



Gap junction–transported cAMP from the niche controls stem cell progeny differentiation

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The niche has been shown to control stem cell self-renewal in different tissue types and organisms. Recently, a separate niche has been proposed to control stem cell progeny differentiation, called the differentiation niche. However, it remains poorly understood whether and how the differentiation niche directly signals to stem cell progeny to control their differentiation. In the *Drosophila* ovary, inner germarial sheath (IGS) cells contribute to two separate niche compartments for controlling both germline stem cell (GSC) self-renewal and progeny differentiation. In this study, we show that IGS cells express *Inx2* protein, which forms gap junctions (GJs) with germline-specific *Zpg* protein to control stepwise GSC lineage development, including GSC self-renewal, germline cyst formation, meiotic double-strand DNA break formation, and oocyte specification. Germline-specific *Zpg* and IGS-specific *Inx2* knockdowns cause similar defects in stepwise GSC development. Additionally, secondary messenger cAMP is transported from IGS cells to GSCs and their progeny via GJs to activate PKA signaling for controlling stepwise GSC development. Therefore, this study demonstrates that the niche directly controls GSC progeny differentiation via the GJ-cAMP-PKA signaling axis, which provides important insights into niche control of stem cell differentiation and highlights the importance of GJ-transported cAMP in tissue regeneration. This may represent a general strategy for the niche to control adult stem cell development in various tissue types and organisms since GJs and cAMP are widely distributed.

niche | stem cell | differentiation | gap junction | cAMP

Stem cells exist in adult tissues to maintain homeostasis through continuous self-renewal and differentiation in organisms ranging from *Drosophila* to humans. Stem cells have also been proposed to treat various human diseases, including diabetes and Parkinson's and Alzheimer's diseases (1). The niche has been demonstrated to control stem cell self-renewal in various systems (2, 3). The differentiation of stem cell progeny has recently been proposed to be also controlled by another niche, the differentiation niche, first in the *Drosophila* ovary and then in the mouse testis (4–6). One of the major obstacles for clinical applications of stem cells in treating human diseases is the lack of a comprehensive understanding of how stem cells are regulated by the niche, particularly their lineage specification. In this study, we have shown that cAMP can be transported from the differentiation niche through gap junctions (GJs) to stem cell progeny to directly control multistep differentiation processes.

The *Drosophila* ovary is an effective model for studying stem cell–niche interactions in the regulation of germline stem cell (GSC) self-renewal and differentiation because of simple anatomy, well-defined cell types, and available versatile genetic tools (7, 8). Every female has a pair of ovaries, each containing 12 to 16 ovarioles. The germarium, a tubular structure located at the apical tip of each ovariole, contains 2 to 3 GSCs and early GSC progeny, including mitotically active cystoblasts (CBs)/cysts (2, 4, 8-cell cysts), newly produced ball-like 16-cell cysts, and oval-shaped 16-cell cysts, which are located at regions 1, 2a, and 2b of the germarium, respectively (9) (Fig. 1 *A* and *B*). The newly formed ball-like 16-cell cysts enter the prophase of meiosis to initiate chromosomal pairing and meiotic recombination (10, 11), whereas those oval-shaped 16-cell cysts begin to choose one permanent oocyte from two phenotypically similar pro-oocytes and then proceed to be covered up by the follicle epithelium. GSCs anteriorly contact cap cells and are wrapped around by cellular processes of laterally localized inner germarial sheath (IGS) cells (aka escort cells), while IGSs localized posterior to those contacting GSCs are situated at the germarial surface to encase CBs and mitotic cysts in region 2a, newly formed ball-like 16-cell cysts in region 2a, and oval-shaped 16-cell cysts in region 2b. Cap cells function as the niche to control GSC self-renewal through BMP signaling and E-cadherin-mediated cell adhesion (12–14), whereas those GSC-contacting IGS cells contribute to the niche for controlling GSC self-renewal (4, 5, 15). The remaining IGS cells directly contact early GSC progeny to form the differentiation niche for regulating their differentiation (4, 5).

Significance

The controlling germline stem cell (GSC) niche is known to control stem cell self-renewal, but it remains poorly understood how the differentiation niche signals directly to stem cell progeny to control their differentiation. In the *Drosophila* ovary, inner germarial sheath cells form the differentiation niche for GSC progeny differentiation. Gap junctions transport the secondary messenger cAMP from the differentiation niche, which activates PKA signaling in early stem cell progeny to control their stepwise differentiation, including cyst formation, meiotic DNA break formation, and oocyte specification. This study represents a significant advance in our understanding of the mechanisms underlying niche-mediated direct control of stem cell progeny differentiation and has significant implications for our understanding of stem cell biology and regenerative medicine.

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The authors declare no competing interest.

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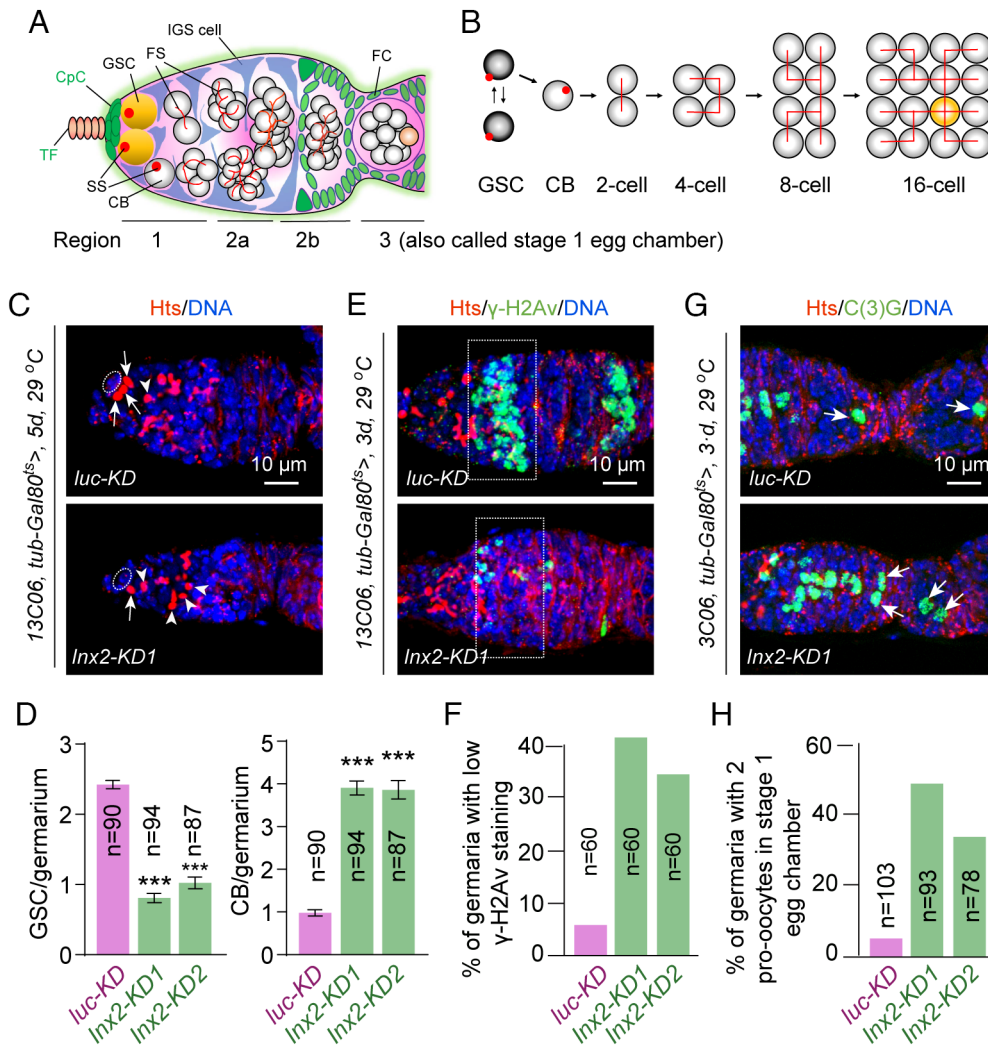


Fig. 1. IGS-expressed *Inx2* controls stepwise GSC development. (A) A schematic diagram illustrating the *Drosophila* germarium, which is divided into three regions based on germ cell developmental stages. Region 1 contains mitotic germ cells, including germline stem cells (GSCs), cystoblasts (CBs), and mitotic cysts (two-cell, four-cell, and eight-cell cysts). Region 2 contains ball-like 16-cell cysts enveloped by IGS cells (2a) and lens-shaped 16-cell cysts surrounded by follicle cells (2b). Region 3 contains a stage 1 egg chamber. Abbreviations: TF, terminal filament; CPC, cap cell; IGS, inner germline sheath; CB, cystoblast; SS, spectrosome; FS, fusome; and FC, follicle cells. (B) A GSC undergoes self-renewing division to generate a GSC and a CB. The CB divides four times with incomplete cytokinesis to form a 16-cell cyst. Different developmental stages of cysts, including 2-cell, 4-cell, 8-cell, and 16-cell cysts, can be easily identified by their distinct branched fusome patterns. (C and D) IGS-specific *Inx2KD* significantly decreases GSCs and increases CB-like cells compared with the control. Cap cells, GSCs, and CBs are highlighted by ovals, arrows, and arrowheads, respectively. D: quantification results. n = number of germaria. (Scale bars, 10 μ m.) Student's t test: *** $P \leq 0.001$. (E–H) IGS-specific *Inx2KD* decreases γ -H2Av levels (E: region 2a by broken rectangle) and increases the incidence of two oocytes (G: arrowheads) in 16-cell cysts compared with the control (with one oocyte). (F and H) quantification results. n = number of germaria.

The mechanisms underlying niche regulation of GSC progeny differentiation have just begun to be elucidated.

One of the mechanisms for IGS cells to promote CB differentiation into cysts is to antagonize Bone Morphogenetic Protein (BMP) signaling. Hedgehog (Hh), Wnt, Epidermal Growth Factor Receptor (EGFR), and Jak–Stat signaling pathways function in IGS cells to prevent BMP signaling from interfering with GSC progeny differentiation (15–24). Recent single-cell analysis has demonstrated the existence of four IGS populations, IGS1–4: IGS1, IGS2, IGS3, and IGS4 represent those directly interacting with GSCs, CBs/mitotic cysts in region 1a, early meiotic 16-cell cyst in region 2a, and oocyte-forming 16-cells in region 2b, respectively (25, 26). In addition, IGS1, IGS2, IGS3, and IGS4 control GSC self-renewal, CB differentiation into cysts, meiotic DSB formation, and timely oocyte determination, respectively (25). Within mammalian ovaries, somatic cells are also known to play critical roles in controlling the differentiation of germ cells, as well as various stages of meiosis, and oocyte maturation (27, 28). However, it remains unclear whether and how these different niche

compartments directly control the differentiations steps. This study demonstrates that GJ-mediated direct signaling from the IGS controls GSC maintenance, cyst formation, meiotic DSB formation, and oocyte determination.

GJs are the intercellular channels formed by two hexameric hemichannels on two adjacent cells to exchange ions and small molecules (29). GJs can be either homotypic (two identical hexamers) or heterotypic (two different hexamers). Among the eight *Drosophila* innexin proteins, *Inx2* has been shown to be expressed and required in IGS cells for germline cyst formation, whereas *Zpg* protein (also known as *Inx4*) is expressed and required in the germline for controlling CB-to-cyst differentiation (30–32). However, it remains to be determined whether *Inx2* and *Zpg* form GJs to mediate direct signaling from the IGS to GSC progeny. This study shows that *Inx2* on the IGS and *Zpg* on the germline form GJs to regulate GSC maintenance, cyst formation, meiotic DSB formation, and oocyte determination by transporting the secondary messenger cAMP. Therefore, this study has uncovered a direct signaling mechanism used by the niche to control stepwise GSC lineage development.

Results

Inx2 Functions in the IGS to Control GSC Maintenance, CB Differentiation, Meiotic DSB Formation, and Oocyte Determination.

Although *Inx2* knockdown in IGS cells throughout their development disrupts cyst formation (32), it remains unknown whether it is also required in adult IGS cells to control CB differentiation into cysts. Our RNA fluorescent in situ hybridization (FISH) results have further confirmed that *Inx2* mRNA is primarily expressed in somatic cells of the adult ovary, including cap cells, IGS cells, and follicle cells (*SI Appendix, Fig. S1A*). To determine whether *Inx2* is required in adult IGS cells to control GSC maintenance and progeny differentiation, we used *13C06-Gal4* (in combination with *tub-Gal80^{ts}: 13C06^{ts}*) to knock down *Inx2* on adult IGS cells for 5 d and then quantified GSCs and CBs. *13C06-Gal4* drives expression of Gal4 protein in IGS cells and follicle progenitors, and also occasionally in one to two cap cells (33). At 25 °C, which allows the temperature-sensitive Gal80^{ts} mutant protein to repress Gal4-mediated shRNA expression, GSCs and their progeny in the females carrying *13C06-Gal4; tub-Gal80^{ts}* and *UAS-Inx2 shRNA* develop normally (*SI Appendix, Fig. S1 B and C*). Compared to the control, *Inx2* knockdown in adult IGS cells causes the significant GSC loss and the accumulation of significantly more CB-like cells 5 d after 29 °C, which activates *Inx2* shRNA expression by inactivating Gal80^{ts}, indicating that *Inx2* functions in adult IGS cells to control GSC maintenance and CB differentiation (Fig. 1 *C* and *D* and *SI Appendix, Fig. S1 D and E*). *Inx2* knockdown by IGS-specific *c587-Gal4* leads to similar mutant phenotypes (*SI Appendix, Fig. S1 F and G*). After 2 wk at 29 °C, *Inx2* knockdown in adult IGS cells causes the accumulation of even more CBs and disrupts egg chamber numbers compared to the control (*SI Appendix, Fig. S1 H–J*). Our results demonstrate that *Inx2* is required in adult IGS cells to maintain GSCs and promote CB differentiation into germline cysts.

To determine whether *Inx2* is required in adult IGS cells to control meiotic double-strand DNA break (DSB) formation and oocyte determination, we proceeded to knock down *Inx2* on adult IGS cells for 3 d, which causes no obvious CB differentiation defects since CB differentiation defects could influence later meiotic entry and oocyte development. Those early meiotic 16-cell cysts in region 2a generate DSBs for meiotic recombination, which can be recognized by an antibody specific to the phosphorylated histone 2A variant (γ -H2Av) (10, 11, 34, 35). Subsequently, those meiotic 16-cell cysts in region 2b proceed to choose one permanent oocyte from the two pro-oocytes, which can be identified by C(3)G protein expression (10, 11, 34). About 90% of the control germlaria contain the high- γ -H2Av meiotic 16-cell cysts with the remaining 10% carrying the low- γ -H2Av meiotic 16-cell cysts, which is still at the earlier phases of the meiosis. Interestingly, knocking down *Inx2* on adult IGS cells for 3 d leads to an increase of low γ -H2Av expression in early 16-cell cysts and an increase in stage-1 egg chambers with two pro-oocytes compared to the control (Fig. 1 *E–H*). These results indicate that *Inx2* is required in adult IGS cells to control GSC maintenance, CB-to-cyst formation, meiotic DSB formation, and timely oocyte determination. It is worth noting that the 3-d *Inx2* knockdown does not result in any obvious IGS cell loss, which helps rule out the IGS cell loss as the cause of those GSC progeny differentiation defects (*SI Appendix, Fig. S1 K and L*).

Zpg on Germ Cells and *Inx2* on IGS Cells form GJs to Control GSC Maintenance, CB-to-cyst Differentiation, Meiotic DSB Formation, and Oocyte Determination. GJ protein Zpg is known to be specifically expressed in germ cells, including GSCs and their early progeny, and its function is important for CB differentiation (30, 31). To visualize endogenous *Inx2* and Zpg protein expression in

the ovary, we generated Bac transgenes expressing the C-terminally GFP-tagged *Inx2* protein (*Inx2-GFP*) or C-terminally Flag-tagged Zpg protein (*Zpg-Flag*) under the control of their endogenous promoters to examine their subcellular localization. *Inx2-GFP* is primarily expressed in somatic cells in the ovarioles, including IGS cells and follicle cells, while *Zpg-Flag* is enriched in germ cells (*SI Appendix, Fig. S2 A and B*). Interestingly, some of *Inx2-GFP* and *Zpg-Flag* proteins are also colocalized at the cellular junctions between IGS cells and GSCs/GSC progeny. Our FISH results have further confirmed the expression of *zpg* mRNA primarily in germ cells labeled by Vasa protein (*SI Appendix, Fig. S3A*). Then, we used the stimulated emission depletion (STED) technique, which can achieve the resolution of 50 nm, to show that *Inx2-GFP* and *Zpg-Flag* are indeed colocalized to form heterologous GJ channels at the interface between IGS cells and GSC progeny (Fig. 2*A*). These results raise the tantalizing possibility that *Inx2* on IGS cells and Zpg on germ cells form heterologous GJ channels for mediating direct IGS-to-GSC/progeny communication and thus regulate GSC lineage development.

To test the possibility, we used *hsFlp; nos>stop>Gal4* and two independent *zpg* RNAi lines to knock down *zpg* specifically in adult germline. In the absence of a heat-shock treatment, the presence of the transcription *stop* cassette effectively prevents the Gal4-mediated activation of shRNA expression so the non-heat-shocked females exhibit normal GSC lineage development (*SI Appendix, Fig. S3 B and C*). Following a heat-shock treatment at 37 °C, Flipase (FLP)-mediated FRT recombination helps remove the FRT-franked *stop* cassette (36), which leads to *nanos* (*nos*)-driven expression of Gal4 specifically in germ cells to activate the shRNA-mediated *zpg* knockdown (*SI Appendix, Fig. S3 D and E*). As in the previous study (30), 5-d-long *zpg* knockdown blocks normal CB differentiation, causing the accumulation of CB-like cells (Fig. 2 *B* and *C*). In addition, its knockdown also causes premature GSC loss, indicating that Zpg also intrinsically maintain GSCs (Fig. 2 *B* and *C*). Moreover, a 2-wk *zpg* knockdown causes more severe germline development defects than 1-wk knockdown (*SI Appendix, Fig. S3 F–H*). In addition, 3-d germline-specific *zpg* knockdown results in the decreased γ -H2Av expression in early 16-cell cysts and the increased frequency of stage-1 egg chambers with two pro-oocytes compared to the control, indicating that Zpg also intrinsically regulates meiotic DSB formation and timely oocyte determination (Fig. 2 *D–G*). Since the GSC developmental defects caused by *zpg* knockdown in germ cells are reminiscent of those caused by *Inx2* knockdown in IGS cells, we propose that *Inx2* on IGS cells and Zpg on germ cells form GJ channel connections for directly controlling GSC maintenance and stepwise GSC progeny differentiation.

***Inx2*-Zpg GJs Transport cAMP from IGS Cells to GSCs and their Progeny.**

Connexin 43-formed GJs can transport cAMP in bone cells to mediate PKA signaling and regulate gene expression (37). First, we investigate the possibility that the cAMP in IGS cells can be transported to GSCs and their early progeny through GJs. Here, we used a commercial rabbit anti-cAMP monoclonal antibody, which specificity is also confirmed by knocking down or overexpressing cAMP synthesis enzyme adenylyl cyclase Rutabaga (*Rut*) in the germlarium (*SI Appendix, Fig. S4 A and B*), to monitor cAMP levels in IGS cells and germ cells using immunofluorescent staining. In the control germlarium, IGS cells produce higher levels of cAMP than cap cells, GSCs and their early progeny, suggesting that IGS cells are likely the major source for cAMP (Fig. 3*A*). Interestingly, either IGS-specific *Inx2KD* or germline-specific *zpgKD* can significantly increase cAMP levels in IGS cells and decrease cAMP levels in GSCs and their early GSC progeny

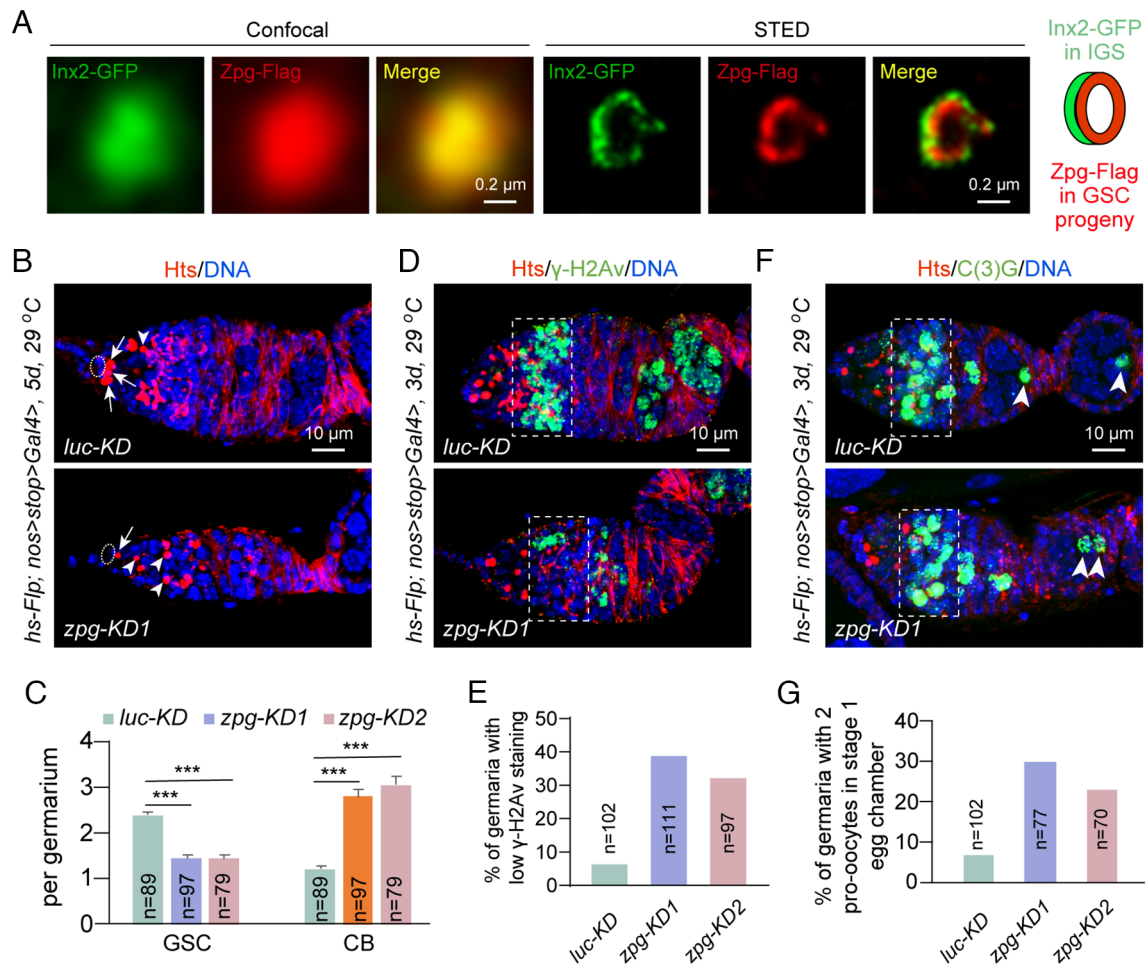


Fig. 2. GJ protein Zpg intrinsically controls GSC maintenance and early GSC progeny differentiation. (A) Confocal images show Inx2 and Zpg colocalization. STED images indicate that Inx2 on IGS cells and Zpg on GSC progeny form a heterologous GJ channel. (Scale bars, 0.2 μ m.) (B and C) Germline-specific *zpgKD* decreases GSC and increases CB-like cells compared to the control. Cap cells, GSCs, and CBs are highlighted by ovals, arrows, and arrowheads, respectively. (C) quantification results. n = number of germaria. (Scale bars, 10 μ m.) Student's t test: *** P \leq 0.001. (D and E) Germline-specific *zpgKD* decreases γ -H2Av expression (broken rectangle) in meiotic 16-cell cysts in region 2a compared to the control. (E) quantification results. n = number of germaria. (Scale bars, 10 μ m.) (F and G) Germline-specific *zpgKD* increases the incidence of two pro-oocytes (arrowheads) cysts in stage-1 egg chambers. (G) quantification results. n = number of germaria. (Scale bars, 10 μ m.)

(Fig. 3 A and B). This result supports the idea that cAMP is indeed transported from IGS cells to GSCs and their early GSC progeny through GJs.

To further confirm cAMP transport from IGS cells to GSC progeny, we next constructed a transgenic strain carrying an engineered bright luminescent protein Nano-lantern fused with the cAMP-binding motif of human EPAC1 under the control of germline-specific *nanos* promoter, *nos*-Nano-lantern-cAMP, which is confirmed to be specifically and highly expressed in germ cells by immunostaining (SI Appendix, Fig. S4C). Nano-lantern is a chimeric protein of an engineered *Renilla* luciferase and GFP variant Venus for producing high bioluminescence, which allows the Nano-lantern-cAMP reporter to perform real-time living imaging for monitoring cAMP levels (38). Based on live imaging on freshly dissected germaria, GSCs and their early progeny generally respond to cAMP throughout the germline (Fig. 3C). Consistently, IGS-specific *Inx2KD* can significantly reduce cAMP responses in GSCs and their progeny (Fig. 3C and D). Carbenoxolone (CBX) is an effective and specific GJ inhibitor (39). After 15-min incubation of freshly dissected germaria with CBX, cAMP responses are significantly down-regulated in GSCs and their progeny compared to the control (Fig. 3E and F). In addition, *Inx2* knockdown in IGS cells and the CBX application do not change the expression

levels of the Nano-lantern protein in germ cells, suggesting that the observed cAMP changes are not due to the changed reporter expression (SI Appendix, Fig. S4 C–F). Furthermore, we have also used the recently developed genetically encoded cAMP reporter *G-Flamp1*, which can monitor cellular cAMP levels in vivo (40), to confirm that IGS cells express much higher levels of cAMP than germ cells (SI Appendix, Fig. S4 G–J). Taken together, our results strongly argue that cAMP in IGS cells is transported to GSCs and their progeny via GJs.

Niche-Initiated cAMP Signaling Is Required for Controlling GSC Maintenance and Stepwise Early Progeny Differentiation.

Based on the *Drosophila* single-nucleus transcriptomic atlas, *rut* is highly expressed in the somatic cells but not the germ cells of the adult ovary (41). Our mRNA FISH result has further confirmed that *rut* mRNAs are expressed in IGS cells at higher levels than in germ cells (SI Appendix, Fig. S5A). Consistent with the expression patterns of *rut*, IGS cells accumulate cAMP at much higher levels than germ cells, including GSCs and their early progeny (Fig. 3A). Knocking down *rut* expression in IGS cells drastically decreases cAMP levels, whereas overexpressing *rut* in those cells dramatically increases cAMP levels, indicating that Rut is the major adenylyl cyclase responsible for cAMP production in IGS

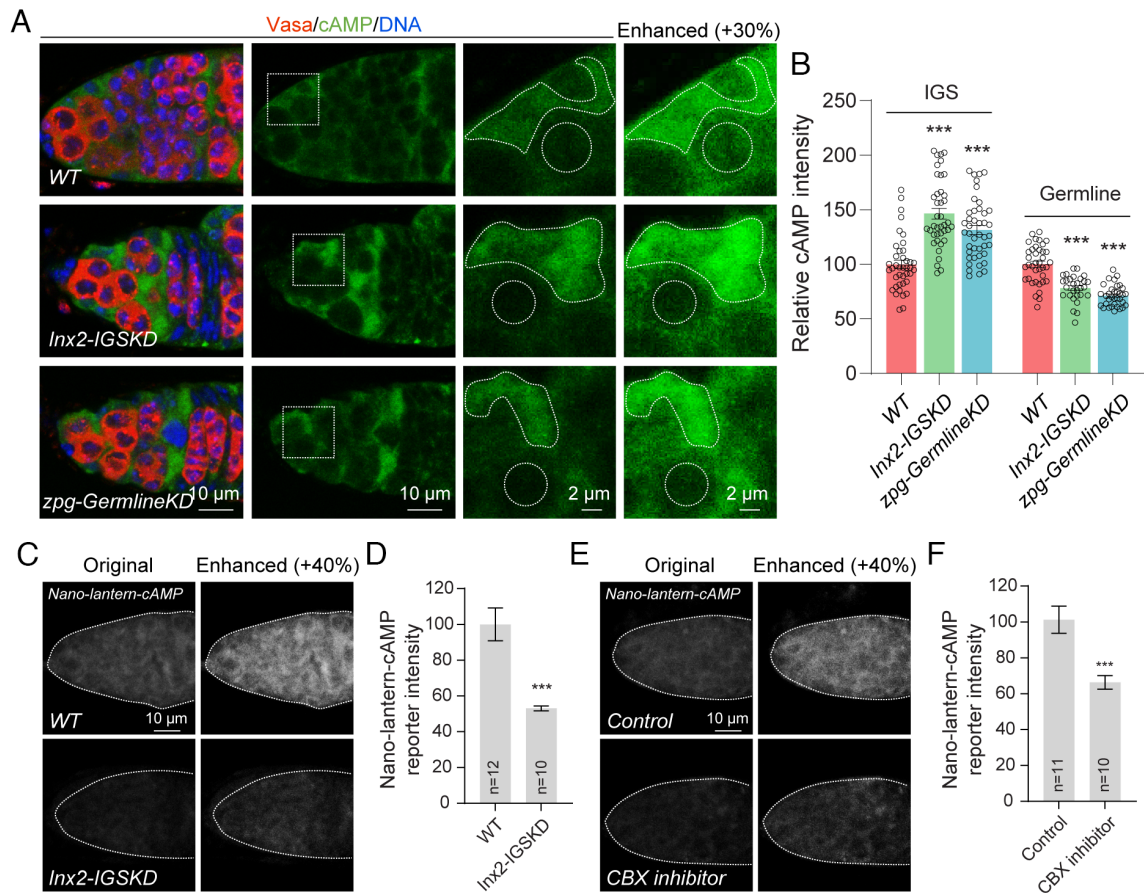


Fig. 3. IGS-expressed cAMP is transported to GSCs and their early progeny via GJs. (A and B) Single section confocal images showing that cAMP is highly enriched in IGS cells and weakly expressed in GSCs and their early progeny. *13C06^{ts}*-driven *Inx2* knockdown and *nos-Geneswitch-Gal4*-driven germline *zpg* knockdown increase cAMP levels in IGS cells and decrease cAMP levels in GSCs and their early progeny. With signal enhancement, cAMP expression can still be detected in GSCs and their progeny. (B) quantification results. (Scale bars, 10 μ m, 2 μ m.) Student's *t* test: ****P* \leq 0.001. (C and D) Live imaging of freshly dissected germlaria expressing the *nos-Nano-lantern-cAMP* reporter. *13C06^{ts}*-driven *Inx2* knockdown decreases the cAMP reporter activity in germ cells. (D) quantification results. (Scale bars, 10 μ m.) Student's *t* test: ****P* \leq 0.001. (E and F) Live imaging of freshly dissected germlaria expressing the cAMP reporter with or without the treatment of the CBX inhibitor. After a 15-min treatment, cAMP levels are significantly reduced in GSCs and their progeny. (F) quantification results. (Scale bars, 10 μ m.) Student's *t* test: ****P* \leq 0.001.

cells (SI Appendix, Fig. S4 A and B). Excitingly, IGS-specific *rut* knockdown disrupts GSC self-renewal and CB differentiation into cysts, meiotic DSB formation, and oocyte determination, indicating that Rut is required in IGS cells for producing cAMP to regulate GSC lineage development (SI Appendix, Fig. S5 B–E and Fig. 4 A–D). Therefore, our findings indicate that IGS-produced cAMP is critical for GSC maintenance and stepwise GSC progeny differentiation.

One of the major cAMP effectors is protein kinase A (PKA), which is composed of two regulatory subunits and two catalytic subunits (42). cAMP binding to the regulatory subunits causes their dissociation from the catalytic subunits, thus releasing active PKA for carrying out the biological functions via phosphorylating target proteins. In *Drosophila*, there are three catalytic subunits, Pka-C1, Pka-C2, and Pka-C3. Based on the *Drosophila* single-nucleus expression atlas, only *Pka-C1*, but not *Pka-C2* and *Pka-C3*, is highly expressed in the germ cells of the adult ovary, although three of them are expressed in the somatic cells of the germlarium (41). To facilitate the efficient knockdown of *Pka-C1* in germ cells, including GSCs and their early progeny at the adult stage, we generated *nos-GeneSwitch-Gal4* (*nos-GS*), which RU486-dependent *GeneSwitch-Gal4* is under the control of the germline-specific *nos* promoter. GeneSwitch-Gal4 is the fusion of yeast transcriptional activator Gal4 with human progesterone receptor, which only enters the nucleus and binds to the UAS-containing promoter to activate

the expression of a target gene in the presence of RU486 (mifepristone) (43). Excitingly, 2 wk after feeding adult females with RU486, germline-specific knockdown of *Pka-C1* in the adult ovary significantly decreases GSCs, increases CB accumulation, and causes defective DSB formation and oocyte determination (SI Appendix, Fig. S5 F–H and Fig. 4 E–H). These results demonstrate that cAMP can activate PKA in the germline to control GSC maintenance, CB differentiation into cysts, meiotic DSB formation, and timely oocyte determination.

Discussion

The *Drosophila* ovary is an effective system for studying stem cell–niche interactions at the molecular and cellular level (7, 8). In the *Drosophila* ovary, cap cells and anterior-row IGS cells function as the niche for controlling GSC self-renewal by activating BMP signaling and promoting E-cadherin-mediated cell adhesion (7, 44, 45), whereas posterior IGS cells promote CB differentiation into cysts partly by preventing BMP signaling (4, 5, 17, 20). Our recent used single-cell RNA analysis demonstrates that IGS cells form four linearly arranged niche compartments (IGS1–IGS4) for controlling GSC self-renewal, CB differentiation, meiotic DSB formation, and timely oocyte determination (25). However, it remains unclear whether posterior IGS cells control stepwise GSC progeny differentiation via direct signaling. In this study, we show

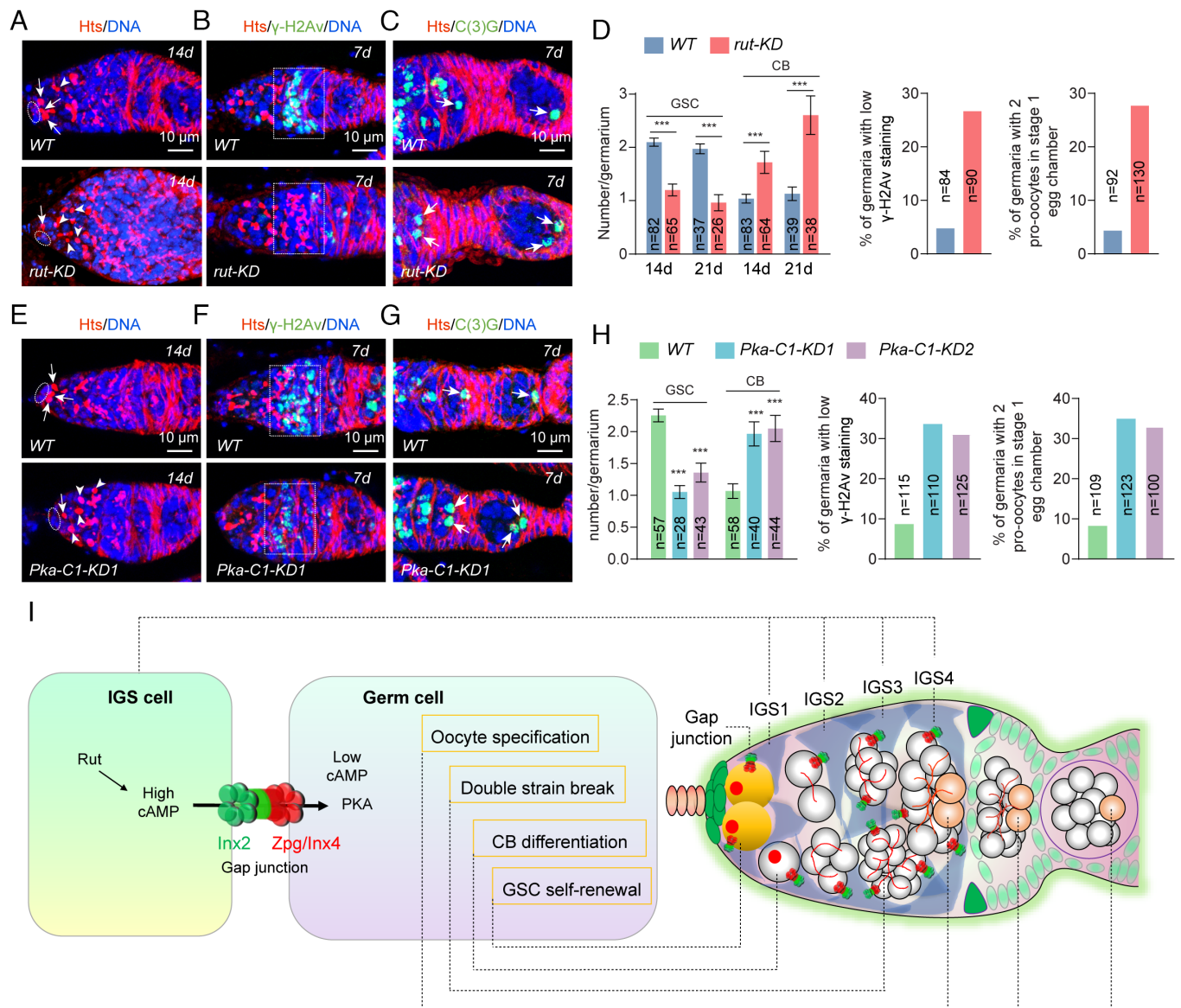


Fig. 4. Rut and Pka-C1 function in the niche and germline, respectively, to control GSC maintenance, CB differentiation, meiotic DSB formation, and pre-oocyte elimination. (A) IGS-specific *rutKD* significantly decreases GSCs and increases CB-like cells compared with the control. Cap cells, GSCs, and CBs are highlighted by ovals, arrows, and arrowheads, respectively. (B and C) IGS-specific *rutKD* decreases γ -H2Av levels in 16-cell cysts (region 2a by broken rectangle) and increases the incidence of 2 oocytes (arrowheads) in 16-cell cysts compared with the control (with one oocyte). (Scale bars, 10 μ m.) (D) Quantification results. n = number of germaria. Student's t test: $***P \leq 0.001$. (E) Germline-specific *Pka-C1-KD* significantly decreases GSCs and increases CB-like cells compared with the control. Cap cells, GSCs and CBs are highlighted by ovals, arrows, and arrowheads, respectively. (Scale bars, 10 μ m.) (F and G) IGS-specific *Pka-C1-KD* decreases γ -H2Av levels in 16-cell cysts (region 2a by broken rectangle) and increases the incidence of 2 oocytes (arrowheads) in 16-cell cysts compared with the control (with one oocyte). (Scale bars, 10 μ m.) (H) Quantification results. n = number of germaria. Student's t test: $***P \leq 0.001$. (I) A working model explaining how GJ-mediated intercellular cAMP/PKA signaling regulates stepwise GSC progeny differentiation.

that *Inx2* on IGS cells and *Zpg* on GSCs and their progeny form GJs for transporting cAMP from the niche to directly control GSC maintenance, CB differentiation, meiotic DSB formation, and timely oocyte determination. IGS-specific *Inx2* knockdown and germline-specific *zpg* knockdown disrupt GSC maintenance, CB differentiation, meiotic DSB formation, and timely oocyte specification. In addition, cAMP synthesized by Rut in IGS cells are transported to GSCs and their early progeny in an *Inx2/Zpg*-dependent manner. Furthermore, IGS-specific *rut* knockdown and germline-specific *Pka-C1* knockdown lead to similar GSC developmental defects caused by genetic ablation of GJs. Taken together, our findings suggest that GJ-mediated direct signaling from the niche to GSCs and their early progeny controls stepwise GSC development (Fig. 4I). Since GJs and the cAMP/PKA pathway are functionally conserved and widely expressed in various

stem cell systems, the regulatory strategy gained from this study could also be applicable to other stem cell systems.

IGS Cells Control GSC Maintenance, CB Formation, Meiotic DSB Formation, and Oocyte Determination Via GJ-Mediated Signaling. *Inx2* has previously been shown to be expressed and required in IGS cells for controlling cyst formation (32). In this study, we have used two independent RNAi lines to knock down *Inx2* on adult IGS cells to demonstrate that *Inx2* functions in the IGS to control GSC maintenance, CB differentiation, meiotic DSB formation, and timely oocyte determination. Similarly, *Zpg* is known to be expressed and required in germ cells to promote CB differentiation (30). Interestingly, we used two independent RNAi lines to knock down *Zpg* in GSCs and their progeny to demonstrate that *Zpg* functions intrinsically to control GSC

maintenance, CB differentiation, meiotic DSB formation, and timely oocyte determination. Excitingly, our super-resolution imaging has demonstrated that *Inx2* on IGS cells and *Zpg* on germ cells are colocalized on their juxtaposed membranes to form GJ channels. These findings have led us to propose that the IGS controls stepwise GSC development via *Inx2/Zpg*-mediated signaling (Fig. 4*I*). In the future, it will be critical to understand how GJs are dismantled and reassembled dynamically when IGS cells disengage a cyst and engage a new cyst. Although Cx43-formed GJs in the niches of hematopoietic stem cells, neural stem cells, and skin cells control stem cell maintenance (46), it remains unclear whether GJs in the niche can also control their progeny differentiation as in the *Drosophila* ovary.

Inx2/Zpg Heterologous GJs Transport cAMP from the Niche to Control Stepwise GSC Development. GJs are known to transport small molecules such as small RNAs, peptides, secondary messengers, and ions (29). For example, connexin 43-formed GJs are capable of transporting cAMP between bone cells (37). In this study, we demonstrate that *Inx2/Zpg* heterologous GJs are capable of transporting cAMP from the niche to control GSC maintenance, CB differentiation, meiotic DSB formation, and timely oocyte determination. First, cAMP produced in IGS cells by adenylyl cyclase *Rut* influences its levels in germ cells. We have used an anti-cAMP antibody and immunofluorescent staining to show that cAMP accumulates in IGS cells at higher levels than in GSCs and their early progeny, which is consistent with the expression patterns of adenylyl cyclase *Rut*. IGS-specific *rut* knockdown significantly and dramatically decreases cAMP levels in IGS cells, GSCs and their early progeny, whereas IGS-specific *rut* overexpression increases cAMP levels in both IGS cells, GSCs and their early progeny. These results indicate that *Rut* is the key adenylyl cyclase in IGS cells for synthesizing cAMP to maintain cAMP levels in GSCs and their early progeny. Second, cAMP is transported from IGS cells to GSCs and their early progeny. Knocking down *Inx2* on IGS cells and *Zpg* on germ cells can decrease cAMP levels in GSCs and their early progeny using the germline-specifically expressed Nano-lantern-cAMP reporter. In addition, blocking the function of GJs by the specific inhibitor CBX can also disrupt cAMP transport from IGS cells to GSCs and their early progeny. Corroborating with the results of IGS-specific *rut* knockdown and overexpression, these findings demonstrate that the niche directly provides cAMP for GSCs and their early progeny via GJs. Third, cAMP production in the niche is critical for controlling multiple steps GSCs and their progeny development. We have used IGS-specific *rut* knockdown to demonstrate that cAMP in IGS cells is critical for controlling GSC self-renewal, CB differentiation, meiotic DSB formation, and timely oocyte determination. Finally, PKA is the major effector of the cAMP pathway in the germline for controlling GSC maintenance and early progeny differentiation. cAMP binds to the regulatory subunits of the PKA kinase complex, which leads to the release active catalytic kinases for carrying out the biological functions (42). Germline-specific knockdown of *Pka-C1*, which is one of the catalytic subunits in *Drosophila*, retards GSC maintenance, CB differentiation, meiotic DSB formation, and timely oocyte determination. Therefore, our study has demonstrated that niche-produced cAMP directly regulates GSC maintenance, CB differentiation, meiotic DSB formation, and timely oocyte determination via GJ-mediated transport and has also reported the existence of direct signaling from the niche for controlling stepwise GSC progeny differentiation (Fig. 4*I*). In the future, we will investigate the detailed mechanisms underlying how PKA

signaling controls stepwise GSC development by identifying its protein targets.

GJs have been implicated in soma-germline and stem cell-niche interactions in various organisms. In *Drosophila*, cAMP/PKA signaling has been shown to regulate germline development by follicle cells during late oogenesis (47), whereas cAMP has also been implicated in the regulation of oocyte maturation by somatic cumulus cells in mammals (48). Since GJs exist between somatic cells and germ cells in the mammalian testis and ovary, they might be responsible for transporting cAMP from the soma to the germline. Cx43-formed GJs in stem cell-niche junctions also regulate stem cell functions in hematopoietic and nervous systems, but the GJ-transmitted signals are still missing (46). cAMP-PKA signaling is known to regulate hematopoietic stem cells (49) and neural stem cells (50). These raise the interesting possibility that the GJ-transported cAMP from the niche also regulates stem cell functions. Therefore, it will be of great interest to investigate whether GJs also transport cAMP from the soma to regulate late germ cell development and from the niche to regulate hematopoietic and neural stem cell functions in mammals.

Materials and Methods

Drosophila Culture. The following *Drosophila* stocks used in this study are described in FlyBase, unless specified: *c587-Gal4*, *13C06-Gal4*, *nos-Gal4*, *hsFlp*, *nos>stop>Gal4*, *tubulin-gal80^{ts}*, *Inx2* RNAi (BL29306 and BL80409), *zpg* RNAi (BL35607 and BL77147), *rut* RNAi (BL80468), *Pka-C1* RNAi (BL35169 and BL57743), and *UAS-G-Flamp1* (a kind gift from Yulong Li). 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 specified days before the analysis of ovarian phenotypes.

Construction of Transgenic Drosophila Stains. The bacterial recombining technology was used to introduce C-terminal insertion of Flag tag into *zpg* in the BAC clone CH321-75N13 and C-terminal insertion of GFP tag into *Inx2* in the BAC clone CH322-22F14, respectively. The clones were integrated on the second chromosome. To generate the *nos-GeneSwitch-Gal4* transgenic strain, the DNA fragments expressing GAL4-DBD, human progesterone receptor-ligand-binding domain, and P65-AD were fused together in the same reading frame and were then cloned into the *Pnos-5'UTR-eGFP-3'UTR* vector (51). To generate the *nos-Nano-lantern-cAMP* and *nos-G-Flamp1* transgenes, the DNA fragment containing *Nano-lantern-cAMP1.6* or *G-Flamp1* were cloned into the *Pnos-5'UTR-eGFP-3'UTR* vector to replace the eGFP coding sequence. The DNA constructs were integrated into the *attp40* site on the second chromosome or into the *attp2* site on the third chromosome via PhiC31-mediated transgenesis, respectively.

Immunostaining and Confocal Imaging. Immunostaining was performed according to our previous procedures (52, 53). The following antibodies were used: mouse monoclonal anti-Hts antibody (1:50, 1B1, DSHB), mouse monoclonal anti-Vasa antibody (1:50, anti-vasa, DSHB), rabbit polyclonal anti-pS137 γ -H2Av antibody (1:1,000, Rockland, #600-401-914), chicken polyclonal anti-GFP antibody (1:500, Invitrogen, #A10262), goat polyclonal anti-GFP antibody (1:500, Rockland, #600-101-215), rabbit polyclonal anti-C3G (1:10,000, gift from Lilly, NICHD/DIR), and rabbit monoclonal anti-cAMP antibody (1:200, Abcam ab134901). For rabbit polyclonal anti-Piwi antibodies, a peptide (MADDQGRRRRPLNC) from *Drosophila* Piwi was synthesized and injected into the rabbit by GeneScript USA Inc., and the Protein A/G purified IgG was used for staining (1:1,000).

All the images except STED were taken with a Leica TCS SP5 or SP8 or Nikon Ti2 confocal microscope. STED images were acquired on a Leica SP8 Gated STED Microscopy with 100 \times , 1.4 oil N.A. objective. Green channel (*Inx2*-GFP, labeled with Abberior STAR 635) was excited with a pulsed white light (80 MHz) tuned to 635 nm; red channel (*Zpg*-Flag, labeled with Alexa Fluor 594) was imaged with the same white laser but tuned to 594 nm. Both channels were depleted with a pulsed 775 nm laser at 80 to 90% maximum output. All STED images were acquired in 2D mode to maximize lateral resolution, and each image was

averaged eight times in the line average mode. Emission photons were collected with internal Leica HyD hybrid detectors with a time gate setting at 1 to 6 ns. Raw STED images were further deconvolved in Huygens Professional software (version 14.10; Scientific Volume Imaging), and a theoretical estimated point spread function was used for deconvolution. Other parameters were using the system default value except that the background was measured from raw image and signal to noise was set in the range of 15 to 20.

FISH. Hybridization chain reaction (HCR) was used to achieve high-sensitivity FISH for *Inx2*, *zpg*, or *rut* mRNA. Probe sets against *Inx2* (LOT: PRC145), *zpg* (LOT: PRD632), *vasa* (LOT: PRG380), or *rut* (LOT: PRR469) mRNAs were ordered from Molecular Instruments, Inc. Immunostaining with anti-Vas, including primary antibody incubation, secondary antibody incubation, postfixation, and dehydration of ovaries, was performed according to the previous publication prior to in situ hybridization. Then, standard steps following the HCR v3.0 protocol for whole-mount fruit fly embryos were as we did previously (25).

GJ Inhibitor CBX Treatment and Live Imaging. CBX, which was used at a 2×10^{-4} g/mL concentration in the Grace medium, was added to newly dissected ovaries for 15 min to maximize blockage efficiency. Control groups were added to pure Grace Medium for the same amount of time. After treatment, ovaries were mounted in PBS and imaged within 10 min.

Quantification and Statistical Analysis. GSCs and CBs were quantified according to our previous studies (52, 53). Briefly, the spectrosume-containing

single germ cells attached to cap cells were counted as GSCs, whereas those spectrosume-containing single germ cells, which are not attached to cap cells, were counted as CBs under the fluorescent microscope. The statistical analysis was done using Microsoft Excel or GraphPad Prism 7 with Student's *t* test method. *P* values are indicated in figure legends, and the results are presented as mean or mean \pm SEM ($***P \leq 0.001$; $**P \leq 0.01$; $*P \leq 0.05$; n.s., no significance).

Data, Materials, and Software Availability. Original data underlying this manuscript can be accessed from the HKUST's public depository at <https://data-space.hkust.edu.hk/bib/V12GOP> (54). For the reagents, please contact Ting Xie (tgx@ust.hk) or Renjun Tu (renjuntu@ust.hk).

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