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Supplemental Information

Novel intrinsic factor Yun maintains female germline stem cell fate through Thickveins

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Supplemental Information

1. Supplemental Figures

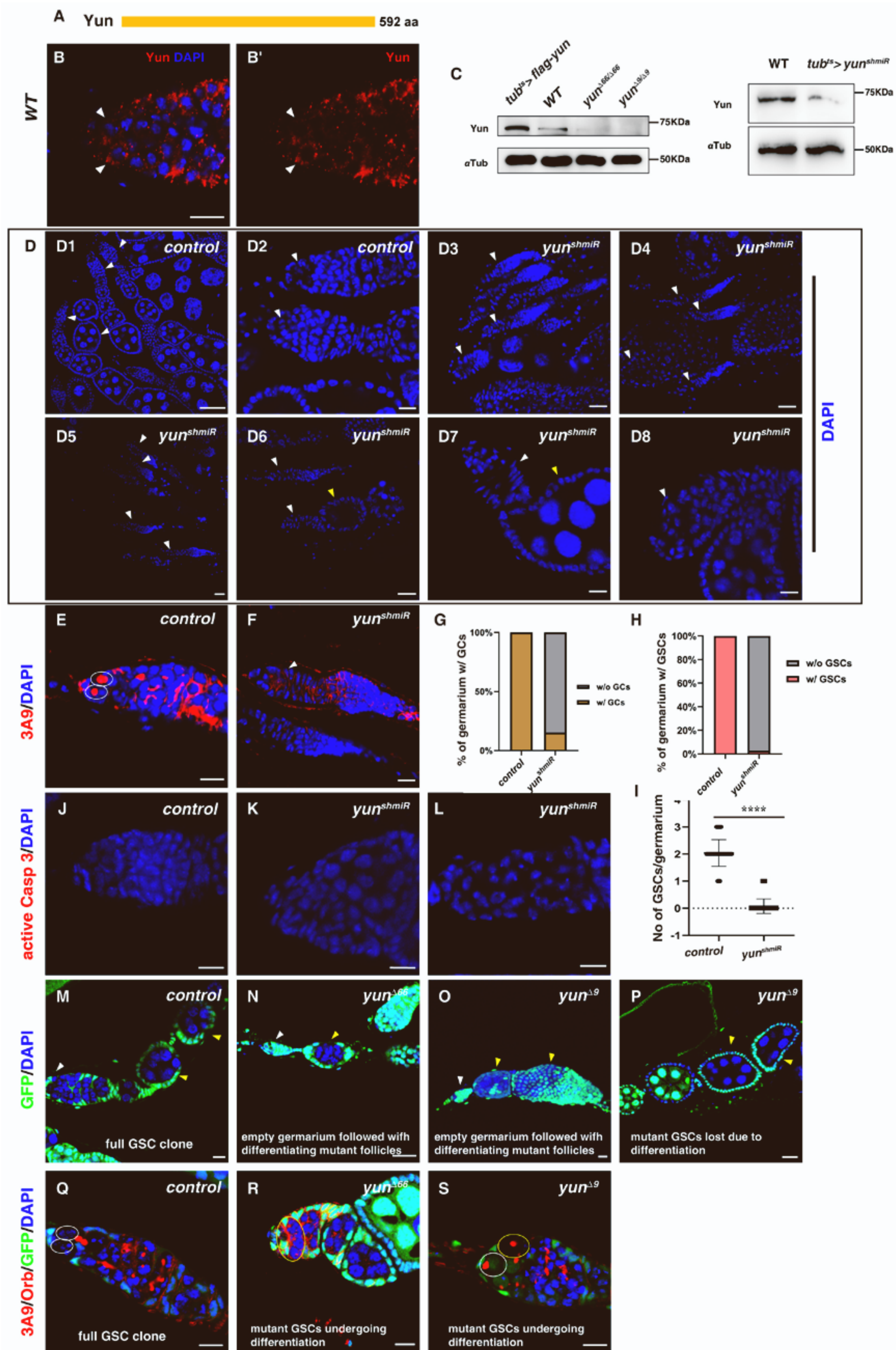


Figure S1. Yun is intrinsically required for female GSC maintenance, related to Figure 1

(A) Scheme of Yun protein. No known domains or motifs are found in Yun.

(B) Yun is expressed in the germarium, especially GSCs (by white arrowheads) and is mainly localized in cytosol. Yun channel is showed separately.

(C) Yun protein in tissues with *yun* overexpression, WT, and *yun* homozygous larva respectively (left panel). Yun protein levels are significantly reduced upon expression of *yun* shmiR construct. Note that *tubGal4* is not expressed in female germline cells and ovaries are included in the samples. ɑTubulin is used as loading control (right panel).

(D) Different ovarioles with germline-specific knockdown of *yun* using *yun*^{shmiR} (*nos>yun*^{shmiR}). Please note that compared with control, most of the ovarioles are devoid of germline cells, with a margin percentage of ovarioles containing germline cells at different differentiation stages (germarium by white arrowheads and egg chambers by yellow arrowheads).

(E) Control germarium labelled with 3A9. The GSCs contain anterior localized spectrosomes (marked by white ovals).

(F) No germline cells can be detected in a *nos>yun*^{shmiR} germarium (white arrowhead).

(G) Quantification of percentage of germarium containing germline cells (GCs) in control and *nos>yun*^{shmiR} ovaries. n⩾75.

(H) Quantification of percentage of germarium containing GSCs in control and *nos>yun*^{shmiR} ovaries. n⩾75.

(I) Quantification of the number of GSCs per germarium in control and *nos>yun*^{shmiR} ovaries. Mean ± SEM is shown. n⩾12. **** $p < 0.0001$.

(J) No apoptosis is detected in control germarium (by active Casp 3 in red).

(K and L) No apoptosis is detected in *nos>yun*^{shmiR} germarium.

(M) Marked control GSCs (GFP$^-$, white arrowhead) can be maintained and constantly produce differentiating progeny (yellow arrowheads, egg chambers).

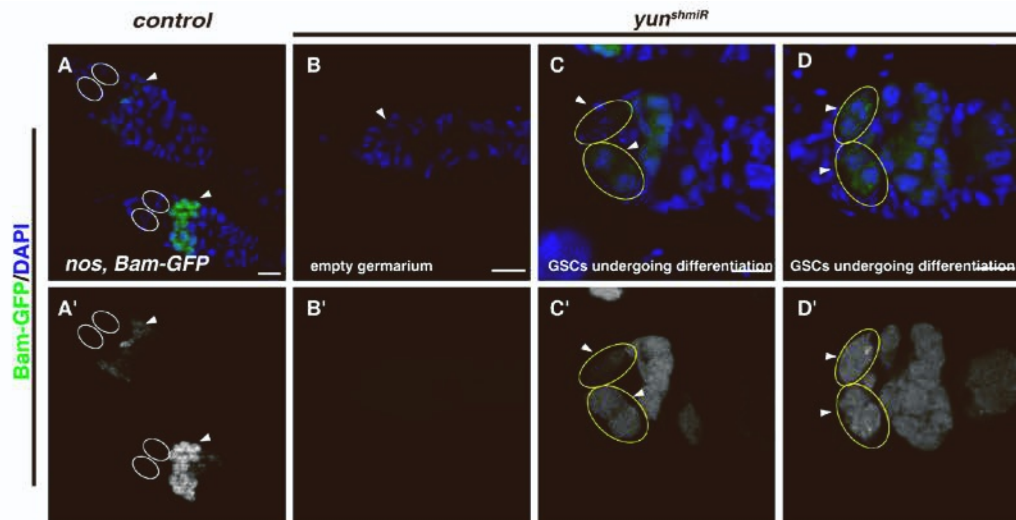
(N and O) Marked *yun* mutants (N: *yun*$^{\Delta66}$ and O: *yun*$^{\Delta9}$) show empty germarium followed by differentiating follicles containing *yun* mutant germline cells (white and yellow arrowheads).

(P) *yun*$^{\Delta9}$ mutant GSCs can not be maintained and differentiate into consecutive developing egg chambers (yellow arrowheads, the corresponding germarium with only wild type GSCs is not shown).

(Q) Marked control GSC (white ovals) clones labelled with 3A9 and Orb.

(R and S) Marked *yun* mutant GSCs (R: *yun*$^{\Delta66}$ and S: *yun*$^{\Delta9}$) (yellow ovals) are no longer be maintained, contain branched fusome, and undergo differentiation.

Scale bars: 10 μm and 50 μm (B, D1, D3, and D4).


control
yun^shmiR
A
B
C
D
nos, Bam-GFP
empty germarium
GSCs undergoing differentiation
GSCs undergoing differentiation
Bam-GFP/DAPI
A'
B'
C'
D'

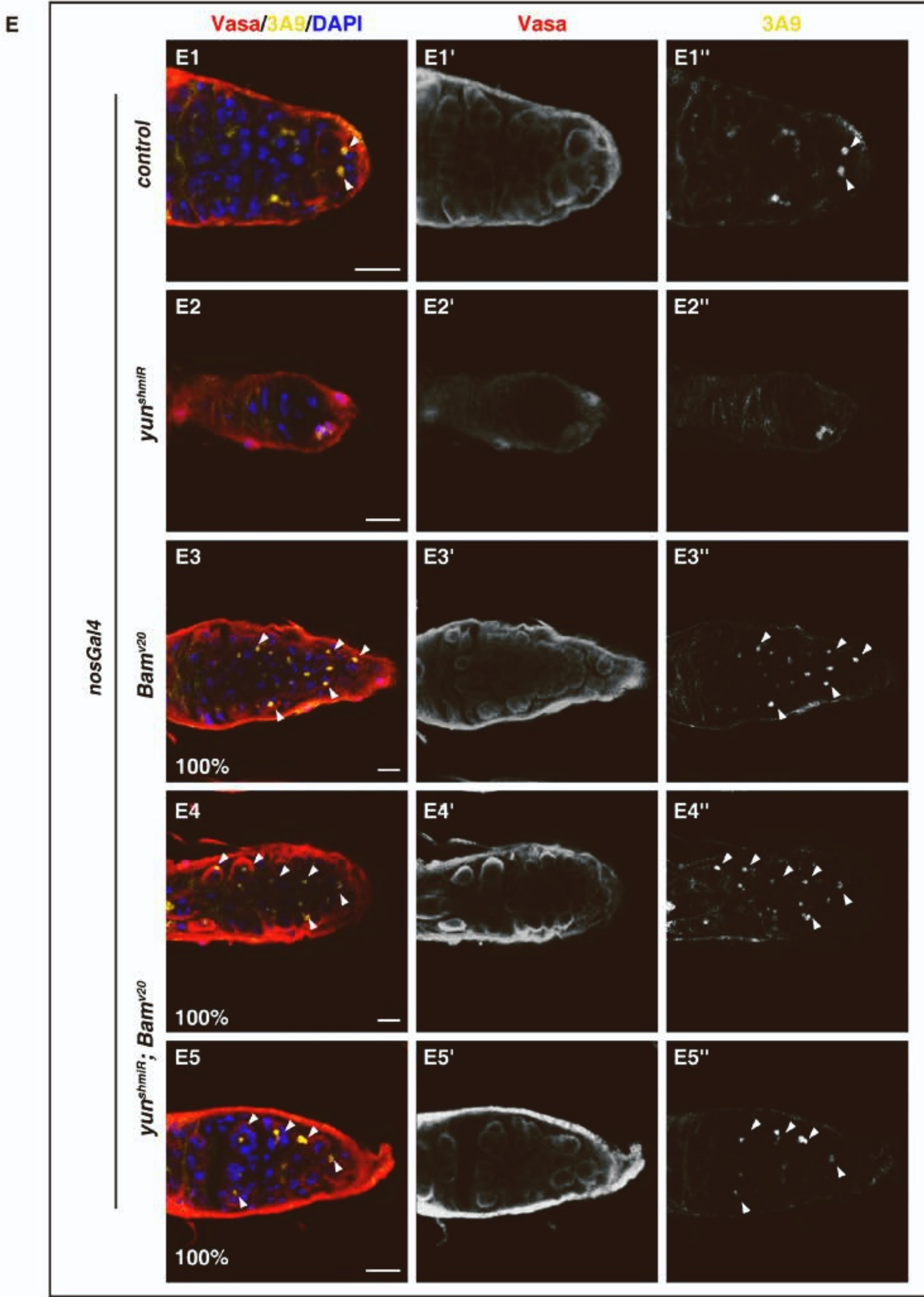

E
Vasa/3A9/DAPI
Vasa
3A9
E1
E1'
E1''
control
E2
E2'
E2''
yun^shmiR
E3
E3'
E3''
nosGal4
Bam^V20
100%
E4
E4'
E4''
100%
E5
E5'
E5''
yun^shmiR; Bam^V20
100%

Figure S2. Yun represses Bam expression for GSC maintenance, related to Figure 2

(A) *Bam-GFP* in control germarium (white arrowheads). Bam-GFP is suppressed in GSCs (labelled by white ovals), but is expressed in developing cysts (white arrowheads). Bam-GFP signal is showed separately in black white.

(B-D) *Bam-GFP* is either not expressed in empty *nos>yun*^{shmiR} germarium (B), or is expressed in the very anterior germline cells in *nos>yun*^{shmiR} germaria (C and D), indicating that these *yun-defective* GSCs are undergoing differentiation (white arrowheads).

(E) Bam functions downstream of Yun. Vasa (red) and 3A9 (yellow, white arrowheads) in control (E1), *nos>yun*^{shmiR} (E2), *nos>Bam*^{v20} (E3), and *nos>yun*^{shmiR}*, Bam*^{v20} (E4 and E5) germaria. Vasa and 3A9 channels are showed separately in black white. Note that GSCs and GSC-like cells are filled in all *nos>Bam*^{v20} and *nos>yun*^{shmiR}*, Bam*^{v20} germaria (white arrowheads).

Scale bars: 10 μm.

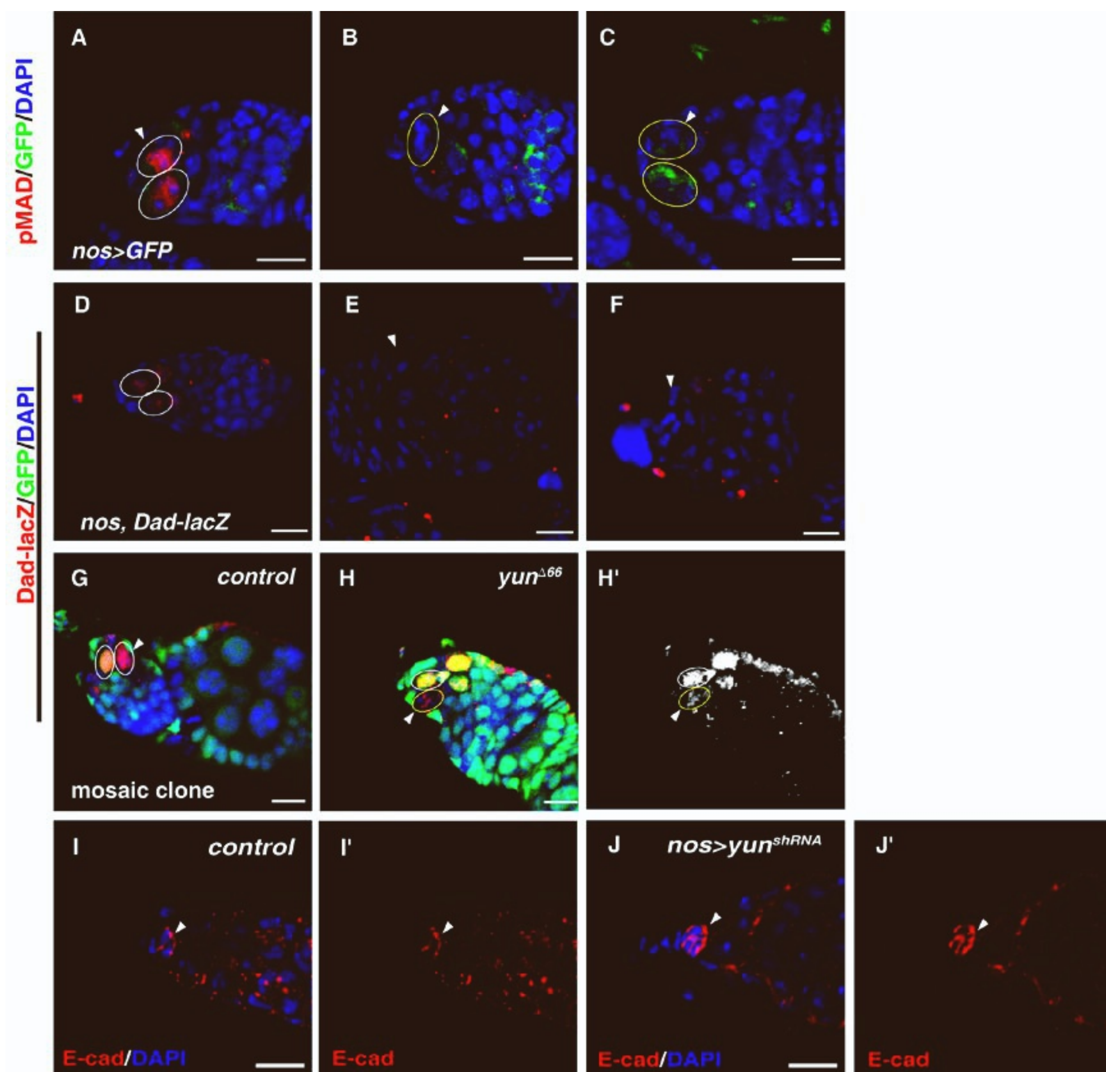


Figure S3. Dpp signaling is inactivated and E-cadherin is unaffected in the absence of

***yun*, related to Figure 2**

(A) BMP signaling (by pMAD) is activated in GSCs (white ovals) in control germarium (white arrowhead).

(B and C) No pMAD signal can be detected in *nos>yun*^{shmiR} germaria (yellow ovals), indicative of BMP signaling inactivation in the absence of *yun*.

(D) The expression of *Dad-lacZ* in control germarium, with GSCs indicated by white ovals.

(E and F) The expression of *Dad-lacZ* in can barely be detectable in *nos>yun*^{shmiR} germaria (white arrowheads).

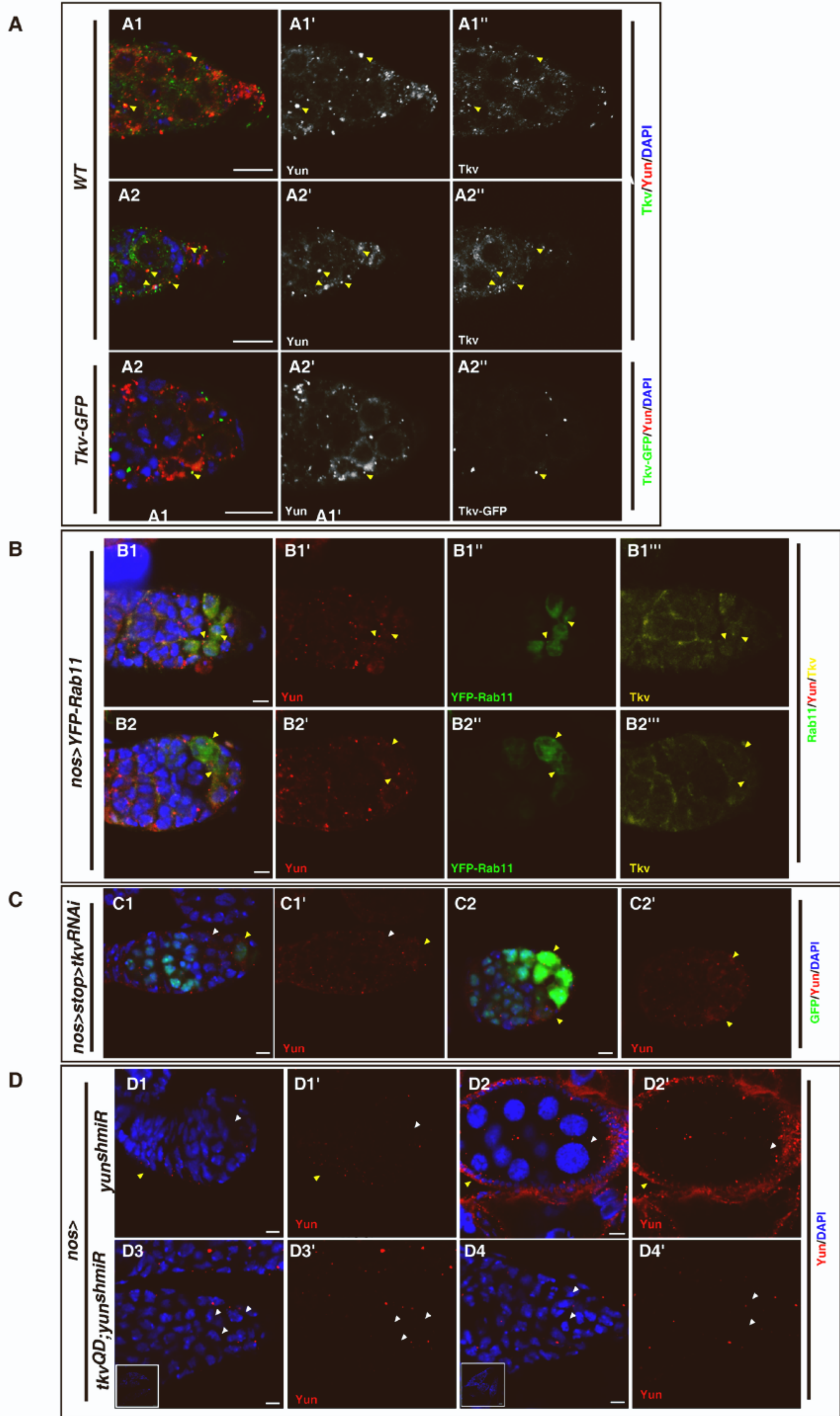
(G) The expression of *Dad-lacZ* in control germarium (GSCs are indicated with white ovals). *Dad-lacZ* expression is not altered in marked control GSC (white arrowhead).

(H) The expression of *Dad-lacZ* is diminished in marked *yun*$^{\Delta 66}$ GSC (yellow oval and white arrowhead). The neighboring wild type GSC is indicated with white oval. Dad-lacZ signal is showed separately in back white (H').

(I) E-cadherin in the GSC-niche junction in control germarium (white arrowhead). E-cadherin signal is showed separately in I'.

(J) E-cadherin in the GSC-niche junction in *nos>yun*^{shmiR} germarium (white arrowhead). E-cadherin signal is showed separately in J'.

Scale bars: 10 μm.



A
A1
A1'
A1''
WT
Yun
Tkv
Tkv/Yun/DAPI
A2
A2'
A2''
Yun
Tkv
Tkv-GFP
Tkv-GFP/Yun/DAPI
Tkv-GFP
B
B1
B1'
B1''
B1'''
nos>YFP-Rab11
Yun
YFP-Rab11
Tkv
Rab11/Yun/Tkv
B2
B2'
B2''
B2'''
C
C1
C1'
C2
C2'
nos>stop>tkvRNAi
GFP/Yun/DAPI
D
D1
D1'
D2
D2'
nos>
yunshmiR
Yun/DAPI
D3
D3'
D4
D4'
tkvQD;yunshmiR

Figure S4. Yun co-localizes with endogenous Tkv in cytosol but not in the recycling endosomes, and the levels of Yun are not affected by *tkv* depletion, related to Figure 3

(A) Some of the Yun puncta (red) co-localize with endogenous Tkv (A1 and A2) and Tkv-GFP (A3)(green) (yellow arrowheads). Yun and Tkv/Tkv-GFP channels are showed separately in black white.

(B) Yun (red) and Tkv (yellow) are rarely observed in Rab11^{+}-recycling endosomes (green). Yun, YFP-Rab11, and Tkv channels are showed separately. Note that when expressed in female germline cells, only a small portion of YFP-Rab11 proteins localizes in recycling endosomes (puncta). The yellow arrowheads indicate YFP-Rab11^{+}-recycling endosomes in which either Tkv, or Yun, or both are absent.

(C) The levels of Yun proteins (red, yellow arrowheads) are largely unaffected in *tkv*-depleted GSCs and germline cells (green). Please refer to Figure S4A for comparison. As Tkv is essential for GSC maintenance, no GSCs and germline cells were observed when tkv^{RNAi} was driven by *nosGal4*. These $nos>tkv^{RNAi}$ ovaries are very small and do not contain any germline cells. Thus the *hsFlp, nos>stop>Gal4* driver was used to deplete *tkv* randomly in germline cells in adult flies.

(D) *yun* is efficiently depleted in $nos>tkv^{QD}; yun^{shmiR}$ germline cells. *yun* is successfully depleted in $nos>yun^{shmiR}$ germline cells (D1 and D2). Note that Yun is barely detectable in $nos>yun^{shmiR}$ germline cells (white arrowheads), but the levels of Yun in somatic cells are not affected (yellow arrowheads). *yun* is efficiently depleted in $nos>tkv^{QD}; yun^{shmiR}$ germline cells (D3 and D4, white arrowheads). The boxed panels in D3 and D4 are germaria taken at low magnifications, in which higher magnifications of germaria are showed in D3 and D4. Please refer to Figure S4A for comparison.

Scale bars: 5 μm and 10 μm (A and D2).

2. Supplemental Experimental Procedures

Fly Lines and Cultures

Flies were maintained on standard media at 25ºC. Crosses were raised at 25ºC in humidity controlled incubators, or as otherwise noted. Flies were transferred to new vials with fresh food every day, and dissected at time points specified in the text. Information for alleles and transgenes used in this study can be found either in FlyBase, or as noted: w^{1118}, *hsFlp, FRT82B-ubi-GFPnls* (for mosaic clonal analysis), *FRT82B*, *nosGal4*, *hsFlp; nos>stop>Gal4; UAS-GFP* (gift from Drs Ting Xie and Jianquan Ni) (Ma et al., 2014), *Bam-GFP* (gift from Drs Dahua Chen and Zhaohui Wang)(Chen and McKearin, 2003), yun^{shmiR}, $yun^{\Delta 66}$, $yun^{\Delta 9}$ (Zhao et al., 2022), *Dad-lacZ*, *Tkv-GFP*, Bam^{v20} (THU0567/HMS00029), $UAS\text{-}tkv^{Q253D}$ (tkv^{QD}), tkv^{RNAi} (THU4804), *UAS-YFP-Rab11* (BL9790)(gift from Jose Pastor-Pareja), $tubGal80^{ts}$, *tubGal4* (tub^{ts}), *esg-lacZ*, and *w* (white) RNAi (BL33623) was used as RNAi control.

Clonal Analysis

The marked mosaic GSC clones were generated using the Flp-mediated FRT recombination technique. The mosaic clones were induced by heat shocking the 3rd instar larva or the 2-3 day-old adult females for 37°C for 60 minutes for 2 days. Flies were maintained at 25°C and transferred to new vials with fresh food every day. The marked clones were assayed at indicated time points after clone induction. The genotypes used for clonal analysis were: (1) *hsFlp*/+; *FRT82B-ubi-GFPnls*/*FRT82B*; (2) *hsFlp*/+; *FRT82B-ubi-GFPnls*/*FRT82B-yun*^{Δ66}; (3) *hsFlp*/+; *FRT82B-ubi-GFPnls*/*FRT82B-yun*^{Δ9}; (4) *hsFlp*/+; *FRT82B-ubi-GFPnls*/*FRT82B-Dad-lacZ*; and (5) *hsFlp*/+; *FRT82B-ubi-GFPnls*/*FRT82B-yun*^{Δ66}, *Dad-lacZ*. No germline cells and egg chambers were observed in ovaries of *nosGal4>tkv*^{RNAi} flies. To deplete *yun* or *tkv* in adult GSCs, *hsFlp; nos>stop>Gal4;UAS-GFP* driver was used (Ma et al., 2014). 2-3 day-old adult females were heat shocked for 37°C for 75 minutes, flies were maintained at 25°C and transferred to new vials with fresh food every day. The flies were dissected at 3 or 7 days after clone induction.

RNAi Knockdown and Overexpression Experiments

To address gene function in GSCs, *nosGal4* was used. For RNAi knock down efficiency, *tubGal80*^{ts}*, tubGal4* (*tub*^{ts}) was used. 2-3 day-old progeny with the desired genotypes were collected after eclosion and maintained at 25°C or at 29°C to inactivate Gal80ts before dissection. As Tkv is essential for GSC maintenance, no GSCs and germline cells were observed when *tkv*^{RNAi} was driven by *nosGal4.* These *nos>tkv*^{RNAi} ovaries are very small and do not contain any germline cells. Thus *hsFlp, nos>stop>Gal4* driver was used to deplete *tkv* randomly in germline cells in adult flies. The flies were transferred to new vials with fresh food every day. Most YFP-Rab11 signals were diffused in the germline cells of *nosGal4>YFP-Rab11* flies.

Generation of Anti-Vasa Antibody

The rabbit anti-Vasa antisera were raised against a MBP fusion protein containing 1-280 aa of vasa with a MBP tag at its N terminus. Fusion proteins were purified according to the manufacturer's protocol and immunizations were performed by Animal Facility, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences.

Immunostainings and Fluorescence Microscopy

For standard immunostaining, ovaries were dissected in 1 X PBS (10 mM NaH_2PO_4/Na_2HPO_4, 175 mM NaCl, pH7.4), and fixed in 4% paraformaldehyde for 25 min at room temperature. Samples were rinsed, washed 3 times, 5 min each with 1 X PBT (0.1% Triton X-100 in 1 X PBS) and blocked in 3% BSA in 1 X PBT for 45 min. Primary antibodies were added to the samples and incubated at 4ºC overnight. The following primary antibodies were used: mouse mAb anti-α-Spectrin (3A9, 1:50, developed by D. Branton, and R. Dubreuil, Developmental

Studies Hybridoma Bank (DSHB)), mouse mAb anti-Orb (4H8, 1:50, developed by P. Schedl, DSHB), mouse mAb anti-Bam (Bag-of-marbles, 1:10, developed by D. McKearin, DSHB), mouse mAb anti-E-cadherin (5D3, 1:50, developed by B.M. Gumbiner, DSHB), mouse mAb anti-FasIII (7G10, 1:50, developed by C. S. Goodman, DSHB), rabbit anti-Vasa (d-260, Santa Cruz, 1:200, Cat No: sc-30210), rabbit anti-Vasa (1:2000, this study), rabbit mAb anti-pMAD3 (Epitomics, 1:200, Cat No: ab92698), rabbit and rat anti-Tkv (1:2000, generous gifts from Dr Cai Yu) (Luo et al., 2015), rabbit anti-Yun (1:1000) (Zhao et al., 2022), rabbit anti-active Caspase-3 (1:200, Abcam, Cat No: ab32042) and rabbit anti-β-glactosidase (1:5000, Cappel, Cat No: 55978). Secondary antibodies were incubated for 2 h at room temperature. DAPI (Sigma, 0.1 μg/mL) was added after secondary antibody staining. The samples were mounted in mounting medium (70% glycerol containing 2.5% DABCO). All images were captured by a Zeiss LSM780 inverted confocal microscope, and were processed in Adobe Photoshop and Illustrator.

Co-immunoprecipitation (co-IP) 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)) on ice for 30 minutes. After centrifugation, lysates were then diluted ten-fold with RIPA buffer and subjected to immunoprecipitation using anti-FLAG M2 affinity gel (Sigma-Aldrich, USA, Cat No: A2220). The immunocomplexes were collected by centrifugation and washed with 1 ml of RIPA buffer three times. For western blotting, immunoprecipitated proteins were separated in SDS-PAGE and then blotted onto PVDF membranes. The membranes were stained with primary antibody overnight at 4°C. Followed by washing, PVDF membranes were incubated with secondary antibodies conjugated with HRP, then the membranes were scanned using Luminescent Image Analyzer (GE, Sweden). Rabbit anti-Yun (1:1000) (Zhao et al., 2022), rabbit anti-Tkv (1:1000, generous gift from Dr Yu Cai)(Luo et al., 2015) and mouse anti-αTubulin (1:1000, Abbkine, Cat No: ABM40037) antibodies were used.

Data Analysis

The numbers of marked GSCs, GSCs per germarium and percentage of germarium with germline cells and GSCs were scored manually under Zeiss Imager Z2/LSM780 microscope for indicated genotypes. The fluorescence intensities of Bam, Bam-GFP, and pMAD were measured by ImageJ software for indicated genotypes. Two parameters, integrated fluorescence density and area, were used in the analysis. As *yun* is essential for GSC maintenance, the *yun*-depleted GSCs could not maintain their stem cell fate and underwent differentiation. Thus the anterior most *yun*-depleted germline cells inside the niche were regarded as GSCs for quantifications of fluorescence intensities of pMAD etc. At least four different images were analyzed for each sample. GraphPad Prism 8 software was used for statistical analyses and graph generation. Statistical analysis was done by the unpaired Student's *t*-test using GraphPad Prism 8 software. The graphs were further modified using Adobe Photoshop and Illustrator. ****$p < 0.0001$.

3. Supplemental References

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Ma, X., Wang, S., Do, T., Song, X., Inaba, M., Nishimoto, Y., Liu, L.-p., Gao, Y., Mao, Y., Li, H.*, et al.* (2014). Piwi Is Required in Multiple Cell Types to Control Germline Stem Cell Lineage Development in the *Drosophila* Ovary. PLOS ONE *9*, e90267.

Zhao, H., Shi, L., Li, Z., Kong, R., Ren, X., Ma, R., Jia, L., Ma, M., Lu, S., Xu, R.*, et al.* (2022). The Yun/Prohibitin complex regulates adult *Drosophila* intestinal stem cell proliferation through the transcription factor E2F1. Proc Natl Acad Sci USA *119*, e2111711119.