

The *Drosophila* cyst stem cell lineage

Partners behind the scenes?

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In all animals, germline cells differentiate in intimate contact with somatic cells and interactions between germline and soma are particularly important for germline development and function. In the male gonad of *Drosophila melanogaster*, the developing germline cells are enclosed by somatic cyst cells. The cyst cells are derived from cyst stem cells (CySCs) of somatic origin and codifferentiate with the germline cells. The fast generation cycle and the genetic tractability of *Drosophila* has made the *Drosophila* testis an excellent model for studying both the roles of somatic cells in guiding germline development and the interdependence of two separate stem cell lineages. This review focuses on our current understanding of CySC specification, CySC self-renewing divisions, cyst cell differentiation, and soma-germline interactions. Many of the mechanisms guiding these processes in *Drosophila* testes are similarly essential for the development and function of tissues in other organisms, most importantly for gametogenesis in mammals.

Introduction

Adult stem cells have been identified from many tissues where they constantly produce highly specialized cells, such as germline, blood, and skin cells.¹⁻⁵ A *Drosophila* testis contains two types of stem cells, germline stem cells (GSCs) and cyst stem cells (CySCs), which give rise to the germline cells (blue in **Fig. 1A**), and cyst cells (yellow in **Fig. 1A**), respectively.^{3,6} Electron-microscopy studies revealed that GSCs and CySCs are arranged in rosettes around a single group of terminally differentiated somatic cells, called the hub, at the apical tip of a wildtype testis.³ Each GSC is flanked by a pair of CySCs which extend cytoplasmic protrusions around the GSC and into the hub (illustrated in **Fig. 1A**). GSCs and CySCs divide asymmetrically to produce daughter cells (gonialblasts and cyst cells, respectively) that form developmental units, termed cysts. During cyst formation, two cyst cells grow cytoplasmic extensions around a single gonialblast to completely enclose it, thereby isolating it from direct contact with any other cell type. Once a cyst is formed, the two cyst cells and the enclosed gonialblast codifferentiate. The gonialblast undergoes four rounds of transit amplification divisions to

produce 16 spermatogonia, which then become spermatocytes, and grow in size. After the growth phase, the spermatocytes undergo meiosis and differentiate into elongated spermatids. During all stages of germline differentiation, the two cyst cells continue to enclose the germline cells, grow tremendously in size, and codifferentiate with the germline. During terminal differentiation, morphological differences between the two cyst cells become apparent, as one cyst cell, the tail cyst cell, becomes much larger than the other cyst cell, the head cyst cell (TCC, HCC; **Fig. 1A**). The developing cysts become progressively displaced toward more basal regions of the testis. This results in a spatio-temporal arrangement of cysts along the apical to basal axis of the wildtype testis.^{3,7-9}

The cyst cell lineage can be visualized easily by immunofluorescence microscopy. Several cell surface markers, such as Semaphorin (green in **Fig. 1B**), are highly expressed on the membranes of cyst cells (Zoller and Schulz, unpublished data) and allow for visualization of the cytoplasmic extensions enclosing the germline cells (red in **Fig. 1B**). Antibodies raised against the transcription factors Zinc finger homeodomain-1 (Zfh-1), Traffic jam (Tj), and Eyes absent (Eya) can be used to assess the developmental stage of the cyst cells. Zfh-1 (red in **Fig. 1C**) is expressed at high levels in CySCs, fading rapidly in early stage cyst cells (cyst cells associated with proliferating germline cells).¹⁰ Tj (green in **Fig. 1C and D**) is expressed at equal levels in CySCs and in early stage cyst cells.¹¹ Eya (red in **Fig. 1D**) shows a very low level of expression in early stage cyst cells and a high level of expression in late stage cyst cells (cyst cells associated with post-mitotic germline cells).¹² In addition to these antibodies, marker genes expressed from transposon insertion lines or driven by tissue-specific Gal4-transactivators provide useful tools for identifying and staging cyst cells throughout the *Drosophila* testis.¹³⁻¹⁵

The arrangement of the germline and the cyst cells in *Drosophila* testes is similar to the arrangement of germline and somatic cells in mammalian testes. Just as in a *Drosophila* testis, the germline cells in a mammalian testis are arranged in a spatio-temporal order along the axis, with the youngest stage germline cells located next to the basal membrane and the more differentiated germline stages located toward the lumen of the seminiferous tubules.^{16,17} Mammalian germline cells are enclosed in large somatic Sertoli cells, which act as a physical barrier as well as a source for nutrients and regulatory molecules. Physical isolation of the differentiating germline cell clusters from each other is achieved via localization of specialized cell junctions between the

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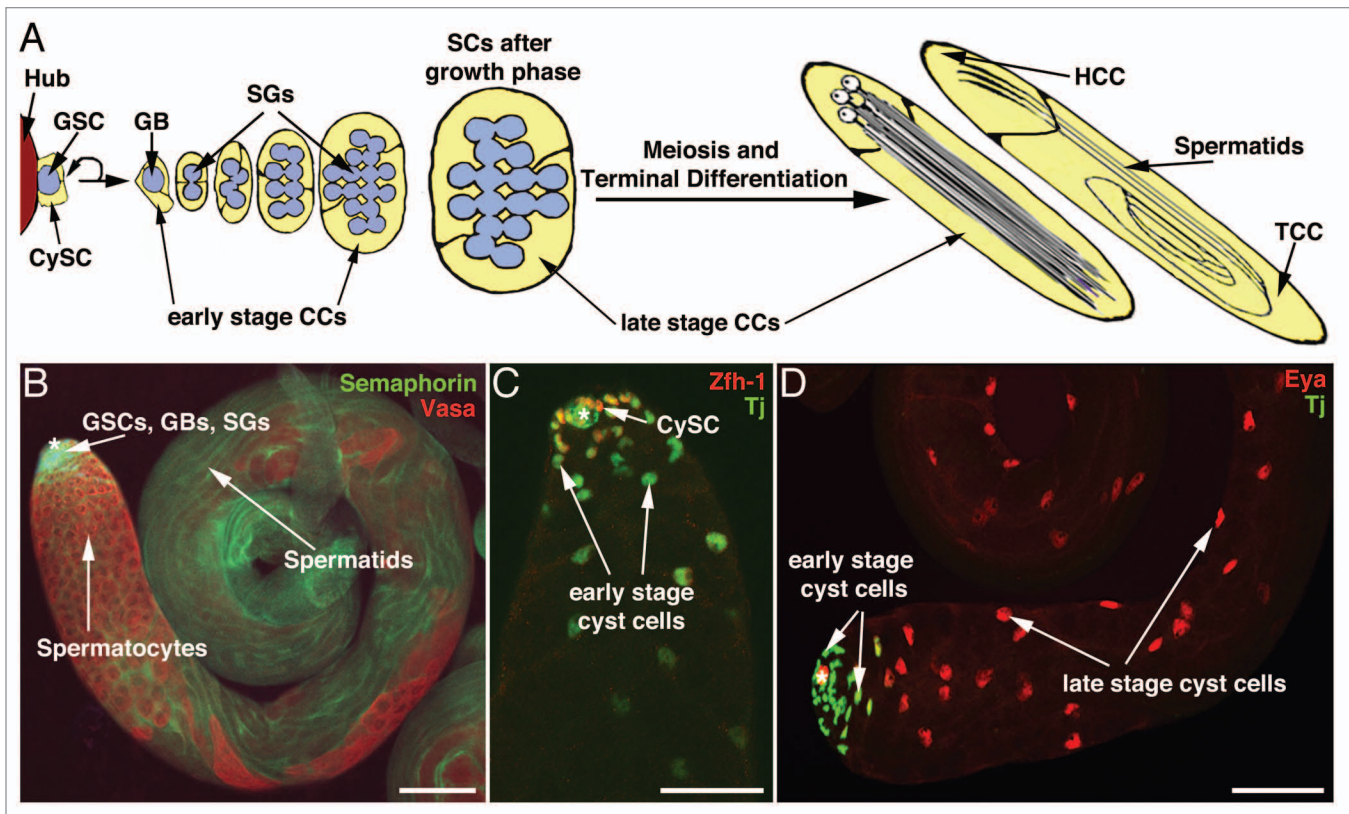


Figure 1. The organization of somatic cells and germline cells in wildtype testes. (A) Graphic depicting the organization and development of the cysts along the apical (left) to basal (right) axes of the testis. CySC, cyst stem cell; GSC, germline stem cell; GB, gonialblast; SG, spermatogonia; SC, spermatocytes; CC, cyst cells; HCC, head cyst cells; TCC, tail cyst cells. (B–D) Immunofluorescent labeling of wildtype testes as indicated. (B) Visualization of cyst cells enclosing the germline cells in a whole testis. (C) The apical tip of a testis showing colocalization of Zfh-1 and Tj. (D) The apical region and the testis coil. Tj is expressed in early stage cyst cells near the tip, and Eya is expressed strongly in late stage cyst cells. *hub cells; scale bars, 50 μm .

Sertoli cells and the germline cells.^{16,18–21} It appears that the enclosure of the developing germline cells, therefore, is dependent upon different mechanisms in *Drosophila* and mammals. While each cluster of germline cells in a *Drosophila* testis has a pair of microenvironment cyst cells generated by CySC divisions, each cluster of germline cells in a mouse testis sits in a compartment within the microenvironment Sertoli cell marked by the localization of junction proteins. The similarity in the tight association between germline and soma in *Drosophila* and mammalian testes, though, suggests that mechanisms and molecules setting up and maintaining the organization, and the communication pathways between germline and soma may be conserved.

The Origin and Identity of CySCs

In most organisms, the germline and somatic cells of the gonad are specified independently and coalesce during development to form the gonad.¹⁷ The *Drosophila* somatic cyst cell lineage originates from somatic gonadal precursor cells (SGPs) specified in parasegments 10–12 during embryogenesis. As the primordial germline cells migrate from the posterior end of the embryo toward the position of the future gonad in parasegment 10, they pass through parasegments 12 and 11 where they associate with the SGPs. Together, the germline and the SGPs from

parasegments 12 and 11 migrate anteriorly and join the SGPs of parasegment 10. There, the germline cells and the SGPs coalesce to form the gonad and the SGPs become specified into the different somatic cell types of the testis.^{22–24} (For a comprehensive review of gonad formation, please visit the review by Mark Van Doren in this issue of *Spermatogenesis*).

A common origin of cyst cells and hub cells from SGPs was shown by lineage tracing experiments. When gonadal precursor cells were labeled in parasegment 11, label-positive progeny were subsequently found among both the hub cells and the cyst cells of adult testes.²⁵ Consistent with the common origin of the two somatic cell types, the cyst cells and the hub cells both express Zfh-1, Tj, and marker genes from enhancer trap lines.^{10,11,13}

The specification of the SGPs as CySCs vs. hub cells was suggested to depend on signaling via the Notch (N), the Epidermal Growth Factor (EGF) and the Sevenless (Sev) signaling pathways. N signaling between the SGPs was sufficient to specify hub fate in SGPs throughout the embryonic gonad. Throughout the gonad, except for the most anterior region, N is opposed by EGF and Sev signaling from the germline to the SGPs. This results in specification of SGPs as hub cells at the anterior tip of the embryonic gonad, and in the specification of SGPs as CySCs in the remainder of the embryonic gonad.^{26–28}

Both specification and maintenance of CySC vs. hub cell fate in *Drosophila* testes requires the antagonistic roles of *lines* (*lin*) and *brother of odd with entrails limited* (*bowl*).²⁵ The *bowl* gene encodes a zinc finger transcription factor and *lin* encodes a cytoplasmic protein with catalytic activity. During *Drosophila* epidermal cell differentiation, Lin binds directly to Bowl to reduce Bowl abundance, possibly by targeting Bowl for degradation. Another protein, Drumstick (*Drm*), competes with Lin for binding to Bowl and, thus, promotes Bowl accumulation.²⁹ Transcription of Bowl was upregulated in testes with excess stem cells based on microarray analysis.³⁰ This observation led to the investigation of a requirement for Bowl and its antagonist Lin in testes. While *bowl* mutant embryonic gonads had fewer hub cells, *lines* mutant embryonic gonads had an increased number of hub cells in comparison to wildtype gonads. Likewise, when CySCs in adult testes were depleted of *lines*, their progeny accumulated in aggregates that expressed a variety of hub-specific marker genes, including a *hedgehog*-reporter and Cactus. Similar to hub cells, these hub-like aggregates appeared to recruit CySCs. The hub-like aggregates failed to recruit functional GSCs based on the expression of several GSC specific marker genes, implying that they are not identical to hub cells.²⁵ How exactly Bowl specifies CySC vs. hub cell fate has not been revealed and no target genes of Bowl have been identified in testes.

Substantiating the common origin of cyst cells and hub cells, CySCs are able to convert into hub cells: they can associate with the hub cells and express hub cell markers. In testes devoid of germline cells, CySCs obtain hub cell identity at a high frequency, suggesting that germline cells play a central role in maintaining the CySC population.⁶ The ability of CySCs to convert into hub cells was also observed in wildtype testes, yet reports on the frequency of this conversion are conflicting.^{25,31} Voog³¹ reported a high frequency of conversion (one converted CySC in 46% of 74 testes examined), while others reported that CySCs very rarely generated daughter cells that adopted hub cell fate. Dinardo²⁵ did not find any converted CySCs out of 40 testes, even though they used the same tools for lineage tracing as exploited by Voog.³¹ It remains to be resolved whether different genetic backgrounds or growth conditions may be the reason for the wide range in conversion frequency.

Lineage tracing experiments confirmed beyond any doubt that CySCs are indeed functional stem cells, as they both self-renew and produce differentiating daughter cells. In a key experiment, a Flippase recombinase was transiently expressed to induce a recombination event that randomly and permanently marked single cells and their progeny by reconstitution of a β -galactosidase (*lacZ*) marker gene. LacZ-positive cells persisting next to the hub had to be either GSCs or CySCs. GSCs produce many clusters of LacZ-positive, proliferating germline cells. CySCs, in contrast, produce daughter cyst cells that do not divide further, but together with a second cyst cell, enclose the developing germline. As expected, LacZ-positive CySCs were observed next to the hub and these stem cells produced a series of single, LacZ-positive cells that associated with developing germline cells.⁶ With these experiments, it was established that the CySCs

in *Drosophila* testes are indeed stem cells, as they self-renew and produce differentiating daughter cells. Subsequent research shifted toward studying the specification of both stem cell populations, the GSCs and the CySCs.

CySC Self-renewal Depends on Signals from the Hub

A series of elegant genetic experiments demonstrated how the cell fate decisions of CySCs to become either new CySCs or cyst cells is regulated (illustrated in Fig. 2A). CySC fate is dependent on signaling from the hub cells via the Janus kinase and Signal Transducer and Activator of Transcription (JAK/STAT) pathway.^{32,33} The highly conserved Jak/STAT signaling pathway is essential in a number of developmental processes in *Drosophila* and in mammals, and has been associated with a variety of cancers.³⁴⁻³⁹ The JAK/STAT signaling pathway was originally discovered as a cytokine-induced signaling pathway required by the myeloid and lymphoid cell lineages and is now known to regulate many stem cell populations, including stem cells in the *Drosophila* ovary and intestine, and murine embryonic stem cells.⁴⁰⁻⁴³ The core JAK/STAT signaling pathway is relatively simple. Binding of the cytokine to its receptor induces conformational changes that lead to activation of associated JAKs. Activated JAKs phosphorylate the cytokine receptors and the tyrosine-phosphorylated motifs in the cytokine receptors serve as docking sites for the SH2 domains of STATs. Once bound to the receptor, the STATs become activated by tyrosine phosphorylation. Phosphorylated STATs dissociate from the receptors, dimerize, and translocate into the nucleus to regulate transcription of target genes.⁴⁴

Drosophila males carrying a temperature sensitive allele of the JAK, *hopscotch* (*hop*), or *stat* were raised at a permissive temperature and then shifted to a restrictive temperature to induce a phenotypic change in the gonad. In testes from shifted animals, CySCs and GSCs were progressively lost. This suggested that GSCs and CySCs failed to self-renew and instead differentiated into cyst cells. Conversely, hyperactivation of JAK/STAT signaling had the opposite effect. Overexpression of the ligand Unpaired (*Upd*), that is normally only expressed in the hub, throughout the germline resulted in the excessive accumulation of CySCs and GSCs, as evident by their expression of stem cell specific marker genes.^{32,33} These studies demonstrated that JAK/STAT signaling induces CySC and GSC fate in *Drosophila* testes. (For a comprehensive review of GSC specification and function, please visit the review by Erika Matunis in this issue of Spermatogenesis).

Additional studies revealed that two proteins act downstream of JAK/STAT to regulate CySC fate in *Drosophila* testes. These are *Zfh-1* and Chronologically inappropriate morphogenesis (*Chinmo*), a protein that may act as a transcriptional regulator or play a role in protein degradation.^{10,45,46} Analogous to *hop* and *stat* mutants, *zfh-1* or *chinmo* deficient CySCs failed to self-renew. Analogous to *Upd* overexpression in the germline, overexpression of *Zfh-1* or *Chinmo* in the cyst cells resulted in an accumulation of CySCs and GSCs.^{10,46} These findings strongly suggest that the activities of *Zfh-1* and *Chinmo* depend on

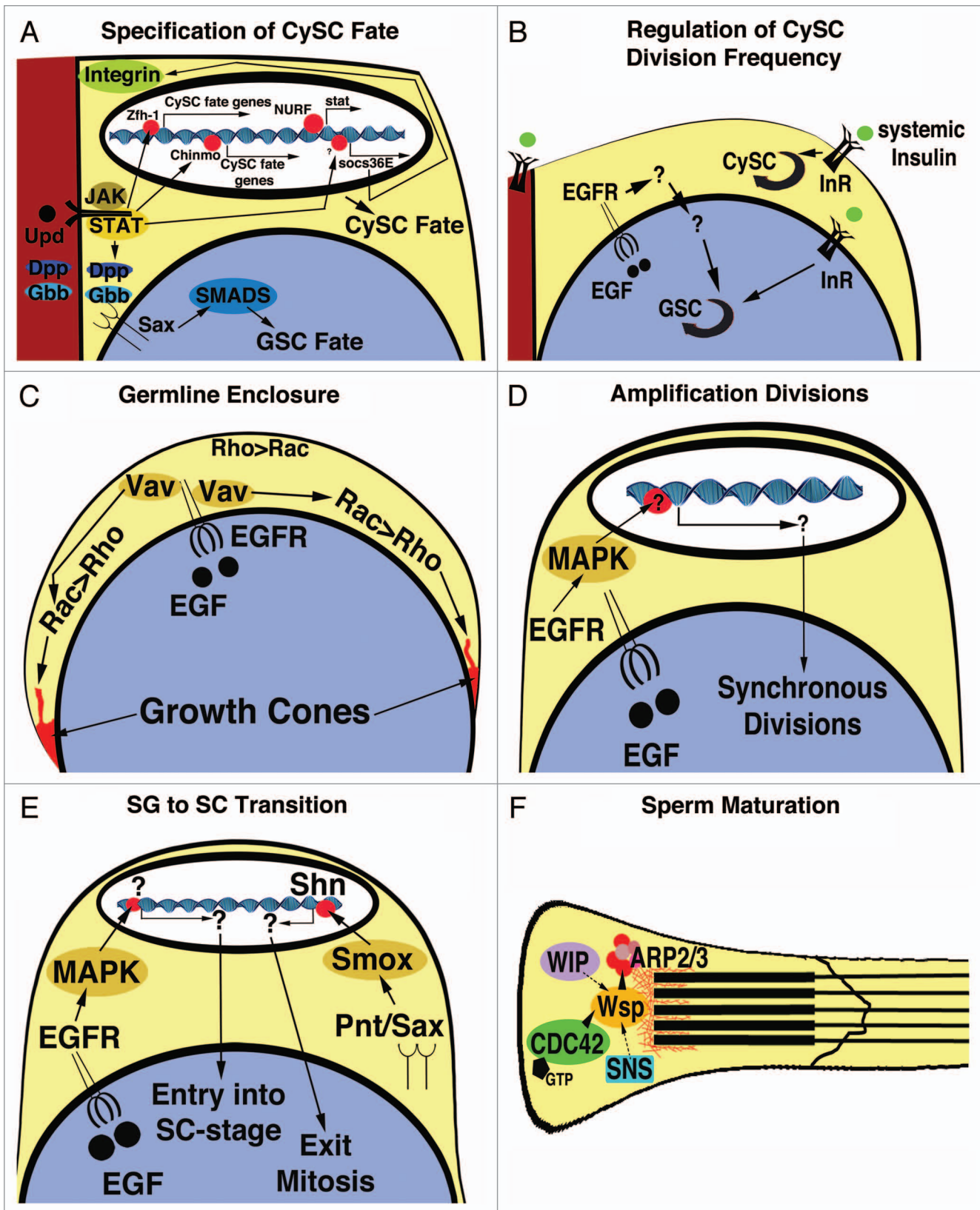


Figure 2. Regulation of cyst development. Graphics depicting specific steps of germline and cyst cell interactions. Molecules and interactions as indicated. See main text for details. (A) Signaling events at the tip of the testes regulating CySC and GSC fate. (B) Signaling events regulating CySC and GSC division frequency (black round arrows). (C) The regulation of germline enclosure by EGF signaling. (D) The regulation of germline proliferation by EGF signaling. (E) Signaling events regulating the transition of the spermatogonia into the spermatocyte stage. (E) Sperm maturation depends on the cap proteins in the head cyst cells.

JAK/STAT signaling and link the JAK/STAT signaling event to transcriptional regulation of target genes. However, *Zfh-1* and *Chinmo* appear to act in an independent manner, based on their expression patterns and genetic interaction. While *Zfh-1* is predominantly expressed in CySCs, *Chinmo* appears to be expressed at similar levels in CySCs and in early stage cyst cells. *Zfh-1* and *Chinmo* expression was unaffected in testes mutant for the other gene and overexpression of *zfh-1* did not restore CySCs in *chinmo* mutants.⁴⁶

In addition to the core signaling event from the hub cells to the CySCs, the levels of JAK/STAT signaling is further regulated cell autonomously within the CySCs.^{47,48} Suppressors Of Cytokine Signaling (SOCS) are highly conserved transcriptional targets of JAK/STAT signaling and antagonize the JAK/STAT pathway via several distinct mechanisms.⁴⁹⁻⁵¹ In *Drosophila* testes, *Socs36E* is expressed in the hub cells and the CySCs. Yet surprisingly, testes from animals carrying a viable allele of *socs36E* showed a defect in the germline. The testes from these *socs36E* mutant animals progressively lost their GSCs from the position next to the hub. Notably, the CySCs in *socs36E* mutant testes had abnormally broad contact regions with the hub and expressed increased levels of β PS-Integrin at the hub-CySC interface. This indicated that JAK/STAT signaling acts via *socs36E* to regulate the expression, stability, or localization of cell adhesion molecules.⁵² The authors further hypothesized that increased cell adhesion between CySCs and hub cells in testes from *socs36E* mutant animals displaces the GSCs away from their position next to the hub. Consistent with these ideas, overexpression of β PS-Integrin in CySCs phenocopied the loss of *socs36E*, and reduction of β PS-Integrin from *socs36E* mutant animals rescued the GSC loss in the testes from *socs36E* mutant animals.⁵² While *socs36E* downregulates JAK/STAT signaling, the Nucleosome-Remodeling Factor (NURF) appears to positively regulate JAK/STAT signaling within the CySCs.^{52,53} Mosaic analysis experiments revealed that CySCs were not maintained when the cells were mutant for subunits of the NURF complex, specifically *nurf301*, *nurf38*, and *iswi*, suggesting a role for the NURF complex in CySC self-renewal. Restoring *STAT* expression in *nurf301* mutant CySCs restored the CySCs loss, showing that *STAT* acts downstream of the NURF complex, and suggesting that *stat* may be a transcriptional target of the NURF complex.⁵³ Following up on the importance of JAK/STAT signaling for stem cells, microarray studies were performed that identified transcripts up- or downregulated in testes with excess stem cells that are now being investigated for their roles in gametogenesis.^{25,30}

CySCs Specify GSC Fate

JAK/STAT signaling is required for the self-renewal of GSCs and CySCs yet specification of GSCs also requires crosstalk between the two stem cell populations.^{32,33,47} When animals were created that expressed *stat* in the CySCs but lacked *stat* specifically in GSCs, surprisingly, the GSCs were maintained. These GSCs had lost contact with the hub yet remained associated with the CySCs and appeared functional. These observations suggested that *stat* activation in the CySCs is sufficient to

mediate self-renewal of the GSCs, and that a second pathway from the CySCs to the GSCs has to induce, or reiterate GSC fate. This other pathway is the Transforming Growth Factor- β (TGF β) signaling pathway.⁴⁷

TGF β is homologous to the Bone Morphogenetic Protein (BMP) and the signaling pathways activated by TGF β and BMP play important roles in development and stem cell function across species.^{4,54-56} BMP signaling has also been implicated in regulating mammalian spermatogenesis, yet studying the role of the BMP pathway in mammals is more difficult due to the high number of BMP family members that may act redundantly.⁵⁷⁻⁶⁰ The core BMP signaling pathway consists of the active receptor and the SMAD transcription factors. The active TGF β family receptor is a transient complex of two receptors with serine-threonine activity and a ligand. The active receptor phosphorylates receptor-regulated Smad type proteins which complex with non-receptor-regulated Smad proteins, enter the nucleus, and regulate transcription of target genes.^{55,56}

The *Drosophila* TGF β ligands Decapentaplegic (Dpp) and Glass bottom boat (Gbb) are expressed in CySCs and hub cells of wildtype testes, and pathway activation has been observed in GSCs of wildtype testes.⁶¹⁻⁶⁴ Leatherman⁴⁷ reported that the TGF β pathway was activated in GSCs from testes with germline-depleted *stat*. The GSCs in these testes had high levels of phosphorylated SMAD in their nuclei, and expression of TGF β antagonists in the *stat*-depleted testes led to a loss of GSCs. This strongly suggested that CySCs signal to neighboring germline cells via the TGF β pathway to induce GSC fate.⁴⁷ It remains to be investigated whether the Dpp and Gbb signals from the hub are also sufficient to induce GSC fate and whether Dpp and Gbb activity in CySCs is directly dependent on the JAK/STAT pathway.

Regulation of CySC Divisions

Stem cells have to adjust to the demand for differentiated cells in order to keep a tissue fully functional. For example, skin stem cells have to produce more skin cells during childhood when an individual grows than they have to produce during adulthood. Likewise, GSCs and CySCs have to adjust to the overall metabolism of the fly and to the demand for sperm. This novel aspect of stem cell biology is only recently being addressed and the *Drosophila* gonad has emerged once again as one of the pioneer systems to approach this unknown territory. A few studies have already addressed the mechanisms by which stem cells regulate their division frequency to produce more or less differentiating germline and cyst cells (illustrated in Fig. 2B). One factor regulating the division frequency of CySCs and GSCs is nutrient availability. When animals were starved, CySCs and GSCs divided at lower frequencies compared with CySCs and GSCs in testes from fed animals.^{65,66} This suggested that insulin signaling regulates the division frequencies of stem cells in the testis in a manner similar as has been proposed for insulin signaling to the GSCs of the ovary.⁶⁷⁻⁶⁹ Complicating matters, CySCs, in turn, influence the division frequency of the enclosed GSCs in an only partially understood feedback loop. In testes from animals

mutant for EGF signaling, GSCs divided at frequencies two to three times higher than GSCs of control testes.⁷⁰

The EGF signaling pathway is highly conserved and plays multiple roles in embryonic development, stem cell biology, and gametogenesis of several species.⁷¹⁻⁷⁴ In *Drosophila*, the major ligand for the pathway, Spitz (Spi), and its receptor, the Epidermal Growth Factor Receptor (EGFR), are assumed to be ubiquitously expressed in many tissues and pathway activation depends on the cell type-specific activity of ligand processing proteases.⁷² In testes, the germline cells process Spi into the active, secreted form via activity of the protease Stet, while EGFR is stimulated on the CySCs and cyst cells.^{9,70,75} Once activated, the tyrosine kinase EGFR phosphorylates cascades of downstream signal transducers.⁷⁶

Removal of either *spi* or *stet* from the germline cells, or removal of the EGFR from the soma resulted in increased division frequencies of GSC but did not affect the division frequencies of CySCs.⁷⁰ This strongly suggested that EGF signaling normally downregulates GSC divisions. However, this effect is indirect as EGF is produced by the germline and received by the CySCs which presumably respond with an unknown return signal to the GSCs. Thus, the division frequency of stem cells depends on both systemic factors and interaction between the CySCs and GSCs.

Germline stem cells show a characteristic orientation of their mitotic spindles during cell division in both mouse and *Drosophila* testes. In *Drosophila* testes, the spindles of the GSCs are oriented perpendicular to the hub.⁷⁷ In rat testes, mitotic spindles of spermatogonia were mainly oriented with angles ranging from 60 to 90° perpendicular in relation to the basement membrane of the seminiferous tubules.⁷⁸ CySCs in *Drosophila* testes also undergo strict asymmetric divisions yet they employ a different cellular mechanism for orienting their plane of division than GSCs do.^{77,79} In GSCs, the mitotic spindle is oriented perpendicular to the hub throughout mitosis.⁷⁷ CySC spindles do not show a consistent orientation. Instead, one of the spindle poles is repositioned to the hub-CySC interface specifically during anaphase.⁷⁹ Spindle positioning in both CySC and GSC divisions requires Centrosomin, as loss of Centrosomin disrupts the spindle orientation in both GSCs and CySCs. While GSCs also require activity of Adenomatous Polyposis Coli (APC), this factor is dispensable for CySC spindles.^{77,79} Instead, CySCs require Moesin, a linker between the membrane and the cytoskeleton.^{79,80} Moesin knockdown via RNA-Interference in CySCs resulted in reduced spindle repositioning during anaphase. In contrast, Moesin knockdown in GSCs had no effect on spindle positioning. In the course of their experiments, the authors noticed that both overexpression and loss of Moesin increased the number of GSCs, CySCs, and cyst cells. Though the mechanism for the dose effect of Moesin is not understood, these results suggested that the correct expression level of Moesin is required for maintaining the correct number of stem cells in wildtype testes.⁷⁹

The above discussed studies showed that a combination of multiple mechanisms, including Upd signaling from the hub, the cell autonomous activity of *lines* and *bowl*, and attachment of the

CySCs to the hub via cell adhesion molecules assure the maintenance and self-renewal of CySCs, as well as proper interactions between CySCs and GSCs in *Drosophila* testes. In the following section, we will focus on discussing cyst cell differentiation and the roles the cyst cells play in guiding germline proliferation and germline differentiation.

Cyst Formation is Regulated by EGF Signaling

The cysts in *Drosophila* testes are one of the few examples in animal development where one cell type completely encloses another. Other examples are the enclosure of nerve cells by the myelin sheath and the formation of biofilm during the mating of yeast.⁸¹⁻⁸³ Research in *Drosophila* testes has revealed insights as to how a specific arrangement between two different cell types is established. Genetic experiments showed that signaling from the germline cells via EGF to the cyst cells is required to induce and organize the growth of the cyst cells around the germline cells (illustrated in Fig. 2C). A conditional allele of *spi* displayed germline enclosure defects of increasing severity with increasing temperature. Removing one copy of the small monomeric GTPase, *rac1*, from *spi* mutant animals raised at an intermediate temperature drastically enhanced the germline enclosure defects resembling testes from *spi* mutant animals raised at higher temperatures. Likewise, reducing the expression of the docking protein and guanidyl exchange factor, Vav, or Rac1 specifically from the cyst cells of *spi* mutant resulted in a similarly strong enhancement of the germline enclosure defects. Together with binding studies of EGFR and Vav, the above genetic data suggested that Vav and Rac1 act in a signaling branch downstream of the EGFR. Conversely, reducing the expression of the small monomeric GTPase Rho1 from the cyst cells had the opposite effect on testes from *spi* mutant animals, restoring germline enclosure even when the animals were raised at high temperatures. This indicated that Rho1 acts in a pathway opposing EGFR for germline enclosure.⁸⁴ In cultured mammalian cells, Rac and Rho play antagonistic roles in regulating cell shape changes and growth via different effects on the actin cytoskeleton.⁸⁵⁻⁸⁸ Based on electron microscopy, expression of dominant negative Rac1- and Rho1-constructs in the cyst cells of otherwise wildtype testes specifically affected the structure of the cyst cell membranes. In wildtype testes, the cyst cell membranes were wavy. Loss of Rac1 from the cyst cells resulted in smoother membranes than those seen in cyst cells of wildtype testes, while loss of Rho1 resulted in the appearance of filopodia in the cyst cell membranes. These findings supported the idea that EGF signaling from the germline cells produces a differential of Rac- and Rho-activities across the cyst cells that, perhaps by organizing the actin cytoskeleton, leads to the directional growth of the cyst cells around the germline cells.⁸⁴ In mammalian testes, the displacement of the differentiating germline cells toward the lumen of the seminiferous tubules requires reorganization of the germline-Sertoli cell contacts.⁸⁹ The Sertoli cells in mouse testes contain a most elaborate cytoskeleton that is essential for their structure and function and likely to play an active role in the constant reorganization of the germline-Sertoli cell contacts.^{19,90} Interestingly, the mammalian

EGFR homolog was reported to localize to Sertoli cells opening the possibility that some of the roles for EGF signaling in somatic cell shape or function are conserved between *Drosophila* and mammals.⁹⁰⁻⁹²

Further studies in *Drosophila* led to the identification of several other conserved proteins that are required for the physical interactions between germline and cyst cells. For example, the large musculo-aponeurotic fibrosarcoma (Maf) factor homolog Tj acts cell autonomously in cyst cells to mediate cyst cell association with the germline by activating the transcription of cell adhesion molecules.¹¹ Similarly, the conserved motor protein Dynein Light Chain 1 (DDL1) is required for the presence of several cell adhesion molecules on the cyst cell membranes and the presence of Eya in the cyst cell nuclei.⁹³ However, it remains to be addressed whether this is due to a role of DDL1 in regulating gene expression, protein localization, or protein stability. Likewise, it would be interesting to learn if loss of *ddl1* has an effect on EGF signaling and whether it plays a parallel role in gametogenesis of other organisms.

Cyst Cells Regulate Germ Cell Proliferation

In most tissues maintained by stem cells, the stem cell daughters undergo several rounds of transit amplifying divisions to increase the numbers of precursors for terminally differentiated cells.^{1,4,5,94} The proliferation of cells needs to be tightly regulated to avoid loss or tumorous growth of a tissue and the mechanisms restricting cell proliferation are of wide interest in development, stem cell biology, and cancer biology. The *Drosophila* testis is an excellent model tissue in which to study tumorigenesis.^{95,96} In *Drosophila* testes, proliferation of the stem cell daughters relies on both germline intrinsic and extrinsic factors.^{6,9,97} The requirement for germline-soma interactions to restrict cell proliferation is most evident in testes that lack either germline cells or germline enclosure by cyst cells. In testes without a germline (agametic testes), the cyst cells lost the ability to differentiate into late stage cyst cells and proliferated as early stage cyst cells instead. The somatic cells appeared to be properly specified and initially behaved normally. However, after five days, the number of early stage cyst cells increased and the number of late stage cyst cells decreased. In addition, the cyst cells underwent mitotic divisions, a trait normally restricted to CySCs. This suggests that germline cells normally restrict the ability of cyst cells to reenter the CySC proliferation program.⁶ Interestingly, cyst cells associated with gonialblasts normally fail to elongate their centrioles and to recruit pericentriolar material, possibly due to downregulation of SAS-6, a main regulator of centriole architecture.⁹⁸ It remains to be investigated whether downregulation of SAS-6 is the cause for the withdrawal of cyst cells from the cell cycle and whether cyst cells in agametic testes continue to express SAS-6.

Conversely, germline proliferation is restricted by surrounding cyst cells. In *spi* or *stet* mutant animals, germline cells and cyst cells were present but the cyst cells did not enclose the germline. In these testes, the germline cells proliferated at early stages and failed to differentiate.^{9,84} Though previous experiments had suggested that EGF signaling produces a feedback loop from

the cyst cells to the enclosed germline cells to restrict germline proliferation, these experiments did not address germline enclosure.^{75,99} Therefore, it remained unclear whether the germline proliferation defects in the *egfr* mutants were due to defects in enclosure or whether EGF also restricts germline proliferation past the enclosure event. Recent studies have shed more light on the specific roles of EGF in restricting germline proliferation and cyst maturation (Hudson and Schulz, unpublished data). By using many alleles and conditions for *spi*, *stet*, *raf*, and *egfr*, as well as overexpression studies, we and others demonstrated that EGF signaling has a range of effects on cyst cells and germline cells. EGF signaling from the germline to the soma regulates the division frequency of GSCs, the enclosure of the gonialblast by cyst cells, the progression of the spermatogonia through amplification divisions, and EGF signaling also promotes the entry of the germline cells into the spermatocyte stage (illustrated in Fig. 2B–E).^{9,70,75,84,99} (Hudson and Schulz, unpublished data) This suggests that the cyst cells send different sets of return signals to the enclosed germline cells at different stages of spermatogenesis. The EGFR also appears to be required cyst cell-intrinsically for their viability, as some EGFR alleles are cell lethal.⁷⁵

In order for the spermatogonia to enter the spermatocyte stage, they first have to exit from the mitotic amplification divisions. The exit of the spermatogonia from mitosis specifically at the 16-cell stage depends on TGF β signaling (illustrated in Fig. 2E). Loss of the TGF β receptors Punt (Pnt) or Saxophone (Sax), the signal transducer Smad on X (Smox), or the downstream transcription factor Schnurri (Shn) from cyst cells resulted in the accumulation of large clusters of spermatogonia of multiples of 16. For example, these cysts contained 32, 64, or 128- spermatogonia.^{100,101} In addition to JAK/STAT, EGF and TGF β signaling, a number of candidate genes have been identified that affected germline proliferation but their mode of action and whether their homologs may play a role in tissue homeostasis in other organisms or cancer is yet to be determined.⁶²

Germline proliferation was suggested to depend on motor-based processes within the cyst cells, based on the phenotypes of mutations in a cytoplasmic dynein, motor proteins and a GTPase.⁹³ Animals carrying a hypomorphic allele of *ddl1* displayed a testes phenotype in which the germline cells overproliferated and accumulated as single cells and 2-cell spermatogonia. In an effort to investigate the mechanism of DDL1 action, the potential binding partners of DDL1 were knocked down via RNA-Interference and the effect on germline and cyst cells investigated.^{93,102} The binding partners for DDL1 are Dhc64C, which encodes a microtubule motor protein, and Myosin V (*didum*), which encodes an actin binding protein.¹⁰³ The mammalian homolog of Myosin V, Myosin 5b, displayed motor protein function during membrane recycling, suggesting a similar role as a motor protein in *Drosophila*.^{93,104} Knockdown of either *didum* or *dhc64C* in the cyst cells resulted in a similar germline overproliferation phenotype as knockdown of *ddl1* and removing one copy of *didum* or *dhc64C* from animals carrying a hypomorphic allele of *ddl1* enhanced the germline proliferation phenotype. This suggested that the three proteins act in a pathway to restrict germline proliferation. However, the effect

on cell adhesion molecules observed in the *ddlc1* mutants was not recapitulated upon reduction of *didum* or *dhc64C*, suggesting that *ddlc1* acts in two distinct pathways, one involved in cell adhesion, and the other in restricting germline proliferation. Finally, cyst cell-reduction of the GTPase, Rab11, which was shown to play a role in Myosin V-dependent secretion in developing photoreceptors, also resulted in germline overproliferation.^{93,105} The authors hypothesized that Rab11 acts in the same pathway as DDLC1, Myosin V, and *Dhc64C*, and that they play a role in the exocytosis of a yet-to-be-identified cyst cell derived signal regulating germline proliferation.⁹³ Interestingly, another Dynein, *Dhc1*, has been associated with EGF secretion in the developing eye and cytoplasmic dynein has been identified in rat Sertoli cells suggesting conserved roles for Dyneins in regulating intracellular processes during development and germline differentiation.^{106,107}

Cyst Cells Regulate Germline Survival

In addition to providing regulatory cues for the development of the germline cells, cyst cells are also required for germline survival, yet for most of the genes mentioned below, their exact mechanisms of action and how they relate to each other is unknown. Germline survival specifically at the spermatocyte stage depends on the transcription factors *Eya* and *Sine oculis* (*So*), suggesting a checkpoint after mitotic amplification divisions. *Eya* and *So* are both expressed in cyst cells associated with spermatocytes and clonal analysis revealed that they act in the cyst cells to prevent the death of spermatocytes prior to their maturation. Mutations in *eya* and *so* show synergistic genetic interactions, suggesting that they function in different pathways.¹²

Several other factors are required for germline survival prior to the spermatocyte stage. The *zero population growth* (*zpg*) gene encodes a gap junction protein which is expressed in the germline. Mutations in *zpg* were associated with underproliferating spermatogonia that eventually died, which suggested that germline cells and surrounding cyst cells exchange small molecules for promoting their viability.¹⁰⁸ Likewise, mutations in the tumor suppressor gene *discs large* (*dlg*), which encodes a septate junction protein, resulted in the death of spermatogonia as well as their enclosing cyst cells, suggesting that germline viability depends on direct connections between the germline and the cyst cells.¹⁰⁹ *slow motion* (*slowmo*), a mitochondrial gene of unknown function, is expressed in cyst cells and affected survival of germline cells at all stages of development.¹¹⁰

When the role of cyst cells in GSC self-renewal and GSC daughter differentiation was first explored, two outstanding studies also suggested a role for EGF signaling in CySC viability or maintenance.^{75,99} This was not recapitulated in testes from animals carrying a temperature sensitive allele of *spi*, animals mutant for the protease *stet*, or animals with CySC-depleted EGFR.^{62,70,84} Kiger⁷⁵ reported a failure to detect *egfr* mutant CySCs or cyst cells in clonal analysis experiments. This failure to detect EGFR mutant somatic cells is not surprising as the mutant alleles used in the clonal analysis experiments had been reported to be cell lethal.^{75,103} Maybe, the EGFR produced from the cell-lethal

alleles folds in a manner interfering with basic cellular functions and, therefore, induces cell death. As the alleles have no effect on the germline, it suggests that the folding defects would depend on ligand binding. We tested the temperature sensitive allele of the EGFR used by Kiger.⁷⁵ We observed that, after the shift to the restrictive temperature, the animals themselves died within a few days supporting the idea that this allele is cell-lethal (Hudson and Schulz, unpublished data).

The other study reported that the somatic cells next to the hub in adult animals depleted of the Mitogen activated protein (Map)-Kinase, Raf, expressed the late cyst cell marker *Eya*, suggesting that the CySCs may have died or differentiated into late stages.⁹⁹ In these animals, Raf activity was systemically reduced, leaving open in which cell type Raf acts to maintain CySCs. As Raf acts downstream of several signaling pathways it is plausible that the observed defects do not reflect loss of EGF signaling from the germline.^{111,112} Consistent with this, we never detected somatic cells next to the hub with high levels of *Eya* expression in testes maternally and zygotically depleted for EGF signaling (Hudson and Schulz, unpublished data). The strongest argument to refute the idea that EGF signaling from the germline to the cyst cells directly prevents CySC death or differentiation is the observation that somatic cells next to the hub in agametic testes did not undergo cell death or express *Eya*.⁶

Cyst Cells Regulate Late Stages of Germline Differentiation

Somatic and germline cells remain intimately associated throughout spermatogenesis in many species, suggesting that interactions between the two cell types also regulate late stages of spermatogenesis.^{1,16,113} An excellent example for this was observed in advanced stages of *Drosophila* spermatogenesis. As spermatids elongate, the cyst cells undergo a series of morphological changes whereby they become structurally distinct from one another. Tail cyst cells grow dramatically in size to accommodate the elongating sperm tails, while the head cyst cells grow to a much lesser extent. Following elongation of the cysts, the head cyst cells associate with the terminal epithelium of the testes, upon which the entire cysts coil to form compact structures.⁸ As the spermatids mature, the head cyst cells grow a cap around the sperm heads (illustrated in Fig. 2F). This cap is rich in F-actin and cell adhesion molecules, and organized by the Arp2/3 complex. A requirement for the cap was shown by phenotypic analysis of testes from animals carrying mutant alleles and expressing RNA-interference-constructs for genes encoding cap proteins specifically in the cyst cells. These studies included knockdown of *Wsp*, the Arp2/3 complex proteins *Sop2* and *Arp3*, *Wasp*-interacting protein (*WIP*), the cell adhesion protein *Sticks* and *stones* (*SNS*), and the small monomeric GTPase *Cdc42*. Any of these mutants resulted in abnormal caps and were associated with an arrest in development of the germline cells prior to cyst coiling. Furthermore, it was shown that release of mature sperm from the cap depends on the activity of the dynamin *Shibire*. Interestingly, spermatogenesis was also arrested when the mammalian *WASp* homologue was disrupted in mouse Sertoli cells,

and the nature of the molecular mechanisms and the degree of parallels have become a future target of this line of research.^{114,115}

Though the cyst cells are clearly required for proper germline proliferation and differentiation *in tissue*, it appears that germline cells have some cell-intrinsic properties for differentiation. An interesting cell culture study of *Drosophila* male germline cells suggested that the entry into terminal differentiation may be independent from surrounding cyst cells. When the authors dissociated 16-cell spermatogonia from the encapsulating cyst cells, the dissociated spermatogonia transitioned into spermatid-like elongated cells. Unfortunately, it was not addressed whether these germline cells had undergone meiosis, whether they had normal DNA-content or chromatin structure, or whether the mitochondria assembled properly to produce the long flagellum of the sperm tail. The authors nevertheless concluded that cyst cells are dispensable for spermatocytes to undergo spermiogenesis.¹¹⁶ Similarly, in some mutant situations, germline cells can enter the spermatocyte stage independent from surrounding cyst cells. For example, germline cells from animals double-mutant for *stet* and the *nucleoporin98–96* locus were not enclosed by cyst cells but developed into spermatocyte-like cells.¹¹⁷

Although the intrinsic properties of germline cells may give them some independence from the soma, the constant and coordinated production of mobile sperm from GSCs remains under cyst cell control. All of the findings discussed in this review (see **Table 1** for overview of all discussed molecules) clearly prove this point and have changed our view of the cyst cell lineage in *Drosophila* testes. While cyst cells were originally thought of as nurturing cells in *Drosophila* testes, the research discussed in this review shows that the CySC lineage in *Drosophila* testes plays an active role in gametogenesis and put them into the spotlight. In *Drosophila* testes, germline and cyst cells undergo coordinated interactions that regulate their codifferentiation and assure the production of functional sperm. A similar interdependence of stem cell lineages was discovered in mammals and similar mechanisms of interactions are being discussed.^{118–121} For example, melanocyte stem cells are closely associated with epithelial stem cells in the hair follicle of the skin, and here as well signaling between the two lineage is an important key in coordinating the differentiation of the two stem cell lineages to make pigmented hair.¹²²

Outlook

The interdependence of germline and cyst cells has been demonstrated for both the early and the final stages of spermatogenesis in *Drosophila*. Cyst cells regulate spermatogonial divisions, germline cell survival, and sperm maturation via several conserved

molecules and pathways. Though research on *Drosophila* cyst cells has dramatically advanced our knowledge in this respect, many questions remain to be addressed. For example, we are only beginning to understand how the divisions of CySCs and GSCs are regulated to assure that two cyst cells are generated for each gonialblast. To start to address these questions, the molecules and pathways regulating stem cell division frequencies need to be identified and studied in detail, followed by the identification of the means by which the two stem cell populations coordinate their activities.

What are the molecular natures of the signals presented by the cyst cells to the enclosed germline cells? Despite the importance of these signals for germline differentiation, not a single one has been identified. A possible explanation is that redundant pathways regulate the behavior of the enclosed germline. Consistent with this idea, attenuation of signaling pathways in sensitized backgrounds can have a strong effect on germline development while not causing an effect in an otherwise wildtype background (Zoller and Schulz, unpublished data; Ng and Schulz, unpublished data).

Molecules and pathways regulating early and late stages of germline and cyst cell development have been described. However, no reports are available that describe interactions between germline and cyst cells during germline meiosis or the initiation of terminal differentiation. Several factors, including Protein Phosphatase Y, the NOA fatty acid elongase, and Ku80, a protein involved in DNA damage repair, are expressed in late stage cyst cells and the study of these genes may shed light on these questions.^{123–125} However, we still need to create a pool of late stage cyst cell-specific markers and transcriptional profiles of differentiating cyst cells in order to study germline and cyst cell differentiation. These tools will ultimately aid us in identifying potential interactions and show whether cyst cells, like germline cells, pass critical cell-intrinsic checkpoints during their differentiation program.

Future research will shed light on these and other key questions. As discussed, many of the principles unraveled by studying gametogenesis in *Drosophila* testes may apply to gametogenesis in other species.

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Table 1. Alphabetical listing of the genes discussed in this review that play roles in or for the CySC lineage

Gene name/Abbreviation	Molecular nature	Discussed function	References
Arp2/3 complex (Sop2, Arp3)	Cytoskeletal regulator	Sperm maturation	114
Brother of odd with entrails limited (bowl)	Transcription factor	CySC/hub cell specification	25
Cdc42	GTPase	Sperm maturation	114
Centrosomin (cnn)	Protein binding	Stem cell fate	79
Chronologically inappropriate morphogenesis (chinmo)	Transcription factor or Protein degradation	CySC fate	46
Decapentaplegic (dpp)	Protein binding/Ligand	GSC fate	61–64
Discs large (dlg)	Protein binding	Cyst survival	109
dhc64C	Microtubule motor activity	GL proliferation	93
Dynein Light Chain 1 (ddlc1)	Cytoplasmic Dynein	GL-Soma adhesion	93
Epidermal growth factor receptor (egfr)	Receptor	GL proliferation	93
		CySC specification	27, 28
		CySC division frequency	70
		GL proliferation	75, 99
Eyes absent (eya)	Transcription factor	Cell viability	75
		Spermatocyte survival	12
F-actin	Cytoskeletal component	Sperm maturation	113, 114
Glass bottom boat (gbb)	Protein binding/Ligand	GSC fate	61–64
Hopscotch (hop) = JAK	Protein tyrosine kinase	CySC fate	32, 33
Insulin	Protein binding/Ligand	GCS + CySC division frequency	65, 66
Insulin Receptor (InR)	Receptor	GSC + CySC division frequency	65, 66
Ku80	Unknown	Unknown	123
Lines (lin)	Catalytic activity	CySC specification	25
Moesin	Protein binding	Stem cell fate	79
Mothers against dpp (mad)	Signal transducer	GSC fate	47
Myosin V (didum)	Actin binding	GL proliferation	93
NOA	Fatty acid elongase	Unknown	124
Notch (N)	Receptor	Hub specification from SGPs	27, 28
NURF complex (nurf301, nurf38, iswi)	Chromatin remodeling	CySC fate	53
Protein Phosphatase (ppy)	Phosphatase	Unknown	122
Punt (pnt)	Receptor	GL proliferation	100
Rab11	Endosome transport	GL proliferation	93
Rac1	GTPase	GL enclosure	84
Rho1	GTPase	GL enclosure	84
Saxophone (sax)	Receptor	GL proliferation	101
SAS-6	N/A	Cyst cell cectriole architecture	98
Schnurri (shn)	Transcription factor	GL proliferation	100
Sevenless (sev)	Receptor	CySC specification from SGPs	26
Shibire (shi)	Actin binding	Sperm release	113
Sine oculis (so)	Transcription factor	GL survival	12
Slow motion (slomo)	N/A	Cyst survival	110
Smad on X	Signal transducer	GL mitosis	102
Signal transducer and activator of transcription (stat)	Signal transducer	CySC fate	32, 33
Suppressor of cytokine signaling 36E (socs36E)	N/A	CySC fate	52
Spitz (spi)	Protein binding/ligand	CySC division frequency	70

CySC, cyst stem cells; GSC, germline stem cells; GL, germline; SGP, somatic gonadal precursors. References that present the research on these genes in *Drosophila* testes are listed.

Table 1. Alphabetical listing of the genes discussed in this review that play roles in or for the CySC lineage

Gene name/Abbreviation	Molecular nature	Discussed function	References
		Germline enclosure	84
		GL proliferation	75, 99
Stet	Protease	CySC division frequency	70
		Germline enclosure	9
		GL proliferation	75, 99
Sticks and Stones (sns)	Protein binding	Sperm maturation	114
Traffic jam (tj)	Transcription factor	GL-soma adhesion	11
Unpaired (upd)	Protein binding/Ligand	CySC fate	32, 33
Zero population growth (zpg)	Gap junction protein	Cyst survival	108
Vav	Guanidyl exchange factor	GL enclosure	84
Wsp	Protein binding	Sperm maturation	114
Wasp-interacting protein (wip)	Actin filament binding	Sperm maturation	114
Zinc finger homeodomain -1 (zfh-1)	Transcription factor	CySC fate	10

CySC, cyst stem cells; GSC, germline stem cells; GL, germline; SGP, somatic gonadal precursors. References that present the research on these genes in *Drosophila* testes are listed.

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