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Supplemental Information

A feedforward loop between JAK/STAT downstream target p115 and STAT in germline stem cells

Ruiyan Kong, Juan Li, Fuli Liu, Yankun Ma, Hang Zhao, Hanfei Zhao, Meifang Ma, and Zhouhua Li

Supplemental Information

1. SUPPLEMENTAL FIGURES

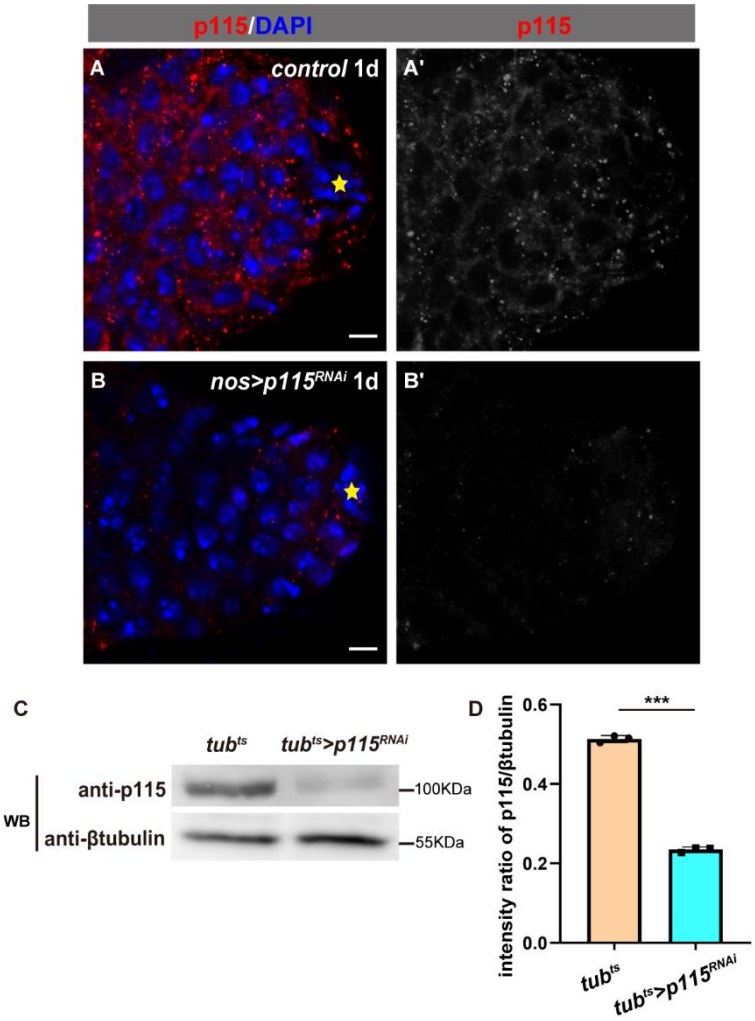

Figure S1. Knockdown efficacy of *p115*^{RNAi} and specificity of p115 antibody.

(A) Anti-p115 (red) staining in control showed p115 is mainly expressed in the cytoplasm as puncta. p115 channel is showed separately in black white.

(B) Knockdown of *p115* in germ cells significantly decreased the protein levels of p115 compared to those of control (A). p115 channel is showed separately in black white. The hubs are indicated by asterisks. Scale bars: 5 μm.

(C) Western blot analysis of p115 and βtubulin levels in lysate from control (*tub*^{ts}) and

tub^{ts} > *p115*^{RNAi}.

(D) Quantification of the ratio of p115/βtubulin from flies with indicated genotypes. Mean ± SD is showed. n=3. ***$P < 0.001$.

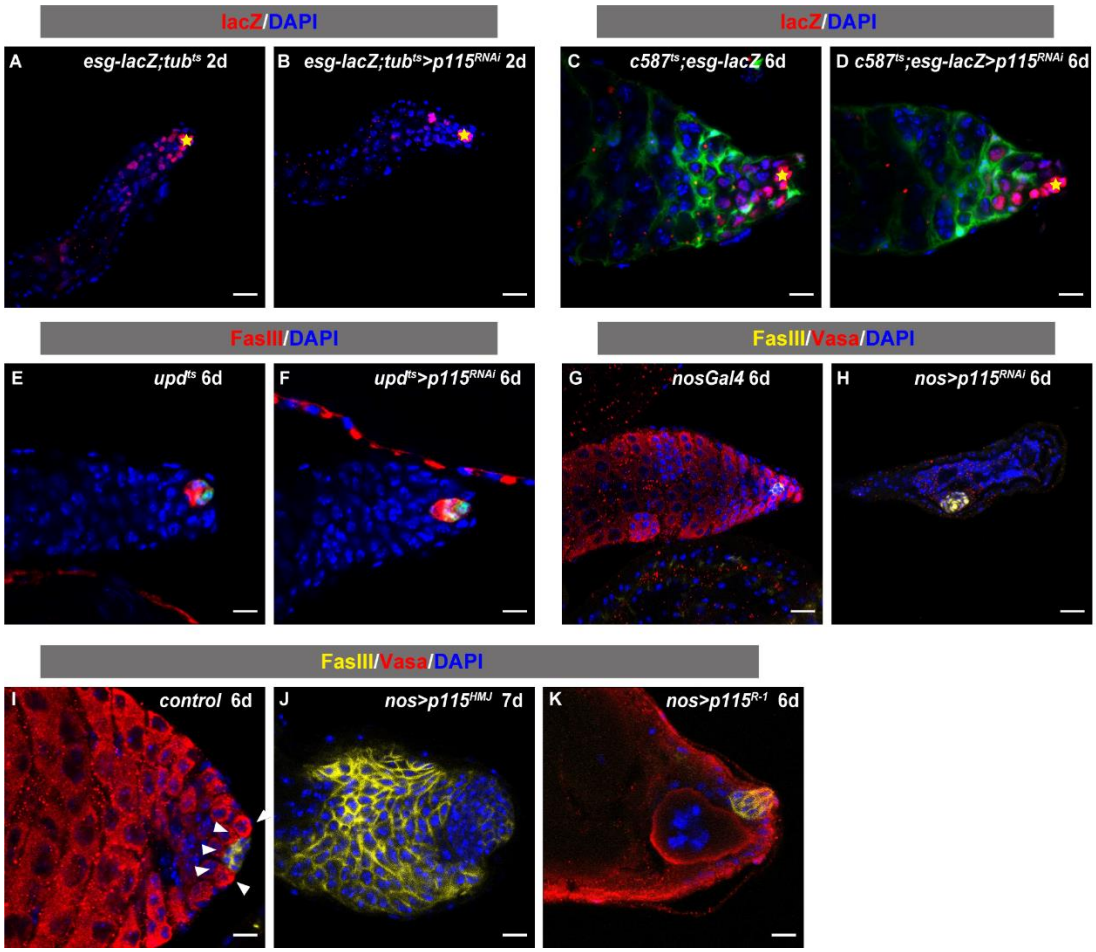

Figure S2. p115 functions in germline cells of adult testis.

(A and B) Systemic knockdown of *p115* using *esg-lacZ; tub*^{ts} led to dramatic reduction of *esg*$^{+}$ cells (*esg-lacZ* in red) (B) compared to control (A). The asterisk denotes the hub.

(C and D) Knocking down of *p115* in somatic cyst cell lineage using *c587Gal4, UAS-GFP, tubGal80*^{ts}*; esg-lacZ (c587*^{ts}*; esg-lacZ)* did not result in obvious defects in testis (*esg-lacZ* in red). The hub is marked by the asterisk.

(E and F) Knocking down of *p115* in the hub using *updGal4, UAS-GFP, tubGal80^{ts} (upd^{ts})* did not cause obvious changes (FasIII in red).

(G and H) Knocking down of *p115* in germline cell lineage using *nosGal4* led to germline cell loss at room temperature 6 d after eclosion.

(I-K) Knocking down of *p115* in germline cells by different RNAi constructs, *p115^{HMJ}* (J) and *p115^{R-1}* (K), caused germline cell loss (red) compared to control (I) (GSCs are indicated by white arrowheads).

Vasa is stained for germline cells, FasIII is stained for the hub, and DAPI is stained for the nucleus. *esg* is highly expressed in the hub and early germ cells. Scale bars: 10 μm (C-F, I-K) and 20 μm (A-B, G-H).

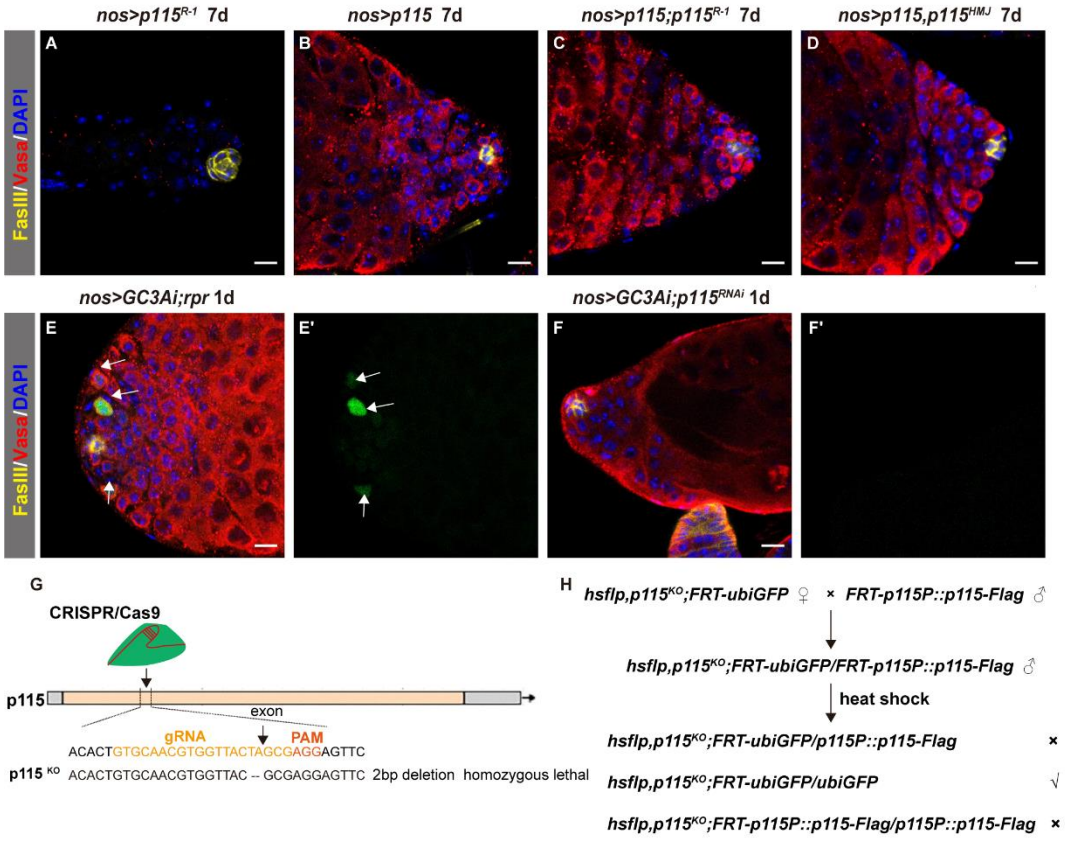

Figure S3. p115 is required for male GSC maintenance.

(A) The germline cells (red by Vasa) in *nos* > $p115^{RNAi}$ adult testis were totally lost at 7 d after eclosion. Please refer to Figures S2I and 2A for control.

(B) Overexpression of *p115* in germline cells results in no obvious phenotype. Please refer to Figure 2A for control.

(C and D) Overexpression of *p115* fully rescued the defects observed in *nos* > $p115^{RNAi}$ testes.

(E) Apoptosis (by GC3Ai in green) could be detected in germline cells expressing *reaper* (*rpr*) (white arrows).

(F) No apoptosis was detected in germline cells in *nos* > $p115^{RNAi}$ testes for 1 d.

(G) Diagram of $p115^{KO}$ mutant generated by CRISPR/Cas9. gRNA and PAM (protospacer adjacent motif) in capital letters are highlighted in light and dark brown, respectively. A 2 bp deletion was generated within gRNA target site in the coding exon of *p115*. Deletion is represented by a dash. $p115^{KO}$ mutant is homozygous lethal.

(H) The workflow to generate mosaic GSC clones of $p115^{KO}$ mutant. $p115^{KO}$ mutant mosaic clones were identified by stronger GFP signals than the neighboring cells (two copies of Ubi-GFP (homozygous $p115^{KO}$ mutant cells) vs one copy/none of Ubi-GFP (heterozygous $p115^{KO}$ mutant cells)).

Vasa is stained for germline cells, FasIII is stained for the hub, and DAPI is stained

for the nucleus. Scale bars: 10 μm.

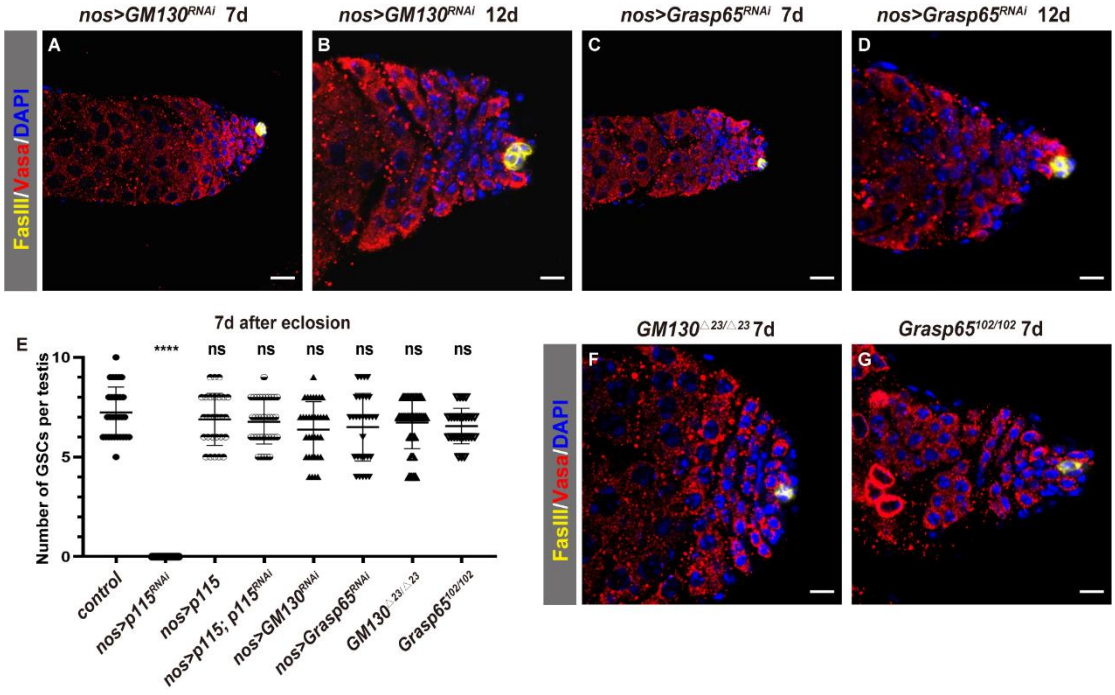

Figure S4. GM130 and Grasp65 are dispensable for male GSC maintenance.

(A and B) No obvious defects were observed in *nos > GM130*^{RNAi} testes at 7 d (A) and 12 d after eclosion (B). Please refer to Figure 2A for control.

(C and D) Knockdown of *Grasp65* in germline cells resulted in no obvious defects at 7 d (C) and 12 d after eclosion (D).

(E) Quantification of the number of GSCs in testes with indicated genotypes from 7-day-old animals (n=30 testes for *nosGal4* as control, n=21 for *nos > p115*^{RNAi}, n=28 for *nos > p115*, n=35 for *nos > p115; p115*^{RNAi}, n=34 for *nos > GM130*^{RNAi}, n=30 for *nos > Grasp65*^{RNAi}, n=33 for *GM130*$^{\Delta 23/\Delta 23}$, n=28 for *Grasp65*$^{102/102}$).

(F and G) No obvious defects were observed in *GM130* and *Grasp65* homozygous mutants. Mean ± SD is showed. Ordinary one-way ANOVA test was used, ns: not significant, ****P<0.0001.

Vasa marks germline cells, FasIII is stained for the hub, and DAPI is stained for the nucleus. Scale bars: 10 μm (B, D, F, G); 20 μm (A, C).

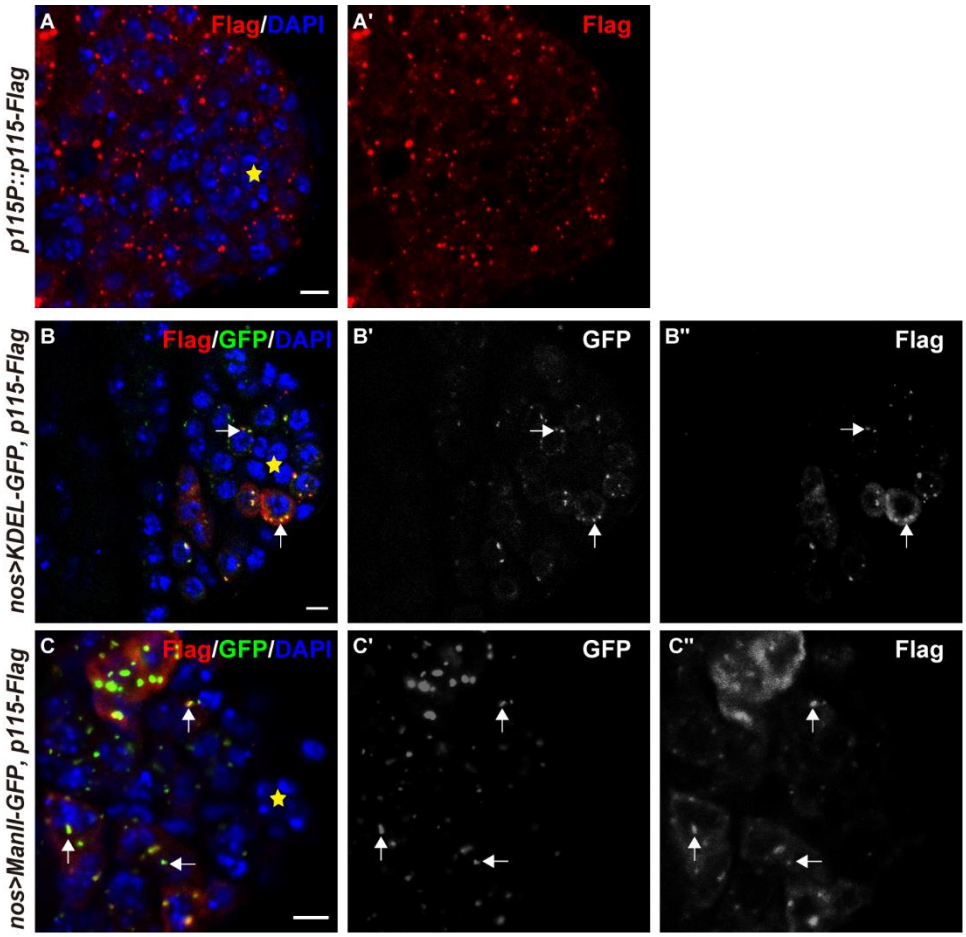

Figure S5. p115 mainly localize in cytosol, the ER and Golgi apparatus.

(A) Expression pattern of p115 is showed by anti-Flag (red) in *p115P::p115-Flag* testis. p115 is mainly expressed in the cytosol as punctate form. The hub is indicated

by asterisk. Flag channel is showed separately.

(B) Anti-Flag (red) and GFP (green) double labeling of *nos > KDEL-GFP, p115-Flag* testis showed co-localization of p115 and the ER (by KDEL-GFP, white arrows). KDEL-GFP and p115-Flag channels are showed separately in black white.

(C) Anti-Flag (red) and GFP (green) double labeling of *nos > Man II-GFP, p115-Flag* testis showed partial co-localization of p115 and the Golgi (by Man II-GFP, white arrows). Man II-GFP and p115-Flag channels are showed separately in black white.

Scale bars: 10 μm (A); 5 μm (B-C).

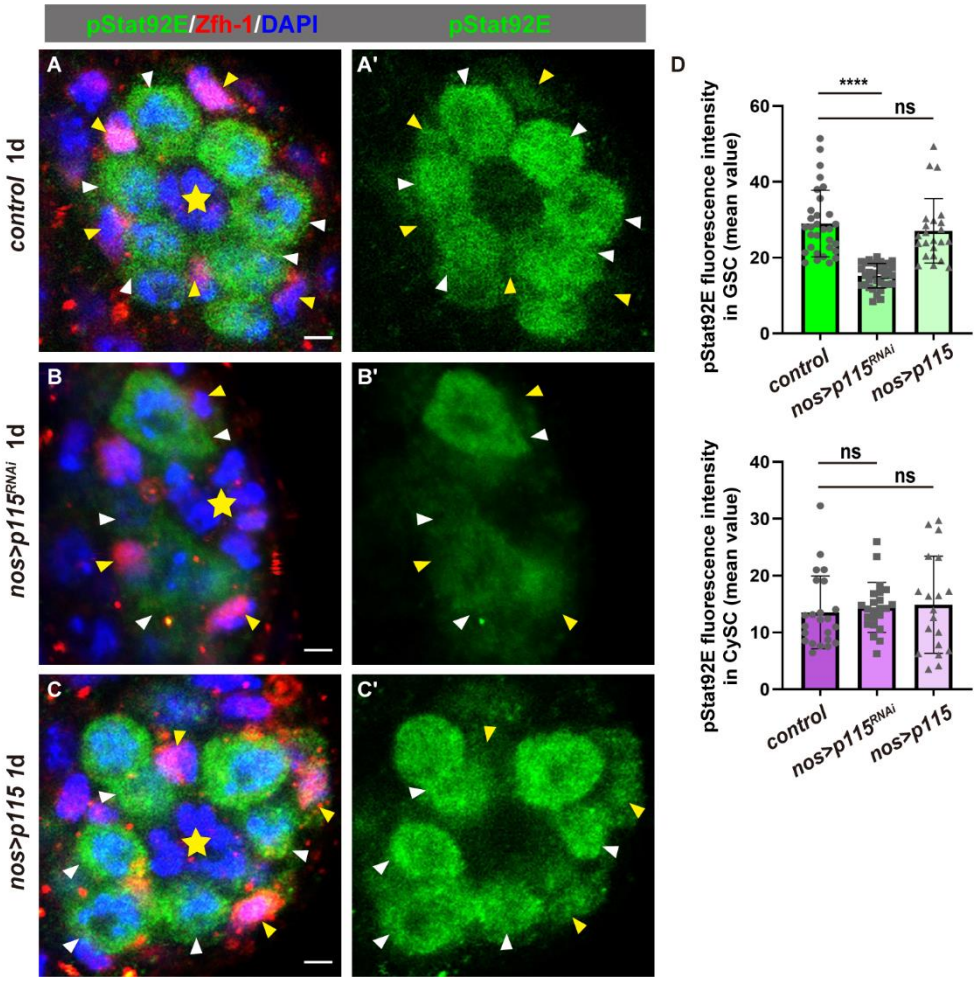

Figure S6. Depletion of p115 in GSCs significantly reduced the pStat92E levels in

GSCs but not in CySCs.

(A-C) Control, *nos > p115*^{RNAi}, and *nos > p115* testes were stained with anti-Zfh-1 antibody (red) to label CySCs and anti-pStat92E (green) antibody to label GSCs. GSCs are indicated by white arrowheads and CySCs are indicated by yellow arrowheads. The hubs are indicated by asterisks. The levels of pStat92E are significantly reduced in *p115*-depleted GSCs. Scale bars: 10 μm.

(D) Quantification of the levels of pStat92E in GSCs and CySCs in testis with indicated genotypes. Mean ± SD is showed. n ⩾ 15. ns, not significant, ****P<0.0001.

2. SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Fly lines and cultures

Flies were maintained on standard diet 25°C with a 12 h/12 h light/dark cycle. The crosses were raised at 18°C and the desired hatched flies were picked up and transferred to new vials with fresh food every day at proper temperature and dissected at time points. Detailed information of the fly stocks used in this study can be found either in FlyBase or as noted: *FRT82B*, *esg-lacZ* (*M5-4*), *nosGal4*, *c587Gal4*, *UAS-GFP, tubGal80*^{ts}*; esg-lacZ (c587*^{ts}*; esg-lacZ)*, *UAS-hop*$^{Tum-l}$, *UAS-Dam* (a generous gift from Dr Andrea H. Brand) (Southall et al., 2013), *updGal4, UAS-GFP, tubGal80*^{ts} *(upd*^{ts}*)*, *esg-lacZ; tubGal4, tubGal80*^{ts} (*esg-lacZ; tub*^{ts}), *p115*$^{R-1}$ (NIG.1422R-1), *p115*^{HMJ} (HMJ23695), *GM130*^{RNAi} (NIG.11061R-1), *Grasp65*^{RNAi} (BL34082/HMS01093), *GM130*$^{\Delta 23}$ (BL65255), *Grasp65*^{102} (BL65257), *Stat92E-Flag-GFP* (BL38670), *UAS-HA-Stat92E* (a generous gift from Dr Erika A. Bach) (Ekas et

al., 2006), *UAS-KDEL-GFP* (BL9898), *UAS-rpr, nosGal4;UAS-GC3Ai* (BL84308), *UAS-Man II-GFP* (a generous gift from Dr Zhou Wei), *hsflp; FRT82B-ubiGFP* was used for mosaic clonal analysis. *w* (*white*) ^{RNAi} (BL33623) and/or $Gal4^{RNAi}$ (HMS504, from TRiP at Harvard Medical School) were used as control.

RNAi knockdown and overexpression experiments

Knockdowns and overexpression were performed by combining the *UAS-shRNA* fly lines and transgenic stocks respectively with *nosGal4*, upd^{ts}, $c587^{ts}$ or tub^{ts} drivers. Crosses were maintained at 18°C. Flies with desired genotype were collected and maintained at 18°C upon eclosion or transferred to 29°C according to experiment need. Flies were transferred to new vials with fresh food every day and dissected at time points. Please note that when the transgene is driven by *nosGal4* in the germline cells, the transgene is not equally expressed in all the germline cells.

Generation of transgenic flies

To identify JAK/STAT signaling downstream targets, *attB-UAST-STAT-Dam* was constructed. *STAT* ORF was amplified from *UAS-HA-Stat92E* (a generous gift from Dr Erika A. Bach) (Ekas et al., 2006) using primer pair (*STAT-Dam*-F:gatctggccggcgc AGATCTGCGGCCGCtcATGAGCTTGTGGAAGCGCATCGCCAGCC and *STAT-Dam*-R: gtaaggttccttcacaaagatccTCTAGATCAAAAGTTCTCAAAGtTTGTAATCGT ATC) and cloned into the NotI and XbaI sites of *attB pUAST-LT3-Dam* (a generous gift from Dr Andrea H. Brand) (Southall et al., 2013). To explore p115 function, two

pairs of oligoes were synthetized (*p115*-F1: ctagcagtGGAGAGCTTCTGTAGCA AACTtagttatattcaagcataAGTTTGCTACAGAAGCTCTCCgcg and *p115*-R1: aattcgc GGAGAGCTTCTGTAGCAAACTtatgcttgaatataactagTTTGCTACAGAAGCTCTCC actg. *p115*-F2: ctagcagtGGAGATGTTCATCAAGACACCtagttatattcaagcataGGTGTC TTGATGAACATCTCCgcg and *p115*-R2: aattcgcGGAGATGTTCATCAAGACACC tatgcttgaatataactaGGTGTCTTGATGAACATCTCCactg). The oligoes were annealed and cloned into the EcoRI and NheI sites of an *attB-pWALIUM20* vector (TRiP) to generate *attB-UAS-p115*^{shRNA}. *UAST-p115-Flag* construct was constructed by cloning the *p115* ORF amplified from gDNA using primer pair (*p115*-ERI-5': CTGAATAGG GAATTGGGAATTCatggagttcctgaagagtggcat and *p115*-ERI-3': CATTTTGGTAC GCCGGAATTCcctgctggcggtgccacttgg) into the EcoRI site of *attB-pUAST-nFlag* vector. *p115P::p115-Flag* was constructed by cloning the promoter and the coding region of *p115* from gDNA using primer pair *p115p*-ERI-5': CTGAATAGGGAATTGGGAATTCggcacacctgaagcgcccggc and *p115p*-ERI-3': CATTTTGGTACGCCGGAATTCcctgctggcggtgccacttggg into the EcoRI site of *attB-pUAST-nFlag* vector. *UAST-Stat92E-Flag-GFP* was constructed by cloning the Stat92E ORF from *UAS-HA-Stat92E* (from Erika A. Bach) (Ekas et al., 2006) using primer pair *STAT*-ERI-5': CTGAATAGGGAATTGGGAATTCatgagcttgtggaagcgcatct cc and *STAT*-XhoI-3': CGCCCTTGCTCACCATCTCGAGAAAGTTCtcaaagtttgtaatc gtatcgaagtcc into the EcoRI and XhoI site of *attB-pUAST-Flag-GFP* vector. Transgenic flies were obtained by standard P-element-mediated germline transformation carrying *attP* site at 86F.

Generation of *p115* knockout mutant

p115 knockout mutant was generated by CRISPR-Cas9 technology. *p115* guide RNA construct was generated by cloning the *p115* guide RNA into the BbsI site of *pCFD4* using primer pair *p115*-sgRNA1-F1: TATATAGGAAAGATATACCGGGTGAACTTC gtgcaacgtggttactagcgGTTTTAGAGCTAGAAATAGCAAG and *p115*-sgRNA1-R1: ATTTTAACTTGCTATTTCTAGCTCTAAAACcgctagtaaccacgttgcacGACGTTAAAT TGAAAATAGGTC. *p115* knockout mutant were generated by injecting *pCDF4-p115-sgRNA* vector into transgenic flies carrying Cas9 protein. The mutants were identified by PCR using primer pair *p115*-KO1-F: GCACCAGGGCTGTCGAGTCGT and *p115*-KO1-R: TTGGATGTTTGAGTTGCCCTTGGTC. PCR products were sequenced to determine the deleted regions.

Mosaic clone analyses

Clones were generated using FLP/FRT system-mediated mitotic recombination. Flies with genotype of *hsflp; FRT82B-ubiGFP* or *hsflp, $p115^{KO}$; FRT82B-ubiGFP* were crossed with flies with genotype of *FRT82B* or *FRT82B-p115P::p115-Flag*. Males with the desired genotypes were selected to conduct time-course clonal analysis after clone induction (ACI). GSC clones were induced by heat shocking two-day-old males with desired genotypes 1 hour at 37°C, twice a day for 2 days. The heat shock-treated flies were maintained at 22°C and transferred to new vials with fresh food daily before dissection.

Generation of mouse anti-p115 antibody

A GST fusion protein containing the region of p115 (101-301 aa) with a GST tag at its N terminal was used as antigen. A DNA fragment encoding 101-301 aa of p115 was amplified and cloned into the BamHI and EcoRI sites of *pGEX-4T-1* vector using primers: *GST-p115*-5': CGGATCTGGTTCGCGTGGATCCgaggaggctgataatcccaccg and *GST-p115*-3': GCTCGAGTCGACCCGGGAATTCctactgattactggagttgttcttg. Fusion proteins were purified according to the manufacturer's protocol and mice were immunized following standard procedure.

Immunostaining and imaging

Testes were dissected in 1×PBS buffer and fixed in 4% PFA for 20 minutes at room temperature. The samples were then rinsed, washed with 1×PBT (0.1% Triton X-100 in 1×PBS) three times, one time for 5mins and blocked in 3% BSA in 1×PBT for 45 min. The samples were then incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: rabbit anti-Zfh-1 (1:50,000) (Xu et al., 2018), rabbit anti-Vasa (d-260, 1:200, Cat No: sc-30210, Santa Cruz, USA), rabbit anti-Vasa (1:2,000) (Zhao et al., 2022), mouse anti-α-Spectrin (3A9, 1:50, developed by D. Branton, and R. Dubreuil, Developmental Studies Hybridoma Bank (DSHB)), mouse anti-FasIII (7G10, 1:100, developed by C. S. Goodman, DSHB), rabbit anti-GFP (1:1,000, Abcam, USA), mouse anti-γ-tubulin (1:1,000, Abbkine, USA), guinea pig anti-Stat92E (1:1,000, a generous gift from Dr Yu Cai), mouse anti-pStat92E (1:2,000,

Abmart, China)(Zhang et al., 2013), rabbit anti-β-glactosidase (lacZ, 1:5,000, Cat No: 55978, Cappel, USA), mouse anti-Flag (1:2,000, Sigma-Aldrich, USA). The rinsing and washing procedures were conducted and samples were then incubated with the secondary antibodies conjugated with Cy3, 488, or Cy5 (Jackson ImmunoResearch, USA) with a dilution of 1:400 for 2 h at room temperature. DAPI (Sigma, 0.1 μg/ml) was added to the secondary antibodies staining. All images were captured using Zeiss LSM 780 laser scanning confocal microscope and processed in Adobe Photoshop and Illustrator.

Transmission electron microscopy

The testes were dissected and fixed in cold 2.5% glutaraldehyde (stored at 4°C), and then washed 4 times with PB buffer (0.1 M). The tissue underwent post-fixation with Osmium tetroxide (1%) and Tetra potassium hexacyanoferrate trihydrate (1.5%) for 1 h at 23℃, followed by ethanol dehydration in graded solutions (50%, 70%, 80%, 90%, 100%, 100%, 100%) for 5min each. Then, 1, 2-Epoxypropane twice for 5min each and gradient infiltration with mixture of 1, 2-Epoxypropane and Epon 812 resin for 8 h (SPI, America). Subsequently, Pure Epon 812 twice and polymerizing in oven (60°C). Blocks of polymerized resin were sectioned using a Leica EM UC7 ultramicrotome (Wetzlar, Germany). Ultra-thin sections (70 nm) were mounted and dried on coated copper grids. Sections were stained on-grid with 2% uranyl acetate (25 min) and lead citrate (5 min). Imaging was carried out using H-7650B transmission electron microscope (Hitachi, Tokyo, Japan).

Quantitative real-time PCR

Total RNA was extracted from ~300 testes using TRIzol (Invitrogen, USA) and was cleaned using RNAeasy (QIAGEN, USA). Extracted total RNA was used to synthesize cDNA through GoTaq® qPCR Master Mix (PROMEGA, USA) according to the manufacturer's instructions. Oligo (dT)15 primer was used for cDNA library synthesis, which was then used for qPCR. NovoStart® SYBR qPCR SuperMix Plus (Novoprotein, China) was used to perform qPCR following the standard protocol provided by QuantStudio 7 Real-Time PCR System (Applied Biosystems, USA). qPCR was performed in duplicate for each of three independent biological replicates. All results are presented as mean ± SD of the biological replicates. Ribosomal gene *RpL11* was used as normalization control. P-values and data significance was calculated according to two-tailed Student's *t* test.

Dam-ID

Dam-ID was carried out according to a previously described method (Gutierrez-Triana et al., 2016). Testes from *c587^{ts};hop^{Tum-l},STAT-Dam* and *c587^{ts};hop^{Tum-l},UAS-Dam* (control) adult fly (about 2,000 flies) were collected after two days expression and immediately preserved on dry ice and kept at -80°C. Genomic DNA was then isolated and amplified as described in (Chen et al., 2018). Dam-ID sequence quality was examined by FastQC (version 0.11.9). Dam-ID-seq reads were aligned using Bowtie2 (version 2.5.0) to build version dm6 of the *D. melanogaster* genome.

MACS2 (version 2.2.7.1) was used to call peaks from alignment results and to identify regions of enrichment. BigWig files were generated for visualization using the bioconductor-chipseeker (Version 1.18.0). Raw data from the Dam-ID were submitted to Gene Expression Omnibus (http://ncbi.nlm.nih.gov/geo) with the access number GEO: GSE226582.

Co-Immunoprecipitation and western blotting

Fly tissues were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, pH 8.0, 0.5% Triton X-100, 0.5% NP-40, 0.5% sodium deoxycholate, and complete protease inhibitor cocktail tablets (Roche, USA)) on ice for 30 minutes. After centrifugation (13,000 rpm,10 minutes at 4°C), lysates were then diluted ten-fold with RIPA buffer and subjected to immunoprecipitation using anti-Flag M2 affinity gel (A2220, Sigma-Aldrich, USA). The immunocomplexes were collected by centrifugation and washed with 1 ml of RIPA buffer three times, one time for 5 mins. For western blotting, immunoprecipitated proteins were separated in SDS-PAGE and then blotted onto PVDF membranes. The membranes were stained with the primary antibody overnight at 4°C. Followed by washing with 1×PBST (0.1% Tween 80 in 1×PBS), PVDF membranes were incubated with the secondary antibodies conjugated with HRP, then the membranes were scanned using Luminescent Image Analyzer (GE, Sweden). Mouse anti-p115 (1:1,000, this study), mouse anti-Flag (1:1,000, Sigma-Aldrich, USA), mouse anti-HA (1:1,000, Sigma-Aldrich, USA), mouse anti-βtubulin (1:1,000, Abbkine, USA) and guinea pig anti-Stat92E (1:1,000, a generous gift from

Dr Yu Cai) antibodies were used.

Signal quantification

Image J software was used for signal quantification (pStat92E and Stat92E). Two parameters, integrated optical density (IOD) and area, were used in the analysis. IOD value per cell was used. At least six different images were analyzed for each sample.

Data analysis

The number of GSCs or spectrosome-containing cells were counted from selected testes under a fluorescence microscope. The number of GSCs in testis was counted according to Vasa staining, α-Spectrin staining, and the position (GSCs are attached to hub cells). A marked GSC clone was calculated by carrying a $GFP^{+/+}$ cell attached to hub cells. The mean fluorescent intensity of Stat92E and pStat92E in GSCs and western blots blands grey values were measured by Image J software. Data processing was analyzed and performed using GraphPad Prism 7.0 (GraphPad Software Inc., USA). P values were determined by two-tailed Student's *t* tests or Ordinary one-way ANOVA test. ^{ns} P > 0.05; *P < 0.05; **P < 0.01; *** P < 0.001; ****P < 0.0001.

qRT-PCR primers used:

p115-S: CACCGCCAGCAGGTAGAAAT

p115-A: TTGCGATTTGCTGCAGTTCC

RpL11-S: GGTCCGTTCGTTCGGTATTCGC

RpL11-A: GGATCGTACTTGATGCCCAGATCG

3. SUPPLEMENTAL REFERENCES

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