Multiple Niche Compartments Orchestrate Stepwise Germline Stem Cell Progeny Differentiation

Highlights

- scRNA-seq identifies four linearly arranged GSC niche compartments, IGS1–IGS4

- IGS1 and IGS2 control GSC maintenance and cyst formation, respectively

- IGS3 and IGS4 regulate meiosis, oocyte development, and shape change of 16-cell cysts

- A population of follicle cell progenitors is transcriptionally defined

In Brief

Tu et al. show that the niche forms linearly arranged compartments (IGS1–IGS4) for controlling multi-step GSC development. IGS1 controls GSC self-renewal, whereas IGS2–IGS4 sequentially regulate germline cyst formation, meiosis, timely oocyte determination, and cyst shape change. One follicle cell progenitor population is also transcriptionally defined.

Authors

Renjun Tu, Bo Duan, Xiaoqing Song, ..., Anoja Perera, Jeff Haug, Ting Xie

Tu et al., 2021, Current Biology 31, 827–839

February 22, 2021 © 2020 The Authors. Published by Elsevier Inc.

https://doi.org/10.1016/j.cub.2020.12.024
Multiple Niche Compartments Orchestrate Stepwise Germline Stem Cell Progeny Differentiation

Renjun Tu,1,3 Bo Duan,1,3 Xiaqing Song,1 Shiyuan Chen,1 Allison Scott,1 Kate Hall,1 Jillian Blanck,1 Dustin DeGraffenreid,1 Hua Li,1 Anoja Perera,1 Jeff Haug,1 and Ting Xie1,2,4,5,*

1Stowers Institute for Medical Research, 1000 East 50th Street, Kansas City, MO 64110, USA
2Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS 66160, USA
3Those authors contributed equally
4Twitter: @TingXieLab
5Lead Contact
Correspondence: tgx@stowers.org

SUMMARY

The niche controls stem cell self-renewal and progenitor differentiation for maintaining adult tissue homeostasis in various organisms. However, it remains unclear whether the niche is compartmentalized to control stem cell self-renewal and stepwise progeny differentiation. In the Drosophila ovary, inner germarial sheath (IGS) cells form a niche for controlling germline stem cell (GSC) progeny differentiation. In this study, we have identified four IGS subpopulations, which form linearly arranged niche compartments for controlling GSC maintenance and multi-step progeny differentiation. Single-cell analysis of the adult ovary has identified four IGS subpopulations (IGS1–IGS4), the identities and cellular locations of which have been further confirmed by fluorescent in situ hybridization. IGS1 and IGS2 physically interact with GSCs and mitotic cysts to control GSC maintenance and cyst formation, respectively, whereas IGS3 and IGS4 physically interact with 16-cell cysts to regulate meiosis, oocyte development, and cyst morphological change. Finally, one follicle cell progenitor population has also been transcriptionally defined for facilitating future studies on follicle stem cell regulation. Therefore, this study has structurally revealed that the niche is organized into multiple compartments for orchestrating stepwise adult stem cell development and has also provided useful resources and tools for further functional characterization of the niche in the future.

INTRODUCTION

Stem cells maintain adult tissue homeostasis through continuous self-renewal and generation of differentiated cells. Their self-renewal is shown to be controlled by the niche in the organisms ranging from Drosophila to mammals.1,3 Recently, stem cell progeny differentiation has also been proposed to be regulated by the niche in the Drosophila ovary.4 The differentiation process often consists of multiple developmental steps for generating one or several functional cell types. However, it remains largely unclear how the niche controls these differentiation steps at the cellular level.

The Drosophila ovary is an effective model for studying niche functions in regulating germline stem cell (GSC) self-renewal and differentiation.5,6 At the tip of the germarium, two or three GSCs contact cap cells anteriorly and inner germarial sheath cells (IGSs) (previously known as escort cells) laterally in region 1 (Figure 1A). Immediate GSC progeny, cystoblasts (CBs), divide four times synchronously with incomplete cytokinesis to form interconnected mitotic cysts (MCs) (2-cell, 4-cell, and 8-cell cysts) and 16-cell cysts.7 IGS cells wrap around CBs and MCs in region 1 as well as 16-cell cysts in region 2a (Figure 1A). Follicle cells begin to surround 16-cell cysts in region 2b and then form stage-1 egg chambers in region 3 (Figure 1A). Cap cells and anterior IGS cells form a niche for controlling GSC self-renewal through Dpp/BMP-mediated signaling and E-cadherin-mediated cell adhesion,8–12 whereas IGS cells form a niche for promoting differentiation partly by preventing bone morphogenetic protein (BMP) signaling.4,12 IGS cells utilize Hh, Wnt, epidermal growth factor receptor (EGFR), and Jak-Stat signaling to prevent BMP signaling in GSC progeny.11,13–21 Long IGS cellular processes are regulated by Hh and Rho-CDC42 small GTPase signaling, and cellular-process-mediated direct interactions are important for CB differentiation and cyst formation.1,14,22 Ecdysone signaling prevents IGS transformation into cap cells during development and is also needed in IGS cells for cyst formation, meiosis, and egg chamber formation.23–25 Therefore, two niches coordinately control GSC development in the Drosophila ovary.

While interacting with IGS cells, newly formed 16-cell cysts in region 2a undergo three important cellular events. First, those 16-cell cysts undergo two important meiotic events: chromosomal pairing and meiotic recombination.26,27 Second, both pro-oocytes form synaptonemal complexes initially, and only one of them becomes the oocyte. Third, 16-cell cysts change their round shape into a lens-like shape to ensure exactly one cyst is packaged into an egg chamber by follicle cells. Thus, it is tantalizing to speculate
that IGS cells function as a niche for regulating these three important germ cell developmental events. Consistently, ecdysone signaling functions in IGS cells to promote meiotic entry and egg chamber formation possibly by maintaining IGS identity. In this study, we use 10x genomics single-cell RNA-sequencing (scRNA-seq) to identify IGS subpopulations, which form linearly arranged niche compartments for orchestrating GSC self-renewal, CB differentiation, meiotic recombination, timely oocyte development, and cyst shape change. Therefore, we propose that the niche forms distinct subcompartments to control GSC maintenance and stepwise progeny differentiation in the Drosophila ovary.
RESULTS

scRNA-Seq Analysis Identifies IGS Subpopulations Interacting with GSCs and Early Progeny in the Adult Ovary

To investigate whether distinct IGS subpopulations physically interact with different developmental stages of GSC progeny, we screened the publicly available Gal4 lines by crossing them with UAS-GFP to identify three Gal4 lines with distinct expression patterns in IGS subpopulations, 31C09, 25A11, and 71E07 (Figure 1B). To visualize GSCs and their early progeny, we immunostained the ovaries for Hu-li tai shao (Hts) protein, which labels spherical spectrosomes in GSCs and CBs and branched fusions in mitotic and 16-cell cysts, as well as membrane cytoskeletons in follicle cells (Figure 1B). Three reporters, UAS-GFP, UAS-mGFP (membrane-tethered green fluorescent protein [GFP]), and UAS-nLacZ (nuclear LacZ) were used to independently confirm that 31C09-, 25A11-, and 71E07-expressing IGS cells cover region 1, regions 1 and 2a, and posterior region 2a, respectively (Figures 1B and S1A). In addition, 71E07 and 31C09 are also expressed in other somatic cells, including follicle cell progenitors (FCPs) and stalk cells (Figure S1A). 31C09, 25A11, and 71E07 express Gal4 under the control of the regulatory sequences of string, wnt4, and dally, respectively. These results support the existence of IGS subpopulations in the adult germarium.

To further define IGS sub-populations, we used 31C09-, 25A11-, and 71E07-driven GFP expression to purify IGS cells for 10x genomics scRNA-seq (Figure S1B). We used the machine-learning algorithm, 1-distributed stochastic neighbor embedding (tSNE), to identify 18 different populations among the 31C09-, 25A11-, and 71E07-expressing ovarian somatic cells (Figure 1C). Because of 71E07 and 31C09 expression in other ovarian somatic cells, we have used our previously identified IGS-specific marker CG7194, which is confirmed by mRNA fluorescent in situ hybridization (FISH), to take out CG7194-expressing clusters for further detailed analysis (Figures 1D and 1E). By unsupervised gene clustering, those CG7194-expressing clusters are composed of five subpopulations, IGS1–IGS5, suggesting the existence of IGS subpopulations (Figures 1F and 1G). Those purified 31C09-expressing IGS cells in region 1 are almost exclusively restricted to the IGS1 and IGS2 subpopulations, whereas those purified 25A11-expressing IGS cells in regions 1 and 2a are distributed to IGS1–IGS4 (Figures 1B and 1G). 71E07-expressing IGS cells in region 1 and somatic cells in regions 2b and 3 are allocated into IGS1, IGS2, and IGS5 subpopulations (Figures 1B and 1G). These results suggest that IGS1 and IGS2, IGS3 and IGS4, and IGS5 are located along the anterior-to-posterior germlar axis.

Our final scRNA-seq dataset has an average of ~5,000 unique molecular identifiers (UMIs) and ~1,500 genes per cell, and each cell type has variable amounts of mRNA content and gene expression (Figure 1H). The hierarchical clustering dendrogram and gene expression heatmap also show that IGS2–IGS4 are much closer to one another, whereas IGS1 and IGS5 are more distant (Figure 1I). By comparing gene expression profiles between different cell populations, IGS2 shows 510 upregulated genes and 427 downregulated genes compared with IGS1, whereas IGS3 exhibits 83 upregulated genes and 70 downregulated genes compared with IGS2; IGS4 shows 229 upregulated genes and 138 downregulated genes compared with IGS3, whereas IGS5 exhibits 827 upregulated genes and 504 downregulated genes compared with IGS4 (Figure 1J). Based on gene ontology (GO) term overrepresentation, IGS1 and IGS5 are also more distinct from the IGS2–IGS4 subpopulations, which are closer to one another (Figures S2A–S2E). The top two GO term categories for the differentially enriched genes in IGS2–IGS5 subpopulations are metabolic processes and biological regulation, which are likely important for IGS cells to support dynamic GSC development. Thus, our results suggest that multiple IGS subpopulations exist to perform distinct biological functions in the regulation of GSC development.

mRNA FISH Identifies Four IGS Subpopulations and One Follicle Cell Progenitor (FCP) Population

To further confirm the cellular locations of IGS subpopulations, we performed mRNA FISH experiments for the IGS subpopulation-enriched genes in adult ovaries. phantom (phm) mRNA, which encodes a cytochrome P450 involved in edcsyne biosynthesis, highlights follicle cells on the basis of its co-expression with known follicle cell marker Fas3 and its complete absence from CG7194-expressing IGS cells (Figures S3A–S3D). Netrin-A (NetA), which encodes a secreted Netrin protein, is expressed in IGS1 and IGS2 interacting with GSCs and CBs/MCs in region 1 (Figure 2A). Based on our observation that IGS1 is more distant from IGS2–IGS5 in gene expression profiles (Figure 1I), the GSC-contacting IGS subpopulation should be IGS1 because IGS2–IGS5 interact with early GSC progeny. Logically, IGS2 should interact with CBs and MCs. Given its overlapped expression with NetA, crocodile (croc) mRNA, which encodes a forkhead transcription factor needed in IGS cells for promoting CB differentiation, must be expressed in IGS1 and IGS2. In addition, croc should also be expressed in IGS3, which posteriorly contacts bangles and beads (bnb)- and Glutathione S transferase S1 (GstS1)–expressing IGS cells (Figures 2B and 2C). Bnb is a protein with unknown functions, whereas GstS1 is a glutathione transerase essential for Drosophila development. Supporting the idea, there is a regional gap between NetA-expressing and bnb-expressing IGS cells, which should be covered by IGS3 (Figure 2A). GstS1- and bnb-expressing IGS cells anteriorly contact the croc-expressing IGS cells, suggesting that GstS1 and bnb are expressed in IGS4 (Figures 2B–2E), santa-maria, which encodes a scavenger receptor important for vitamin A synthesis, is expressed in IGS5 given that its expressing region is anteriorly adjacent to GstS1- and bnb-expressing IGS4 (Figures 2B–2E), wunen2 (wun2), which encodes a lipid phosphatase needed for germ cell migration, is expressed in the IGS cells that posteriorly contact phm-expressing cells and anteriorly overlap with bnb-expressing cells, indicating that wun2 is expressed in IGS4 and IGS5 (Figure 2F), Helical factor (Hf), which encodes a secreted immune-regulated cytokine, shares a similar expression pattern in IGS1–IGS3 with croc (Figure 2G). Therefore, the five IGS subpopulations are successfully mapped onto different regions of the germarium with overlapped marker gene expression in adjacent IGS subpopulations (Figures 2H and 2I). It is worth noting that scRNA-seq and mRNA FISH results on NetA, croc, and wun2 are very consistent,
but the results on bnb and GstS1 do not quite match, suggesting that scRNA-seq results need validation by RNA FISH (Figure S4).

Previous studies have identified an FCP population located between IGS cells and Fas3-expressing follicle cells. Given its germarial location, the santa-maria+ IGS5 could be the FCP population (Figures 2D, 2E, and S5A). Because PZ1444 is a widely used IGS marker, the most posterior PZ1444+ IGS cells also express IGS4 marker bnb, indicating that IGS4 is the most posterior IGS subpopulation (Figure S5B). As predicted, the highly santa-maria-expressing IGS5 anteriorly contacts the most posterior PZ1444+ IGS cells anteriorly and Fas3+ follicle cells posteriorly (Figures S5B–S5D). On the basis of these results, IGS5 is indeed the same as the FCP population, which specifically expresses santa-maria (Figures 2D and 2E). Therefore, this study has identified four IGS subpopulations and the FCP (Figures 2H and 2I).

**Four IGS Subpopulations Dynamically Express dpr17**

On the basis of the scRNA-seq result, dpr17 is expressed in IGS4 at higher amounts than other IGS subpopulations (Figure 3A). Surprisingly, mRNA FISH results show that dpr17 exhibits four different expression patterns in IGS subpopulations in addition to terminal filament cells: (1) no expression in any IGS cells, (2) IGS1 and IGS2 cells, (3) IGS3 and IGS4 cells, and (4) IGS1–IGS4 cells (Figure 3B). We used Cas9/CRISPR to knock the yeast Gal4 gene into dpr17 to generate dpr17-Gal4 to confirm its dynamic IGS expression patterns. By using the UAS-nLacZ reporter, we show that dpr17-Gal4 gene into dpr17 to generate dpr17-Gal4 to confirm its dynamic IGS expression patterns. By using the UAS-nLacZ reporter, we show that dpr17-Gal4 and dpr17 mRNA are comparable (Figures 3C and 3D). dpr17 encodes an immunoglobin (lg)-domain-containing protein involved in synapse recognition and specificity determination by engaging with another Ig-domain-containing protein DIP-ε or
and FCP (Figure 4B). In addition, they are also lowly expressed in the Fas3+ follicle cells (Figure 4B). Further, we used the dnc-Neurotactin (Nrt), CG42458, and dnce (dnc) encode neuregulin-like EGFR ligand, transmembrane adhesion protein, homeobox transcription factor, and cyclic adenosine monophosphate (cAMP)-specific phosphodies- terase, respectively, which are known to regulate neuronal development and functions.43–47 These observations suggest that IGS cells use various pathways to control their interaction with GSCs and their progeny.

IGS-Enriched Genes, bin and vn, Are Needed Extrinsically for Controlling CB Differentiation

Then, we used c587-Gal4 and tub-Gal80ts (c587-Gal80ts) to knock down the IGS-enriched genes, bin and vn, to delineate their functions in adult IGS cells for regulating GSC progeny differentiation. c587-Gal4 is highly expressed in all the IGS cells,48 tub-Gal80ts ubiquitously expresses a temperature-sensitive mutant GAL80 protein in all cells, including IGS cells; the mutant GAL80 protein is functional at 18°C to repress c587-driven UAS-shRNA expression in IGS cells, but 29°C inactivates its function to allow short hairpin RNA (shRNA) expression in IGS cells.49 When the newly eclosed adult c587ts, UAS-shRNA females at 18°C are shifted to 29°C, the expression of a specific shRNA in adult IGS cells can be achieved as we have done previously.34,50 PZ1444 is ex- pressed in both IGS cells and cap cells, which can be easily distinguished based on morphology and location.41 Spectro-some-containing GSCs and CBs can be distinguished from each other based on the fact that cap cells physically contact GSCs, but not CBs,28 IGS-specific bin knockdown (binKD) for 7 days does not affect GSC maintenance because the binKD germaria and the luciferase knockdown (lucKD) control germaria (firefly luc, a non-Drosophila gene, is used as the control for non-specific RNAi effect) have normal 2 or 3 GSCs (Figures 5A, 5B, 5C to repress c587-Gal4-driven UAS-shRNA expression in IGS cells (Figures 5A and 5B). These results demonstrate that our previous studies have shown that a severe IGS cell loss also causes the germ cell differentiation defect.4,12,51 we then examined and quantified PZ1444+ IGS cells in the control and binKD germaria. In contrast with the control germaria carrying 30–35 PZ1444+ IGS cells, most of the binKD germaria completely lose their IGS cells (Figures 5A and 5B). These results demonstrate that homeobox transcription factor Bin promotes IGS maintenance and consequently GSC progeny differentiation. Given that the IGS loss disrupts GSC progeny differentiation by elevating BMP signaling,4,12,51 we then used two BMP signaling reporters, Dad-lacZ and bam-GFP, to verify BMP
signaling in the control and binKD germaria. In the control germaria, Dad-lacZ is expressed in GSCs, whereas bam-GFP expression is repressed in GSCs and upregulated in differentiated GSC progeny (Figures 5C and 5D). By contrast, the binKD germaria show Dad-lacZ upregulation and bam-GFP repression in the accumulated SGCs located a few cells away from cap cells (Figures 5C and 5D). These results further confirm that IGS cells are needed for preventing BMP signaling in GSC progeny.

EGFR signaling is needed in IGS cells for their maintenance and for promoting GSC progeny differentiation, whereas Vn is a neuregulin-like ligand activating EGFR in Drosophila. Consistently, 28 days after knockdown in IGS cells, the vn knockdown (vnKD) germaria gradually lose most of IGS cells, still leaving a few IGS cells in the anterior germarial region (Figures 5E, 5F, S6C, and S6D). The vnKD germaria show slightly reduced GSCs, increased SGCs, and
excess 16-cell cysts (Figures 5E and 5F). Given that EGFR signaling has previously been shown to be important for FSC regulation,52 the accumulated 16-cell cysts are likely caused by facultative follicle cell development due to the loss of IGS-expressed Vn in the regulation of FSCs and FCPs. Given that EGFR signaling in IGS cells is known to be activated by ligands in underneath germ cells,53 this study suggests that IGS-expressed Vn can also contribute to EGFR signaling for promoting IGS maintenance and GSC progeny differentiation.

Anterior IGS-Expressed Genes, NetA and Hf, Are Needed Extrinsically for Maintaining GSCs

To further validate NetA protein expression in the gerarium, we analyzed the Drosophila strain carrying a recombination-mediated cassette exchange of a Mi[MIC] insertion, which results in the expression of NetA protein tagged with EGFP-FlAsH-StrepII-TEV-3xFlag. Consistently, NetA-GFP is expressed in IGS1 and IGS2, but at lower amounts in IGS2 (Figure 6A). NetA-GFP is absent in germ cells, which are marked by Vasa expression (Figure 6A). c587ts-driven expression of two UAS-shRNA lines against NetA can knock down NetA mRNA expression effectively and specifically in adult IGS cells (Figures S6E and S6F). Compared with the control gerarium, the NetAKD gerarium carry significantly fewer GSCs and consequently fewer SGCs because SGCs are produced by GSCs (Figures 6B and 6C). In addition, the NetAKD gerarium have the normal number of IGS cells compared with the control, indicating that NetA is dispensable for IGS maintenance (Figures 6D and 6E). These results indicate that NetA is needed in IGS1 and possibly IGS2 for maintaining GSCs. Because NetA is a secreted signaling molecule, it remains to be determined whether NetA maintains GSCs by direct signaling in the future.

Although Hf functions like a cytokine to induce anti-bacteria immune response in S2 cells, the in vivo biological function remains unclear.39 c587ts-driven expression of a UAS-Hf shRNA can knock down Hf expression effectively in adult IGS cells (Figures S6G and S6H). Compared with the control gerarium, the HfKD gerarium show significantly fewer GSCs and SGCs (Figures 6F and 6G). Interestingly, IGS cells are also significantly reduced in the knockdown gerarium, indicating that Hf also maintains IGS cells. Along with the NetA findings, these results have further supported the idea that IGS1 is also a part of the niche for maintaining GSCs.
Posterior IGS-Enriched Genes, \textit{wun}2, and \textit{GstS}1, Are Needed for Meiotic Recombination, Timely Oocyte Specification, and Cyst Shape Change

Early 16-cell cysts in region 2a produce double-stranded DNA breaks (DSBs) for meiotic recombination, which can be identified by H2AvD, a phosphorylated form of histone variant H2A; these DSBs are successfully repaired in 16-cell cysts in regions 2b and 3 (Figure 7A).\(^{54}\) Then, we used these DSBs to knock down their expression in IGS4 cells efficiently to investigate whether they regulate early meiotic entry (Figures S6I–S6L).\(^{54}\) Interestingly, \textit{wun}2 or \textit{GstS}1 knockdown in IGS cells decreases H2AvD expression in early 16-cell cysts compared with the control, indicating that \textit{wun}2 and \textit{GstS}1 are needed in IGS4 cells for meiotic recombination in 16-cell cysts (Figures 7A and 7B).\(^{54}\) In addition, IGS-specific \textit{wun}2 or \textit{GstS}1 knockdown also results in the accumulation of round-shaped 16-cell cysts moving pairwisely to region 2b, where normally only one lens-shaped 16-cell cyst waits to be covered by follicle cells, suggesting that \textit{wun}2 and \textit{GstS}1 are needed in IGS4 cells for promoting cyst shape changes (Figures 7C and 7D).\(^{54}\) However, because of \textit{wun}2 expression in FCPs, we could not rule out the possibility that \textit{wun}2 also regulates FCPs to change cyst shape. These results suggest that \textit{wun}2 and \textit{GstS}1 are needed in IGS4 and possibly IGS3 (due to their low expression in IGS3) for regulating meiosis and cyst shape changes.

Bin and Smoothened (Smo) Are Required for Posterior IGS4 for Meiosis and Oocyte Specification

A recent study has identified \textit{H2126-switchGal4} as a posterior IGS-specific \textit{Gal4}.\(^{57}\) \textit{switchGal4} is an inducible \textit{Gal4} system, in which the yeast \textit{Gal4} gene is fused with human progesterone receptor (GR) to produce a \textit{Gal4}-GR fusion protein capable of binding to the UAS promoter upon progesterone analog RU486 administration.\(^{58}\) We first used \textit{UAS-nLacZ} to confirm that \textit{H2126-switchGal4} is expressed in posterior IGS cells and at lower amounts in FCPs upon RU486 administration (Figure S7A).\(^{58}\) Then, we applied mRNA FISH in combination with Fas3 antibody immunostaining to show that \textit{H2126-switchGal4} is expressed in IGS4 cells and also a few IGS3 cells (Figures S7B and S7C). Bin is described earlier shown to, whereas Smoothened (Smo), a Hh receptor, is previously shown to maintain IGS cells and promote CB

| Figure 6. Anterior IGS-Enriched NetA and Hf Are Required for Maintaining GSCs |
|---|
| Cap cells and GSCs are highlighted by broken ovals. (A) Confocal images showing high expression of NetA-GFP in IGS1 and its weak expression in IGS2. Scale bars, 10 μm. (B and C) c587ts+-driven NetA knockdown decreases GSCs and SGCs (B). Shown in (C) are quantification results; n = number of germaria). Student’s t test: ***p < 0.001. (D and E) c587ts+-driven NetA knockdown does not significantly affect IGS cells (D). Shown in (E) are quantification results; n = number of germaria). Scale bars, 10 μm. Student’s t test: ***p < 0.001, **p < 0.01. Scale bars, 10 μm. See also Figure S6. |

| Figure 7. Stages of Meiotic Recombination and Oocyte Specification |
|---|
| (A) Confocal images showing high expression of NetA-GFP in IGS1 and its weak expression in IGS2. Scale bars, 10 μm. (B and C) c587ts+-driven NetA knockdown decreases GSCs and SGCs (B). Shown in (C) are quantification results; n = number of germaria). Scale bars, 10 μm. Student’s t test: ***p < 0.001, **p < 0.01, n.s., no significance. (D and E) c587ts+-driven Hf knockdown decreases GSCs, SGCs, and IGS cells (F). Shown in (G) are quantification results; n = number of germaria). Scale bars, 10 μm. Student’s t test: ***p < 0.001, **p < 0.01, n.s., no significance. |

| Table 1. Quantification of IGS1 and IGS2 Cells |
|---|
| | NetA-GFP/Fas3 | NetA-GFP/Fas3 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
| | | IGS1 | IGS2 |
| | IGS1 | IGS2 |
To further define the function of IGS3 and IGS4 in the regulation of meiosis and oocyte formation, we used H2126-switchGal4 and previously validated UAS-shRNA lines to knock down bin and smo in adult IGS3 and IGS4 cells for 4 days, which did not cause severe IGS loss on the basis of the morphologies of the knockdown germaria (Figures 7 G–7J). Consistently, knocking down bin or smo in adult IGS3 and IGS4 also decreases H2AvD expression in early 16-cell cysts in region 2a, and increases the presence of two oocytes in the 16-cell cysts of stage 1 egg chambers (Figures 7G–7J). Therefore, these results further confirm that IGS3 and IGS4 control meiotic recombination and timely oocyte determination in 16-cell cysts.
DISCUSSION

Although IGS cells and their cellular processes form a niche for controlling GSC progeny differentiation, including CB differentiation, cyst formation, and the meiotic entry, it remains unclear whether IGS cells form distinct niche compartments that control different differentiation steps. In this study, we used scRNA-seq to identify four IGS subpopulations, IGS1–IGS4, which are organized linearly along the gerarium to interact with GSCs and their early progeny. We have further used genetic manipulations to show that IGS1–IGS4 subpopulations form functionally separate compartments for controlling GSC maintenance, CB differentiation, meiotic recombination, timely oocyte specification, and cyst shape change. In addition, we have molecularly defined the FCP population, which remain poorly studied because of the lack of suitable molecular markers. Therefore, this study has identified four IGS subpopulations that form subsequential niche compartments for controlling different steps of GSC progeny differentiation and has also molecularly defined the poorly studied FCP population, which opens the door for in-depth studies of IGS and FCP populations in the future.

Four IGS Subpopulations Form Linearly Arranged Niche Compartments

Two recent scRNA-seq studies on the whole Drosophila ovary have identified different somatic cell types important for oogenesis. However, those two studies failed to identify the IGS subpopulations because of high complexities of somatic cell types and similarities of IGS cells. In this study, we have used GFP-based cell sorting and scRNA-seq to successfully identify four IGS subpopulations, IGS1–IGS4, and have further used gene-specific Gal4 lines and mRNA FISH to show their linear arrangement in the anterior gerarium. IGS1 and IGS2 reside in region 1 to interact with GSCs and CBs/MCs, whereas IGS3 and IGS4 are located in region 2a to contact 16-cell cysts. IGS4 is the most posterior population directly contacting FSCs and has been identified by uncovering new markers and functions and has also provided important insight into GSC maintenance because of the lack of IGS1-specific Gal4 lines. Although we show that IGS cells are needed for CB differentiation and IGS2 directly contacts CBs/MCs, we could not directly demonstrate that IGS2 directly controls the differentiation of CBs and MCs into 16-cell cysts because of the lack of IGS2-specific Gal4 lines.

This study also shows that IGS3 and IGS4 control meiosis, oocyte specification, and cyst shape. IGS3 and IGS4 in region 2a extend their long cellular processes to wrap around newly formed H2AvD+ pre-meiotic and meiotic 16-cell cysts. These 16-cell cysts still have two pro-oocytes, but they only retain one oocyte and also undergo the round-to-lens shape change when surrounded by follicle cells in region 2b. Knocking down IGS4-expressing wun2 and GstS1 decreases the H2AvD+ 16-cell cysts in region 2a and increases the presence of round 16-cell cysts in region 2b, which fails to become lens-shaped, suggesting that IGS4 cells regulate meiotic recombination and cyst shape change. In addition, IGS-specific knockdown of GstS1, but not wun2, causes the presence of 2 pro-oocytes in stage 1 egg chambers. Consistently, H2126-mediated bin and smo knockdown in IGS4 can also decrease the H2AvD+ 16-cell cysts and increase the frequency of stage 1 egg chambers with two pro-oocytes, further supporting that IGS4 regulates meiosis and oocyte specification. However, we could not completely rule out the possibility that IGS3 cells might also regulate meiotic recombination and timely oocyte specification because of the lack of IGS3-specific Gal4 lines given that IGS3 expresses low amounts of wun2 and GstS1 and some of them also express H2126. In addition to one previous study showing that Edcsyone signaling in IGS cells regulates meiotic entry, this study has, for the first time, shown that IGS cells regulate meiotic recombination in 16-cell cysts. Given that the oocyte was previously thought to be determined entirely by the intrinsic mechanism, the differential RNA and protein transport caused by asymmetrically localized microtubules and fusions is also the first time to show that IGS cells influence timely oocyte determination in Drosophila. Therefore, this study has provided
important insight into IGS subpopulations in the regulation of GSC maintenance, cyst formation, meiotic recombination, timely oocyte determination, and cyst shape change, but the underlying signaling mechanisms await future investigation through generation of new genetic tools (Figure 7K).

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead Contact
  - Materials Availability
  - Data Availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - *Drosophila* culture
- **METHOD DETAILS**
  - Generation of Gal4 lines using Cas9/CRISPR
  - Immunostaining and confocal imaging
  - Fluorescence-activating cell sorting (FACS) of GFP-positive IGS cells
  - Fluorescent RNA *in situ* hybridization (FISH)
  - 10x Chromium single-cell RNA-seq library construction (v2)
  - Single cell RNA-seq 10X data preprocessing
  - Single cell RNA-seq data analysis
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.12.024.

**ACKNOWLEDGMENTS**

We would like to thank the members of the Xie laboratory for advice and discussion; Flybase; Bloomington *Drosophila* Stock Center; the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947); Developmental Studies Hybridoma Bank; and H.S. Hawley, H.J. Hsu, and M. Lilly for reagents. This work was supported by Stowers Institute for Medical Research and a grant from National Institutes of Health (R01HD097664) to T.X.

**AUTHOR CONTRIBUTIONS**

R.T., B.D., X.S., S.C., A.S., K.H., J.B., D.D., H.L., A.P., and J.H. carried out the experiments and collected and analyzed the data. R.T., B.D., and T.X. designed the project. R.T. and T.X. wrote the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no conflicts of interest.

Received: February 3, 2020
Revised: November 17, 2020
Accepted: December 15, 2020
Published: December 23, 2020

**REFERENCES**

1. Li, L., and Xie, T. (2005). Stem cell niche: structure and function. Annu. Rev. Cell Dev. Biol. 21, 605–631.

2. Losick, V.P., Morris, L.X., Fox, D.T., and Spradling, A. (2011). *Drosophila* stem cell niches: a decade of discovery suggests a unified view of stem cell regulation. Dev. Cell 21, 159–171.

3. Morrison, S.J., and Spradling, A.C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell 132, 598–611.

4. Kirilly, D., Wang, S., and Xie, T. (2011). Self-maintained escort cells form a germline stem cell differentiation niche. Development 138, 5087–5097.

5. Spradling, A., Fuller, M.T., Braun, R.E., and Yoshida, S. (2011). Germline stem cells. Cold Spring Harb. Perspect. Biol. 3, a002642.

6. Xie, T. (2013). Control of germline stem cell self-renewal and differentiation in the *Drosophila* ovary: concerted actions of niche signals and intrinsic factors. Wiley Interdiscip. Rev. Dev. Biol. 2, 261–273.

7. de Cueva, M., Lilly, M.A., and Spradling, A.C. (1997). Germline cyst formation in *Drosophila*. Annu. Rev. Genet. 31, 405–428.

8. Song, X., Wong, M.D., Kawase, E., Xi, R., Ding, B.C., McCarthy, J.J., and Xie, T. (2004). Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the *Drosophila* ovary. Development 131, 1353–1364.

9. Xie, T., and Spradling, A.C. (1998). decapentaplegic is essential for the maintenance and division of germline stem cells in the *Drosophila* ovary. Cell 94, 251–260.

10. Song, X., Zhu, C.H., Doan, C., and Xie, T. (2002). Germline stem cells anchored by adherens junctions in the *Drosophila* ovary niches. Science 296, 1855–1857.

11. Wang, X., and Page-McCaw, A. (2018). Wnt6 maintains anterior escort cells as an integral component of the germline stem cell niche. Development 145, dev158527.

12. Wang, X., Pan, L., Wang, S., Zhou, J., McDowell, W., Park, J., Haug, J., Staehling, K., Tang, H., and Xie, T. (2011). Histone H3K9 trimethylase Egless controls germline stem cell maintenance and differentiation. PLoS Genet. 7, e1002426.

13. Tseng, C.Y., Su, Y.H., Yang, S.M., Lin, K.Y., Lai, C.M., Rastegari, E., Amartuvshin, O., Cho, Y., Cai, Y., and Hsu, H.J. (2018). Smad-Independent BMP Signaling in Somatic Cells Limits the Size of the Germline Stem Cell Pool. Stem Cell Reports 11, 811–827.

14. Lu, T., Wang, S., Gao, Y., Mao, Y., Yang, Z., Liu, L., Song, X., Ni, J., and Xie, T. (2015). COP9-Hedgehog axis regulates the function of the germline stem cell progeny differentiation niche in the *Drosophila* ovary. Development 142, 4242–4252.

15. Huang, J., Reilein, A., and Kazdor, D. (2017). Yorkie and Hedgehog independently restrict BMP production in escort cells to permit germline differentiation in the *Drosophila* ovary. Development 144, 2584–2594.

16. Mottier-Pavie, V.I., Palacios, V., Eliazer, S., Scoggin, S., and Buszczak, M. (2016). The Wnt pathway limits BMP signaling outside of the germline stem cell niche in *Drosophila* ovaries. Dev. Biol. 417, 50–62.

17. Wang, S., Gao, Y., Song, X., Ma, X., Zhu, X., Mao, Y., Yang, Z., Ni, J., Li, H., Malanowski, K.E., et al. (2015). Wnt signaling-mediated redox regulation maintains the germ line stem cell differentiation niche. eLife 4, e08174.

18. Luo, L., Wang, H., Fan, C., Liu, S., and Cai, Y. (2019). Wnt ligands regulate Tkv expression to constrain Dpp activity in the *Drosophila* ovarian stem cell niche. J. Cell Biol. 209, 595–608.

19. Hamada-Kawaguchi, N., Nore, B.F., Kuvada, Y., Smith, C.I., and Yamamoto, D. (2014). Btk29A promotes Wnt4 signaling in the niche to terminate germ cell proliferation in *Drosophila*. Science 343, 294–297.

20. Upadhyay, M., Martino Cortez, Y., Wong-Deyrup, S., Tavares, L., Schowalter, S., Floria, P., Hill, C., Nasraiah, M.A., Chittur, S., and Rangan, P. (2016). Transposon Dysregulation Modulates dWnt4 Signaling to Control Germline Stem Cell Differentiation in *Drosophila*. PLoS Genet. 12, e1005918.

21. Maimon, I., Popliger, M., and Gilboa, L. (2014). Without children is required for Stat-mediated zfh1 transcription and for germline stem cell differentiation. Development 141, 2602–2610.

Current Biology 31, 827–839, February 22, 2021 837
61. Sahai-Hernandez, P., and Nystul, T.G. (2013). A dynamic population of stromal cells contributes to the follicle stem cell niche in the Drosophila ovary. Development 140, 4490–4498.
62. Dionne, H., Hibbard, K.L., Cavallaro, A., Kao, J.C., and Rubin, G.M. (2018). Genetic Reagents for Making Split-GAL4 Lines in Drosophila. Genetics 209, 31–35.
63. Liu, M., Lim, T.M., and Cai, Y. (2010). The Drosophila female germline stem cell lineage acts to spatially restrict DPP function within the niche. Sci. Signal. 3, ra57.
64. de Cuevas, M., and Spradling, A.C. (1998). Morphogenesis of the Drosophila fusome and its implications for oocyte specification. Development 125, 2781–2789.
65. Koch, E.A., and Spitzer, R.H. (1983). Multiple effects of colchicine on oogenesis in Drosophila: induced sterility and switch of potential oocyte to nurse-cell developmental pathway. Cell Tissue Res. 228, 21–32.
66. Theurkauf, W.E., Alberts, B.M., Jan, Y.N., and Jongens, T.A. (1993). A central role for microtubules in the differentiation of Drosophila oocytes. Development 118, 1169–1180.
67. Grieder, N.C., de Cuevas, M., and Spradling, A.C. (2000). The fusome organizes the microtubule network during oocyte differentiation in Drosophila. Development 127, 4253–4264.
68. Deng, W., and Lin, H. (1997). Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarized microtubule array for oocyte specification in Drosophila. Dev. Biol. 189, 79–94.
69. Diao, F., Ironfield, H., Luan, H., Diao, F., Shropshire, W.C., Ewer, J., Marr, E., Potter, C.J., Landgraf, M., and White, B.H. (2015). Plug-and-play genetic access to Drosophila cell types using exchangeable exon cassettes. Cell Rep. 10, 1410–1421.
70. Zimmerman, S.G., Peters, N.C., Altaras, A.E., and Berg, C.A. (2013). Optimized RNA ISH, RNA FISH and protein-RNA double labeling (IF/FISH) in Drosophila ovaries. Nat. Protoc. 8, 2158–2179.
71. Zou, F., Tu, R., Duan, B., Yang, Z., Ping, Z., Song, X., Chen, S., Price, A., Li, H., Scott, A., et al. (2020). Drosophila YBX1 homolog YPS promotes ovarian germ line stem cell development by preferentially recognizing 5-methylcytosine RNAs. Proc. Natl. Acad. Sci. USA 117, 3603–3609.
72. Ma, X., Zhu, X., Han, Y., Story, B., Do, T., Song, X., Wang, S., Zhang, Y., Blanchette, M., Gogol, M., et al. (2017). Aubergine Controls Germline Stem Cell Self-Renewal and Progeny Differentiation via Distinct Mechanisms. Dev Cell 41, 157–169.
## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-Hts | Developmental Studies Hybridoma Bank | Cat#hts RC; RRID: AB_528289 |
| Rabbit polyclonal anti-β-galactosidase antibody | MP BIOMEDICALS | Cat#08559761; RRID: AB_2335269 |
| Rabbit polyclonal anti-pS137 H2AvD antibody | Rockland | Cat#600-401-914; RRID: AB_828383 |
| Rat polyclonal anti-Vasa antibody | Developmental Studies Hybridoma Bank | Cat#anti-vasa; RRID: AB_760351 |
| Chicken polyclonal anti-GFP antibody | Invitrogen | Cat#A10262; RRID: AB_2534023 |
| Mouse monoclonal anti-C3G | gift from Dr. Scott Hawley | PMID: 15767569 |
| Rabbit polyclonal anti-C3G | gift from Dr. Mary Lilly | PMID: 12588841 |
| **Deposited Data** |        |            |
| scRNA-sequencing dataset | GEO: GSE143817 | This study |
| **Experimental Models: Organisms/Strains** |        |            |
| D. melanogaster: c587-Gal4 | The Xie lab | FlyBase ID: FBII0003776 |
| D. melanogaster: tubP-Gal80ts (ChrIII) | Bloomington Drosophila Stock Center | RRID: BDSC_7017 |
| D. melanogaster: tubP-Gal80ts (ChrIII) | Bloomington Drosophila Stock Center | RRID: BDSC_7019 |
| D. melanogaster: PZ1444 | The Xie lab | FlyBase ID: FBII0003776 |
| D. melanogaster: UAS-mGFP | Bloomington Drosophila Stock Center | RRID: BDSC_5137 |
| D. melanogaster: UAS-GFP | Bloomington Drosophila Stock Center | RRID: BDSC_5431 |
| D. melanogaster: UAS-NetA-GFP | Bloomington Drosophila Stock Center | RRID: BDSC_59409 |
| D. melanogaster: mini-GFP | Bloomington Drosophila Stock Center | RRID: BDSC_68183 |
| D. melanogaster: bam-GFP | The Xie lab | FlyBase ID: FBII0003776 |
| D. melanogaster: dnc-GFP | Bloomington Drosophila Stock Center | RRID: BDSC_60535 |
| D. melanogaster: GMR31C09-Gal4 | Bloomington Drosophila Stock Center | RRID: BDSC_49067 |
| D. melanogaster: GMR25A11-Gal4 | Bloomington Drosophila Stock Center | RRID: BDSC_49106 |
| D. melanogaster: GMR25A11-Gal4 | Bloomington Drosophila Stock Center | RRID: BDSC_49106 |
| D. melanogaster: GMR71E07-Gal4 | Bloomington Drosophila Stock Center | RRID: BDSC_49106 |
| D. melanogaster: GMR71E07-Gal4 | Bloomington Drosophila Stock Center | RRID: BDSC_49106 |
| D. melanogaster: UAS-lacZ | Bloomington Drosophila Stock Center | RRID: BDSC_31603 |
| D. melanogaster: RNAi of luc | Bloomington Drosophila Stock Center | RRID: BDSC_31288 |
| D. melanogaster: RNAi of NetA | Bloomington Drosophila Stock Center | RRID: BDSC_31665 |
| D. melanogaster: RNAi of Hfs | Bloomington Drosophila Stock Center | RRID: BDSC_34718 |
| D. melanogaster: RNAi of bin | Bloomington Drosophila Stock Center | RRID: BDSC_67844 |
| D. melanogaster: RNAi of vn | Bloomington Drosophila Stock Center | RRID: BDSC_56950 |
| D. melanogaster: RNAi of GstS1 | Bloomington Drosophila Stock Center | RRID: BDSC_28885 |
| D. melanogaster: RNAi of GstS1 | Bloomington Drosophila Stock Center | RRID: BDSC_53238 |
| D. melanogaster: RNAi of wun2 | Bloomington Drosophila Stock Center | RRID: BDSC_32381 |
| D. melanogaster: RNAi of smo | Bloomington Drosophila Stock Center | RRID: BDSC_32423 |
| D. melanogaster: dpr17-Gal4 | This study | This study |
| D. melanogaster: H2126-SwitchGal4 | gift from Dr. Hwei-Jan Hsu | PMID: 31018943 |
| D. melanogaster: RNAi of smo | Bloomington Drosophila Stock Center | RRID: BDSC_27037 |
| D. melanogaster: RNAi of smo | Bloomington Drosophila Stock Center | RRID: BDSC_62987 |
| **Software and Algorithms** |        |            |
| GraphPad Prism 7 | GraphPad Software | RRID: SCR 002798 |
| Adobe Illustrator | Adobe Inc. | Adobe Illustrator 2020 24.0.1 |
| ImageJ | NIH | N/A |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ting Xie (tgx@stowers.org).

**Materials Availability**
New reagents generated in this study are available via the lead contact.

**Data Availability**
Original scRNA sequencing data have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession number GEO: GSE143817). Original data underlying this manuscript can be accessed from the Stowers Original Data Repository at http://www.stowers.org/research/publications/libpb-1575.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Drosophila culture**
Flies were maintained and crossed at room temperature on standard cornmeal/molasses/agar media unless specified. For maximizing the effect of RNAi-mediated knockdown or gene overexpression, newly eclosed flies were shifted to 29°C for the specified time before analyzing ovarian phenotypes. For the GeneSwitch Gal4-mediated knockdown, RU486 administration was performed at adult stage according to the previous publication.57

**METHOD DETAILS**

**Generation of Gal4 lines using Cas9/CRISPR**

*dpr17*-Gal4 were generated according to previous publication.69 Briefly, homology arms of approximately 800-1000 bp were amplified by PCR from the *Drosophila* genomic DNA. The sgRNA sequence for *dpr17* is gaaattatgctgatctgtgccgg. ggtttatcccgttaaggaagc and gatcagcataatttcatttgcat primers were used to amplify the left arm, while acaagcgaaggcagaatcag and caggtgaactttggcactca primers were used to amplify the right arm.

**Immunostaining and confocal imaging**
Immuno staining was performed according to our previous published procedures (Song et al.,2002; Xie and Spradling, 1998). The following antibodies were used in this study: mouse monoclonal anti-Hts antibody (1:50, 1B1, DSHB), rabbit polyclonal anti-j1-galactosidase antibody (1:500, #08559761, MP Biomedical), rabbit polyclonal anti-pS137 H2AvD antibody (1:2000, #600-401-914S, Rockland), chicken polyclonal anti-GFP antibody (1:500, Invitrogen, #A10262), mouse monoclonal anti-C3G (1:500, gift from Dr. Hawley, Stowers Institute), rabbit polyclonal anti-C3G (1:10000, gift from Dr. Lilly, NICHD/DIP).
**Fluorescence-activating cell sorting (FACS) of GFP-positive IGS cells**

31C09-Gal4, 25A11-Gal4, 71E07-Gal4 were used to drive UAS-GFP expression in different IGS cell populations. After being cultured for 1 week at 25 °C, Drosophila ovaries were dissected and placed in the Grace’s medium (Sigma-Aldrich; G9771), washed twice by 1 × PBS and centrifuged at 700 × g for 1 min. The ovaries were incubated with a prewarmed Collagenase solution (50D11833; Worthington) in a 15 mL conical tube at a 37 °C water bath for 3 min with gentle shaking. Enzyme reaction was stopped after 3 min of incubation following the addition of the cold 1 × DPBS+2% FBS. Dissociated samples were washed by 1 × DPBS and then centrifuged at 700 × g and 4 °C for 5 min. The cell pellet was resuspended in 1 × DPBS and filtered with the 70 μm Ficon (BD; 340605). Cells were centrifuged and then resuspended in 200 μl of 1 × DPBS for sorting the GFP-positive cells at 45 psi with 70 μm tip (BD; InFlux) immediately. The samples were processed with the PrimeFlow RNA Assay kit (ThermoFisher) following the manufacturers protocol.

**Fluorescent RNA in situ hybridization (FISH)**

Hybridization chain reaction (HCR) v3.0 method was used to achieve mRNA FISH at high sensitivity and specificity. Probe sets against mRNA were ordered from Molecular Instruments, Inc. For the combined immunostaining and FISH staining experiments, the immunostaining procedures, including primary antibody incubation, secondary antibody incubation, and ovary postfixation and dehydration, were performed according to the previous publications.

10x Chromium single-cell RNA-seq library construction (v2)

After the dissociated cells were sorted into the Schneider’s media, they were further assessed for their concentration and viability via a Nexcelom Cellometer Auto T4. Only when the sorted cells were over 40% viable, they were loaded on a Chromium Single Cell Controller (10x Genomics, Pleasanton, CA), based on live cell concentration, with a target of 3,000–5,000 cells per sample. Libraries were prepared using the Chromium Single Cell 3’ Library & Gel Bead Kit v2 (10x Genomics) according to manufacturer’s directions. Resulting short fragment libraries were checked for quality and quantity using an Agilent 2100 Bioanalyzer and Thermo Fisher Qubit Fluorometer. Libraries were pooled at equal molar concentrations and sequenced to a depth necessary to achieve at least 50,000 mean reads per cell—~130M reads each—on an Illumina HiSeq 2500 instrument using Rapid SBS v2 chemistry with the following paired read lengths: 26 bp Read1, 8 bp I7 Index and 98 bp Read2. Approximately 700-2500 cells were captured and used for analysis.

Single cell RNA-seq 10X data preprocessing

cDNA libraries were sequenced as paired-end reads on the Illumina HiSeq 2500 machine. Raw sequencing data were processed using 10x Genomics Cell Ranger pipeline v2.1., and the reads were demultiplexed into Fastq file format using cellranger mkfastq. Genome index was built by cellranger mkref using Drosophila genome dm6, ensembl 84 gene model. Data were aligned by STAR aligner and cell counts tables were generated using cellranger count function with default parameters.

Single cell RNA-seq data analysis

Cellranger’s raw gene count matrices were further analyzed using the Seurat (v2.3.3) R package in standard protocols. The cells that have less than 1000 UMIs were excluded from downstream analysis. Gene expression results were log-normalized, and then re-gressed on the number of UMIs. Principle component analysis (PCA) was done using the highly variable genes, and the first 25 principle components (PCs) were used for clustering analysis to identify distinct cell clusters based on PCElbowPlot. tSNE plots were used to visualize the clustering results. Known and de novo markers were used to classify the cells into different IGS subpopulations.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

GSCs, SGCs and IGS cells were quantified according to our previous studies under the fluorescent microscope. Briefly, the spectrosome-containing single germ cells attached to the cap cells are defined as GSCs, whereas those single germ cells away from cap cells are identified as differentiating SGCs; PZ1444 is used to label IGS cells and cap cells, which can be easily distinguished based on size and location. The statistical analysis was done using GraphPad Prism 7 with the Student’s t test method. p values are indicated in figure legends, and the results are presented as mean or mean ± SEM (**p ≤ 0.001; **p ≤ 0.01; *p ≤ 0.05; n.s., no significance).