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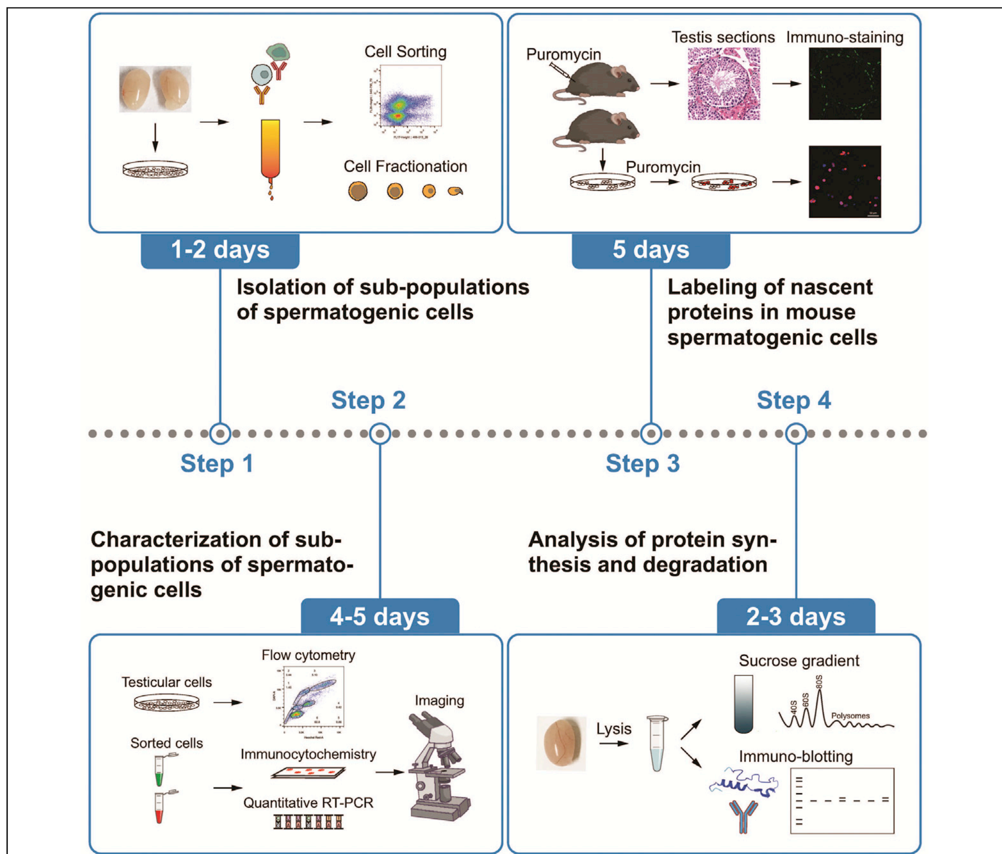
Protocol

Protocol for isolation and proteostatic analysis of sub-populations of spermatogenic cells in mouse



Spermatogenesis generates heterologous cell populations which, if not distinguished clearly, often hinder mechanistic and etiological studies. Here, we present a protocol to identify and isolate populations of mouse spermatogenic cells, including spermatogonial stem cells (SSCs), spermatocytes, and haploid spermatids. We also describe absolute quantification of mRNA copy numbers in SSCs. The isolated cells can be used for analyzing nascent protein synthesis and protein degradation, two main events that maintain cellular proteostasis important for healthy and long-term production of male gametes.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for isolation of sub-populations of SSCs

Protocol for determination of absolute copy numbers of mRNAs in SSCs

Identification of spermatogenic cells at defined developmental stages

In vivo and *in vitro* labeling of nascent proteins of mouse spermatogenic cells

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Protocol for isolation and proteostatic analysis of sub-populations of spermatogenic cells in mouse

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SUMMARY

Spermatogenesis generates heterologous cell populations which, if not distinguished clearly, often hinder mechanistic and etiological studies. Here, we present a protocol to identify and isolate populations of mouse spermatogenic cells, including spermatogonial stem cells (SSCs), spermatocytes, and haploid spermatids. We also describe absolute quantification of mRNA copy numbers in SSCs. The isolated cells can be used for analyzing nascent protein synthesis and protein degradation, two main events that maintain cellular proteostasis important for healthy and long-term production of male gametes.
For complete details on the use and execution of this protocol, please refer to Zou et al. (2021).

BEFORE YOU BEGIN

Spermatogenesis encompasses multiple steps, ranging from mitosis, meiosis to cellular morphogenesis. The progressive nature of development of spermatogenic cells entails multiple cell types with distinguished cellular states, of which identification and isolation are prerequisite of the study of underlying molecular mechanisms. How proteins, the functional units of cells, are synthesized and maintained in spermatogenic cells at various developmental stages remain to be fully understood. Here we describe protocols that can be used to isolate sub-populations of mouse spermatogenic cells and to analyze nascent protein synthesis and protein degradation, two main events that maintain cellular proteostasis that are important for the regulation of spermatogenesis.

Preparation one: Animal usage

Both wild type C57BL/6 mice and transgenic mouse lines under the same genetic background were used for the isolation of spermatogenic cells. Male mice at the age of 10-dpp (days-post-partum) or 2–3-month old adults were used for dissection of testes and preparation of testicular cells. All animal husbandry and handling should be carried out according to the guidelines of IACUC and that of local institutes. The usage of mice in this protocol was approved by the animal care committee at the Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences (Permit No. 2020240).

Preparation two: Generation of gene edited mouse lines

Gene editing is an essential step to generate genetically modified animals in order to study the functional roles of a gene, especially when *in vitro* systems are lacking for the study of spermatogenic

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cells. Conventional gene editing method using embryonic stem cells is time consuming and the efficiency is low. Since the discovery of CRISPR/Cas9 (Shalem et al., 2015; Doudna and Charpentier, 2014), gene editing in mouse can be achieved via various means of nucleic acid delivery in mouse pre-implantation embryos at different stages (van der Weyden et al., 2021; Henao-Mejia et al., 2016; Kim et al., 2017; Hur et al., 2016). A study using microinjection of mouse 2-cell embryos for generating targeted large insertions has also been described previously (Gu et al., 2018). We routinely use an alternative approach of cytoplasmic injection of *in vitro* transcribed RNAs in mouse 2-cell embryos in order to generate mouse lines with targeted gene knockout. Injection of both blastomeres of 2-cell embryos helps to increase the efficiency of mutagenesis that occurs in embryos. Founder mice carrying genetic mutations can be generated with high efficiency in relatively shorter period of time (4–5 months).

Preparation three: Buffers and equipment

Most of the buffers needed can be prepared and stored at 4°C unless otherwise noted. Equipment, including FACS machine, ultracentrifuge and sucrose gradient fractionator should be turned on 30 min prior to use accordingly (see below).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
eFluor 780-conjugated anti-cKIT (1:500)	eBioscience	Cat#47117180
PE-conjugated anti-PLZF (1:200)	eBioscience	Cat#12932080
Rabbit anti-SYCP3 (1:400)	Abcam	Cat#ab15093
Mouse anti-γH2AX (1:500)	Millipore	Cat#05-636
Mouse anti-Puromycin (1:1000 for IF, 1:10000 for WB)	Millipore	Cat#MABE343
Rabbit anti-DNAJB6 (1:100 for IF, 1:2000 for WB)	Proteintech	Cat#11707-1-AP
Rabbit anti-DNAJB8 (1:100 for IF, 1:2000 for WB)	Proteintech	Cat#17071-1-AP
Rabbit anti-Ubiquitin (1:1000)	Abcam	Cat#ab7780
Mouse anti-αTubulin (1:5000)	Proteintech	Cat#66031-1-lg
Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:1000)	Invitrogen	Cat#A-11036
Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000)	Invitrogen	Cat#A-11001
Alexa Fluor 568-conjugated goat anti-mouse IgG (1:1000)	Invitrogen	Cat#A-11004
HRP-conjugated goat anti-mouse IgG (1:5000)	MultiSciences	Cat#GAM007
HRP-conjugated goat anti-rabbit IgG (1:5000)	MultiSciences	Cat#GAR007
Chemicals, peptides, and recombinant proteins		
Taq DNA polymerase	TIANGEN	Cat#ET101
Reverse Transcriptase XL, AMV	Takara	Cat#2620A
TB Green Premix ExTaq	Takara	Cat#PR420A
Paraformaldehyde (PFA)	Sigma-Aldrich	Cat#P6148
DMEM basic media	Gibco	Cat#C11995500CP
Collagenase IV	Worthington	Cat#LS004188
DNase I	Worthington	Cat#LS002139
Trypsin	Worthington	Cat#LS003703
EKRB, Krebs Ringer Buffer	AMSBIO	Cat#KRB-1000
Hank's Balanced Salt Solution (HBSS, 1×)	Gibco	Cat#14025092

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fetal Bovine Serum (FBS)	Gibco	Cat#16140071
Non-essential amino acids (NEAA, 100×)	Gibco	Cat#11140-050
Penicillin/Streptomycin (100×)	Gibco	Cat#15140-122
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat#A2153-500G
Sucrose	Sigma-Aldrich	Cat#V900116-500G
Phosphate buffered saline (PBS, Ca^{2+}/ Mg^{2+} free, 1×), pH7.4	Servicebio	Cat#G4202-500mL
Hoechst 33342	Sigma-Aldrich	Cat#14533
4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich	Cat#D8419-1MG
Propidium Iodide (PI)	Sigma-Aldrich	Cat#P4170
Giemsa	Sigma-Aldrich	Cat#GS500
Photo-Flo 200 solution	Kodak Professional	Cat#1464510
Protease Inhibitor cocktail	Roche	Cat#4693132001
Puromycin	Gibco	Cat#A1113803
Recombinant ribonuclease inhibitor (RRI)	Takara	Cat#2313B
RIPA buffer	Beyotime	Cat#P0013E
TRIzol® Reagent	Invitrogen	Cat#15596-038
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich	Cat#40718-5ML
1,4-Dithiothreitol (DTT)	Sigma-Aldrich	Cat#1114740001
Triton™ X-100	Sigma-Aldrich	Cat#X100-100ML
TERGITOL™ solution (Type NP-40)	Sigma-Aldrich	Cat#NP40S-100ML
Tween® 20	Sigma-Aldrich	Cat#P9416-50ML
Glycerol	Sigma-Aldrich	Cat#G5516-100ML
Xylenes, histological grade	Sigma-Aldrich	Cat#534056-500ML
Sodium citrate	Sigma-Aldrich	Cat#C8532
5× loading buffer	CWBIO	Cat#CW0027
Neutral Balsam	Biosharp Life Sciences	Cat#BL704A
Critical commercial assays		
RNAsimple ToTal RNA Kit	TIANGEN	Cat#DP419
TIANgel Midi Purification Kit	TIANGEN	Cat#DP209
Proteostat Protein Aggregation Assay Kit	Enzo Life Sciences	Cat#ENZ-51023-KP050
Enhanced BCA Protein Assay Kit	Beyotime	Cat#P0010
ECL Detection Kit	Tanon	Cat#180-501
Experimental models: Organisms/strains		
Mouse: C57BL/6J (Male, 10-day to 2–3-month old)	The Jackson Laboratory	JAX: 000664
Mouse: B6; CBA-Tg(Pou5f1-EGFP) 2Mnn/J (OG2) (Male, 10-day to 2–3-month old)	The Jackson Laboratory	JAX: 004654
Mouse: C57BL/6J-*Rpl39l* (Male, heterozygous and homozygous mutants, 10-day to 2–3-month old)	This study	Rpl39l-KO
Oligonucleotides		
Rpl39l-RT-F	This study	5′CACCCTGCCTTTCCAAGTTCAA3′
Rpl39l-RT-R	This study	5′GTTCGTCTCCAATGTCTCCGCT3′
Software and algorithms		
ImageJ	NIH (Schneider et al., 2012)	https://imagej.nih.gov/ij/download.html
Others		
MoFlo XDP Cell Sorter	Beckman Coulter	Moflo Astrios EQ
BD LSRFortessa™ Cell Analyzer	BD Biosciences	BD LSR II
Confocal Laser Scanning Microscope	ZEISS	LSM T-PMT

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
EVOS^{fl} Microscope	Advanced Microscopy Group	AMF5000
BioComp Gradient Master^{TM}	BioComp	Model 108
BioComp Fractionator	BioComp	N/A
Avanti^{TM} Ultracentrifuge	Beckman Coulter	Optima L-80 XP
Rotor	Beckman Coulter	SW41
Ultra-Clear Centrifuge Tube (14 × 89 mm)	Beckman Coulter	Cat#344059
Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell	Bio-Rad	Cat#1703940
Chemiluminescent Imaging System	Sinsitech	MiniChemi^{TM} 610
Nitrocellulose membrane	GE Healthcare Life Sciences	Cat#10-401-196
Glass Tissue Homogenizer	Canfort Laboratory and Education Supplies Co., Ltd.	Cat#LG109
Falcon® 40-μm Cell strainer	Life Sciences	Cat#352340
Micro-dissecting scissors	Sigma-Aldrich	Cat#S3146-1EA
Tweezers	Sigma-Aldrich	Cat#T5790-1EA
Hemacytometer Set	Hausser Scientific	Cat#1483
5-mL Falcon tube	BD Falcon	Cat#352058
15-mL Centrifuge Tube	Corning	Cat#430790
50-mL Centrifuge Tube	Corning	Cat#430828
35-mm cell culture dish	Greiner Bio-One	Cat#627160
60-mm cell culture dish	Greiner Bio-One	Cat#628160
6-well cell culture plate	Greiner Bio-One	Cat#657160
ImmHis^{TM} Hydrophobic Barrier Pen	Yeason	Cat#36310ES64

MATERIALS AND EQUIPMENT

DMEM complete media

Reagent	Final concentration	Amount
DMEM basic media	–	88 mL
FBS (100%)	10%	10 mL
NEAA (100×)	1×	1 mL
Penicillin/Streptomycin (100×)	1×	1 mL
Total	n/a	**100 mL**

Sterilize by filtering through 0.22-μm filter and store at 4°C up to 1 month.

Hypotonic buffer

Reagent	Final concentration	Amount
Tris-HCl (300 mM, pH8.2)	30 mM	5 mL
Sucrose (500 mM)	50 mM	5 mL
Trisodium citrate dihydrate (170 mM)	17 mM	5 mL
EDTA (100 mM)	5 mM	2.5 mL
PMSF (100 mM)	1 mM	0.5 mL
Protease inhibitor cocktail (PIC, 100×)	1×	0.5 mL
ddH_2O	–	31.5 mL
Total	n/a	**50 mL**

Sterilize by filtering through 0.22-μm filter and store at 4°C up to 6 months. PIC is added before use.

Isotonic sodium citrate

Reagent	Final concentration	Amount
Sodium Citrate	2.2%	2.2 g
ddH_2O	–	100 mL
Total	n/a	**100 mL**

Sterilize by filtering through 0.22-μm filter and store at 4°C up to 6 months.

Hypotonic sodium citrate		
Reagent	Final concentration	Amount
Sodium Citrate	1.0%	1.0 g
ddH_2O	–	100 mL
Total	n/a	**100 mL**
Sterilize by filtering through 0.22-μm filter and store at 4°C up to 6 months.		

Fixation buffer		
Reagent	Final concentration	Amount
Paraformaldehyde (PFA, 10%)	1%	5 mL
Triton X-100 (3%)	0.15%	2.5 mL
Sodium borate (100 mM, pH9.2)	10 mM	5 mL
PBS (1×)	–	37.5 mL
Total	n/a	**50 mL**
Sterilize by filtering through 0.22-μm filter and store at 4°C up to 1 month.		

Cell and tissue lysis buffer		
Reagent	Final concentration	Amount
NaCl (1.5 M)	150 mM	20 mL
HEPES (200 mM, pH7.4)	20 mM	20 mL
$MgCl_2$ (500 mM)	5 mM	2 mL
NP-40 (10%)	1%	20 mL
DTT (100 mM)	1 mM	2 mL
Protease inhibitor cocktail (PIC, 100×)	1×	2 mL
Recombinant RNase inhibitor (RRI, 40 U/μL)	0.2 U/μL	1 mL
DEPC treated ddH_2O	–	133 mL
Total	n/a	**200 mL**
Sterilize by filtering through 0.22-μm filter, store at 4°C up to 6 months. PIC, RRI and DTT are added before use.		

Alternatives: Other brands of reagents at biological grades are also suitable.

STEP-BY-STEP METHOD DETAILS

Fluorescence-activated cell sorting of spermatogonial stem cells

Timing: 6 h

Spermatogonial stem cells (SSCs) are able to self-renew and differentiate, two characteristics of tissue stem cells (Kanatsu-Shinohara and Shinohara, 2013). During these processes, SSCs change their cellular states progressively, thus generating a heterogeneous population of SSCs that possess varied gene expression and functionalities (Hermann et al., 2018; Shami et al., 2020; Guo et al., 2018). To understand the regulatory mechanisms of SSC self-renewal and differentiation, it is essential to isolate SSCs at their distinctive cellular states (La et al., 2018). The following protocol describes experimental steps taken to isolate sub-populations of SSCs via fluorescence-activated cell sorting (FACS), using a transgenic (Og2) mouse line that expresses GFP under the control of *Pou5f1* promoter (Yoshimizu et al., 1999; Yeom et al., 1996) and marker protein cKIT. *Pou5f1* encodes the stem cell marker OCT4 and cKIT is a marker for differentiating SSCs, thus $GFP^{H}cKIT^{-}$, $GFP^{H}cKIT^{+}$ and $GFP^{L}cKIT^{+}$ can be used to represent SSCs at varied proliferating and differentiating states (Figures 1A and 1B).

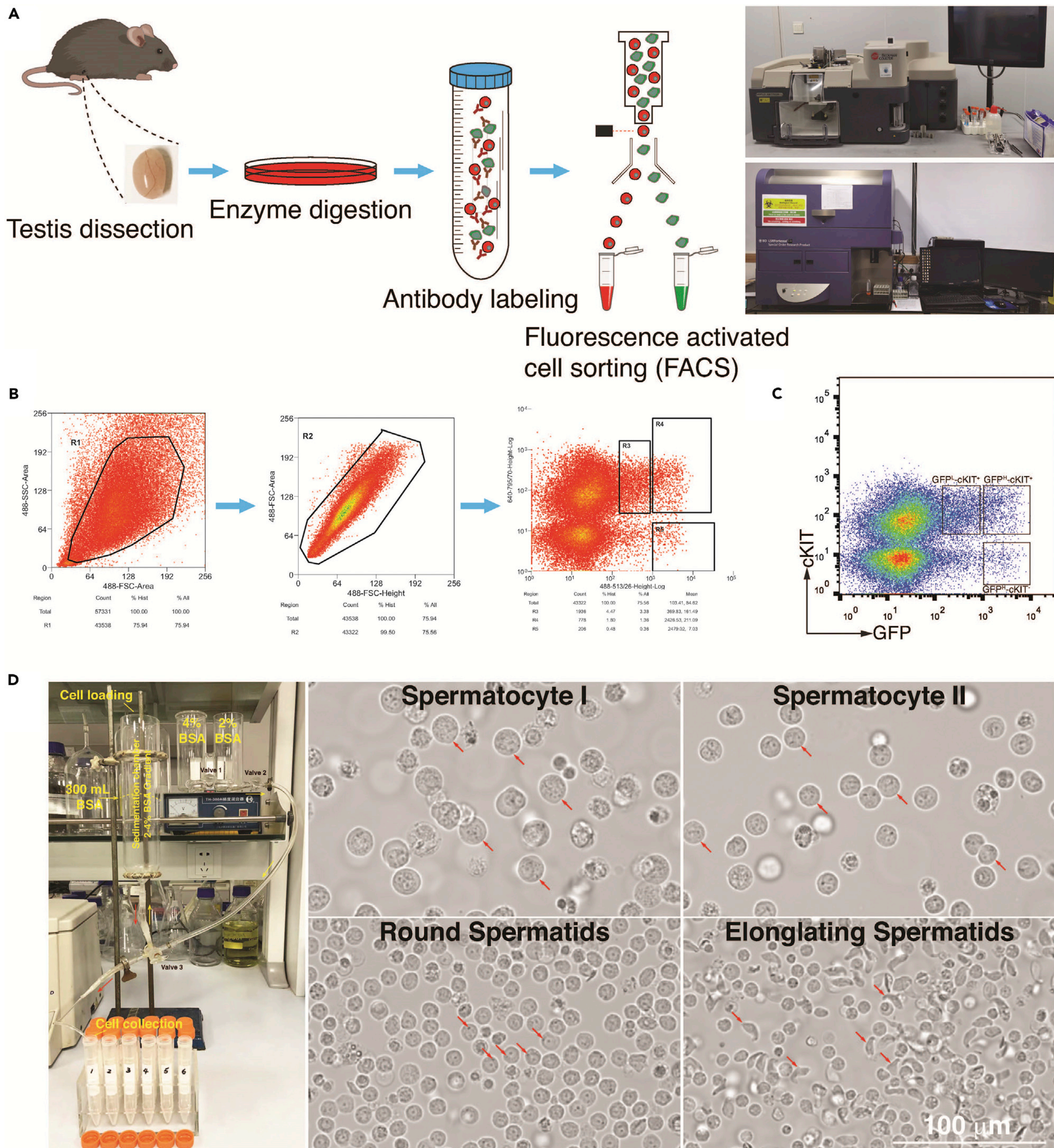

Figure 1. Isolation of sub-populations of spermatogenic cells in mouse

(A) Experimental design of obtaining single cell suspension from mouse testes by enzymatic digestion. Cell sorting system and flow cytometer used are shown on right.

(B) Representative images of FACS showing the gating of labeled spermatogonial stem cells.

(C) A representative image of FACS showing the isolation of labeled spermatogonial stem cells.

(D) Images adapted from (D) Experimental set-up of BSA gradient sedimentation (left panel) and representative images of isolated spermatocytes and spermatids (right panels). Images of cells are adapted from (Xu et al., 2014). Red arrows indicate cells positive for respective fractions. Note each round spermatid contains a single dark particle (the chromatoid body) adjacent to the nucleus.

1. Enzymatic digestion of testicular cells.
   a. The enzymatic digestion of testicular cells is modified from previously published method (Bellve, 1993). Prepare three 35-mm dishes, each containing 2 mL DMEM basic media; one 35-mm dish containing 2 mL DMEM basic media with 2 mg/mL Collagenase IV and 5 μg/mL DNase I, pre-warm media for 30–60 min in a 37°C humidified incubator with 5% CO_2.
   b. Sacrifice four male juvenile mice (10–12-dpp) or one adult mouse (2–3-month) by cervical dislocation after anesthetization with CO_2.
   c. Sterilize abdomen using 70% ethanol, open the abdominal cavity with micro-dissecting scissors and tweezers.
   d. Dissect testes with tweezers and scissors, place testes in pre-warmed DMEM basic media, remove tunica albuginea, dismantle seminiferous tubules and remove attached blood vessels as much as possible. Wash seminiferous tubules by moving them through dishes containing clean DMEM basic media.
   e. Transfer cleaned seminiferous tubules to the dish containing media with Collagenase IV and DNase I, mince seminiferous tubules with a micro-dissecting scissor into small pieces, incubate the dish in the 37°C incubator for 10 min, disperse cells once by pipetting up and down gently.
   f. Add 20 μL Trypsin (50 mg/mL stock), continue incubation for another 15 min, pipet gently to dissociate cells every 5 min.

   ⚠ CRITICAL: The timing of Trypsin digestion is not constant, partly due to the number of testes processed at one time and the age of mice. Insufficient digestion will reduce the yield of spermatogenic cells, whereas excessive digestion will increase cell mortality. Depending on the actual situation, incubation can be stopped when no observable tubule pieces are found.

   Note: Clean scissors and tweezers with 70% ethanol between mice during dissection, wash seminiferous tubules free of hair before transferring them into enzyme-containing media.

   g. Filter dispersed cells into a 50-mL Corning tube through a 40-μm cell strainer, collect cells by centrifugation at 150 × *g*, 4°C, for 10 min, using a refrigerated table-top centrifuge.
   h. Discard supernatant and resuspend cell pellets with 2 mL HBSS, count cell numbers with a hemacytometer, centrifuge again at 400 × *g*, 4°C, for 5 min.
   i. Resuspend cells in HBSS containing 5% FBS to a concentration of 2×10^6 cells/mL.

   Note: Throughout the protocol, cell washing steps are referring to repeated centrifugation and resuspension of cells in desired buffer. Cells are usually spun down at 400 × *g*, 4°C, 5 min, unless otherwise indicated. After discarding supernatants, cells are resuspended with 1 mL buffer and incubated for 5 min on ice.

2. FACS of sub-populations of SSCs troubleshooting 1.
   a. Disperse testicular cells from four 10-day old Og2 mice as described in step 1.
   b. Place 1 mL dispersed cells (2×10^6) in a 1.5-mL Eppendorf tube.
   c. Add 2 μL eFluor 780-conjugated anti-cKIT antibody (1:500), incubate 45 min on ice in dark (wrap tubes in tinfoil).

   Note: Resuspending cells once with a pipette during antibody incubation will help to fully conjugate antibodies to cells.

   d. Wash cells once with 1 mL ice-cold HBSS.
   e. Spin down cells and resuspend stained cells in 1 mL HBSS containing 5% FBS.

   Optional: Before sorting, cell suspension can be filtered through 40-μm strainer again to remove cell clumps and thereby to avoid plugging during sorting process.

f. Turn on the accelerated cell sorting system MoFlo XDP Cell Sorter 30 min before running samples, adjust sheath stream and laser alignment.
g. Run 5000–10,000 cells, set gate-1 (R1) in FSC-Area/SSC-Area to exclude cell debris and aggregates, set gate-2 (R2) in FSC-Height/FSC-Area to exclude non-single cell clusters. Apply excitation/emission at 488 nm/507 nm for GFP and 647 nm/780 nm for cKIT, respectively. Set gate-3 to 5 (R3 to R5) using green and red signals that separate labeled cells on X and Y axis of the contour plot (Figures 1B and 1C). Collect sample cells and examine them under a fluorescent microscope.
h. Run all cells using the same gating parameters to sort out $GFP^{H}cKIT^{-}$, $GFP^{H}cKIT^{+}$ and $GFP^{L}cKIT^{+}$ SSCs.
i. Collect sorted cells into 5-mL Falcon tubes containing 1 mL HBSS, centrifuge cells at 700 × *g*, 4°C, 5 min.
j. Proceed to steps 13 or 17–19 for analyses of proteostasis in SSCs. Alternatively, discard supernatant, store cells at −80°C for subsequent RNA extraction and quantitative RT-PCR.

Optional: Before store cells at −80°C, adding 1 mL TRIzol reagent into cells is beneficial for maintaining RNA stability.

Note: It is advised to proceed for RNA preparation right after cell sorting to ensure the quality and preservation of messenger RNAs.

Pause point: Cells can be stored at −80°C for up to one month.

Velocity sedimentation of spermatocytes and haploid spermatids

Timing: 1 day

Meiotic and post-meiotic spermatogenic cells (*a.k.a.* spermatids) represent major cell populations in adult testes. Although some specific marker proteins are available, cell sorting methods using FACS may not be suitable for obtaining large quantity of haploid cells for biochemical analyses. In this step, we describe the conventional method that has been developed in the past to separate spermatocytes and haploid spermatids (Bellve, 1993). This step can be combined with the fluorescence activated cell sorting of SSCs to comprehensively characterize spermatogenic cells at various developmental stages.

3. Velocity sedimentation at unit gravity with BSA gradient troubleshooting 2.
   a. Make 200 mL 2% and 200 mL 4% BSA in HBSS.
   b. Use 150 mL of each BSA solution to prepare 300 mL 2%–4% consecutive BSA gradient using a gradient mixer. This usually takes about 1 h (Figure 1D).

CRITICAL: Air bubbles can damage the consecutive gradient of BSA. They should be avoided by filling the plastic pipeline with 2% BSA solutions gently before preparing the gradient. Although DMEM basic media or HBSS is used for enzyme digestion of testicular cells, EKRB buffer can also be used. Cell viability should be examined by Trypan Blue staining after cell collections. Using this method, we routinely obtain viable cells above 90%. 1× PBS was sometimes used during velocity sedimentation when collected cells are processed for subsequent biochemical analyses immediately or short-term experiments, however, it is suggested to avoid usage of PBS in order to better maintain cell viability.

Note: Autoclave BSA solutions to sterilize in order to use testicular cells for further experimentation, including transient cell culture.

Note: Bottoms of 2% and 4% BSA chambers should be placed higher than the upper limit of BSA gradient in the sedimentation chamber.

c. Disperse testicular cells from 2 adult male mice as described in step 1.
d. Resuspend cells in 10 mL HBSS, slowly lay cells on top of the BSA gradient, stand for 2.5 h at RT.
e. Manually collect cell fractions into 15-mL Corning tubes, 5 mL/per tube.
f. Examine spermatocytes, round and elongating spermatids using a light microscope (EVOS^{fl}), according to their cellular morphologies.
g. Pool fractions containing the same cell types together for further experimentation.

Note: Depending on the volume of BSA gradient and number of testicular cells used, collected fractions containing spermatocytes, round and elongated spermatids may vary from time to time. Fractions to pool together should be determined each time by examining cells under a light microscope. The most abundant cells fractioned from adult mice using the velocity sedimentation at unit gravity are usually spermatocytes and spermatids. Pachytene spermatocytes (SPCY I) contain large nuclei with a thin rim of cytoplasm, whereas spermatocytes at meiotic II (SPCY II) are smaller and come in fractions after pachytene spermatocytes. Round spermatids are smaller and have circular morphology with a distinctive dark particle (the Chromatoid body) next to the nucleus, whereas elongating spermatids have oval shaped nuclei with overlaying hooked tips. Residue bodies that are shed from maturing sperm usually come in the last few fractions and are difficult to separate from elongated spermatids due to their similar sizes. These can be discarded if more pure fractions of spermatids are required for subsequent experiments. Spermatogonial stem cells are difficult to obtain using BSA gradient due to their scarce content. Representative images of fractionated cells are shown in Figure 2D (Xu et al., 2014).

Analytical flow cytometry of spermatogenic cells

Timing: 5 h

Spermatogenic cells can be analyzed without collecting them. Protocol described here is modified from a previous method (Bastos et al., 2005), using the varied contents of DNA in spermatogenic cells at different developmental stages. Comparing to wild type mice, this step can provide a glance at whether genetically modified mice are compromised in the process of spermatogenesis.

4. Flow cytometry of testicular cells.
   a. Prepare testicular cell suspensions as described in step 1.
   b. Place 1 mL single cell suspension in a 1.5-mL Eppendorf tube (2×10^6 cells).
   c. Add Hoechst 33342 at a concentration of 10 μg/mL, wrap the tube with tinfoil to avoid light, incubate in a 32°C water bath for 20 min.
   d. Wash cells once with 1 mL HBSS, resuspend cells in 1 mL HBSS containing 5% FBS and 2 μg/mL propidium iodide (PI), incubate 10 min.
   e. Wash cells once with 1 mL HBSS, resuspend cells in 1 mL HBSS containing 5% FBS.

   Note: Due to the integrity of plasma membrane, live cells are not stained by PI, which can be used to discriminate live and dead cells.

   f. Turn on LSRFortessa 30 min prior to use. Run 5000–10,000 cells, set gate-1 in FSC-A/SSC-A to exclude cell debris and aggregates, set gate-2 in PI/FSC-A to exclude PI$^+$ cells, set gate-3 in Hoechst Red/Hoechst Blue contour plot to exclude cell debris and clusters for PI$^-$ cells. Run cells using Hoechst Red/ Hoechst Blue. Collect data for further analyses.

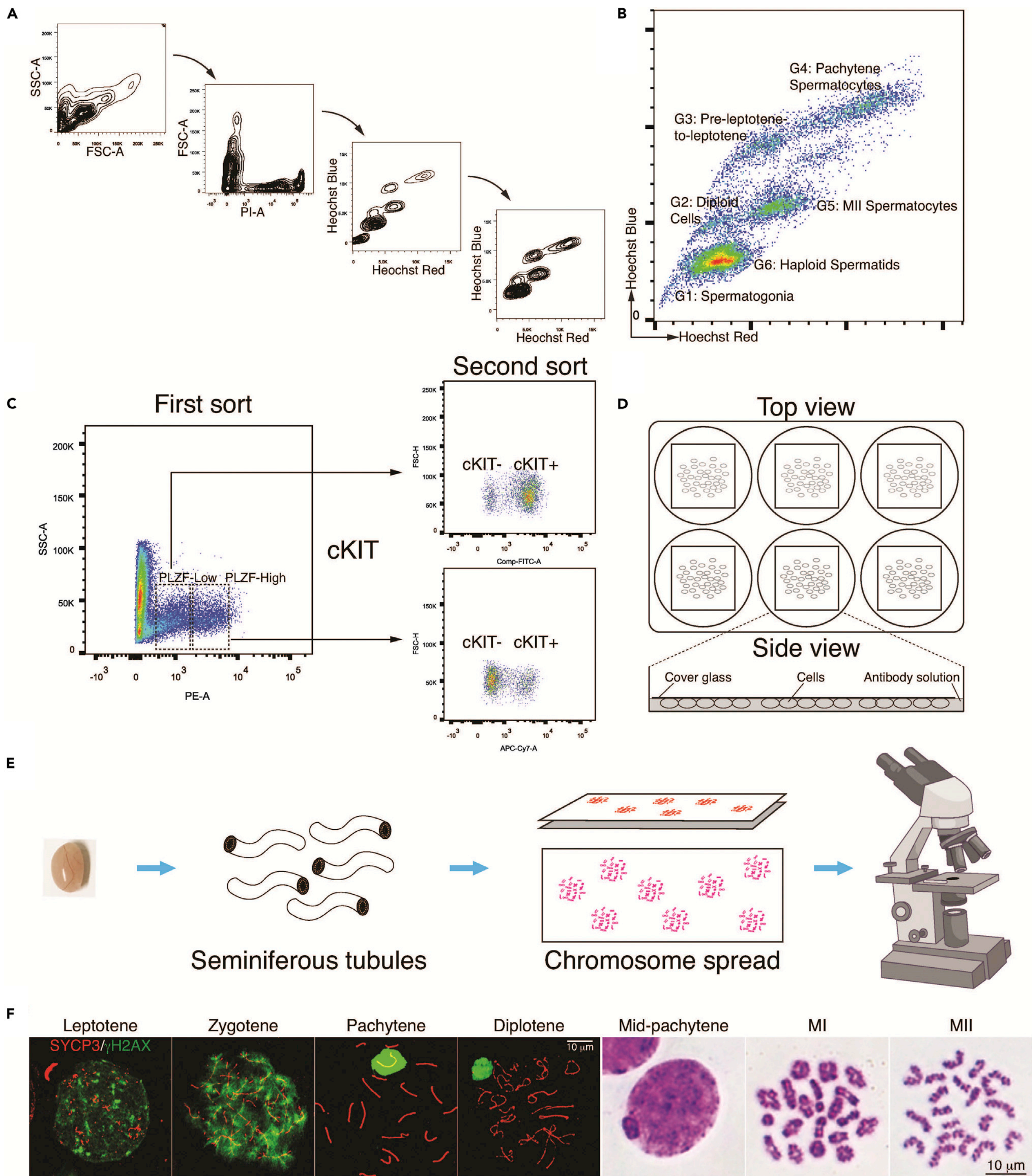

Figure 2. Analysis of sub-populations of spermatogenic cells in mouse

(A) Representative images showing the gating of flow cytometry of DNA labeled spermatogenic cells.

(B) A representative image of flow cytometry of spermatogenic cells using Hoechst 33342 staining. Image adapted from (Zou et al., 2021).

(C and D) (C) Representative images of flow cytometry of spermatogonial stem cells. Images adopted from (D) Schematic drawing of immuno-staining using cover-glasses up-side-down for incubation of attached cells with antibodies.

Figure 2. *Continued*
(E) Experimental scheme of making chromosome spread for identification of spermatocytes.
(F) Representative confocal images of immunocytochemistry and Giemsa staining showing spermatocytes at different meiotic stages. Images adapted and modified from (Zou et al., 2021).

g. Haploid spermatids can be distinguished by their DNA contents. Round and elongating spermatids can be further separated using forward scattering (FSC) parameter according to their sizes.
h. Flow cytometry data are processed using FlowJo software, the ratios of 2N spermatogonia, 4N spermatocytes and 1N spermatids in total testicular cells can be calculated accordingly. Troubleshooting 3.

Note: Testicular cells stained with Hoechst 33342 can be divided into six populations, including spermatogonia, diploid cells, pre-leptotene to leptotene spermatocytes, pachytene spermatocytes, MII spermatocytes and haploid spermatids (Figures 2A and 2B).

Analytical flow cytometry of spermatogonial stem cells

Timing: 7 h

It is desired that sometimes SSCs are analyzed for their compositions of various sub-populations without isolating them using flow cytometry.

5. Flow cytometry analyses of spermatogonial stem cells.
   a. To analyze SSC sub-populations, use wild-type mice to disperse testicular cells as described in step 1. Chill 1× PBS on ice.
   b. Transfer 2×10^6 dispersed testicular cells (1 mL) into a 1.5-mL Eppendorf tube, centrifuge at 400 × *g*, 4°C, 5 min.
   c. Resuspend cells in 1 mL 4% paraformaldehyde (PFA), incubate on ice for 30 min.
   d. Wash cells once with 1 mL 1× PBS, then incubate cells in 1 mL 1× PBS containing 0.2% Triton X-100 on ice for 15 min.
   e. Wash cells once with 1 mL 1× PBS, resuspend cells in 1 mL 1× PBS containing 2% fetal bovine serum (FBS).
   f. Spin down cells, resuspend cell pellet with 0.5 mL 1× PBST (1× PBS containing 0.01% Triton X-100, freshly made), add 2.5 μL PE-conjugated anti-PLZF (1:200) and 1 μL eFluor 780-conjugated anti-cKIT (1:500), wrap tubes with tin-foil, incubate on ice for 60 min.

   Note: Gently pipetting cells up and down to mix them once during incubation helps to conjugate antibodies to cells.

   g. Wash cells three times, with 1 mL 1× PBS, 10 min on ice each time.
   h. Spin down cells at 400 × *g*, 4°C, 5 min, discard supernatant, resuspend cells in 0.5 mL 1× PBST, run ~5000 cells first to select gating parameters as described in step 2g on LSRFortessa.
   i. Applying excitation/emission at 507 nm/561 nm for PLZF and 647 nm/780 nm for cKIT, respectively.
   j. Flow cytometry data are processed by FlowJo software.

   Note: PLZF is a marker for proliferating SSCs and cKIT is a marker for differentiating SSCs. $PLZF^H cKIT^-$, $PLZF^H cKIT^+$ and $PLZF^L cKIT^+$ can be used to represent proliferating, early differentiating and late differentiating SSCs, respectively (Figure 2C). Other marker proteins that have suitable antibodies for fluorescently staining SSCs can also be applied for analyses of SSC subpopulations using this protocol.

Quantitative RT-PCR of gene expression in spermatogonial stem cells

Timing: 1.5 days

Changes of gene expression often precede changes of cellular states during development. Although current high throughput technologies, such as single cell RNA sequencing, have helped to unveil the dynamic changes of gene expression of developing cells, they sometimes are not practical for the analysis of individual genes. In this step, we describe procedures that would allow absolute quantitation of mRNA copy numbers of specific genes in sub-populations of SSCs, using *Rpl39l* as an example. This can be used as a quality control of sorted SSCs, as well as to characterize unknown genes in SSC sub-populations.

6. Total RNA extraction from sorted SSC sub-populations.
   a. Following step 2, extract total RNAs from sorted cells using RNAsimple ToTal RNA Kit. Also prepare total RNA from testes of a juvenile mouse using the same kit (https://en.tiangen.com/content/details_68_4278.html).
   b. Dissolve RNAs in RNase-free ddH_2O, determine RNA concentrations via spectrophotometry.
7. Generation of standard curve of gene expression troubleshooting 4.
   a. Set up reverse transcription using total RNA from the juvenile mouse in a 0.5-mL Eppendorf tube. We used AMV reverse transcriptase for the RT reaction, other reverse transcriptase such as SuperScript IV can also be used.
   b. Set up PCR reaction using gene specific primers accordingly, use high-fidelity Taq DNA polymerase or equivalent DNA polymerase. This PCR reaction generates control samples for constructing the standard curve of a specific gene.
   c. Subject PCR products to agarose gel electrophoresis.
   d. Excise gel strips containing the PCR products under UV light.
   e. Purify DNA fragments using TIANgel Midi Purification Kit (https://en.tiangen.com/content/details_69_5485.html).
   f. Determine concentrations of purified DNAs using spectrophotometry, calculate copy numbers according to the molecular weight of DNA: copies/μL = $(6.02 \times 10^{23}$ copies/mol) × (ng/μL (DNA concentration) $\times 10^{-9})$/(bp (DNA length) × 660 gram/mol.
   g. Make 10× dilutions of a series DNA templates as: $2 \times 10^{7,\,6,\,5,\,4,\,3,\,2,\,1,\,0}$ copies/μL, set up PCR reactions as following:

Reagents	Stock concentration	Amount
Template	$2 \times 10^{7,\,6,\,5,\,4,\,3,\,2,\,1,\,0}$ copies/μL	1 μL
Forward primer (Rpl39l-RT-F)	10 μM	0.4 μL
Reverse primer (Rpl39l-RT-R)	10 μM	0.4 μL
TB Green Premix Ex Taq	2×	10 μL
ddH_2O	–	8.2 μL
Total	–	**20 μL**

   h. Run PCR on a CFX96 Real-Time system as following:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	30 s	1
Denaturation	95°C	5 s	40 cycles
Annealing/Extension	60°C	30 s (+ plate reads)	
Denaturation	95°C	10 s	1
Melt Curve	65°C–95°C	Increment of 0.5°C for 5 s (+ plate reads)	

i. Draw standard curves using DNA copy numbers vs. CT values, using equation: copy number of *Rpl39l* = $2 \times 10^{(37.46\text{-CT})/3.595}$.

Note: A linear relationship between DNA copy numbers and CT values can be established, in which higher DNA copy numbers correspond to lower CT values.

8. Determination of mRNA copy numbers of a specific gene in SSCs using quantitative RT-PCR.
   a. Set up reverse transcription reaction using total RNAs from sorted SSC sub-populations following steps 7a and 7b.
   b. Use 1 μL RT samples to run quantitative PCR reactions following steps 7i and 7j.
   c. Calculate mRNA copy numbers according to the CT values using the standard curve.

⚠ CRITICAL: Manipulation of RNA-containing samples and materials should be carried out on a clean bench and use RNase-free Eppendorf tubes and pipette tips.

Immunocytochemistry of spermatocytes undergoing meiotic cell division

⏲ Timing: 2 days

During meiosis, chromosomes in spermatogenic cells, namely spermatocytes, undergo one round of DNA replication and two consecutive rounds of cell division. Changes of chromosomal states, such as their morphology and the distribution of specific DNA binding proteins, allow the identification of spermatocytes at different meiotic stages. This is important for studies of genes that play regulatory roles during meiosis. This step describes procedures for quantifying the progression of meiotic cell cycle, using chromosomal spread of testicular cells, as well as immuno-staining of cell nuclei (Evans et al., 1964; Peters et al., 1997). In particular, SYCP3 that decorates chromosomal synaptonemal complex and γH2AX that localizes to XY chromosomal pairing are used for immunofluorescent staining of meiotic cells and classifying stages of meiotic cell cycle (Figures 2E and 2F).

9. Immuno-staining of spermatocytes with anti-SYCP3 and anti-γH2AX.
   a. Dissect out testes from adult mice following steps 1a–1d.
   b. Transfer 15-cm long seminiferous tubules into a 35-mm dish containing 1 mL hypotonic solution, incubate at RT for 1 h.
   c. Transfer 3-cm long seminiferous tubules into a 40-μL drop of 100 mM sucrose solution on a glass slide, tear the tubule into small pieces with two fine-tipped tweezers.
   d. Pipet up and down to release testicular cells onto the glass slide with a 20-μL pipette-man.
   e. Wet 18 mm × 18 mm cover glass slides by dipping them in fixation buffer.
   f. Transfer a drop of cell suspension (20 μL) onto the upper left corner of a cover slide, slowly spread cells first in the horizontal direction then in the vertical direction by tilling the slide with hand in a swirling motion.
   g. Process the rest of seminiferous tubules following steps c–f.

⚠ CRITICAL: Spreading cells evenly on the cover glasses is important for thoroughly fixing cells at an appropriate density.

Note: Hypotonic solution helps to avoid contamination of blood cells attached to the tubules and dissociation of testicular cells.

   h. Dry cover glasses in a moist chamber (a plastic box containing a layer of water) for 2 h, RT.
   i. Wash cover glasses twice by incubating them in 2 mL 0.4% Photo-Flo 200 solution, 3 min each time. Photo-Flo solution helps to clear the cover glasses for better visualization under confocal microscope.

j. Place cover glasses in 6-well plates, block cells with 1 mL 2% BSA/PBST for 2 h at RT.
k. Remove blocking buffer, add 1 mL 1% BSA/PBST containing anti-SYCP3 (1:400) and anti-γH2AX (1:500), incubate overnight (12–16 h) at 4°C.

Optional: Turn slides up-side down during incubation with antibodies can reduce volumes of solution used and thus save antibodies (Figure 2D).

l. Wash cover glasses the next morning three times by incubating them in 2 mL 1× PBST, 10 min each time.
m. Add 1 mL 1% BSA/PBST containing appropriate fluorescein-tagged secondary antibodies (1:1000), wrap 6-well plate in tinfoil, incubate for 1 h, RT.
n. Wash cover glasses three times with 1× PBST, 2 mL, 10 min each time, then mount cover glasses onto slides in 50% glycerol/PBST.
o. Examine slides under a Confocal Laser Scanning Microscope (CLSM), take images covering at least 500 spermatocytes.

10. Giemsa staining of chromosomes.
 a. Dissect out testes from adult mice following steps 1a–1d.
 b. Transfer 6-cm long seminiferous tubules into a 35-mm dish containing 1.5 mL isotonic sodium citrate (2.2%).
 c. Squeeze out spermatogenic cells from seminiferous tubules with tweezers.
 d. Collect cells by centrifuge at 100 × *g*, RT, 5 min, discard supernatant, resuspend cells in 1.5 mL hypotonic sodium citrate (1%), mix and incubate cells at RT for 12 min.
 e. Centrifuge cells at 100 × *g*, RT, 5 min, discard supernatant, resuspend cells in 0.5 mL fixation buffer made of 74% ethanol, 25% acetic acid and 1% chloroform, mix and incubate cells at RT for 5 min. Repeat once.
 f. Add 10-μL drop of cell suspensions onto glass slides, air dry.
 g. Stain cells with Giemsa diluted in ddH_2O (1:20) for 30 min at RT.
 h. Wash slides under gently flowing water for 10–20 s, air dry slides.

⚠ CRITICAL: Check the intensity of Giemsa under a light microscope during washing every 5 s helps to acquire staining with clear and appropriate intensities.

Note: Sodium citrate is an alkalinizing reagent that can help to chelate Ca^{2+} and helps to dissociate tubules for release of spermatogenic cells, may also prevent blood cells from clotting on the tubules.

 i. Seal slides with neutral balsam.
 j. Examine stained cells under a light microscope equipped with a CCD camera, take adequate images that include at least 1000 mid-pachytene nuclei.

11. Counting spermatocytes at various stages of meiotic cell cycle.
 a. For prophase I spermatocytes, from confocal images, count cells with distinct SCYP3 and γH2AX staining patterns to distinguish cells at leptotene, zygotene, pachytene and diplotene stages. Calculate percentages of spermatocytes as: Number of spermatocytes at a particular stage/Total number of all spermatocytes counted X 100%.
 b. For meiosis I (MI) and meiosis II (MII) spermatocytes, from Giemsa stained images, count cells with distinct chromosome morphology in defined areas that contain 1000 nuclei at mid-pachytene stage. Calculate the ratio of MII/MI spermatocytes, which reflects the progression of meiosis I to meiosis II.

Note: Prophase I spermatocytes can be distinguished by the appearance of chromosomes in their nuclei when stained for SCYP3 and γH2AX, the markers for synaptonemal complex formation. For example, leptotene spermatocytes have diffused distribution of SCYP3 in nuclei, zygotene spermatocytes have thin thread of SCYP3 formed and γH2AX foci start to form,

pachytene spermatocytes have thick strings of SCYP3 and a clear XY body decorated with γH2AX, whereas diplotene spermatocytes have SCYP3 positive threads with separated crosses, γH2AX positive XY body is still present at this stage. MI and MII spermatocytes have no nuclear envelopes and contain condensed intact homologous chromosomes and thinner sister chromatids, respectively (Figure 2F).

In vivo and *in vitro* labeling of nascent proteins in spermatogenic cells

Timing: 5 days

In vivo labeling of nascent proteins facilitates the study of how different cell types of a tissue express cell's functional units, proteins. In this step, we describe methods for *in vivo* labeling of nascent proteins in mouse testis using intraperitoneally injected puromycin, as well as *in vitro* labeling of nascent proteins in transiently cultured spermatogenic cells that are dispersed from mouse testes (Figures 3A and 3B).

12. Puromycin labeling of nascent proteins in mouse testes troubleshooting 5.
    a. *In vivo* labeling of nascent proteins with puromycin.
        i. Weigh male mice with a scale, an adult male mouse typically weighs about 30 grams.
        ii. Inject puromycin (10 mg/mL) intraperitoneally at a dose of 65 mg/kg body weight.
        iii. Prepare two 60-mm dishes contain 5 mL 1× PBS.
        iv. At 1.5 h post-injection of puromycin, sacrifice mice and dissect out testes in 1× PBS. For each mouse, use one testis for preparing tissue sections and another one for preparing tissue lysates (go to step 12c).
        v. Wash testes with 1× PBS briefly, cut small openings at both ends of testes using a microdissecting scissor, place testes in 4% paraformaldehyde, incubate overnight (12–16 h) at 4°C.

    Optional: Other amino acid analogs, including HPG and AHA, can also be examined for labeling nascent proteins in mouse testis.

    Note: Poking a few holes on lateral sides of testes with a 30-gauge needle is beneficial for complete fixation of testes.

        vi. Dehydrate fixed testes through a series of ethanol with increasing concentrations: 30%, 50%, 70%, 80%, 90%, 95% and 100%, 5 min each, embed testes in paraffin.
        vii. Cut embedded testes into 5-μm thin sections with a microtome, starting from the middle of a testis. Collect sections with glass slides (two sections on one standard 2.6 × 7.6 cm slide), typically at least 10 sections need to be collected for each testis.
        viii. Bake slides in a 56°C oven for 10 min, cool to RT, store sections at 4°C.

    Pause point: Sections can be stored at 4°C for up to three months.

    b. Immuno-staining with anti-puromycin antibody.
        i. Take slides out of 4°C storage, warm to RT, soak slides in xylene (100%) twice to de-wax, 5 min each.
        ii. Rehydrate sections by soaking slides through 100% ethanol twice, 3 min each, then 95% ethanol once, 3 min, 1× PBS twice, 5 min each and ddH_2O once, 2 min.
        iii. Place slides in 0.01 M citrate, heat with a microwave using medium power for 3 min, then switch to low power and maintain for 20 min.
        iv. Cool slides to RT, then wash them twice with 1× PBS, 5 min each.
        v. Incubate slides by submerging them in 0.3% Triton X-100 in 1× PBS for 15 min at RT.

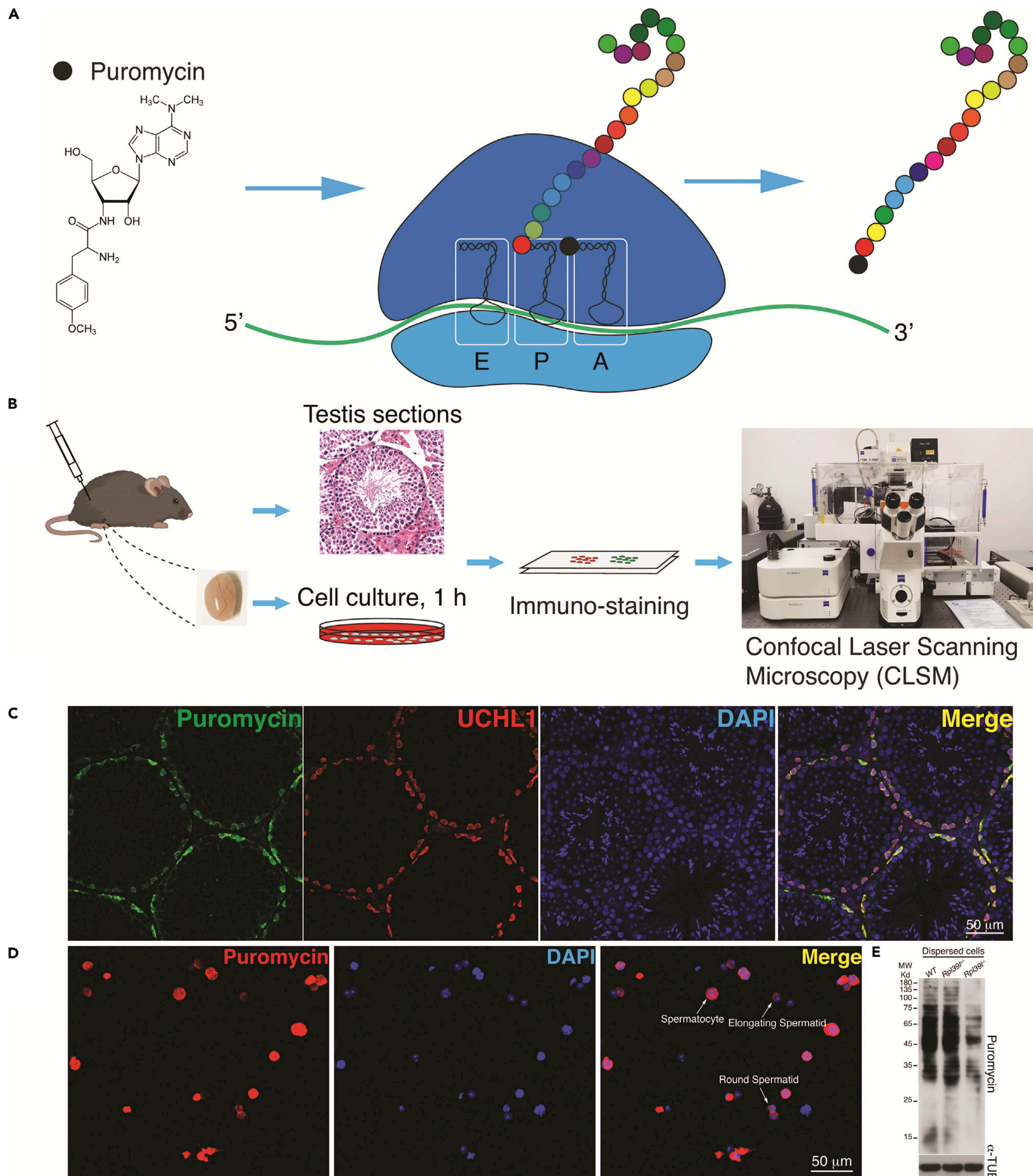

Figure 3. Nascent protein labeling of mouse spermatogenic cells

(A) Schematic of puromycin labeling of nascent polypeptide. Incorporation of puromycin causes release of newly synthesized polypeptides with puromycin attached at the C-terminal ends.

(B) Experimental design of nascent protein labeling in both mouse testis and dispersed testicular cells. The confocal laser scanning microscope is shown on right.

Figure 3. *Continued*

(C) Representative confocal images of mouse testis section immuno-stained with anti-puromycin (green) and spermatogonia marker UCHL1 (red). Nuclei were stained with DAPI. Scale bar: 50 μm.

(D) Representative confocal images of testicular cells that were transiently cultured in puromycin-containing media *in vitro*. Cells were immuno-stained with anti-puromycin (red). Nuclei were stained with DAPI. Spermatocytes and spermatids are indicated with arrows. Scale bar: 50 μm.

(E) Representative western blotting of puromycin labeled testicular cells. The image is adapted from (Zou et al., 2021).

vi. Wash briefly in 1× PBS, submerge slides in 2% BSA/PBST to block for 2 h at RT.
vii. Remove blocking buffer, dry slide area surrounding tissue sections with paper towel, draw circle around sections with a hydrophobic pen.
viii. Add 100 μL 1% BSA/PBST containing anti-puromycin (1:1000) to each circle, incubate 2 h at 4°C.
ix. Wash slides three times by submerging them in 2 mL 1× PBST, 10 min each time.
x. Add 100 μL 1% BSA/PBST containing appropriate fluorescein-tagged secondary antibodies (1:1000) to each circle, incubate for 1 h at RT in dark.
xi. Wash slides three times by submerging them in 2 mL 1× PBST, then incubate them in 1× PBST containing 1 μg/mL DAPI for 30 min at RT in dark (100 μL/circle).
xii. Wash slides three times with 1× PBST, then mount slides in 50% glycerol/PBS, examine slides with CLSM.
xiii. From confocal images, count the number of puromycin-positive spermatogenic cells (Figure 3C). The intensity of puromycin signals reflecting the quantity of nascent proteins can also be measured.

⚠ CRITICAL: To compare the fluorescent intensity of puromycin signals among samples, it is critical to maintain constant imaging parameters during CLSM.

Note: Timing for incubation with the primary and secondary antibodies can be determined experimentally, ranging from 2 h to overnight (12–16 h). Longer time incubation should be carried out at 4°C.

c. Immuno-blotting of puromycin labeled nascent proteins.
i. Following step 12a-iv, weigh testes.
ii. Remove tunica albuginea, wash seminiferous tubules briefly in 1× PBS, place them in a glass homogenizer on ice, add ice-cold RIPA buffer containing 1× protease inhibitor cocktail at 100 mg tissue/mL, typically 1 mL for one testis.
iii. Homogenize testes with glass pestle for 5 min on ice, transfer the crude homogenates into a 1.5-mL Eppendorf tube, seal the tube with paraffin film, rotate for 30 min at 4°C.
iv. Centrifuge lysates at 12,000 × *g* for 10 min at 4°C, transfer supernatants to fresh Eppendorf tubes.
v. Measure protein concentration using Enhanced BCA Protein Assay Kit (https://www.beyotime.com/product/P0010.htm), aliquot and store lysates at −80°C.

Note: RIPA buffer can be replaced by cell lysis buffer used for other steps in this protocol.

⏸ **Pause point:** Testis lysates can be stored at −80°C for up to three months.

vi. Mix lysates containing 40 μg proteins with 5× reducing SDS loading buffer at 4:1 volume ratio. Samples were boiled for 5 min in a water bath and subjected to denaturing SDS-PAGE (SDS Polyacrylamide gel electrophoresis).
vii. Transfer proteins from polyacrylamide gels to nitrocellulose membrane using semi-dry transfer apparatus (Bio-Rad) at 20 V, 1.5 h, RT.
viii. Block membranes with 5% BSA/TBST (1× Tris buffered saline containing 0.1% Tween 20) at RT for 2 h on a horizontal shaker.

Optional: Before blocking, protein bands and quantities in each lane resolved by SDS-PAGE can be visualized by staining the membranes with Ponceau S (0.2%) for 5 min.

ix. Incubate membranes in 1% BSA/TBST containing primary antibodies (anti-puromycin, 1:10000, anti-α-Tubulin, 1:5000) at 4°C for overnight (12–16 h).
x. Wash membranes three times with 1× TBST by vigorously shaking at RT, 10 min each.
xi. Incubate membranes in 1% BSA/TBST containing appropriate secondary antibodies (1:5000) at RT for 1 h.
xii. Wash membranes three times with 1× TBST by vigorously shaking at RT, 10 min each.
xiii. Reveal protein bands using ECL Detection kit (https://orioner.com/High-sig-ECL-Western-Blotting-Substrate-ECL-180-501), image them with a Chemiluminescence imager.
xiv. Analyze intensities of protein bands using ImageJ software.

13. Puromycin labeling of nascent proteins in dispersed spermatogenic cells troubleshooting 6.
    a. Prepare testicular cell suspension as described in step 1.
    b. Incubate 2×10^6 cells in DMEM complete media containing 5 μg/mL puromycin for 1 h in a 37°C humidified incubator, with 5% CO_2.
    c. Collect cells into 1.5-mL Eppendorf tubes, centrifuge at 400 × *g*, 4°C, 5 min.
    d. Wash cells twice with 1× PBS, 5 min each, resuspend cells in 1 mL 1× PBS.
    e. Transfer 20 μL cells onto poly-lysine coated slides, adhere at RT for 30 min.
    f. Fix cells by dropping 50 μL 4% PFA on top of adhered cells, incubate at RT 10 min.
    g. Remove 4% PFA, add 50 μL 0.2% Triton X-100 in 1× PBS on top of cells, incubate at RT for 15 min to permeabilize cells.
    h. Proceed to immuno-staining and CLSM as described in steps 12b-vi–xiii.
    i. Centrifuge down remaining cells from 13d, remove supernatant, add 200 μL cell lysis buffer for cells from one testis, pipet to mix well and rotate 30 min at 4°C.
    j. Centrifuge at 12,000 × *g*, 4°C, for 10 min, transfer supernatant to fresh tubes, determine protein concentrations using Enhanced BCA Protein Assay Kit, aliquot and store lysates at −80°C.
    k. Run SDS-PAGE and immuno-blotting with anti-puromycin antibody as described in steps 12c-vi–xiv (Figure 3D).

Polysome profiling of mouse testis lysate

Timing: 7 h

Different stages of spermatogenic cells contain varied proteomes that are regulated by both protein synthesis and degradation. Changes of ribosomes on mRNAs entail differential translational status of mRNAs. In this step, we describe procedures that can be undertaken to analyze translational status of mRNAs via sucrose gradient sedimentation (Figures 4A and 4B).

14. Lysates preparation from mouse testes.
    a. Dissect testes from one adult male mouse, weigh testes, wash briefly in 1× PBS, remove tunica albuginea and blood vessels, place seminiferous tubules in a glass homogenizer on ice.
    b. Add ice-cold tissue lysis buffer at a ratio of 1 mL per 100 mg tissue.
    c. Homogenize testes with a glass pestle on ice for 2 min, transfer crude lysates to 1.5-mL RNase-free Eppendorf tubes, rotate 30 min at 4°C.
    d. Centrifuge at 2,000 × *g* for 10 min at 4°C, using a refrigerated table-top centrifuge, transfer supernatants to fresh 15-mL ultracentrifuge tubes, keep tubes on ice.
    e. Centrifuge at 20,000 × *g* for 10 min at 4°C. Transfer supernatants to 1.5-mL RNase-free Eppendorf tubes, keep on ice.
    f. Resolve pellets from d and e in 0.5% SDS, sonicate to lyse, examine samples using Western blotting along with the fractions from sucrose gradient (see below).

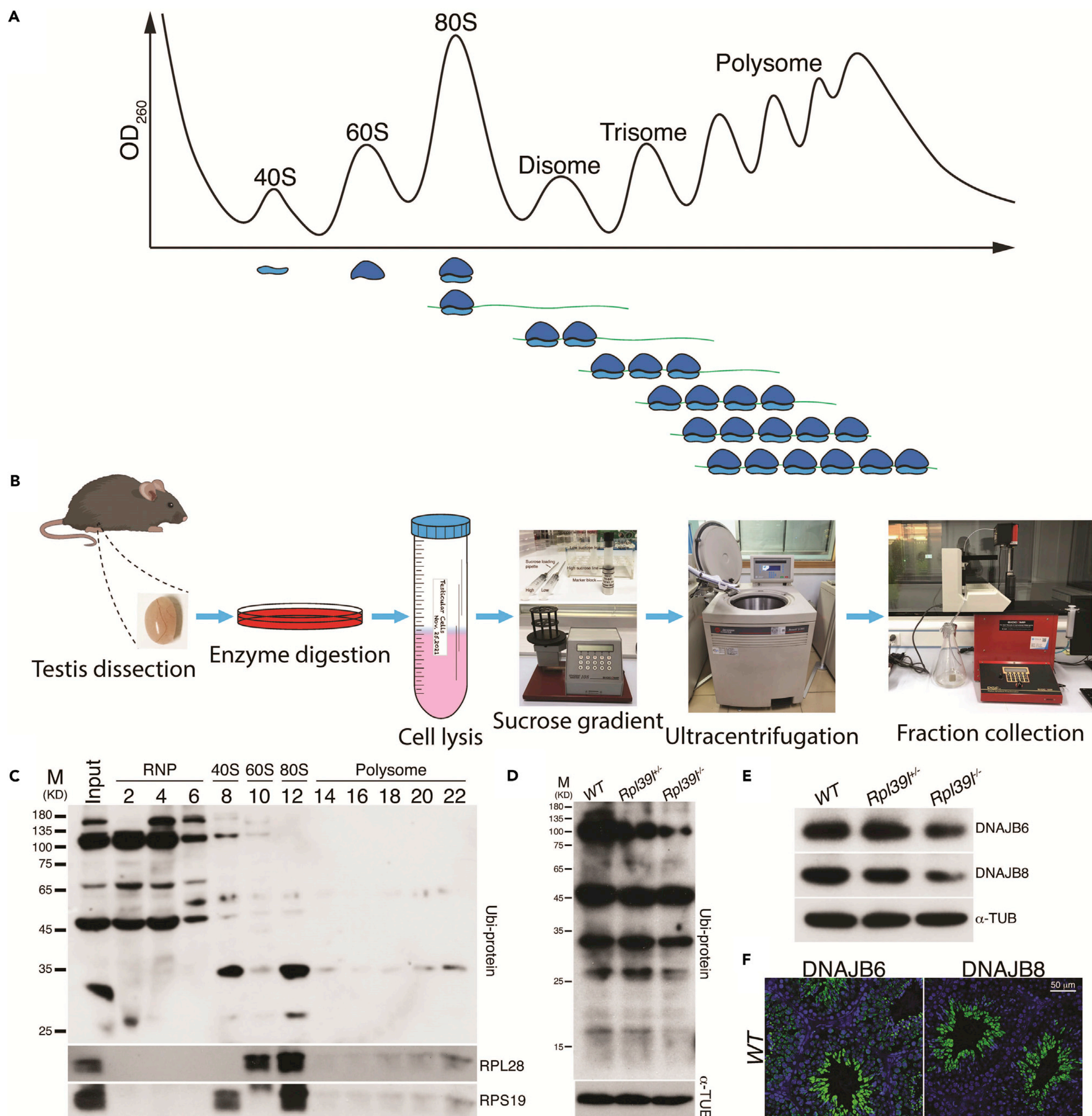

Figure 4. Evaluation of proteostasis in mouse spermatogenic cells

(A) Schematic of sucrose gradient sedimentation showing a typical profile representing different states of ribosomes.

(B) Experimental design of sucrose gradient for separating ribosome-RNA complexes from mouse testis lysates.

(C–E) Representative western blotting of fractions collected following sucrose gradient (C), as well as changes of expression of ubiquitinated proteins (D) and molecular chaperones (E) in *Rpl39l* null testes.

(F) Representative confocal images of mouse testis sections immuno-stained with anti-DNAJB6 or DNAJB8 (green). Nuclei were stained with DAPI. Scale bar: 50 μm. Images in C-F are adapted from (Zou et al., 2021).

Note: Testicular cell suspensions or FACS isolated cells could also be subjected to preparation of lysates, however, collecting enough cells from FACS isolation could be difficult for SSCs.

15. Sucrose gradient preparation troubleshooting 7.
    a. Prepare 10% and 50% sucrose solutions with lysis buffer.
    b. Accessories of Gradient Master 108 contain Marker blocks and Stoppers for dividing 13-mL ultracentrifuge tubes into heavy and light sucrose fractions, draw borders on tubes using the Marker block (Figure 4B).
    c. Using the low sucrose loading pipette, add 10% sucrose from the bottom of the tube to the high sucrose line.
    d. Insert high sucrose loading pipette to the bottom of the tube, add 50% sucrose until it reaches the high sucrose line. The 10% sucrose should be pushed upward to the low sucrose line, leave ~0.5 cm sample space on top.
    e. Close the tube with the Stopper, place Stopper gently with a tilted angle in order to drive air bubbles out through the air hole. Keep tubes in ice.

Note: Air bubbles can disrupt sucrose gradient if not completely removed. Adding extra 5–10 μL 10% sucrose on top helps to push air bubbles out when closing the lid with the Stopper.

Note: Sucrose solution is prepared using lysis buffer made with DEPC-treated ddH_2O.

    f. Turn on the Gradient Master 108. Level the magnetic platform with a Bubble Level.
    g. Select appropriate time, angle and speed to use according to the provided index table. Place sucrose tubes on the tube holder, start run. For SW41 rotor, we used time/angle/speed of 0.05/87/30 to make 10%–50% gradient.
    h. Stand sucrose gradients on ice.

Alternatives: Other devices can be used for generating sucrose gradients, including Hoefer™ SG Series Gradient Makers, CBS Scientific Gradient Maker with proper sizes and Bio-Rad Model 385 Gradient Former.

16. Sucrose gradient sedimentation.
    a. Layer 0.5 mL testes lysates on top of the sucrose gradient.
    b. Balance tubes with lysis buffer by weighing on a Balancer.
    c. The Optima L-80 XP Ultracentrifuge should be turned on 30 min prior to run, cooled to 4°C along with the rotor. Place sucrose gradients in SW41 rotor.
    d. Centrifuge at 210,000 × *g*, 4°C, for 2 h, with the fastest acceleration and the slowest deceleration speed.
    e. Carefully take out tubes without disturbing the gradients, collect 0.5-mL fractions into RNase-free Eppendorf tubes, using the BioComp Fractionator. Clean the tubing on the fractionator with DEPC-ddH_2O before and after collecting fractions.
    f. Fractions are taken out from top to bottom, OD_{260} is monitored and recorded simultaneously.
    g. Draw curves of OD_{260} for ribosome profiles, peaks of 40S, 60S subunits, monosomes and polysomes should be easily distinguishable.
    h. Measure protein concentrations of fractions using Enhanced BCA Protein Assay Kit (https://www.beyotime.com/product/P0010.htm).
    i. Proteins contained in fractions can be examined by SDS-PAGE and Western blotting.
    j. RNAs contained in fractions can be extracted using RNAsimple ToTal RNA Kit (https://en.tiangen.com/content/details_68_4278.html). Reverse transcription and PCR can be used to generate cDNA library if RNA sequencing is required. More detailed procedures can be found in a recent publication (Galmozzi et al., 2019).
    k. Store fractions at −80°C if not used immediately.

⚠ CRITICAL: All buffers and devices come in contact with lysates should be RNase free.

Note: Fractions can also be taken out at 1 mL intervals. Vacuum cryo-vaporizer can be used to bring down the volume of fractions.

Pause point: Fractions can be stored at −80°C for up to three months.

Detection of molecular chaperones and ubiquitinated proteins

Timing: 2 days

Proteins are folded by molecular chaperones during synthesis. Unfolded proteins are often degraded through proteasome or lysosome systems, of which the former recognizes poly-ubiquitinated proteins. Thus, expression of molecular chaperones and protein ubiquitination indicate protein quality control and turnover of cellular proteomes. In this step, we describe procedures that can be used to examine the distribution of molecular chaperones, ubiquitinated proteins as well as protein aggregations in various spermatogenic cells, including SSCs, as indicators of disrupted cellular proteostasis.

17. Immuno-blotting of testis lysates.
    a. Prepare testes lysates, perform SDS-PAGE and immuno-blotting as described in step 12c, using antibodies against DNAJB6 (1:2000), DNAJB8 (1:2000) and Ubiquitin (1:1000).
    b. Compare relative quantities of proteins using ImageJ. In our case, molecular chaperones (DNAJB6 and DNAJB8) and ubiquitinated proteins were decreased in *Rpl39l* null mice, comparing to their wild type counterparts (Figures 4C–4E).

Note: DNAJB6 and DNAJB8 are members of HSP40 chaperone family that often binds unfolded or mis-folded proteins to prevent them from forming aggregates in cells. Changes of their expression levels can be used as an indicator of changes in cellular proteostasis.

18. Immuno-staining of testis sections.
    a. Prepare testes sections as described in steps 12a-iv–viii.
    b. Perform immuno-staining with antibodies against DNAJB6 (1:100) and DNAJB8 (1:100) following steps described in 12b.
    c. Examine stained sections with CLSM.
    d. To identify varied status of proteome in spermatogenic cells, cell types can be identified according to their locations on testis sections, or sections can be immuno-stained with marker proteins of spermatogenic cells simultaneously with proteostatic indicators. In our case, it was found that DNAJB6 was localized in spermatogenic cells at earlier developing stages, whereas DNAJB8 was more enriched in haploid spermatids at later stages (Figure 4F).
19. Protein aggregation assay.
    a. Weigh testes extracted from adult mice.
    b. In 60-mm dishes containing 1× PBS, remove tunica albuginea and attached blood vessels, dismantle seminiferous tubules, wash through fresh 1× PBS.
    c. Transfer seminiferous tubules to glass homogenizer pre-chilled on ice, add lysis buffer, 1 mL per testis.
    d. Homogenize with a glass pestle briefly, transfer the crude lysates to 1.5-mL Eppendorf tubes, rotate for 30 min at 4°C.
    e. Spin at 6,000 × *g* for 5 min, 4°C. Transfer supernatants to fresh tubes.
    f. Dilute 20× Detection Reagent to 1× with 1× assay buffer, both provided in the Proteostat Protein Aggregation Assay Kit (https://www.enzolifesciences.com/fileadmin/files/manual/ENZ-51023_insert.pdf).
    g. Mix 98 μL testis lysate with 2 μL 1× Detection Reagent, add the mix to 96-well plate, one mix per well.
    h. Incubate 15 min in dark at RT.

i. Read fluorescent signals on a multimode microplate reader with excitation/emission wavelengths at 550/600 nm, respectively.

EXPECTED OUTCOMES

Using microinjection of RNAs in mouse 2-cell embryos, followed by transplantation of injected embryos into oviducts of foster female mice, genetically manipulated founder mice would be generated in a short period of time (4–5 months). Typically, above 50% new-born F0 pups carry varies mutations that occur at the sgRNA targeting sites, including deletion/frame-shift and insertion/frame-shift mutations. Among them, only the mutation that disrupts the main open reading frame is needed for further breeding and expansion of the mouse colony. Selecting sgRNAs with the lowest scores of off-targeting often ensure that there are little un-expected mutations occurred in the genome. This can be verified by PCR and direct DNA sequencing.

Sub-populations of SSCs are expected to be isolated using marker proteins, such as GFRα1, OCT4, PLZF and cKIT, representing self-renewing, transient amplifying, proliferating and differentiating SSCs. From one neonatal mouse, about 1–2 × 10^4 SSCs can be isolated for downstream cell biological and biochemical analyses (Figures 1A and 1B). Combinations of these marker proteins allow separation of SSCs that are in the transition states, which are characteristic to the heterogeneous SSCs (Figure 2C).

Nascent protein labeling using puromycin is a sensitive method for detection of newly synthesized polypeptides in testis in a short period of time (1–2 h). Combined with immuno-fluorescent staining, state of protein synthesis can be analyzed at single-cell level for SSCs (Figure 3). Although labeling of spermatogenic cells at later stages may be hindered by the blood-testis-barrier, dispersed testicular cells can be cultured *in vitro* briefly for labeling of nascent proteins. Fair amounts of cells (up to 1 × 10^7 cells/per testis) can be processed for biochemical analyses, including ribosomal profiling, Western blotting and immunoprecipitation (Figure 4).

The procedures described in this protocol can facilitate analyses of spermatogenic cells at various developmental stages, including SSCs, spermatocytes and haploid spermatids. These would be the starting points for study of gene functions that are involved in the regulation of mouse spermatogenesis.

LIMITATIONS

Procedures described should be used along with functional studies of spermatogenic cells, for example, for SSCs, transplantation of isolated SSCs is a standard method for qualifying stem cell functions (Brinster and Zimmermann, 1994). Also, analyses of sperm motility and fertility of the animals that may be caused by genetic mutations should be conducted in order to assess if targeted genes ultimately affect male fertility.

The quantity of cells obtained by FACS is relatively limited for large scale downstream biochemical analyses, especially for SSCs. New markers should be tested for the separation of sub-populations of SSCs. Measurements of RNA copy numbers should also be verified by experiments using high resolution fluorescent imaging at single cell level, including scFISH.

The development and growth of spermatogenic cells require the support of testicular somatic cells, which produce hormones and growth factors through steroidogenesis (Griswold, 1998; Engeli et al., 2018), a situation that lacks when germ cells are processed and cultured alone *in vitro*. Thus, the conditions for processing and culturing spermatogenic cells *in vitro* should be further optimized. These include the comparisons of temperature effects as well as other factors (Kubota et al., 2004).

The normal testis temperature (32°C–33°C) is ~4°C lower than the body temperature. In this protocol, we used a 37°C incubation temperature for the enzymatic digestion of testicular cells. Although the 37°C condition has been shown to be suitable for the isolation and long-term culture of spermatogonial stem cells (Kanatsu-Shinohara et al., 2003), the possibility that 32°C–33°C may be more optimal for the processing of testicular cells should be examined. The heat shock effect for spermatogenesis has been under intensive studies. However, how temperature takes effects on various stages of spermatogenic cells in different species remain to be fully understood.

TROUBLESHOOTING

Problem 1

Low yield of viable cells during FACS for SSCs (step 2).

Potential solution

Disperse cells thoroughly during enzymatic digestion of seminiferous tubules. Filter cells before FACS so that cell aggregates are not formed during the run. Limiting the time of manipulating cells *in vitro* often helps to preserve the viability of cells.

Problem 2

Purities of haploid round spermatids and elongating spermatids are low during BSA gradient sedimentation (step 3).

Potential solution

Disperse testicular cells thoroughly, filter cell suspension before loading cells onto BSA gradient, use less cells so that cell aggregation can be avoided. We typically use 1–2 adult mice for preparation of testicular cell suspensions and isolation of round and elongating spermatids with purities above 95%.

Problem 3

There are no apparent cell clusters when using flow cytometry to analyze Hoechst 33342 stained testicular cells (step 4).

Potential solution

Adjust the voltage intensity of Hoechst Red and Hoechst Blue parameters and maintain linear axis. Typically, six populations of spermatogenic cells can be sorted, including spermatogonia, diploid cells, pre-leptotene to leptotene spermatocytes, pachytene spermatocytes, MII spermatocytes and haploid spermatids. Haploid spermatids often occupy the majority of entire spermatogenic cells in adult mice (Figure 3B).

Problem 4

Lower efficiency of PCR amplification leads to higher CT values (step 7).

Potential solution

The efficiency of PCR amplification for rare samples during real-time quantitative PCR can be improved by: 1) increasing the concentration of primers used; 2) use 3-step PCR cycle instead of 2-step PCR cycle, for example, using denaturation, annealing and extension at 95°C, 5 s, 55°C, 30 s and 72°C, 30 s, respectively.

Problem 5

Spermatocytes and spermatids are not labeled during 1.5-h pulse of intraperitoneally injected puromycin (step 12).

Potential solution

This is probably caused by the blood-testis-barrier which prevents puromycin from entering during the short time period of labeling. Dispersed spermatogenic cells could be used for this purpose. In addition, optimization could be achieved by using different concentrations of puromycin and label testes with longer time. However, puromycin inhibits protein synthesis, which could also be toxic to cells. Longer time should be avoided to reduce mortality of cells. In addition, alternative amino acid analogs, such as AHA and HPG, should also be tested for nascent protein labeling in mouse testis.

Problem 6

Many cells fell from glass slides after fixation with 4% PFA (step 13).

Potential solution

The peeling effect could be minimized by increasing the concentration of cells used, prolonging the time of adhering cells to poly-lysine coating and using ice-cold acetone instead of 4% PFA to fix cells.

Problem 7

Resolution of ribosomal profiling is low (step 15).

Potential solution

Tissue or cell lysates should be prepared thoroughly to release RNA-protein (RNP) complexes while still maintaining their integrity. Tissue or cell lysates should be cleared of non-specific aggregates by repeating centrifugation a couple of times. Sucrose gradient using different concentrations of sucrose can also be tested for different tissues or cells. All devices that come into contact with cell lysates should be RNase free.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Huayu Qi (qi_huayu@gibh.ac.cn).

Materials availability

This study did not generate new unique reagents. Transgenic mouse line and *Rpl39l* gene null mouse line will be available upon request.

Data and code availability

The protocol does not contain any new datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization and design: Q.Z., H.Q.; Investigation: Q.Z., L.Y.; Data analysis: Q.Z., L.Y., H.Q.; Writing: Q.Z., H.Q.; Supervision: H.Q.; Funding acquisition: H.Q. All authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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