Wnt Signaling-Mediated Redox Regulation Maintains the Germline Stem Cell Differentiation Niche

by

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Abstract

To remain self-renewal or to differentiate, this is one of the most important question in the stem cell field. Both stem cell maintenance and differentiation are regulated by intrinsic and extrinsic signals from surrounding stromal cells or niche. The *Drosophila* ovary is a simple but elegant model to study stem cells and their niche. At the tip of *Drosophila* ovary, a cluster of somatic cap cells form the germline stem cell niche and provide self-renewal signal to maintain GSC self-renewal. When GSC divides, its daughter cell, cystoblast leaves the GSC niche and undergoes differentiation. Outside the self-renewal niche, inner sheath cells are required for germ cell differentiation by functioning as a germ cell differentiation niche.

Compared to the self-renewing niche, relatively little is known about the maintenance and function of the differentiation niche. Here we show that the cellular redox state regulated by Wnt signaling is critical for the maintenance and function of the differentiation niche to promote germ cell differentiation. Defective Wnt signaling results in the loss of the differentiation niche, and thus a germ cell differentiation defect. Mechanistically, Wnt signaling controls the expression of multiple *glutathione-S-transferase* family genes and the cellular redox state. Finally, *Wnt2* and *Wnt4* function redundantly to maintain active Wnt signaling in the differentiation niche. Therefore, this study has revealed a novel strategy for Wnt signaling in regulating the cellular redox state and maintaining the differentiation niche.

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List of Abbreviations

TF: terminal filament

CPC: cap cell

GSC: germline stem cell

CB: cystoblast

ISC: inner sheath cell

FSC: follicle stem cell

FC: follicle cell

FS: fusome

DC: developing cyst

SS: spectrosome

BMP: bone morphogenetic protein

Mad: mothers against dpp

Tkv: thick veins

Shg: shotgun

Arm: armadillo

Dsh: dishevelled

Axn: axin

Sgg: shaggy

TCF: T-cell factor

Fz: frizzled

Dpp: decapentaplegic

Gbb: glass bottom boat

Bam: bag-of-marbles

Dad: daughters against dpp

Pum: pumilio

Nos: nanos

Upd: unpaired

GST: glutathione-S-transferase

ROS: reactive oxygen species

DHE: Dihydroethidium

List of Publications

Wang S, Gao Y, Song X, Ma X, Zhu X, Mao Y, Yang Z, Ni J, Li H, Malanowski K, Anoja P, Park J, Haug J, Xie T: Wnt Signaling-Mediated Redox Regulation Maintains the Germline Stem Cell Differentiation Niche. *eLife*. 2015 Oct 9; 4. doi: 10.7554/eLife.08174

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Chen S, **Wang S**, Xie T: Restricting self-renewal signals within the stem cell niche: multiple levels of control. Curr Opin Genet Dev. 2011 Dec; 21(6):684-9.

Manuscript in Revision

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Ma X, Do T, Song X, **Wang S**, Weng C, Li H, Story B, Blanchette M, Gogol M, Peak A, Anoja P, Xie T. Aubergine and Bam Cooperatively Control Germline Stem Cell Lineage Differentiation and piRNA-Mediated Transposon Repression.

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Chapter 1: Introduction to Dissertation

The *Drosophila* Ovary is an Effective Model System to Study Stem Cell Regulation

Most adult tissues are maintained by stem cell throughout whole lifetime such as blood, testis, skin and β cells [1-3]. Stem cells can proliferate continuously to replenish the lost cells throughout the lifetime. Adult stem cells are maintained in a niche to remain self-renewal [1, 2]. The stem cell daughter cell undergoes differentiation while leaving the self-renewal niche. Loss of stem cells leads to malfunction or degeneration of the whole tissue or organisms [4, 5] while the over proliferation or differentiation defects of stem cells causes tumor formation [6, 7]. The mechanism that controls the balance of self-renewal and differentiation of stem cells helps find a potential way to cure tissue degeneration or cancer [8-10].

It has been 15 years since the GSC and its niche were first studied in the *Drosophila* ovary[11]. Sophisticated genetic tools make it convenient to study stem cells in *Drosophila* ovary. The FLP-mediated FRT stem cell marking system is used to generate marked clones and determine the effect of a mutation on the maintenance, proliferation and differentiation of different types of cells [12-14]. Moreover, the GAL4-UAS system can be used to manipulate specific gene expression levels in particular cells or even particular time points by overexpression, knockdown or knockout [15]. And with fluorescent protein labeled cells, we can perform live imaging, FACS and the followed microarray or RNA-Seq analysis.

The *Drosophila* female has two ovaries (Figure 1.1), and each ovary contains 12-16 ovarioles. The most anterior part of the ovariole is germarium. The tip of germarium contains two types of somatic cells, TFs and CPCs. Two or three GSCs are anchored to CPCs posteriorly. The GSC generates two new daughter cells while dividing. If the daughter cell stays at the niche,

it remains a GSC, while the daughter cell leaving the niche undergoes differentiation into a CB. The CB divides four times without complete cytokinesis, and forms a 2-, 4-, 8- or 16-cell cyst. During this process, these differentiated germ cells interact with another population of somatic cells, ISCs. And then, 16-cell cysts are wrapped up by s, which are produced by FSCs.

The Self-renewal Niche in the *Drosophila* Ovary

The GSC self-renewal niche is responsible for stem cell maintenance (Figure 1.2). Notch signaling regulates niche formation and maintenance. When TF cells are newly formed at the late instar larvae stage, they express a Notch ligand, Delta, to activate Notch signaling in adjacent somatic cells and turn them into CPCs [16]. To expand Notch signaling in the somatic precursor cells leads to increased CPC number or ectopic CPCs [16, 17]. These extra CPCs expand the GSC niche so that increased CPCs leads to more GSCs and ectopic CPCs will result in ectopic GSCs [16, 17]. Notch signaling still function in adult stage [16]. Loss of Notch signaling in the adult stage causes CPC loss and further GSC loss [16]. Notch expression is regulated by brain-derived insulin as well as ecdysone signaling [18, 19]. Therefore, Notch signaling is required for GSC niche formation and maintenance under control of insulin and ecdysone signaling.

The self-renewal Niche provides signals to maintain GSCs by repressing differentiation. TFs produce Upd to activate Jak/Stat signaling in CPCs and anterior ISCs, further inducing two Bmp ligand production, Gbb and Dpp [20, 21]. BMP activates the tetrameric receptor complex formed by two type I receptors Tkv and two type II receptors Punt and Sax [22, 23]. The constitutively active type II receptor Punt phosphorylates the Tkv receptor results in production of phosphorylated Mad (pMad) [24, 25]. The p-Mad forms a complex with Medea and then

enters the nucleus to regulate gene transcription. The P-Mad-Medea complex represses the transcription of master differentiation gene *bam*, preventing GSCs from differentiation [26-28]. On the other hand, this complex activates the transcription of *Dad*, which in turn inhibits BMP signaling by either targeting active receptors for degradation or by competitive binding to SMAD-responsive elements [29]. BMP, unlike the wing disk or embryo, functions as a short-range niche signal. Consequently, BMP is not strong enough to inactivate bam expression, which promotes differentiation in CBs and other germline cyst cells outside of GSC niche. To keep BMP inside of the GSC niche is required for GSC asymmetric division, so it must be correctly regulated by multiple mechanisms. Dally, a glypican family protein, accumulates at high levels around the CPCs. Dally binds and stabilizes Dpp to activate BMP signaling [30, 31].

Piwi and Yb in TF cells and CPCs are essential for GSC maintenance. *Yb* encodes a novel protein and *piwi* encodes a highly conserved protein involved in piRNA production [32-38]. Yb regulates Hedgehog (Hh) production in niche cells, which functions redundantly with a Piwi-regulated pathway in GSC regulation [39]. In addition, the Piwi-regulated niche signal maintains GSCs by repressing *bam* transcription. When piwi is removed from the niche cells, *bam* expression is derepressed in GSCs [40]. Corto, a chromatin-associated protein [41], is identified to antagonize Piwi function in niche cells so it is likely that Piwi cooperates with Corto to control niche signal production via regulating piRNA production or chromatin remodeling.

Intrinsic factors also function to regulate GSC maintenance in parallel with extrinsic signals. In GSCs, differentiation factors are repressed by regulatory complex which contains Pumilio (Pum) and Nanos (Nos) [42-47]. In addition, eIF4A, the translation initiation factor, also

maintains GSC self-renewal and proliferation via repressing Bam and promoting E-cadherin expression directly[48]. Pelota, a translation release factor-like protein is essential for GSC self-renewal and proliferation by repressing a Bam-independent pathway [49]. Interestingly, microRNAs, which regulate gene expression by affecting mRNA stability or translation, are also essential for controlling GSC self-renewal [50]. All together, these data demonstrate a vital role of translational regulation in maintaining GSC self-renewal.

Adherens junctions play an important role in GSC maintenance. At the tip of germarium, GSCs are anchored to the niche via E-cadherin [51]. E-cadherin is accumulated at the anterior side of the GSC to form adherens junctions with CPCs. GSCs have to adhere to the CPCs strongly to avoid being expelled from the self-renewal niche. E-cadherin deficiency in GSCs leads to their loss. Interestingly, GSCs with E-cadherin overexpression can push wild-type GSCs out of niche, which indicates that E-cadherin is required for GSC maintenance via a niche occupancy competition mechanism [52]. E-cadherin levels in germ cells are regulated by *bam*. In GSCs, whereas differentiation-promoting mRNAs are repressed by the Pum-Nos complex. Outside GSC niche, *bam* expression is up-regulated, and Bam protein associates with the Benign Gonial Cell Neoplasm (Bgcn) and other proteins to inhibit E-cadherin mRNA translation and further activate differentiation. The Bam/Bgcn repressive complex binds to the 3'UTR of E-cadherin to repress its expression in CBs [48]. As expected, β-catenin, encoded by *armadillo*, a partner of E-cadherin, is required for GSC niche anchorage [53]. Rab11, a regulator of recycling endosomes, is required for controlling GSC self-renewal possibly via regulation of E-cadherin accumulation [54].

The Differentiation Niche in the *Drosophila* Ovary

In the germarium, differentiated germ cells, from CBs to 16-cell cysts, interact with ISCs (Figure 1.1, Figure 1.2). Previously, anterior ISCs contacting CPCs are proposed to generate all ISCs via a stem cell mechanism like cyst stem cells in the testis [12]. However, the results from long-term live imaging and clone analysis all indicate that lost ISCs are replenished by neighboring ISCs rather than stem cells [55, 56]. ISCs are different from cyst cells in the testis that accompany germ cells moving posteriorly, because they remain at the same place without moving [55, 56].

Interestingly, ISCs form long cellular processes, based on the size and morphology of germ cells they interact with, to surround and separate each single germ cell cluster [56]. When Rho1 function is disrupted in ISCs to inhibit long cellular processes formation, germ cell differentiation is also blocked due to BMP signaling expansion, suggesting that ISCs' normal morphology is necessary for germ cell differentiation by restricting BMP signaling activity [56]. Reversely, if germ cell differentiation is blocked by *bam* mutation or *dpp* overexpression, long cellular processes are disrupted [56]. Therefore, EC long cellular morphology and germ cell normal differentiation require and dependent on each other.

ISC population is required to promote germ cell differentiation. Two recent studies showed that a H3K9 methyltransferase *eggless* (*egg*) and piRNA components *piwi* are required for ISC population maintenance [57, 58]. ISC loss leads to germ cell differentiation defects by increasing BMP signaling in the differentiation niche [56-58].

Previous studies discovered multiple molecular mechanisms in ISCs to govern GSC lineage differentiation. EGFR-MAPK signaling has been shown to function in ISCs to restrict BMP signaling inside the GSC niche by repressing Dally production [59, 60]. Forced *dally*

expression or disrupting Egfr or Map kinase components in ISCs causes BMP to increase in the differentiation niche, blocking CB from differentiation [60]. Lsd1 functions in ISCs to repress dpp transcription to restrict Bmp signaling [61]. Egg and Vreteno regulate piRNA production in ISCs and regulate CB differentiation by controlling BMP signaling [57, 61, 62]. Ecdysone signaling also functions to regulate CB differentiation via regulating E-cadherin level in ISCs to modulate ISC-CB interaction [63].

Gap junctions form between differentiated germ cells and ISCs. The progeny of mutant GSCs for *zero population growth* (*zpg*), encoding the gap junction protein innexin-4, cannot differentiate normally [64], probably due to the failure to transport necessary signals from their surrounding ISCs.

All of the above findings indicate that ISCs help to prevent self-renewal signaling and provide signals for CB differentiation, functioning as germ cell differentiation niche. More detailed mechanisms the differentiation niche operates by remain to be discovered in the future.

Canonical Wnt Signaling

The Wnt signaling pathway is one of the evolutionarily-conserved signal transduction pathways during animal development, from Hydra to humans [65-68]. Wnt signals control the proliferation, fate specification, polarity, and migration of cells throughout development. In addition, overactivation of Wnt signaling is a major factor in oncogenesis in the human colon and other tissues [69]. Studies in *Drosophila* and vertebrates have shown that Wnt signals are transduced in at least two distinct ways; a 'canonical' β-catenin dependant pathway, and a noncanonical β-catenin independent pathway.

Transcription factor β-catenin is the major effector of the canonical Wnt signaling pathway (Figure 1.3). In the absence of ligand, cytoplasmic β-catenin interacts with APC and Axin scaffold proteins and is a substrate for the kinases CKI and GSK3β. Phosphorylated β-catenin is then ubiquitinated and destroyed by the proteosome. When Wnt ligand binds to a Fz family receptor and a coreceptor of the LRP-5/6/arrow family, the APC/Axn/CK1/GSK3β destruction complex is inhibited, the stabilized β-catenin is translocated to the nucleus where it interacts with TCF/LEF family transcription factors. In the absence of Wnt signal, TCF/LEF factors repress Wnt-responsive genes by interacting with co-repressors. The β-catenin/TCF/LEF complex activates target gene expression.

ROS and Oxidative Stress

ROS are chemically reactive molecules containing oxygen which is natural byproduct of the normal metabolism of oxygen. They have important roles in cell signaling and homeostasis. At high concentrations, ROS is hazardous for living organisms and damage all major cellular constituents. At moderate concentrations, ROS play an important role as regulatory mediators in signaling processes. Many of the ROS response genes protect the cells against oxidative stress and help reestablish "redox homeostasis." In tissues or organisms, different oxidative stress response genes co-operatively function together to protect cells from ROS-induced damage (Figure 1.4). Superoxide dismutases (SODs) are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. SOD is an important antioxidant defense in nearly all cells exposed to oxygen. GSTs comprise a family of eukaryotic and prokaryotic phase II metabolic isozymes best known for their ability to catalyze the conjugation of the reduced form of glutathione (GSH) to consume peroxide in oxidative stress defense. Catalase (Cat) is

also a very important enzyme in protecting the cell from oxidative damage by consuming peroxide.

Figure 1.1 *Drosophila* ovary and germarium

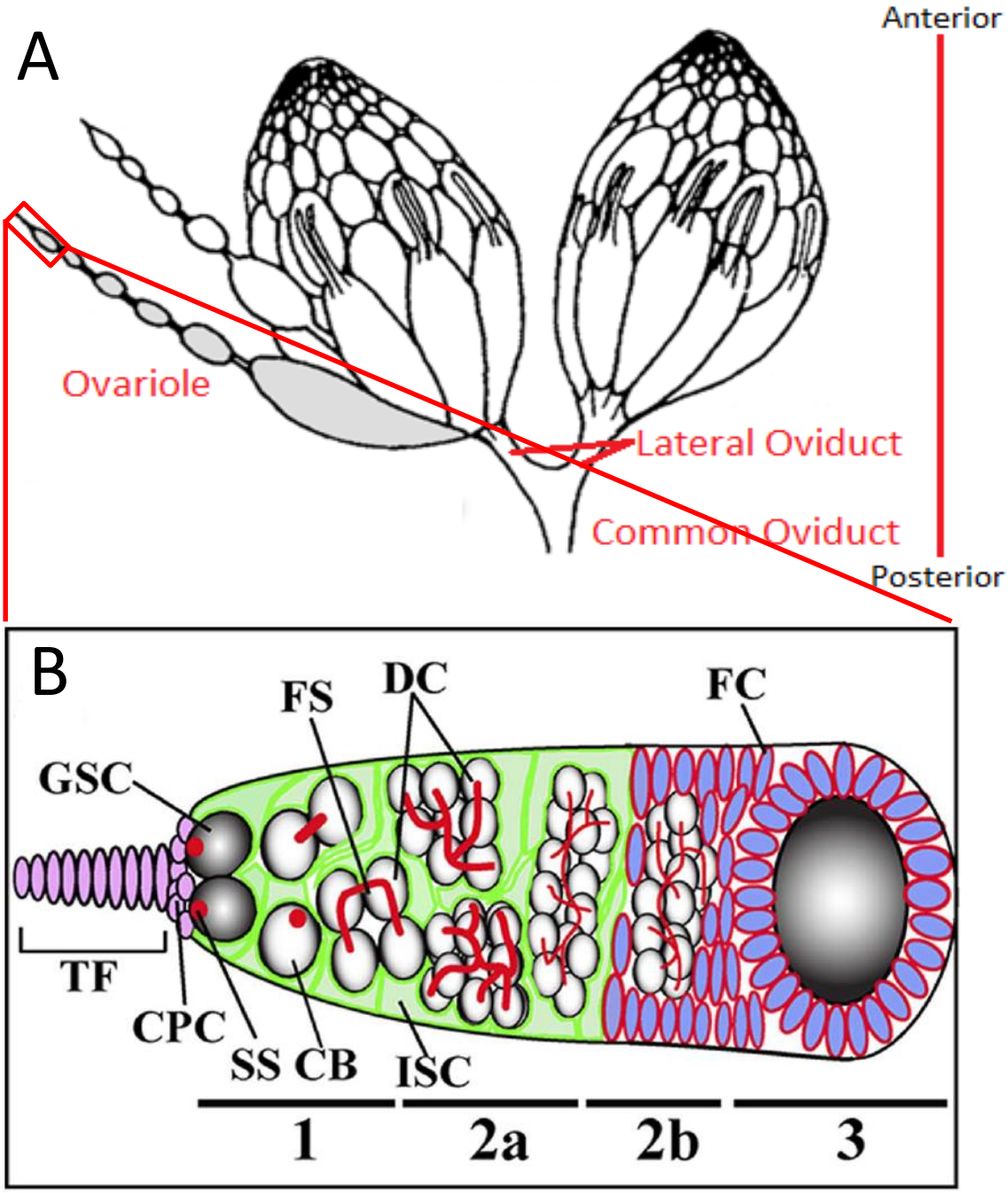


Figure 1.2 Self-renewal and differentiation niche in *Drosophila* ovary

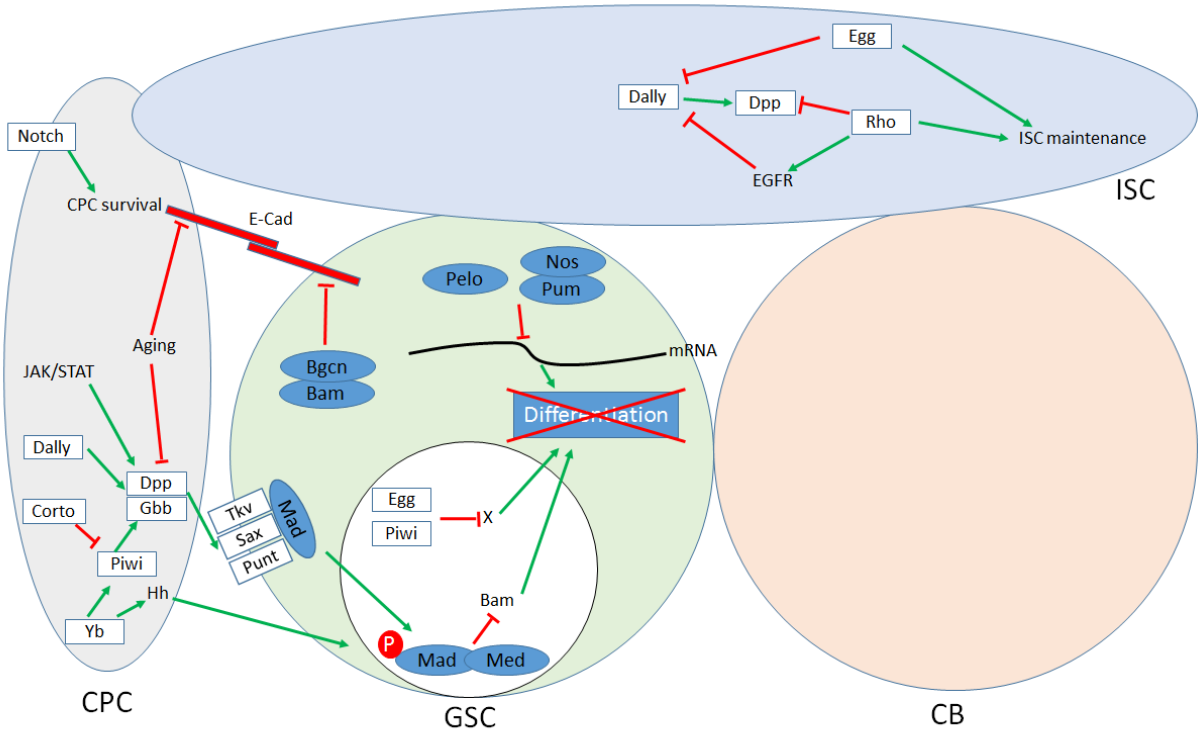


Figure 1.3 Canonical Wnt signaling pathway

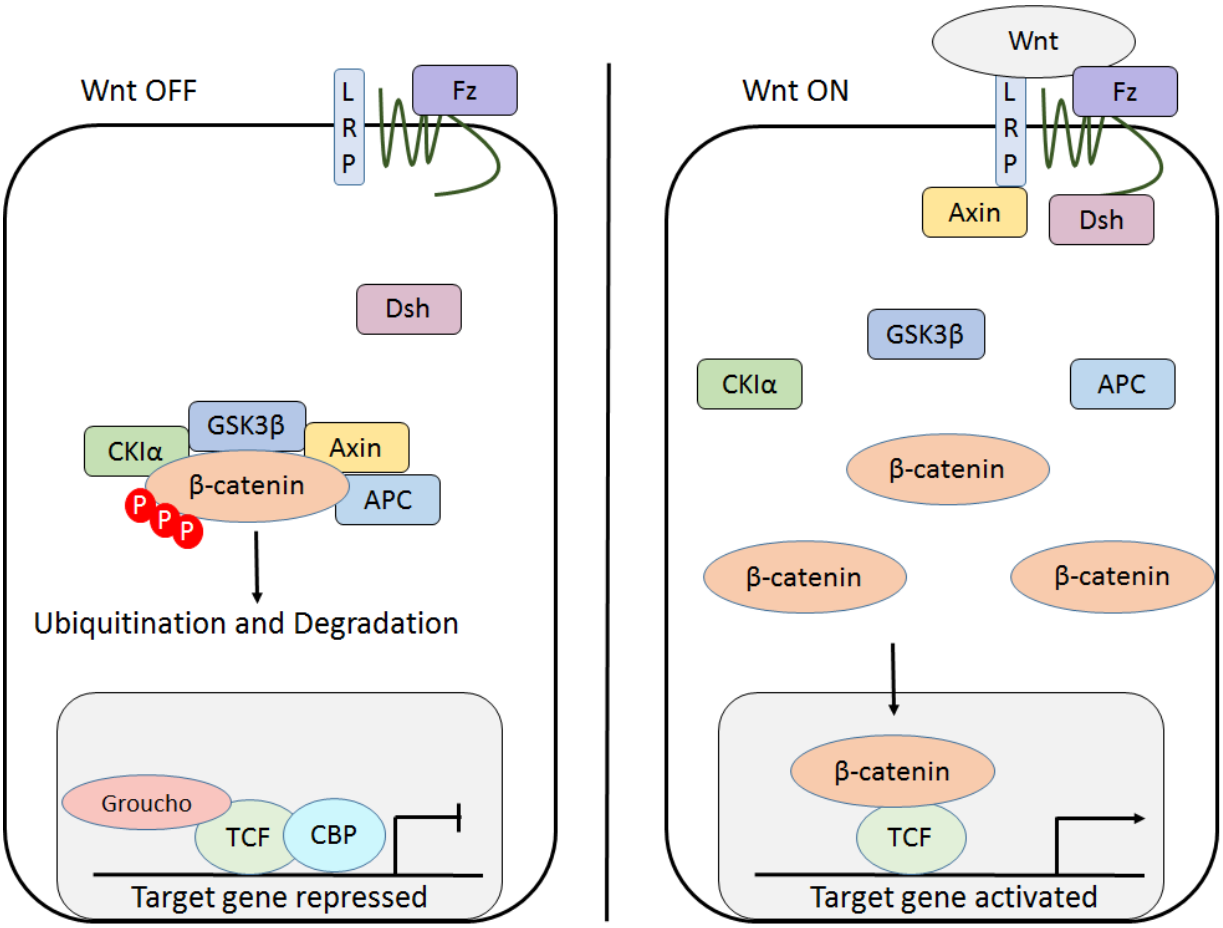
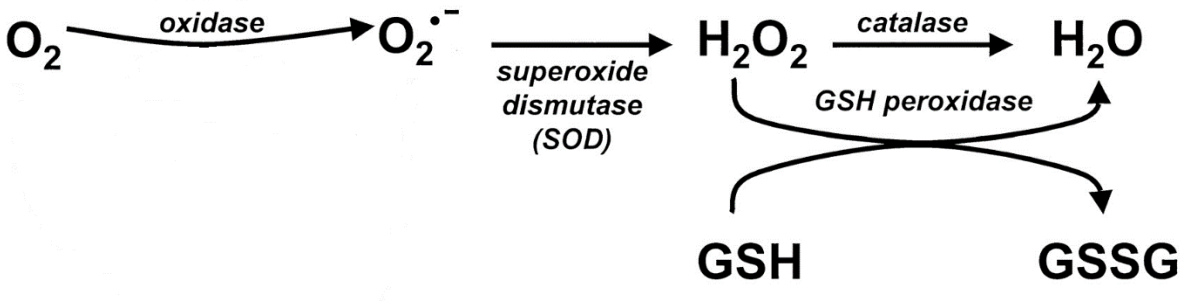


Figure 1.4 Pathways of ROS production and clearence



Chapter 2: Material and Method

Drosophila Strains

The *Drosophila* stocks used in this study include: c587 [70], *PZ1444/Cyo* [70], *UAS-SOD1/TM3,sb* [71], *UAS-axn/TM3,sb* (*w*; KrIf-1/CyO; P{UAS-Axn.GFP}3/TM3, Sb* BL7225)[72], *UAS-sgg* (*w1118; P{UAS-sgg.S9E}2* BL6818), *armRNAi-1/TM3, Ser* (*y^1 v^1; P{TRiP.JF01251}attP2/TM3, Ser^1* BL31304) ; *armRNAi-2* (*y^1 v^1; P{TRiP.JF01252}attP2* BL31305), *dshRNAi-1* (*y^1 v^1; P{TRiP.JF01253}attP2* BL31306), *dshRNAi-2* (*y^1 v^1; P{TRiP.JF01254}attP2* BL31307), *axnRNAi* (*y^1 v^1; P{TRiP.HM04012}attP2* BL31705), *sggRNAi* (*y^1 sc* v^1; P{TRiP.GL00277}attP2* BL35364), *Wnt2RNAi-1* (*y^1 sc* v^1; P{TRiP.HMS01613}attP2* BL367220) [73], *Wnt2RNAi-2* (TH00483), *Wnt2RNAi-3* (TH00484) and *Wnt4RNAi-1* (*y^1 v^1; P{TRiP.JF03378}attP2* BL29442), *Wnt4RNAi-2* (TH00485), *Wnt4RNAi-3* (TH00486). TH lines for *Wnt2* and *Wnt4* were constructed for this study according to the published procedure [74]; the coding region of Gst2 and Cat were cloned into a UASp vector to generate *UAS-Gst2* and *UAS-Cat* using standard molecular biology techniques. *Drosophila* strains were maintained and crossed at room temperature on standard cornmeal/molasses/agar media unless specified. To maximize the RNAi-mediated knockdown effect, newly enclosed flies were cultured at 29 ^{o}C for a week before the analysis of ovarian phenotypes.

Immunostaining and Confocal Imaging

Immunohistochemistry was performed according to our previously published procedures [75]. 10-50 flies are dissected in Grace's Medium (Sigma; G1842). Then fix samples with 4% paraformaldehyde in 1xPBS for 15 minutes. Wash samples three times and 15 minutes each

time. Block with 5% normal goat serum in 1xPBS+0.1% Triton X-100 for one hour. Incubate with primary antibody in 1xPBS overnight. Wash samples four times and 15 minutes each time. Block for 1 hour. Then incubate with secondary antibody overnight. Incubate samples with 1% DAPI in 1xPBS+0.1% Triton X-100 for 15 minutes. Wash five times and 15 minutes each time. Preserve samples in Vectashield mounting medium. The following antibodies were used in this study: rabbit polyclonal anti-galactosidase antibody (1:100, Cappel), mouse monoclonal anti-Hts antibody (1:50, DSHB), Guinea pig polyclonal anti-Piwi antibodies (1:100; provided by H. Lin), rabbit monoclonal anti-pS423/425 Smad3 antibody (1:100, Epitomics) and rat monoclonal anti-Vasa antibody (1:50, DSHB). All images were taken with a Leica TCS SP5 confocal microscope.

For BrdU incorporation, female flies were fed on food with yeast paste containing BrdU (10 mg/ml) for three consecutive days, and then on food with BrdU-free yeast paste for 7 days. The ovaries were then fixed and processed for BrdU label detection along with other protein markers according to our previously published procedures [51]. Samples are fixed in 4% polyformaldehyde in 1xPBS for 15 minutes. And fix an additional 15 miutes in 4% polyformaldehyde in 1xPBS+0.6%Triton X-100. Wash twice in DNaseI buffer and 5 minutes each time. Incubate samples for one hour at 37 ^{o}C in DNaseI (Roche; 4716728001, 10 units/ml). Wash twice with 1xPBS+0.3% Triton X-100. Incubate overnight in BrdU primary antibody (1:100, Becton-Dickinson). The rest procedure is the same as normal immunohistochemistry.

TUNEL labeling was performed with Apoptosis Detection Kit (Milipore; S7110). Samples are fixed with 4% polyformaldehyde in 1xPBS. Wash ovaries three times with 1xPBS+0.1% Triton X-100, 15 minutes each time. Add 150 µl Equilibration Buffer for 2 minutes, then remove it. Incubate samples with 50 µl Working Strangth TdT Enzyme for 1 hour at 37 ^{o}C. Apply

Stop/Wash Buffer and agitate for 15 seconds. Incubate for 10 minutes at room temperature. Wash three times with 1xPBS+0.1% Triton X-100, 5 minutes each time. Apply Working Strength Anti-Digoxigenin Conjugate for 30 minutes at room temperature avoiding light. Wash four times with 1xPBS and 2 minutes each time. Block samples for 1 hour with 5% normal goat serum in 1xPBS+0.1% Triton X-100. Then incubate overnight with primary antibodies. The rest procedure is the same as normal immunohistochemistry.

For DHE staining, according to the published procedure [53], ovaries are dissected and incubated in Grace's medium at room temperature to allow optimal respiration. Reconstitute 1mg DHE dye (Invitrogen; D11347) right after dissection and immediately before use in 100 µl anhydrous DMSO. Dissolve 1 µl of the reconstituted dye in 1ml Grace's Medium to give a final concentration of approximately 30uM. Vortex to evenly disperse the dye. Incubate the tissue with the dye for 3 to 7 minutes in a dark chamber, on an orbital shaker at room temperature. Perform three 5-minute washes in Grace's Medium in a dark chamber, on an orbital shaker at room temperature. Fix slightly for 4 to 8 minutes in 7% formaldehyde in 1xPBS. Rinse once in 1xPBS right after fixation. Mount immediately in Vectashield. At last capture images immediately using a confocal microscope.

ISCs purification and RNA-Seq

Three hundred pairs of Drosophila ovaries were dissected for each replicate and placed in Grace's medium (Sigma-Aldrich; G9771) and then washed twice by adding 1X DPBS and centrifuged at 700xg for one minute. The ovaries were incubated with pre-warmed Collagenase (Worthington, Collagenase Type II, Lot# 50D11833) in 15ml conical tube at 37˚C water bath for

three minutes with gentle shaking. Enzyme reaction was stopped after three minutes by adding cold 1X DPBS + 2% FBS. 35. Dissociated sample was washed by adding 1X DPBS and centrifuged at 700xg, 4 ˚C for five minutes. The cell pellet was re-suspended in 1X DPBS and filtered with 70um Filcon (BD; 340605) in to 5ml flow tubes. 10k-200k ISCs can be harvested after sorting, which depends on samples' phenotype. Cells were centrifuged and then re-suspended in 200ul of 1X DPBS for sorting at 45psi with 70um tip (BD; InFlux) immediately in to TRIzol (life technologies; 15596-018). Total RNAs were extracted with Trizol and purified by organic extraction followed by isopropanol precipitation. These RNA samples were sent to Molecular Biology Facility in Stowers Institute for further processing. Following manufacturer's directions and using the Illumina TruSeq library construction kits (Illumina, Cat. No. RS-122-2001/2), mRNA was isolated from 150ng (100 ng for minimum amount) of total RNA per sample and short fragment libraries were constructed. The resulting libraries were purified using Agencourt AMPure XP system (Backman Coulter, Cat. No. A63880), and were then quantified using a LabChip® GX (PerkinElmer) and a Qubit Fluorometer (Life Technologies). All libraries were pooled, re-quantified and run as 50bp single-end lanes on an Illumina HiSeq 2500 instrument, using HiSeq Control Software 2.0.5 and Real-Time Analysis (RTA) version 1.17.20.0. Secondary Analysis version CASAVA-1.8.2 was run to demultiplex reads and generate FASTQ files.

Statistical analysis

All data statistical analysis are processed in Excel. The error bars in all bar figure indicate the standard deviation. And all P-values are calculated by 2-tailed type-1 Student's t-test.

Chapter 3: Wnt Signaling-Mediated Redox Regulation Maintains the Germline Stem Cell Differentiation Niche

Abstract

Adult stem cells continuously undergo self-renewal and generate differentiated cells. In the *Drosophila* ovary, two separate niches control GSC self-renewal and differentiation processes. Previous researches focused more on the GSC niche than the differentiation niche. It is still relatively unclear that how differentiation niche, which is formed by ISCs, functions and is maintained. In this study, we show that the cellular redox state regulated by Wnt signaling is criticle for the maintenance and function of the differentiation niche to promote germ cell differentiation. Defective Wnt signaling leads to the loss of the differentiation niche, and thus a germ cell differentiation defect. Wnt signaling controls the expression of multiple *glutathione-S-transferase* family genes, and thus the cellular redox state. *Wnt2* and *Wnt4* function redundantly to maintain Wnt signaling activity in the differentiation niche. Therefore, this study has revealed a novel strategy for Wnt signaling to regulate the cellular redox state and maintain the differentiation niche.

Introduction

Stem cells have two important properties, self-renewal and differentiation, which are critical for continuously generating new functional cells to maintain tissue homeostasis. The self-renewal property is controlled in various stem cell systems by interplays between signals from the niche and intrinsic factors [1, 2, 76]. Germline stem cells (GSCs) in the *Drosophila* ovary

and testis are attractive systems for studying stem cell self-renewal at the molecular and cellular level [77, 78]. Although stem cell differentiation was widely thought to be a developmentally default state, we have recently proposed that GSC lineage differentiation is also controlled extrinsically by a differentiation niche formed by ISCs. However, it remains unclear how the maintenance and function of the differentiation niche are regulated at the molecular level. In this study, we show that autocrine Wnt2/4 signaling maintains the differentiation niche by regulating ISC proliferation and survival via redox regulation.

In the *Drosophila* ovary, two or three GSCs at the tip of the germarium, the most anterior region of the *Drosophila* ovary, continuously self-renew and generate differentiated GSC daughters, CBs. The CBs further divide four times synchronously with incomplete cytokinesis to form 2-cell, 4-cell, 8-cell or 16-cell cysts [79]. GSCs and their differentiated progeny can be reliably identified by their unique morphology of germline-specific intracellular organelles known as fusomes: GSCs and CBs contain a spherical fusome known as the spectrosome, whereas differentiated germ cell cysts contain a branched fusome [80]. GSCs can be reliably distinguished from CBs by their direct contact with cap cells (Figure 3.1A). Cap cells function as the self-renewing niche to maintain GSCs by activating BMP signaling and maintaining E-cadherin-mediated cell adhesion [51, 75, 81]. In addition, various classes of intrinsic factors work with BMP signaling and E-cadherin to control GSC self-renewal [77]. Therefore, GSC self-renewal is controlled by coordinated functions of niche-initiated signaling pathways and intrinsic factors.

Following GSC division, differentiating GSC daughters, CBs, are always positioned away from the self-renewal niche. ISCs sit on the surface of the germarium to send their cellular processes to wrap up underneath CBs, mitotic cysts and early 16-cell cysts, which move

posteriorly [55, 70, 82]. Our recent study suggests ISCs and their associate long cellular processes act as the differentiation niche to promote GSC progeny differentiation in the *Drosophila* ovary because disrupting long ISC processes leads to an accumulation of CB-like cells, indicative of a germ cell differentiation defect [70]. A series of genetic studies have further supported the existence of the differentiation niche.

The epidermal growth factor (EGF) signaling pathway is active in ISCs to promote GSC lineage differentiation partly by repressing *dally* expression [60, 83]. In addition, Rho signaling is also required in ISCs to promote GSC differentiation partly by repressing *dally* and *dpp* expression. *dally* encodes a proteoglycan protein, which is capable of promoting Dpp/BMP diffusion to the differentiation niche [30, 31]. Ecdysteroid signaling also operates in ISCs to promote germ cell differentiation because inactivating ecdysteroid receptors EcR and Usp in ISCs disrupts cyst formation [84]. One potential mechanism is that ecdysteroid signaling controls the formation of ISC cellular processes, thereby promoting the interaction between ISCs and germ cells [85]. Gap junction protein Inx2 functions in ISCs to promote germ cell differentiation, but its transmitted substances between ISCs and germ cells remain identified [86]. The importance of gap junctions between ISCs and germ cells could also explain why ISC cellular processes are important for germ cell differentiation. Therefore, physical interactions and signaling-mediated communications between ISC cellular processes and GSC progeny likely contribute to GSC progeny differentiation collectively.

In addition, chromatin regulators are also important in ISCs to promote GSC differentiation. Eggless, a *Drosophila* H3K9 trimethyltransferase, maintains ISCs and represses *dally* and *dpp* expression in ISCs, thereby promoting germ cell differentiation [57]. Similarly, Piwi functions in ISCs likely as a chromatin regulator to control germ cell differentiation partly

by repressing *dpp* expression [58, 87]. dBre1 (a E3 ubiquitin ligase) and dSet1 (a H3K4 trimethylase) together control H3K4 trimethylation in ISCs and promote germ cell differentiation partly by limiting BMP signaling from the differentiation niche [88]. The potential chromatin factor Without children (Woc) maintains the ISC-germ cell physical interaction via regulation of Stat-Zfh1 [89]. The histone demethylase Lsd1 is required in ISCs to promote germ cell differentiation by maintaining ISC survival, maintaining ISC morphology and preventing BMP signaling from the differentiation niche [90, 91]. Therefore, ISCs function as the differentiation niche by preventing BMP signaling and direct communication.

A recent study showed that tyrosine kinase Btk29A maintains Wnt signaling in ISCs by phosphorylating *Drosophila* β-catenin homolog Armadillo (Arm) [92]. It also argues that Wnt4 activates Wnt signaling to maintain Piwi expression and repress E-cadherin expression in ISCs, thereby promoting germ cell differentiation. In contrast, this study has demonstrated that both ISC-expressing Wnt2 and Wnt4, but not Wnt4 alone, act through known Wnt pathway components to maintain active Wnt signaling, promoting germ cell differentiation. More importantly, we show that Wnt signaling is required to maintain ISCs by promoting ISC survival and proliferation. Surprisingly, Wnt signaling is dispensable for Piwi expression. Instead, we demonstrate that Wnt signaling controls the expression of four *Gst* genes to maintain the reduced redox, thereby promoting ISC maintenance and germ cell differentiation. Finally, knocking down one of the *Gst* gene, *GstD2*, in ISCs leads to the germ cell differentiation defect. Therefore, our study has revealed a novel mechanism autocrine Wnt signaling utilizes to maintain ISCs and promote germ cell differentiation.

Results

Canonical Wnt signaling is required in the differentiation niche to promote germ cell differentiation

To identify the genes that function in ISCs to promote germ cell differentiation, we used *c587*-driven *UAS-RNAi* expression to knock down individual genes in ISCs. *c587* is a GAL4 line that is specifically expressed in ISCs and early follicle cell progenitors based on *UAS-GFP* expression [28] (Figure 3.1B). To facilitate the identification of GSCs and differentiated germ cells, spectrosomes and fusomes are labeled by Hts staining [80], and germ cells are visualized by Vasa staining [93]. In contrast to the control germarium containing one or two CBs, knocking down Wnt downstream genes *armadillo* (*arm*) and *disheveled* (*dsh*) in ISCs leads to an accumulation of many sptrosome-containing CB-like cells (collectively referred to single germ cells at least one cell diameter away from cap cells) (Figure 3.1C and 1D). Based on the fact that control germaria rarely contain three CBs, the germaria containing four or more CBs are considered to exhibit a germ cell differentiation defect. Over 90% of *arm* and *dsh* knockdown germaria (*armKD1* and *dshKD1*) exhibit the germ cell differentiation defect (Figure 3.1E). Similarly, *c587*-driven expression of the RNAi lines against different *arm* or *dsh* sequences (*armKD2* and *dshKD2*) also generates the similar germ cell differentiation defect (Figure 3.1E). In *Drosophila*, Wnt ligands bind the receptor complex composed of Frizzled (Fz), Frizzled 2 (Fz2) and Arrow to activate Dsh and stabilize Arm, which forms a protein complex with a TCF-like Pangolin in the nucleus to activate target gene expression, whereas Axin (Axn) and Shaggy (Sgg) negatively modulate Wnt signaling by promoting Arm degradation [94]. Simultaneous knockdown down of both *fz* and *fz2* (*fzKD+fz2KD*) in ISCs recapitulate the germ cell differentiation defect caused by either *armKD* or *dshKD*, although either *fzKD* or *fz2KD* yields a

much weaker germ cell differentiation defect (Figure 3.1E and 3.1F; Figure 3.2). Consistently, overexpression of *axn* and *sgg* in ISCs also leads to the germ cell differentiation defect similar to that caused by *armKD* and *dshKD* (Figure 3.1E, 3.1G and 3.1H). These results indicate that Wnt signaling is required in ISCs to promote germ cell differentiation.

The expression of a constitutively active mutant arm^{S10} (*arm**) leads to hyperactive Wnt signaling independently of Wnt ligands [95]. *c587*-driven *arm** expression alone does not cause any obvious GSC maintenance and germ cell differentiation defects, but results in severe egg chamber budding defects likely due to defective follicle cell development (Figure 3.1I). Interestingly, ISC-specific *arm** expression can fully rescue the germ cell differentiation defect caused by the two independent *dshRNAi* knockdowns, indicating that Arm functions downstream of Dsh in ISCs to promote germ cell differentiation (Figure 3.1J-L). These results further suggest that the two *dsh*RNAi lines are highly specific. Taken together, our findings demonstrate that canonical Wnt signaling works in ISCs to promote GSC lineage differentiation.

Wnt signaling is required in adult ISCs to promote GSC lineage differentiation

The *c587-gal4* driver is known to be expressed in somatic precursor cells in the developing female gonad, which give rise to terminal filament, cap cells, adult ISCs and follicular stem cells [96]. To determine if adult ISCs require active Wnt signaling for GSC lineage differentiation, we took advantage of *c587*-deriven expression of a temperature-sensitive *gal4* repressor *gal80* (*c587>>UAS-*$gal80^{ts}$) to allow the expression of *UAS-RNAi* lines against *dsh* and *arm* only in adult ISCs using temperature shift. At 25°C, $gal80^{ts}$ is functional to prevent *gal4*-driven expression of a *UAS* transgene, but at 29°C, $gal80^{ts}$ is inactivated to allow *gal4*-driven gene expression [97]. After the *c587>>*$gal80^{ts}$ control and *c587>>gal80ts UAS-dshRNAi*

or *UAS-armRNAi* females eclosed at 25°C (RNAi expression is extremely low or not expressed), they either continued to be kept at 25°C for one week (keeping RNAi expression repressed), or were moved to 29°C for one week (turning on the RNAi expression due to *gal80* inactivation). For the *c587>>gal80^{ts}* control females, which were cultured at 25°C or 29°C as adults for one week, their germaria contain the normal number of CBs (Figure 3.3A and 3.3B). Interestingly, for the *c587>>gal80^{ts} UAS-dshRNAi* or *UAS-armRNAi* females, which were cultured at constant 25°C, their germaria contain the normal or close to normal numbers of CBs (Figure 3.3B-F). In contrast, for the *c587>>gal80^{ts} UAS-dshRNAi* or *UAS-armRNAi* females, which were cultured at constant 29°C, their germaria contain excess CBs, indicative of the germ cell differentiation defect (Figure 3.3B and 3.3C'-F'). These results demonstrate that Wnt signaling is required in adult ISCs to promote germ cell differentiation.

Wnt signaling maintains ISCs by promoting proliferation and survival

Our previous studies have shown that a severe ISC loss also causes the similar germ cell differentiation defect [57, 70]. Then we determined if Wnt signaling controls ISC maintenance by using the PZ1444 reporter to quantify ISC numbers in control and Wnt signaling-defective germaria. PZ1444 expresses nuclear LacZ in ISCs and cap cells, which can be distinguished based on nucleus size and location [81]. In the one-week-old control germaria, PZ1444 labels 30-35 ISCs in addition to cap cells (Figure 3.4A and 3.4C). In the one-week-old *arm* and *dsh* knockdown germaria, only fewer than 5 ISCs remain (Figure 3.4B-D). Some knockdown germaria have completely lost ISCs (Figure 3.4B), whereas others retain one or a few ISCs (Figure 3.4D). Consistently, overexpression of *axn* and *sgg* also leads to a severe ISC loss

(Figure 3.4E and 3.4F). These results demonstrate that Wnt signaling is required to maintain ISCs.

Wnt signaling maintains ISCs possibly by promoting cell proliferation, survival or both. Interestingly, *arm**-expressing germaria contain significantly more ISCs (Figure 3.4G). In contrast with the one-week-old control germaria containing 32 ISCs, the one-week-old *arm**-expressing germaria carry 130 ISCs (Figure 3.4C). Consistently, ISC-specific *axn* and *sgg* knockdown, which increases Wnt signaling activity [98, 99], also leads to more ISCs (Figure 3.5). Interestingly, germ cell differentiation proceeds normally in the germaria carrying extra ISCs, suggesting that ISCs provide a permissive environment for germ cell differentiation (Figure 3.4G; Figure 3.5).

To further investigate how Wnt signaling maintains the ISC population, we examined ISC proliferation and apoptosis in the control, *armKD*, *dshKD* and *arm**-expressing germaria using BrdU incorporation and TUNEL labeling assays, respectively. In order to avoid severe ISC loss in these experiments, we purposely cultured the control, *armKD*, *dshKD* and *arm**-expressing females at 29^{o}C for shorter time than earlier experiments. BrdU incorporation labels replicating ISCs in the S-phase of the cell cycle, whereas TUNEL labeling detects fragmented DNA in dying cells. Based on BrdU labeling results, defective Wnt signaling significantly decreases ISC proliferation, whereas hyperactive Wnt signaling significantly increases ISC proliferation (Figure 3.4H-L). Based on TUNEL labeling results, defective Wnt signaling significantly increases ISC apoptosis, whereas hyperactive Wnt signaling significantly decreases ISC apoptosis (Figure 3.4M-Q). These results suggest that Wnt signaling maintains the ISC population by promoting proliferation and increasing survival.

Wnt signaling is required in ISCs to prevent BMP signaling in differentiated GSC progeny and maintain long ISC cellular processes

Our previous studies have also shown that ISC loss disrupts germ cell differentiation by increasing BMP signaling [57, 58, 70]. In the *Drosophila* germarium, BMP signaling leads to production of phosphorylated Mad (pMad) and activation of *Dad-lacZ* reporter expression in GSCs [27, 28, 100, 101]. In the control germaria, pMad and *Dad-lacZ* expression is restricted to GSCs (Figure 3.6A-A' and 3.6D-D'). In the *armKD* and *dshKD* germaria, pMad and *Dad-lacZ* expression is activated in the accumulated CBs locating away from cap cells in addition to GSCs (Figure 3.6B-B', 3.6C-C', 3.6E-E' and 3.6F-F'). These results indicate that BMP signaling is spread to the differentiation zone in the Wnt signaling-defective germarium.

To investigate how Wnt signaling regulates germ cell differentiation at the molecular level, we compared the gene expression changes in FACS-purified GFP-labeled control, Wnt signaling-defective *dsh* knockdown and *axn*-overexpressing (AxnOE) ISCs using deep RNA sequencing (RNA-seq). Our RNA-seq results show that known *Drosophila* BMP signaling components and regulators, including *dally* and *dpp*, which are often upregulated in defective ISCs [57, 58, 60, 70, 87], remain unchanged in the *dshKD* and *AxnOE* ISCs in comparison with the control ISCs (Figure 3.6G). To further determine if upregulated BMP signaling is responsible for the germ cell differentiation defect resulted from defective Wnt signaling, we determined the effect of heterozygous *dpp* mutations on the differentiation defect caused by *dshKD* because they have been shown to suppress the differentiation defect caused by BMP signaling upregualtion [57, 70]. Our results indicate that *dpp* heterozygous mutations do not show robust rescue effect

on the germ cell differentiation (Figure 3.6H). These results suggest that Wnt signaling in ISCs regulates germ cell differentiation not mainly by preventing BMP signaling in the differentiation niche.

Long ISC cellular processes are also required to promote germ cell differentiation [70]. They can be easily visualized by *c587*-driven expression of membrane-tethered GFP, CD8GFP. In the control germaria, ISC cellular processes wrap up differentiated germ cells (figure 3.6I-I'). In contrast, cellular processes in the remaining ISCs of the *armKD* and *dshKD* germaria fail to extend into the accumulated CBs (Figure 3.6J-K'). These results indicate that Wnt signaling is required to maintain ISC cellular processes.

Wnt signaling is dispensable for maintaining Piwi protein expression in ISCs

A recent study proposes that Wnt signaling upregulates *piwi* expression in ISCs, thereby promoting germ cell differentiation [92]. To verify if Piwi protein is indeed down-regulated in Wnt signaling-defective ISCs, we quantified the expression of Piwi protein in the PZ1444-labeled *dshKD* or *armKD* ISCs. Piwi protein shows comparable expression levels among the examined 203 control ISCs, 48 *armKD1* ISCs, 58 *armKD2* ISCs and 46 *dshKD2* ISCs (Figure 3.7A-D). Surprisingly, Piwi protein levels are significantly elevated in the *dshKD1* ISCs (93 examined) in comparison with the control ISCs (Figure 3.7D). In addition, we also used Fluorescence-Activated Cell Sorting (FACS) to purify the GFP-labeled control ISCs, *axnOE* ISCs and *dshKD1* ISCs for RNA-sequencing. The RNA-seq results show that the *piwi* mRNA levels are slightly upregulated in the *axnOE* ISCs, and are significantly upregulated in the *dshKD* ISCs in comparison to the control ISCs (Figure 3.7E). Therefore, our results indicate that Wnt

signaling promotes germ cell differentiation unlikely by maintaining Piwi expression in ISCs. However, Wnt signaling possibly works with Piwi in an unknown means to promote germ cell differentiation because the loss of their functions in ISCs lead to similar germ cell differentiation defects [58, 87, 92].

Wnt signaling maintains the cellular redox state in ISCs, GSCs and early differentiated germ cells by maintaining *Glutathione-S-transferase* (*Gst*) gene expression

Our RNA-seq results also show that four *Gst* genes, *GstD2*, *GstD4*, *GstD10* and *GstE3*, decrease their mRNA expression levels significantly in both *dshKD* and *axn*-overexpressing ISCs in comparison with the control (Figure 3.8A). GST proteins comprise a family of eukaryotic metabolic enzymes for eliminating hydrogen peroxide (H_2O_2) and catalyzing the conjugation of the reduced form of glutathione (GSH) to oxidized substrates for the purpose of detoxification. Thus, downregulated expression of the *Gst* genes in the Wnt signaling-defective ISCs might cause the increase in cellular reactive oxygen species (ROS), which can be detected by dihydroethidium (DHE) fluorescence. In the anterior half of control germaria, ISCs and underneath differentiating germ cells show low DHE fluorescence, indicating that these cells have low cellular ROS levels, including ISCs (Figure 3.8B; Figure 3.9A-B'). In contrast, *dshKD* and *armKD* ISCs elevate DHE fluorescence, and germ cells underneath also increase DHE fluorescence, further supporting the idea that defective Wnt signaling results in the increased cellular ROS in ISCs and their interacting germ cells (Figure 3.8B'; Figure 3.9C-G). As a distinct GST family member from the GST-D family members, GST2 has similar function in regulating the cellular redox state [102]. Consistently, GST2 overexpression in the *dshKD* ISCs can also

restore low DHE fluorescence in the anterior half of the germaria, suggesting that Wnt signaling controls the cellular redox state in ISCs, GSCs and early GSC progeny by regulating *Gst* gene expression (Figure 3.8B"; Figure 3.9H). Catalase (CAT) can also help eliminate cellular H_2O_2 by converting it to free oxygen and water. As expected, CAT overexpression in the *dshKD* ISCs also restores low cellular ROS in the anterior half of the germaria (Figure 3.8B'"; Figure 3.9I). These results suggest that Wnt signaling regulates the cellular redox state in ISCs by controlling *Gst* gene expression.

Then, we determined if the restoration of the reduced redox state in *dshKD* ISCs could rescue the germ cell differentiation defect. Cytoplasmic superoxide dismutase1 (SOD1) is also important for the clearance of cellular ROS. The germaria overexpressing GST2, CAT and SOD1 in ISCs contain normal CB numbers (Figure 3.8C; Figure 3.10A-C). GST2, CAT and SOD1 overexpression in the *dshKD* ISCs drastically and significantly reduces CB numbers in the germaria, but does not change GSC numbers significantly, indicating that increased cellular ROS levels in *dshKD* ISCs is largely responsible for the germ cell differentiation defect (Figure 3.8C-F; Figure 3.10D-G). Similarly, GST2, CAT and SOD1 overexpression in the *dshKD* ISCs significantly and drastically rescue the ISC number, but not to the wild-type ISC number, indicating that increased ROS is at least partly responsible for the loss of *dshKD* ISCs (Figure 3.8G-J; Figure 3.10H-M). Taken together, these results demonstrate that Wnt signaling in ISCs maintains the reduced redox state, which is partly responsible for ISC maintenance and germ cell differentiation.

***GstD2* works with *Cat* in ISCs to control germ cell differentiation**

Our RNA-seq results indicate that *GstD2* is the most abundantly expressed *Gst* genes in ISCs. In addition, it is also the most severely downregulated *Gst* gene in the Wnt signaling-defective ISCs, which prompted us to further investigate its function in promoting germ cell differentiation (Figure 3.8A). We generated two independent RNAi lines against *GstD2*, among which Line 1 (*GstD2KD1*) efficiently knocks down *GstD2* mRNA but Line 2 (*GstD2KD2*) does not based on quantitative RT-PCR results on the purified ISCs (Figure 3.8K). In contrast with the control germaria containing one CB on average, the *GstD2KD1* germaria contain two CBs, which are significantly more than the control (Figure 3.8L and 3.8O). Also expected, the *GstD2KD2* germaria behave like the control, containing one CB on average (Figure 3.8O). Interestingly, ISC-specific expression of *Gst2* and *Cat* can also rescue the moderate germ cell differentiation defect caused by *GstD2KD1*, indicating that the differentiation defect is caused by *GstD2* knockdown in ISCs (Figure 3.8O). Considering the fact that additional three *Gst* genes are downregulated in the Wnt signaling-defective ISCs, these results indicate that *Gst* genes are required in ISCs to promote germ cell differentiation.

Both *Cat* and *Gst* genes work together to remove cellular hydrogen peroxide (H_2O_2). The ISC-specific *Cat* knockdown (*CatKD*) germaria behave like the control germaria, containing one CB on average (Figure 3.8M and 3.8O). Interestingly, ISC-specific *CatKD* significantly enhances the germ cell differentiation defect caused by *GstD2KD1*, and consequently the double knockdown germaria accumulate significantly more CBs than the single knockdown germaria (Figure 3.8N and 3.8O). In addition, the *CatKD* germaria carry a normal number of ISCs, whereas the *GstD2KD* germaria carry slightly fewer ISCs (Figure 3.8P, 3.8Q and 3.8S). Consistently, the *GstD2KD1 CatKD* germaria carry significantly fewer ISCs than the *GstD1KD1* or *CatKD* germaria (Figure 3.8R and 3.8S). These results indicate that *Cat* and *Gst* genes work

together in ISCs to maintain ISCs and promote germ cell differentiation, and further suggest that redox control is important for ISC maintenance.

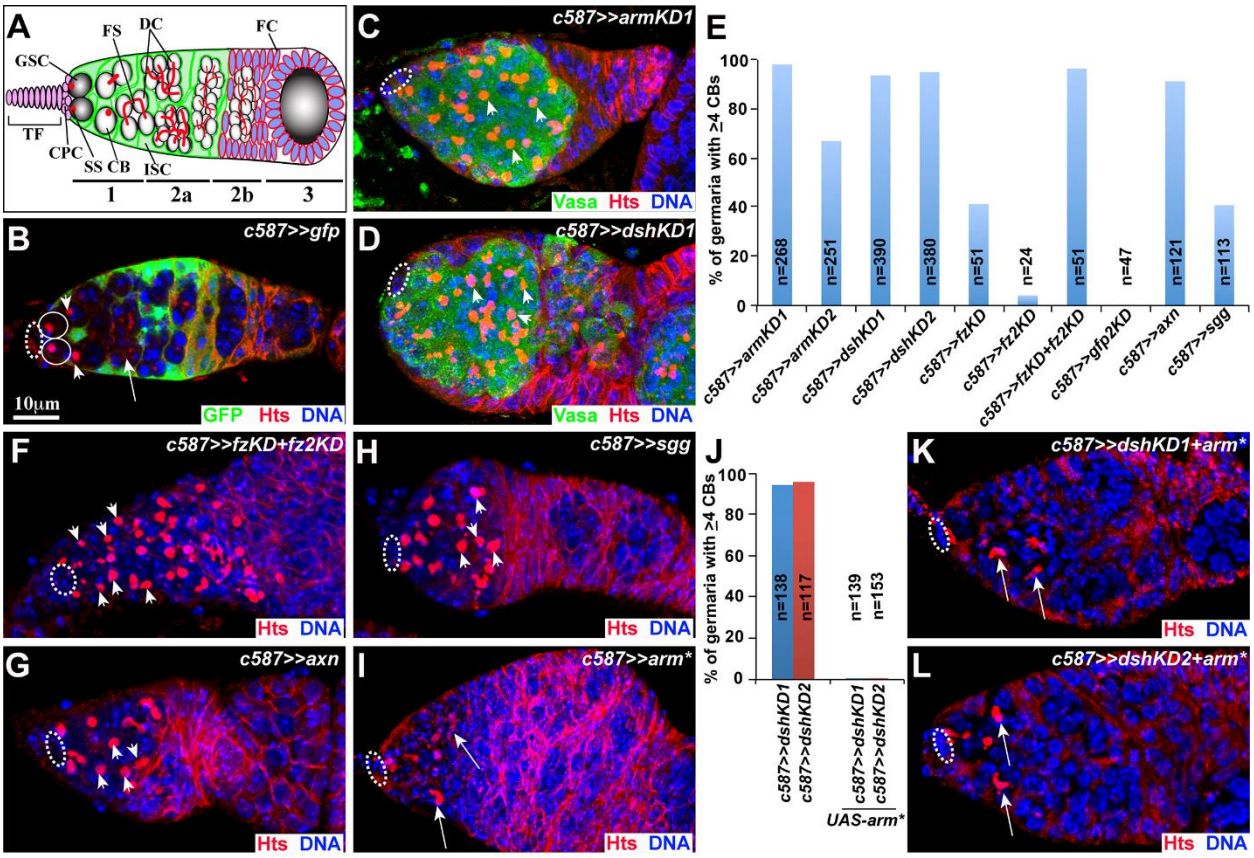
Autcrine Wnt2 and Wnt4 function redundantly to maintain active Wnt signaling in ISCs

Our RNA-seq results indicate that four *Wnt*-like genes are expressed in the purified ISCs, but *wingless* shows little expression (Figure 3.11A). *Wnt2* and *Wnt4* are expressed at high levels, whereas *Wnt5* and *Wnt6* are present in much lower levels (Figure 3.11A). A recent study has shown that *wnt4* mRNAs are indeed restricted to ISCs, whereas *wnt2* mRNAs are also present in ISCs [103]. Knocking down *Wnt2* or *Wnt4* alone in ISCs by two or three independent RNAi lines results in no or slight germ cell differentiation defect based on CB numbers (Figure 3.11B-D). This is in contrast with the recent study claiming that Wnt4 alone in ISCs is responsible for germ cell differentiation [92]. Consistent with the idea that Wnt2 and Wnt4 function redundantly in ISCs, simultaneous knockdown of *Wnt2* and *Wnt4* via different combinations of RNAi lines leads to a severe germ cell differentiation defect, which is similar to that caused by *dshKD* or *armKD* (Figure 3.11D-H). These results demonstrate that Wnt2 and Wnt4 in ISCs serve as redundant autocrine signals for promoting germ cell differentiation.

Figures and Legends

Figure 3.1. Canonical Wnt signaling in ISCs promotes germ cell differentiation. (**A**) The *Drosophila* germarium dividing into three regions 1, 2a, 2b and 3. Abbreviations: TF-terminal filament; CPC-cap cell; ISC-inner germarial sheath cell; FC-follicle cell; GSC-germline stem cell; CB-cystoblast; DC-developing cyst; SS-spectrosome; FS-fusome. In **B**-**L**, cap cells are highlighted by broken ovals, whereas CBs and cysts are indicated by arrowheads and arrows, respectively. (**B**) In the *c587>>UAS-GFP* germarium containing two GSCs (spectrosomes indicated by arrowheads) close to cap cells, differentiated cysts are surrounded by GFP-positive ISCs. (**C**-**E**) In *armKD1* (**C**) *dshKD1* (**D**) germaria, many spectrosome-containing CBs accumulate far away from cap cells. **E** shows quantification results on the percentages of the germaria exhibiting the germ cell differentiation defect ($\geq$4 CBs). (**F**-**H**) *fz fz2* double knockdown (**F**), *axn*- (**G**) and *sgg*-overexpressing (**H**) germaria contain excess CBs. (**I**-**K**) In *arm**-overexpressing *dshKD* germaria (**K**, **L**), GSC progeny differentiate into cysts containing branched fusomes (arrows) as those in the *arm**-overexpressing germarium (**I**). **J** shows quantification results.

Figure 3.1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FS
DC
FC
GSC
TF
CPC
SS CB
ISC
1
2a
2b
3
B
c587>>gfp
10μm
GFP Hts DNA
C
c587>>armKD1
Vasa Hts DNA
D
c587>>dshKD1
Vasa Hts DNA
E
% of germaria with ≥4 CBs
n=268
n=251
n=390
n=380
n=51
n=24
n=51
n=47
n=121
n=113
c587>>armKD1
c587>>armKD2
c587>>dshKD1
c587>>dshKD2
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F
c587>>fzKD+fz2KD
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H
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Hts DNA
J
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c587>>dshKD2
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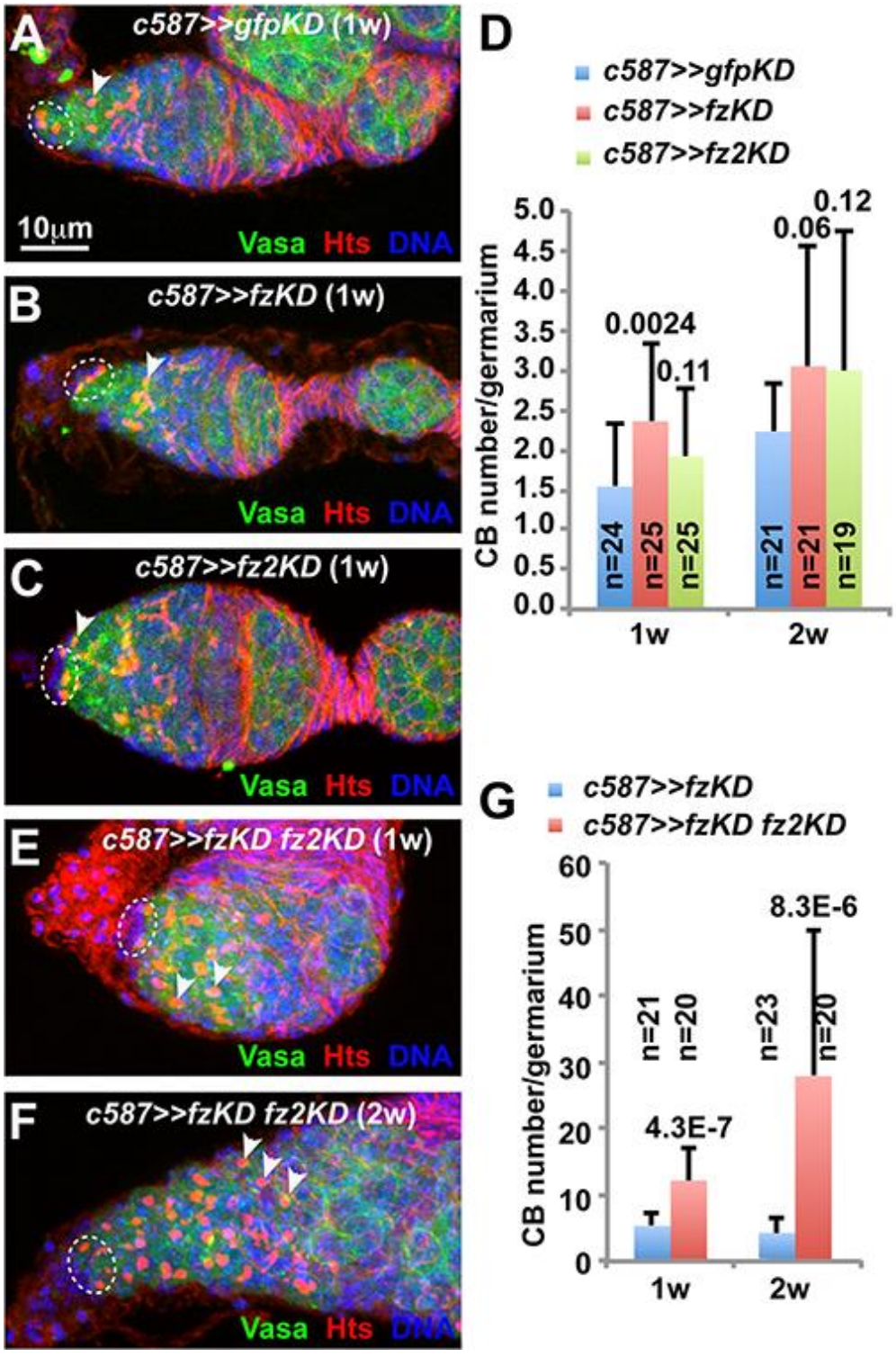
c587>>dshKD2
UAS-arm*
K
c587>>dshKD1+arm*
Hts DNA
G
c587>>axn
Hts DNA
I
c587>>arm*
Hts DNA
L
c587>>dshKD2+arm*
Hts DNA

Figure 3.2 Wnt receptors FZ and FZ2 function redundantly in ISCs to promote germ cell differentiation. Broken ovals highlight cap cells and GSCs, while arrowheads denote spectrosomes in CBs (**A**-**C**, **E** and **F**). (**A**-**D**) *fzKD* (**B**) or *fz2KD* (**C**) germaria contain normal CBs in comparison with the *gfpKD* germarium (**A**). **D** shows the quantification results on CB numbers in one-week-old (1w) and two-week-old (2w) control and knockdown germaria. (**E**-**G**) *fzKD fz2KD* (**E**, **F**) germaria contain significantly more CBs than *fzKD* germaria (**B**). **G** shows the quantification results on CB numbers.

Figure 3.2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c587>>gfpKD (1w)
10μm
Vasa Hts DNA
B
c587>>fzKD (1w)
Vasa Hts DNA
C
c587>>fz2KD (1w)
Vasa Hts DNA
E
c587>>fzKD fz2KD (1w)
Vasa Hts DNA
F
c587>>fzKD fz2KD (2w)
Vasa Hts DNA
D
c587>>gfpKD
c587>>fzKD
c587>>fz2KD
CB number/germarium
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0024
0.11
0.06
0.12
n=24
n=25
n=25
n=21
n=21
n=19
1w
2w
G
c587>>fzKD
c587>>fzKD fz2KD
CB number/germarium
60
50
40
30
20
10
0
4.3E-7
8.3E-6
n=21
n=20
n=23
n=20
1w
2w

Figure 3.3. Wnt signaling is required in adult ISCs to promote germ cell differentiation. Broken ovals highlight GSCs, whereas arrowheads indicate CBs. Two experimental regimens 25-25 and 25-29 mean the females cultured under 25ºC or 29ºC for one additional week after reaching adulthood at 25ºC, respectively. (**A**, **A'**) Control *c587>>gal80ts* germaria contain one or two CBs under the 25-25 or 25-29 condition. (**B**) Quantification results on the percentages of the germaria carrying 4 or more CBs show that adult stage-specific *arm* or *dsh* knockdown causes an accumulation of more CBs in comparison with the control. (**C**-**F**) *armRANi*- (**C, D**) or *dshRNAi*- (**E, F**) carrying germaria contain one or two CBs under 25-25, indicating that RNAi-mediated *arm* or *dsh* knockdown in adult ISCs under 25ºC is very limited based on the germ cell differentiation phenotype. (**C'**-**F'**) *armRANi*- (**C', D'**) or *dshRNAi*- (**E', F'**) carrying germaria contain many more CBs under 25-29, indicating that RNAi-mediated *arm* or *dsh* knockdown under 29ºC in adult ISCs leads to the severe germ cell differentiation defects.

Figure 3.3

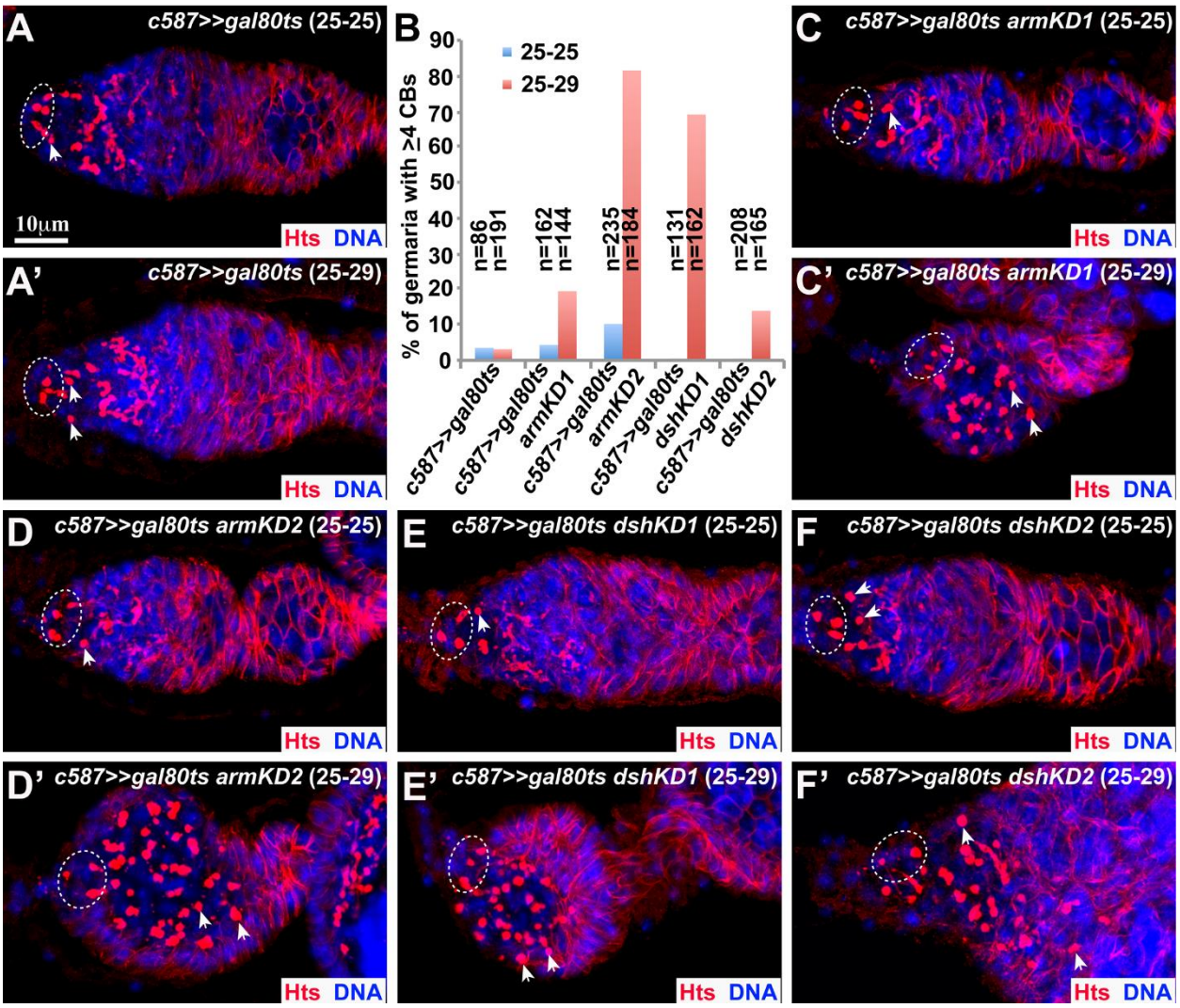

A
c587>>gal80ts (25-25)
10μm
Hts DNA
A'
c587>>gal80ts (25-29)
Hts DNA
B
25-25
25-29
% of germaria with ≥4 CBs
90
80
70
60
50
40
30
20
10
0
n=86
n=191
n=162
n=144
n=235
n=184
n=131
n=162
n=208
n=165
c587>>gal80ts
c587>>gal80ts armKD1
c587>>gal80ts armKD2
c587>>gal80ts dshKD1
c587>>gal80ts dshKD2
C
c587>>gal80ts armKD1 (25-25)
Hts DNA
C'
c587>>gal80ts armKD1 (25-29)
Hts DNA
D
c587>>gal80ts armKD2 (25-25)
Hts DNA
E
c587>>gal80ts dshKD1 (25-25)
Hts DNA
F
c587>>gal80ts dshKD2 (25-25)
Hts DNA
D'
c587>>gal80ts armKD2 (25-29)
Hts DNA
E'
c587>>gal80ts dshKD1 (25-29)
Hts DNA
F'
c587>>gal80ts dshKD2 (25-29)
Hts DNA

Figure 3.4. Wnt signaling maintains the differentiation niche by promoting cell proliferation and survival. Cap cells are highlighted by broken ovals. (**A**) The control germarium contains PZ1444-labeled cap cells and ISCs (one by arrow). (**B**-**D**) The *armKD* (**B**) and *dshKD* (**D**) germaria maintain zero and one ISC (arrow), respectively. **C** shows quantification results on ISC numbers (mean $\pm$ standard deviation; *Student*'s t-test is used to calculate *P* values). (**E**, **F**) *axn*- (**E**) and *sgg*- (**F**) expressing germaria contain no ISCs (**E**) and a few ISCs (**F**, arrows), respectively. (**G**) ISC-specific *arm** expression drastically increases the ISC number (one by arrow). (**H**-**L**) ISC-specific knockdown of *arm* (**I**) and *dsh* significantly decreases BrdU-positive PZ1444-labeled ISCs (arrows), whereas ISC-specific *arm** expression (**K**) significantly increases BrdU-labeled ISCs in comparison with the control (**H**). The arrowhead in **I** indicates a BrdU-positive germ cell cyst. **J** and **L** show quantification results. (**M**-**Q**) ISC-specific knockdown of *arm* and *dsh* (**N**) significantly increases TUNEL-positive PZ1444-labeled ISCs (arrows), whereas ISC-specific *arm** expression (**P**) significantly decreases TUNEL-positive ISCs in comparison with the control (**M**). **O** and **P** show quantification results.

Figure 3.4

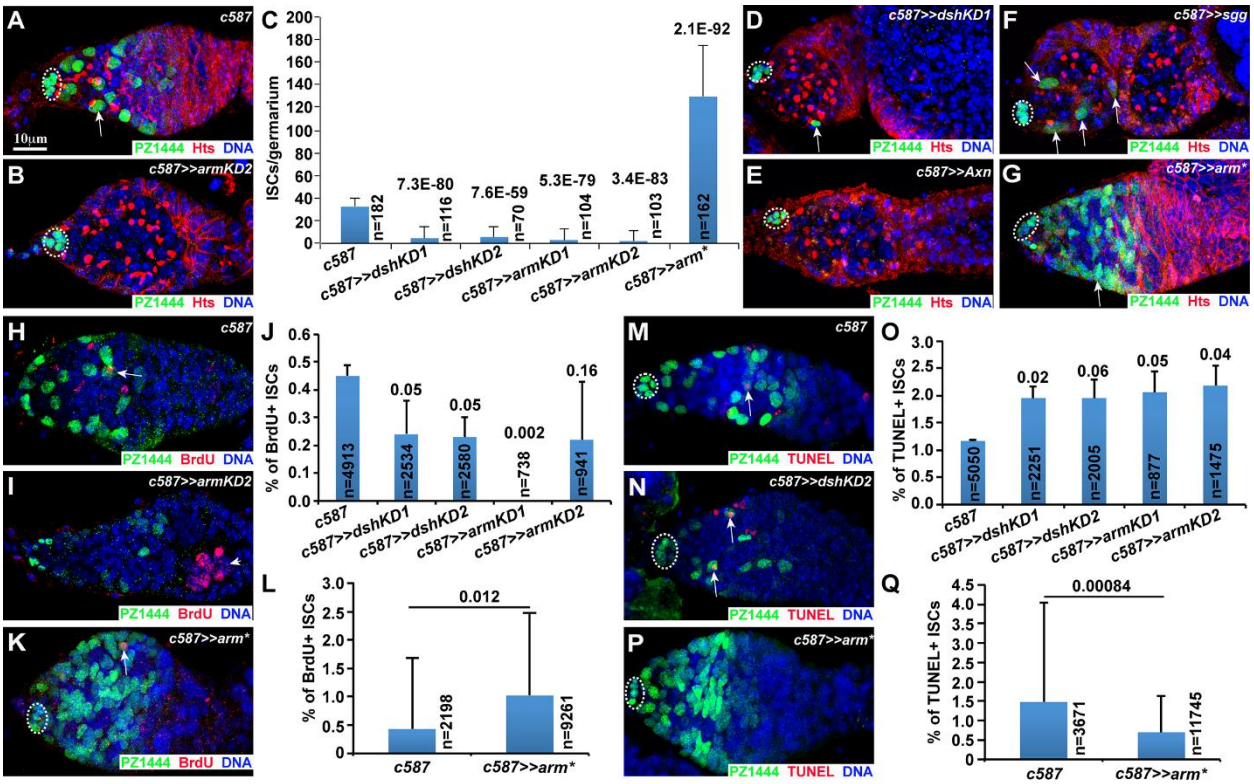

A
c587
10μm
PZ1444 Hts DNA
B
c587>>armKD2
PZ1444 Hts DNA
C
ISCs/germarium
2.1E-92
7.3E-80
7.6E-59
5.3E-79
3.4E-83
n=182
n=116
n=70
n=104
n=103
n=162
c587
c587>>dshKD1
c587>>dshKD2
c587>>armKD1
c587>>armKD2
c587>>arm*
D
c587>>dshKD1
PZ1444 Hts DNA
E
c587>>Axn
PZ1444 Hts DNA
F
c587>>sgg
PZ1444 Hts DNA
G
c587>>arm*
PZ1444 Hts DNA
H
c587
PZ1444 BrdU DNA
I
c587>>armKD2
PZ1444 BrdU DNA
K
c587>>arm*
PZ1444 BrdU DNA
J
% of BrdU+ ISCs
0.05
0.05
0.002
0.16
n=4913
n=2534
n=2580
n=738
n=941
c587
c587>>dshKD1
c587>>dshKD2
c587>>armKD1
c587>>armKD2
L
% of BrdU+ ISCs
0.012
n=2198
n=9261
c587
c587>>arm*
M
c587
PZ1444 TUNEL DNA
N
c587>>dshKD2
PZ1444 TUNEL DNA
P
c587>>arm*
PZ1444 TUNEL DNA
O
% of TUNEL+ ISCs
0.02
0.06
0.05
0.04
n=5050
n=2251
n=2005
n=877
n=1475
c587
c587>>dshKD1
c587>>dshKD2
c587>>armKD1
c587>>armKD2
Q
% of TUNEL+ ISCs
0.00084
n=3671
n=11745
c587
c587>>arm*

Figure 3.5. Hyperactive Wnt signaling increases the ISC population. Cap cells are highlighted by ovals, while some of the ISCs are denoted by arrowheads. (**A**) In the control germarium, PZ1444 labels cap cells and ISCs. (**B**-**D**) The *axnKD* (**B**) and *sggKD* (**C**, **D**) germaria accumulate more ISCs.

Figure 3.5

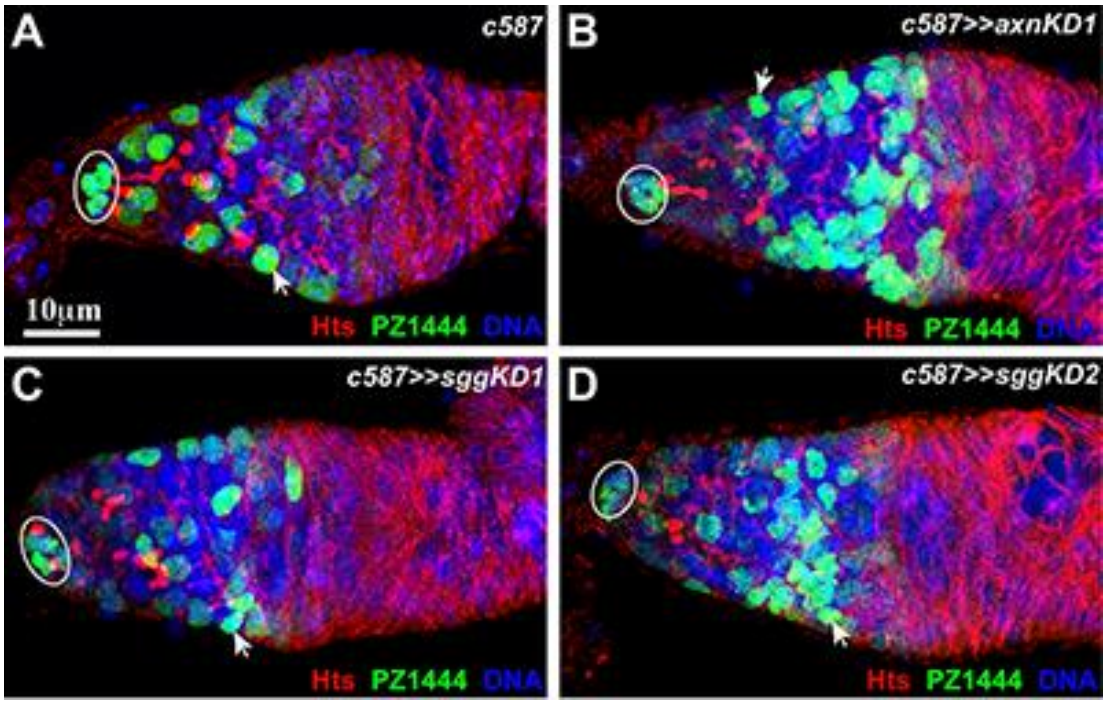

A
c587
10μm
Hts PZ1444 DNA
B
c587>>axnKD1
Hts PZ1444 DNA
C
c587>>sggKD1
Hts PZ1444 DNA
D
c587>>sggKD2
Hts PZ1444 DNA

Figure 3.6. Wnt signaling in ISCs prevents BMP signaling in the differentiated germ cell zone and maintains long ISC cellular processes. Ovals indicate GSCs, whereas arrowheads denote CBs or CB-like cells. **A'-F'** images only show green fluorescence of **A**-**F**. (**A**, **A'**) In the control germarium, GSCs are positive for pMad, but one CB is negative. (**B**-**C'**) In the *armKD* (**B**, **B'**) and *dshKD* (**C**, **C'**) germaria, GSCs are pMad-positive as in the control. Although most of the accumulated CBs are negative for pMad (arrows), some rare CBs are pMad-positive (arrowheads). (**D**, **D'**) The control germarium shows that GSCs are positive for *Dad-lacZ* expression, but one CB is negative. (**E**-**F'**) In the *armKD* (**E**, **E'**) and *dshKD* (**F**, **F'**) germaria, GSCs are *Dad-lacZ*-positive as in the control. Some of the accumulated CBs are negative for *Dad-lacZ* (arrows), but the other ones exhibit low *Dad-lacZ* expression (arrowheads). (**G**) RNA-seq results on the purified ISCs show that the known components in the BMP signaling pathway, including *dpp*, *gbb* (also encoding a BMP ligand) and *dally* mRNAs, remain largely unchanged in *axn*-overexpressing ISCs or *dskKD* ISCs in comparison with the control (FKPM=reads per kilobase per million mapped reads). (**H**) The heterozygous dpp^{hr56} mutation has some weak suppression, while the heterozygous dpp^{hr4} mutation has no suppression, on the germ cell differentiation defects caused by *dshKD* based on the germaria containing 4 or more CBs. (**I**-**K'**) *armKD* (**J**, **J'**) and *dshKD* (**K**, **K'**) ISCs lack their CD8GFP-positive cellular processes extending into the accumulated CBs (arrowheads) in contrast with the control ISCs extending the cellular processes wrapping up underneath germ cells (arrowheads, **I'**). **I'**-**K'** represent a higher magnification of highlighted areas in **I**-**K**.

Figure 3.6

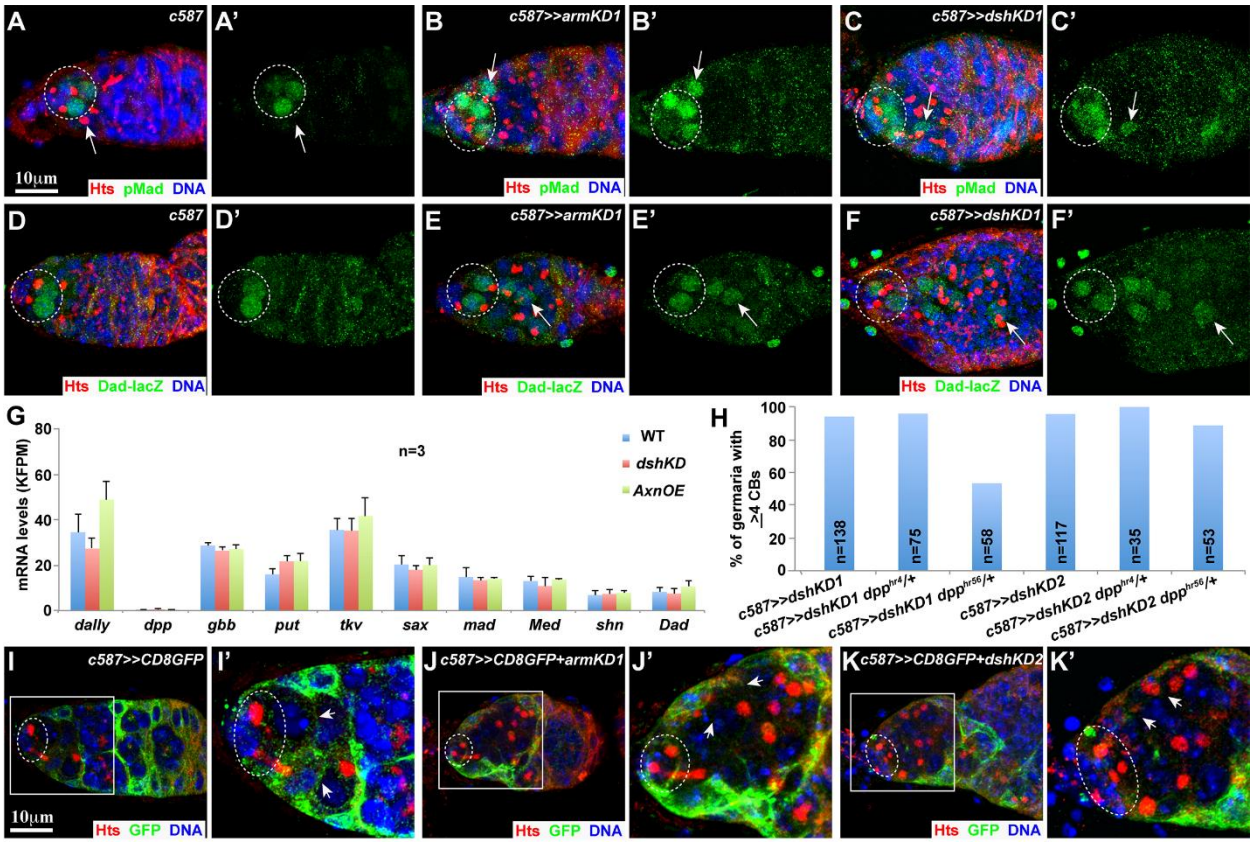

A
c587
A'
B
c587>>armKD1
B'
C
c587>>dshKD1
C'
10μm
Hts pMad DNA
D
c587
D'
E
c587>>armKD1
E'
F
c587>>dshKD1
F'
Hts Dad-lacZ DNA
G
mRNA levels (KFPM)
n=3
WT
dshKD
AxnOE
dally
dpp
gbb
put
tkv
sax
mad
Med
shn
Dad
H
% of germaria with ≥4 CBs
n=138
n=75
n=58
n=117
n=35
n=53
c587>>dshKD1
c587>>dshKD1 dpp^hr4/+
c587>>dshKD1 dpp^hr56/+
c587>>dshKD2
c587>>dshKD2 dpp^hr4/+
c587>>dshKD2 dpp^hr56/+
I
c587>>CD8GFP
I'
J
c587>>CD8GFP+armKD1
J'
K
c587>>CD8GFP+dshKD2
K'
Hts GFP DNA

Figure 3.7. Piwi mRNA and protein expression remain normally expressed in the Wnt signaling-defective ISCs. PZ1444 is used to highlight ISCs. (**A**) The control germarium shows that ISCs (arrowhead) express more Piwi proteins than cap cells (oval) and underneath germ cells. (**B**, **C**) The remaining *armKD* (**B**) and *dshKD* (**C**) ISCs (arrows) still retain high Piwi protein expression. (**D**) Quantification results show that Piwi protein levels remain largely unchanged in the remaining *armKD1*, *armKD2* and *dshKD2* ISCs in comparison with the wild-type control ISCs, but it appears to increase its expression in *dshKD1* ISCs. (**E**) RNA sequencing results on the purified ISCs show that *piwi* mRNA expression levels are not changed in *axn*-overexpressing ISCs, but increase in *dskKD* ISCs, in comparison with the wild-type control ISCs.

Figure 3.7

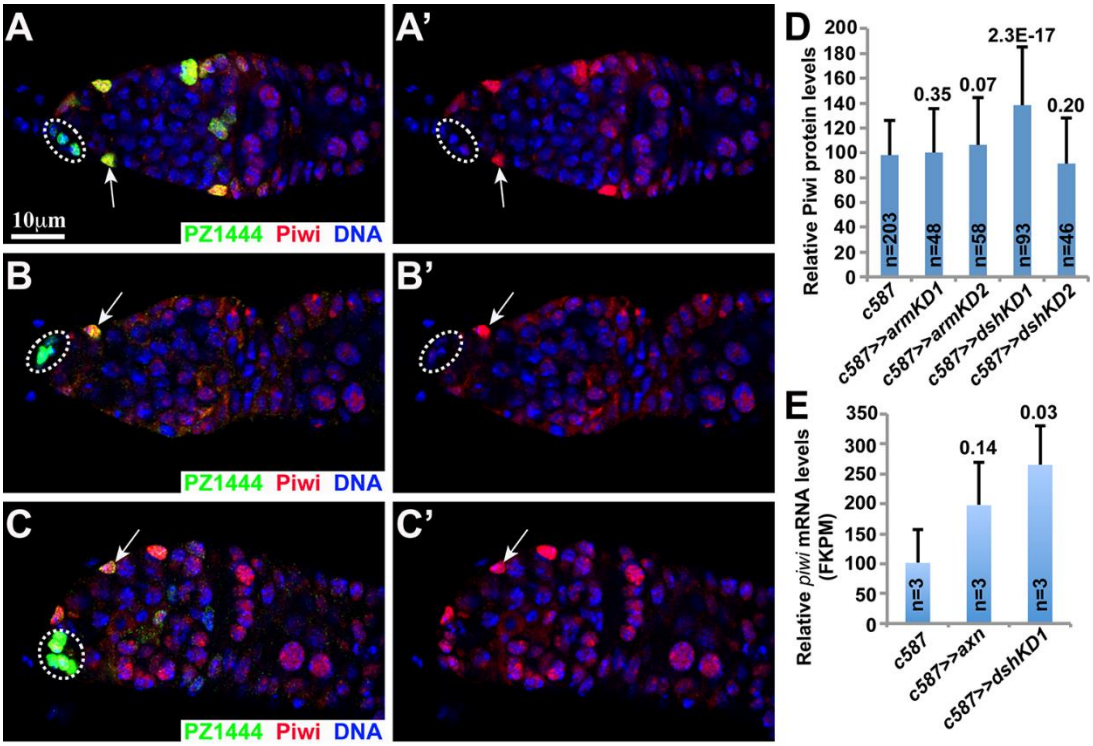

A
A'
B
B'
C
C'
10μm
PZ1444 Piwi DNA
D
Relative Piwi protein levels
200
180
160
140
120
100
80
60
40
20
0
2.3E-17
0.35
0.07
0.20
n=203
n=48
n=58
n=93
n=46
c587
c587>>armKD1
c587>>armKD2
c587>>dshKD1
c587>>dshKD2
E
Relative piwi mRNA levels (FKPM)
350
300
250
200
150
100
50
0
0.14
0.03
n=3
n=3
n=3
c587
c587>>axn
c587>>dshKD1

Figure 3.8. Wnt signaling maintains the reduced redox state in ISCs, promoting germ cell differentiation. (**A**) RNA sequencing results of the purified ISCs show that *GstD2*, *GstD4*, *GstD10* and *GstE3* mRNA expression levels are significantly lower in *axn*-overexpressing or *dskKD* ISCs than the control ISCs. In **B**-**B'"**, **D**-**I** and **L**-**Q**, cap cells and GSCs are highlighted by broken ovals, some of the CBs are indicated by arrowheads and some PZ1444-positive ISCs are denoted by arrows. (**B**-**B'"**) The *dshKD* germarium (**B'**) exhibits a drastic increase of DHE fluorescence in the anterior region, including ISCs, GSCs and early GSC progeny, in comparison with the control germarium (**B**). GST2 (**B"**) or CAT (**B'"**) overexpression in ISCs restores low DHE fluorescence in the d*shKD* germariun. (**C**-**J**) GST2 (**D**, **G**), CAT (**E**, **H**) or SOD1 (**F**, **I**) overexpression in *dshKD* ISCs significantly decreases CBs (arrowheads; **D**-**F**) and significantly increase ISCs (arrows; **G**-**I**). **C** and **J** show quantification results on CB and ISC numbers, respectively (for each genotype, 50 or more germaria examined). (**K**) Quantitative RT-PCR results on the purified ISCs show that *GstD2* knockdown by RNAi Line 1 (*GstD2KD1*), but not the line 2 (*GstD2KD2*), significantly decreases *GstD2* mRNA levels in comparison to the control (*c587*). (**L**-**O**) *GstD2KD1* (**L**) and *CatKD* germaria contain 4 CBs and 0 CB, respectively, but the *GstD2KD1 CatKD* germarium (**N**) contains 5 CBs (**O**: CB quantitative results). (**P**-**S**) *GstD2KD1 CatKD* germarium (**R**) contains fewer ISCs (arrows) than *GstD2KD1* (**P**) and *CatKD* (**Q**) germaria (**S**: ISC quantitative results). Note: PZ1444 expression appears to be downregulated in *GstD2KD1 CatKD* ISCs.

Figure 3.8

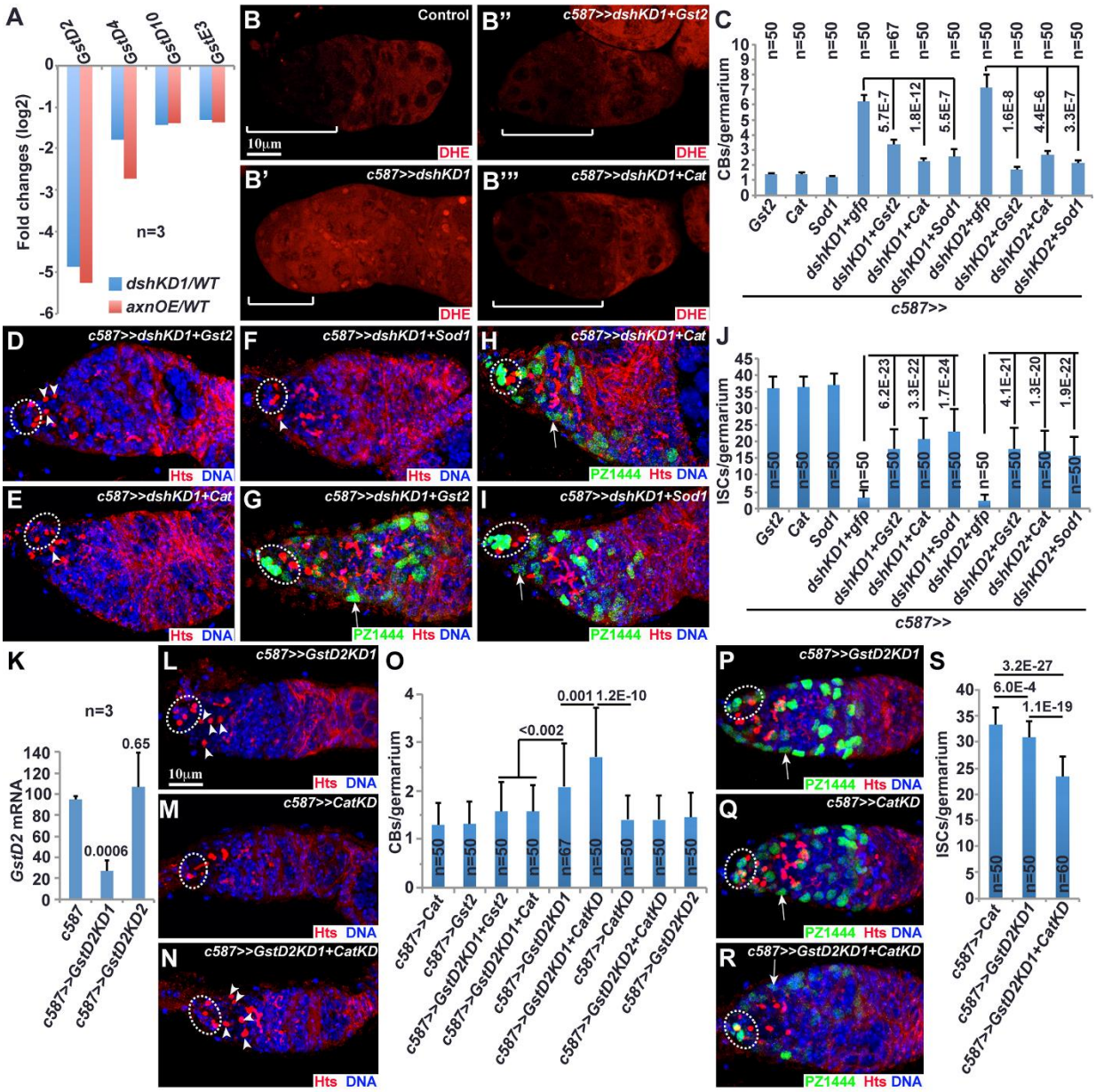

A
Gst2
Gst4
GstD10
GstE3
Fold changes (log2)
n=3
dshKD1/WT
axnOE/WT
B
Control
B''
c587>>dshKD1+Gst2
B'
c587>>dshKD1
B'''
c587>>dshKD1+Cat
10μm
DHE
C
CBs/germarium
n=50
n=67
5.7E-7
1.8E-12
5.5E-7
1.6E-8
4.4E-6
3.3E-7
Gst2
Cat
Sod1
dshKD1+gfp
dshKD1+Gst2
dshKD1+Cat
dshKD1+Sod1
dshKD2+gfp
dshKD2+Gst2
dshKD2+Cat
dshKD2+Sod1
c587>>
D
c587>>dshKD1+Gst2
F
c587>>dshKD1+Sod1
H
c587>>dshKD1+Cat
E
c587>>dshKD1+Cat
G
c587>>dshKD1+Gst2
I
c587>>dshKD1+Sod1
Hts DNA
PZ1444 Hts DNA
J
ISCs/germarium
6.2E-23
3.3E-22
1.7E-24
4.1E-21
1.3E-20
1.9E-22
K
n=3
GstD2 mRNA
0.65
0.0006
c587
c587>>GstD2KD1
c587>>GstD2KD2
L
c587>>GstD2KD1
M
c587>>CatKD
N
c587>>GstD2KD1+CatKD
O
CBs/germarium
<0.002
0.001
1.2E-10
c587>>Cat
c587>>Gst2
c587>>GstD2KD1+Gst2
c587>>GstD2KD1+Cat
c587>>GstD2KD1
c587>>GstD2KD1+CatKD
c587>>CatKD
c587>>GstD2KD2+CatKD
c587>>GstD2KD2
P
c587>>GstD2KD1
Q
c587>>CatKD
R
c587>>GstD2KD1+CatKD
S
ISCs/germarium
3.2E-27
6.0E-4
1.1E-19
c587>>Cat
c587>>GstD2KD1
c587>>GstD2KD1+CatKD

Figure 3.9. Wnt signaling maintains the reduced redox state in ISCs. (**A**) The control ovariole exhibit low DHE staining in the germarial region, and gradually upregulates DHE staining in differentiated germ cells in egg chambers. **B'**-**F'** only show DHE staining in **B**-**F**. In **B**-**F'**, broken ovals highlight cap cells, whereas arrowheads point to PZ1444-labeled ISCs. (**B**, **B'**) Control germarium shows low DHE staining in ISCs and cap cells (broken oval). (**B**-**F'**) *armKD* (**C**-**D'**) and *dshKD* (**E**-**F'**) ISCs as well as their underneath germ cells increase DHE staining. (**G**-**I**) GST2 (**H**) or CAT (**I**) overexpression in ISCs restores low DHE fluorescence in the anterior region of the d*shKD2* germariun (**G**). Bars in **A** and **B** (**B**-**I** in the same scale) represent 20μm and 10μm, respectively.

Figure 3.9

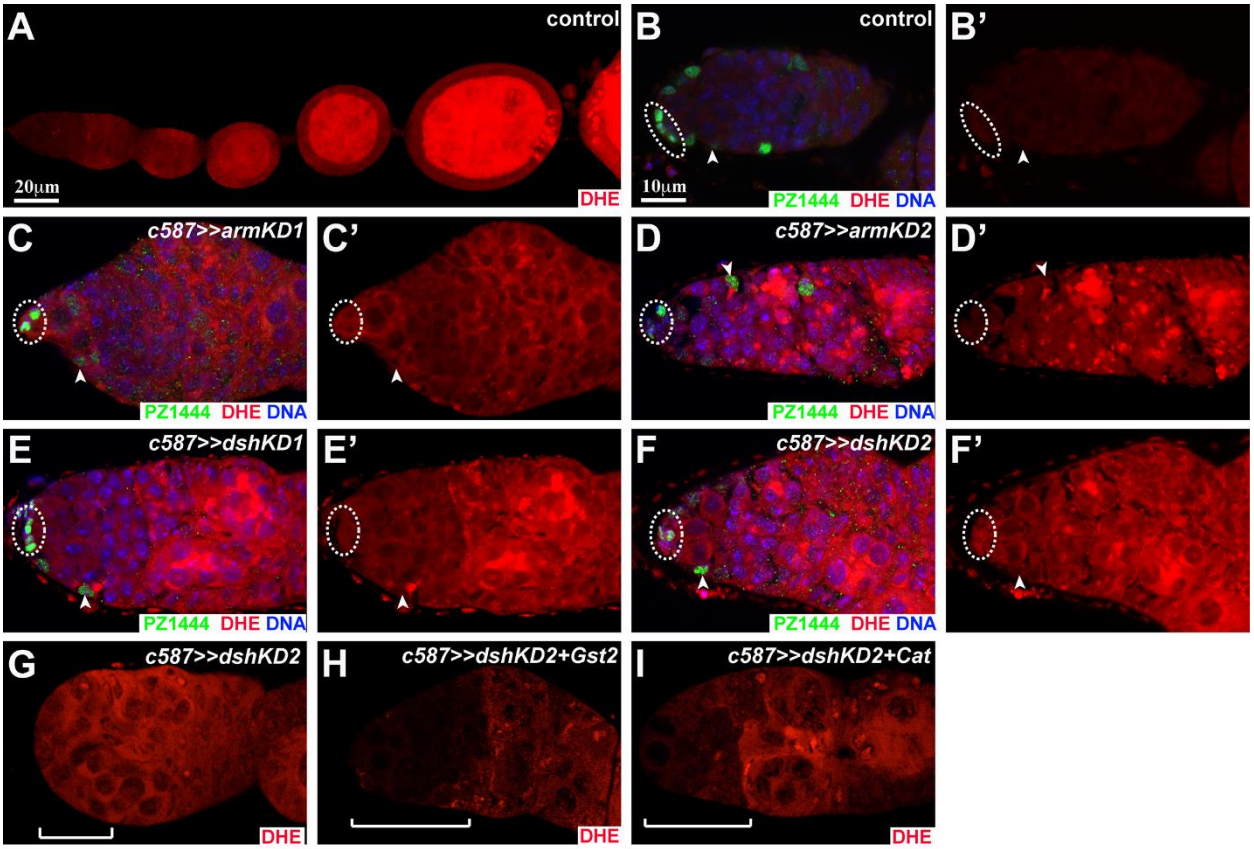

A
control
20μm
DHE
B
control
10μm
PZ1444 DHE DNA
B'
C
c587>>armKD1
PZ1444 DHE DNA
C'
D
c587>>armKD2
PZ1444 DHE DNA
D'
E
c587>>dshKD1
PZ1444 DHE DNA
E'
F
c587>>dshKD2
PZ1444 DHE DNA
F'
G
c587>>dshKD2
DHE
H
c587>>dshKD2+Gst2
DHE
I
c587>>dshKD2+Cat
DHE

Figure 3.10. Reduced redox state is important for ISC maintenance. In **A**-**F**, broken ovals highlight cap cells and GSCs, whereas arrowheads point to CBs. (**A**-**G**) Overexpressing GST2 (**A**), CAT (**B**) or SOD1 (**C**) in ISCs does not affect GSC maintenance and germ cell differentiation, but drastically reduces CBs in the *dshKD2* germaria (**D**-**F**). **G:** GSC quantification results. In **H**-**M**, broken ovals highlight cap cells and GSCs, whereas arrowheads point to PZ1444-labeled ISCs. (**H**-**M**) (**N**-**P**) Overexpressing GST2 (**H**), CAT (**I**) or SOD1 (**J**) in ISCs does not affect ISC maintenance, but drastically rescues the ISC loss phenotype in the *dshKD2* germaria (**K**-**M**).

Figure 3.10

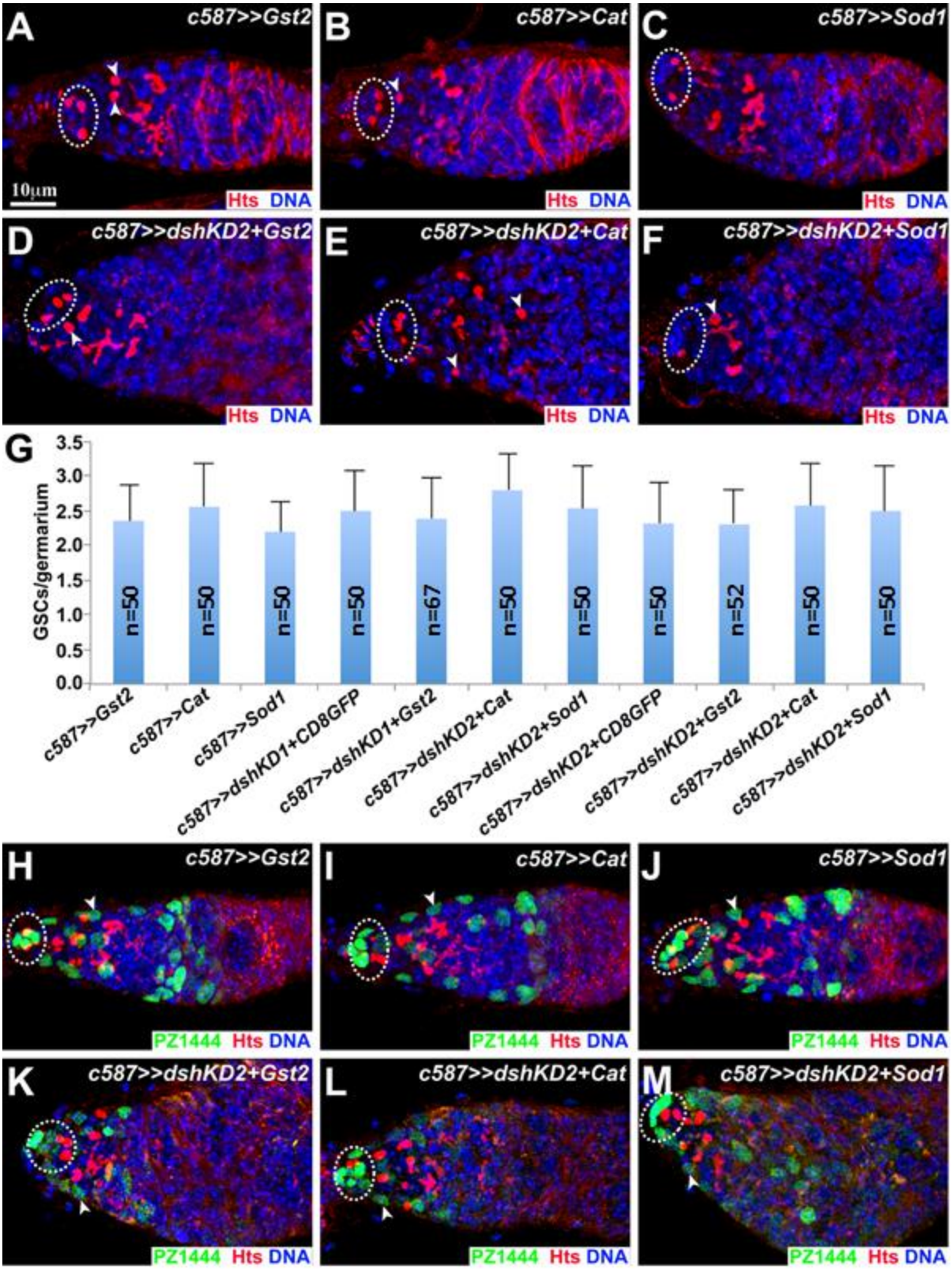
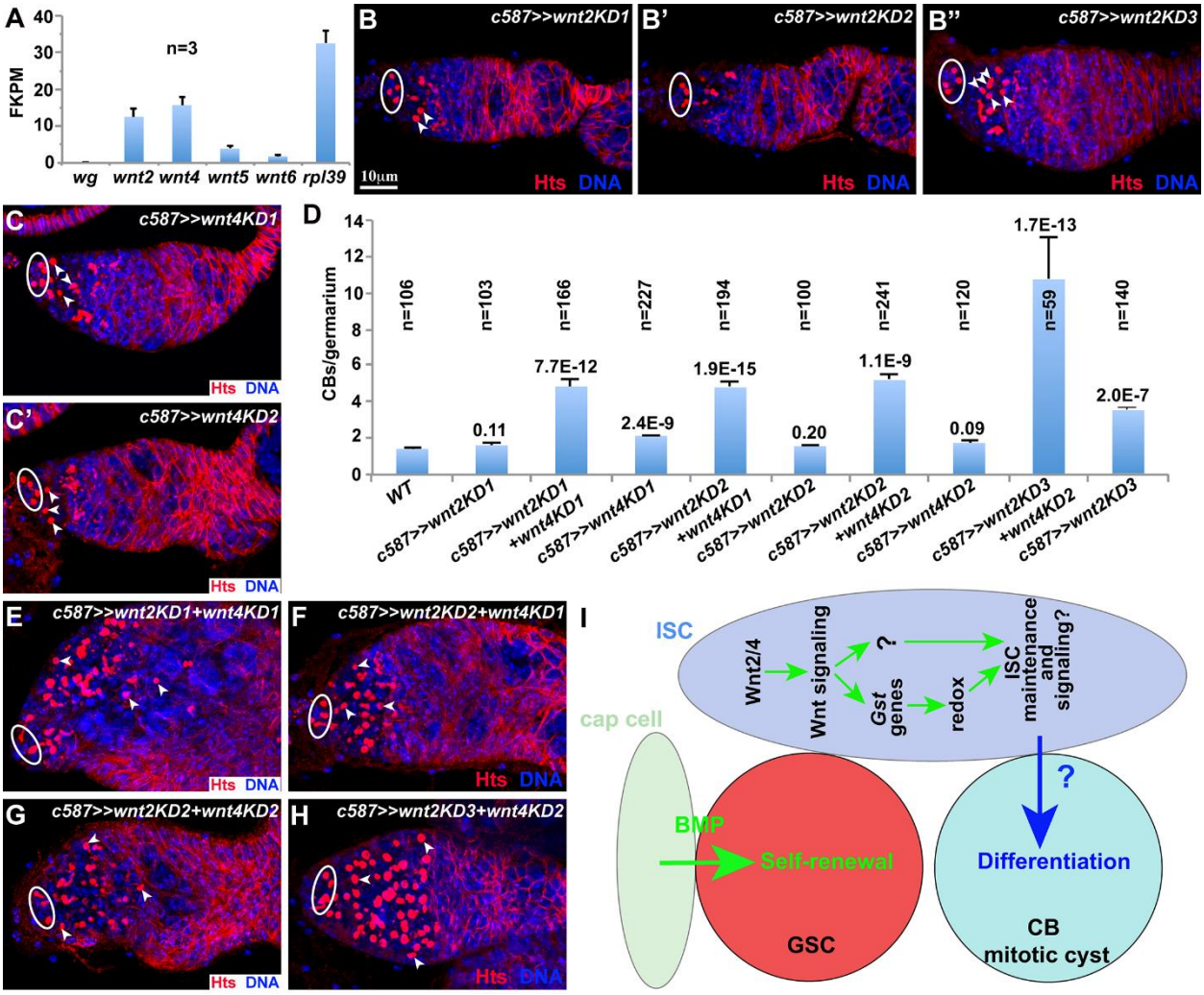

A
c587>>Gst2
10μm
Hts DNA
B
c587>>Cat
Hts DNA
C
c587>>Sod1
Hts DNA
D
c587>>dshKD2+Gst2
Hts DNA
E
c587>>dshKD2+Cat
Hts DNA
F
c587>>dshKD2+Sod1
Hts DNA
G
GSCs/germarium
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
n=50
n=50
n=50
n=50
n=67
n=50
n=50
n=50
n=52
n=50
n=50
c587>>Gst2
c587>>Cat
c587>>Sod1
c587>>dshKD1+CD8GFP
c587>>dshKD1+Gst2
c587>>dshKD2+Cat
c587>>dshKD2+Sod1
c587>>dshKD2+CD8GFP
c587>>dshKD2+Gst2
c587>>dshKD2+Cat
c587>>dshKD2+Sod1
H
c587>>Gst2
PZ1444 Hts DNA
I
c587>>Cat
PZ1444 Hts DNA
J
c587>>Sod1
PZ1444 Hts DNA
K
c587>>dshKD2+Gst2
PZ1444 Hts DNA
L
c587>>dshKD2+Cat
PZ1444 Hts DNA
M
c587>>dshKD2+Sod1
PZ1444 Hts DNA

Figure 3.11. Wnt2 and Wnt4 function redundantly in ISCs to promote germ cell differentiation. (**A**) RNA sequencing results show that *Wnt2* and *Wnt4* are highly expressed in the purified ISCs in comparison with *Wnt5* and *Wnt6*. In **B**-**C'** and **E**-**H**, cap cells and GSCs are highlighted by ovals. (**B**-**D**) *Wnt2* (**B**-**B"**) or *Wnt4* (**C**, **C'**) knockdown by independent RNAi lines causes a slight accumulation of CBs (arrowheads). **D** shows quantification results on CB numbers. (**E**-**H**) Knocking down both *Wnt2* and *Wnt4* significantly increases CBs (some by arrowheads). (**I**) A schematic diagram showing that autocrine *Wnt2* and *Wnt4* signals control ISC maintenance and promote germ cell differentiation at least in part by maintaining the reduced redox state.

Figure 3.11


A
FKPM
n=3
wg
wnt2
wnt4
wnt5
wnt6
rpl39
B
c587>>wnt2KD1
B'
c587>>wnt2KD2
B"
c587>>wnt2KD3
10μm
Hts DNA
C
c587>>wnt4KD1
C'
c587>>wnt4KD2
D
CBs/germarium
n=106
n=103
n=166
n=227
n=194
n=100
n=241
n=120
n=59
n=140
0.11
7.7E-12
2.4E-9
1.9E-15
0.20
1.1E-9
0.09
1.7E-13
2.0E-7
WT
c587>>wnt2KD1
c587>>wnt2KD1 +wnt4KD1
c587>>wnt4KD1
c587>>wnt2KD2 +wnt4KD1
c587>>wnt2KD2
c587>>wnt2KD2 +wnt4KD2
c587>>wnt4KD2
c587>>wnt2KD3 +wnt4KD2
c587>>wnt2KD3
E
c587>>wnt2KD1+wnt4KD1
F
c587>>wnt2KD2+wnt4KD1
G
c587>>wnt2KD2+wnt4KD2
H
c587>>wnt2KD3+wnt4KD2
I
ISC
Wnt2/4
Wnt signaling
?
Gst genes
redox
ISC maintenance and signaling?
cap cell
BMP
Self-renewal
GSC
?
Differentiation
CB
mitotic cyst

Chapter 4: Discussion

Although the differentiation niche is critical for promoting GSC progeny differentiation, little is known about its regulation. Here we have identified Wnt2 and Wnt4 as autocrine signals to maintain the GSC differentiation niche partly through redox regulation (Figure 3.11I). First, Wnt signaling is required in ISCs for their maintenance by promoting cell proliferation and survival. Defective Wnt signaling causes a severe ISC loss, thereby preventing germ cell differentiation. Second, Wnt signaling is required to maintain the reduced redox state by sustaining the expression of *Gst* genes. This represents a novel connection between Wnt signaling and redox control. In addition, this study has also revealed that the reduced redox state is critical for ISC survival and thus for promoting germ cell differentiation. Third, Wnt2 and Wnt4 represent redundant autocrine signals for maintaining Wnt signaling in the germ cell differentiation niche. Wnt signaling has been shown to control stem cell self-renewal directly [104], whereas ROS has shown to prime hematopoietic progenitor differentiation in *Drosophila* [105]. This study has demonstrated the importance of Wnt signaling in maintaining the GSC differentiation niche by reducing ROS and thus promoting GSC lineage differentiation. Therefore, this study not only has identified critical signals for maintaining the GSC differentiation niche but also has revealed a novel function of Wnt signaling in the regulation of cellular redox.

This study has demonstrated that autocrine Wnt signaling controls ISC maintenance, thereby promoting germ cell differentiation. First, canonical Wnt signaling is required in ISCs to promote germ cell differentiation. ISC-specific knockdown of Wnt signal transducers *Fz/fz2*, *dsh* and *arm* as well as ISC-specific overexpression of Wnt signaling inhibitors *sgg* and *axn* lead to similar germ cell differentiation defects. Moreover, ISC-specific overexpression of a

constitutively active form of *arm** can fully rescue the germ cell differentiation defect caused by *dsh* knockdown. Second, Wnt signaling maintains ISCs by promoting proliferation and survival. ISC-specific knockdown of *dsh* and *arm* leads to a severe ISC loss, whereas ISC-specific knockdown of *sgg* and *axn* or ISC-specific *arm** overexpression expands the ISC population. In addition, hyperactive Wnt signaling increases ISC proliferation and decreases apoptosis, whereas Wnt signaling downregulation increases ISC apoptosis and decreases proliferation. Thus, canonical Wnt signaling maintains the differentiation niche by promoting ISC proliferation and survival. Third, ISC-expressing Wnt2 and Wnt4 function redundantly in the differentiation niche to promote germ cell differentiation. Our RNA-seq results show that *wnt2* and *wnt4* mRNAs are present in the purified ISCs at high levels, while other *wnt* genes are expressed at much lower levels. ISC-specific *wnt2* and *wnt4* double knockdown results in more severe germ cell differentiation defects than *wnt2* or *wnt4* single knockdown.

Piwi has recently been shown to be required in the differentiation niche for promoting germ cell differentiation partly by repressing *dpp* expression [58, 87]. Although a recent study proposes that Wnt signaling controls germ cell differentiation by regulating *piwi* expression [92], this study shows that Piwi protein and mRNAs are not downregulated in Wnt signaling-defective ISCs, suggesting that Wnt signaling does not control *piwi* expression in the differentiation niche. Instead, this study has further revealed that Wnt signaling maintains the differentiation niche by controlling the cellular redox. First, our RNA-seq results show that *GstD2*, *GstD4*, *GstD10* and *GstE3* mRNA expression levels are significantly downregulated in the purified Wnt signaling-defective ISCs in comparison with the control ISCs. Second, defective Wnt signaling in ISCs elevates ROS levels in themselves and underneath germ cells, indicating that Wnt signaling is required in the differentiation niche to maintain low ROS levels. Third, ISC-specific GST2,

SOD1 and CAT overexpression can dramatically suppress the ROS elevation, the germ cell differentiation retardation and the ISC loss caused by defective Wnt signaling, indicating that ROS elevation is responsible for the germ cell differentiation defect and the ISC loss. Finally, ISC-specific knockdown of *GstD2* and *Cat* results in the ISC loss and the germ cell differentiation defect, indicating that ROS elevation in ISCs is sufficient to cause ISC loss and retard germ cell differentiation. This study has, for the first time, demonstrated that autocrine Wnt signaling controls cellular redox state in the differentiation niche and thus promotes germ cell differentiation.

So far, various studies have revealed a number of important signaling pathways and factors in ISCs to promote germ cell differentiation by maintaining ISC cellular process-mediated germ cell-soma interaction and preventing BMP signaling [77]. This study shows that Wnt signaling is required for preventing BMP signaling in differentiated germ cells and for maintaining long ISC cellular processes. Rho, Eggless, Woc, Lsd1, Piwi, EGFR signaling and ecdysone signaling have been shown to be required in ISCs to maintain long ISC cellular processes encasing germ cells [57, 58, 70, 83, 85, 89, 90]. Since properly differentiated germ cells are also required to maintain long ISC cellular processes [70], it is difficult to distinguish the cause and effect of the germ cell differentiation defect and the ISC cellular process loss. In contrast, three known strategies operate in the differentiation niche to prevent BMP signaling, thereby producing a permissive environment for germ cell differentiation. First, Lsd1, Rho and Piwi are required in ISCs to repress *dpp* mRNA expression, thereby directly preventing BMP signaling in the differentiation niche [58, 70, 87, 91]. *dpp* encodes a BMP ligand, which activates BMP signaling important for GSC self-renewal [75]. Second, Rho, Eggless and EGFR signaling function in ISCs to repress the expression of *dally,* preventing BMP diffusion from the self-

renewal niche to the differentiation niche [57, 60, 70]. Dally, which is a proteoglycan protein facilitating BMP differentiation and signaling, is expressed in cap cells, the GSC self-renewal niche, to restrict BMP signaling to GSCs [30, 106]. Third, a recent study has proposed that cap cell-initiated Wnt signaling maintains the expression of *tkv* encoding a type I BMP receptor in ISCs, thereby preventing BMP signaling in differentiated germ cells [103]. However, this study shows that inactivating Wnt signaling in ISCs does not affect the mRNA expression of *tkv* and other BMP pathway components. It remains possible that Wnt signaling controls Tkv protein expression at the level of translation or post-translation. Therefore, future research will be needed to investigate the potential molecular mechanisms for Wnt signaling in the differentiation niche to prevent BMP signaling and maintain long ISC cellular processes.

Like all scientific researches, this study also contains several potential caveats when interpreting the data.

In this study, we used *c587-gal4* to express all UAS lines in ISCs. However, based on *c587>gfp* staining (figure 3.1 A), besides ISCs, *c587-gal4* also express UAS lines in anterior FCs, although the expression level is very low. Such expanded expression might lead to some misinterpretation to our data. To avoid this possibility, we should use other more specific gal4 lines. *13C06-gal4* was initially constructed for *Drosophila* neuroanatomy and neurogenetics research [107]. And in *Drosophila* germarium, it has more specific ISC expression pattern as identified in previous study [108]. So when compared with *c587-gal4*, this line might be better to study genes or signaling function in differentiation niche.

Our data has demonstrated that Wnt signaling is critical for differentiation niche maintenance as well as germ cell differentiation. However, our previous research has addressed

that germ cell differentiation and differentiation niche formation are required for each other. So it is still unclear in our study, Wnt signaling regulate differentiation niche directly or germ cell differentiation. In another word, which one is the cause and which one is the consequence. To investigate this, we could generate one or a few Wnt defective ISC clones via the positively marked mosaic lineage (PMML) system [109]. Only one or a few ISC mutant clones are not enough to lead to severe germ cell differentiation. So we will detect whether in these mutant clones, the proliferation, apoptosis and cellular process are disrupted. If they are changed in mutant clones, it is likely that the Wnt signaling regulates differentiation niche maintenance directly. If not, it is possible that Wnt signaling controls germ cell differentiation directly.

Chapter 5: Conclusions and Future Directions

Conclusions

The main conclusions of this study are summed up in the following:

Wnt signaling is required in adult ISCs to promote GSC lineage differentiation

In the *Drosophila* ovary, differentiation niche consisted of ISCs is essential for normal germ cell differentiation. In this study, we show that Wnt signaling, activated by Wnt2 and Wnt4, is responsible for germ cell differentiation by maintaining ISC long cellular processes as well as population via both proliferation and survival. Reducing Wnt signaling in adult flies by knocking down *dsh*, *arm*, or overexpressing *axn*, *sgg* or double knocking down *wnt2* and *wnt4* leads ISC loss, defective ISC long cellular processes and the germ cell differentiation defect.

Wnt signaling maintains the cellular redox state in ISCs, GSCs, and early differentiated germ cells by maintaining Gst gene expression.

In this study, we performed RNA-Seq experiment and identified that in ISCs, Wnt signaling function to maintain Gst gene expression level, which further regulates ISC cellular redox state. We confirmed this conclusion by performing ROS and ISC double staining in Wnt defective germarium.

GstD2 works with Cat in ISCs to control germ cell differentiation

Oxidative stress response genes in ISCs maintain the cellular redox state down in the differentiation niche to regulate germ cell differentiation. Knocking these oxidative stress

response genes in ISCs lead to high ROS level, and consequently ISC loss and germ cell differentiation defects. Overexpressing these genes can partially rescue the germ cell differentiation phenotype caused by defective Wnt signaling.

Future Directions

Using *Drosophila* genetics as our base, we have isolated a new role for Wnt signaling in the ovary. New insights into developmental biology, Wnt biology and stem cell research have been revealed. However, a number of questions still remain unanswered and to be explored in future projects.

Wnt and BMP Signaling in Differentiation Niche

In Chapter 3, our results indicate that Wnt signaling in ISCs regulates germ cell differentiation and maintains the differentiation niche. In the *Drosophila* germarium, BMP has been addressed to be the most critical component functioning in GSC niche to directly control GSC self-renewal by repressing differentiation. In this study, we also performed multiple BMP signaling activity marker staining showing that BMP expands outside the GSC niche in the Wnt signaling defective germarium. However, in the purified ISC RNA-Seq result, we didn't observe significant change of BMP involved genes. Furthermore, we also tried to rescue differentiation defect phenotype in mutant gemarium by reducing BMP activity in ISCs. The rescue effect is not dramatic. So it is still unclear whether Wnt could affect germ cell differentiation directly through In the future, we would continue to investigate whether there is interaction between Wnt and BMP in ISCs by studying more other BMP singaling components.

Gst Genes and the Cellular Redox State in Differentiation Niche

In this study, we show that *Gst* family genes act downstream of the Wnt signaling pathway in ISCs to maintain the differentiation niche and regulate germ cell differentiation. And low ROS

level is required in the gemarium, at least in anterior half, to promote germ cell differentiation. However, it is still unknown why germ cell early differentiation requires low ROS state. It is possible that high oxidative stress may lead to more DNA damage which may disrupt genetic information transfer. So to maintain low ROS might be a protective mechanism in productive system and even more, maybe this is also related to aging-related stem cell malfunction. This could be interesting to study in future projects.

In this study, we also demonstrated that to overexpress anti-oxidant genes can partially rescue the tumor phenotype in defective Wnt germarium. And similar to Wnt signaling, other genes, e.g. *egg*, *RhoI*, also function in ISCs to maintain ISC population, morphology. It is interesting to investigate whether *Gst* family genes or other oxidative stress response genes are the common downstream of these genes or signaling pathways. We can verify this hypothesis by performing the same experiments. We can detect the oxidative stress response genes expression level in other tumor mutant samples. And also we could perform anti-oxidant gene overexpression to rescue the tumor germarium. If results are consistent to this study, which means that to maintain low ROS level is critical for germ cell differentiation in the ovary.

The Key in Differentiation Niche

Signaling pathways and genes are continuously identified in the differentiation niche to promote germ cell differentiation. However, all these researches are almost independent to each other. So far there is no mechanism that can correlate all reported mutant phenotypes, which is important to the differentiation niche study. To pursue the answer, we could perform RNA-Seq on ISCs mutant to identify common target genes. It is possible that there are some genes whose

expression levels are changed consistently in all, or most, mutant ISCs, similar to *bam* in germ cells. These genes might be critical for germ cell differentiation regulation. To determine such genes' function, we could perform genetic experiments to confirm the result from RNA-Seq. For example, we can manipulate these genes' expression level to detect whether changing these genes can lead to germ cell differentiation defects, or can rescue the reported mutant phenotype. All these results could help us to explore the mechanism that hides behind all the scattered discoveries in *Drosophila* ovarian differentiation niche.

Apply to other systems

In this study, we have explored the relations between Wnt signaling in ISCs, germ cell differentiation and cellular redox state in whole germarium, which discovered a novel mechanism that how somatic differentiation niche function to regulate stem cell maintenance and differentiation. The results and conclusions of this study could be applied to other systems and further to clinical trials.

Because of the complexity of interactions between germ cells and surrounding somatic cells *in-vivo*, it is also important to promote stem cell differentiation *in-vitro* before applying to clinical use. With the successfully established *Drosophila* ovarian germ cell and somatic cell co-culture system [110], we can apply our result and conclusions to *in-vitro* system to indicate a new clinical medicine target of germline stem cell differentiation regulation in somatic differentiation niche.

Furthermore, the interactions between stem cell and surrounding cells are quite common in other stem cell systems. For example, in mouse testis, the sertoli cells behave similarly to ISCs in

Drosophila ovary. It is likely that sertoli cell also function as differentiation niche to spermatogenic stem cell. So if we could apply this study to mouse testis system, it would be very helpful to study how stem cell differentiation is regulated in high level animal models as mammals.

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