Chinmo Function During Cyst Stem Cell Establishment in the Drosophila Testis

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Chinmo Function During Cyst Stem Cell Establishment in the Drosophila Testis

A thesis submitted to the Department of Biology at

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ABSTRACT

Stem cells are crucial for organogenesis and maintenance of healthy tissues. They also have potential use in stem cell therapies and regenerative medicine. Yet, our understanding of how stem cells develop during organ formation is limited. *Drosophila* testes provide one of the most tractable, and thoroughly characterized systems for studying stem cell behavior. The stem cell niche that forms in the testis is a model for the microenvironments present in other organs where there is an asymmetrically dividing population of stem cells. In the testis niche, cyst stem cells (CySCs) and germline stem cells (GSCs) are arrayed around somatic hub cells. Signaling from hub cells regulates the equilibrium of stem cell maintenance and differentiation. The Wawersik Lab has shown that the Jak-STAT signaling pathway is necessary and sufficient for CySC maintenance shortly after GSC establishment (Wawersik et al. 2012). However, the function of downstream mediators of the Jak-STAT pathway are not entirely known. One of these downstream mediators is *chinmo* (Bach et al, 2011). In the adult *Drosophila* testis, *chinmo* has been shown to be required for maintenance of cyst stem cells (CySC) (Bach et al, 2011). Additionally, *chinmo* is responsible for maintenance of male sexual identity of CySCs in the adult testis (Ma, Wawersik, Matunis, 2014). Prior studies have not assessed *chinmo* expression or function during development. This thesis will study *chinmo*'s role in CySC maintenance and the initial determination of male sexual identity in *Drosophila* gonad during the embryonic and larval development. Specifically, the temporal and spatial expression of *chinmo* was characterized in developing gonads. Loss and gain of function studies were also performed. Results indicate that *chinmo* is both necessary and sufficient for establishment, but not maintenance, of functional CySCs in
the developing testis. These data suggest that *chinmo* has a different function in CySCs during development than in an adult *Drosophila testis*.

**INTRODUCTION**

Stem cells are essential for multi-cellular development, as they provide the cells that make up our bodies and continue to renew damaged cells throughout life. During development, a complex series of signals are required to establish a stem cell niche and turn these few cells into a functioning organ. Failure to properly regulate these niches can lead to a loss of stem cells or flawed differentiation, which is linked to numerous diseases including osteoporosis and cardiomyopathy (Pekovic and Hutchinson, 2008). Specific mechanisms controlling stem cell development, however, are not well understood and it is unclear the degree to which signals controlling stem cell maintenance in adult tissues control stem cell formation. In this thesis, I will examine the mechanisms that control specification, establishment, and maintenance of functioning stem cells during organ development using the model system of the *Drosophila melanogaster* testis.

*Stem cells are essential for organogenesis*

Organogenesis is the process by which the three primary germ layers develop into the body's internal organs. In order for this process to occur, it requires stem cells to be able to undergo two types of division (Fig. 1). First, stem cells need to be capable of symmetric division. This is when a stem cell divides and each daughter cell maintains the same cell fate as the original cell. This is important for expanding the pool of stem cells that the body can draw from. The second type of division is asymmetric division.
This occurs when a stem cell divides and one daughter cell maintains the same stem cell fate, while the other cell takes on a more differentiated fate. Asymmetric divisions not only allow for the production of the different cell types that make up the body, but by leaving one of the daughter cells with the fate of a stem cell, it ensures that the population of stem cells is continuously maintained.

Inside the blastocyst that forms early during development are pluripotent inner mass cells (Robertson et al., 1986). These embryonic stem cells have the potential to give rise to all three germ layer types: ectoderm, endoderm, and mesoderm. As development progresses, progenitor stem cell populations are specified from cells in the different germ layers. From these progenitor cells, functional, asymmetrically dividing stem cells must be established in order to produce cells that carry out the function of developing the organ, while also maintaining stem cells. These stem cells must then be maintained throughout development and the adult lifespan of the organism. In adult tissues, the balance between stem cell maintenance and differentiation is controlled by the stem cell niche. While the adult and developing stem cell niche may not always be the same in all instances, it is likely that stem cell development is coordinated by the development of stem cell niches from progenitor cells that interact with stem cell precursors and promote their development into functional stem cells.

While the process of stem cell specification, establishment, and maintenance is not entirely known in most organs, even less is understood about the signals that mediate genetic changes through each step of development. Loss of stem cell regulation has been attributed to infertility, cancer, and neurodegenerative disorders (Vlajkovic et al., 2012; Pardal et al., 2005; Bonfanti et al., 2011). Because stem cells can self-renew and produce
healthy differentiated cells, stem cell therapy holds great potential with replacing damaged or dead cells (Fox et al., 2014). In order to properly implement stem cell therapy, more needs to be understood about the function of the stem cell niche so that therapeutic stem cells can be successfully integrated. A major problem with studying stem cells is being able to find stem cell populations in the body. The stem cell population in the *Drosophila* testis in comparison is a highly tractable system, where stem cells can be located and studied in the context of the stepwise processes of stem cell development.

**Drosophila Melanogaster as a model for stem cell niches**

Several advantages make *Drosophila* an ideal organism for understanding mechanisms controlling tissue development. Their small size and quick generation time from egg to adult in ~11 days (at 25 °C), means that animals can be reared with little equipment in a short time-period (Ashburner and Roote, 2007). At peak fertility, a *Drosophila* female produces 100 fertilized eggs a day (Sang, 2001). This high fecundity helps to ensure there are large sample sizes to work with. While the *Drosophila* genome contains only 5% the size of the average mammalian genome, fundamental signaling pathways and homologs of proteins that are found throughout development of all vertebrates are conserved (Ashburner, 2005). Information gleaned from studying *Drosophila* genetics can, therefore, be applied to developmental processes in all vertebrate model organisms, including humans.
Drosophila's manageable genome size has allowed several genetic tools to be developed, which make Drosophila one of the most tractable model systems for manipulating genotypes. One of these is the development of balancer chromosomes. Multiple, naturally occurring inversions in these chromosome prevent meiotic recombination between paired chromosomes (Kile, 2003). This allows for heterozygotes of specific genes to be maintained in a population. Another important genetic tool is the Gal4-UAS tissue-specific gene overexpression system (Fig. 2). This system is comprised of the yeast protein Gal4 and a target gene under control of a response element, Upstream Activating Sequences (UAS), analogous to an enhancer element of eukaryotes (Duffy, 2002). Because transcription of the UAS- response element requires Gal4 to be present, any transgene controlled by this element is transcriptionally silent until mated to flies expressing the Gal4, termed the driver. This means that transgenic responder lines can be made for gene products that are lethal or have reduced viability when expressed. The Gal4 driver can be placed under certain native gene promoters so its expression and, therefore, the expression of the responder element are upregulated in specific tissue types and times in development. One category of target genes that can be induced tissue specifically is RNAi, which can be used to create gene knockdowns by inhibiting transcription of targeted genes. The Gal4-UAS system can then be used to see the phenotype that occurs when that particular protein is knocked down in a certain cell type or at a specific time point in development.
**Development of the Drosophila testis**

The *Drosophila* testis offers one of the best models for studying stem cell behavior and regulation. The testis has an easily located stem cell niche and population of stem cells. The testis can be visualized in embryos and larvae using immunostaining and epifluorescence microscopy (Fidler, Boulay et al, 2014). Furthermore, markers have been developed for the identification of stem cell precursors, the stem cell niche microenvironment, and their differentiating progeny (LeBras & Van Doren, 2006; Sheng et al, 2009; Sinden et al, 2014). Moreover, various Gal4 drivers have been developed for the testis, making the model highly tractable (Duffy, 2002).

The embryonic gonad consists of two primary cell types, primordial germ cells (PGCs) and somatic gonadal precursor cells (SGPs). At ~2 hours after egg laying (AEL) (stages 4–5) in of embryogenesis, PGCs arise from maternally deposited cytoplasm at an embryo's posterior, called the germ plasm (Mahowald, 1962; Jemic, 2011) (Fig. 3). At ~2 hours AEL (stage 7) mid-gut invagination pulls PGCs towards the interior of the embryo. This changes at ~4 hours AEL (stage 9) when PGCs begin to migrate through the wall of the midgut towards somatic gonadal precursor (SGP) cells derived from the dorsolateral mesoderm (Kunwar et al., 2008; Boyle et al., 1997). Additionally, male-specific SGPs (msSGPs), are specified in both males and females, but only persist in developing testis (DeFalco et al., 2003). As the PGCs migrate bilaterally, the two cell populations begin to interact at ~8hrs AEL (stage 12). At ~9hrs AEL (stage 13), SGPs send out extensions to ensheath the PGCs, and the PGCs once again take on a rounded morphology. One of the transcription factors responsible for mediating enseathment is the MAF family transcription factor Traffic Jam (TJ) (Kawashima et al., 2003). At ~12
hrs AEL (stage 15) the gonad forms a compact structure and msSGPs undergo apoptosis in developing ovaries.

As testis development progresses, the stem cell niche forms from a sub-population of anterior SGPs at ~23 hrs AEL during the last stage of embryogenesis (stage 17; Le Bras and Van Doren, 2006). At the time of hub formation anterior PGCs develop into germline stem cells (GSCs), and make connections with the hub through the protein DN-cad (Jenkins et al., 2003; Sheng et al 2009). Similarly, a sub-population of SGPs give rise to cyst stem cell progenitors that then become established as functional cyst stem cells (CySCs) (Fig. 4). The functional CySCs are docked at the hub, ensheath the GSCs and have the capacity to undergo asymmetric stem cell divisions (Sinden, Badgett et al, 2012). In early larval and adult testes, there are typically 6-10 GSCs and 11 CySCs docked at the hub (Wang and Jones, 2011; Sheng et al, 2009; Sinden & Badgett et al, 2012). The hub is responsible for controlling stem cell maintenance and differentiation. It does so in GSCs by orienting the centrosome so that the mitotic spindle forms perpendicular to the hub and stem cell division is oriented away from the hub (Inaba et al., 2010; Yamashita et al., 2003). By the first larval instar stage (L1), spermatogenesis begins (Sheng et al, 2009). As one GSC daughter (called a gonialblast) is pushed away from the hub by oriented division, it no longer has access to signals secreted by the hub, which promote maintenance of stem cell identity, and it proceeds to differentiate. In addition to oriented GSC division, two CySCs divide asymmetrically away from the hub. Each CySC asymmetrically divides to produce one daughter cell that remains a CySC, while the other daughter cell differentiates into a cyst cells that ensheaths the newly formed gonialblast and supports it’s differentiation into sperm. At the start of
spermatogenesis, the goniablast undergoes four rounds of cell division with incomplete cytokinesis. This results in a 16-cell spermatogonia surrounded by cyst cell pairs that initiates meiosis during pupal development to produce functional sperm by the time an adult fly encloses from it’s pupal case.

**Jak-STAT signaling pathway**

One of the primary pathways for regulating maintenance, or self-renewal, of stem cells around the hub is the Jak-STAT signaling pathway. The pathway is activated by the ligand, Unpaired (Upd), which is secreted by the hub to activate the Jak-STAT pathway in adjacent stem cells ( Cuevas and Matunis, 2011 ) (Fig. 5). Upd binding to a cell surface receptor, called Domeless, resulting in activation of the receptor-associated tyrosine kinase, Janus-activated kinase (Jak; also called Hopscotch (Hop) in *Drosophila*). Jak/Hop activation leads to recruitment of the Signal Transducer and Activator of Transcription (STAT) protein from the cytoplasm to the receptor, and subsequent phosphorylation of STAT by Jak. In its phosphorylated form, STAT dimerizes and translocates into the nucleus where it acts as a transcription factor to modulate expression of target genes.

Jak-STAT activation is critical for hub-mediated maintenance of stem cell populations in developing and adult testes (Tulina and Matunis, 2001; Leatherman and DiNardo, 2010). GSCs and CySC docked at the hub are exposed to the Upd ligand that is expressed and secreted by hub cells, but daughter gonialblasts and cyst cells that reside away from the hub due to asymmetric cell division are not. In adults and newly formed GSCs in the testis, Jak-STAT activation promotes formation of Cadherin-based cell adhesions that dock GSCs to the hub and likely provide a scaffold for the asymmetrically
oriented mitotic spindle (Yamashita et al., 2003). In CySCs, Jak-STAT signaling appears to repress cyst differentiation, while also promoting expression of signals from the CySC to adjacent GSCs that repress germ cell differentiation in the niche (Sinden et al, 2012; Leatherman and DiNardo, 2010). Indeed, the Wawersik Lab recently showed that Jak-STAT signaling is necessary and sufficient for CySC maintenance shortly after stem cell niche formation and that expansion of CySCs in the niche represses differentiation of GSC daughters into gonialblasts (Sinden, Badgett et al., 2012).

The Jak-STAT signaling pathway is also involved in germ cell sex determination in the Drosophila testis. In order for sexual identity to be properly established, the sex of the soma and germline need to match. While it is not completely understood, there are mechanisms by which the soma mediates non-autonomous, or dependent, sex determination in the germline. Even before hub formation, Upd is expressed in a male-specific manner in the somatic gonad (Wawersik et al., 2005). This activates the JAK/STAT pathway in male germ cells during the time of gonad formation. The transcription factor STAT92E, the Drosophila STAT homologue, that becomes activated in these male germ cells has been shown to be necessary for male-specific germ cell in early development and sufficient to promote some aspects of male germ cell behavior in the ovaries (Wawersik et al., 2005).

**chinmo function in adult Drosophila testis**

Two targets of STAT92E are the genes zinc finger homeodomain 1(zfh-1) and chronologically inappropriate morphogenesis (chinmo) (Fig. 6). Zfh-1 has been shown to be expressed in CySCs and is required for CySC maintenance (Leatherman and
Recently, experiments analyzing *chinmo*'s function in the adult *Drosophila* testis, found that Chinmo protein is detected in CySCs, early cyst cells, and also at lower levels in GSCs and gonialblast cells (Flaherty, et al., 2011). Chinmo was detected in the hub, where STAT92E is not expressed (Flaherty, et al., 2011). This suggests that unknown factors, in addition to STAT92E, regulate *chinmo* expression.

Through experiments where marked cell clones were made mutant with a null allele of *chinmo*, it was shown that, while Chinmo is expressed in both GSCs and CySCs, it is only required in CySCs maintenance (Flaherty, et al., 2011). Additionally, while the Jak-STAT pathway is known to control expression of the transcription factor, zinc finger homeodomain-1 (*zfhl*) that is also required for CySC maintenance in the adult, it was shown that *chinmo* does not act through *zfhl* to maintain CySCs.

Additional studies in adult testes revealed an additional phenotype that results from partial loss of function allele mutants of *chinmo*, called *chinmo*\(^{ST}\) (Ma et al. 2014). In these mutants it was found that loss of Chinmo in CySCs causes them to undergo a sex reversion (Fig. 7). The CySCs and their progeny had morphological structures resembling follicle cells found in the *Drosophila* ovary. These cells also expressed female somatic markers consistent with conversion of the male CySCs into female somatic stem cells. Furthermore, it was shown that ectopic expression of the canonical male sex determination factor DoublesexM (DsxM) in the CySC lineage was able to partially rescue the chinmo sex reversion phenotype. This indicates that *chinmo* acts upstream of DsxM and is needed to maintain the male identify in somatic stem cells.

The goal of this thesis is to examine the role of chinmo during *Drosophila* testis development, which has never been studied. This prospective is needed to determine if
*chinmo* has different functions throughout development and to more fully understand the phenotypes seen in *chinmo* mutants of the adult testis. In order to examine *chinmo*'s function we: 1) characterized *chinmo*'s expression during embryonic and larval stages of development, 2) assessed if *chinmo* is necessary for CySC specification/establishment and maintenance, and 3) assessed if *chinmo* is sufficient to establish functional CySCs. Our results show that Chinmo is expressed in SGPs as early as stage 15 embryos. Additionally, *chinmo* is found to be necessary for CySC establishment, but unlike in adults, it is not necessary for CySC maintenance. Lastly, overexpression of *chinmo* in SGPs and CySCs results in defective germ cell differentiation, which is likely due to a defect in the function of cyst cells.

**METHODS**

*Fly Stocks*

Fly stocks were obtained from the Bloomington Stock Center (http://flystocks.bio.indiana.edu/) or donated from a collaborating *Drosophila* lab as specified. The strain *y¹, w¹¹¹⁸* flies were used as controls. *UAS-chinmoRNAi* was used to produce knockdowns of *chinmo* (E. Matunis; (Ma et al., 2014)). *UAS-lacZ* was employed as a reporter gene. *twist24B-Gal4* was used to induce expression of UAS-transgenes in SGPs starting at stage 11 (Brand and Perrimon, 1993). *dsx-Gal4* was used to induce expression of UAS-transgenes in SGPs starting at stage 15 (S. Goodwin; Robinett et al., 2010). *c587-Gal4* was used to induce expression of UAS-transgenes in CySCs starting at late stage 17 (A. Spradling; (Kai and Spradling, 2003)). Partial loss of function *chinmoST* mutants were used to analyze Chinmo specifically depleted in the CySC lineage (E. Matunis; (Ma et al., 2014)). *dsx-Gal4* homozygous females mated to
*Pin/Cyo; UAS-chinmo<sup>FL</sup>/TM3 Sb* males was used to produce progeny with overexpression of Chinmo in the somatic cell lineage (E. Matunis; Qing Ma; Ma et al., 2014).

**Collection of embryos and larvae and preparation for immunostaining**

Flies of the desired genotype were obtained using the Gal-4-UAS system, as described (Duffy, 2002; also seen introduction). For example, male *UAS-chinmoRNAi* flies are mated to female virgin *twist24B-Gal4* flies to obtain *twist24B-Gal4; UAS-chinmoRNAi* progeny. This method was employed in all experiments to obtain progeny with the proper genotype, except for the control *y<sup>1</sup>, w<sup>1118</sup>* wild type and *chinmoST* flies which were mated to themselves. 100-120 adult flies are collected at a 4:1 ratio of females to males in a range from 2-7 days of age (Fidler, Boulay et al, 2014). These flies were then put into a cylindrical cage with an apple juice agar plate attached using parafilm. This cage is placed into an incubator set for 24°C. There is an initial 24 hour accumulation period, then the first agar plate is discarded and replaced with a new apple juice agar plate that's been incubated at 24°C. In specific cases, flies were raised at 29°C to assess the phenotype of a hyperactive Gal-4 driver. Next, embryos were collected in time increments of either embryo (0-24 hr), 1<sup>st</sup> instar or L1 larvae (24-48 hr), 2<sup>nd</sup> instar or L2 larvae (48-72 hr), or 3<sup>rd</sup> instar or L3 larvae (72-96 hr) after egg laying (AEL), collecting each agar plate after removing it at the desired time. At this time embryos and larvae are subjected to fixation in 37% formaldehyde followed by methanol (Fidler, Boulay et al, 2014). To prepare samples for immunostaining, samples were rehydrated in a 50% methanol/PBTw solution followed by washing with BBTw as per Fidler, Boulay et al, 2014. Samples were then sonicated in order to remove cuticle layers that interfere
with antibody penetration. Sonication times generally used were: 2 sonications for stage 17/early-L1, 5 sonications for early/mid-L1, 9 sonications for mid/late-L1, 11 sonications for early/mid-L2, 13 sonications for mid/late-L2, 15 sonications for early-L3, 22 sonications for mid/late-L3 (Fidler et al, 2013).

**Immunostaining**

Immunostaining was performed as per Fidler, Boulay et al, 2015. Following sonication, samples were rinsed twice in BBTw, and then washed three times with BBTw for 3 minutes. Sample was then blocked in a 5% normal goat serum (NGS)/BBTw to reduce nonspecific antibody binding. Primary antibodies were then added and incubated overnight at 4°C and a minimum of 4 hours at room temperature (~24°C). These steps of BBTw rinses, washes, and 5% NGS/BBTw blocks were repeated for additional antibodies that needed to be incubated by themselves (see below). Following staining with the primary antibodies, fluorophore-labeled secondary antibodies were applied in a similar protocol following BBTw rinses, washes, and 5% NGS/BBTw blocks. After immunostaining with primary and secondary antibodies, samples were stored in 100 µl of DABCO solution (Fidler, Boulay et al, 2015) and -20°C until being mounted onto a slide.

The following primary antibodies were used: rabbit anti-Vasa at 1:5000 (R. Lehmann); rabbit anti-ZFH1 at 1:5000 (R. Lehmann); guinea pig anti-Traffic Jam at 1:2500 (D. Godt); mouse anti-Fasciclin 3 at 1:10 (C. Goodman; Developmental Studies Hybridoma Bank [DSHB]); mouse anti-EYA at 1:25 (S. Benzer/N. Bonini; DSHB); mouse anti-1B1 at 1:4 (H. Lipshitz; DSHB); mouse anti-β-galactosidase at 1:5000 (Promega); rat anti-vasa 1:20; rat-anti- Chinmo at 1:500 (N. Sokol), rat anti-N-Cadherin at 1:20 (T. Uemura; DSHB). Secondary antibodies (Molecular Probes) used were: goat
anti-mouse 488, goat anti-mouse 546, goat anti-mouse 633, goat anti-rabbit 633, goat anti-rat 546, goat anti-rat 633, goat anti-guinea pig 633, goat anti-rat 488, goat anti-rabbit 546. All secondary antibodies were used a final concentration of 1:500. Additionally, DAPI (Roche) was used to stain nuclei at a concentration of 1µg/mL, applied for 3 minutes.

Modifications to the protocol were made in some experiments to enhance the staining quality with specific antibodies. Both the EYA and ZFH-1 primary antibodies were rocked by themselves for 48 hours at 4°C to increase antibody binding. The antibodies mouse anti-EYA, rabbit anti-ZFH1, guinea pig anti-Traffic Jam, and rat-anti-Chinmo were applied by themselves and not as part of a mixture of all primary antibodies. This allowed them to be reused without contamination from other antibodies in future experiments.

Confocal microscopy and analysis

Embryo and larvae samples were mounted on a glass microscope slide and in a solution of 70% glycerol containing 2.5% DABCO solution (Fidler, Boulay et al, 2015) and p-phenylenediamine anti-fade agent (Sigma) at a 0.2 mg/mL final concentration. Embryos and larvae were staged based on their morphologies. Embryos were arranged from stage 5 to late stage 17 (Campos-Ortega and Hartenstein, 1985), while larvae were staged according to physical size (Sinden, Badgett et al, 2012). Slides were viewed using an Olympus BX51 microscope equipped with a DSU spinning disc confocal system and Q-imaging RETIGA-SRV CCD camera. Slidebook 5.0 software by 3I was used to
capture and analyze all images using 3 dimensional capture in 3 wavelength channels. The size of the gonad and size of cells was analyzed using Slidebook's ruler tool.

Male embryos and larvae were distinguished from females by immunostaining for the presence of a coalesced hub. To determine for the expression pattern of Chinmo, all cells were analyzed for weak or strong expression of the rat anti-Chinmo antibody. To determine the morphologies of the differentiation defective phenotype, the presence of dot fusomes and vacuolization, or the formation of areas that lack germ and cyst cells, was assessed. The significance of the difference between the number of chinmo knockdown, Chinmo overexpression, and wild type testis having this defective differentiation was determined using a standard two-tailed student t-test. This was then used to obtain a p-value, where it was assumed there was significance at P<0.05. To determine the number of somatic cells expressing high-levels of ZFH-1, CySC cells arrayed within 1–2 cell diameters from the hub were analyzed. A two-tailed student t-test was employed to determine significance. To analyze EYA expression cyst cells located from 2 cell diameters away from the hub to the posterior in the gonad were analyzed. β-Gal expression of c587 expression assessed expression of β-Gal in SGPs, germ cells, CySC, hub cells, and the testis sheath (somatic cells surrounding the testis). In the preliminary data from the Matunis lab, Fas3 was used as a marker for the formation of follicle cells found in ovaries.

RESULTS

Characterization of Chinmo expression in embryonic and larval stages
Chinmo expression has previously been characterized in the adult *Drosophila* testis. Experiments have shown that Chinmo is present in CySCs and early cyst cells (Flaherty, et al., 2010). In addition, Chinmo is detected in the hub and at lower levels in the GSCs and early gonialblasts. Chinmo expression in the CySC, early cyst cells, and germline matches the expression of stabilized Stat92E, which is known to upregulate the expression of *chinmo* (Flaherty, et al., 2009; Okegbe and DiNardo, 2011). However, STAT92E is not detected in the hub, suggesting that *chinmo* is regulated by additional factors. These results were consistent with Chinmo's role in maintenance of CySCs, as there is no Chinmo expression detected in differentiated cyst cells that reside away from the hub. While Chinmo's expression pattern has been well characterized in the adult *Drosophila* testis, it is unknown if Chinmo is expressed during the development of the testis. If Chinmo were detected during development in stem cell precursors, it would suggest a function during specification and/or establishment of the testis stem cell niche.

To assess for a possible role for Chinmo during stem cell development in the *Drosophila* gonad, we determined when Chinmo expression is first detected in the developing testis and further assessed its expression pattern as development progresses. A Chinmo antibody was used to detect Chinmo in wild type embryos and larvae. The sample was co-immunostained to detect the germline-specific protein, Vasa (Fujiwara et al, 1994), as well as Traffic Jam (TJ), which is present in somatic gonadal precursors (SGPs), hub cells, CySC, and early cyst cells (Li et al., 2003; Sinden, Badgett et al, 2012). We find that Chinmo expression is not detected in embryos ~11 hrs after egg laying (AEL; embryonic stage 12) and any stages younger than this age (Fig. 8A-8A", n=48). Chinmo expression is first detected in SGPs at ~12 hrs AEL (stage 15; Fig. 8B-
8B", n=14) just after gonad coalescence is complete (Jenkins, et al., 2003). Chinmo continues to be expressed in all SGPs during hub cell establishment at ~20 hrs AEL (early stage 17 embryos; Fig. 8C-8C", n=14). Once a functional stem cell niche has formed, Chinmo is readily detected in hub cells, CySCs, and to a lesser extent in somatic cells in the testis posterior which may be early cyst cells (~24 hrs AEL, Fig. 8D-8D", n=21). In larvae aged ~30 hrs AEL (mid L1 stage Fig. 8E-8E", n=21) and ~48 hrs AEL (L2 stage, 8F-8F", n=19) Chinmo is detected in hub, CySC and early cyst cells. Moreover, Chinmo is faintly detected in GSCs at ~48 hrs AEL. At ~64 hrs (late stage L2), Chinmo expression in the soma becomes restricted to only CySCs and hub cells. This pattern remains into the late larval stages up to 72-96 hrs AEL (L3 stage, 8G-8G", n=72). Additionally, beginning at ~72 hrs AEL, Chinmo expression becomes detected in gonialblasts, in 8-cell spermatogonia, and staining is more distinct in GSCs. This final pattern of expression resembles that found in the adult testis (Flaherty et al., 2010; Ma et al., 2014).

**Chinmo is necessary for CySC specification/establishment, but not maintenance during development**

Studies in adult *Drosophila* testis have shown that *chinmo* is necessary for CySC maintenance (Flaherty et al., 2011). As Chinmo is expressed in SGPs prior to establishment of functional CySCs, we wanted to determine if Chinmo regulates the specification and/or establishment of CySCs from a pool of embryonic SGPs. We also assessed whether Chinmo functions to promote CySC maintenance in the newly formed stem cell niche.
We first assessed whether *chinmo* is necessary for aspects of CySC development. To do this, we performed tissue-specific *chinmo-RNAi* knockdown in developing testes using the Gal4-UAS system (Duffy, 2002). While a number of Gal4 gene expression drivers have been shown to be expressed in newly formed SGPs (*double sex (dsx) -Gal4 and twist,24B-Gal4; Robinett et al., 2010;*) or in adult CySCs (*c587-Gal4; Kawase et al.,2004*), the activity of these drivers had not been characterized throughout testes development. Therefore, we examined induction of a UAS- target gene (*UAS-lacZ::nls*) by these different drivers during embryonic and larval testis development. As previously reported (Robinett et al., 2010), the *dsx-Gal4* driver was found to be active specifically in SGPs starting at ~9 hrs AEL (stage 11; Fig. 9A-D). Importantly, we found that testis-specific expression persisted into later stages of development, where the *dsx-Gal4* driver remained active in the CySC lineage (i.e CySCs and cyst cells), as well as hub cells. While the *twist,24B-Gal4* driver showed broader expression in mesodermal cells surrounding embryonic SGPs, it too showed high levels of expression in embryonic SGPs and in the newly formed stem cell niche (Fig. 9E-G). Interestingly, in later stages of larval development, activity was only observed in hub cells and in cells that begin to form the testis sheath (Fig. 9H). In contrast to these drivers with early/embryonic activity, we found that the *c587-Gal4* driver was not active in embryonic SGPs (Fig. 9I-J) and LacZ induction is first observed at ~24hrs AEL (just after stem cell niche formation) where it is expressed in CySCs, hub cells and also in somatic cells in the testis posterior (Fig. 9K). This expression pattern persists into larval development (Fig. 9L) and is similar to that observed in the adult testis (Kawase et al.,2004). Thus, we have characterized Gal4 drivers with three different activation patterns: (*dsx-Gal4*) early SGP induction with
activity persisting broadly in larval testes, (\textit{twist,24B-Gal4}) early SGP induction with activity diminishing in CySCs and cyst cells in larval testes, and (\textit{c587-Gal4}) late induction with activity present in the CySC lineage, as well as hub cells only after the stem cell niche has become function.

Having characterized these drivers, we used them to knockdown \textit{chinmo} in somatic gonadal cells at different stages of testes development. Because presence of functional CySCs and cyst cells are critical for GSC maintenance and differentiation (Leatherman and DiNardo, 2010; Sinden et al., 2012), we looked for germ cell differentiation defects after somatic \textit{chinmo} knockdown as a first screen for defective CySC development. To do so, larval testes with \textit{chinmo} knocked down using different Gal4 drivers were immunostained to detect a germline-specific organelle, called the fusome. Fusomes, which are believed to orient the mitotic spindle during cell division (Grieder et al., 2000), form cytoplasmic extensions that elongate and then branch between the shared cytoplasm of 2, 4-, 8-, and 16- cell spermatogonia that undergo incomplete cytokinesis. As fusomes mature from a spherical/dot structure in GSCs and early gonialblasts, changes in fusome morphology from a “dot” to elongated, and then branched structures, can be used to assess germ cell differentiation status. Thus, by co-immunostaining ~48 hrs (L2) larvae to detect fusomes (1B1), germ cells (Vasa) as well as hub cells (Fas3 on N-Cadherin), larval testes with altered germ cell differentiation due to somatic \textit{chinmo} knockdown can easily be detected. Furthermore, by knocking down \textit{chinmo} at different stages of development, we can determine whether \textit{chinmo} functions during CySC specification/establishment vs. CySC maintenance after the stem cell niche has formed. For example, if larval testes are defective as a result of \textit{chinmo} knockdown
by the c587-Gal4 driver, but not the early twist,24B-Gal4 driver, it would indicate that
chinmo is required for CySC maintenance and/or production of cyst cells, but not for the
specification or establishment of functional CySC. Alternatively, if a defect is observed
due to early dsx-Gal4 and twist,24B-Gal4 drivers, but not the c587-Gal4 driver, it would
indicate that chinmo is required for CySC specification or establishment, but is not
involved in CySC maintenance.

To assess whether chinmo is required for CySC specification and/or
establishment, we first assessed for germ cell differentiation defects after chinmo
knockdown using the early twist,24B-Gal4 driver. 92% of testes in twist24B-Gal4;
UAS-chinmoRNAi knockdown larvae (Fig. 10B-10B', n=61) showed a differentiation
defective phenotype, compared to 16% in age-matched twist24B-Gal4 controls lacking
the UAS-chinmoRNAi transgene (Fig. 10A-10A', n=25). This phenotype was
characterized by dot fusomes in the posterior of the testis and the formation of vacuoles,
or large gaps where germ cells are absent, throughout the testis. Occasionally, branched
fusomes were seen in isolated groups of spermatogonia in the testis posterior of twist24B-
Gal4; UAS-chinmoRNAi larvae; indicating that germ cell differentiation is not entirely
inhibited.

To verify that Chinmo is indeed absent in these samples, twist24B-Gal4; UAS-
chinmoRNAi larvae at ~30 hrs and ~48 hrs AEL were stained with a Chinmo antibody.
None of the ~30 hr AEL larvae showed any Chinmo expression in developing testes (Fig.
11A-A', chinmoRNAi: n=27; y1 w1118: n=21). Conversely, in ~48 hr larvae when the
twist,24B-Gal4 is no longer active in CySCs/cyst cells, Chinmo expression is observed,
but at much lower levels compared to controls (Fig. 11B-1B', chinmoRNAi: n=12;
Chinmo expression was also detected in GSCs at comparable levels to controls in ~48 hrs larvae. Further, in chinmo knockdown larvae at both ages, Chinmo expression remained detectable in the brain, where Chinmo is known to be expressed but the twist,24B-Gal4 driver is inactive. Thus, loss of Chinmo expression correlates both spatially and temporally with activity of the twist,24B-Gal4 driver; consistent with the hypothesis that chinmo knockdown is caused by a defect in CySC specification and/or establishment.

As further confirmation that early somatic chinmo causes the germ cell differentiation phenotype described above, we also examined ~48 hr larvae expressing UAS-chinmoRNAi under control of the dsx-Gal4 driver. 48\% of testes in dsx-Gal4; UAS-chinmoRNAi larvae showed a germ cell differentiation phenotype similar to twist24B-Gal4; UAS-chinmoRNAi larvae (Fig. 10C-10C'; n=16), while 0\% of testes from dsx-Gal4 controls lacking the knockdown transgene were phenotypic (Fig. 10D-10D'; n=24). Together, these data are consistent with the hypothesis that chinmo functions during early SGP development to promote either CySC specification and/or establishment.

To determine if Chinmo is also necessary for CySC maintenance after stem cell niche formation, we examined ~48 hr larvae expressing UAS-chinmoRNAi under control of the late-activated c587-Gal4 driver. Interestingly, 0\% of testes in c587-Gal4; UAS-chinmoRNAi larvae displayed differentiation defective germ cells (Fig. 12B-B',n=31), while 24\% of testes from c587-Gal4 controls lacking the knockdown transgene showed altered germ cell differentiation (Fig. 12A-A',n=34). It must still be determined why some controls show testis development defect under these circumstances. However, the regular observation of a phenotype due to early/embryonic chinmo knockdown in the
somatic gonad, but not after late/larval knockdown, suggests that *chinmo* is necessary to promote CySC specification and/or establishment, but not CySC maintenance in the developing testis. As *chinmo* was previously shown to promote CySC maintenance in the adult testes (Flahtery, et al., 2009), these data suggest a different function during development than observed in adults.

While early knockdown of *chinmo* causes a phenotype that correlates with reduced Chinmo expression, we were interested to determine if loss of *chinmo* by means other than RNAi would alter testes development. While loss of function *chinmo* mutations are embryonic lethal, recent work by the Matunis lab elucidated a partial loss-of-function *chinmo* allele (*chinmo*^Sex Transformation^ or *chinmo*^ST^) that lacks Chinmo expression in the CySC lineage (Ma et al., 2014). Therefore, we examined the impact of the *chinmo*^ST^ allele on testis development. For these studies, we co-immunostained ~30 hr (mid-L1) and ~72 hr (late-L2) old larvae for TJ to detect CySC/early cyst cells, Vasa to detect germ cells, and Chinmo to determine if Chinmo protein was reduced/absent. While germ cell differentiation was not directly assessed by fusome staining, 0% of testes from *chinmo*^ST^ mutant larvae displayed hallmarks of the *chinmo* knockdown phenotype, including vacuolization (*n*=57,25,32 for 30- 48- & 72- hr AEL larvae, respectively), and all *chinmo*^ST^ testes showed enlargement of posterior germ cells associated with onset of spermatogonial differentiation in controls (*n*=57,25,32 for 30-, 48- & 72- hr AEL larvae, respectively). However, unlike adult testes lacking Chinmo protein in CySCs and daughter cyst cells, somatic Chinmo expression in *chinmo*^ST^ testes matched that of controls in ~30 hr and ~48 hr larvae (Fig. 13A-D; *chinmo*^ST^: *n*=21 and 19, respectively; *control*: *n*=57 and 25, respectively). Interestingly, at ~72 hrs AEL, the number of testes
staining for Chinmo in the CySC lineage began to decline in chinmo\textsuperscript{ST} larvae as compared to controls; Chinmo was detected in 72% of chinmo\textsuperscript{ST} testes compared to 98% of age-matched controls (Fig. 13E-F; n=42 & 32 respectively; P=0.001). Thus, while chinmo\textsuperscript{ST} mutant testes do not appear to be phenotypic shortly after stem cell niche formation, this may result from the fact that Chinmo protein remains present during embryonic and early larval development. As a result, we focused our remaining studies on tissue specific chinmo knockdown. The reduction of Chinmo expression in CySCs at ~72 hrs AEL, however, suggests that a developmental transition may occur at ~72 hrs AEL where the stem cell niche begins to behave more like the adult testes.

Having established that chinmo knockdown is necessary in the somatic gonad during testis stem cell niche formation, but not stem cell maintenance, we also sought to characterize onset of the knockdown phenotype. Specifically, we quantitated pervasiveness of this phenotype in twist24B-Gal4;UAS-chinmoRNAi larvae at ~30 hrs (mid-L1), ~48 hrs (late L1) and ~72 hrs (late L2) AEL. As controls, age-matched y\textsuperscript{1}, w\textsuperscript{1118} larvae, as well as c587-Gal4;UAS-chinmoRNAi larvae (which we previously showed to be non-phenotypic at ~48 hrs AEL; Fig. 12B-B') were examined. In twist24B-Gal4;UAS-chinmoRNAi larvae, the chinmo knockdown phenotype was observed in 50% of ~30 hr AEL testes (Fig. 14A-14A',n=23), 92% of ~48 hr AEL testes (Fig. 14B-14B',n=61), and 63% of ~72 hr AEL testes (Fig. 14C-14C',n=12). In c587-Gal4;UAS-chinmoRNAi controls, a phenotype was observed in only 7% of ~30 hr testes (Fig. 14J-14J',n=30), 0% of ~48 hr testes (Fig. 14K-14K',n=9), and 21% of ~72 hr testes (Fig. 14L-14L',n=14). Similarly, y\textsuperscript{1}, w\textsuperscript{1118} controls were phenotype in 6% of ~30 hr testes (Fig. 14G-14G',n=18) and 7% of ~48 hr testes (Fig. 14H-14H',n=14). Unfortunately, staining from ~72 hr
$y^1, w^{1118}$ was not obtained. These results further support the hypothesis that Chinmo is necessary for specification and establishment of CySC, but not for their maintenance.

The decline in the number of testes showing a phenotype between ~48 hrs and ~72 hrs in twist24B-Gal4; UAS-chinmoRNAi ($p=0.0059$) is consistent with a decrease in chinmoRNAi due to the driver becoming inactive in CySCs after ~48 hrs AEL.

**Chinmo knockdown leads to increased CySC number and defective cyst differentiation**

The observation that Chinmo is required in embryonic SGPs for normal CySC development suggests that, in the absence of Chinmo, CySC progenitors may never be specified from the pool of SGPs, preventing formation of functional CySCs.

Alternatively, functional CySCs may fail to be established from CySC progenitors, leading to defects in cyst cell differentiation. To test the hypothesis that chinmo is required for specification of CySC progenitors from embryonic SGPs, we directly tested for presence of CySCs in larval testes after chinmo knockdown. A Zinc Finger Homeodomain-1 (ZFH-1) antibody was used to detect the presence of CySCs after early soma-specific chinmo knockdown (dsx-Gal4; UAS-chinmoRNAi). Larvae were costained to detect germ cells (Vasa) and the CySC lineage (TJ). ZFH-1 positive cells were detected in dsx-Gal4; UAS-chinmoRNAi testes, indicating that CySC are specified in the developing stem cell niche (Fig. 15B-15B'). Interestingly, there was a slight increase in the average number of ZFH-1 positive CySCs detected after early knockdown as compared to controls. An average of 11 CySCs was observed in dsx-Gal4 larvae lacking the chinmoRNAi transgene (Fig. 15A-15A', n=15), compared to 14 in dsx-Gal4; UAS-chinmoRNAi larvae (Fig. 15B-15B', n=34). This statistically significant increase in CySC
number after early *chinmo* knockdown (*p*=0.0058) suggests that an increased number of CySCs may be specified from the SGP pool. Alternatively, it is possible that this increase may arise from CySC over-proliferation and/or an increase in symmetric stem cell divisions due aberrant CySC establishment.

To test for defects in establishment of functional asymmetrically dividing CySCs, we assessed whether CySCs were capable of giving rise to differentiated progeny. An Eyes Absent (EYA) antibody was used to detect the presence of cyst cells that have been shown to arise from newly formed CySCs (Sinden, Badgett et al, 2012). Early soma-specific *chinmo* knockdown was performed (*dsx*-Gal4; *UAS-chinmoRNAi*) and resultant larvae co-immunostained to detect germ cells (VASA) and the CySC lineage (TJ). In control larvae lacking the RNAi construct, EYA is detected in nuclei of cyst cells, which are interspersed between the differentiating spermatogonia in middle and posterior regions of the testis (Fig. 16A-A'). However, in *dsx*-Gal4; *UAS-chinmoRNAi* larvae, EYA positive cells were found to be clustered around vacuoles within the testes. EYA staining was also excluded from the nucleus in many instances (Fig. 16B,C). Specifically, 55% of *dsx*-Gal4; *UAS-chinmoRNAi* (n=31) larvae showed clustering of EYA positive cells adjacent to vacuoles, compared to 5% in *dsx*-Gal4 controls (n=21). Additionally, nuclear-exclusion of EYA staining was detected in 87% of *dsx*-Gal4; *UAS-chinmoRNAi* testes (n=31), compared to none detected in controls (n=21). Interestingly, when EYA was excluded from the nucleus, it remained nearby the nucleus and did not extend around the differentiating spermatogonia, which cyst cells normally ensheath. As nuclear excluded EYA would be expected to stain diffusely throughout the cyst cell cytoplasm, this suggests that some cyst cells are unable to grow to ensheath spermatogonia.
Combined with the clustering of multiple EYA positive cells around testis vacuoles, these data suggest a defect in the capacity for cyst cells to differentiate. As this defect correlates with the germ cell differentiation phenotype observed after chinmo knockdown in embryonic SGPs, but not after stem cell niche formation, it is possible that chinmo functions to promote a gene program that enables CySCs to produce functional cyst cells. Alternatively, although an increased number of CySCs in the developing stem cell niche suggests otherwise, we do not rule out the possibility that chinmo knockdown is somehow affecting the long-term health of the CySCs and their progeny.

A recent report also indicates that chinmo functions in aging adult CySCs to prevent sex-reversion of male CySCs into female ovarian stem cells (Ma et al, 2014). Reversion of male CySCs to ovarian somatic stem cells is associated with cell aggregation. We, therefore, tested if the chinmo knockdown phenotype arose from altered somatic sex determination. Because CySC sex reversion in adult chinmo mutant testes leads to production of follicle cells that arise from ovarian somatic stem cells, we immunostained dsx-Gal4; UAS-chinmoRNAi larvae to detect the follicle cell marker, Fasciclin 3 (Fas3). Similar to controls, Fas3 expression is detected exclusively in hub cells in dsx-Gal4; UAS-chinmoRNAi larvae at ~48 hrs AEL (Fig. 17A, n=16). Together, these data suggest that chinmo is not required for CySC sex maintenance, but that it plays a critical role in the specification and/or establishment of functional CySCs from the pool of SGPs.

**Chinmo overexpression also causes testes development defects**

To further examine the function of Chinmo during development of embryonic SGPs, we also assessed the impact of Chinmo over-expression on CySC development.
Specifically, we examined the impact of Chinmo over-expression in SGPs, CySCs and cyst cells on germ cell development and differentiation by examining fusome morphology. Control and \textit{dsx-Gal4;UAS-chinmo}\textsuperscript{wild type} larvae were co-immunostained for the fusome marker, 1B1, and to detect germ cells (Vasa) and the CySC lineage (TJ). In the control larvae, only 3% of the testis ∼48 hr AEL showed abnormal germ cell differentiation (Fig 18A-A', n=34), while 71% of the testis in \textit{dsx-Gal4;UAS-chinmo}\textsuperscript{wild type} larvae showed germ cell differentiation defects (Fig 18B-B', n=38)(p<0.0001).

Importantly, this phenotype differs from the previously described phenotype caused by \textit{chinmo} knockdown. Instead of germ cell vacuolization, branched fusomes were not detected at the testis posterior. Moreover, posterior germ cells displayed dot fusomes and a spherical morphology similar to that found in undifferentiated GSCs and daughter gonialblasts. As expansion of CySCs throughout the developing testes causes a similar phenotype (Sinden, Badgett et al, 2012), this suggests that increased Chinmo expression also expands the CySC population.

**DISCUSSION**

Our data indicate that \textit{chinmo} has a significant role in regulating somatic gonadal development during formation of the \textit{Drosophila} testis stem cell niche. It was found that Chinmo is first expressed in embryonic SGPs and this expression is maintained in hub cells, CySCs, and newly differentiated cyst cells during and after stem cell niche formation (Fig. 8). Chinmo expression then becomes restricted to hub cells and CySCs as development progresses. Additionally, Chinmo becomes expressed in GSCs, then in gonialblasts and later stage spermatogonia during late stages of larval testis development.
This suggests that Chinmo has a role in regulating development of SGPs into functional CySCs, and that it may also play a role in regulation of both CySC and GSC homeostasis. Consistent with a role in CySC formation, *chinmo* knockdown in SGPs and CySC progenitors prior to stem cell niche formation causes defective germ cell differentiation. This phenotype correlates with a slight increase in CySC number as well as defects in the ability of cyst cells (that arise from CySCs) to differentiate properly. Furthermore, over-expression of wild-type Chinmo in early SGPs appears to inhibit germ cell differentiation in a manner consistent with an expansion of CySCs throughout the developing testes. Interestingly, knockdown of *chinmo* after stem cell niche formation does not induce a germ cell differentiation phenotype. Together, these data indicate that *chinmo* is necessary and sufficient for the proper specification and subsequent establishment of functional CySCs during *Drosophila* testes development (Fig. 19).

**Role of chinmo in Cyst Stem Cell specification/establishment**

To explain our data, we hypothesize that *chinmo* functions to promote aspects of CySC identity during CySC progenitor specification. Specifically, *chinmo* may be required to endow newly established CySCs with the capacity to differentiate into functional cyst cells. Indeed, while expression of CySC markers are present after *chinmo* knockdown and these cells appear capable of asymmetric division, most of the cyst cell progeny from these divisions appear to have physiological differences that prevent them from fully ensheathing differentiating gonialblasts/spermatogonia. As a result, the cyst cells cluster together and fail to nurture early stages of spermatogenesis, resulting in the formation of vacuoles in the testes, likely caused by zones of germ cell death (Fig. 10).
This defect could arise either during CySC specification or CySC establishment. To further examine this phenotype, *chinmo* knockdown testes should be analyzed for presence of both apoptosis as well as necrotic death. This can be assessed through immunostaining for either the apoptotic marker cleaved Caspase-3 (Chinnaiyan and Dixit, 1996) or the necrotic marker Eiger, the unique *Drosophila* tumor necrosis factor (Igaki et al, 2002).

In addition to the proposed role in promoting CySC progenitors with the capacity to produce functional cyst cells, our data suggest that *chinmo* may be required to endow functional CySCs with the capacity to regulate the balance between symmetric and asymmetric division. The increased number of cells expressing CySC markers in the newly formed testis niche after *chinmo* knockdown is consistent with this interpretation. However, it is also possible that early cyst cells, which retain expression of the CySC marker ZFH-1 (though at lower levels than CySCs), fail to leave the stem cell niche after *chinmo* knockdown. Cell tracking experiments performed through live cell imaging may be useful to help resolve these questions.

A role of *chinmo* in promoting CySC progenitor identity is also supported by Chinmo overexpression. As indicated above, germ cell differentiation defects after Chinmo over-expression phenocopy defects observed after expansion of CySCs throughout the testes. We, therefore, hypothesize that Chinmo overexpression in CySC progenitors leads to an excess of CySCs throughout the testes. While it is unclear why this might occur at the expense of cyst cell differentiation, it may be that abnormally high levels of Chinmo in posterior somatic cells represses cyst cell differentiation. Alternatively, high levels of Chinmo may promote symmetric CySC divisions that
expand the CySC population at the expense of asymmetric, cyst producing, divisions. Further studies to test these hypotheses include staining for the pattern of Chinmo expression in after Chinmo overexpression, as well as direct assessment for CySC expansion at the expense of cyst cell differentiation using EYA and ZFH-1 and cyst or CySC markers, respectively.

A caveat to these experiments is that the *dsx-Gal4* and *twist,24B-Gal4* larvae displayed defective differentiation phenotypes at low levels in the absence of RNAi. This is possibly from general sickness due to insertion of the Gal4 transgene, which could be exacerbated by presence of a *chinmo-RNAi* transgene. However, a statistically significant reduction in phenotype induction in larvae expressing the *chinmoRNAi* transgene using the late *c587-Gal4* driver indicates this is not the case. A future experiment to confirm that *chinmoRNAi* is responsible for the phenotype would be to assess whether different levels of *chinmo* knockdown correlate with more or less phenotype induction. To do this, flies expressing *chinmoRNAi* under control of a Gal4 driver could be reared at different temperatures. Because Gal4 activity is temperature-dependent (Duffy, 2002), the severity of the differentiation defective phenotype should increase when incubated at higher temperatures and attenuate at lower temperatures if *chinmoRNAi*, and not the location of the transgene insertion, is responsible for the phenotype. It will also be important to determine why phenotypic testes are even observed in control samples.

An additional caveat to analysis of Chinmo overexpression is that a higher than expected number of larvae exhibited a phenotype. For these experiments larvae were generated by mating *dsx-Gal4* homozygous females with males heterozygous for the *UAS-chinmoFL* transgene. This couldn't result in progeny with more than 50% of larvae
carrying the UAS-chinmoFL transgene. However, 71% of testes examined from this mating were scored as phenotypic. While this discrepancy must be explored further, it is possible that the higher than expected number of phenotypic testes was observed due to testes development defects occasionally observed in controls. Lower than normal sample sizes could also skew these observations further.

Furthermore, the conclusion that that Chinmo is required for specification and/or establishment but not maintenance is based on the fact that Chinmo knockdowns using dsx-Gal4 and twist,24B-Gal4 drivers showed a phenotype, but a phenotype was not seen using the c587-Gal4 driver. A caveat to this is that we have not formally confirmed that the c587-Gal4 driver is indeed knocking down Chinmo activity from the time it becomes active at ~24 hours AEL and into later larval stages. A Chinmo stain of c587-Gal4;chinmoRNAi testes from embryonic stages to ~48 hours AEL is in progress. However, since it is known that c587-Gal4;chinmoRNAi causes a phenotype in adults and due to the fact that we see no phenotype in ~48 hours AEL larvae, we predict that our hypothesis is correct. In addition, dsxGal4;chinmoRNAi testes of the same ages are also being stained to confirm that there is a loss of Chinmo in these knockdown testes.

**Role of chinmo in stem cell maintenance/self-renewal**

As our data indicate that chinmo is not required for CySC maintenance in the newly formed stem cell niche, our data emphasizes that chinmo's function is different during developmental stages than in adult Drosophila. In the transition from larval to adult development stages, there can be significant transitions in regulation. An example of this is the attenuation of the BMP signaling that occurs in pupal and adult testes (Lim
and Fuller, 2012). It is hypothesized that \textit{chinmo} may take on new functions when changes in signaling pathways of the \textit{Drosophila} testis occur around this time. This would explain why \textit{chinmo} can promote CySC maintenance in the adult testes (Flaherty, et al., 2009), but not during development (Fig. 12). Further analyses are required to understand what could change in the regulation of \textit{chinmo} to alter its function at different developmental stages. Possible experiments include elucidation of binding partners for Chinmo that might affect its capacity for gene regulation, as well as epigenetic changes in the developing CySCs that impact accessibility of \textit{chinmo} to different gene promoters.

In addition to \textit{chinmo}'s role in somatic stem cell lineage during development, \textit{chinmo} may have a role in GSC development. This is supported by the induction of Chinmo expression in GSCs and immediate progeny during late stages of larval testis development. It remains to be determined what this role might be because GSC clones lacking \textit{chinmo} function are not lost in the adult testis (Flaherty et al., 2010). While there is only one known \textit{chinmo} ortholog in the \textit{Drosophila} genome, it is possible that another gene plays a redundant function in adult and developing germ cells to promote GSC maintenance. As we also find that Chinmo expression is absent in the germline of developing ovaries (Fig. 20), it is possible that Chinmo expression is somehow linked to sex-specific germ cell development.

\textit{chinmo’s role in regulating somatic sexual identity during testis development}

\textit{chinmo} has been shown to function in the maintenance of male sexual identity in the adult \textit{Drosophila} testis (Ma et al., 2014). This was shown through analysis of markers of female-specific cell structures. Among these was Fas3, a marker for ovarian
follicle cells, detected interstitially between germ cells in $\text{chinmo}^{ST}$ mutant testes.

However, lack of Fas3 expression outside the hub after $\text{chinmo}$ knockdown suggests absence of female somatic differentiation (Fig. 17). At the same time, $\textit{Drosophila}$ ovary development is delayed compared to testes, and follicle cells do not normally form in normally developing ovaries until ~96 hrs AEL (Gilboa and Lehmann, 2004). Therefore, it is formally possible that defective gonad development observed after $\text{chinmo}$ knockdown is the result of improper assignment of sexual identity in the somatic gonad. Further analysis of the impact of Chinmo over-expression in late-stage larval ovaries may help resolve this question. Additionally, immunostaining for additional markers of ovarian development in older larval testes may be useful.

Overall, the data from the $\text{chinmo}$ knockdown and overexpression experiments suggests that a certain level of Chinmo expression needs to be maintained to ensure CySCs are properly established from CySC progenitor cells. Any significant increase or decrease of Chinmo from normal levels of expression will likely results in loss of germ cell differentiation, which causes diminished fertility. This information is not only important for understanding the regulation of CySC development and homeostasis in $\textit{Drosophila}$, but provides a model for how functional stem cells are established from progenitor cells in all stem cell niches. As $\text{chinmo}$ homologs exist in mammals (Gissel et al., 2005), this work may also yield insight into function of related transcription factors in human tissues.

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FIGURES

Asymmetric Division

Symmetric Division

Self Renewal

Committed Progenitor

Stem Cell Maintenance

Stem Cell Niche

Stem Cell Niche

S

S

S

P

S
Fig. 1: Characteristic stem cell symmetric and asymmetric divisions

Stem cells can undergo symmetric division to amplify stem cell populations. Through asymmetric divisions, stem cells can be maintained while producing a committed progenitor cell that goes on to differentiate. The stem cell niche regulates the balance between differentiation and maintenance.
**Fig. 2:** The temporal and tissue-specific control of gene expression through the Gal4/UAS overexpression system.

A fly with a yeast Gal4 transgene under control of a tissue-specific gene promoter is mated to a fly containing the UAS that controls expression of a target gene. In their progeny, the transcription factor Gal4 binds to the UAS regulatory element and upregulates expression of a target gene that is under control of the UAS promoter.

Fig. 3: Embryonic testis development

Blue, hindgut; red, midgut; yellow, germ cells; green, somatic gonadal precursor cells; dark blue, male-specific somatic gonadal precursor cells, light blue, germ cells in stages 15/17 male gonad; pink, germ cells in stages 15/17 female gonad; red, hub cells in stage 17 male gonad; gray, pigment cells. PGCs migrate from the posterior of the embryo during invagination of the mesoderm. The PGCs are ensheathed by SGPs starting at ~11 hrs AEL (stage 13), and form a coalesced gonad at ~12 hrs AEL (stage 15). mSGPs are lost in the ovareis at this stage. At the end of embryogenesis (~24 hr AEL; stage 17), a cluster of 10-12 non-mitotic cells (red) form the hub of the testis stem cell niche.

Fig. 4: Stem cell specification, establishment, and maintenance

SGPS are specified to take on either a hub progenitor or CySC progenitor cell fate. Following this, these progenitor cells are established into functional hub cells and CySCs. CySCs and GSCs undergo asymmetric division to produce differentiated cyst cells and gonialblast cells respectively, while maintaining a stem cell population around the hub. Two cyst cells ensheath the gonialblast as it undergoes divisions to become spermatogonia.
Secretion of the ligand unpaired (upd) from hub cells promotes activation of the Jak-STAT pathway in CySCs and GSCs. Jak-STAT activation is needed to maintain stem cell fate, and cells that divide away from the hub loose Jak-STAT signaling and differentiate. In the GSCs, bag of marbles (bam) is repressed to prevent differentiation. In the CySCs, targets may include zinc finger homeodomain-1 and chinmo.

Fig. 6: chinmo function in the testis stem cell niche

*chinmo* and *zf*-*h*-1 are up regulated by phosphorylated STAT92E. *chinmo* is expressed in both GSC and CySCs. Chinmo is only necessary for CySC self-renewal, while STAT92E is necessary for self-renewal of both CySCs and GSCs.
**Fig. 7: Sex reversion phenotype in adult chinmo loss of function mutants**

Aged adult *chinmo*\textsuperscript{ST} mutants testis exhibit ovarian structures. (O) Control testis where Fas3 is expressed only in hub cells. (Q) *chinmo* mutants develop cells displaying markers and morphologies of female specific follicle stem cells, follicle cells, and stalk cells develop in the. (S) Adult ovary with Fas3 staining of follicle cells. Model K depicts the results that support young adult *chinmo*\textsuperscript{ST} mutant testis being to develop follicle stem cell, which later develop into stalk-like and follicle-like cells.

**Fig. 8: Chinmo is expressed in SGPs and becomes progressively restricted to CySCs after testis niche formation.**

Wild type embryonic and larval testes immunostained with anti-Chinmo (green), anti-Vasa (red) to detect germ cells, and anti-TJ to detect SGPs, hub, and CySCs. Samples aged (A-A’’) ~11 hrs AEL/stage 13 (A-A''), 12 hrs AEL/stage 15 (B-B''), 20 hrs/early stage 17(C-C''), 24 hrs/late stage 17(D-D''), 30 hrs/L1 (E-E''), 50 hrs/L2 (F-F''), and 80 hrs/L3 (G-G'') AEL. Testes are outlined by white dotted line, hub oriented to left and indicated by asterisk. (A-A'') Chinmo was not detected in the SGPs prior to gonad coalescence. (B-B'') Chinmo is first detected in all SGPs at the time of gonad coalescence (white arrow; Chinmo expression) and remains present in all somatic gonadal cells (C-C'') just prior to hub formation and (D-D'') after hub formation and stem cell establishment. (E-E'') Chinmo remains detectable in hub cells, CySCs, and is also present in newly differentiated cyst cells but is not detected in germ cells or somatic cells at the testis posterior. (F-F'') At around 48 hrs (L2) Chinmo expression restricts to only the CySC cells and the hub, and Chinmo becomes detected in faintly in GSCs (blue arrow; germline Chinmo expression). (G-G'') By 80 hrs Chinmo expression is detected in the hub, CySC, and faintly in GSCs, gonialblasts, and 8-cell spermatogonia. Scale bars at 20 µm.
**Fig. 9: Characterization of dsx-Gal4, twist24B-Gal4, and c587-Gal4 drivers**

Embryonic and larval testes obtained by mating either dsx-, twist24B-, or c587-Gal4 females with males carrying a UAS-β-galactosidase (β-gal) transgene. Samples of the indicated ages immunostained with anti-Vasa, to detect of germ cells (red), anti-DN-Cadherin to detect the hub (blue), and anti-β-gal (green; shown alone in grayscale) to detect reporter gene expression. Testes are outlined by the white dotted line, the hub is oriented to the left and indicated with an asterisk, and β-gal expression of note indicated with a white arrow. (A-B) dsx-Gal4 activity is first detected in SGPs starting at ~9 hrs AEL. (C-D) dsx-Gal4 remains active in CySCs, cyst cells, and hub cells into ~48 hrs AEL. (E-F) twist24B-Gal4 activity is detected in SGPs starting at ~9 hrs AEL, and remains active in CySCs just after hub establishment (G). (H) By ~48 hrs AEL twist24B-gal4 activity is only detected in the hub and in cells that begin to form the testis sheath. (K) c587-Gal4 activity is first detected at ~24hrs AEL where it is expressed in CySCs, hub cells, and in somatic cells in the testis posterior. (L) This expression pattern persists into larval development.
### Percent of Testis with Differentiation Defective Phenotype

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent</th>
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<tbody>
<tr>
<td>Control</td>
<td>16%</td>
<td>25</td>
</tr>
<tr>
<td>twist24B-Gal4;chinmoRNAi</td>
<td>92%</td>
<td>61</td>
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<tr>
<td>Control</td>
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<td>16</td>
</tr>
<tr>
<td>dsx-Gal4;UAS-chinmoRNAi</td>
<td>58%</td>
<td>24</td>
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*p-values:*
- p<0.0001
- p=0.0002
Fig. 10: Early soma specific loss of Chinmo results in defective germ cell differentiation.

Larval testes aged 48 hours AEL immunostained with anti-1B1(green) to detect fusomes, anti- Fasciclin 3 (Fas3; green) to detect the hub, and anti-Vasa (red) to detect germ cells. The testes are outlined by the white dotted line, and the hub is oriented to the left and indicated with an asterisks. (A-A') A control (twist24B-Gal4 mated to self, or twist24B-Gal4 only) shows proper dot fusomes in the testis anterior (white arrow) and elongated fusomes forming in the posterior (yellow arrow). (B-B') Early soma-specific knockdown of chinmo (twist24B-Gal4; UAS-chinmoRNAi) shows a differentiation defective testis phenotype characterized by vacuolization (blue arrow) in between germ cells and spherical fusomes (white arrow) present in the testis posterior. (C-C', D-D') Soma-specific knockdown using the dsx-Gal4 driver (dsx-Gal4; UAS-chinmoRNAi) also display the same phenotype. Scale bars at 20 µm.
**Fig. 11: Loss of Chinmo expression in early soma specific knockdown**

Larval testes aged ~30 and ~48 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-Chinmo (green) to detect Chinmo. The testes are outlined by the white dotted line, and the hub is oriented to the left and indicated with an asterisk. (A,A') In *twist24B-Gal4;UAS-chinmoRNAi* testis at 30 hours no Chinmo is detected. (A'') Chinmo is able to be detected in these larvae from the presence of Chinmo in neural cells (white arrow) (B,B') *twist24B-Gal4;UAS-chinmoRNAi* testis 40 hours showed Chinmo expression in anterior germ cells and faint expression in the hub. Scale bars for 20 μm.
48 hours (L2)

48 hours (L2)

48 hours (L2)

<table>
<thead>
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<th>Percent of Testis with Differentiation Defective Phenotype</th>
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<tbody>
<tr>
<td>100%</td>
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- 0% (n=31, c587-Gal4; chinmoRNAi)
- 24% (n=34, dsx-Gal4; UAS-chinmoRNAi)
- 100% (Control)
Fig. 12: Defective germ cell differentiation phenotype observed only after early soma specific chinmo knockdown.

Larval testes aged ~48 hours AEL immunostained with anti-1B1 (green) to detect fusomes, anti- Fasciclin 3 (Fas3; green) to detect the hub, and anti-Vasa (red) to detect germ cells. The testes are outlined by the white dotted line, and the hub is oriented to the left and indicated with an asterisks. (A,A') The control (c587-Gal4 only) testis show dot fusomes in the testis anterior (white arrow) and elongated fusomes forming in the posterior (yellow arrow). (B,B') Soma specific knockdown of chinmo after CySC establishment (c587-Gal4;UAS-chinmoRNAi) shows also appears normal with elongated fusomes forming in the posterior. (C',C") Soma specific knockdown of chinmo in SGPs show differentiation defective phenotype characterized by vacuolization (blue arrow) and dot fusomes (white arrow). Scale bars at 20 µm.
**Fig. 13:** Absence of phenotype in chinmo$^{ST}$ mutant testes is consistent with chinmo's function in CySC maintenance only in adult testis.

Larval testis aged $\sim$30 hours, 48 hours, and 72 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-TJ (blue) to detect hub cells, CySC and early cyst, and anti-Chinmo (green) to detect CySCs. The testes are outlined by the white dotted line, and the hub is oriented to the left and indicated with an asterisks. (A-A",B-B") At the age of $\sim$30 hours both wild type ($y^1,w^{1118}$) and chinmo$^{ST}$ testis show Chinmo expression in the hub, CySC, and early cyst cells. (C-C",D-D") At the age of $\sim$48 hours wild type and chinmo$^{ST}$ testis, Chinmo expression is observed in the hub, CySC, early cyst cells, and GSCs (white arrow; germ cell Chinmo). (E-E",F-F") At the age of 72 hours wild type testis Chinmo is detected in the hub, CySC, early cyst cells, GSCs, gonialblasts, and 8-cell spermatogonia. At this age a significant number of chinmo$^{ST}$ lose Chinmo expression in CySC compared to controls and lose Chinmo expression in germ cells ($P=0.001$). Additionally, no abnormal defective germ cell differentiation morphology was observed in the chinmo$^{ST}$ testis. Scale bars for 20 $\mu$m.
**1B1 Vasa Hub**

- 30 hrs (L1)
- 48 hrs (L2)
- 72 hrs (L3)

**1B1 Hub**

**1B1 Vasa Hub**

**1B1 Hub**

**Graph:**

- Percent of tests with differentiation defective phenotype
- 30hr: n=23, n=18, n=10
- 48hr: n=61, n=14, n=14
- 72hr: n=12, n=14, n=11

**Legend:**

- twist24B-Gal4; UAS-chinmoRNAi
- c587-Gal4; UAS-chinmoRNAi
- y1,w1118
Fig. 14: Time course of phenotype induction after early and late soma specific chinmo knockdown.

c587-Gal4; UAS-chinmoRNAi and twist24B-Gal4; UAS-chinmoRNAi larval testis aged 30 hours, 48 hours, and 72 hours AEL. c587-Gal4; UAS-chinmoRNAi immunostained with anti-1B1 (green) to detect fusomes, anti-Vasa (red) to detect germ cells, and anti-Fasciclin 3 (Fas3; green) detect the hub. twist24B-Gal4; UAS-chinmoRNAi are labeled with anti-N-Cadherin (green) instead of anti-Fas3 to detect the hub. The testes are outlined by the white dotted line, and the hub is oriented to the left and indicated with an asterisks. (A-C) twist24B-Gal4; UAS-chinmoRNAi begin to exhibit the differentiation defective phenotype characterized by vacuolization (blue arrow) and dot fusomes (white arrow) in the posterior of the testis beginning at ~30 hrs AEL. (D-E) c587-Gal4; UAS-chinmoRNAi tesits with wild type patterning including branching fusomes (yellow arrow) and absence of vacuolization. Scale bars at 20 µm.
48 hrs (L2)

**A**

control

Max Z projection

48 hrs (L2)

**B**

dsx-Gal4;UAS-chinmoRNAi

Max Z projection

**A’**

**B’**

Number of ZFH1 positive CySC

![Bar chart](image)

- Dsx-Gal4;chinmoRNAi: n=34, p=0.0058
- Dsx-Gal4 only: n=15

---

*ZFH-1 Vasa TJ*
**Fig. 15: Chinmo knockdown leads to overproliferation of CySCs around the hub**

Larval testes aged ~48 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-TJ (blue) to detect hub cells, CySC and early cyst, and anti-ZFH-1 (green) to detect CySCs. The testes are outlined by the white dotted line, and the hub is oriented to the left and indicated with an asterisks. All images are max z projections of 10 z-planes above and below the hub. (A,A',B,B') Control (*dsx*-only) and *dsx*-Gal4; *UAS-chinmoRNAi* testis show ZFH-1 positive CySCs adjacent to the hub. *dsx*-Gal4; *UAS-chinmoRNAi* testis have a slight increase in the number of CySCs adjacent to the hub compared to controls (p=0.0058). Scale bars for 20 µm.
dsx-Gal4;UAS-chinmoRNAi

EYA Vasa TJ

48 hrs (L2)

Control

A

A'

B

B'

dsx-Gal4;UAS-chinmoRNAi

48 hrs (L2)

C

C'

dsx-Gal4;UAS-chinmoRNAi

48 hrs (L2)
**Fig. 16: chinmo knockdown leads to cytoplasmic EYA expression and clustering of cyst cells around testis vacuoles**

Larval testes aged ~48 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-TJ (blue) to detect hub cells, CySC and early cyst, and anti-EYA (green) to detect cyst cells. The testes are outlined by the white dotted line, and the hub is oriented to the left and indicated with an asterisks. (A,A') A control testis (y¹,w¹¹¹⁸) shows age-appropriate EYA staining (arrowhead) in cyst cells throughout the medial and posterior of the testis . (B,B') dsx-Gal4; UAS-chinmoRNAi testis show cytoplasmic EYA staining (white arrow) and clustering of EYA positive cyst cells around testis vacuoles (blue arrow). There is an increase in the number of testes showing this phenotype in dsx-Gal4; UAS-chinmoRNAi compared to controls (p=0.0002). Scale bars for 20 µm.
**Fig. 17: Expression of Fas3 is only detected in hub cells**

Larval testes aged ~48 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-Fas3 (green) to detect hub cells and follicle cells, and anti-1B1 (green) to detect fusomes. (A,A’) Fas3 is detected only in hub cells and is not detected elsewhere in the testes as would be predicted if follicle cell formation had been induced. Scale bars for 20 µm.
1B1 Vasa Fas3

48 hrs (L2)

1B1 Fas3

48 hrs (L2)

dsx-Gal4;UAS Chinmo FL
**Fig. 18: Overexpression of Chinmo causes loss of germ cell differentiation.**

Larval testes aged ~48 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-TJ (blue) to detect hub cells, CySC and early cyst, and anti-1B1 (green) to detect fusomes. The testes are outlined by the white dotted line, and the hub is oriented to the left and indicated with an asterisks. (A,A') A control testis (dsx-Gal4 only) showing normal spermatogonial differentiation with branched fusomes. (B,B') Testis in progeny produced by mating of dsx-Gal4 homozygous females to UAS-chinmoFL/TM3 Sb males show dot fusomes in the posterior (white arrow) and isolated spherical germ cells indicative of defective differentiation (blue arrow). The increase in number of testis showing this phenotype is statistically significant as compared to controls (p<0.0001). Scale bars for 20 µm.
Fig. 19: *chinmo* knockdown and overexpression data indicate a function of *chinmo* in establishment of functional CySCs from progenitors

In a wild type testis CySC progenitors become established CySCs that differentiate into cyst cells, which supports gonialblast differentiation into spermatogonia. When *chinmo* is knockdown in CySC progenitors, CySCs are established that cannot produce functional cyst stem cells. Additionally, an increased number of CySC are detected at the hub, possibly due to symmetric division. When Chinmo is overexpressed in CySC progenitors an excess of CySCs are established, which leads to an expansion of CySCs that inhibit germ cell differentiation.
Fig. 20: Chinmo detected in the somatic cells of ovaries

Larval ovaries aged 30 hours AEL immunostained with anti-Vasa (red) to detect germ cells, anti-TJ (blue) to detect hub cells, CySC and early cyst, and anti-Chinmo (green) to detect Chinmo. (A,A’) Chinmo expression is detected in the somatic cells of the ovary (arrow). This indicates that Chinmo in development is not expressed sex specifically in the testis. Scale bars for 20 µm.