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

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SUMMARY

Germline stem cells (GSCs) are critical for the reproduction of an organism. The self-renewal and differentiation of GSCs must be tightly controlled to avoid uncontrolled stem cell proliferation or premature stem cell differentiation. However, how the self-renewal and differentiation of GSCs are properly controlled is not fully understood. Here, we find that the novel intrinsic factor Yun is required for female GSC maintenance in *Drosophila*. GSCs undergo precocious differentiation due to de-repression of differentiation factor Bam by defective BMP/Dpp signaling in the absence of *yun*. Mechanistically, Yun associates with and stabilizes Thickveins (Tkv), the type I receptor of Dpp/BMP signaling. Finally, ectopic expression of a constitutively active Tkv (Tkv^{QD}) completely suppresses GSC loss caused by *yun* depletion. Collectively, these data demonstrate that Yun functions through Tkv to maintain GSC fate. Our results provide new insight into the regulatory mechanisms of how stem cell maintenance is properly controlled.

INTRODUCTION

The self-renewal (maintenance) and differentiation of adult stem cells are critical for proper tissue homeostasis, so the balance of self-renewal and differentiation of adult stem cells must be tightly controlled. Disruption of this balanced control leads to either excessive stem cell proliferation or precocious stem cell depletion, eventually resulting in various diseases, including cancer and aging (Lin, 2008; Morrison and Spradling, 2008). Therefore, understanding of the underlying mechanisms controlling stem cell self-renewal and differentiation is important for the development of potential therapeutics for human diseases.

The adult *Drosophila* ovary is an excellent system to study the regulation of stem cell self-renewal and differentiation *in vivo* (Hsu et al., 2019; Ishibashi et al., 2020; Zhang and Cai, 2020). A structure, termed germarium, locates at the anterior end of a *Drosophila* ovariole, which contains the GSC niche, GSCs, and developing cysts (Figure 1A). Two or three GSCs are located in the niche at the anterior tip of the germarium, which is composed of several types of somatic stromal cells including terminal filaments (TFs), cap cells (CPCs), and escort cells (ECs). GSCs can be easily identified by their location and the intracellular organelle known as the spectrosome: they physically contact with cap cells and contain an anteriorly anchored spherical-shaped spectrosome (Figure 1A). GSCs normally undergo asymmetric self-renewing divisions, producing a self-renewed GSC daughter and a differentiating daughter, called a cystoblast (CB). CB is displaced from the niche (one cell diameter away from cap cells and containing a randomly localized spectrosome) to initiate differentiation. CB undergoes four rounds of synchronized divisions with incomplete cytokinesis, producing a 2- to 16-cell developing cyst. The developing cyst contains a branched organelle (fusome) that interconnects individual cystocytes (Lin et al., 1994). The niche utilizes several extrinsic signals, such as bone morphogenetic protein (BMP) (Dpp and Gbb), Upd cytokine, and Wnt, to maintain GSC self-renewal (Luo et al., 2015; Wang et al., 2008a; Xie and Spradling, 1998, 2000). The short-range BMP/Dpp signaling is the major niche signal, which functions in one cell diameter in the niche to maintain GSC self-renewal and repress the transcription of the differentiation factor Bam (Bag-of-marbles) (Chen and McKearin, 2003; Losick et al., 2011; Tabata and Takei, 2004; Tu et al., 2020). Upon binding of BMP/Dpp ligands to the hetero-dimeric receptor composed of type I receptor (like Tkv) and type II receptor (like Punt), Tkv is activated and results in the phosphorylation of MAD (Mothers against dpp, co-SMAD in *Drosophila*, pMAD), pMAD forms a complex with Med (Medea, R-SMAD in *Drosophila*), and they translocate in the nucleus of GSCs to regulate the expression of their target genes such as *Dad* (*Daughters against dpp*) (Araujo et al., 2011; Chen and McKearin, 2003; Decotto and Spradling, 2005; Losick et al., 2011; Tabata and Takei, 2004; Xie and Spradling, 1998, 2000). Additionally, niche-expressing E-cadherin is required to anchor GSCs in the niche for long-term maintenance (Song and Xie, 2002). Somatic and germline cells deploy several mechanisms acting in concert to spatially restrict BMP/Dpp signaling within the niche (Chen et al., 2010; Guo and Wang, 2009; Harris and Ashe, 2011; Hayashi et al., 2009; Liu et al., 2010; Losick et al., 2011; Luo et al., 2015; Ting, 2013; Xia et al., 2010, 2012). As BMP/Dpp signaling is inactivated in multiple ways in CBs, *bam* expression is de-repressed, which further drives germline cell

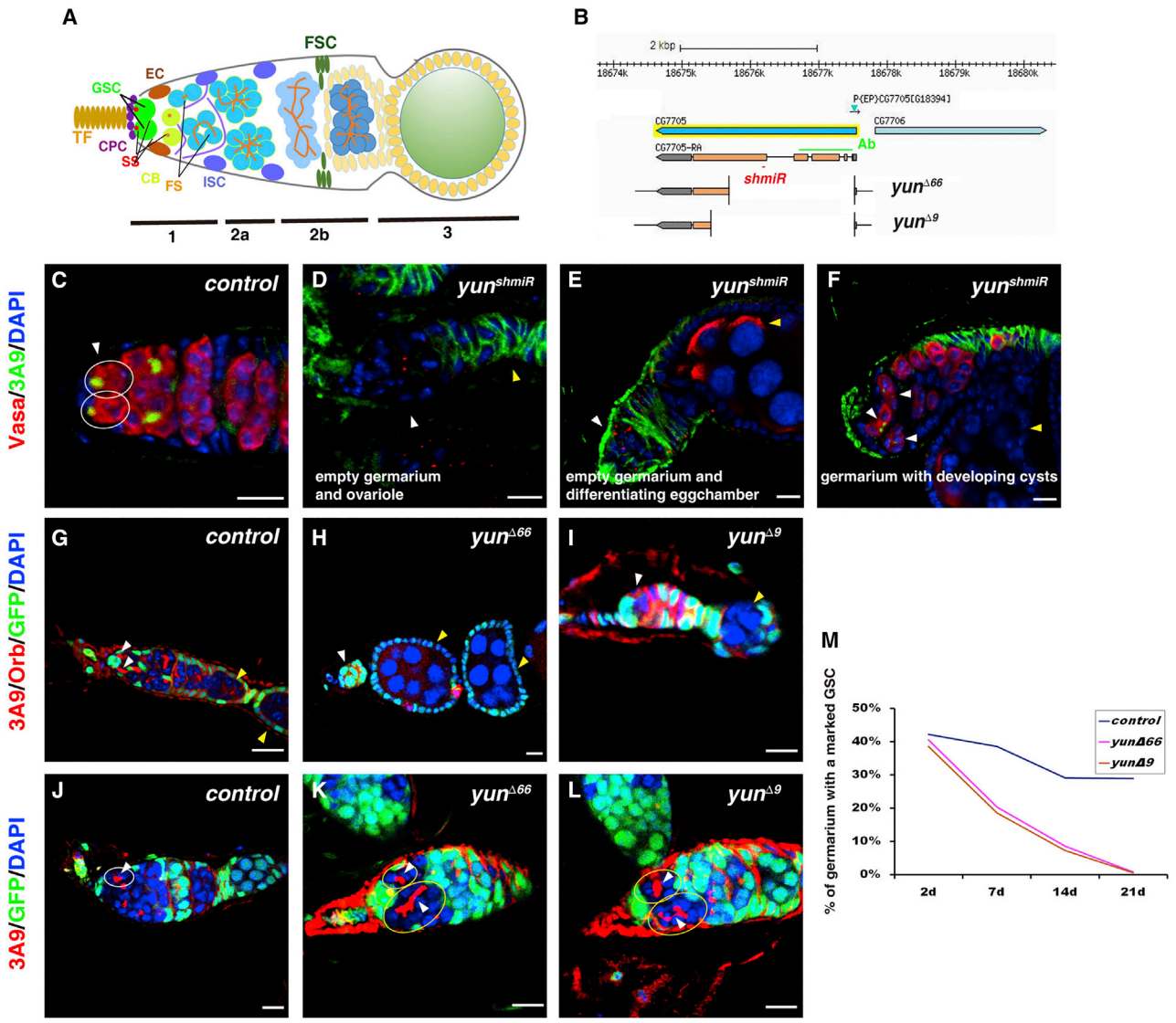


Figure 1. Yun is required for GSC maintenance

(A) Schematic cartoon of the germarium. TF: terminal filament, CPC: cap cell; GSC: germline stem cell; CB: cystoblast; EC: escort cell; SS: spectrosome; FS: fusome; ISC: inner sheath cell; FSC: follicle stem cell.

(B) The schematic cartoon of *yun* reagents used.

(C) Vasa (red) and 3A9 (green) in control germarium (white ovals and arrowhead).

(D–F) Germaria of *nos* > yun^{shmiR} (white and yellow arrowheads).

(G) 3A9 and Orb (red) in marked control clones induced from larva.

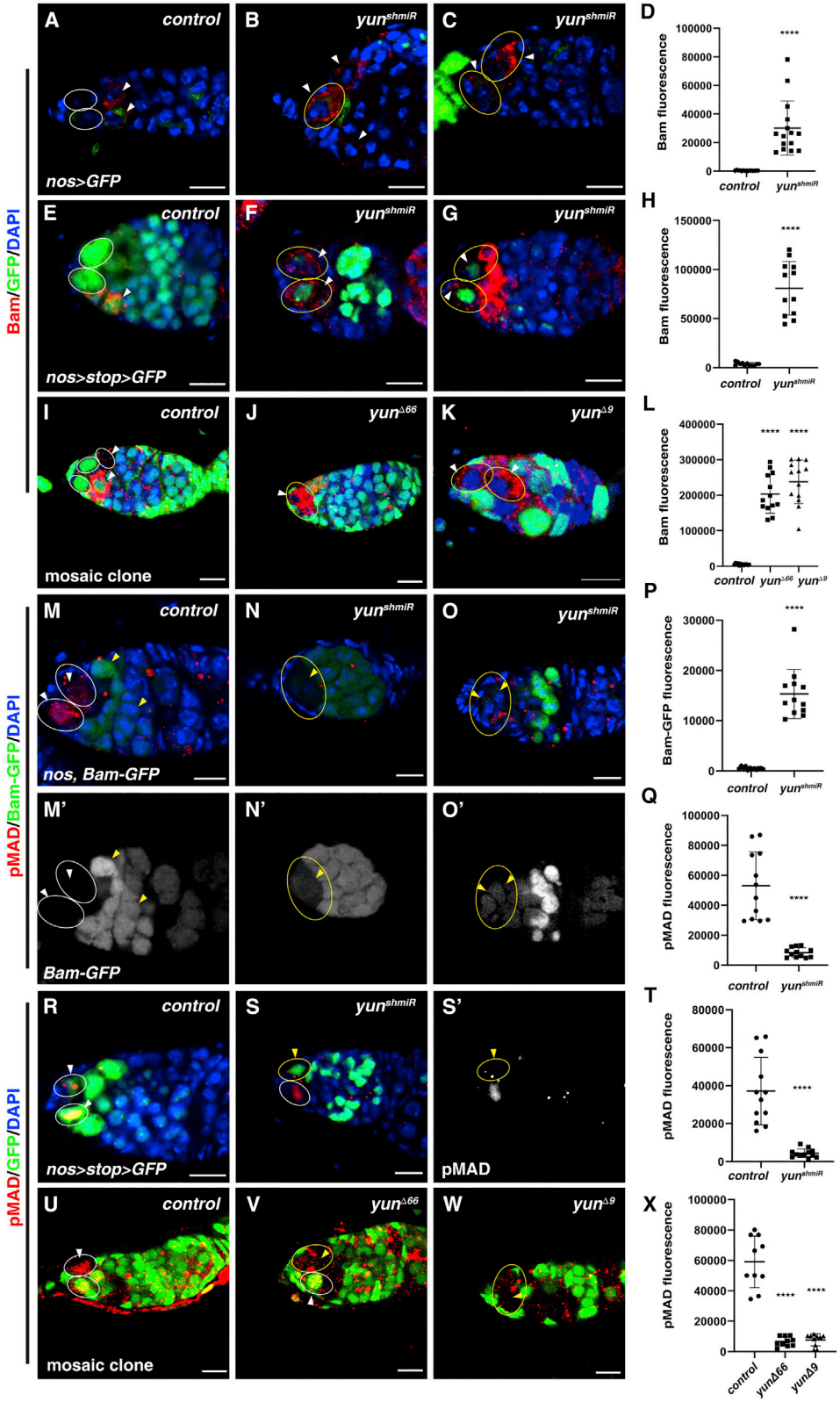
(H and I) 3A9 and Orb (red) in *yun* mutant mosaic germline clones induced from larva (white and yellow arrowheads).

(J) 3A9 in marked control clones induced in adult flies (white oval and arrowhead).

(K and L) 3A9 in *yun* mutant mosaic germline clones induced in adult flies (yellow ovals and white arrowheads).

(M) Percentages of the germaria carrying a marked WT or *yun* mutant GSC clone over time (2, 7, 14, and 21 days after ACI). Three replicates. Scale bars: 10 μm.

differentiation through multiple independent mechanisms (Casanueva and Ferguson, 2004; Chen and McKearin, 2003; Chen et al., 2011; Fu et al., 2015; Li et al., 2009, 2013; McKearin and Ohlstein, 1995; McKearin and Spradling, 1990; Ohlstein and McKearin, 1997; Pan et al., 2014; Shen et al., 2009; Song et al., 2004). However, how BMP/Dpp signal is precisely controlled in GSCs for GSC maintenance is not fully understood.



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RESULTS AND DISCUSSION

We identified a novel factor that we named Yun ("luck" in Chinese) in a large-scale RNAi screen for regulators of adult intestinal stem cell maintenance, proliferation, and differentiation (Liu et al., 2022; Ren et al., 2022; Zhao et al., 2022a, 2022b). *yun* (*CG7705*) encodes a novel protein of 592 aa without any known domain and motif (Figures 1B and S1A). During the course of our study, another group showed that it is implicated in cell proliferation in larval brain and spermatogenesis and named it *diamond* (*dind*) (Graziadio et al., 2018), but its mechanism in brain cell proliferation and spermatogenesis remained unexplored, and it is unclear whether Yun functions in GSCs regulation. As Yun could be detected in female germline cells, including GSCs, we asked whether Yun plays any role in GSC regulation (Figure S1B). Depleting *yun* in GSCs using a functional yun^{shmiR} by *nosGal4* in germline resulted in almost complete elimination of germline cells and GSCs, indicating that Yun may be required for GSC maintenance (Figures 1B, S1C, and S1D) (Zhao et al., 2022b). Detailed examinations showed that most of the *nos* > yun^{shmiR} germaria was devoid of germline cells compared with control germaria (Figures 1C–1E). And no GSCs were observed in those *nos* > yun^{shmiR} germaria containing a few germline cells, indicating that these germline cells are developing cysts (Figures 1F and S1E–S1I). GSC loss could be due to defective GSC self-renewal or apoptosis. However, no increased apoptosis was observed in *yun*-depleted germline cells, excluding the possibility that *yun*-defective GSCs are lost due to apoptosis (Figures S1J–S1L).

To further confirm that Yun intrinsically controls GSC maintenance, we performed mosaic clonal analysis of *yun* null mutants using Flp/FRT system (Figure 1B) (Zhao et al., 2022b). We first examined the mosaic clones that were induced at larval stages in adult animals. Control GSC clones underwent constant self-renewal divisions and produced developing egg chambers; although no GSCs were observed in *yun* mutant clones, many empty germaria were followed by differentiating egg chambers containing *yun* mutant germline cells, indicating that *yun*-defective GSCs could not be maintained and underwent differentiation (Figures 1G–1I and S1M–S1P). We then induced mosaic clones in adult flies. Marked control GSCs were constantly observed, while *yun* mutant GSCs could not maintain their stem cell fate and differentiated (Figures 1J–1L and S1Q–S1S). We further investigated GSC maintenance by examining the percentage of marked GSCs at different time points after clone induction (ACI). Compared with the marked control GSCs that still remained in the niche 2 and 3 weeks ACI, respectively, *yun* mutant GSCs were rapidly lost, and no marked *yun* mutant GSCs could be observed 3 weeks ACI (Figure 1M). Collectively, these data indicate that Yun is intrinsically required for GSC maintenance.

The differentiation factor Bam plays major role in GSC self-renewal and differentiation (McKearin and Spradling, 1990). We then examined the expression of Bam in the absence of *yun* using Bam antibody and a *bam-GFP* reporter, respectively (Chen and McKearin, 2003). Bam protein could not be detected in control GSCs, while Bam protein was detected within the *nos* > yun^{shmiR} niche, indicating that elevated Bam protein may cause the loss of *yun*-defective GSCs (Figures 2A–2D). Furthermore, Bam protein was detected within the niche of *nos* > *stop* > *Gal4*>yun^{shmiR} germaria, which bypassed the possible side effects of early-on

Figure 2. Yun represses GSC differentiation

(A) Bam (red) in control germarium (GSCs, white ovals).
(B and C) Bam (red) in *nos* > yun^{shmiR} germarium (GSCs, white ovals).
(D) Quantification of Bam fluorescence in control and *nos* > yun^{shmiR} GSCs. Mean ± SEM is shown. n = 15. ****p < 0.0001.
(E) Bam (red) in *nos* > *stop* > *GFP* germarium (GSCs, white ovals).
(F and G) Bam (red) in *nos* > *stop* > yun^{shmiR} germarium (yellow ovals and white arrowheads).
(H) Quantification of Bam fluorescence in control and *nos* > *stop* > yun^{shmiR} GSCs. Mean ± SEM is shown. n = 12. ****p < 0.0001.
(I) Bam (red) in marked control clones (oval) induced in adult flies.
(J and K) Bam (red) in marked *yun* mutant clones (yellow ovals).
(L) Quantification of Bam fluorescence in control and *yun* mutant GSCs. Mean ± SEM is shown. n ≥ 12. ****p < 0.0001.
(M) pMAD (red) and Bam-GFP (green) in control germarium (GSCs, white ovals). Bam-GFP channel is showed separately in black and white.
(N and O) pMAD (red) and Bam-GFP (green) in *nos* > yun^{shmiR} germarium (GSCs, yellow ovals).
(P) Quantification of Bam-GFP fluorescence in control and *nos* > yun^{shmiR} GSCs. Mean ± SEM is shown. n = 12. ****p < 0.0001.
(Q) Quantification of pMAD fluorescence in control and *nos* > yun^{shmiR} GSCs. Mean ± SEM is shown. n = 12. ****p < 0.0001.
(R) pMAD (red, white arrowheads) in marked *nos* > *stop* GSCs (white ovals).
(S) pMAD (red, arrowheads) in marked *nos* > *stop* > yun^{shmiR} GSCs (yellow oval). pMAD channel is shown separately.
(T) Quantification of pMAD fluorescence in control and *nos* > *stop* > yun^{shmiR} GSCs. Mean ± SEM is shown. n = 12. ****p < 0.0001.
(U) pMAD (red, white arrowhead) in marked control GSCs (white ovals).
(V and W) pMAD (red, white arrowheads) in marked *yun* mutant clones (yellow ovals).
(X) Quantification of pMAD fluorescence in control and *yun* mutant GSCs. Mean ± SEM is shown. n = 10. ****p < 0.0001. Scale bars: 10 μm.

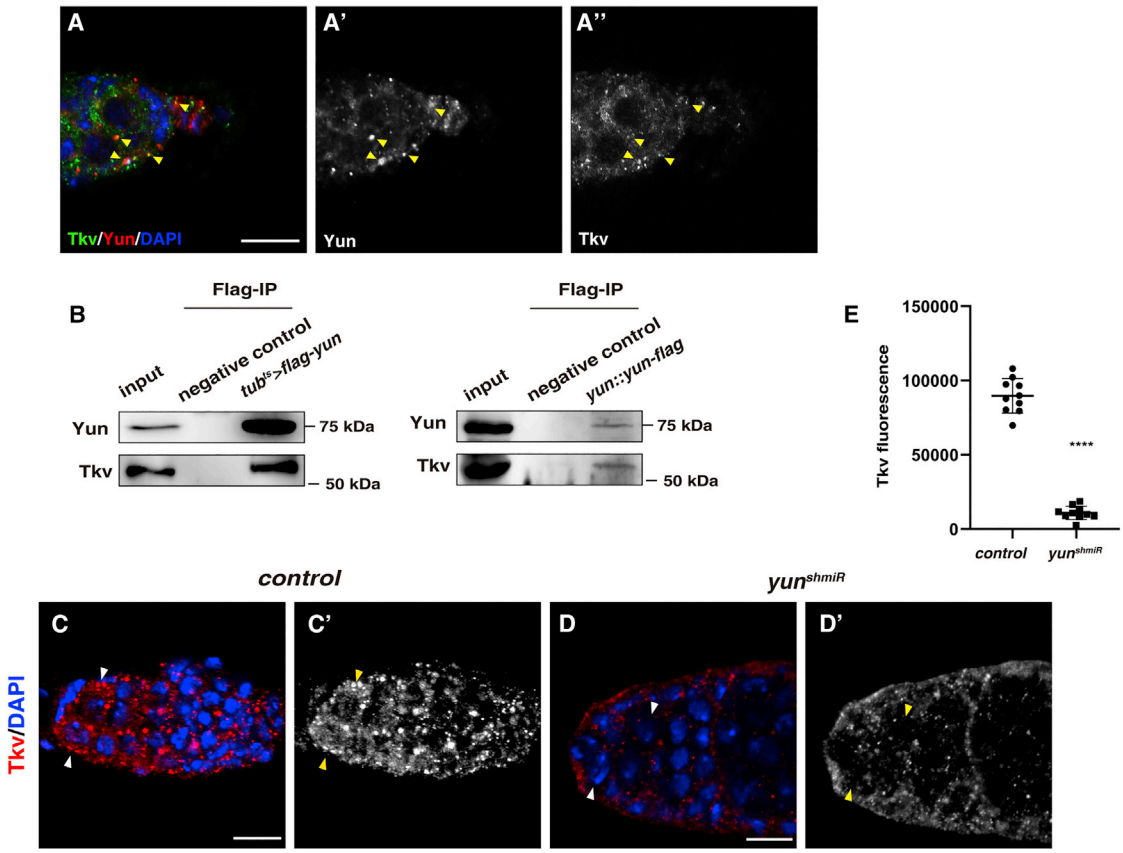

Figure 3. Yun interacts with and stabilizes Tkv

(A) Some endogenous Yun puncta (red) co-localize with endogenous Tkv (green) (yellow arrowheads). Yun and Tkv channels are shown separately in black and white.

(B) Endogenous Tkv associates with both overexpressed and endogenous Yun by coimmunoprecipitation.

(C) Tkv (red) in control GSCs (white arrowheads). Tkv channel is showed separately in black and white.

(D) The levels of Tkv (red) are dramatically reduced in *nos* > yun^{shmiR} germarium (white arrowheads).

(E) Quantification of Tkv fluorescence in control and *nos* > yun^{shmiR} GSCs. Mean ± SEM is shown. n = 10. ****p < 0.0001. Scale bars: 10 μm.

depletion of *yun* using *nosGal4* (Figures 2E-2H) (Ma et al., 2014). Moreover, Bam protein was detected in *yun* mutant GSCs within the niche (Figures 2I–2L). *bam-GFP* is repressed in control GSCs, while it was detected in putative GSCs in the *nos* > yun^{shmiR} niche (Figures 2M–2P and S2A–S2D). Altogether, these data show that upregulation of Bam in *yun*-defective GSCs is likely the cause of GSC loss. Consistently, simultaneous depletion of Bam in *nos* > yun^{shmiR} germaria completely rescued GSC loss observed in *nos* > yun^{shmiR} germaria, and *nos* > Bam^{RNAi}, yun^{shmiR} germaria were identical to *nos* > Bam^{RNAi} germaria (Figure S2E). Collectively, these data show that Yun is intrinsically required for GSC maintenance by repressing *Bam* expression.

Niche-derived BMP/Dpp signaling is necessary and sufficient for GSC self-renewal and *Bam* suppression (Casanueva and Ferguson, 2004; Chen and McKearin, 2003; Kai and Spradling, 2003; Song et al., 2004; Xie and Spradling, 1998, 2000). Next, we determined the activation of Dpp signaling in *yun*-defective GSCs. The levels of pMAD were strongly diminished in *nos* > yun^{shmiR} and *nos* > *stop* > yun^{shmiR} germaria compared with those in control GSCs (Figures 2M–2O, 2Q–2T, and S3A–S3C). The levels of pMAD were also dramatically reduced in marked *yun* mutant GSCs compared with those in control GSCs (Figures 2U–2X). The expression of *Dad-lacZ,* the downstream target of Dpp signaling, was almost undetectable in *nos* > yun^{shmiR} germline cells compared with that of control (Figures S3D–S3F). The expression of *Dad-lacZ* in *yun* mutant GSCs was also dramatically diminished (Figures S3G–S3H′). Previous study showed that adhesion of GSCs to cap cells by E-cadherin is important for GSC maintenance (Song and Xie, 2002), so we asked whether the levels of E-cadherin were affected upon Yun depletion.

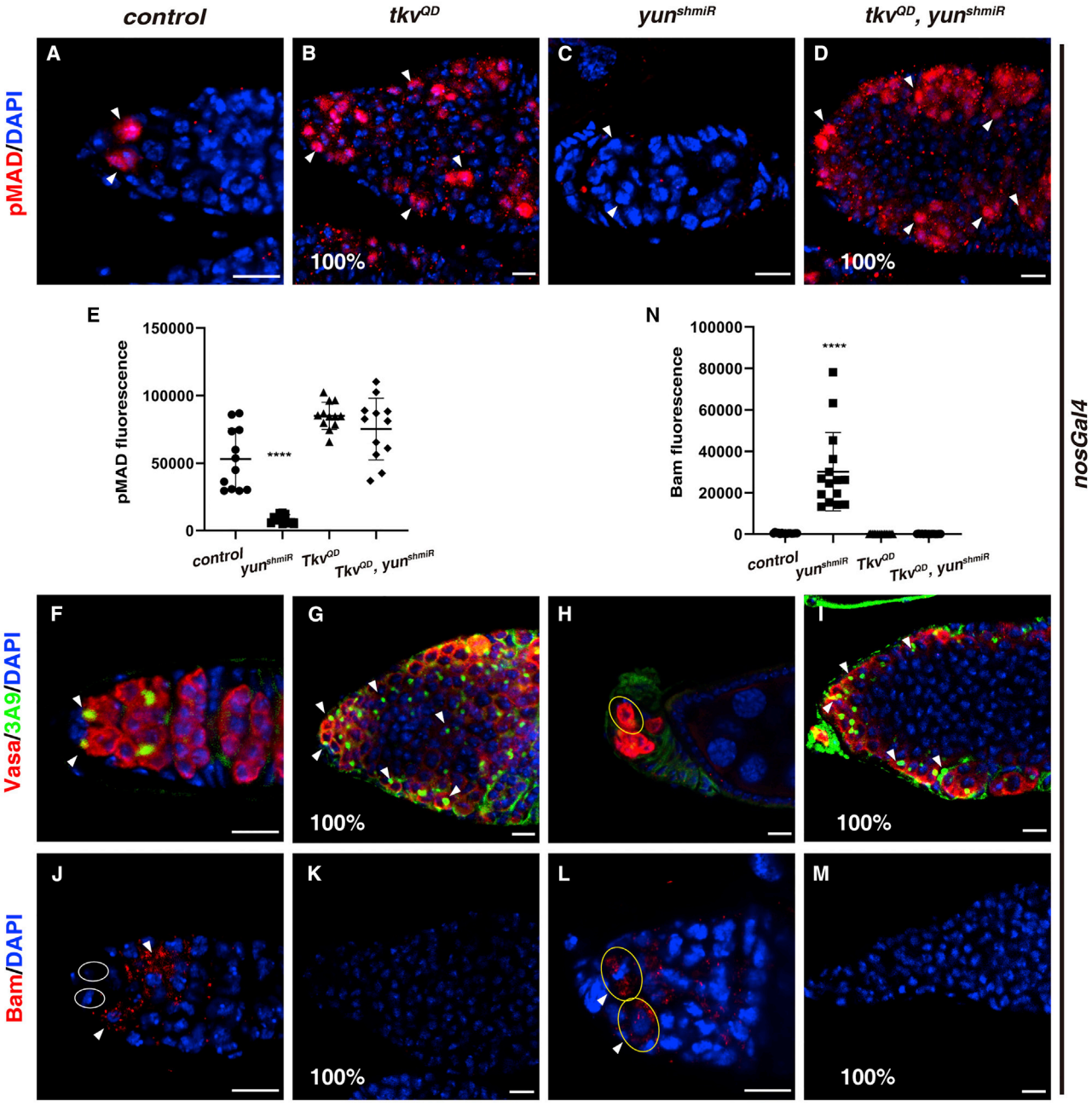

Figure 4. Yun functions through Tkv to maintain GSC

(A) GSCs (by pMAD in red, white arrowheads) in control germarium.

(B) *nos* > tkv^{QD} germaria contain numerous GSC-like cells (white arrowheads).

(C) No GSCs (by pMAD in red, white arrowheads) can be detected in *nos* > yun^{shmiR} germarium.

(D) *nos* > tkv^{QD}, yun^{shmiR} germaria contain numerous GSC-like cells (white arrowheads).

(E) Quantification of pMAD fluorescence in GSCs with indicated genotypes. Mean ± SEM is shown. n = 12. ****p < 0.0001.

(F) Vasa (red) and 3A9 (green) in control germarium (white arrowheads).

(G) Vasa (red) and 3A9 (green) in *nos* > tkv^{QD} germarium. All germaria contain numerous GSC-like cells (white arrowheads).

(H) Vasa (red) and 3A9 (green) in *nos* > yun^{shmiR} germarium that contains developing cysts (yellow oval).

(I) Vasa (red) and 3A9 (green) in *nos* > tkv^{QD}, yun^{shmiR} germarium. All germaria contain numerous GSC-like cells (white arrowheads).

(J) Bam (red, white arrowhead) in control germarium.

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The results showed that no reductions in the levels of E-cadherin were observed in the *nos > yun^{shmiR}* niche compared with those in the control, indicating that the loss of *yun-defective* GSCs is not caused by reduced adhesion of GSCs to cap cells (Figures S3I and S3J′). Altogether, these data show Yun is intrinsically required for Dpp signaling activation to suppress Bam expression for GSC maintenance.

How does Yun regulate GSC self-renewal through Dpp signaling? Since Dpp signaling is defective in the absence of Yun, we asked whether Yun functions through the key components of the Dpp signaling pathway. Interestingly, we found the subcellular localization of Yun in GSCs is similar to that of Tkv, and Yun often co-localizes with Tkv (Figure 3A and S4A). Similar phenomena were observed between endogenous Yun and GFP-tagged Tkv under its endogenous promoter in the germarium (Figure S4A). These data suggest that Yun may function through Tkv to maintain GSC fate. We found that Yun and Tkv could not be detected in the recycling endosomes at the same time, indicating that the receptor recycling pathway may not account for the GSC maintenance defects observed in the absence of *yun* (Figure S4B). Furthermore, the coimmunoprecipitation results showed that both overexpressed and endogenous Yun associate with endogenous Tkv (Figure 3B). We then examined whether Yun affects Tkv levels. Interestingly, the levels of Tkv in the niche of *nos > yun^{shmiR}* germaria were dramatically reduced compared with those in control GSCs, indicating that Yun affects Tkv protein levels (Figures 3C and 3D′) (Luo et al., 2015). We also examined whether Tkv affects the levels of Yun. However, the levels of Yun proteins were largely unaffected in the absence of *tkv*, indicating that Tkv is not likely to affect the levels of Yun proteins (Figure S4C). Collectively, these data indicate that Yun functions through Tkv to regulate Dpp signaling, thereby maintaining GSC fate.

To further confirm this conclusion, we carried out rescue experiments. Expression of a constitutively active Tkv (*tkv^{QD}*) resulted in strong Dpp signaling activation in the germ cells (Figures 4A and 4B). Compared with diminished Dpp signaling in the *nos > yun^{shmiR}* germaria, Dpp signaling was ectopically activated in all *nos > tkv^{QD}, yun^{shmiR}* ovarioles, identical to that of *nos > tkv^{QD}* ovarioles (Figures 4A–4E). The levels of Yun proteins were significantly eliminated in germline cells of *nos > tkv^{QD}, yun^{shmiR}* ovarioles (Figure S4D). These data support the notion that Yun functions through Tkv to regulate GSC self-renewal. Consistently, the germaria and developing follicles in *nos > tkv^{QD}, yun^{shmiR}* ovarioles were filled with GSCs and GSC-like cells, identical to those of *nos > tkv^{QD}* ovarioles (Figures 4F–4I). Moreover, precocious *bam* expression observed in *nos > yun^{shmiR}* GSCs was completely suppressed in *nos > tkv^{QD}, yun^{shmiR}* ovarioles, identical to *nos > tkv^{QD}* ovarioles (Figures 4J–4N). Altogether, these data show that Yun acts through Tkv to ensure Dpp signaling in GSCs, thereby maintaining GSC fate within the niche.

As the major niche-derived extrinsic signal, Dpp is spatially restricted within the niche to ensure GSC maintenance and proper GSC lineage development. To achieve such a tight spatial control of Dpp activity, multiple strategies are deployed (Chen et al., 2011). Extrinsically, cap cell-expressed glypican Dally limits Dpp diffusion to confine high Dpp concentrations within the niche (Guo and Wang, 2009; Hayashi et al., 2009; Liu et al., 2010). Viking, the type IV collagen, sequesters Dpp around GSCs to limit the functional range of Dpp (Wang et al., 2008b). Escort cell-expressed Tkv functions as a receptor sink/trap to remove excess diffusible Dpp to restrict Dpp activity locally within the niche (Luo et al., 2015; Xu et al., 2018). Intrinsically, different mechanisms were identified to promote Dpp signal activation in GSCs but rapidly dampen it in CBs (Chen et al., 2011). These mechanisms involved the post-translational regulation of Dpp signaling components in CBs: (1) the degradation of activated Tkv by the Fused-Smurf complex (Xia et al., 2010), (2) the translation repression of MAD by the Brat-Pumilo complex (Harris et al., 2011), and (3) the regulation of Sax levels by miR-184 (Iovino et al., 2009). It is also reported that Bam acts redundantly with Smurf to turn down Dpp signaling through an unknown mechanism (Casanueva and Ferguson, 2004). Inside GSCs, a variety of mechanisms are deployed to ensure proper Dpp signal activation for GSC self-renewal. Many intrinsic factors have been identified to regulate Dpp signaling. Petola and TSC1/2 (tuberous sclerosis complex $^{1}/_{2}$) are required to maintain Dpp signaling but are dispensable for *bam* repression (Sun et al., 2010; Xi et al., 2005). Lissencephaly-1 (Lis-1) directly binds to MAD to stabilize it and facilitate its phosphorylation, thereby regulating Dpp signaling (Chen et al., 2010). Here we provide evidence that Yun associates with and stabilizes Tkv to ensure Dpp signal activation for GSC maintenance. These data suggest that in GSCs the Dpp signaling pathway is regulated at multiple levels to ensure GSC maintenance.

(K) Bam (red) is barely detected in all *nos > tkv^{QD}* germaria.
(L) Bam (red, white arrowheads) is expressed in putative GSCs (yellow ovals) in the niche of *nos > yun^{shmiR}* germarium.
(M) Bam (red) is barely detected in all *nos > tkv^{QD}, yun^{shmiR}* germaria.
(N) Quantification of Bam fluorescence in GSCs with indicated genotypes. Mean ± SEM is shown. n = 15. ****p < 0.0001. Scale bars: 10 μm.

Dpp signaling also acts as a short-range signal to maintain self-renewal of male GSCs, another well-established system to stem cell biology (Kawase et al., 2004; Shivdasani and Ingham, 2003). We are interested to examine whether Yun is also required for male GSC maintenance. Interestingly, Yun is not expressed in the testis niche, and no obvious defects were observed upon *yun* depletion in male GSCs (not shown). These data suggest that although Dpp signaling is required in both niches, these two niches utilize different mechanisms to ensure GSC self-renewal.

EXPERIMENTAL PROCEDURES

Fly lines and cultures

Flies were maintained on standard media at 25°C. Crosses were raised at 18°C in humidity-controlled incubators or as noted. Information for alleles and transgenes used can be found either in FlyBase or as noted.

Immunostainings and fluorescence microscopy

The following primary antibodies were used: mouse mAb anti-α-Spectrin (3A9, 1:50, Developmental Studies Hybridoma Bank [DSHB]), mouse mAb anti-Orb (4H8, 1:50, DSHB), mouse mAb anti-Bam (Bag-of-marbles, 1:10, 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 (1:2,000, generous gifts from Dr Yu Cai) (Luo et al., 2015), rabbit anti-Yun (1:1,000) (Zhao et al., 2022a, 2022b), and rabbit anti-lacZ (1:5,000, Cappel, Cat No: 55,978). All images were captured by a Zeiss LSM780 confocal microscope and processed by Adobe Photoshop and Illustrator.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.07.014.

AUTHOR CONTRIBUTIONS

Z.H.L. conceived the study and designed the experiments. H.Z., Z.R.L., R.K., and L.S. performed experiments and data analysis. R.M. and X.R. assisted with experiments. Z.H.L. wrote the manuscript.

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CONFLICTS OF INTEREST

The authors declare no competing interests.

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Stem Cell Reports, Volume *17*

Supplemental Information

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

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Supplemental Information

1. Supplemental Figures

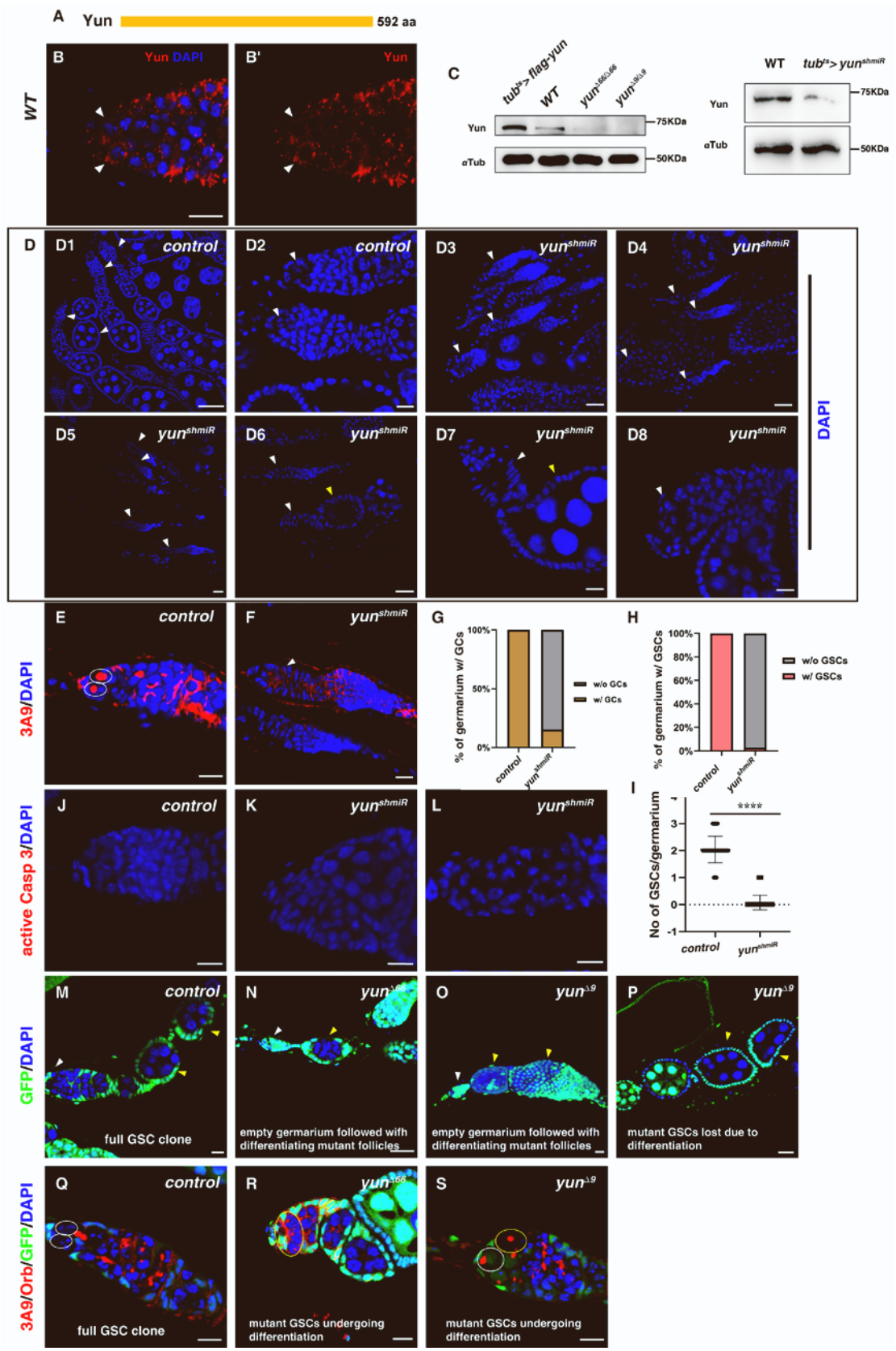

Figure S1. Yun is intrinsically required for female GSC maintenance, related to Figure 1

(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. αTubulin 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: $yun^{\Delta 66}$ and O: $yun^{\Delta 9}$) show empty germarium followed by differentiating follicles containing *yun* mutant germline cells (white and yellow arrowheads).

(P) $yun^{\Delta 9}$ 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: $yun^{\Delta 66}$ and S: $yun^{\Delta 9}$) (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).

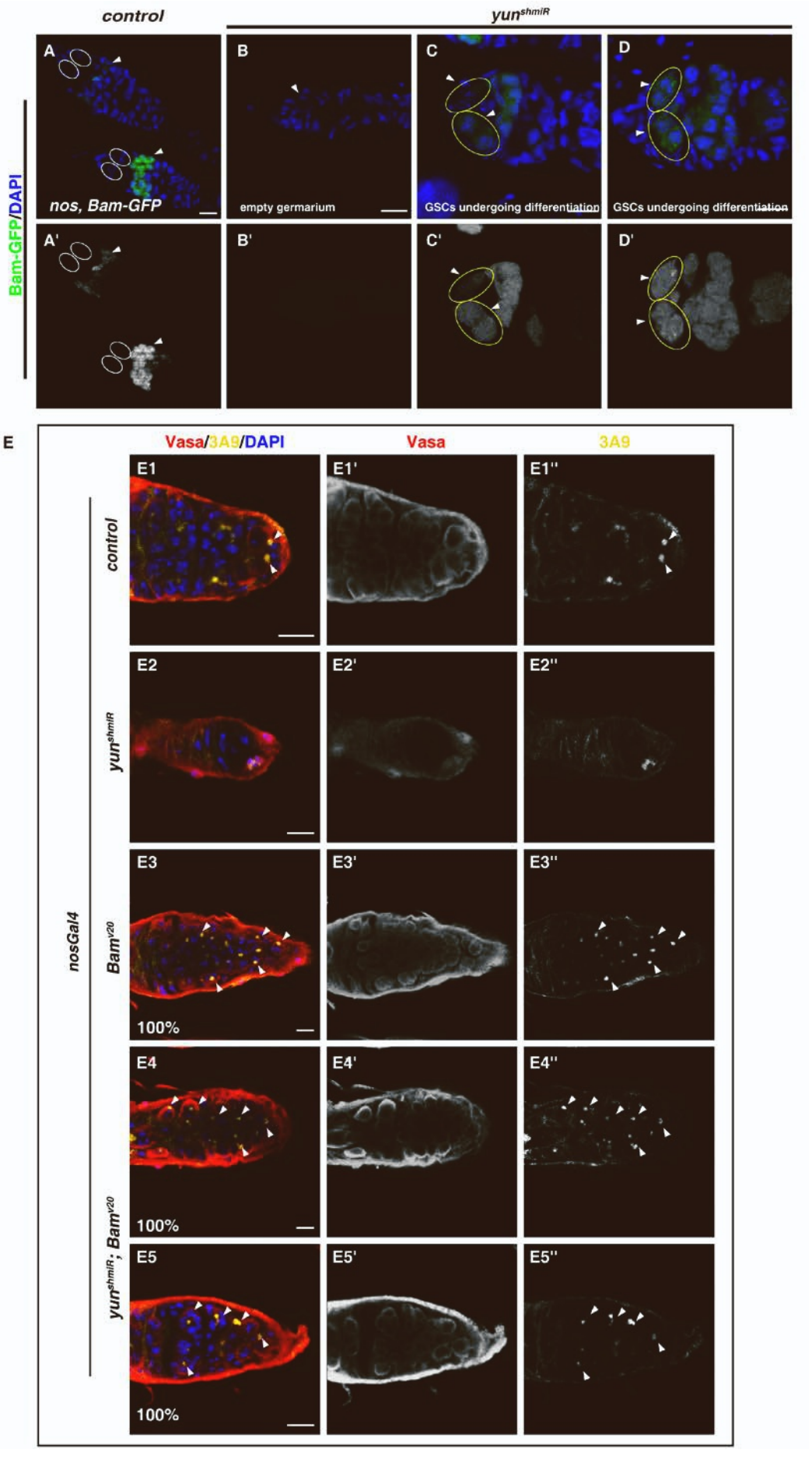
control
yunshmiR
A
B
C
D
Bam-GFP/DAPI
nos, Bam-GFP
empty germarium
GSCs undergoing differentiation
GSCs undergoing differentiation
A'
B'
C'
D'
E
Vasa/3A9/DAPI
Vasa
3A9
E1
E1'
E1''
control
E2
E2'
E2''
yunshmiR
E3
E3'
E3''
Bamv20
100%
E4
E4'
E4''
100%
nosGal4
yunshmiR; Bamv20
E5
E5'
E5''
100%

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.

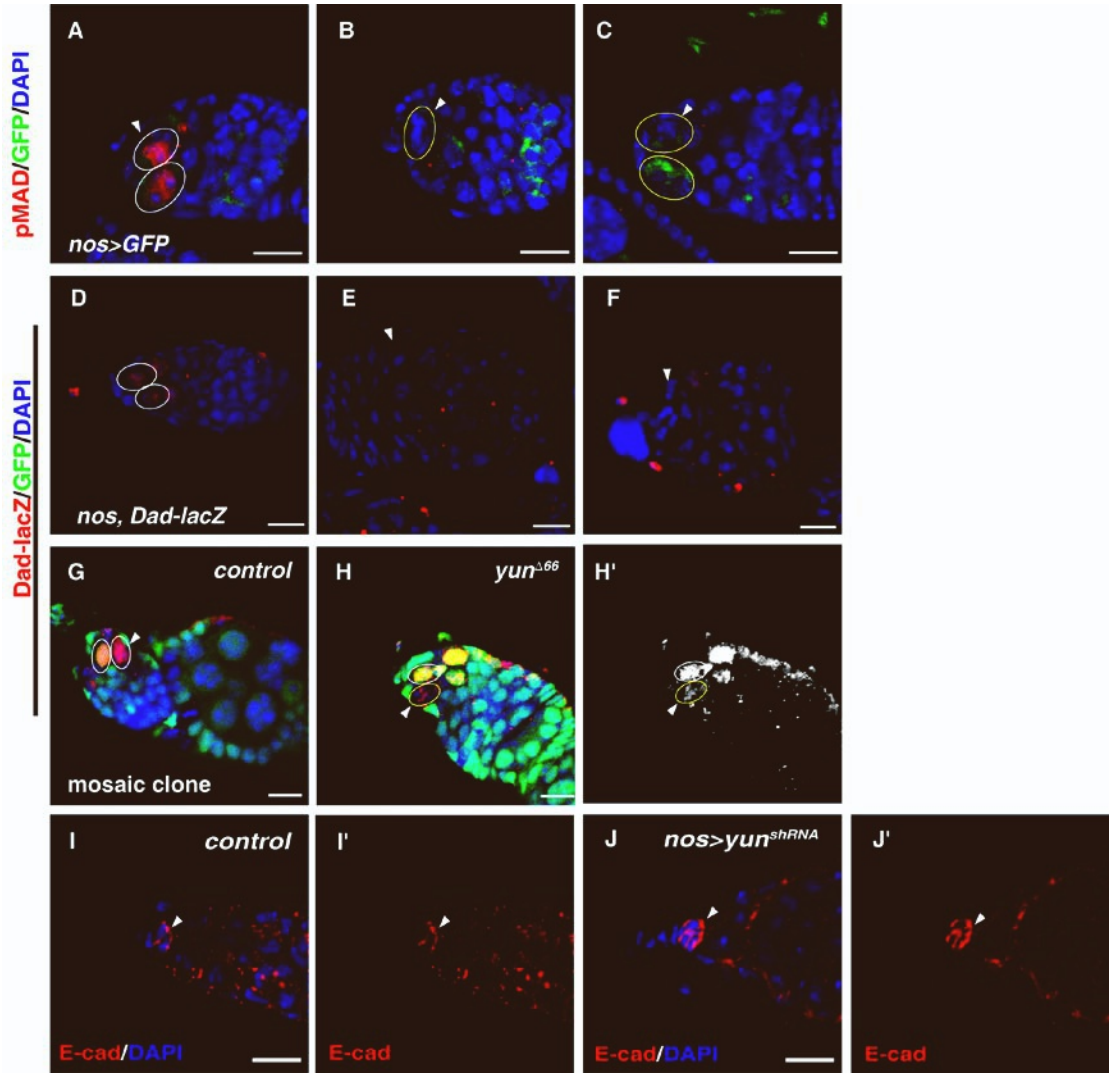

Figure S3. Dpp signaling is inactivated and E-cadherin is unaffected in the absence of

***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.

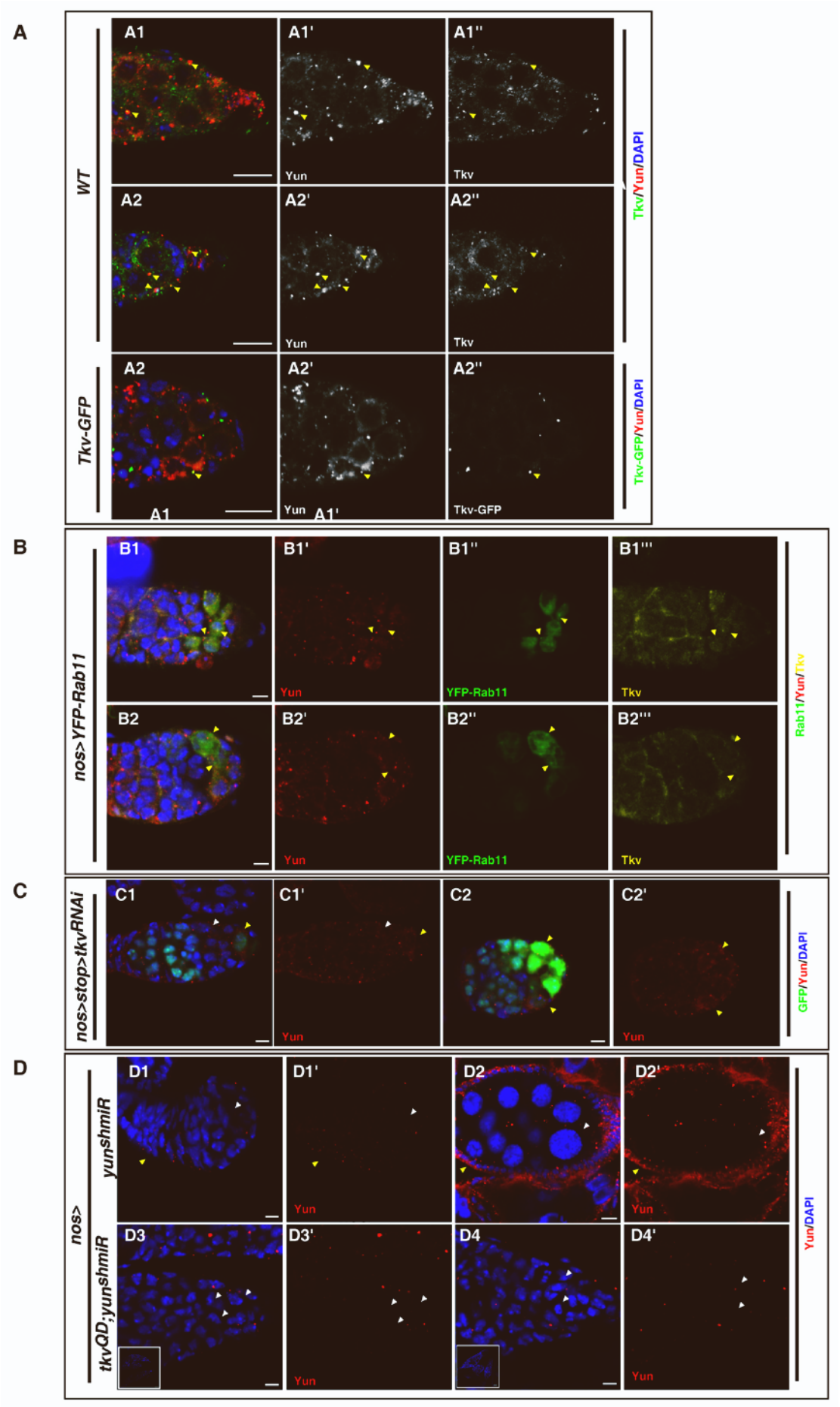
A
WT
A1
A1'
A1''
Yun
Tkv
Tkv/Yun/DAPI
A2
A2'
A2''
Yun
Tkv
Tkv-GFP
A2
A2'
A2''
Yun
Tkv-GFP
Tkv-GFP/Yun/DAPI
A1
A1'
B
nos>YFP-Rab11
B1
B1'
B1''
B1'''
Yun
YFP-Rab11
Tkv
Rab11/Yun/Tkv
B2
B2'
B2''
B2'''
Yun
YFP-Rab11
Tkv
C
nos>stop>tkvRNAi
C1
C1'
C2
C2'
Yun
GFP/Yun/DAPI
D
nos>
yunshmiR
D1
D1'
D2
D2'
Yun
tkvQD;yunshmiR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

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^{Δ66}*, *yun^{Δ9}* (Zhao et al., 2022), *Dad-lacZ*, *Tkv-GFP*, *Bam^{v20}* (THU0567/HMS00029), *UAS-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*$^{\Delta 66}$; (3) *hsFlp*/+; *FRT82B-ubi-GFPnls*/*FRT82B-yun*$^{\Delta 9}$; (4) *hsFlp*/+; *FRT82B-ubi-GFPnls*/*FRT82B-Dad-lacZ*; and (5) *hsFlp*/+; *FRT82B-ubi-GFPnls*/*FRT82B-yun*$^{\Delta 66}$, *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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