Fig. S3

A. sgRNA-1 and sgRNA-2 targeting Exon2, Exon3, Exon4, Exon5, and Exon6. Primer sets 1, 2, and 3.

B. Gel electrophoresis results:
- Primer set 1: 1 kb target, 925 bp
- Primer set 2: 700 bp target
- Primer set 3: 605 bp target

C. Genomic DNA sequence comparison:
- wt allele: TTAGCAACAGTAACCTGGAGGTCT
- Nrip14 KO: TTAGCAACAGTAACCTGGAGGTCT

D. Immunostaining of NANOG, OCT4, and SOX2.

E. AB2.2 Chr:
- Day 1
- Day 2

F. Nrip14 KO:
- Day 1
- Day 2
- Day 3
- Day 4
- Day 5
- Day 6

G. Immunostaining of STELLA and Hoechst.

H. Relative expression level:
- Nrip14+/+
- Nrip14-/-

I. Relative expression level:
- Posit
- Nrip14
- STELLA
- Negative
Fig. S4

A. Target Sequence: 5'-GCTGCAAGCATATTTTGATGAGG-3' NGG: PAM sequence

B. WT catatatgat gaggatat
   +2 catatatgATgaggatat
   -5+1 catatatggaggtgattat

C. Nlrp14+/+ Nlrp14+/- Nlrp14-/-

D. Number of Pups
   - Male
   - Female

E. Nlrp14+/+ Nlrp14+/- Nlrp14-/-

F. Gonad-somatic index (%)

G. Western Blot
   - Nlrp14+/+
   - Nlrp14+/-
   - Nlrp14-/-
   - Actin

H. Western Blot
   - Nlrp14+/+
   - Nlrp14+/-
   - Nlrp14-/-
   - Procaspase-3
   - Cleaved Caspase-3
   - Actin

I. Relative motility of sperm

J. NS
Fig. S5
Fig. S6

A. Anti-DDX4
B. SYCP3
C. Anti-MSY2
D. Sox9
E. Relative number of Sox9+ cells

Legend: NS = Not Significant
Fig. S1. Induction and characterization of PGCLC differentiation in mouse ESCs.

(A) Schematic of PGCLC differentiation procedure. (B) Representative images of mESCs in naïve, EpiLC, and PGCLC states. Scale bar: 200µm. (C) The induction of EpiLC and PGCLC markers was confirmed in samples from different differentiation stages. mESCs were differentiated following procedures listed in (A). EpiLCs were harvested at day 2 post differentiation while PGCLC spheres were harvested on day 8. Total RNAs were prepared by the Trizol method. Error bar represented data from three independent experiments. (D) Schematic of the experimental flow to identify potential regulators in PGCLC differentiation. (E) The knockdown of Nlrp14 strongly inhibited STELLA expression in PGCLC spheres. Representative images were shown for PGCLC spheres differentiated from shRNA transduced mESCs. Spheres were harvested at day 8 post differentiation and subject to immunostaining with the anti-STELLA antibody. Non-targeting shRNA was used as the control group. IgG was used as the negative control for immunostaining. Hoechst was used for nuclear staining. “□” showed the shNlrp14 group immunostaining. Scale bar: 50µm.

Fig. S2. Nlrp14 was specifically expressed in mouse gonads and
induced during PGCLC differentiation.

(A) Two transcripts for NLRP14 were indicated in the Ensembl database.
(B-D) Both Nlrp14 transcripts were specifically expressed in mouse ovary and testis. Error bar represented data from two mice. (E) Nlrp14 was induced in EpiLC and PGCLC stages. Error bar represented data from three independent experiments. (F) Nlrp14 knockdown did not affect the overall appearance of cells in the EpiLC state. Representative images for shNlrp14 cells in different EpiLC-induction stages were shown. Scale bar: 200µm.
(G) Nlrp14 knockdown did not affect the overall appearance of PGCLC spheres. Representative images for shNlrp14 cells in different PGCLC-induction stages were shown. Scale bar: 200µm.

Fig. S3. CRISPR/Cas9-mediated Nlrp14 knockout strongly inhibited PGCLC differentiation.

(A) Schematic of Nlrp14 knockout strategy in mouse ES cell line (AB2.2). sgRNA-1 and sgRNA-2 were located on Exon3 and Exon5, respectively. The arrows indicated the location of the primers for clone identification.
(B) Nlrp14 KO monoclonal cell lines were identified by PCR. “□” showed the candidate cell line. (C) The deletion mutation in Nlrp14 KO cells was confirmed by Sanger sequencing. An 8.2 kb deletion was present in the genomic DNA sequence. (D) Nlrp14 KO cells maintained their pluripotent state with positive immunostaining results of Nanog (green), OCT4 (red),
SOX2 (red), and Hoechst (blue). Scale bar: 50µm. (E-F) The knockout of Nlrp14 did not affect the overall appearance of cells/spheres in EpiLC and PGCLC differentiation. Representative images were shown. Scale bar: 200µm. (G) A significant decrease of STELLA expression was detected in PGCLC spheres from Nlrp14 KO cells. Representative images were shown. Normal IgG was used as the negative control for immunostaining. Scale bar: 50µm. (H) Nlrp14 deficiency had no significant effects on EpiLC marker expression. Cells at the EpiLC stage (day 2 post differentiation) were harvested for total RNA extraction and qRT-PCR. Fgf5, Wnt3, and Dnmt3b were used as EpiLC markers. Error bar represented data from three independent experiments. Statistics: Independent-sample t-test. NS: not significant. (I) Nlrp14 deficiency led to a significant decrease in gene expression for most of the PGCLC markers. PGCLC spheres were harvested for RNA extraction and qRT-PCR at day 8 post differentiation. Prdm1, Prdm14, Stella, and Nanos3 were used as PGCLC markers. Error bar represented data from three independent experiments. Statistics: Independent-sample t-test. ** p< 0.01, *** p< 0.001. NS: not significant.

Fig. S4. Generation and characterization of Nlrp14 knockout (KO) mice.

(A) The overall strategy for CRISPR/Cas9-mediated Nlrp14 knockout in mice. A sgRNA targeting early part of Nlrp14 CDS was injected with Cas9
to induce frameshift mutations. (B) Two mutant lines of Nlrp14 knockout mice were identified and used for downstream analysis. “□” shows about mutant location in knockout mice. (C) No obvious difference was identified in body shape and size among Nlrp14+/+, Nlrp14+-/-, and Nlrp14-/- male mice. Mouse age: three months. (D) The sex and genotype ratios followed Mendelian rule among offspring from Nlrp14+-/- breeding pairs. (E) Nlrp14 KO male mice showed no significant difference from wt ones in the general appearance of testis and gonad-somatic index. Error bar represented data from six mice. Scale bar: 2mm. Statistical analysis: one-way ANOVA followed by Tukey post hoc multiple comparisons. NS: not significant. (F) Representative images and quantitative analysis for sperm motility measurement in Nlrp14 wt, heterozygous, and KO animals under HTF medium stimulation. Scale bar: 50μm. (G, H) Increased expression of cleaved caspase 8 and caspase 3 was detected in the Nlrp14 KO testis. Protein expression levels of procaspase 8, cleaved caspase 8, procaspase 3 and activated caspase 3 were examined by western blotting using total lysate from testis of wt, hetero and homo mice. Each group contained samples from two adult animals (2-3 months) of each genotype. ACTIN was used as the loading control. (I, J) The sperm motility had no significant difference among each genotype under HTF medium stimulation. The motility of sperms within cauda epididymis of each genotype was measured by computer-assisted sperm analysis (CASA). Error bar
represented data from six mice. Statistics: one-way ANOVA followed by Tukey post hoc multiple comparisons. NS: not significant.

Fig. S5. Verification of all the antibodies used for IHC analysis.

(A-H) Antibody verification of anti-DDX4, anti-SCYP3, anti-Cyclin D2, anti-MSY2, anti-ACE, anti-Sox9, anti-HSPA2, anti-HSP70, respectively. Scale bar: 50µm in A, B, D, G, and H; 100µm in C, E, and F.

Fig. S6. Nlrp14 deficiency compromised germ cell differentiation, but not Sox9+ Sertoli cells.

(A) DDX4 (red) expression level was significantly increased in Nlrp14 KO testis. Testis sections were immunostained with anti-DDX4 and images were taken at different exposure time (121ms, upper panel or 66ms, middle panel). Hoechst was used for nuclear staining. Scale bar: 100µm. (B) The expression and distribution of SYCP3 (green) were significantly changed in the Nlrp14 KO testis. Scale bar: 100µm. (C) Expression of MSY2 (red) level was significantly decreased in Nlrp14 KO testis. Representative images were taken at different exposure time (121ms, upper panel or 61ms, middle panel) exposure time. Scale bar: 100µm. (D) Sox9+ Sertoli cells were not significantly changed in Nlrp14 KO mice. Representative images for Sox9 immunostaining in testis sections of each genotype were shown. Scale bar: 50µm. (E) Quantitative analysis of Sox9+ Sertoli cells indicated
no significant change in Nlrp14 KO mice. Testis sections from each genotype were immunostained with the anti-Sox9 antibody. Sox9+ Sertoli cells per seminiferous tubule were quantified and normalized to wt control. Error bar represented data from 15 seminiferous tubules for each genotype. Statistics: one-way ANOVA followed by Tukey post hoc multiple comparisons. NS, not significant.

**Fig. S7. Characterization of Spermatogonial stem cell line and NLRP14 interacting proteins**

**(A)** C18-4 cells were positive for Oct4 expression. Representative images for Oct4 immunostaining confirmed that the C18-4 spermatogonial stem cell line (SSC) kept its original characteristics. Hoechst was used as nuclear staining. Normal IgG was used as the negative control. Scale bar: 50μm.

**(B)** Expression of other SSC markers such as Ddx4, Dazl, and Plzf was also confirmed in C18-4 cells. Total RNAs were extracted using the Trizol method. Testis cDNA was used as the positive control. PCR products were analyzed at 30 cycles (actin) or 35 cycles (all other genes).

**(C)** Reverse IP confirmed the interaction between HSP70 and NLRP14. C18-4 cells were transfected with FLAG-tagged NLRP14 for 48hrs before harvested for IP analysis. The expression of transgenes was first confirmed by anti-FLAG antibody and Western blotting. WCL: whole cell lysate.

**(D)** Schematic of wt and truncation mutants of mouse HSP70.

**(E)** NBD domain of HSP70.
was required for NLRP14 binding. C18-4 cells were transfected with
different combinations of vectors for 48hrs before harvest for IP analysis.
All transgene expression was confirmed by Western blotting. WCL: whole
cell lysate. (F) Hspa2 mRNA levels were similar among Nlrp14 wt,
heterozygous, and KO animals. Error bar represented data from six mice.
Mice age: 3 months. Statistics: one-way ANOVA followed by Tukey post
hoc multiple comparisons. NS, not significant. (G) Nlrp14 overexpression
did not affect the poly-ubiquitination of HSP70 in 293FT cells. The
experimental procedure was the same as in (E). MG-132 was added at
42hrs post-transfection (6hrs before harvest) at 10µM final concentration.
(H) Interaction with HSP70 could protect NLRP14 from poly-
ubiquitination. The experimental procedure was the same as in E). MG-
132 was added at 42hrs post-transfection (6hrs before harvest) at 10µM
final concentration. (I) Immunofluorescence and statistical analysis
inferred that HSP70 expression level was slightly decreased in Nlrp14 KO
testis. Testis sections were prepared from mice of each genotype at 3
months of age. Hoechst was used as nuclear staining. Relative fluorescence
intensity per area was analyzed by ImageJ software and normalized with
wt signal. For each section, 5-6 seminiferous tubules were analyzed. Error
bar represented data from three animals for each genotype. Statistics: one-
way ANOVA followed by Tukey post hoc multiple comparisons (by SPSS).
* p< 0.05; ** p< 0.01. Scale bar: 100µm.
**Video S1:** Normal sperm concentration and motility in wt mice.

**Video S2:** Normal sperm concentration and motility in Nlrp14 heterozygous mice.

**Video S3:** Reduced sperm concentration and compromised motility were detected in Nlrp14 KO mice.
Materials and Methods

Bioinformatic analysis

To identify testis-specific genes, RNA-sequencing datasets from 8-week-old mice covering 24 tissues and 236 samples were downloaded from ENCODE (1). Genes with FPKM (fragments per kilobase of transcript per million fragments mapped) value > 20 in testis were selected. Those with a fold change between testis and other tissues ≥ 2 were considered as testis specifically expressed genes. The RNA-sequencing data for PGCLC differentiation was from GEO (GSE30056) (2). The differentially expressed genes in EpiLCs (day2) to ESCs (2i-LIF), PGCLCs to ESCs (2i-LIF), and PGCLSCs to EpiLCs (day2) were analyzed and identified using R and affy package with the cutoff line at logFold Change >1 (3).

For single-cell sequencing re-analysis, the original dataset (gene expression matrices of mouse spermatogenesis) was downloaded from NCBI's Gene Expression Omnibus (GEO) (accession ID: GSE107644(4)). The UMI (unique molecular identifiers) counts of genes at each developmental stage were averaged on the merged gene expression matrix. The smooth lines were predicted on averaged UMI counts using a generalized additive model (GAM). The data were processed in R language and figures were plotted by R and ggplot2 package.
Cells culture and PGCLCs differentiation

Mouse embryonic stem cells (mESCs) were cultured and maintained on 0.01% gelatin (G1890, Sigma) coated plates in 2i+LIF medium to maintain the naïve stage and in DMEM with 15% FBS (10099-141, Gibco), 1% NEAA(11140050, Gibco), 1% Pen-Strep (15140122, Gibco), 1% Glutamax (35050061, Gibco), 0.1 mM 2-Mercaptoethanol, and 1,000 U/ml LIF (C017, Novoprotein) for regular culture. The epiblast-like cells (EpiLCs) were induced by plating 1.0 x 10^5 naïve mESCs per well on 12-well plates coated with 16.7 μg/ml human plasma fibronectin (FC010, Millipore) in N2B27 medium containing 20 ng/ml Activin A (C687, Novoprotein), 12 ng/ml bFGF (C046-B, Novoprotein) and 1% KSR (A3181502, Gibco). The medium was refreshed every day. The PGCLCs were induced in suspension by plating 1.0 x 10^3 EpiLCs per well in the ultra-low attachment U-bottom 96-well plates (7007, Costar) in GMEM (11710-035, Gibco) with 15% KSR (A3181502, Gibco), 0.1 mM NEAA (11140050, Gibco), 1 mM sodium pyruvate (113600670, Gibco), 0.1 mM 2-Mercaptoethanol, 1% Pen-Strep (15140122, Gibco), 2 mM Glutamax (35050061, Gibco), 500 ng/ml BMP4 (314-BP/CF, R&D Systems), 500 ng/ml BMP8A (1073-BPC, R&D Systems), 1,000 U/ml LIF (C017, Novoprotein), 100 ng/ml SCF (CX53, Novoprotein), and 50 ng/ml EGF (C029, Novoprotein). Medium was refreshed every day. C18-4 and 293FT cell lines were cultured in DMEM with 10% FBS (SFBE, NATOCOR),
and 1% Anti-anti (15240062, Gibco).

**shRNA vector and lentiviral production**

pLKO-Non-targeting shRNA was purchased from Sigma (SHC016). For PGCLC candidate genes, three pairs of shRNA oligos for each gene were designed (Table S1). shRNA oligos were annealed and ligated into pLKO.1-puro lentiviral vector by Age I and EcoR I. To generate lentivirus, 293FT cells were seeded at $7.5 \times 10^5$ per well in 6-well plates, 1.5 $\mu$g packaging mix ($M_{REV}: M_{GAG}: M_{VSVG} = 3: 2: 1$) and 500 ng shRNA plasmid were co-transfected the next day using Lipofectamine 3000 (L3000-015, Invitrogen). 600 $\mu$l virus supernatant was harvested 2 days later by centrifuge at 4,000 rpm for 5min at room temp and then concentrated with Lenti-X concentrator (631232, Takara) following the manufacturer’s suggestions. For lentiviral transduction of mESCs, cells were seeded at 3.0 $\times 10^5$ per well in 12-well plates and transduced in suspension with lentiviruses (3 shRNA viruses of each gene were used at a volume ratio of 1:1:1, supplemented with 4$\mu$g /ml Polybrene (TR-1003-G, Millipore). The medium was refreshed the next day and 1$\mu$g/ml puromycin was added for drug selection. Three days post-transduction, the transduced mESCs were switched to 2i+LIF medium and induced for EpiLC/PGCLC differentiation.

**Immunohistochemistry and immunostaining**
Tissue sections were first deparaffinated and rehydrated. Antigen retrieval was performed with Tris/EDTA pH9.0 buffer (microwave to boil over for 15-20 min and cooled at room temperature). After PBS wash, sections were treated with 3% hydrogen peroxide for 10 min, followed by 0.3% Triton X-100 for 10 min and PBS wash for 3 times. Sections were then blocked with 5% BSA (A1933 Sigma) in PBS for 1hr at room temperature. The primary antibody was diluted at 1:200 in 2.5% BSA-PBS according to the manufacturer’s suggestion and incubated with tissue sections overnight at 4 °C. Then, the sections were washed for 3 times with PBS. The secondary antibody was diluted at 1:400 and incubated with sections for 1hr at room temperature followed by PBS wash for 3 times. Then the sections were stained by DAB (1:50) for 70s at room temperature. After washed by PBS, the sections were also stained by hematoxylin for 5min at room temperature followed by 1% ammonia treatment for 1min. Images of sections were then taken on IX73 (Olympus, Japan). Antibodies and reagents used in the study were: anti-Cyclin D2 (3741s, Cell Signaling), Mouse/rabbit polymer detection system (PV-6000, ZSBG-BIO), and DAB colorimetric kit (ZLI-9017, ZSBG-BIO). For immunofluorescence, the primary antibody was diluted from 1:100 to 1:400 following manufacturer’s suggestions in 2.5% BSA-PBS with 0.1% Triton X-100 (T9284, Sigma), and incubated with sections overnight at 4 °C. The sections were then washed for 3 times with PBS. The secondary antibody
was diluted 1:400 and applied for 1hr at room temperature followed by PBS wash for 3 times. Hoechst was diluted 1:1000 and stained for 20 min followed by PBS wash for 3 times. Images were taken on TCS SP5II (Leica, Germany). Antibodies used in the study were: anti-DDX4 (ab13840, Abcam), anti-SYCP3 (ab205846, Abcam), anti-MYS2 (EM1701-40, HuaAn Biotechnology), anti-ACE (NBP2-67111, Novus), Embryonic Stem Cell Marker Panel (ab107156, Abcam), anti-SOX9 (ET1611-56, HuaAn Biotechnology), anti-HSPA2 (12797-1-AP, Proteintech), anti-HSP70 (10995-1-AP, Proteintech), normal rabbit IgG (12-370, Millipore), normal mouse IgG (N103, Millipore), Hoechst (H3570, Invitrogen), donkey anti-mouse IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 568 (A10037, Invitrogen), and donkey anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 488 (A10037, Invitrogen).

For immunostaining of PGCLC spheres, cells were harvested at day 8, washed 3 times in PBS, and then fixed in 4% PFA for 30 min at room temperature. 0.3% Triton-X 100 in PBS was used to permeabilize the cells for 30 min at room temperature. Then the cells were washed with 0.1% PBST for 3 times and then blocked in 5% BSA (A1933 Sigma) in 0.1% PBST for 1hr at room temp. Anti-STELLA antibody (ab19878, Abcam) was diluted at 1:200 and incubated with cells overnight at 4 °C followed
by PBS wash for 3 times. The secondary antibody was diluted at 1:400 and incubated with cells for 1hr at room temp followed by PBS wash for 3 times. Hoechst was diluted 1:1000 and stained for 20 min followed by PBS wash for 3 times. Images were taken on FV1200 (Olympus Japan).

**Male germ cells isolation and FACS analysis**

Testes were dissected into a dish containing ice-cold sterile PBS. For each genotype, testes from 2 animals (2-6 months) were pooled and processed. Each testis was decapsulated by making an incision and forcing the content of the testis through the incision by sterile tweezers into 15ml tube containing 5ml ice-cold PBS (with Ca$^{2+}$ and Mg$^{2+}$) with 0.25mg/ml collagenase type I (C0130, Sigma). Then the seminiferous tubules were incubated for 15min at 37℃ with gently shake every 5min. The tubules were then allowed to sediment to the bottom of the tube and washed twice by PBS. Washed tubules were then incubated in 5ml 0.25% Trypsin-EDTA (25200072, Gibco) with 1µg/ml DNaseI (04716728001, Roche) at 37℃ for 5min with gently shake. Trypsin digestion was terminated by adding an equal volume of culture medium (DMEM with 10% FBS (SFBE, NATOCOR), and 1% Anti-anti (15240062, Gibco)). The suspension was centrifuged at 400g for 5min at 4℃ and the tubules were resuspension in culture medium and disaggregated into a single-cell suspension by pipetting up and down. Aggregates were removed by filtering the cell
suspension through a 70 μm filter (352350, FALCON). Cells were counted by hemocytometer.

Germ cells were stained both for DNA content and expression of the c-Kit cell surface marker. For DNA content, $1 \times 10^6$ germ cells were washed once with PBS, resuspended in 1ml of ice-cold PI staining solution (10mM Tris (0497, VWR), pH 8.0, 1mM NaCl (71386, Sigma), 0.1% Nonidet P40 (I8896, Sigma), 50μg/ml PI (P4170, Sigma), 10μg/ml RNase A (S8028, TIANGEN)), pipetting up and down and incubated on the ice at least for 10min. For c-Kit surface staining, $1 \times 10^6$ germ cells were washed once by PBS with 2% FBS (SFBE, NATOCOR), and then resuspension in 100μl PBS with 2% FBS. The anti-c-Kit-PE (105807, BioLegend) and PE Rat IgG2b control (400607, BioLegend) were diluted following the manufacturer’s suggestions. After washed twice by PBS with 2% FBS, the signal was analyzed. The DNA content and expression of c-Kit were assessed on a FACS analyzer (BD LSRfortessa) as described (5) (6).

**mRNA extraction, quantitative PCR and RT-PCR**

Total RNAs were extracted using Trizol (T9424, Sigma). 1μg total RNAs were used for reverse transcription by Superscript II (18064014, Invitrogen). Quantitative PCR was performed using a BIO-RAD CFX384 real-time system and the SYBR Green Supermix from BIO-RAD (172-
5124). The primers used were listed in Table S3. RT-PCR was performed using PrimeSTAR® Max DNA Polymerase (R045Q, Takara) with 2ng cDNA in 10µl volume. The primers used to analyze the marker genes expression profile in C18-4, adult testis (as positive control) and H2O (as negative control) were also listed in Table S3 (Ddx4, Dazl and Plzf).

**Mice**

C57BL/6 strain mice were housed in standard cages in an Assessment and Accreditation of Laboratory Animal Care credited SPF animal facility on a 12-h light/dark cycle. All animal protocols are approved by the Animal Care and Use Committee of the Model Animal Research Center, College of Life Sciences, Sichuan University.

**Knockout mouse model construction**

Nlrp14 knockout mouse model was created by IDMO (Beijing, China) using the CRISPR/Cas9 system. The 3rd exon of mouse Nlrp14 was chosen as the sgRNA target. sgRNA sequence was 5’-GCTGCAGCATATATTGATGAGG-3’ (PAM sequence is underlined). The target genomic regions were amplified by PCR and sequenced to verify the presence of indel mutations. The primers used in this part were listed in Table S3.

**Morphological and histological analysis**

Adult mice of each genotype were assessed for phenotype at around 2-3
months age. Body weight and gonad weight of mice from each genotype were measured. The Gonad-Somatic Index (GSI) was calculated as (gonad weight/body weight) x 100%. Images of testis of each genotype were taken using SZX10 (Olympus, Japan), and then fixed by modified Davidson’s fluid. The sections of the testis from 3 mice of each genotype were stained with hematoxylin and eosin, and images were taken on IX73 (Olympus, Japan). In order to assess the reproductivity, continuous mating for 10 weeks was carried out for every 6 groups of female (+/+)/male (+/+), female (+/-)/male (+/-), and female (+/+)/male (-/-). The average number of pups per female was calculated. The reproductivity of wt mice was confirmed first before mating with KO ones.

**Sperm analysis and scanning electron microscopy**

Sperms were collected from the cauda epididymites of adult male mice (2-3 months old, 6 mice for each genotype). Each cauda was carefully trimmed to remove adipose and other tissues, washed in PBS, and placed in 1ml of pre-heated PBS or HTF on a heater at 37 °C for 3 min to release the sperm. After incubation, the tissue was removed and the suspension was mixed gently by pipetting. This suspension was then diluted at 1:10 in PBS or HTF for Computer-assisted sperm analysis (CASA, AndroVision, Carl Zeiss, Germany) to assess the motility. 6 view field was assessed by CASA for each sperm sample. Rest of the diluted suspension was then used
to quantify the sperm number and prepare for electron microscopic analysis. The morphology of sperm was observed under the microscope after hematoxylin stain, and the ratio between normal and abnormal sperm was calculated for each genotype. For electron microscopic analysis, the sperms were fixed in 2.5% glutaraldehyde solution in phosphate buffer, collected on poly-L-lysine coated glass overslips, post-fixed in osmium tetroxide, dehydrated through a series of ethanol gradient and subjected to critical point drying and then coated with gold/palladium. Finally, samples were analyzed by Inspect F50 (FEI, USA).

**TUNEL assay**

TUNEL assay was detected by Click-iT™ Plus TUNEL Assay (C10617, Invitrogen) and performed according to the manufacturer’s suggestion.

**Plasmids**

The coding sequence of mouse Nlrp14 was cloned into pPB backbone with CAG promoter, N-terminal FLAG tag, and C-terminal BSD cassette (Blasticidin drug selection cassette). The mutant mouse FLAG-Nlrp14 was constructed by mutating the 412th A to T which introduced a stop codon at the 138th amino acid of the Nlrp14 gene. Δ-81-403, Δ-404-627, and Δ-628-993 deletion plasmids were constructed by deleting the corresponding sequences on full-length FLAG-Nlrp14 plasmid using KOD-Plus-
Mutagenesis kit (SMK-101, Toyobo). The coding sequence of mouse Hspa2 and Hsp70 was cloned into the pEDNA backbone with EF1α promoter and N-terminal Myc tag, respectively. Δ-2-389, Δ-397-633, Δ-2-386, and Δ-394-642 deletion plasmids were constructed by deleting the corresponding sequences using KOD-Plus-Mutagenesis kit (SMK-101, Toyobo), respectively. The coding sequence of mouse Bag2 was cloned into pLVX backbone and mouse CHIP was cloned into pEDNA backbone with N-terminal Myc tag. The coding sequence of human NLRP14 was synthesized from Qinglan Biotech (Wuxi, China) based on the sequence from NCBI and sub-cloned into pBEP backbone with EF1α promoter and N-terminal FLAG tag. The mutant human FLAG-Nlrp14 was constructed by mutating the 322th A to T which also introduced a stop codon at 108th amino acid of the human Nlrp14 gene. The coding sequence of human Hspa2 was cloned from human testis cDNA into pBEP backbone with EF1α promoter and N-terminal Myc tag. The coding sequence of human BAG2 was cloned from human testis cDNA into pBEP backbone with EF1α promoter.

**Immunoprecipitation & Western Blotting**

48hrs post-transfection, cells from 6-well plates were collected by centrifuge at 5,000 rpm for 3 min. The cell pellet was re-suspended in 250μl NP-40 lysis buffer (P0013F, Beyotime) with 2.5μl protease inhibitor
cocktail (100x, P8340, Sigma). Cells were gently mixed by pipetting and incubated on ice for 6 min (repeat the procedure three times). After that, cell debris was removed by centrifuge at 13,000 rpm for 30 min at 4°C and the lysate was then used for protein concentration measurement with Pierce™ BCA protein Assay Kit (23227, Thermo). 20μl whole cell lysate was used for Western blotting to check transgene expression. For immunoprecipitation (IP), an equal amount of whole-cell lysate (WCL) for each sample was adjusted to the total volume of 350μl with NP-40 lysis buffer. 10μl magnetic Protein-G beads (1004D, Dynabeads, Invitrogen) per sample were first prepared by washing with 150μl NP-40 lysis buffer for 3 times and added to WCL for 15 min incubation at 4°C to remove the non-specific binding of cellular proteins. After that, beads were removed and primary antibodies were added into WCL for overnight incubation at 4°C. The next day, 15μl Protein-G beads (washed 3 times with NP-40 buffer before) were added to the antibody-containing WCL for IP. The mixture was then incubated for 2 hrs at 4°C. Antibody and protein-bound magnetic beads were then collected and washed with 150μl NP-40 lysis buffer for 5 times. Finally, 45μl 2x loading buffer (with DTT, P1040, Solarbio) was added to each sample (beads) for denaturing at 100°C for 10 min before the lysate was used for SDS-PAGE and Western blotting analysis.

For SDS-PAGE and Western blotting, an equal volume of lysates was
loaded onto 10% SDS-PAGE gels, and proteins were transferred to PVDF membranes (Bio-Rad, 1620177) using the semi-dry system (Bio-Rad). Membranes were blocked with 5% milk in TBST for at least 1hr at room temp. Antibodies used include: anti-FLAG (F1804, Sigma), anti-HSPA2 (12797-1-AP, Proteintech), anti-HSP70 (10995-1-AP, Proteintech), anti-BAG2 (A304-751A, BETHYL), anti-His (A00186, GeneScript), normal rabbit IgG (12-370, Millipore), normal mouse IgG (N103, Millipore), anti-Actin (sc-1616, Santa Cruz), goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz), goat anti-mouse IgG-HRP (sc-2005, Santa Cruz), donkey anti-mouse IgG (H+L) cross-adsorbed secondary antibody, DyLight 800 (SA5-10172, Invitrogen), donkey anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, DyLight 680 (SA5-10042, Invitrogen), and donkey anti-goat IgG (H+L) cross-adsorbed secondary antibody, DyLight 800 (SA5-10092, Invitrogen). Silver staining was performed by following the manufacturer’s instructions (C500021, Sangon Biotech).

For analysis of procaspases and cleaved caspases expression, each testis from two adult animals of each genotype was incubated in 1×SDT (1% SDS (0227, VWR), 0.1M DTT (11583786001, Sigma), 0.1M Tris (0497, VWR)) with 1× protease inhibitor cocktail (100x, P8340, Sigma) for 30min on ice after dissociated with homogenizer. Then cell debris was removed by centrifuge at 13,000 rpm for 30min at 4°C and the lysate was then used
for protein concentration measurement with Pierce BCA Protein Assay Kit (23227, Thermo) before Western blotting analysis. Antibodies used include: anti-Caspase-3 (9662S, CST), anti-cleaved-Caspase-3 (9661S, CST), anti-Actin (sc-1616, Santa Cruz), goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz), goat anti-mouse IgG-HRP (sc-2005, Santa Cruz), anti-Caspase-8 (RT1099, HuaAn Biotechnology).

Mass Spectrometry Analysis
To identify NLRP14 interacting proteins, three independent samples of wt NLRP14, IgG control, and mutant NLRP14 were prepared. The Immunoprecipitation procedure was the same as described above. The Mass Spectrometry analysis was carried out by the Mass Spectrometry Core facility in the College of Life Sciences (QE-Plus, Thermo Fisher, USA). Differential proteins among wt NLRP14 and IgG control and mutant NLRP14 groups were identified by the LFQ intensity from analyzed samples. P values were calculated using the \( t \)-test. Cutoff line: \( \log_2 \text{FoldChange} > 0 \) and \( p \)-value < 0.06. The LFQ intensity was z-score transformed for the heatmap.

Poly-Ubiquitination Assay
Transfected cells were treated with 10mM MG-132 (S2619, Selleckchem) for 6hrs before harvest. 48hrs post-transfection, cells from a 6-well plate
were collected by centrifuging at 5,000 rpm for 3 min. The cell pellet was re-suspended by vortex in 100µl NP-40 lysis buffer (P0013F, Beyotime) with 1x protease inhibitor cocktail (100x, P8340, Sigma) and 0.1% SDS (L3771, Sigma), then incubated on ice for 10 min. After that, the cell lysate was incubated at 95 ℃ for 5 min before adding 350µl NP-40 lysis buffer with 1x protease inhibitor cocktail and sonication. The lysate was cleared by centrifuge at 13,000 rpm for 30min at 4℃ and total protein concentration was measured with Pierce™ BCA protein Assay Kit (23227, Thermo). 20 µl lysate was used as input for checking transgene expression. The rest of the IP procedure was the same as mentioned above. Antibodies used include: anti-Myc (sc-40, Santa Cruz), anti-HA (11867423001, Roche), anti-FLAG (F1804, Sigma), anti-BAG2 (A304-751A, BETHYL), anti-Actin (sc-1616, Santa Cruz), goat anti-rat IgG-HRP (sc-2006, Santa Cruz), goat anti-mouse IgG-HRP (sc-2005, Santa Cruz), donkey anti-mouse IgG (H+L) cross-adsorbed secondary antibody, DyLight 800 (SA5-10172, Invitrogen), donkey anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, DyLight 680 (SA5-10042, Invitrogen), and donkey anti-goat IgG (H+L) cross-adsorbed secondary antibody, DyLight 800 (SA5-10092, Invitrogen).

Nuclear and cytoplasmic protein extraction

Nuclear and cytoplasmic proteins were extracted using Nuclear and
Cytoplasmic Protein Extraction Kit (P0027, Beyotime) and performed according to the manufacturer’s suggestion. The data was analyzed by immunoblotting. Antibodies used include: anti-FLAG (F1804, Sigma), anti-HSPA2 (12797-1-AP, Proteintech), anti-GAPDH (AB0037, Abways), anti-LAMIN (sc-7292, Santa Cruz), donkey anti-mouse IgG (H+L) cross-adsorbed secondary antibody, DyLight 800 (SA5-10172, Invitrogen), donkey anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, DyLight 680 (SA5-10042, Invitrogen).

Statistical analysis

All statistical analysis was done using SPSS software. Data were presented as the mean values and standard error of the mean (±SEM). P < 0.05 was considered statistically significant using the \( \chi^2 \) test, independent-sample \( t \)-test, and one-way analysis of variance (ANOVA) in the SPSS software.

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Table S1: shRNA used in this study.

| Gene name | shRNAs | Sequence (5’-3’) |
|-----------|--------|-----------------|
| Adcy7     | 1      | gccatcattgagcgccctcaaa |
|           | 2      | cctgaagatcatggttaacctt |
|           | 3      | caattaggccattctggtaaat |
| Crlf1     | 1      | gacatttcacatatcaatta |
|           | 2      | gcaacaaagaaacctaccaga |
|           | 3      | ctgcaagctacctctgtctata |
| Ddx25     | 1      | gcttcgtgttgttggtgcta |
|           | 2      | ccaatgttatcaagttgcgaa |
|           | 3      | caagaccacttcataagcaat |
| Grip1     | 1      | cgccctctgatttcctatat |
|           | 2      | cctctgttaccttatgga |
|           | 3      | gacctggtttagcagaaat |
| Hspa4l    | 1      | cgacagacttgggtcagtt |
|           | 2      | ccaggttgaaaccacctttta |
|           | 3      | gtaatggatcaagctactta |
| Mael      | 1      | cctccaacaacatccatagat |
|           | 2      | caggggttgaaaccaccttttt |
|           | 3      | caatagtaaatgtcactccta |
| Nlrp14    | 1      | taaactgtgtgatagagattgc |
|           | 2      | tacagatccatttcatacgg |
|       |   | DNA Sequence           |
|-------|---|-----------------------|
| **Slc25a31** | 1 | cgtaactacctgttctggaat |
|       | 2 | gctcaatctgactacctgt   |
|       | 3 | cttgatgaaacaccatataa |
| **St6galnac2** | 1 | cggaaacctctgccagtaaat |
|       | 2 | gaataatctgagcgcagcaca |
|       | 3 | ccaagctgattaacacagct |
| **Vash2** | 1 | gatttaactgcaacgggttact |
|       | 2 | geggttcctatcagctttta |
|       | 3 | geggttcctatcagctttta |
Table S2: sgRNA used in this study.

| Gene name | sgRNA name | Sequence (5’-3’) |
|-----------|------------|------------------|
| Nlrp14    | Cell-line-1| tgaggatatccaaaacctccg |
|           | Cell-line-2| acgggctacattcaggtcgc |
|           | KO Animal  | gctgcagcatatatgagt |

## Table S3: Primers used in this study.

| Gene name | Primer name | Sequence (5’-3’) |
|-----------|-------------|------------------|
| Oct4      | forward     | gatgctgtgagccaaggcaag |
|           | reverse     | ggctcctgatcaacagcatcac |
| Sox2      | forward     | catgagagcaagtactggcaag |
|           | reverse     | ccaacgatatacaacctgcatgg |
| Nanog     | forward     | ctttcacctattaaggtgettg |
|           | reverse     | tggcctcgtctcatcatgg |
| Fgf5      | forward     | aaagtcgatggcctcag |
|           | reverse     | cttcgtctgatcttctact |
| Wnt3      | forward     | caagcacaacaatgaagcag |
|           | reverse     | tgggaactcagggtttt |
| Dnmt3b    | forward     | ctcgcaaggtggtggccaac |
|           | reverse     | cttgggcattgcatcatcttgc |
| Prdm1     | forward     | agcatgacctgacatggc |
|           | reverse     | ctcacacttcatgtaagag |
| Prdm14    | forward     | acagccaagcaattgcatctac |
|           | reverse     | ttacctggccatttcatttgctc |
| Stella    | forward     | aggcctgaaggaatgagtttg |
|           | reverse     | tctaatctcccgatttcteg |
| Naons3    | forward     | cactacggccctaggaggtg |
|           | reverse     | tgatcgcgtgacaagactgtgg |
|           | forward          | reverse          |
|-----------|------------------|------------------|
| Actin     |                  |                  |
|           | forward caccctgctgtttccagaac | reverse cccaccttactgaagccaga |
|           |                  |                  |
| Nlrp14 transcript I |                  |                  |
|           | forward caaagcactggcctagcag | reverse getttcaaaggagaatccag |
|           |                  |                  |
| Nlrp14 transcript II |                  |                  |
|           | forward ccctccagttgatgctcttt | reverse ggctttgtgcatgagagaac |
|           |                  |                  |
| Nlrp14 both transcripts |                  |                  |
|           | forward acgtcttagaagatgaagggataag | reverse tgaactgcaagacctaagcag |
|           |                  |                  |
| Nlrp14 primer set 1 |                  |                  |
|           | forward agtagcagtcagcatgtggt | reverse agctttgggtttgtagcctga |
|           |                  |                  |
| Nlrp14 primer set 2 |                  |                  |
|           | forward tggagttatatagccaagagtagacc | reverse agaggagactgctgggttag |
|           |                  |                  |
| Nlrp14 primer set 3 |                  |                  |
|           | forward tcaagggctgtatgtctcag | reverse gacagaccccctctcacagggc |
|           |                  |                  |
| Nlrp14 sequence primer |                  |                  |
|           | forward gaaaattttgtttgatcatgtgtgttctttg | reverse atctgggtccagttttacacagt |
|           |                  |                  |
| Ddx4      |                  |                  |
|           | forward ctactccaggaggtagtgatg | reverse tgtgtaacatctctgcaagc |
|           |                  |                  |
| Dazl      |                  |                  |
|           | forward ctgatcgaactggttgtggtcg | reverse ggttggaggtgcatgtaaag |
|           |                  |                  |
| Plzf      |                  |                  |
|           | forward gagacacacagacagacacca | reverse gtggcagagtttgcactcaa |
