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The Jak-STAT target Chinmo prevents sex transformation of adult stem cells in the *Drosophila* testis niche

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Abstract

Local signals maintain adult stem cells in many tissues. Whether the sexual identity of adult stem cells must also be maintained was not known. In the adult *Drosophila* testis niche, local Jak-STAT signaling promotes somatic cyst stem cell (CySC) renewal through several effectors, including the putative transcription factor Chronologically inappropriate morphogenesis (Chinmo). Here, we find that Chinmo also prevents feminization of CySCs. Chinmo promotes expression of the canonical male sex determination factor Doublesex^M (Dsx^M) within CySCs and their progeny, and ectopic expression of Dsx^M in the CySC lineage partially rescues the *chinmo* sex transformation phenotype, placing Chinmo upstream of Dsx^M. The Dsx homologue DMRT1 prevents the male-to female conversion of differentiated somatic cells in the adult mammalian testis, but its regulation is not well understood. Our work indicates that sex maintenance occurs in adult somatic stem cells, and that this highly conserved process is governed by effectors of niche signals.

Introduction

Male versus female fate is controlled by a variety of mechanisms across taxa (Kopp, 2012). In mammals, this choice was recently found to be labile even in adults; loss of sex-specific transcriptional regulators in the adult mouse gonad causes differentiated somatic cells to transdifferentiate into somatic cells of the opposite sex (Matson et al., 2011; Uhlenhaut et al., 2009). This indicates that sexual identity must continuously be maintained in specific differentiated cell types long after sex determination has occurred. Whether sexual identity is plastic in undifferentiated adult stem cells remains unknown. Since adult stem cells have the capacity to rebuild entire adult organ systems, altering a stem cell's sexual identity could conceivably cause widespread changes to the tissue.

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In *Drosophila*, a well-studied sex determination cascade culminates in the sex-specific splicing of mRNA encoding the conserved transcription factor Doublesex (Dsx); this binary choice cell-autonomously dictates somatic sexual identity (Whitworth et al., 2012). While upstream regulation of sex determination differs between flies and mammals, downstream control converges on *Dsx/mab-3 related transcription factor (Dmrt)* genes (Matson and Zarkower, 2012). The Janus kinase-Signal Transducer and Activator of Transcription (Jak-STAT) pathway is also required for the initial choice of somatic sexual identity in *Drosophila* embryos and promotes male germline sexual behavior in embryonic testes (Jinks et al., 2000; Wawersik et al., 2005). However, it is not known whether Jak-STAT signaling is required for sex maintenance in *Drosophila*, and the link between the Jak-STAT pathway and the canonical sex determination pathway is unknown.

The *Drosophila* ovary and testis provide excellent models for studying adult stem cell behavior *in vivo* (Fuller and Spradling, 2007; Matunis et al., 2012). In the testis, Jak-STAT signaling maintains two types of stem cells: sperm-producing germline stem cells (GSCs) and supporting somatic stem cells called cyst stem cells (CySCs). Both of these cell types attach to a single niche created by quiescent somatic hub cells at the testis apex and divide asymmetrically to produce differentiating progeny (spermatogonia and cyst cells, respectively) that are displaced from the niche (Matunis et al., 2012). Several factors, including the Jak-STAT targets Zinc-finger homeodomain-1 (Zfh-1) and Chinmo, are required for CySC self-renewal (Amoyel et al., 2013; Flaherty et al., 2010; Issigonis and Matunis, 2012; Leatherman and Dinardo, 2008; Michel et al., 2012). Here we reveal an unexpected function of Chinmo: it acts through the canonical sex determinant Dsx^M to maintain the male identity of adult CySCs.

Results

Reduction of Chinmo triggers the appearance of cells resembling ovarian follicle cells in the adult niche, then throughout the testis

While screening for *Drosophila* testis phenotypes, we identified a spontaneous mutation causing a striking transformation of the adult testis. Adult mutant males are fertile, indicating testes develop normally. Consistent with this observation, testes from young males (0–1 day) are indistinguishable from wild type testes in overall morphology (Figures 1C–D, I–J). With age, however, a progressive change in the testis morphology occurs. Initially, subtle changes are detected at the testis apex, where aggregates of epithelial somatic cells (defined as 8 or more closely apposed cells expressing high levels of adhesion proteins) appear adjacent to the hub, while the remainder of the tissue is unaffected (Figures 1E, K, P–Q). With time, somatic cell aggregates acquire additional cells and extend away from the testis apex, while older differentiating germ cells and cyst cells are displaced toward the basal end of the testis (Figures 1F–G, L–M). In 7–9 day old males, an obvious transformation is apparent throughout the testis: somatic cell aggregates adjacent to the hub remain but now a monolayer of columnar epithelial cells lines the testis periphery, while germ cells are restricted to the lumen of the tissue (Figures 1G, M, R). The progression of this phenotype from the testis apex to the basal end suggests a stem cell origin. This testis phenotype had not been described before. However, the somatic cells bear a striking

resemblance to the arrangement of somatic follicle cells within the *Drosophila* ovary, which form a columnar monolayer surrounding developing germ cells (Mahowald and Kambyzellis, 1980) (Figures 1B, H, N, S). Therefore, we refer to these somatic cells in the mutant testes as “follicle-like cells”. We also find that germ cells in 7–9 day old mutant testes are arrested as early male germ cells (spermatogonia) based on their morphology, branching fusomes (de Cuevas et al., 1997; Hime et al., 1996; Lin and Spradling, 1994) (open arrowheads, Figure 1R), expression of the male-specific early germ cell marker M5-4 (Tran et al., 2000) (Figures S1A–C) and the undifferentiated germ cell marker Bam (data not shown) (McKearin and Spradling, 1990). By 2–3 weeks, degeneration of differentiation-arrested germ cells is observed (Figure S1D). Since germ cell arrest occurs when the germline and somatic sex are mismatched (Whitworth et al., 2012), this phenotype is consistent with a defect in somatic sexual identity maintenance in the adult testis.

Sex maintenance has not been characterized previously in the adult *Drosophila* testis or ovary, but was recently found to occur in the adult mammalian testis and ovary (Matson et al., 2011; Uhlenhaut et al., 2009). The underlying mechanisms are of interest, since defects in this process may cause testicular cancer or disorders of sexual development (Hanson and Ambaye, 2011). Therefore, we sought to identify this spontaneous mutant. Our genetic complementation (Table S1, Figure 2A) and rescue experiments (Figures 2B–C, Table S2) indicate that the mutation is a partial loss-of-function allele of the essential BTB-Zinc finger cell fate determinant *chinmo* (Flaherty et al., 2010; Zhu et al., 2006), which we denote *chinmo*^{Sex Transformation}, or *chinmo*ST. Furthermore, *Chinmo* is specifically depleted in the CySC lineage in *chinmo*ST mutant testes (Figures 2D–H, S1E–F). Together, these data support the hypothesis that *Chinmo* promotes male sexual identity in the adult testis soma.

Reduction of *Chinmo* in adult testes causes somatic stem cells and their progeny to acquire female somatic identity

Since testes with reduced *chinmo* acquire somatic cells that morphologically resemble ovarian follicle cells, we directly tested whether these follicle-like cells underwent a male-to-female sexual transformation. Specifically, female-specific gene expression and cell behavior was examined in *chinmo*ST testes. *Castor*, *Cut*, and a *Slbo-GFP* gene reporter are normally expressed in adult ovaries (Chang et al., 2013; Jackson and Blochlinger, 1997) (described below), but not testes (Figures 3A, D, G). *Castor*, the earliest marker in the ovarian follicle stem cell lineage, marks follicle stem cells and their earliest progeny (Figure 3C), while more differentiated follicle cells express high levels of *Cut* and then *Slbo-GFP* (Figures 3F, I). In *chinmo*ST testes, these three markers are sequentially expressed as the phenotype progresses (Figures 3B, E, H). In testes from young (1–3 day) *chinmo*ST males, follicle-like cells have not formed yet (described above). However, the earliest ovarian marker, *Castor*, is detected in most testes (61.7%, n=47). Importantly, *Castor* is always restricted to a subset of somatic cells within the testis at this time-point: CySCs and their earliest daughters (Figure 3B). To ask whether *Castor* expression originates in CySCs, their earliest daughters, or both, we next stained testes from even younger males (newly eclosed virgins). At this time-point, fewer testes contained *Castor*-positive cells (38.8% of testes, n=49), and the number of *Castor*-positive cells ranged from 3 to around 30 (data not shown). *Castor* was detected in at least one CySC in all testes where it was expressed, further

supporting the hypothesis that CySCs, but not their differentiating progeny (cyst cells) which have exited the stem cell niche, are the cells within the testis that undergo sexual transformation. These data further suggest that follicle-like cells arise from sex transformed CySCs. Consistent with this hypothesis, the ovarian somatic cell marker *Cut* becomes strongly expressed in follicle-like cells in 7–9 day old *chinmoST* testes (Figure 3E). In contrast, the earlier marker, *Castor*, becomes depleted from follicle-like cells (described below, Figure 4), a pattern consistent with its absence in ovarian follicle cells (Chang et al., 2013). Finally, the *Slbo-GFP* reporter, which normally becomes highly expressed in follicle cells at stage 10 of oogenesis (Cai et al., 2014) (Figure 3I), is also expressed in a subset of follicle-like cells in older *chinmoST* testes (Figure 3H). Together these data suggest that follicle-like cells can progress through the normal follicle cell differentiation program to a surprising extent, in an otherwise male gonad. Consistent with this hypothesis, *Yolk protein1* (*Yp1*) transcripts, which become highly expressed in follicle cells at stage 10 but are not transcribed in the testis (Brennan et al., 1982; Logan et al., 1989), are present in testes with reduced *chinmo* (Figure 3J).

In addition to distinct morphological and molecular characteristics, somatic cells in the testis and ovary differ in potency and proliferation status. CySCs are unipotent, giving rise to cyst cells that exit the cell cycle (Gönczy and Dinardo, 1996). In contrast, follicle stem cells are multipotent, yielding both stalk cells and mitotically active follicle cells (Margolis and Spradling, 1995). By examining markers of cell proliferation in *chinmoST* testes, we find that follicle-like cells express mitotic markers (Figures 3K–O). Furthermore, somatic cells with the molecular and morphological characteristics of stalk cells become apparent in older *chinmoST* testes (Figure 4), suggesting that reduction of *Chinmo* causes CySCs to transdifferentiate into cells resembling multipotent female follicle stem cells. Together, these data support the hypothesis that *chinmo* actively maintains a male sexual identity in adult CySCs.

Chinmo autonomously maintains male sexual identity in adult somatic stem cells

The above data indicate that CySCs specifically require *Chinmo* for maintenance of male sexual identity. However, this requirement could be direct or indirect. To distinguish between these possibilities, we used cell-type specific RNA-interference (RNAi) knockdown of *chinmo*. Consistent with previous mosaic analysis (Flaherty et al., 2010), knockdown of *chinmo* in the germ line during development does not yield a testis phenotype (Figure 5F), ruling out a germline requirement for *chinmo*. We next allowed testes to develop normally, and then conditionally induced *chinmo* RNAi in subsets of adult somatic cells. Knockdown of *chinmo* in adult hub cells does not yield testes with the *chinmoST* phenotype (Figure 5G). In contrast, the *chinmoST* phenotype is fully recapitulated by knockdown of *chinmo* in all adult CySCs and early cyst cells (Figures 5A–E, S2A–D, Table S3a and b). To determine whether *chinmo* is required primarily in CySCs or in their cyst cell progeny, we compared the phenotypes that developed when various CySC lineage drivers were used to induce *chinmo* RNAi. Drivers expressed in CySCs and their immediate cyst cell daughters are sufficient to recapitulate the *chinmoST* phenotype, while drivers expressed solely in later cyst cells are not (Figures 5H, S2E–I, Table S3a). Together these data indicate

that *chinmo* is required in CySCs, but not in their differentiated progeny, for the maintenance of adult somatic sexual identity.

Follicle-like cells arise from the adult somatic stem cell lineage, not hub cells

The fact that CySCs autonomously require *chinmo* for maintenance of adult sexual identity suggests that follicle-like cells arise directly from CySC lineage cells. However, it is also possible that *chinmo* is required in CySCs to prevent hub cells, the only other somatic cells in the testis niche, from giving rise to follicle-like cells. To distinguish between these possibilities, we used genetic lineage tracing to permanently mark hub cells or CySCs and early cyst cells in *chinmoST* males during development, and then assayed for the presence of permanently marked cells in adult testes. Permanently marked hub cells do not produce marked follicle-like cells in *chinmoST* testes (Figures 6A–B, Table S4), ruling out hub cells as a source of follicle-like cells. In contrast, testes with permanently marked CySCs and early cyst cells acquire marked follicle-like cells over time (Figures 6C–E). Since follicle-like cells do not express the CySC and early cyst cell driver used in this experiment (Figures S2J–L), the marked follicle-like cells must be descendants of CySCs and early cyst cells. We conclude that CySCs and early cyst cells, but not hub cells, give rise to follicle-like cells when *chinmo* is reduced. Consistent with this finding, hub cells within *chinmoST* testes remained quiescent when assayed for proliferation using extended in-vivo BrdU labeling (Figures 6F–H). Since all CySC drivers are also expressed in early cyst cells, we cannot exclusively mark CySCs via lineage tracing. However, since CySCs but not cyst cells require *chinmo*, and CySCs are the earliest cells to express ovarian somatic markers, our data strongly support the hypothesis that somatic stem cells, rather than differentiated cells, undergo sex transformation when levels of Chinmo are reduced.

Chinmo maintains male sexual identity through the canonical sex determination pathway

Sex determination in both mammals and *Drosophila* converges on the transcription factor Dsx/Dmrt1. Our finding that *chinmo* promotes adult somatic sex maintenance prompted us to ask whether Chinmo works in concert with the canonical sex determination pathway, in which a splicing cascade mediated by Sex-lethal (*Sxl*) and Transformer (*Tra*) promotes the formation of either a female or male isoform of Dsx (*Dsx^F* or *Dsx^M*, respectively) (Whitworth et al., 2012). Although female forms of *Sxl*, *tra* and *dsx* mRNA were not detected in *chinmoST* testes (Figure S3A), changes in *Dsx^M* protein expression were observed. *Dsx^M* is absent from the ovary (Figure S3B) but present in the somatic cells of the testis including hub cells, CySCs and cyst cells (Hempel and Oliver, 2007) (Figure 7A). Testes from young *chinmoST* males closely resemble wild type testes, but a few CySCs and their immediate progeny lack *Dsx^M*, while older cyst cells retain this male determinant. As somatic cell aggregates and follicle-like cells arise, they all lack *Dsx^M*, and the remaining older *Dsx^M*-positive somatic cells become displaced from the testis apex (Figures 7B–D). Similar results were obtained in testes with *chinmo* RNAi knockdown in the CySC lineage (data not shown). Although *Dsx^M* protein is decreased in the CySC lineage in *chinmoST* testes, a decrease in *dsx^M* mRNA is not detectable at the whole testis level (Figure S3A), suggesting that a decrease in *dsx^M* mRNA in CySCs and cyst cells might be masked by the presence of *dsx^M* mRNA from other cells in the testis, such as sheath, basal epithelial, and seminal vesicle cells. Therefore, we used *dsx-Gal4* to detect changes in *dsx* transcription

levels specifically in CySC lineage cells (Robinett et al., 2010). Two independent *dsx-Gal4* lines are active in CySC lineage cells in control testes (Figure 7E, G) but not in follicle cells in normal ovaries (Figure S3C–D). In *chinmoST* testes, however, somatic aggregates and follicle-like cells no longer express these transgenic reporters (Figure 7F, H). These results suggest that Chinmo either directly or indirectly regulates *dsx^M* transcription levels. Since Dsx^M directly represses *Yp1* expression (Burtis et al., 1991; Coschigano and Wensink, 1993), the appearance of ectopic *Yp1* transcripts in *chinmoST* testes likely reflects loss of Dsx^M (Figure 3J). Taken together, these results suggest that Chinmo is required for maintenance of *dsx^M* expression in the CySC lineage, and that loss of Dsx^M contributes to the male-to-female somatic sex transformation in *chinmoST* testes. In support of this hypothesis, expression of Dsx^M in the CySC lineage in *chinmo-RNAi* testes partially rescues the phenotype (Table 1). We have also found, using two independent *dsx-RNAi* lines, that knockdown of *dsx* in the CySC lineage partially phenocopies the *chinmo* mutant phenotype. In most *dsx-RNAi* testes, germ cells overproliferate and arrest at early spermatogonial stages (Figure 7J, Figures S3I–J) as do germ cells in *chinmoST* testes (Figure 1R), consistent with a mismatch of germline and somatic sex. Some testes also contain small aggregates of somatic cells (Figure S3J, arrows) that resemble the follicle-like cells in *chinmo* mutant testes, but they never develop a full layer of follicle-like cells. Together, these results suggest that Chinmo has targets in addition to Dsx^M that maintain other aspects of CySC fate and prevent them from transforming into follicle stem cell-like cells. We conclude that male sexual identity is actively maintained in the CySC lineage of the adult testis, and that this requires the concerted action of Chinmo and Dsx^M.

Discussion

The male-to-female sex transformation phenotype that we observe in *Drosophila* testes with reduced Chinmo demonstrates that adult somatic stem cells actively maintain their sexual identity in a cell autonomous manner (Figure 7K). In vertebrates, sexual phenotypes were thought to be determined exclusively by systemic hormones, but recent studies have found exceptions to this rule. In birds, for example, somatic sexual identity may be largely cell autonomous, as illustrated by lateral gynandromorph chickens; these male:female chimeras have one side that appears male and the other female, even though both sides are exposed to the same blood system and hormone environment (Zhao et al., 2010). Another example is the adult mouse gonad, where loss of sex-specific transcriptional regulators can cause differentiated somatic cells to transdifferentiate into cells of the opposite sex (Matson et al., 2011; Uhlenhaut et al., 2009). Other recent work has found that adult stem cells can respond differently to the different levels of hormones found in male and female mice: hematopoietic stem cells, which were thought to function similarly in both sexes, respond to the higher levels of estrogen in females, resulting in differences in self-renewal ability (Nakada et al., 2014). We now show, using the *Drosophila* testis as a model, that adult somatic stem cells can also autonomously maintain their sexual identity. Chinmo regulates sexual identity by promoting expression of the male sex determination factor Doublesex^M (Dsx^M) in CySCs and their progeny. Since *dsx*-family members play central roles in the maintenance of somatic sexual identity in diverse organisms including mice, fish and amphibians (Masuyama and Matsuda, 2012; Matson et al., 2011; Shibata et al., 2002), but their upstream

regulators are not well understood, it will be interesting to determine whether *Chinmo* is required for sex maintenance at other times in development and in other tissues in *Drosophila*, and whether *Chinmo* homologues function more broadly in sex maintenance in other organisms.

Our data suggest that *Chinmo* is the central regulator of CySC sex maintenance and implicate *Dsx^M* is an important target of *Chinmo* in this process. However, since expression of *Dsx^M* only partially rescues the sex maintenance phenotype in *chinmo* mutant testes, and *dsx* knockdown only partially phenocopies the *chinmo* mutant phenotype, *Dsx^M* is unlikely to be the only target of *Chinmo*. Together, our data support a model in which *Chinmo* promotes expression of *Dsx^M*, which in turn regulates its own set of target genes that are critical for male sex identity. In addition to promoting *Dsx^M* expression, *Chinmo* likely affects the expression of *Dsx*-independent target genes that are important for additional aspects of CySC identity. In this case, resupplying *Dsx^M* does not fully rescue the *chinmo*-RNAi phenotype because these testes still lack *Chinmo* targets that are independent of *Dsx* but required for CySC self-renewal. Similarly, CySCs lacking *Dsx* may only partially recapitulate the *Chinmo* loss-of-function phenotype because they lack expression of male determinants downstream of *Dsx*. However, expression of *Dsx*-independent *Chinmo* targets may permit maintenance of CySCs lacking *Dsx*, and may also prevent the complete conversion of these mutant CySCs into Follicle Stem or progenitor-like cells. It is also possible that additional sex determination factors, such as the female determinant *Dsx^F*, will be required for a full conversion of CySCs lacking *Dsx^M* into follicle stem or progenitor-like cells. Assessing the role of this and other sex determination pathway members in sex maintenance will be of interest. Furthermore, since our data suggest that *Chinmo* regulates *Dsx^M* at the level of transcription, comparison of targets of *Chinmo* and *Dsx^M* in the CySC lineage should be informative.

It is intriguing that removing *chinmo* clonally in a few CySCs leads to rapid loss of the mutant CySCs (Flaherty et al., 2010), while depleting *chinmo* from all CySCs (via RNAi or the *chinmoST* mutation) allows them to remain but become feminized. We hypothesize that CySC clones lacking *chinmo* (either weak or strong alleles) are out-competed by wild type CySCs, and that the full sex transformation phenotype only develops when the possibility of competition is removed. (i.e., when most or all CySCs lack *chinmo*). Individual CySCs lacking *Chinmo* may also undergo sex transdifferentiation, but we have not been able to determine whether or not this is the case, since such CySCs are lost quite rapidly. Comparing testes that contain either a few or many CySCs that lack *chinmo* could be informative, as it should reveal the number of stem cells that must be mutant in order to allow the sex transformation of the testis soma to occur. The ability of stem cells to compete for niche access is a poorly understood yet important aspect of adult stem cell biology in general (Stine and Matunis, 2013), and this work provides an avenue for studying the underlying mechanisms.

Our finding that stem cells undergo sexual transformation may provide unique insight into how transdifferentiation is regulated at a cellular and molecular level more generally. Sex transformation of stem cells in adult *Drosophila* testes provides a highly tractable genetic system to study cellular mechanisms by which highly conserved *dsx*-related transcription

factors and their target genes actively maintain somatic sexual identity. This may provide insight into human testicular cancers, such as granulosa cell tumors, that may be linked to altered somatic sexual identity (Hanson and Ambaye, 2011). Analysis of *Chinmo* and *Dsx*/*Dmrt1*-mediated sex maintenance pathways in somatic stem cells may also yield insight into the maintenance of sexual dimorphism in other organs, such as the mammalian liver, and provides a unique model to study adult stem cell transdifferentiation *in vivo*.

Experimental procedures

Fly stocks and cultures

Fly stocks were raised at 25 °C on standard molasses/yeast medium unless otherwise indicated. The following fly stocks were used: *UAS-FL-chinmo* and *chinmo*¹ (Zhu et al., 2006), *eyaA3-Gal4* (Leatherman and DiNardo, 2008), *M5-4* (Gönczy and DiNardo, 1996), *c587-Gal4* (Kai and Spradling, 2003), *nanos-Gal4-VP16* (Van Doren et al., 1998), *tj-Gal4* (Drosophila Genetic Resource Center), *UAS-dsx^M* (Lee et al., 2002), and *UAS-lifactin-GFP* and *slbo-lifactin-GFP* (from X. Wang and D. Montell), *UAS-dsx RNAi-1* (*P[KK111266]VIE-260B*, From Vienna Drosophila RNAi Center (VDRC)), *UAS-dsx RNAi-2* (from T. Shirangi and M. Mckeown), *dsx-Gal4-1* (*w*¹¹¹⁸; *P[GMR40A05-Gal4]attP2*, From Bloomington Drosophila Stock Center (BDSC)), *dsx-Gal4-2* (*w*⁻; *dsx-Gal4/TM3, Sb, Ser, twist-gal4, UAS-2xEGFP*, from B. Baker). *y w* and *chinmo*ST/*CyO* flies were used as control flies. Other fly stocks were from the VDRC or BDSC including the *SOD[x39]/TM3* line from which *chinmo*ST was isolated.

Immunostaining

Testes and ovaries were dissected, fixed, and stained as described previously (Matunis et al., 1997). Tyramide signal amplification (Invitrogen) was used to increase sensitivity of rat anti-*Dsx*^M (from B. Oliver, 1:500 dilution). Antibodies used are described in Supplemental Experimental Procedures.

Lineage analysis

For lineage analysis, *chinmo*ST; *actin>stop>lacZ* or *chinmo*^{KG05386}; *actin>stop>lacZ* males were mated to *E132-Gal4; chinmo*ST; *UAS-FLP* or *c587-Gal4; chinmo*ST; *UAS-FLP* females to permanently mark hub cells or the CySC lineage, respectively. Newly eclosed flies were then aged prior to testis dissection and immunostaining. To determine whether the *c587-Gal4* and *E132-Gal4* drivers become re-expressed in follicle-like cells in aged *chinmo*ST testes, *chinmo*ST; *UAS-lifactin-GFP* males were crossed to *E132-Gal4; chinmo*ST; *tub-Gal80[ts]* or *c587-Gal4; chinmo*ST; *tub-Gal80[ts]* females at 18 °C. Newly eclosed progeny were aged at 18 °C for 1 week to allow development of the follicle-like cell phenotype, and then switched to 29 °C to assess Gal4 activity in *chinmo*ST testes.

mRNA extraction and PCR

mRNA extraction and reverse transcription-PCR were performed as previously described (Issigonis and Matunis, 2012). Primer sequences are in Supplemental Experimental Procedures.

In vivo BrdU incorporation

Groups of about 20 age-matched adult males were incubated at 25 °C in empty vials for 4 hours, and then transferred to vials of fly food overlaid with disks of filter paper soaked with 120 µl of 2.5 mM 5-bromo-2'-deoxyuridine (BrdU) (Life Technologies) and 5 µl of green food coloring (McCormick). After 24 hours, flies that had not eaten the BrdU (and therefore did not have green guts) were discarded, and BrdU was detected in testes from the remaining flies as described (Brawley and Matunis, 2004).

Quantification of cell division and severity of the *chinmo*ST phenotype

CySC mitoses were quantified by counting the number of PH3⁺ Vasa⁻ cells within two cell diameters of the hub. Cyst cell mitoses were quantified by counting the number of PH3⁺ Vasa⁻ cells more than two cell diameters from the hub. To categorize the severity of the *chinmo*ST phenotype, we used the following criteria. Morphologically wild-type testes were scored as normal. Testes with epithelial aggregates and/or follicle-like cells at the apex near the hub, but not away from the apex, were scored as having a mild phenotype; these testes often contained overproliferating germ cells. Testes with epithelial aggregates and/or follicle-like cells throughout the entire testis were scored as having a severe phenotype; germ cells in these testes were often arresting at early spermatogonial stage or degenerating. The severity of the *chinmo*ST and *chinmo* RNAi phenotypes both progress with age. Therefore, the presence of a mild vs. severe phenotype was used to estimate testis age when not clearly indicated.

Microscopy and Image Analysis

Fixed testes were mounted in Vectashield (Vector Labs), imaged with a Zeiss LSM 5 Pascal or LSM 510 Meta, and analyzed using the Zeiss LSM Image Browser software; panels are single confocal sections unless stated otherwise. Statistical analysis of pH3 positive cell number was performed with GraphPad Prism 5 software and averages were compared using a two-tailed Student's t test assuming unequal variances. Percentages were analyzed for statistical significance using a chi-squared test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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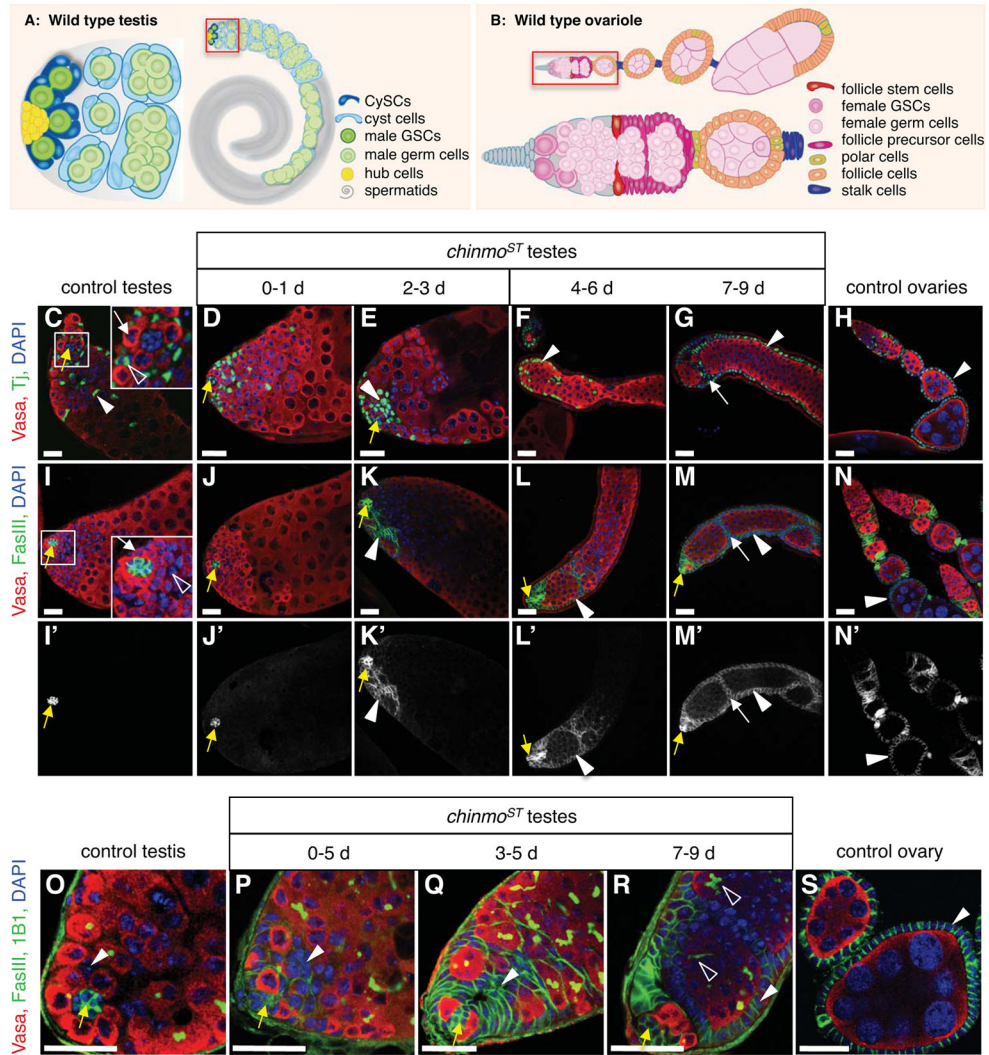


Figure 1. Reduction of Chinmo causes somatic cells in adult testes to be gradually replaced by cells resembling ovarian follicle cells
(A) Illustration of a wild-type *Drosophila* testis (right) with the apex magnified (left). Germline stem cells (GSCs, dark green) and somatic cyst stem cells (CySCs, dark blue) adhere to the hub (yellow). GSCs produce differentiating male germ cells (spermatogonia and spermatocytes, green) that are displaced from the hub and form elongated spermatids (grey) and mature sperm (not shown). Approximately two CySCs flank each GSC; CySCs produce squamous, quiescent cyst cells (light blue), which encase differentiating germ cells.
(B) Illustration of a wild-type *Drosophila* ovariole (top) comprised of a germarium (magnified, bottom) followed by a series of developing egg chambers. In the germarium, anterior niche cells cap cells (grey) support GSCs (dark pink), which produce differentiating female germ cells (light pink). Two somatic follicle stem cells (red), located near the middle of the germarium, produce follicle precursor cells (magenta), which differentiate into follicle cells (orange), stalk cells (purple), and polar cells (yellow). Each egg chamber contains 16 germ cells surrounded by a monolayer of columnar epithelial follicle cells. Polar cells are located at each end; egg chambers are linked by chains of stalk cells. **(C–S)**

Immunofluorescence detection in adult testes and ovaries of Tj (C–H, green) to visualize somatic cell nuclei, or FasIII (I–S, green) to highlight the hub in all testes (yellow arrows), and somatic cell membranes in ovaries and *chinmo* mutant testes (arrowheads). Panels I'–N' show the FasIII signal alone. Vasa (red) marks germ cells and DAPI (blue) marks nuclei in all panels. In control testes (C, I, O), somatic CySC lineage cells (arrowheads) are squamous and interspersed among germ cells. Insets (C, I) show GSCs (white arrows) and CySCs (open arrowheads) surrounding the hub. In *chinmo*ST testes (D–G, J–M, P–R), a distinct phenotype develops over time. Testes from young mutant males (D–E, J–K, P–Q) resemble those from controls except that most (~77%, n = 61) contain aggregates of 8 or more somatic cells (arrowheads); these always appear near the hub (yellow arrows). As flies age (F–G, L–M, R), aggregates expand beyond the testis apex and become columnar and peripheral (arrowheads) in 82% of testes (n = 545), forming FasIII-positive “follicle-like cells” that resemble somatic follicle cells (arrowheads) in control ovaries (H, N, S). Follicle-like cells occasionally invaginate (G, M, white arrows) to envelop groups of germ cells. 1B1 (O–R, green) marks fusomes; branching fusomes in older germ cells in *chinmo*ST testes indicate spermatogonial arrest (R, open arrowheads). Scale bars = 20 μm. See also Figure S1.

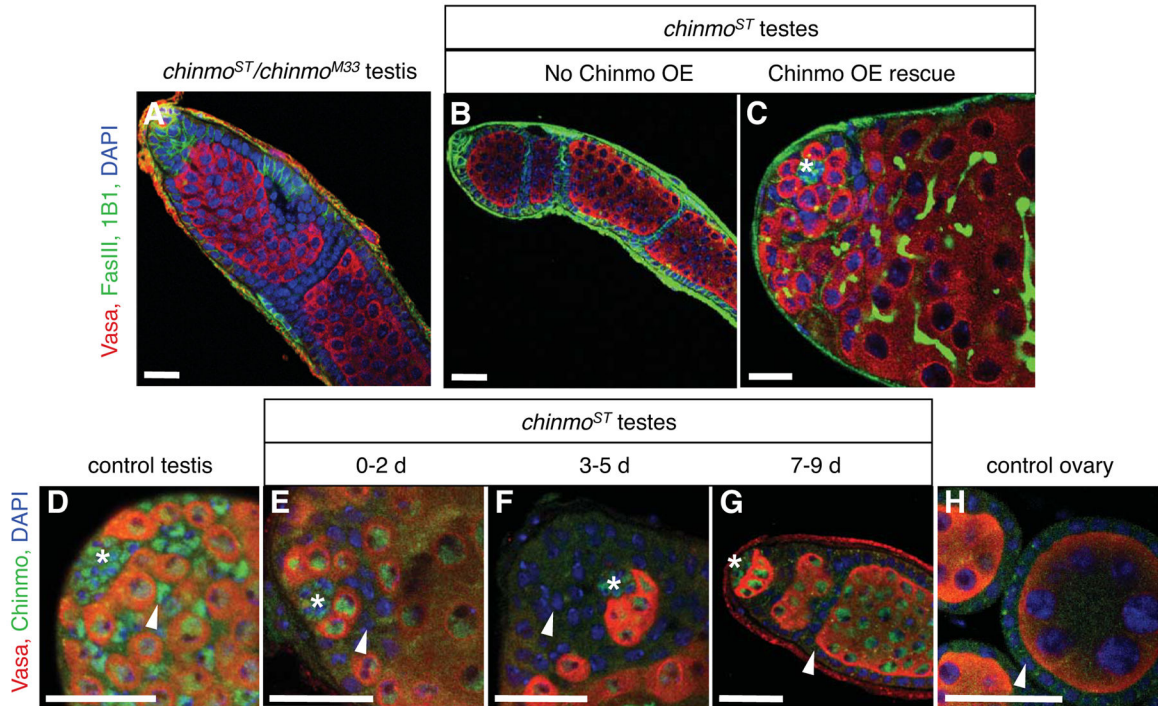


Figure 2. Genetic complementation and rescue experiments indicate that *chinmoST* is a partial loss-of-function allele of *chinmo*

(A) Immunofluorescence detection of FasIII (green at cell periphery) to visualize follicle-like cells in testes, and Vasa (red) to visualize germ cells. Testes from *chinmoST chinmo^{M33}* males resemble *chinmoST* homozygous testes (compare to Figure 1R). (B–C) The follicle-like cell phenotype in *chinmoST* testes (B) can be rescued by overexpressing *chinmo* in the CySC lineage (*c587-Gal4; chinmoST; UAS-chinmo/tub-Gal80^{ts}*) (C). The position of the hub is marked with an asterisk. (D–H) Immunofluorescence detection of Chinmo (green) in testes and ovaries. In control testes (D), Chinmo is found in the nuclei of hub cells (asterisk), CySC lineage cells (arrowhead), and germ cells (Vasa, red) (Flaherty et al., 2010). In both young and old *chinmoST* testes (E–G), Chinmo is present in hub cells and germ cells but is absent from CySC lineage cells (arrowhead). Chinmo is absent from follicle cells (arrowhead) in control ovaries (H). DAPI marks nuclei (blue). Hubs marked by asterisk. Scale bars = 20 μ m. See also Figure S1, Table S1, Table S2.

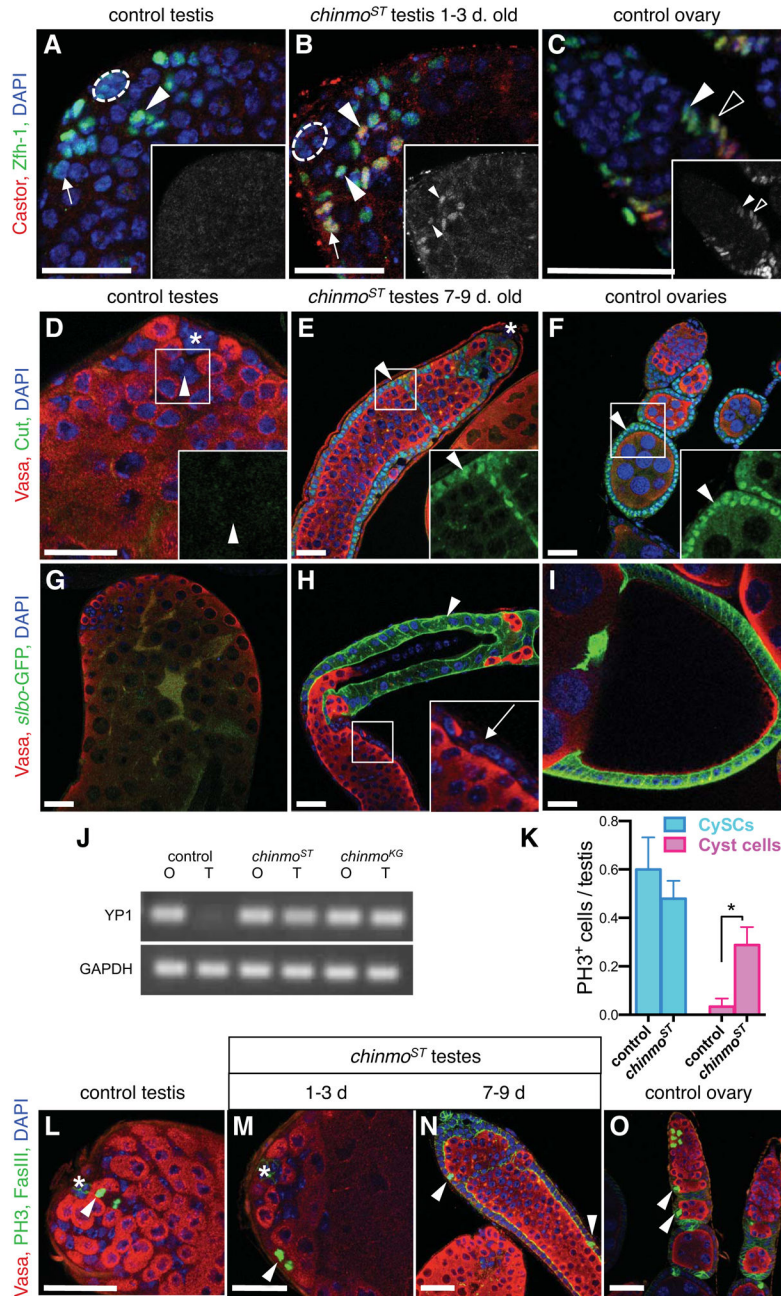


Figure 3. Reduction of Chinmo in adult testes causes somatic stem cells and their progeny to acquire female somatic markers
 (A–C) Immunofluorescence detection of Zfh-1 (green) and Castor (red) in testes and ovaries. Insets, red channel only shown in grayscale. In control testes (A), Zfh-1 marks CySCs (arrowhead) and early cyst cells (arrow), but Castor is not detected. In testes from young (1–3 day) *chinmoST* males (B), some CySCs (arrowheads) and cyst cells (arrow) express both Zfh-1 and Castor, as do ovarian follicle stem cells (solid arrowhead) and their early progeny (follicle cell progenitors, open arrowhead) in control ovaries (C). (D–F) Immunofluorescence detection of Cut (green) and Vasa (red). Insets, green channel only,

magnified. In control testes (D), Cut is absent from all cells including CySCs (arrowhead), but it is present in follicle-like cells in *chinmoST* testes (E, arrowhead) and follicle cells in control ovaries (F, arrowhead). (G–I) Immunofluorescence detection of *slbo*-GFP (green). *slbo*-GFP is not expressed in control testes (G) but marks later somatic follicle cells (around stage 10) in control ovaries (I). In *chinmoST* testes (H), *slbo*-GFP marks a subset of follicle-like cells (arrowhead); adjacent follicle-like cells (inset, arrow) are not marked. (J) *Yolk protein 1 (YPI)* expression is detected by RT-PCR in ovaries (O) and testes (T) from 7–9 day *chinmoST* and *chinmo^{KG}* adults but not from control adults. GAPDH is used as a control. (K) Bar graph showing the number of CySCs and cyst cells containing the mitotic marker phospho-histone H3 (PH3) in testes from 1–3 day old control or *chinmoST* flies. Somatic cells within 2 cell diameters of the hub were scored as CySCs; those more than 2 cell diameters away were scored as cyst cells. Significantly more mitotic cyst cells are found in *chinmoST* testes than in control testes (*, $p=0.0168$). There is no significant difference in the number of mitotic CySCs between *chinmoST* and control testes. Data represented as mean \pm standard error of the mean. (L–O) Immunofluorescence detection of PH3 (green in nuclei). FasIII (green) marks cell membranes in the hub (asterisk, L–M), follicle-like cells (N), and follicle cells (O). In control testes (L), PH3 is detected in CySCs (arrowhead); cyst cells away from the hub are non-mitotic and do not express PH3. Cyst cells in young *chinmoST* testes (M) and follicle-like cells in older *chinmoST* testes (N) express PH3 (arrowheads), as do follicle cells (arrowheads) in control ovaries (O). Hubs marked by dashed line (A–B) or asterisk (D–E, L–M). DAPI marks nuclei (blue, all panels). Scale bars = 20 μ m.

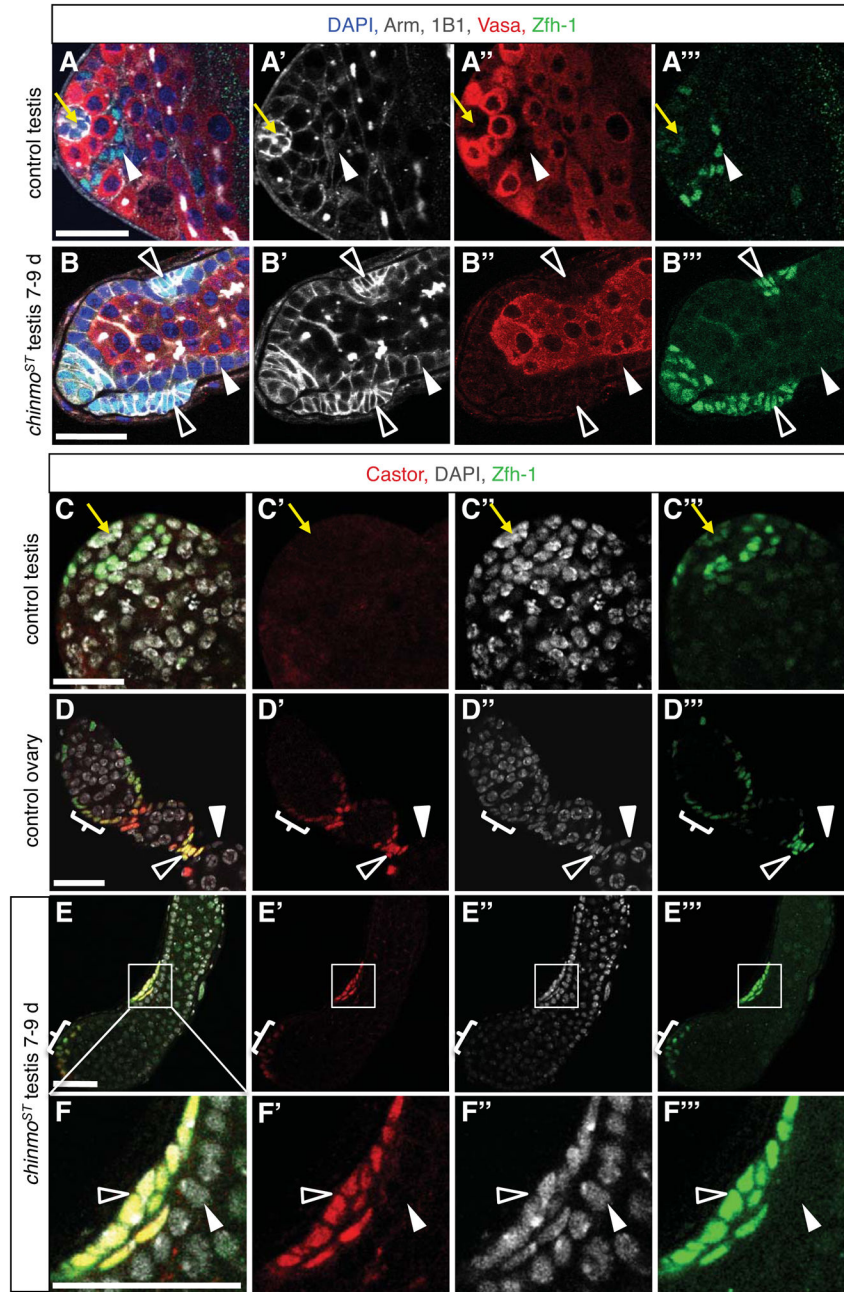


Figure 4. Ovarian stalk cell markers are expressed in a subset of somatic cells in *chinmo*ST testes (A–B) Immunofluorescence detection of Arm (white) and Zfh-1 (green) reveals stalk-like cells in *chinmo*ST testes. In control testes (A), hub cells (yellow arrow) express high levels of Arm and low levels of Zfh-1, and CySCs and their immediate daughters (arrowhead) express low levels of Arm and high levels of Zfh-1. In all other cyst cells, Arm is low and Zfh-1 is absent. In *chinmo*ST testes (B), Arm is low and Zfh-1 is absent in follicle-like cells lining the periphery (solid arrowhead). Other somatic cells (open arrowheads) form aggregates that resemble ovarian stalk cells morphologically and express high levels of Arm and Zfh-1, which are characteristic of stalk cells (data not shown). These aggregates are

typically located just beneath the testis sheath, sandwiched between follicle-like cells and the basement membrane. (C–F) Immunofluorescence detection of Castor (red) and Zfh-1 (green) reveals stalk-like cells in *chinmo*ST testes. Castor is absent from control testes (C; also see Fig. 2A). Hubs marked by yellow arrow. In control ovaries (D), Castor is expressed in follicle stem cells and early follicle cell progenitors (bracket; also see Fig. 2C). After egg chamber formation, Castor is restricted to polar cells and Zfh-1⁺ stalk cells (open arrowhead) and is no longer expressed in main-body follicle cells (solid arrowhead). In *chinmo*ST testes (E–F), Castor is expressed in Zfh-1⁺ cell aggregates at the testis apex that resemble follicle cell progenitors (bracket) and in stalk-like cells (open arrowheads) but not in Zfh-1⁻ follicle-like cells (solid arrowhead). Panel F is an enlargement of the boxed area in panel E. Scale bars = 20 μm.

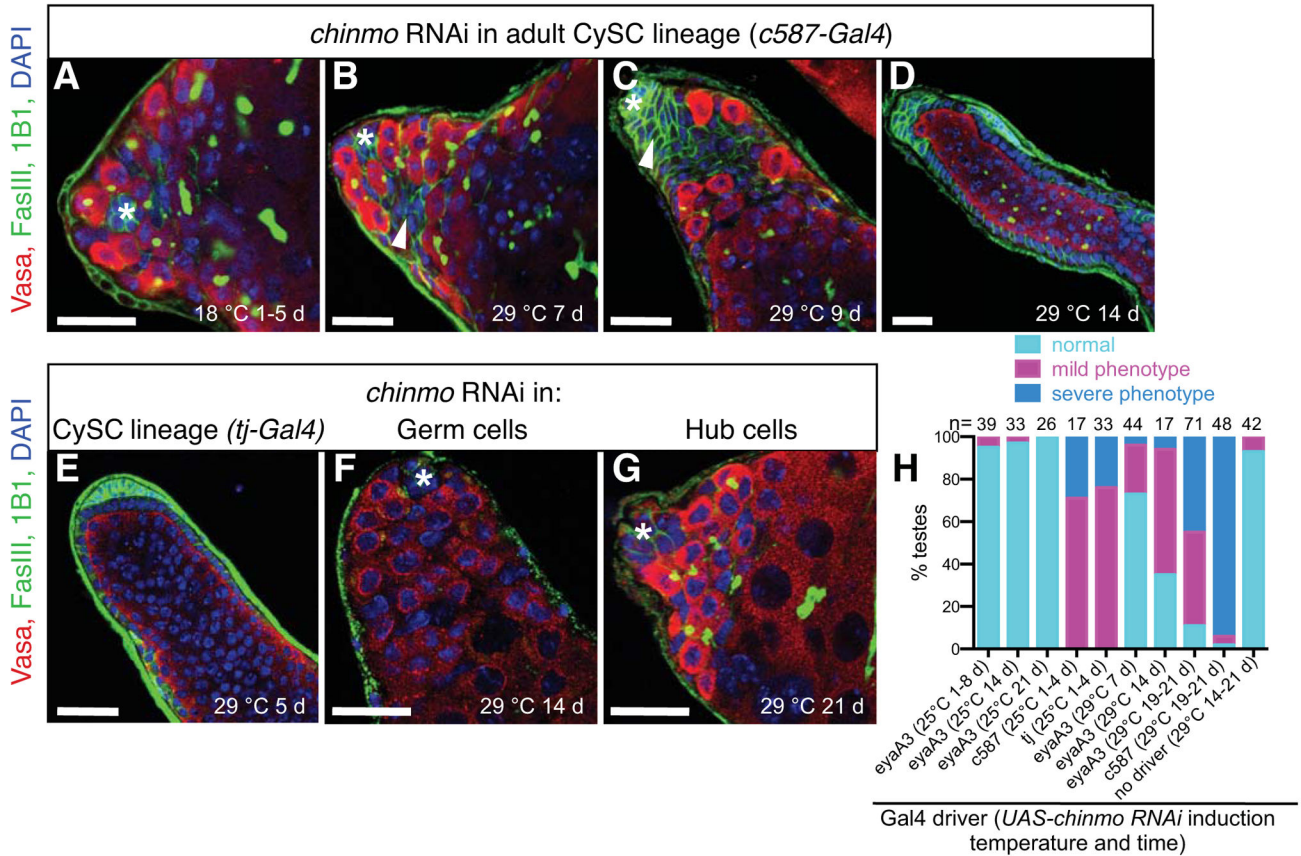


Figure 5. Chinmo is required autonomously in adult CySC lineage cells to prevent their transformation into female soma

(A–G) Immunofluorescence detection of FasIII (green at cell periphery) and the germ cell marker Vasa (red) to visualize the morphology of adult testes after expression of *chinmo*-RNAi in different cell types. Before RNAi induction in the CySC lineage (A), testes appear normal. After RNAi induction in the CySC lineage, testes resemble *chinmo*ST testes: somatic cells initially form aggregates (97% of testes, n = 36/37) (B–C, arrowheads) and then follicle-like cells (76% of testes, n = 68/90) (D). RNAi induction in the CySC lineage with a different Gal4 driver also phenocopies *chinmo*ST testes (E). RNAi induction in germ cells (F) or in hub cells (G) does not phenocopy *chinmo*ST. Hubs marked by asterisk. Scale bars = 20 μm. (H) Composite bar graph showing the percentage of testes with normal, mild, or severe phenotypes after expression of *chinmo*-RNAi with different somatic drivers. Testes remain morphologically wild type after *chinmo*-RNAi expression only in cyst cells (*eyaA3-Gal4* at 25 °C), but after expression in both CySCs and cyst cells (*eyaA3-Gal4* at 29 °C, *c587-Gal4*, or *tj-Gal4*), testes display a range of *chinmo*ST phenotypes, as defined in Methods. See also Figure S2, Table S3.

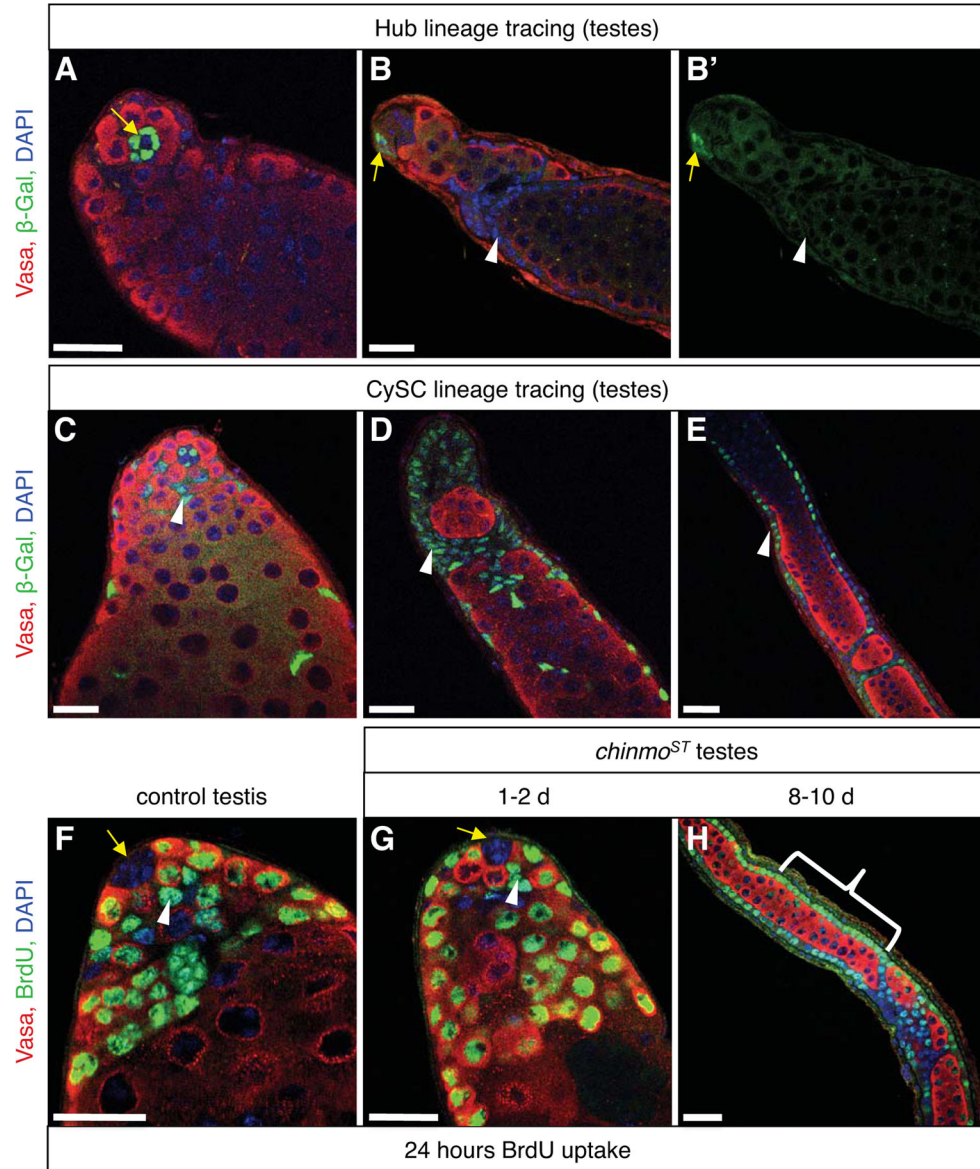


Figure 6. Follicle-like cells come from the cyst stem cell lineage but not from hub cells (A–E) Immunofluorescence detection of β -gal (green), which permanently marks either hub cells alone (A–B) or CySC lineage and hub cells (C–E) in *chinmoST* testes. Somatic cell aggregates and follicle-like cells (B, D–E, arrowheads) are derived from CySC lineage cells (C, arrowhead) but not from hub cells (A–B, arrows) because they express β -gal in testes with marked CySC lineage cells but not in testes with only marked hubs. (F–H) Immunofluorescence detection of the thymidine analog bromodeoxyuridine (BrdU, green). Adult males were fed BrdU for 24 hr prior to dissection to label all cells that traversed S-phase during this time. In control (F) and young *chinmoST* (G) testes, BrdU is not found in any hub cells (arrows), but many germ cells (red) and CySCs (arrowheads) are BrdU⁺. BrdU is also found in most follicle-like cells in older *chinmoST* testes (H, bracket). In all panels,

DAPI marks nuclei (blue) and Vasa marks germ cells (red). Scale bars = 20 μm . See also Table S4.

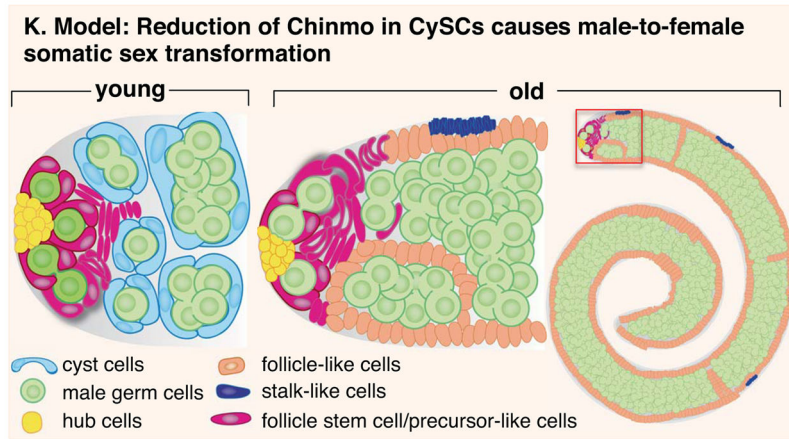
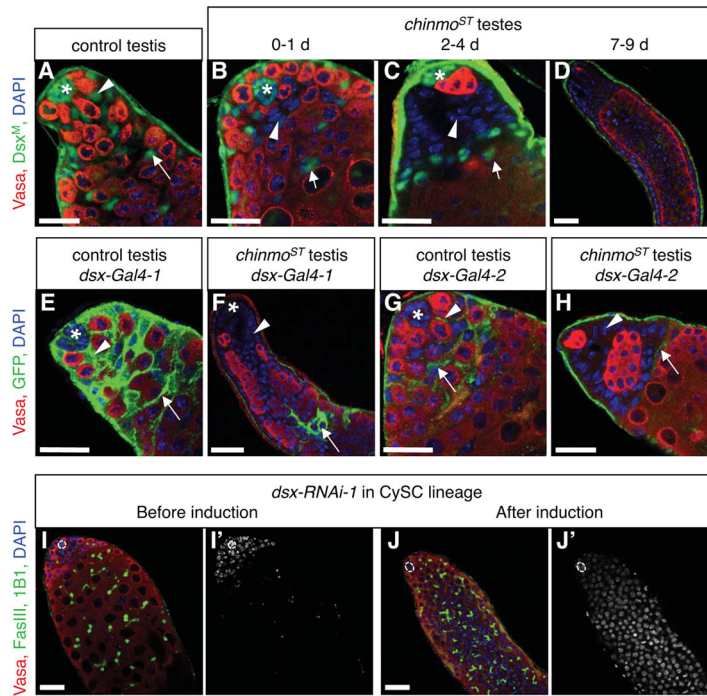


Figure 7. Chinmo maintains the male identity of adult somatic stem cells through the canonical sex determination pathway

(A–D) Immunofluorescence detection of Dsx^M (green) in adult testes. In control testes (A), Dsx^M is expressed in the nuclei of hub cells (asterisk), CySCs (arrowhead), and cyst cells (arrow) (n=59 testes). In young *chinmo*ST testes that are still morphologically normal (B), Dsx^M levels are low in some CySCs and early cyst cells (arrowhead) but remain high in differentiated cyst cells away from the apex (arrow) (n=35 testes). In *chinmo*ST testes containing somatic cell aggregates (C), Dsx^M levels are low in aggregates (arrowhead) but remain high in differentiated cyst cells (arrow) (n=37 testes). In *chinmo*ST testes with follicle-like cells (D), Dsx^M is absent from all follicle-like cells (n=42 testes). (E–H) Immunofluorescence detection of cytoplasmic GFP (green) in adult testes to reflect the transcription of *dsx-Gal4* (two different lines). *dsx-Gal4-1* is expressed in control testes (E) at high levels in CySCs (arrowhead) and cyst cells (arrow), and at lower levels in some hub cells (asterisk) (n=15 testes). In *chinmo*ST testes (F), *dsx-Gal4-1* expression is absent from

somatic aggregates and follicle-like cells (arrowhead) but remains high in differentiated cyst cells away from the apex (arrow) (n=19 testes). *dsx-Gal4-2* is expressed in control testes (G) in early cyst cells (arrow) and in some CySCs (arrowhead) but is not detectable in hub cells (asterisk) (n=21 testes). In *chinmoST* testes (H), *dsx-Gal4-2* expression is absent from somatic aggregates (arrowhead) but present in some differentiated cyst cells away from the apex (arrow) (n=28 testes). (I–J) Immunofluorescence detection of FasIII and 1B1 (green) and DAPI (blue) in adult testes before and after induction of *dsx-RNAi* in the CySC lineage. Panels I' and J' show the blue channel (DAPI) only in grayscale. Before RNAi induction (I), testes look normal, and cells that stain brightly with DAPI (early germ cells and somatic cells) are restricted to the apex of the testis (n = 32 testes). After RNAi induction (29 °C for 10 days) (J), testes fill with germ cells that arrest as early spermatogonia, based on the expansion of the bright DAPI zone and presence of branching fusomes (94% of testes, n = 51). In all panels, nuclei are marked with DAPI (blue); germ cells are marked with Vasa (red); Hubs marked by asterisk (A–C, E–G) or dashed line (I–J). scale bars = 20 μm. (K) Schematic drawings of a young (left) and older (center) *chinmo* mutant testis apex, and an older *chinmo* mutant testis (right) with the apex indicated (red box). In young *chinmo* mutant testes, CySCs and their early progeny lose male fate and adopt a follicle stem cell/progenitor-like cell identity (magenta). As flies age, follicle stem cell/progenitor-like cells produce follicle-like cells (orange) and stalk-like cells (purple), which gradually displace normal cyst cells from the niche. The germ cells, which become restricted to the lumen of the tissue, maintain characteristics of their male identity, but over-proliferate and arrest as spermatogonia since the germline and somatic sex are mismatched. See also Figure S3.

Table 1

Overexpressing DsxM in the CySC lineage rescues Chimmo knock down phenotype

Use <i>c587-Gal4</i> to express: [1]	% testes with FasIII ⁺ somatic aggregates [2]						
	0 d	4 d	7 d	9 d	11 d	14d	
<i>UAS-chimmoRNAi-1</i>	0 (n=31)	6.3 (n=64)	73.8 (n=103)	80.5 (n=133)	97.1 (n=35)	100 (n=59)	
<i>UAS-chimmoRNAi-1, UAS-DsxM</i>	0 (n=25)	5.3 (n=76)	36.6 (n=153)	55.9 (n=145)	39.4 (n=33)	86.3 (n=124)	
<i>UAS-DsxM</i>	0 (n=18)	N/A	0 (n=24)	5.3 (n=19)	0 (n=21)	N/A	

[1] *UAS-chimmoRNAi-1* = chimmo^{HMO4048}

[2] All flies were raised at 18 °C, which suppresses RNAi induction. After eclosion, adult flies were shifted to 29 °C for the indicated amount of time to induce RNAi

[3] For DsxM rescue, p<0.0001 at 7, 9, 11days, P<0.01 at 14 days