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Original Article

Dcun1d3 is dispensable for spermatogenesis and male fertility in mice

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Abstract: Background: DCUN1D3, a member of the DCNL (defective in cullin neddylation-like) protein family, has been implicated in ultraviolet (UV) radiation-induced cell cycle checkpoints, cell growth, survival, and neddylation. However, its specific function in male germ cells and potential involvement in spermatogenesis remain poorly understood. Methods: To investigate the role of Dcun1d3 in male reproduction, we generated *Dcun1d3* knockout (KO) mice. Sperm parameters were evaluated using computer-assisted sperm analysis (CASA), while histological and immunohistochemical analyses were performed to assess spermatogenesis. Results: *Dcun1d3*-KO mice exhibited no significant differences in testicular histology, sperm quality, levels of germ cell apoptosis, or fertility outcomes compared to wild-type controls. Conclusions: These findings indicate that Dcun1d3 is not essential for spermatogenesis or male fertility in mice. This study provides evidence to streamline future investigations by excluding Dcun1d3 as a critical regulator of male germ cell development and offers useful insights for human fertility gene research.

Keywords: Dcun1d3, testis, spermatogenesis, male fertility

Introduction

Spermatogenesis is a complex and dynamic process that occurs in the seminiferous tubules (STs) and ultimately results in the production of mature male gametes [1, 2]. This intricate process involves a series of stages, including a series of processes such as the generation of differentiated spermatogonia, spermatocytes, entry into meiosis, and the formation of spermatids [3]. This biological cascade requires stringent regulation of post-translational modifications, among which the neddylation pathway emerges as a critical molecular mechanism governing meiotic recombination and cellular homeostasis [4]. Central to this regulatory network are cullin-RING ligases (CRLs), a family of ubiquitin E3 enzymes whose catalytic activity depends on covalent attachment of the ubiquitin-like modifier NEDD8 to cullin subunits - a process termed cullin neddylation [5].

The DCNL (Defective in Cullin Neddylation) protein family, comprising evolutionarily conserved cochaperones, orchestrates cullin neddylation by facilitating NEDD8 transfer to specific cullin subpopulations [6]. Notably, DCUN1D1 (DCNL1) has been implicated in spermatogenic regulation through its canonical role in promoting Cul3 neddylation, thereby modulating CRL-mediated ubiquitination events essential for germ cell development [7]. Intriguingly, recent studies reveal functional antagonism within the DCNL family: DCUN1D3 (DCNL3) counteracts DCUN1D1-mediated neddylation and demonstrates tumor-suppressive properties through

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regulation of DNA damage responses [8, 9]. Despite these advances, the reproductive functions of DCUN1D3 remain enigmatic, particularly regarding its potential involvement in mammalian spermatogenesis.

This study aimed to explore the potential role of Dcun1d3 in the process of spermatogenesis in mice. Through generation and phenotypic characterization of *Dcun1d3*-knockout (*Dcun1d3*-KO) mice, we systematically evaluated the protein's contribution to spermatogenic progression and male fertility. Contrary to expectations derived from its paralog Dcun1d1, our findings demonstrate that genetic ablation of Dcun1d3 does not significantly impair testicular development, spermatogenic efficiency, or reproductive capacity in murine models.

Materials and methods

Bioinformatic analysis

The scRNA-seq dataset datasets GSM5563668 and GSE149512 were downloaded from the GEO database. These two datasets respectively contain information regarding normal adult testicular samples from mice and humans. We employed the "Seurat" software package for data analysis. The standardized data were integrated by means of the "Harmony" function, and the distribution was displayed using "Dotplot" and "Featureplot" [10-13].

Animals

Dcun1d3$^{(+/-)}$ mice and C57BL-6J were purchased from Cyagen Biosciences Inc. (Suzhou, China). These mice had a background of C57BL/6J. The mice were raised under specific pathogen-free conditions at the Animal Center of Nanjing Medical University. All animal experiments were approved by the Animal Ethics and Welfare Committee of Nanjing Medical University (Approval No. 2402015).

Fertility test

In the fertility experiments, *Dcun1d3*$^{(-/-)}$-KO males were paired with WT females (both aged 8 weeks) at a ratio of 1:2 for three months. The number of all litters was recorded.

Polymerase chain reaction (PCR) genotyping

Genotyping of *Dcun1d3*-wild-type (WT, +/+), heterozygous (+/-), and KO (-/-) mice was performed by PCR and agarose gel electrophoresis of DNA. The primers used for genotyping were as follows: F1: 5'-CTGCTACCTCTGTATTGTTCTGCT-3; F2: 5'-AATGAGGAATTGGACCTTTCTGGA-3'; R1: 5'-TAAATTAGCTTAAAGGGACGGGGT-3'.

Histology

The modified Davidson's fluid was employed to fix the testes and epididymides of adult *Dcun1d3*-WT (+/+) and KO (-/-) mice for 48 hours. The specimens were dehydrated with a series of ethanol concentrations, and then the samples were embedded in paraffin. The specimens were sectioned into 5-micrometer-thick slices, rehydrated, and stained with periodic acid-Schiff (PAS) or hematoxylin and eosin (H&E), as previously described [14-16]. Finally, images were captured using an optical microscope, and histological analysis was conducted.

Computer-assisted sperm analysis (CASA)

Sperm were collected from the cauda epididymides of adult *Dcun1d3*-WT (+/+) and KO (-/-) mice, and suspended in human tubal fluid medium (InVitroCare, Inc., Frederick, Maryland, USA). They were incubated at 37°C for 5 minutes, and then the Ceros™ II Sperm Analysis System (Hamilton Thorne, Beverly, Massachusetts, USA) was used to assess the semen quality using.

Immunofluorescence

Immunofluorescence staining was performed as previously described [17-20]. The sections were placed in 10 mM citrate buffer (pH 6.0) for antigen retrieval, and blocked with 1% (w/v) bovine serum albumin for 2 hours. Subsequently, the sections were incubated with the following primary antibodies: anti-3β-hydroxysteroid dehydrogenase (3β-HSD) (1/500, Santa), anti-gamma H2A.X Variant Histone (γ-H2AX) (1/200, Abcam), anti-LIN28 (1/200, Abcam), anti-SRY-box transcription factor 9 (SOX9) (1/200, Millipore) and peptide nucleic acid (PNA) (1/500, Vector) at 4°C overnight. On the next day, the sections were washed three times with phosphate-buffered saline (PBS) at room temperature and then incubated with AlexaFluor secondary antibodies (Thermo Scientific, Waltham, USA) for 1 hour at 37°C. Eventually, the sections were

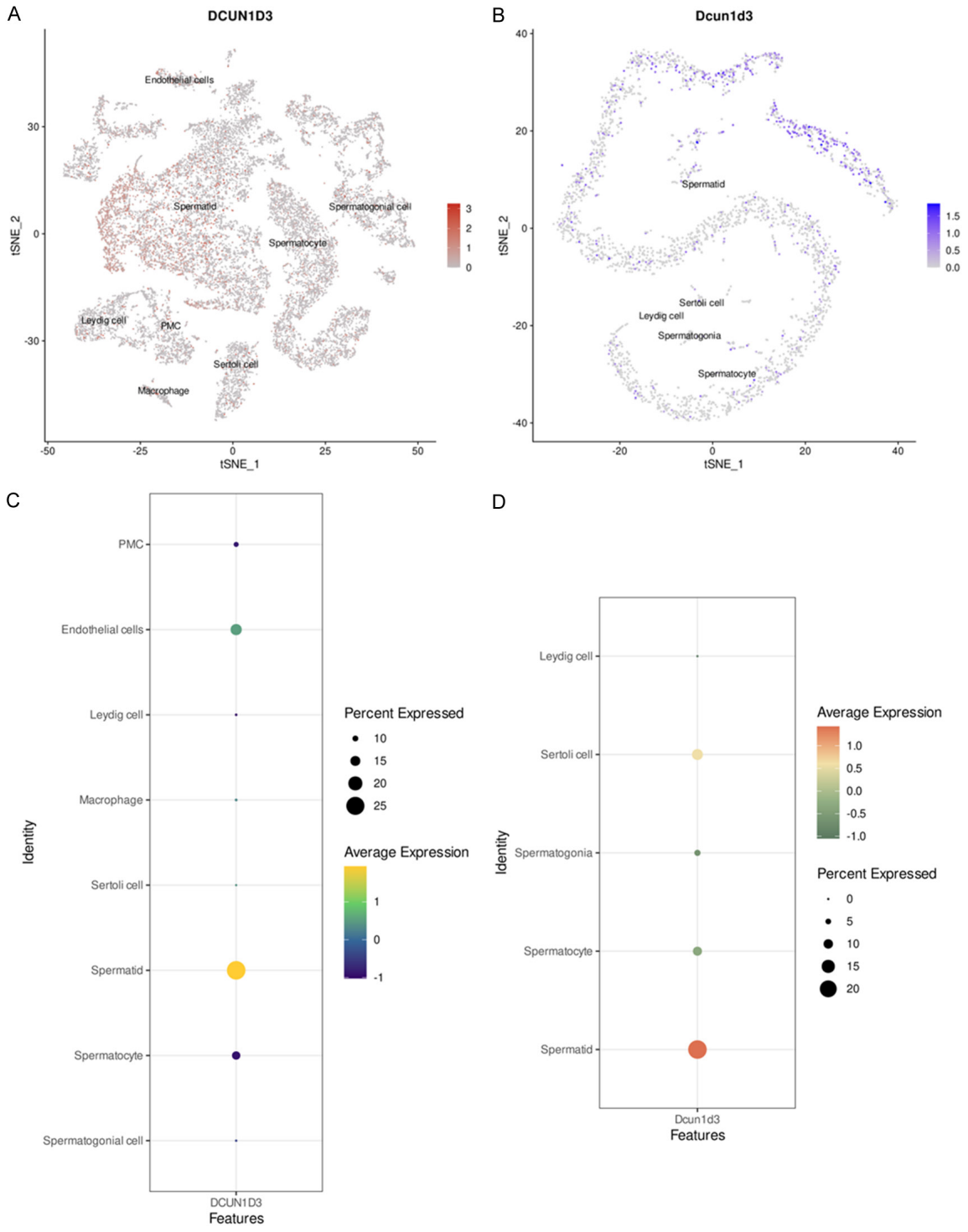

Figure 1. DCUN1D3/Dcun1d3 expression patterns in human and mouse testes. A, B. Featureplots showing the distribution of DCUN1D3/Dcun1d3 in human and mouse testes. C, D. Dotplots illustrating DCUN1D3/Dcun1d3 expression in various cell types from the human and mouse testis samples.

stained with 4',6-diamidino-2-phenylindole (DAPI). The images were captured using a confocal microscope (LSM800, Zeiss, Oberkochen, Germany) for fluorescence analysis.

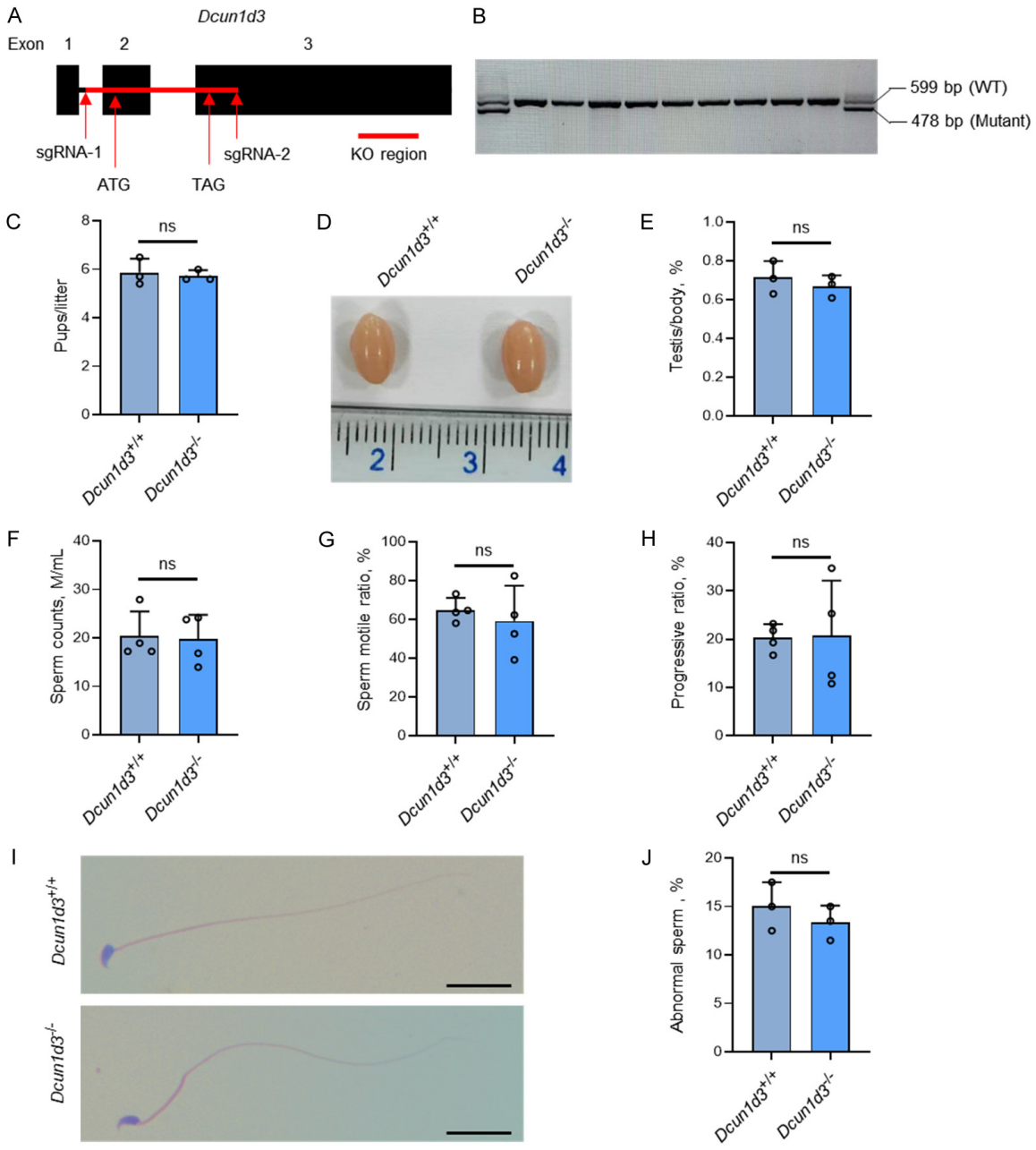

Figure 2. Generation of *Dcun1d3*-KO mice. (A) Diagram of CRISPR/Cas9-mediated Dcun1d3 genome editing. The whole coding sequences were deleted. (B) Identification of mouse genotypes by PCR. (C) Fertility testing of WT (n = 3) and *Dcun1d3*-KO (n = 3) mice. (D) Gross morphology of the testes of WT, *Dcun1d3*-KO mice. (E) Testis/body weight ratios of WT (n = 3) and *Dcun1d3*-KO (n = 3) mice. (F-H) CASA results for the sperm counts (F), motility (G), and progressive ratios (H) in WT, *Dcun1d3*-KO mice. M, million. n = 4 for each group. (I) H&E staining of sperm cells in the cauda epididymides of WT, *Dcun1d3*-KO mice. Scale bar = 20 μm. (J) Quantitative analysis of the results shown in (I). n = 3 for each group. n.s., not significant.

Terminal deoxynucleotidyl transferase-dUTP nick-end labeling (TUNEL) assay

Apoptotic cells were detected in accordance with the method described earlier [21], namely, paraffin testicular sections were treated with proteinase K (10 μg/mL) for 10 minutes and then incubated in equilibration buffer for 30 minutes. Subsequently, the sections were incubated with BrightRed-labeled buffer (Vazyme, Nanjing, China) at 37°C for 1 hour, washed three times with PBS, stained with 4',6-diamid-

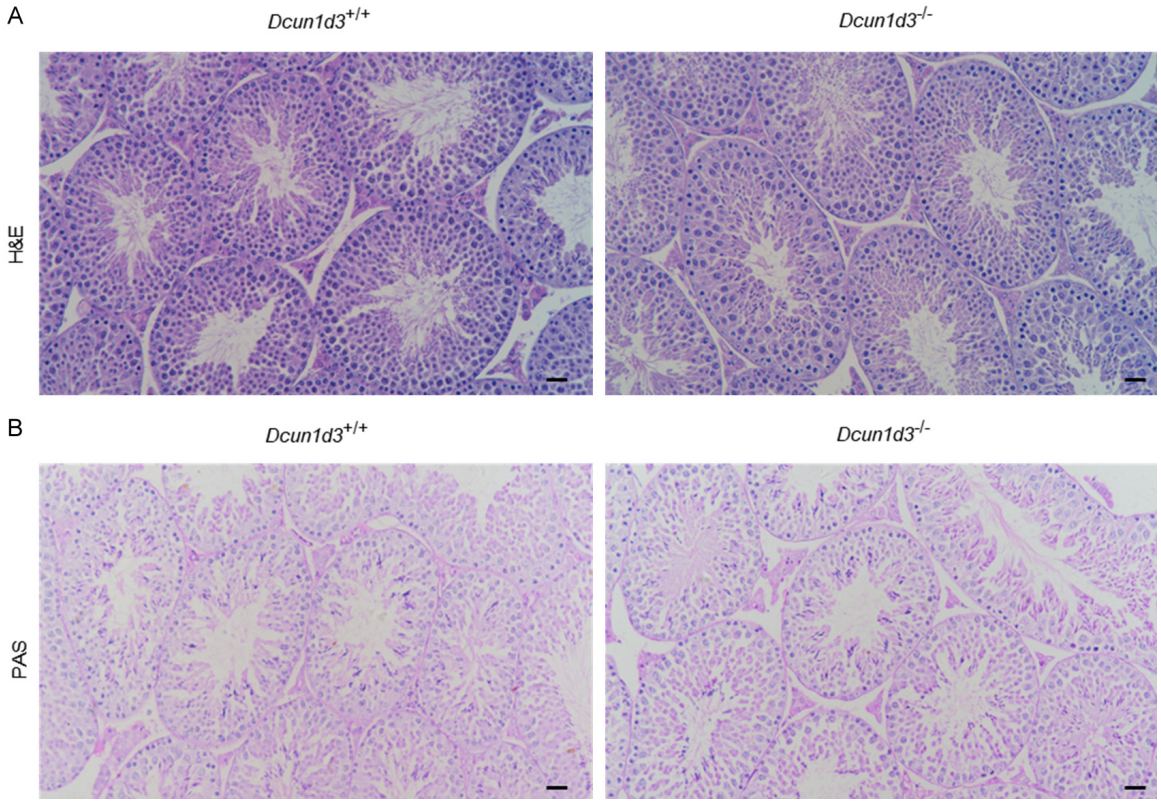

Figure 3. Testicular Histology. A. H&E staining of testicular sections from WT and *Dcun1d3*-KO mice. B. Periodic Acid Schiff (PAS) staining of testicular sections from WT and *Dcun1d3*-KO mice. Scale bar = 25 μm.

ino-2-phenylindole (DAPI), and finally, the images were analyzed using a confocal laser microscope (LSM800, Zeiss, Oberkochen, Germany).

Statistical analysis

The data were analyzed by GraphPad Prism 9. Results were expressed as mean ± SD. Student's t-test and one-way ANOVA were employed for comparisons. A *p*-value of less than 0.05 was deemed statistically significant [22, 23].

Results

DCUN1D3/Dcun1d3 expression patterns in human and mouse testes

DCUN1D3 belongs to the DCNLs family and is highly conserved in vertebrates. We relied on bioinformatics predictions to ascertain the expression and distribution of DCUN1D3 within the testis. Utilizing the publicly available single-cell RNA sequencing (scRNA-seq) data from human and mouse testes, we observed that DCUN1D3/Dcun1d3 was predominantly expressed in spermatid (**Figure 1**).

Generation of Dcun1d3-KO mice

We employed the CRISPR/Cas9 genome editing technique to generate a *Dcun1d3* global KO mouse line (**Figure 2A**), and conducted PCR amplification to rapidly identify the genotypes of *Dcun1d3* WT (+/+), KO (-/-), and heterozygous (+/-) mice (**Figure 2B**).

Dcun1d3-KO mice are fertile

Fertility assessments of *Dcun1d3*-KO mice showed that their litter sizes were similar to those of wild-type (WT) mice (**Figure 2C**). No significant differences were observed in testis dimensions or weight between WT and *Dcun1d3*-KO mice (**Figure 2D**, **2E**). Sperm analyses using computer-assisted sperm analysis (CASA) revealed that the sperm concentration, motility, and progressive velocity in *Dcun1d3*-KO mice were comparable to those in WT mice (**Figure 2F-H**). Additionally, H&E staining for sperm morphology evaluation indicated no notable distinctions between WT and *Dcun1d3*-KO mice (**Figure 2I**, **2J**). In summary, these findings suggested that Dcun1d3 did not influence the fertility or semen quality of mice.

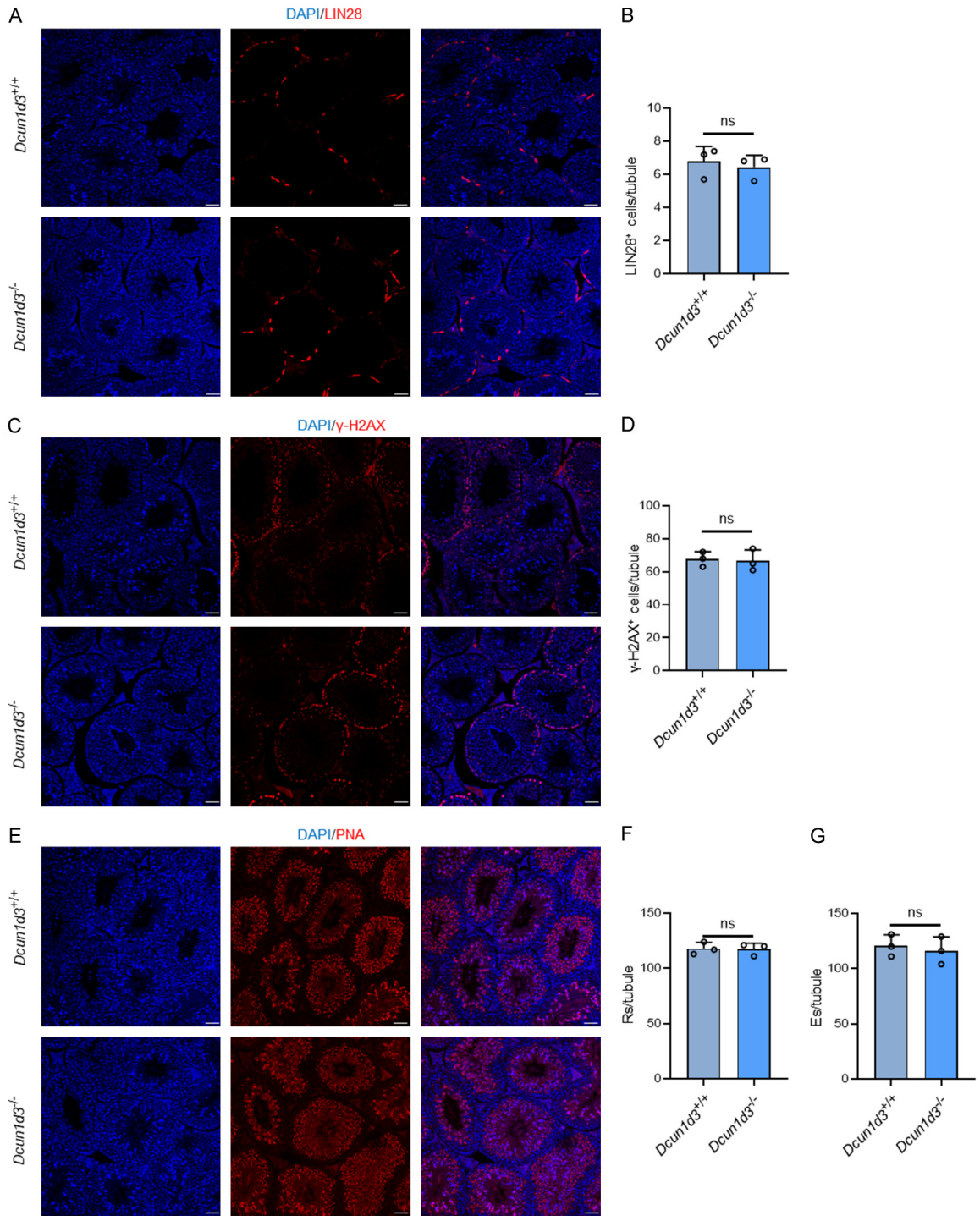

Figure 4. Immunostaining of stage-specific markers during spermatogenesis in WT and *Dcun1d3*-KO mice. (A) Immunostaining of LIN28 in paraffin-embedded testicular sections from WT and *Dcun1d3*-KO mice. Scale bar = 50 μm. (B) Quantification of (A). n = 3 for each group. (C) Immunostaining of γ-H2AX in paraffin-embedded testicular sections from WT and Dcun1d3-KO mice. Scale bar = 50 μm. (D) Quantification of (C). n = 3 for each group. (E) Immunostaining of PNA in paraffin-embedded testicular sections from WT and *Dcun1d3*-KO mice. Scale bar = 50 μm. (F) Quantification of round spermatids (Rs) in (E). n = 3 for each group. (G) Quantification of elongating/elongated spermatids (Es) in (E). n = 3 for each group. n.s., not significant.

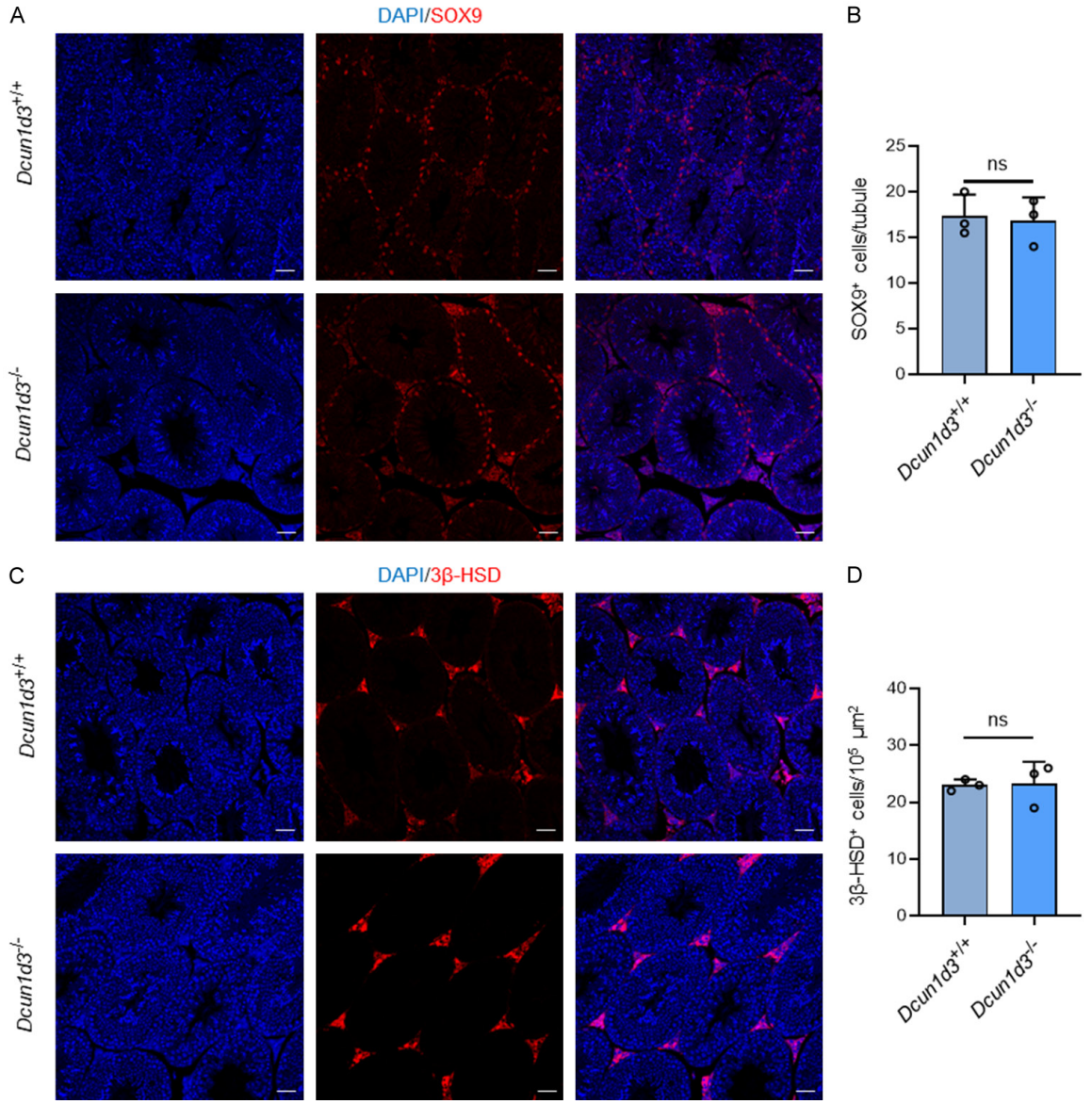

Figure 5. Immunostaining of SOX9 and 3β-HSD in WT and *Dcun1d3*-KO mice. (A) Immunostaining of SOX9 in paraffin-embedded testicular sections from WT and *Dcun1d3*-KO mice. Scale bar = 50 μm. (B) Quantification of (A). n = 3 for each group. (C) Immunostaining of 3β-HSD in paraffin-embedded testicular sections from WT and *Dcun1d3*-KO mice. Scale bar = 50 μm. (D) Quantification of (C). n = 3 for each group. n.s., not significant.

Dcun1d3-KO mice show normal spermatogenesis

The testicular morphology was assessed via H&E staining. Both WT and *Dcun1d3*-KO mice presented intact seminiferous tubules, which contained spermatogenic cells at all stages (**Figure 3A**). Meanwhile, the development of germ cells within the seminiferous epithelium was inspected by PAS staining. The testes of *Dcun1d3*-KO mice, similar to those of WT mice, manifested complete spermatogenesis, revealing all stages of the spermatogenic cycle (**Figure 3B**). Thus, the deletion of Dcun1d3 had no influence on spermatogenesis.

Furthermore, to better observe spermatogenesis, we quantified the spermatogonial stem cells, spermatocytes, acrosomes, Sertoli cells, Leydig cells, and apoptotic cells in the testes of WT and *Dcun1d3*-KO mice by LIN28, γ-H2AX, PNA, SOX9, 3β-HSD, and TUNEL signals, respectively. The outcomes demonstrated that there were no disparities in the numbers of

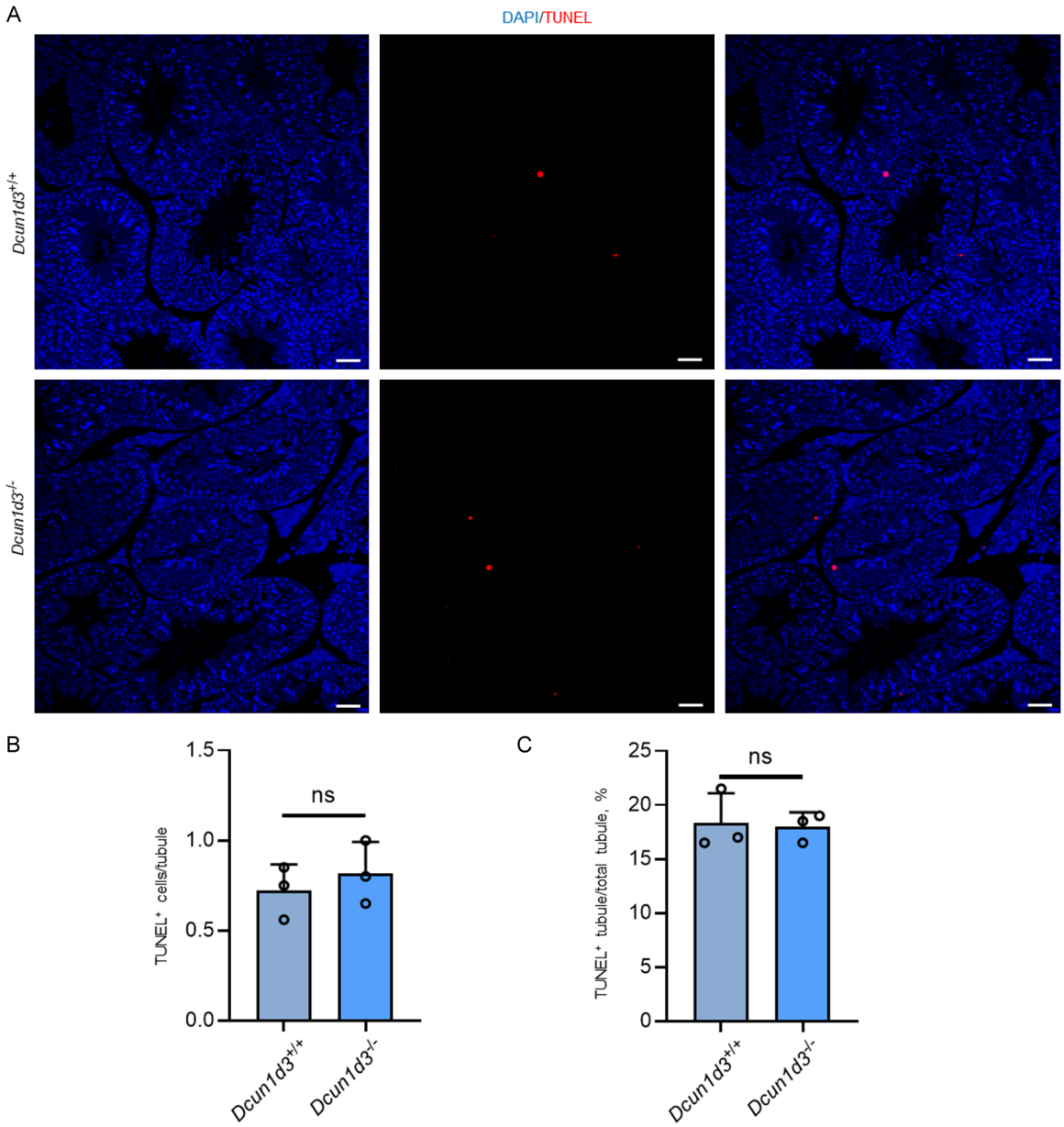

Figure 6. TUNEL assay in WT and *Dcun1d3*-KO mice. A. TUNEL assay. Scale bar = 50 μm. B. Quantification of TUNEL$^+$ cells (indicating apoptosis) in the testes from *Dcun1d3*-KO and WT mice by TUNEL assay. n = 3 for each group. C. Quantification of TUNEL$^+$ tubule/total tubule (expressed as a percentage) in the testes from *Dcun1d3*-KO and WT mice by TUNEL assay. n = 3 for each group. n.s., not significant.

these cell types between the two groups of mice (**Figures 4**-**6**). These discoveries indicated that the deficiency of Dcun1d3 does not impact spermatogenesis in mice.

Discussion

Mammalian spermatogenesis requires precise coordination of mitotic proliferation, meiotic recombination, and post-meiotic differentiation - processes fundamentally dependent on ubiquitin-proteasome system (UPS)-mediated protein regulation [24, 25]. The Cullin-RING ligase (CRL) family, the largest group of multi-subunit E3 ligase complexes, plays a central role in this regulation [26]. Emerging evidence underscores the spermatogenic indispensability of CRL components. For example, Cul4A is of paramount importance for spermatogenesis, and it participates in the repair of double-

strand breaks via homologous recombination during the process of male meiosis [27]. CRLs also play a crucial role in neddylation, a ubiquitin-like modification that is vital for the stability and function of CRL complexes [28, 29]. DCUN1D1, a regulator of neddylation, can facilitate the neddylation of cullin family components within the E3-CRL complex [30]. Research also has demonstrated that DCUN1D1 is essential for spermatogenesis, possibly involving its role in promoting Cul3 neddylation [7].

Notably, the functional dichotomy between DCNL family members remains poorly characterized in reproductive contexts. While DCUN1D1 demonstrates essential spermatogenic functions, our investigation reveals that its homolog DCUN1D3 - previously implicated in UV stress responses and DCUN1D1 antagonism [8, 9] - does not share this reproductive significance. Through CRISPR/Cas9-mediated generation of *Dcun1d3*-knockout (*Dcun1d3*-KO) mice, we observed preserved testicular histology, complete spermatogenic progression, and normal fertility parameters. This phenotypic resilience aligns with emerging recognition that numerous testis-enriched genes, including FBXO22 [31], ASB15 and ASB17 [32]. Prove dispensable for murine fertility through compensatory mechanisms.

The functional redundancy observed in Dcun1d3-KO mice likely reflects evolutionary adaptations safeguarding gametogenesis. Potential compensatory mechanisms may involve: 1) Functional overlap with DCUN1D1 or other DCNL homologs maintaining baseline neddylation activity; 2) Activation of alternative UPS regulatory pathways. Precedent exists for such adaptive responses. For example, the average expression of Slc26a5 and Slc26a11 in the testes of *Slc26a1*-KO mice was higher than that in *Slc26a1*-WT mice [33]. While our study did not identify the specific compensatory pathways, the preserved CRL functionality in knockout models suggests robust molecular buffering capacity within neddylation networks.

In conclusion, our study provides important insights into the role of DCUN1D3 in spermatogenesis and fertility. First, they establish DCUN1D3 as non-essential for murine spermatogenesis despite structural homology with DCUN1D1, highlighting functional divergence within the DCNL family. Second, the demonstration of uncompromised fertility in *Dcun1d3*-KO models provides critical negative data to prevent redundant investigations, redirecting research efforts toward bona fide fertility regulators. While our study suggests limited involvement of DCUN1D3 in baseline spermatogenesis, its established role in DNA damage response raises a critical caveat: our current assessment under physiological conditions may fail to capture context-dependent phenotypes. Future investigations may prioritize evaluating DCUN1D3's reproductive relevance in stress scenarios or in combination with genetic perturbations, which could unmask latent roles in maintaining genomic stability during germ cell development.

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Disclosure of conflict of interest

None.

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