofluorescence staining was performed to detect the expression of PLZF (a spermatogonial stem cell marker) in testicular sections (scale bar = 50 μ m).

(K) The number of PLZF⁺ cells per seminiferous tubule was statistically analyzed, revealing a significant depletion of PLZF⁺ cells in the *Vasa*-cKO group.

(L) Additionally, the percentage of Sertoli cell-only (SCO) tubules was calculated, demonstrating a statistically significant increase in *Vasa*-cKO mice compared to controls.

*(Nuclei were counterstained with DAPI. Data in C and E are presented as mean $\pm$ SD. Data in K are presented as a scatter plot with the median line. ** $P < 0.01$, *** $P < 0.001$ compared with the Ctrl group. Statistical significance was determined using Student's *t*-test for C and E, one-way ANOVA followed by Tukey's post hoc test for H, I, and K, and Chi-square test for L).*

Figure 4: Radiation-induced REV7 nuclear foci formation and 53BP1 co-localization in SSCs.

(A) Immunofluorescence staining was utilized to evaluate the spatiotemporal dynamics of REV7 in mouse testis cross-sections at various repair time points (control, 30 min, 2 h, and 24 h) following 2 Gy irradiation. Sections were co-stained for REV7, GFR α 1 (a specific marker for mouse spermatogonial stem cells, mSSCs), and DAPI. The subcellular localization and nuclear foci formation of REV7 were specifically observed within GFR α 1-positive mSSCs, with magnified views highlighting the merged channels (scale bars indicated in images).

(B, C) The average number of REV7 foci per GFR α 1-positive mSSC, alongside the percentage of REV7-positive mSSCs among the total GFR α 1-positive population, were quantitatively analyzed across the indicated repair times.

(D) Furthermore, *in vitro* isolated and cultured primary mSSCs were subjected to 2 Gy irradiation and assessed at identical repair time points. The cultured cells were co-stained for REV7 (green) and 53BP1 (red) to evaluate their spatiotemporal relationship. Representative images demonstrate that REV7 nuclear foci are robustly induced post-irradiation and exhibit prominent co-localization with 53BP1 (scale bars indicated in images).

(E, F) The average number of REV7 foci per cell and the proportion of REV7-positive cells were statistically analyzed.

*(Nuclei were counterstained with DAPI. Data are presented as mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns: no statistical significance. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test).*

Figure 5: Effects of *Rev7* knockdown on SSC proliferation, apoptosis, and cell cycle.

(A) Western blot analysis was utilized to validate the shRNA-mediated REV7 knockdown efficiency in C18-4 spermatogonial stem cells. Three independent targeting sequences (sh*Rev7* -1, sh*Rev7* -2, and sh*Rev7* -3) were compared against a scramble negative control (shCtrl), with GAPDH used as an internal control.

(B) The CCK-8 assay was performed to measure cell proliferation by assessing the absorbance at 450 nm at 24, 48, 72, and 96 h post-seeding. Notably, the inclusion of an irradiated knockdown group (sh*Rev7* +IR) demonstrated that *Rev7* depletion significantly enhanced the radiosensitivity of C18-4 cells; the profound flattening of the growth curve indicates severe susceptibility to IR-induced growth arrest and apoptosis.

(C) Flow cytometry with Annexin V-FITC/PI double staining was employed to evaluate cell apoptosis rates in the shCtrl and sh*Rev7* groups. Cells were categorized into necrotic, late apoptotic, early apoptotic, and viable populations.

(D) A statistical analysis of the total apoptotic cell percentages was conducted.

(E) Additionally, flow cytometric analysis of PI-stained cells was used to determine the DNA content distribution.

(F) The percentages of cells in the G0/G1, S, and G2/M phases were quantitatively analyzed.

*(Data are presented as mean $\pm$ SD, n = 3. **** P < 0.0001).*

Figure 6: DNA damage accumulation and transcriptomic changes in *Rev7* - depleted SSCs.

(A) Western blot analysis was utilized to detect the protein levels of REV7, Ub-PCNA (K164) (a translesion synthesis [TLS] marker), and γ H2AX (a double-strand break [DSB] marker) in C18-4 cells from the shCtrl and sh*Rev7* groups.

(B) The relative band intensities of these proteins were quantitatively analyzed using GAPDH as the internal control.

(C) To verify specificity, γ H2AX protein levels were analyzed by Western blot following the re-expression of Flag-tagged wild-type REV7 (Flag-REV7) in sh*Rev7* cells.

(D) The corresponding band intensities were statistically analyzed.

(E) Furthermore, Western blot was used to assess γ H2AX and REV7 protein levels in cells exposed to 2 Gy ionizing radiation (IR) at different repair time points (0, 1, 2, 6, and 24 h).

(F, G, H) The relative grayscale ratios for γ H2AX and REV7 at these time points were quantitatively analyzed and plotted to compare the differences between the two groups.

(I) To investigate transcriptomic alterations, RNA sequencing was performed. A volcano plot was generated to show differentially expressed genes (DEGs) between the sh*Rev7* and shCtrl groups.

(J, N) Gene Ontology (GO) Biological Process enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted to display the most significantly enriched terms and signaling pathways.

(M) A hierarchical clustering heatmap was utilized to visualize the Z-score normalized expression levels of DEGs across samples.

(K, L, O, P) Finally, Gene Set Enrichment Analysis (GSEA) plots were generated to assess pathways including "Meiotic cell cycle", "Chromosome segregation", "Male meiotic nuclear division", and "Meiotic chromosome segregation".

(Data are presented as $\text{mean} \pm \text{SD}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. NES: normalized enrichment score).

Figure 7: Evaluation of DSB repair pathways in *Rev7*-depleted SSCs.

- (A) The alkaline comet assay was utilized to evaluate DNA damage accumulation in C18-4 cells from the shCtrl and sh*Rev7* groups (scale bar = 50 μ m).
- (B) The comet tail moment was quantitatively analyzed.
- (C) Western blot analysis was performed to detect the protein expression levels of NHEJ pathway-related components (53BP1, RIF1, SHLD1, and SHLD2) under basal conditions.
- (D) NHEJ (Ku70, Ku80) and HR (BRCA1, pRPA2) pathway proteins were detected following 2 Gy ionizing radiation (IR) treatment for 2 h.
- (E, F) The relative band intensities of these proteins were statistically analyzed using GAPDH as an internal control.
- (G) To specifically evaluate DSB repair efficiency, dual-reporter gene systems were employed.
- (H) The NHEJ-GFP reporter, which relies on I-SceI endonuclease-induced DSBs, was used to assess NHEJ repair efficiency, with successful events yielding functional GFP. Flow cytometric analysis was performed to determine the GFP-positive cell percentage in shCtrl and sh*Rev7* groups.
- (I) The repair efficiency (GFP/dsRed ratio) was statistically analyzed.
- (J, K) Similarly, the HR-GFP reporter system was utilized to evaluate HR repair efficiency, where successful repair via homologous templates reconstructs wild-type GFP.
- (Data are presented as mean $\pm$ SD. ** $P < 0.01$, **** $P < 0.0001$. Statistical significance in B was determined using the Mann-Whitney U test, $U = 219$).*

Figure 8: REV7 interaction with the Shieldin complex and recruitment to targeted DSBs.

(A) An endogenous co-immunoprecipitation (Co-IP) assay was utilized to investigate protein interactions. Total lysates from C18-4 cells were immunoprecipitated with an anti-REV7 antibody or a control IgG, and the eluates were subsequently analyzed by Western blot to detect REV7, 53BP1, and SHLD2.

(B) A targeted DSB induction system utilizing an ER-AsiSI fusion protein was employed to study REV7 recruitment. The schematic diagram illustrates the construct and the mechanism by which 4-hydroxytamoxifen (4-OHT) induces the nuclear translocation of AsiSI to generate DSBs at specific genomic recognition sites.

(C) Representative high-magnification immunofluorescence images of individual C18-4 cells were utilized to display the co-staining of REV7 and γ H2AX following DOX and 4-OHT induction, visually demonstrating their significant co-localization at specific DSB sites (scale bar = 10 μ m).

(D, E) The number of REV7 foci per nucleus and their average fluorescence intensity were quantitatively analyzed between the Ctrl and 4-OHT groups.

(F) Line profile analysis was utilized to map the immunofluorescence signal intensity distribution of γ H2AX and REV7 along the selected linear region, demonstrating highly consistent overlapping peaks at multiple spatial positions.

(G) Furthermore, immunofluorescence was performed to co-stain REV7 and 53BP1 across three conditions: untreated shCtrl, DOX/4-OHT-induced shCtrl, and DOX/4-OHT-induced shRev7 cells.

(H) Quantitative analysis was conducted to evaluate the co-localization dynamics and foci formation of 53BP1 in response to Rev7 knockdown.

(I) ChIP-qPCR analysis was utilized to assess the relative enrichment (% Input) of γ H2AX, REV7, 53BP1, and SHLD2 at a specific AsiSI-induced DSB locus versus a negative control locus (*ACTB*). The enrichment levels were compared across the three experimental conditions: untreated shCtrl, DOX/4-OHT-induced shCtrl, and DOX/4-OHT-induced shRev7 cells.

(J) Finally, REV7 ChIP-seq was conducted to generate a genomic enrichment profile following AsiSI-induced DSBs, comparing the Ctrl group and the 4-OHT group.

*(Data are presented as mean $\pm$ SD, $n = 3$ independent biological replicates for I. * $P < 0.05$, *** $P < 0.001$, ns: no statistical significance. Statistical significance was determined using the Mann-Whitney U test for D and E, and one-way ANOVA or Student's t -test for H and I).*

Figure 9: Transcriptomic alterations in adult SSCs after acute *Rev7* deletion.

(A) A schematic diagram was used to illustrate the tamoxifen induction regimen and the subsequent experimental workflow for the adult conditional knockout model (H11-Cre; *Rev7*^{fl/fl}) and the control group.

(B) Following administration, fluorescence-activated cell sorting (FACS) was performed to isolate THY1⁺ spermatogonial stem cells (SSCs) from testicular tissues, with representative scatter plots shown for the Control (Ctrl) and Knockout (KO) groups.

(C) The percentage of THY1⁺ SSCs was statistically analyzed between the two groups.

(D) RNA sequencing (RNA-seq) was subsequently performed on the sorted SSCs. The mRNA expression levels of *Rev7* were quantified (FPKM) to validate the knockout efficiency.

(E) Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) was performed, and a dot plot displays the top enriched non-redundant terms across biological process (BP), cellular component (CC), and molecular function (MF) categories, highlighting a strong association with spermatid differentiation, sperm motility, and motile cilium structures.

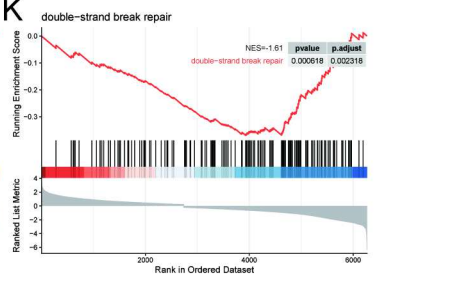
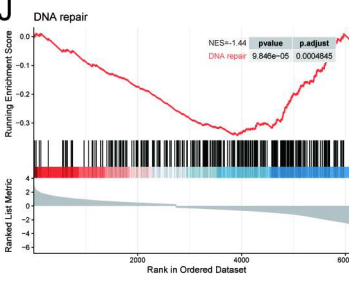
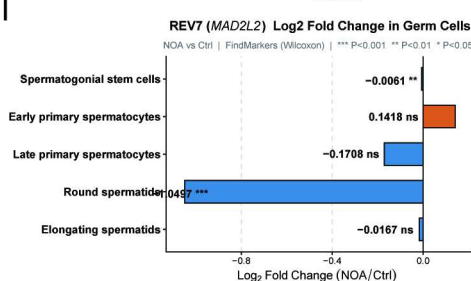
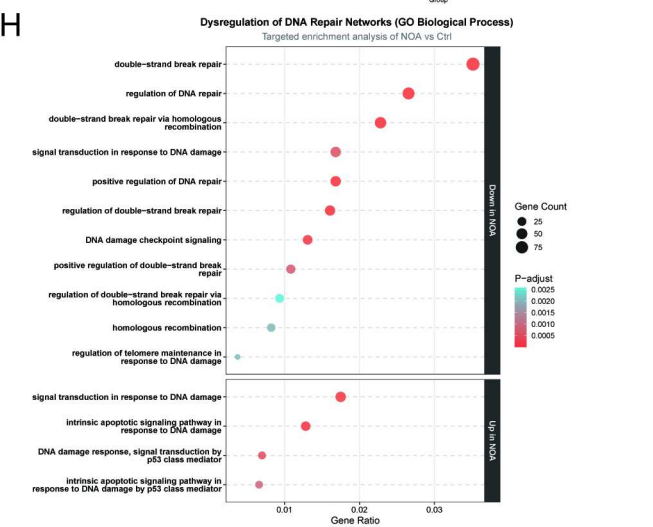
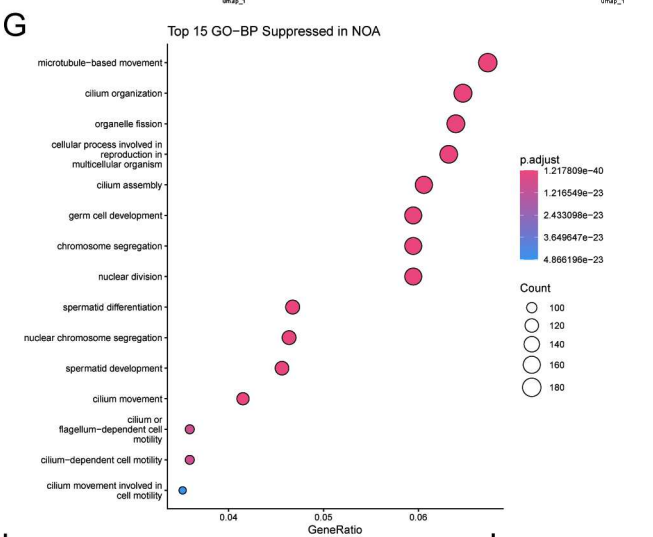
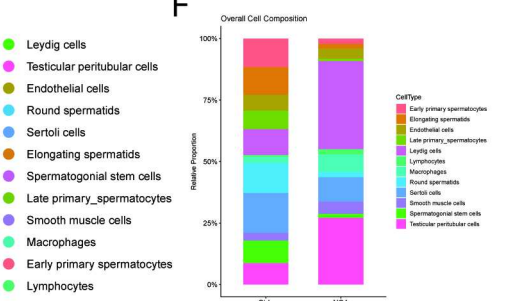
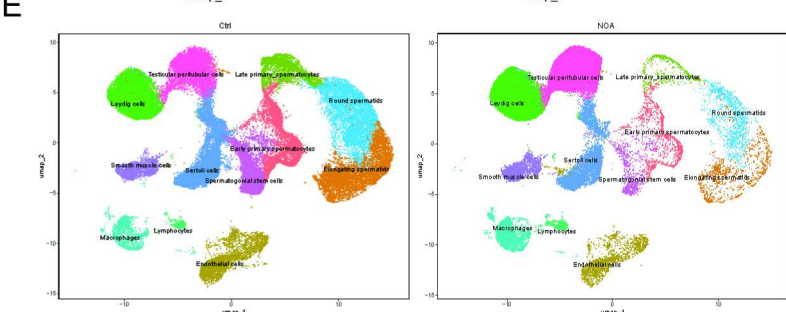
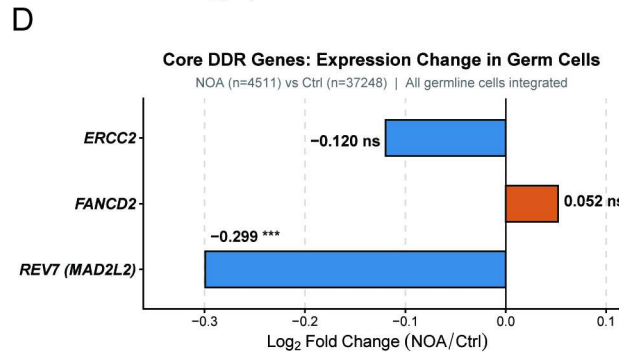
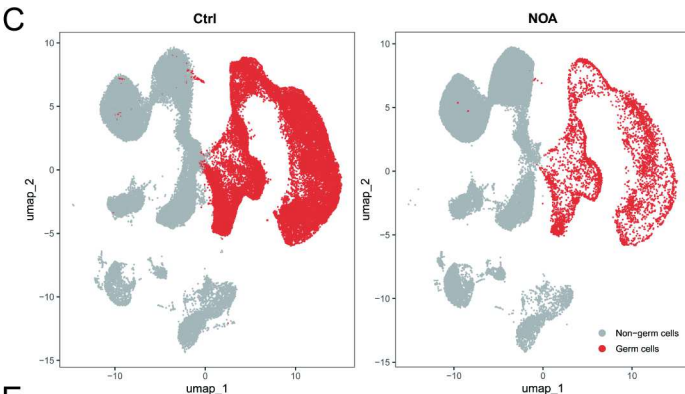
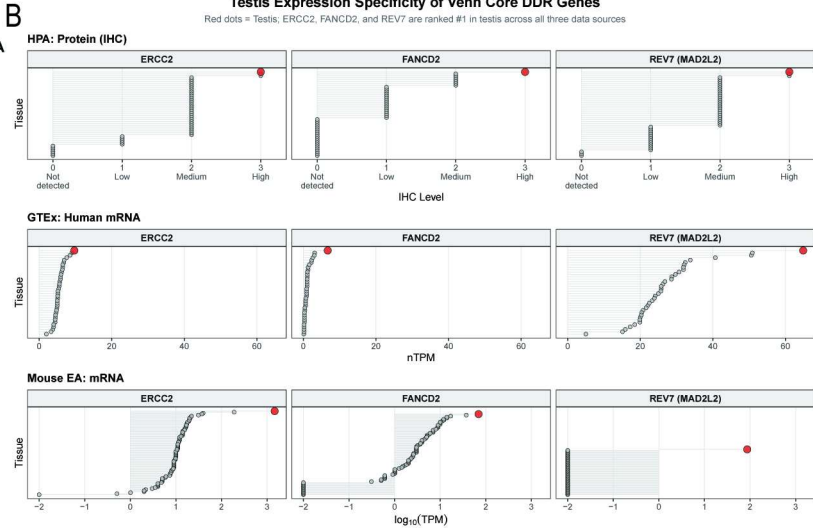
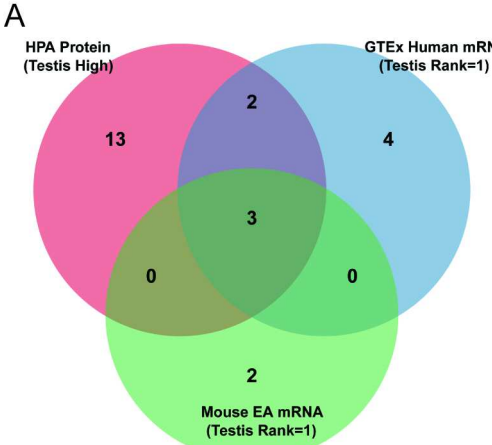
(F) Furthermore, Gene Set Enrichment Analysis (GSEA) demonstrated a significant and coordinated negative enrichment (downregulation) of gene sets specifically associated with cilium movement, flagellated sperm motility, sperm flagellum assembly, and overall sperm motility in the KO group.

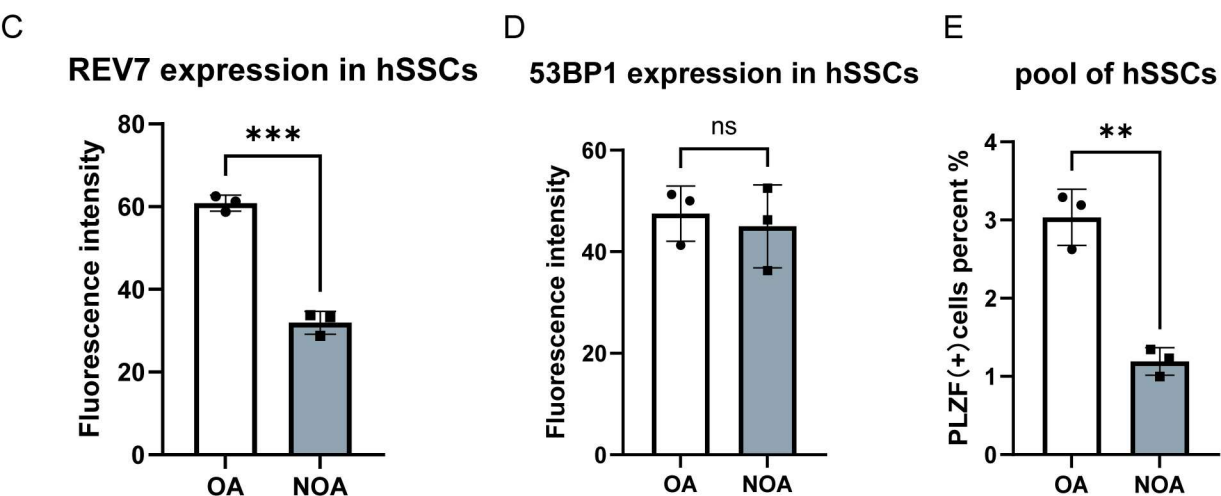
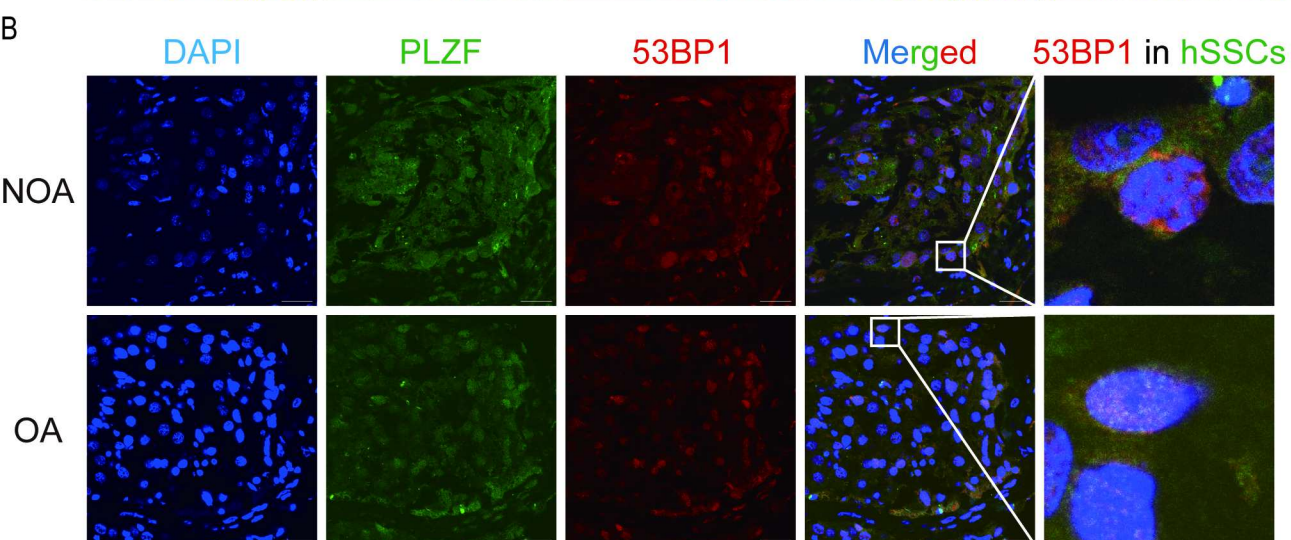
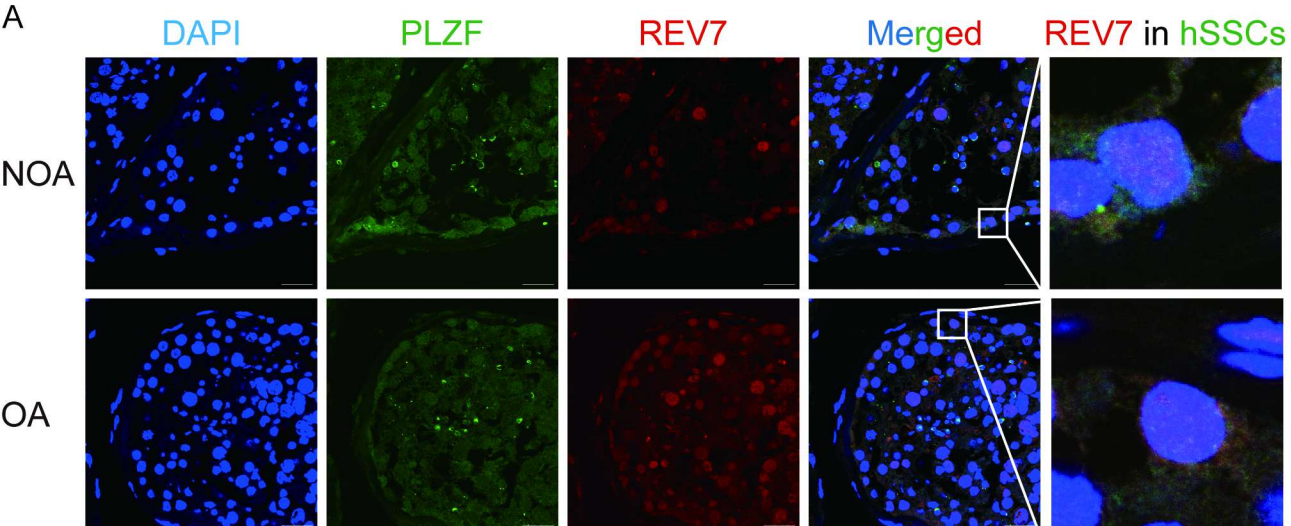
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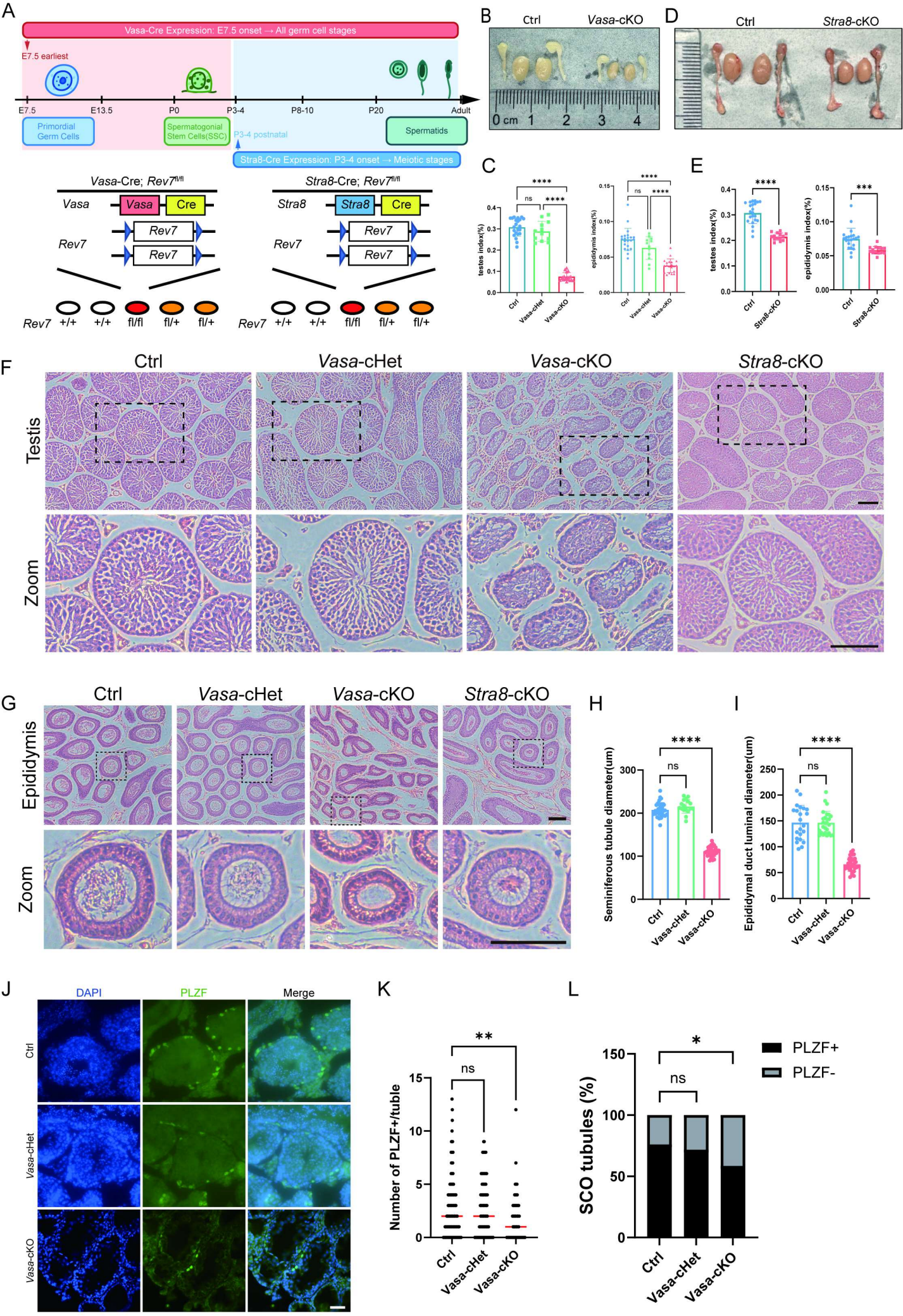
Graphical abstract

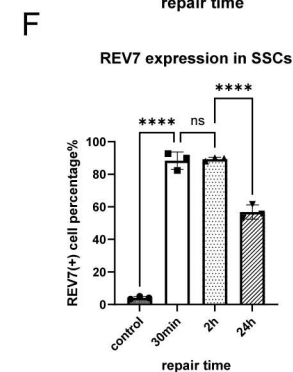
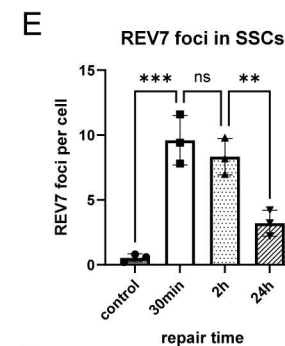
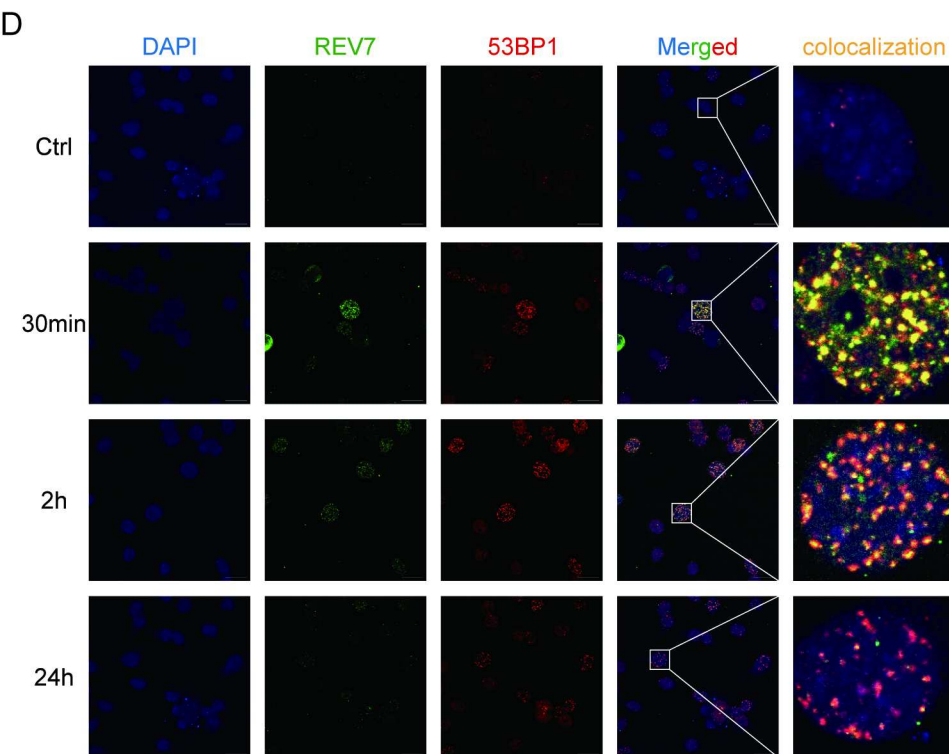
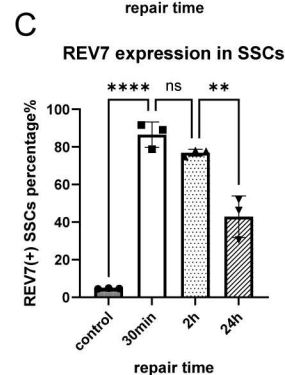
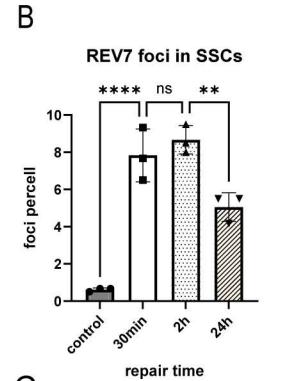
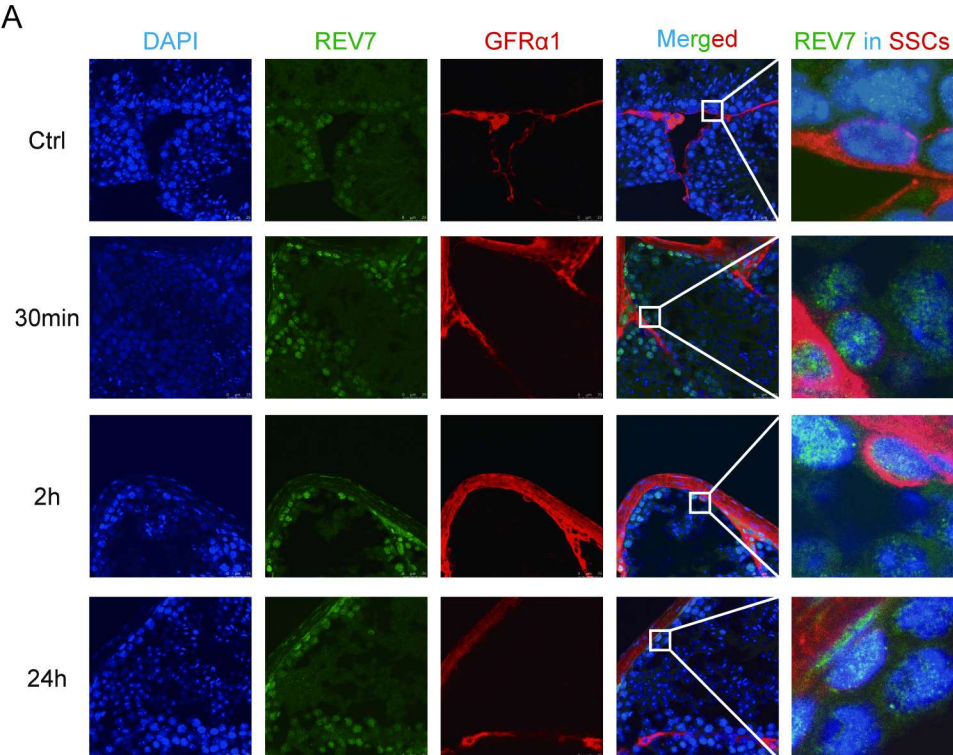
Graphical Abstract: Collectively, the data support a working model in which REV7 couples DSB recognition to pathway-appropriate repair in SSCs. In the presence of REV7 (**left panel**), the 53BP1-RIF1 axis engages REV7 and the SHLD2-containing Shieldin complex at broken DNA ends. This DNA-end protection limits BRCA1-associated resection and favors NHEJ, thereby enabling timely damage resolution, preserving genome stability, and sustaining SSC self-renewal and downstream spermatogenesis. When REV7 is depleted (**right panel**), SHLD1/2 abundance and NHEJ activity decrease, whereas BRCA1, phosphorylated RPA2, and HR-reporter activity increase. Despite this shift toward resection and HR, DNA lesions persist, as evidenced by elevated γ H2AX and comet tail moments. The unresolved damage is accompanied by p53-associated transcriptional responses, cell-cycle disruption, and apoptosis, ultimately depleting the SSC pool and compromising spermatogenesis. We therefore propose that failure of the REV7-Shieldin-NHEJ axis is a mechanistic link between defective SSC genome maintenance and NOA-associated male infertility.

Testis Expression Specificity of Venn Core DDR Genes
Red dots = Testis, ERCC2, FANCD2, and REV7 are ranked #1 in testis across all three data sources

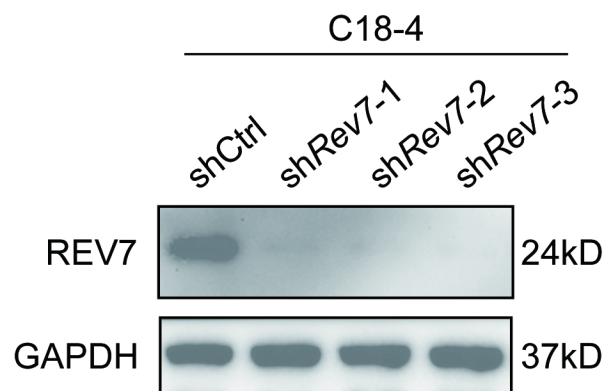




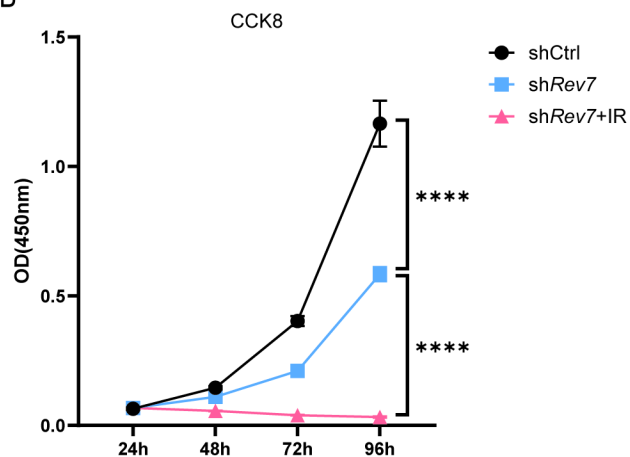




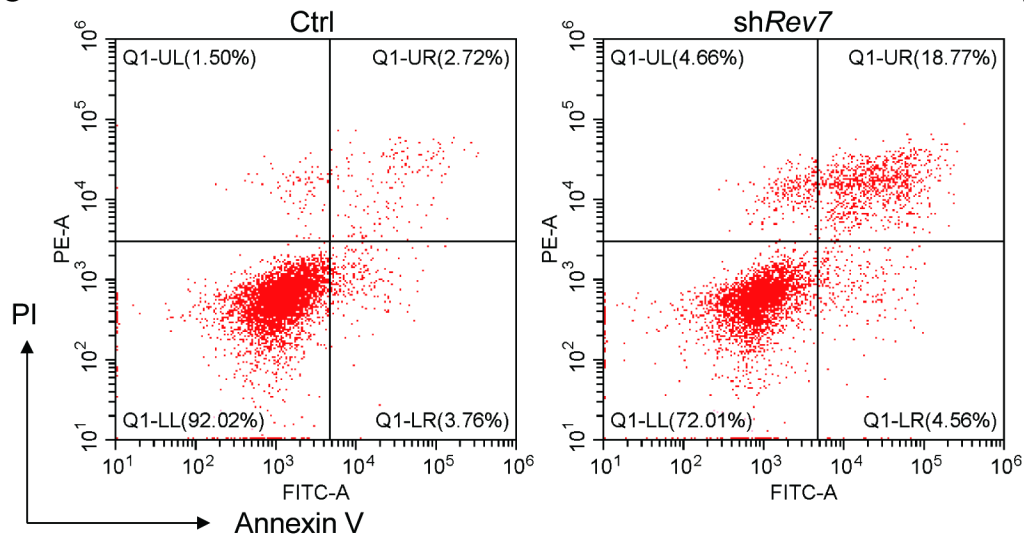
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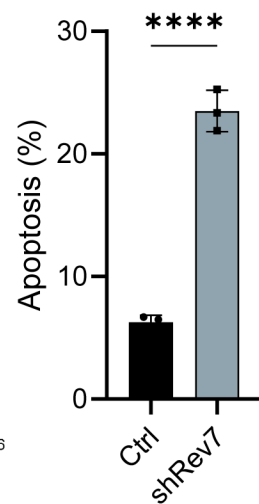
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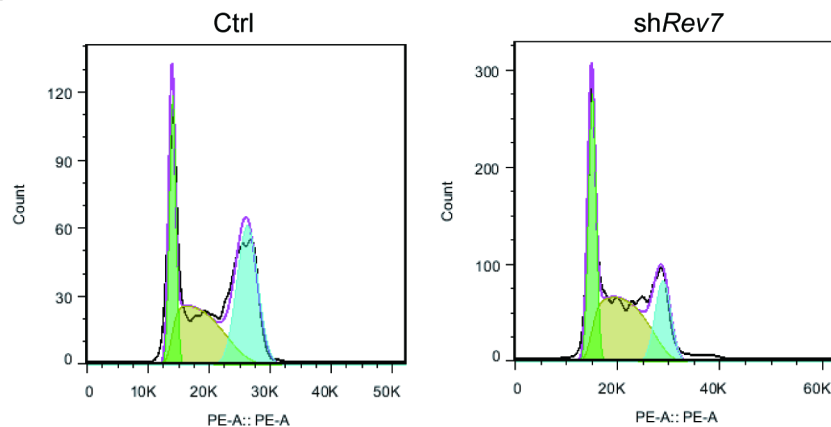
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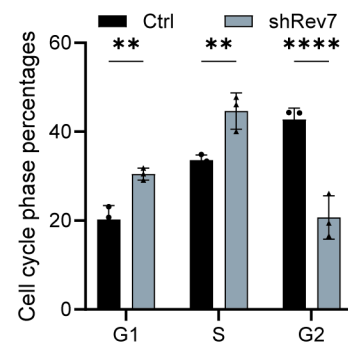
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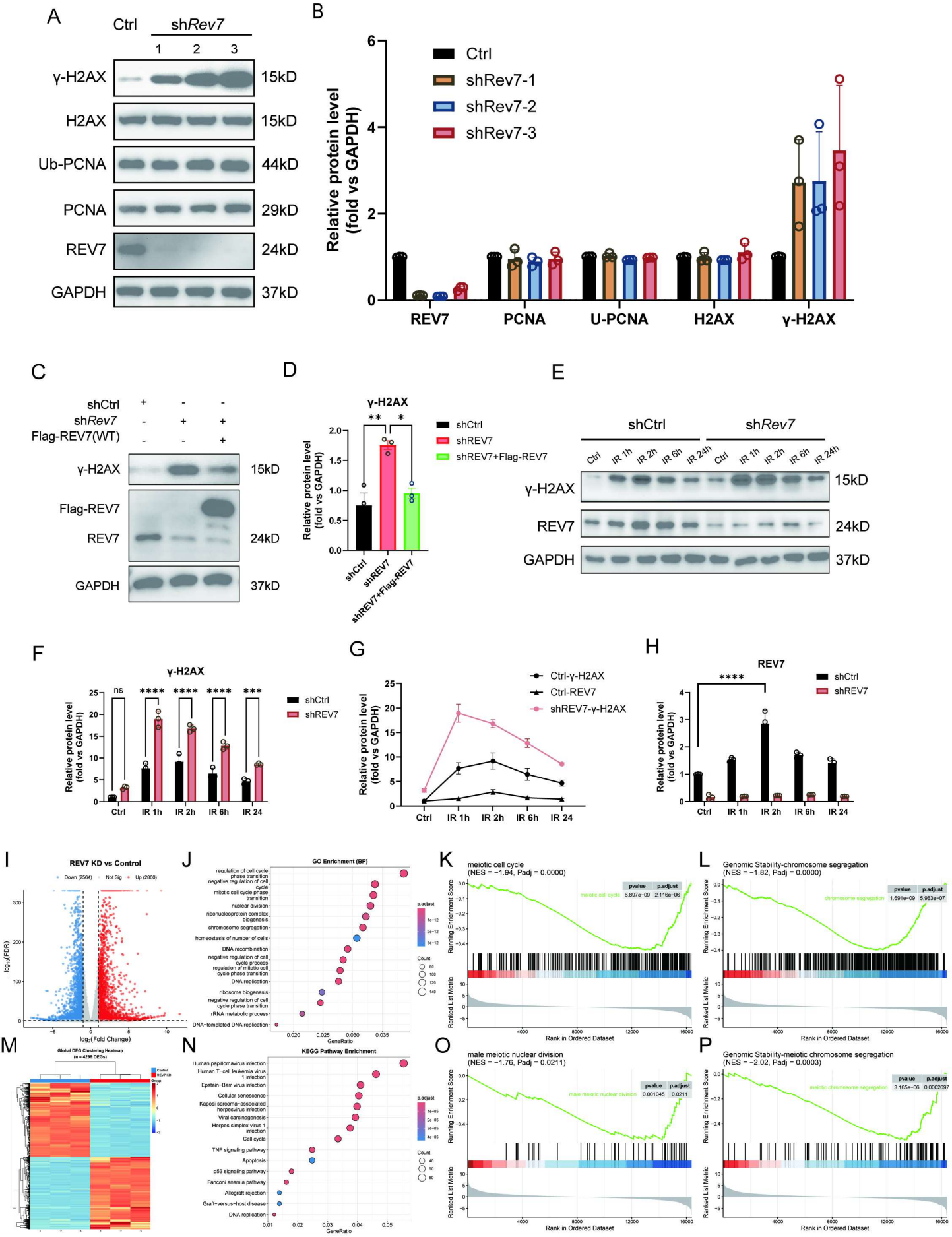


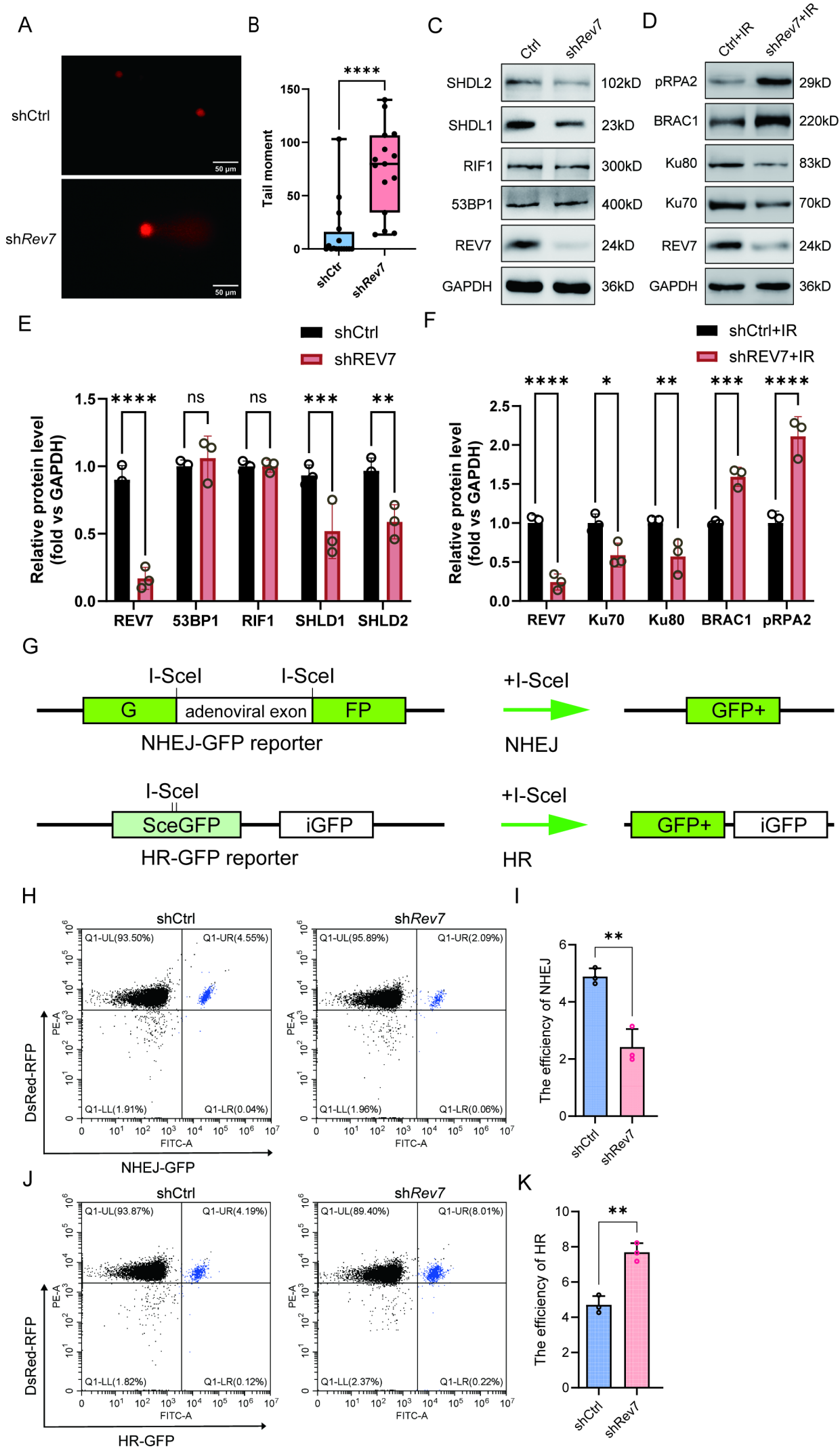
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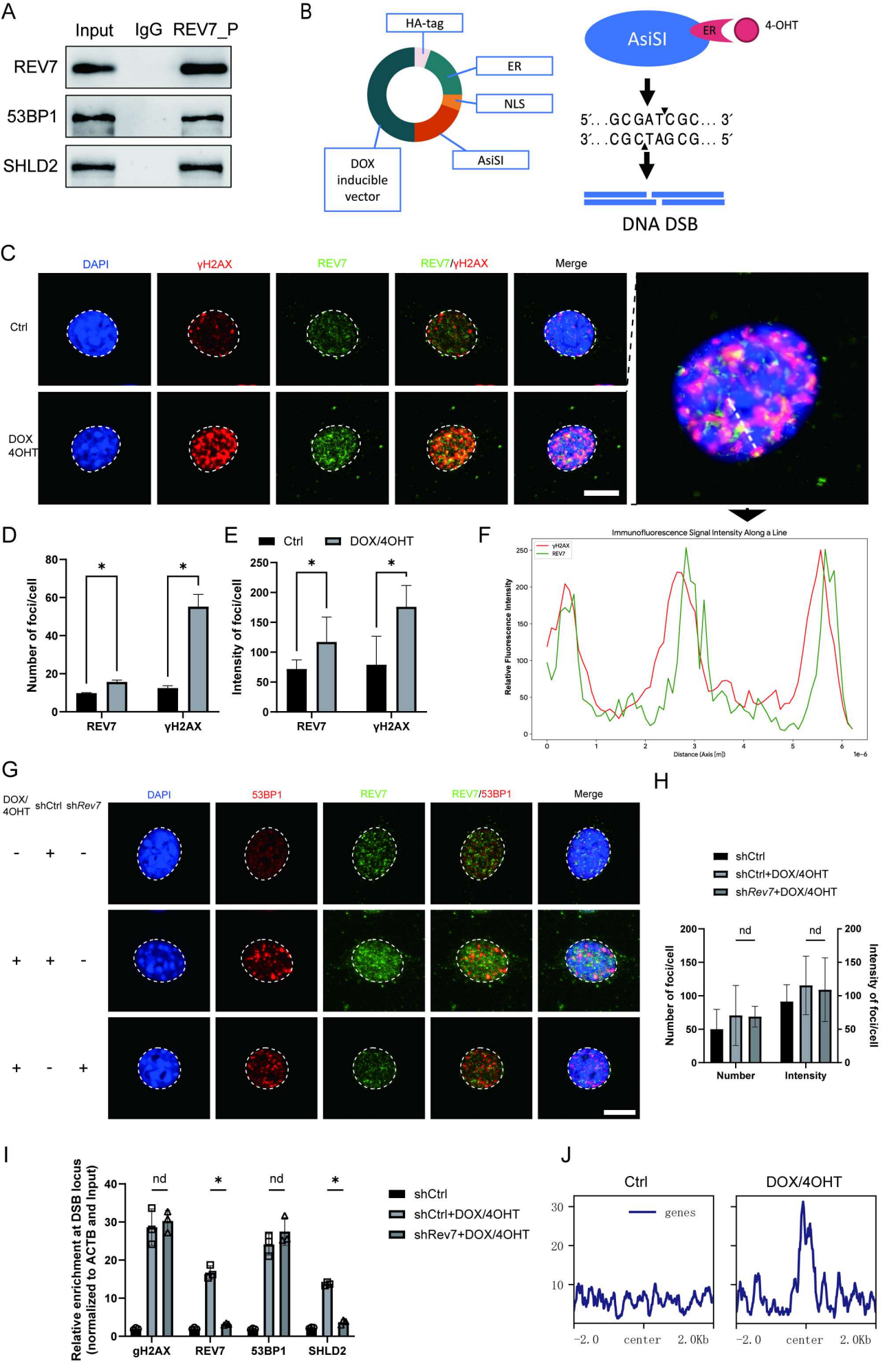


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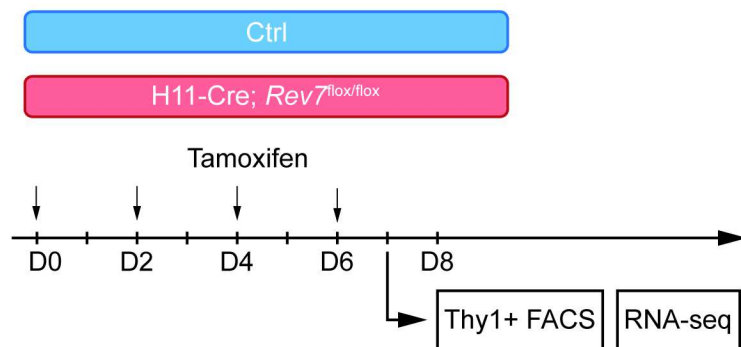




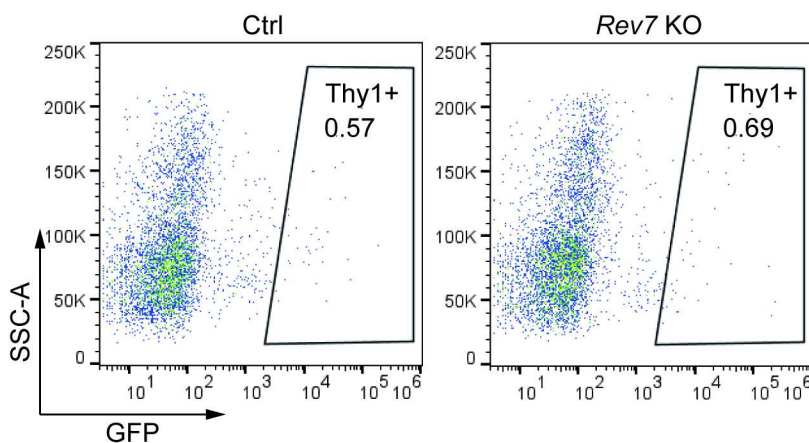




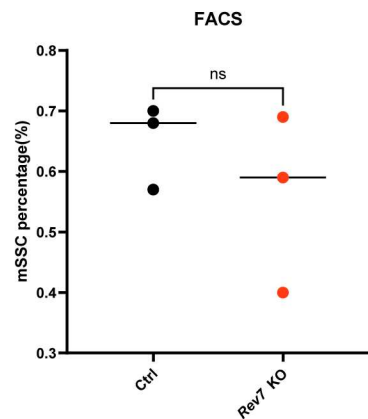
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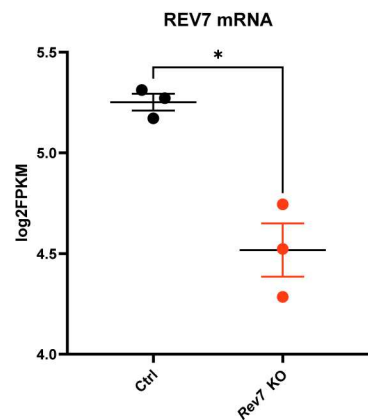
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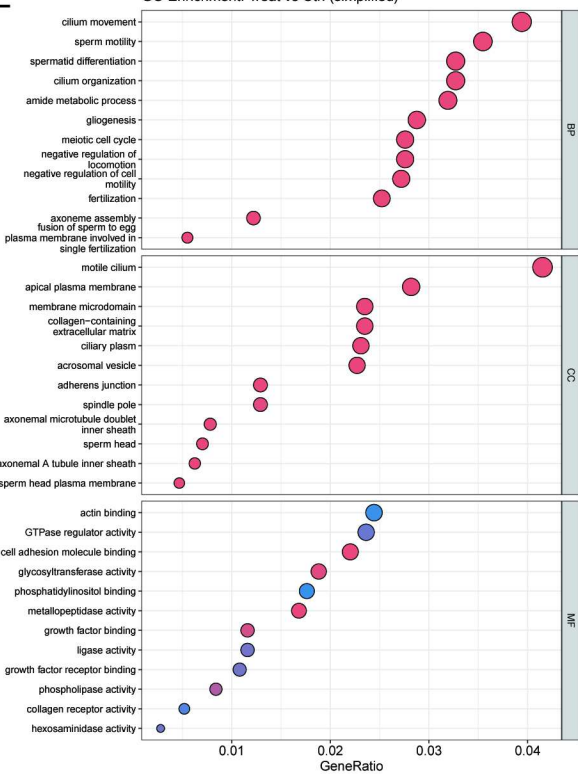


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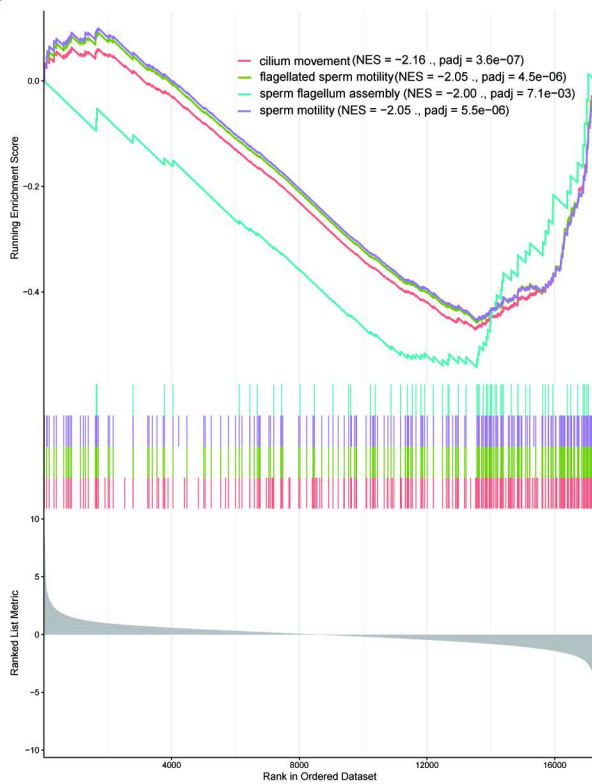


E

GO Enrichment: Treat vs Ctrl (simplified)



F



Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementary.pdf](#)
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