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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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SUMMARY

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 DoublesexM (DsxM) within CySCs and their progeny, and ectopic expression of DsxM in the CySC lineage partially rescues the chinmo sex transformation phenotype, placing Chinmo upstream of DsxM. The Dsx homolog 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. Because adult stem cells have the capacity to rebuild entire adult organ systems, altering the sexual identity of a stem cell could conceivably cause widespread changes to the tissue.

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 Chronologically inappropriate morphogenesis (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 DoublesexM (DsxM) 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 and 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 days old) are indistinguishable from wild-type testes in overall morphology (Figures 1C, 1D, 1I, and 1J). 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...
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(defined as eight or more closely apposed cells expressing high levels of adhesion proteins) appear adjacent to the hub, whereas the remainder of the tissue is unaffected (Figures 1E, 1K, 1P, and 1Q). With time, somatic cell aggregates acquire additional cells and extend away from the testis apex, whereas older differentiating germ cells and cyst cells are displaced toward the basal end of the testis (Figures 1F, 1G, 1L, and 1M). In 7- to 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, whereas germ cells are restricted to the lumen of the tissue (Figures 1G, 1M, and 1R). 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 arrangement of the somatic cells in the mutant testes bears a striking resemblance to that of somatic follicle cells within the wild-type Drosophila ovary: both are arranged in a columnar monolayer that surrounds developing germ cells (Mahowald and Kambysselis, 1980) (Figures 1B, 1H, 1N, and 1S). Therefore, we refer to these somatic cells in the mutant testes as “follicle-like cells.” We also find that germ cells in 7- to 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 et al., 1994) (open arrowheads, Figure 1R), expression of the male-specific early germ cell marker M5-4 (Tran et al., 2000) (Figures S1A–S1C available online), 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). Because 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, because 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 (Figure 2A; Table S1) and rescue experiments (Figures 2B and 2C; 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 as chinmoSex Transformation or chinmoST. We did not find any changes in the coding region (data not shown), suggesting that the mutation is in a regulatory region of the gene. Consistent with this hypothesis, Chinmo is only depleted in the CySC lineage in chinmoST mutant testes (Figures 2D–2H; Figures S1E and S1F). 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

Because 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 sex transformation. Specifically, female-specific gene expression and cell behavior was examined in chinmoST testes. Castor, Cut, and a sibo-GFP gene reporter are normally expressed in adult ovaries (Chang et al., 2013; Jackson and Blochlinger, 1997) (described below), but not in testes (Figures 3A, 3D, and 3G). Castor, the earliest marker in the ovarian follicle stem cell lineage, marks follicle stem cells and their early progeny (Figure 3C), whereas more differentiated follicle cells express high levels of Cut and then sibo-GFP (Figures 3F and 3I). In chinmoST testes, these three markers are sequentially expressed as the phenotype progresses (Figures 3B, 3E, and 3H). In testes from young (1- to 3-day-old) chinmoST 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 sex 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- to 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 older ovarian follicle cells (Chang et al., 2013). Finally, the sibo-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 tests (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–3O). 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
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 (gray) 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; gray) 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.

(legend continued on next page)
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 germline during development does not yield a testis phenotype (Figure 5F), ruling out a germline requirement for chinmo. Next, we 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

![Image](image_url)
knockdown of chinmo in all adult CySCs and early cyst cells (Figures 5A–5E; Figures S2A–S2D; Tables S3A and S3B). 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, whereas drivers expressed solely in later cyst cells are not (Figure 5H; Figures S2E–S2I; 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.

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- to 3-day-old) 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–J) 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 (YP1) expression is detected by RT-PCR in ovaries (O) and testes (T) from 7- to 9-day-old chinmoST and chinmoKG adults, but not in control testes. 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- to 3-day-old chinmoST flies. Somatic cells within two cell diameters of the hub were scored as CySCs; those more than two 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 ± SEM.

(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 nonmitotic 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 and B) or asterisk (D, E, L, and M). DAPI marks nuclei (blue, all panels). Scale bars, 20 μm.
Follicle-like Cells Arise from the Adult Somatic Stem Cell Lineage, Not from 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 chinmo<sup>ST</sup> males during development, and then we assayed for the presence of permanently marked cells in adult testes. Permanently marked hub cells do not produce marked follicle-like cells in chinmo<sup>ST</sup> testes (Figures 6A and 6B; 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–6E). Because follicle-like cells do not express the CySC and early cyst cell driver used in this experiment (Figures S2J–S2L), 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 chinmo<sup>ST</sup> testes remained quiescent when assayed for proliferation using extended in vivo bromodeoxyuridine (BrdU) labeling (Figures 6F–6H). Because all CySC drivers are also expressed in early cyst cells, we cannot exclusively mark CySCs via lineage tracing. However, because 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<sup>x</sup> or Dsx<sup>M</sup>, respectively) (Whitworth et al., 2012). Although female forms of Sxl, tra, and dsx mRNA were not detected in chinmo<sup>ST</sup> testes (Figure S3A), changes in Dsx<sup>M</sup> protein expression were observed. Dsx<sup>M</sup> 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 chinmo<sup>ST</sup> males closely resemble wild-type testes, but a few CySCs and their immediate progeny lack Dsx<sup>M</sup>, whereas older cyst cells retain this male determinant. As somatic cell aggregates and follicle-like cells arise, they all lack Dsx<sup>M</sup>, and the remaining older Dsx<sup>M</sup>-positive somatic cells become displaced from the testis apex (Figures 7B–7D). Similar results were obtained in testes with chinmo RNAi knocked down in the CySC lineage (data not shown). Although Dsx<sup>M</sup> protein is decreased in the CySC lineage in chinmo<sup>ST</sup> testes, a decrease in dsx<sup>M</sup> mRNA is not detectable at the whole-testis level (Figure S3A), suggesting that a decrease in dsx<sup>M</sup> mRNA in CySCs and cyst cells might be masked by the presence of dsx<sup>M</sup> 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 (Figures 7E and 7G), but not in follicle cells in normal ovaries (Figures S3C and S3D). In chinmo<sup>ST</sup> testes, however, somatic aggregates and follicle-like cells no longer express these transgenic reporters (Figures 7F and 7H). These results suggest that Chinmo either directly or indirectly regulates dsx<sup>M</sup> transcription levels. Because Dsx<sup>M</sup> directly represses Yp1 expression (Burtis et al., 1991; Coschigano and Wensink, 1993), the appearance of ectopic Yp1 transcripts in chinmo<sup>ST</sup> testes likely reflects loss of Dsx<sup>M</sup> (Figure 3J). Taken together, these results suggest that Chinmo is required for maintenance of dsx<sup>M</sup> expression in the CySC lineage and that loss of Dsx<sup>M</sup> contributes to the male-to-female somatic sex transformation in chinmo<sup>ST</sup> testes. In support of this hypothesis, expression of Dsx<sup>M</sup> 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 (Figures 7J, S3I, and S3J), as do germ cells in chinmo<sup>ST</sup> 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<sup>M</sup> 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<sup>M</sup>.

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
Chinmo Maintains Sex Identity of Adult Stem Cells

**Figure Legend:**

A - A'' - A''' - A''''

B - B' - B'' - B'''

C - C' - C'' - C'''

D - D' - D'' - D'''

E - E' - E'' - E'''

F - F' - F'' - F'''

**Legend:**
- DAPI, Arm, 1B1, Vasa, Zfh-1
- Castor, DAPI, Zfh-1

**Supplementary Material:**
- Additional figures and analysis supporting the text.
stem cells can also autonomously maintain their sexual identity. Chinmo regulates sexual identity by promoting expression of the male sex determination factor Doublesex\textsuperscript{M} (Dsx\textsuperscript{M}) in CySCs and their progeny. Because dsx-family members play central roles in the maintenance of somatic sexual identity in diverse organisms, including mice, fish, and amphibians (Masuyama et al., 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 homologs 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\textsuperscript{M} as an important target of Chinmo in this process. However, because expression of Dsx\textsuperscript{M} only partially rescues the sex maintenance phenotype in chinmo mutant testes, and dsx knockdown only partially phenocopies the chinmo mutant phenotype, Dsx\textsuperscript{M} is unlikely to be the only target of Chinmo. Together, our data support a model in which Chinmo promotes expression of Dsx\textsuperscript{M}, which in turn...
regulates its own set of target genes that are critical for male sexual identity. In addition to promoting DsxM expression, Chinmo likely affects the expression of Dsx-independent target genes that are important for additional aspects of CySC identity. In this case, resupplying DsxM does not fully rescue the chinmo-RNAi phenotype because these testes still lack Chinmo targets.

Figure 6. Follicle-like Cells Come from the Cyst Stem Cell Lineage, but Not from Hub Cells

(A–E) Immunofluorescence detection of β-galactosidase (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, and E, arrowheads) are derived from CySC lineage cells (C, arrowhead), but not from hub cells (A and 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 DsxM (green) in adult testes. In control testes (A), DsxM is expressed in the nuclei of hub cells (asterisk), CySCs (arrowhead), and cyst cells (arrow) (n = 59 testes). In young chinmoST testes that are still morphologically normal (B), DsxM levels are low in some CySCs and early

(legend continued on next page)
that are independent of Dsx but are required for CySC self-renewal. Similarly, CySCs lacking Dsx may only partially recapitulate the Chinmo loss-of-function phenotype because, although they lack expression of male determinants downstream of Dsx, they continue to express Dsx-independent Chinmo targets. These 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. Furthermore, because our data suggest that Chinmo regulates DsxM at the level of transcription, comparison of targets of Chinmo and DsxM 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), whereas 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 outcompeted 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, because such CySCs are lost quite rapidly. Comparing testses 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.

Because Chinmo is a Jak-STAT effector, we expected that loss of Jak-STAT signaling might give a similar phenotype to loss of Chinmo. However, reduction of Jak-STAT via mutations or RNAi causes only rapid stem cell differentiation and no apparent sex conversion phenotype (Leatherman and DiNardo, 2010; data not shown). Loss of Jak-STAT signaling causes loss of other targets besides Chinmo, for example Zfh-1 (Leatherman and DiNardo, 2008). We speculate that loss of these targets causes stem cells to differentiate before they are able to undergo a sex conversion, which our data suggest can arise only from stem cells.

Our finding that stem cells undergo sex 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 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 provide 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-chimino and chimino (Zhu et al., 2006), eya3-Gal4 (Leatherman and DiNardo, 2008), MSL-4 (Gonczy and DiNardo, 1996), c587-Gal4 (Kai and Spradling, 2003), nanos-Gal4-VP16 (Van Doren et al., 1998), t-JGal4 (Drosophila Genetic Resource Center), UAS-dsxM (Lee et al., 2002), UAS-iflactin-GFP and sibo-iflactin-GFP (from X. Wang and D. Montell), UAS-dsx RNAi-1 (PJKK11126B), from Vienna Drosophila RNai Center [VDRC], UAS-dsx RNAi-2 (from T. Shirangi and M. McKeeown), dsx-Gal4-1 (w1118; P{GRM40A05-Gal4}attP2; from Bloomington Drosophila Stock Center [BDSC]), and dsx-Gal4-2 (w; dsx-Gal4/TM3,Sb.Ser, twist-gal4, UAS-2xEGFP; from B. Baker), w and chiminoST/CyO flies were used as control flies. Other fly stocks were from the VDRC or BDSC, including the SOD(x39)/TM3 line from which chiminoST 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 (from B. Oliver; 1:900 dilution). Antibodies used are described in the Supplemental Experimental Procedures.

**Lineage Analysis**
For lineage analysis, chiminoST; actin > stop > lacZ or chiminoGQ538; actin > stop > lacZ males were mated to E132-Gal4; chiminoST; UAS-FLP or c587-Gal4; chiminoST; 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 chiminoST testes, chiminoST; UAS-iflactin-GFP males were crossed to E132-Gal4; chiminoST; tub-Gal80[ts] or c587-Gal4; chiminoST; 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 were then switched to 29°C to assess Gal4 activity in chiminoST testes.

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

**In Vivo BrdU Incorporation**
Groups of 20 age-matched adult males were incubated at 25°C in empty vials for 4 hr and then transferred to vials of fly food overlaid with disks of filter paper soaked with 120 μl of 2.5 mM BrdU (Life Technologies) and 5 μl of green food coloring (McCormick). After 24 hr, flies that had not eaten the BrdU (and therefore did not have green guts) were discarded, and BrdU was detected in the hub. To categorize the severity of the chiminoST phenotype, we used the following criteria. Morphologically wild-type testes were 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 testes were scored as having a severe phenotype; germ cells in these testes were often arrested at an early spermatogonial stage or degenerating. The severity of the chiminoST and chimino RNAi phenotypes both progress with age. Therefore, the presence of a mild versus 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-square test.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, three figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.10.004.

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