RESEARCH ARTICLE

Chinmo prevents transformer alternative splicing to maintain male sex identity

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Abstract

Reproduction in sexually dimorphic animals relies on successful gamete production, executed by the germline and aided by somatic support cells. Somatic sex identity in Drosophila is instructed by sex-specific isoforms of the DMRT1 ortholog Doublesex (Dsx). Female-specific expression of Sex-lethal (Sxl) causes alternative splicing of transformer (tra) to the female isoform traF. In turn, TraF alternatively splices dsx to the female isoform dsxF. Loss of the transcriptional repressor Chinmo in male somatic stem cells (CySCs) of the testis causes them to “feminize”, resembling female somatic stem cells in the ovary. This somatic sex transformation causes a collapse of germline differentiation and male infertility. We demonstrate this feminization occurs by transcriptional and post-transcriptional regulation of traF. We find that chinmo-deficient CySCs upregulate tra mRNA as well as transcripts encoding tra-splice factors Virilizer (Vir) and Female lethal (2)d (Fl(2)d). traF splicing in chinmo-deficient CySCs leads to the production of DsxF at the expense of the male isoform DsxM, and both TraF and DsxF are required for CySC sex transformation. Surprisingly, CySC feminization upon loss of chinmo does not require Sxl but does require Vir and Fl(2)d. Consistent with this, we show that both Vir and Fl(2)d are required for tra alternative splicing in the female somatic gonad. Our work reveals the need for transcriptional regulation of tra in adult male stem cells and highlights a previously unobserved Sxl-independent mechanism of traF production in vivo. In sum, transcriptional control of the sex determination hierarchy by Chinmo is critical for sex maintenance in sexually dimorphic tissues and is vital in the preservation of fertility.

Author summary

Sexually dimorphic adult tissues, like ovaries and testes, require continuous sex-specific instruction for proper function. Establishment of female somatic sex identity in Drosophila is controlled by an alternative splicing cascade wherein Sex-lethal (Sxl) produces the female-specific protein TransformerF (TraF). By contrast, males lack Sxl and undergo
default splicing, preventing Tra^F production. Since Tra^F expression in males causes sex transformation and impairs tissue function, males must have evolved robust protection against feminization. Here, we investigate the role of a single factor, Chinmo, in protecting male sex identity in the testes: loss of Chinmo in male somatic stem cells causes them to acquire female identity. We demonstrate that this feminization occurs through the induction of Tra^F and its downstream targets. Surprisingly, Sxl is not induced in these sex transformed cells. Instead, two other alternative splice factors, Virilizer and Female lethal (2)d, are enriched in chinmo-mutant somatic cells and are required for their feminization. Our work demonstrates that transcriptional repression of female-biased alternative splice factors prevents sex transformation in the somatic gonad and that tra^F production can occur independently of Sxl. Given the importance of sex maintenance in tissue homeostasis, such protective mechanisms may exist in other tissues.

Introduction

Sexual dimorphism, or the differences between male and female individuals in a species, is observed in many organisms, including insects, reptiles, and mammals. Sex-specific tissue development is essential for proper gonadogenesis, and sexual dimorphism has also been observed in other tissues such as brain, adipose tissue, and intestine [1–4]. While extensive literature has dissected the mechanism of sex determination in early development, recent studies have demonstrated that maintenance of sex identity is also essential for adult tissue homeostasis [5–7]. It is therefore critical to determine the signals that both specify and maintain sex identity.

Differential gene expression via alternative splicing establishes the sex-specific differences observed in the fruit fly Drosophila melanogaster. In flies, the sex of an organism is determined by its number of X chromosomes [8–10]. In XX flies, a positive autoregulatory mechanism activates and maintains expression of the RNA-recognition motif (RRM) containing protein Sex-lethal (Sxl) [11]. In female somatic cells, Sxl binds directly to a polyuridine (poly(U)) tract upstream of exon 2 in transformer (tra) pre-mRNA [12, 13]. This results in the skipping of exon 2, which contains an early stop codon, and synthesis of full-length Tra (Tra^F) in females. In XY flies, which lack Sxl, tra mRNA incorporates exon 2, resulting in premature translational termination and a presumptive small peptide with no known function [13]. Several other factors have been shown to act in concert with Sxl in sex-specific alternative splicing, such as Virilizer (Vir), Female lethal (2)d (Fl(2)d), and Spenito (Nito). All three proteins have an RRM and are required for sex-specific and non-sex-specific functions in Drosophila [14–19].

One of the best characterized targets of the RNA-binding protein Tra^F is doublesex (dsx), which can yield one of two functional isoforms [20]. In XX flies, Tra^F is required for the alternative splicing of dsx and fruitless (fru) pre-mRNAs, generating female-specific Dsx^F and preventing Fru synthesis [21, 22]. In XY flies, which lack Tra^F, dsx and fru pre-mRNA undergo default splicing and generate male-specific Dsx^M and Fru^M. The Dsx^F and Dsx^M transcription factors regulate the majority of known sex-specific differences in gene expression and external appearance in Drosophila, often by direct transcriptional regulation of critical sex-specific genes [20, 23, 24]. Dsx^F and Dsx^M have identical DNA binding sites and bind regulatory sites in many common target genes, and it is generally believed that Dsx isoform association with sex-specific co-factors determines whether the target gene is activated or repressed [20, 25–28].

Loss of sex identity in sexually dimorphic tissues has profound effects on organ development and function [1–4, 29–31]. In the gonad, sex identity is specified autonomously in both the germline and the soma; somatic gonadal cells additionally send essential non-autonomous
cues to instruct germline sex identity [29, 31–34]. Proper gonadogenesis is impeded when the sex identity of the germline does not match that of the soma, and such a mismatch frequently causes sterility [31, 32]. Despite the importance of maintaining sex identity for tissue development and homeostasis, regulation of canonical sex determinants at the transcriptional level has remained relatively unexplored.

In *Drosophila* gonads, germline stem cells (GSCs) divide to produce daughters that ultimately differentiate into sperm and oocytes, respectively. Proper gametogenesis proceeds through the ensheathment of GSC daughters by somatic support cells that exhibit sex-specific differences. In the testis, a niche of quiescent somatic cells termed the hub supports GSCs and somatic cyst stem cells (CySCs), which produce somatic support cells (Fig 1A, left). GSCs divide with oriented mitosis, and daughter cells that are displaced from the niche differentiate through 4 rounds of transit-amplifying mitotic divisions. CySCs are the only mitotically active somatic cells in wild type testes, and they divide to produce post-mitotic cyst cells. Two cyst cells ensheath a single GSC daughter and remain associated with the germ cell cluster throughout its transit-amplifying divisions. During somatic differentiation, cyst cells grow dramatically to accommodate the enlarging spermatogonia [35–38].

In the ovary, GSCs also divide to produce differentiating daughter cells that undergo 4 mitotic divisions to give rise to 16-cell interconnected germ cysts (Fig 1A, right). The developing germ cyst is surrounded by a layer of somatic follicle cells, which are produced by follicle stem cells (FSCs). CySCs and FSCs require similar self-renewal signals, and both male and female somatic gonadal cells exhibit similar cellular behaviors [39–50]. However, their differentiating offspring exhibit distinct behaviors and markers: cyst cells are quiescent as they differentiate, while follicle cells continue to cycle. Additionally, follicle cells form an epithelium to ensheathe the germline, while cyst cells grow in volume and express tight junction proteins to encapsulate spermatogonia [35, 37, 38, 51–53].

Sex-specific anatomical differences are achieved by differential expression of transcription factors [2]. In particular, the transcription factor Chinmo is expressed in male but not female somatic gonadal cells [29, 54, 55]. Chinmo contains a Broad, Tramtrack, and Bric-à-brac/Poxvirus and Zinc finger (BTB/POZ) domain and two C2H2-Zinc fingers (ZFs). Many BTB-ZF proteins in *Drosophila* and mammals have characterized roles as transcriptional repressors [56–58]. However, while clonal loss of *chinmo* from imaginal tissue leads to ectopic gene expression in a cell-autonomous manner [54], no direct targets of Chinmo have been identified. Congruent with its dimorphic expression in the somatic gonad, *chinmo* has no apparent requirement in follicle cells but is essential for CySC niche occupancy [54, 55]. Chinmo is also required for the maintenance of male sex identity in CySCs, as loss of *chinmo* from all CySCs causes them to lose male sex identity, express markers of ovarian follicle cells and adopt an epithelial-like organization [29]. These data have led to a model in which single CySC clones lacking *chinmo* are outcompeted by wild type CySC neighbors, but *chinmo* depletion in all CySCs removes this competitive environment and leads to sex transformation [29, 54]. We have also observed that *chinmo*-mutant CySC clones that lack the JAK/STAT and EGFR pathway inhibitor *Socs36E* can form aggregates, suggesting that CySCs lacking *chinmo* can feminize so long as they are given a chance to proliferate [59]. This sex transformation was reportedly due in part to a transcriptional loss at the *dsx* locus, leading to a loss of *DsxM*; however, sustained expression of UAS-*dsxM* could not prevent the acquisition of female sex identity in *chinmo*-mutant CySCs, indicating that the molecular mechanism by which these cells feminize is still unclear [29].

Our work supports an alternate model whereby male sex identity is maintained not by preventing transcriptional loss of *dsxM*, but by preventing alternative splicing of *dsx* pre-mRNA into *dsxF*. Since *TraF* is responsible for *dsx* alternative splicing in canonical sex determination, we investigated a possible role for *TraF* in CySC feminization upon *chinmo* loss. Here, we
report that Chinmo maintains male sexual identity by preventing the expression of the female sex determinant \( \text{tra}^F \) through a two-step mechanism. We first show that Chinmo represses both expression and alternative splicing of \( \text{tra} \) pre-mRNA. Next, we demonstrate that feminization of \( \text{chinmo} \)-mutant CySCs does not require Sxl. We instead find that RNA binding proteins Vir and Fl(2)d, which are necessary to alternatively splice \( \text{tra}^F \) in the adult ovary, are important for the feminization of \( \text{chinmo} \)-mutant CySCs. Thus, we uncover a novel mode of sex maintenance involving previously unreported regulation of \( \text{tra} \) transcription and a Sxl-independent mechanism of \( \text{tra}^F \) splicing in the somatic gonad.

**Results**

**Chinmo is expressed dimorphically in the somatic gonad and is required for male identity in CySCs**

We found dimorphic expression of Chinmo in *Drosophila* gonads. While Chinmo protein was expressed in all cell types of the adult testis stem cell niche (Fig 1B and 1B’, arrowheads; S1I

**Fig 1. Chinmo is expressed dimorphically in *Drosophila* gonads.** (A) Schematic of the adult *Drosophila* testis (left) and ovary (right). In the testis, the niche (green) supports two populations of stem cells, germline stem cells (GSCs, dark pink) and somatic cyst stem cells (CySCs, dark blue). The GSC divides to produce differentiating daughter cells (light pink) that undergo four transit-amplifying divisions. The CySC divides to produce cyst daughter cells (light blue) that exit the cell cycle and ensheath the differentiating GSC daughter. Cyst cells continue to ensheath the associated spermatogonial cyst during transit-amplifying divisions. In the ovary, the niche (green) supports GSCs (dark pink), which divide to give rise to differentiating daughters (light pink) that undergo 4 mitotic divisions. The developing germline cyst is ensheathed by an epithelial layer of follicle cells (light blue). Follicle cells are proliferative descendants of follicle stem cells (FSCs, dark blue) located in the anterior part of the ovary. (B) Chinmo (green) is present in CySCs (B’, green arrowhead), GSCs (B’, magenta arrowhead), and niche cells (B’, cyan arrowhead) in a wild type testis. (C) Chinmo is not expressed in follicle cells of a wild type ovary. Vasa (red) marks the germline and Zfh1 (blue) marks somatic cells in the testis and ovary. Scale bars = 10 \( \mu \)m.

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Chinmo represses transformer alternative splicing

Fig), it was not detectable in somatic cells of the adult ovary (Fig 1C; S1J Fig). We next confirmed that loss of Chinmo expression in CySCs leads to the acquisition of female identity. When *chinmo* was depleted in the CySC lineage by RNAi using the somatic driver *tj-gal4* (*tj>*chinmo*RNAi; S1K Fig), expression of the male sex determinant DsxM was lost (Fig 2A–2C), and the follicle cell marker Castor (Cas), normally absent from the testis, was ectopically expressed (Fig 2D–2F). In wild type testes, Fasciclin 3 (Fas3) was expressed in niche cells but not in CySCs (Fig 2G). However, in *tj>*chinmo*RNAi* testes, we observed Fas3-expressing somatic aggregates resembling epithelial follicle cells that eventually organized at the periphery (Fig 2H and 2I). A marker of late-stage follicle cell maturation, Slow border cells (Slbo), was absent from wild type CySCs (S1A and S1B Fig) but was ectopically expressed in *tj>*chinmo*RNAi* testes (S1C Fig). Finally, transcripts of the DsxF target *Yp1* were upregulated in *chinmo*-deficient testes.

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This sex transformation phenotype is due to loss of chinmo in the CySC lineage and not the niche, as depletion of chinmo specifically in niche cells produced no overt phenotype (S1E and S1F Fig; [29]).

Because CySCs serve a critical role in maintaining GSCs, as well as producing somatic support cells, the stem cell niche in tj>chinmoRNAi testes frequently becomes agamic even at relatively early time points after depletion (S1G and S1H Fig; [29, 60]). Based upon these observations, we hypothesized that tj>chinmoRNAi males would become sterile. To test this, we mated successively tj>chinmoRNAi males to OregonR virgin females and scored the number of progeny. Upon each of two mating rounds, tj>chinmoRNAi males exhibited a significant reduction in fertility (25% and 55% compared to control males, p<0.05 and p<0.001, respectively) (Fig 2I). By the third successive mating, tj>chinmoRNAi males were completely sterile whereas control males were not (p<0.0001). Taken together, our results align with previous work showing that Chinmo is required in adult CySCs to preserve male sex identity [29]. Additionally, we demonstrate that CySC male identity is essential for fertility.

CySC feminization upon loss of chinmo is dependent on the female sex determinant dsxF

We next sought to determine the mechanism by which CySCs undergo feminization upon loss of chinmo. According to a previous report, dsxM mis-expression in chinmo-deficient CySCs (c587>chinmoRNAi; >dsxM) delays feminization [29], suggesting that dsxM transcription was reduced in chinmo-mutant CySCs. However, at the time point when all c587>chinmoRNAi testes contained Fas3-positive aggregates, nearly all c587>chinmoRNAi; >dsxF testes were also feminized [29], indicating a delay but not an abrogation of the phenotype. Additionally, depletion of all dsx transcripts using an RNAi transgene (dsxKK111266) targeting the common region of dsxM and dsxF did not recapitulate the defect seen upon loss of chinmo [29]. These data indicate that the loss of DsxM alone cannot fully account for the phenotype of chinmo-mutant CySCs. We reasoned that the loss of DsxM protein observed in chinmo-mutant CySCs could result from alternative splicing of the dsx pre-mRNA into dsxF rather than from a transcriptional decrease at the dsx locus (Fig 3A). If this were true, we would expect to find in chinmo-deficient CySCs: 1) active transcription of the dsx locus; 2) expression of alternatively-spliced dsxF transcripts; and 3) expression of DsxF protein. To assess dsx transcription levels, we surveyed 4 independently-generated dsxRNAi transcriptional reporters: two Gal4 knock-in reporters in the dsx locus (dsx-gal4; [2] and dsx-gal4Δ2; [61]), one MiMIC allele at the dsx locus (dsxMIO3050·GFS1.3; [62]) and one Janelia transgene containing a 2.5 kb dsx regulatory element (GMR40A05·gal4; [63]. We selected dsx-gal4 for further use because it was the only line that was robustly expressed in both adult testes and adult ovaries and therefore accurately reflected dsx transcription (Fig 3B and 3C). By contrast, the other 3 lines displayed male-biased or very low expression in gonads (S2A–S2F Fig). We then assessed dsx-gal4 activity as a proxy for transcription of the dsx locus upon chinmo depletion. We used a genetic approach to remove chinmo from the CySC lineage by analyzing testes homozygous for the chinmoST allele [29] in the dsx-gal4 background. While chinmoST/CyO testes express normal levels of Chinmo, chinmoST/chinmoST males lack Chinmo in the CySC lineage [29]. As expected, GFP was expressed in somatic cells in control chinmoST/CyO; dsx-gal4/UAS-GFP testes and ovaries (Fig 3B and 3C). Importantly, GFP was also expressed in chinmoST/chinmoST; dsx-gal4/UAS-GFP mutant testes (Fig 3D), demonstrating that dsx is still transcribed in chinmo-deficient cyst cells. We also visualized dsx transcript abundance in tj>chinmoRNAi testes using semi-quantitative RT-PCR. Primers that recognize both dsx mRNA isoforms (dsxCOMMON, or dsxF) reveal that dsx is still present in tj>chinmoRNAi testes (Fig 3E). We confirmed that dsxF is produced...
specifically by chinmo-deficient somatic cells by performing RT-PCR on FACS-sorted CySCs and early cyst cells. As expected, a \( dsx^F \)-specific band was observed in RNA extracts from wild type ovaries (Fig 3F, left lane). We also observed \( dsx^F \) in FACS-purified chinmo-deficient cyst
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To confirm these results, we monitored 
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expression [54], we used a temperature-sensitive gal80 allele (tj-gal4, tub-gal80TS or tjTS) and reared flies at the permissive temperature (18˚C). Adult F1 females were then shifted to the restrictive temperature (29˚C) for 5 days before ovaries were homogenized. We observed a 2.2-fold decrease in total tra mRNA abundance (p<0.001) and a 1.8-fold decrease in traF.
Fig 5. The female sex determinant Tra\textsuperscript{f} is required for feminization of chinmo-mutant CySCs. (A) Semi-quantitative RT-PCR of total \textit{tra} and \textit{tra}\textsuperscript{f} mRNA in control \textit{tj}\textsuperscript{+} testes (first lane), control \textit{tj}\textsuperscript{+} ovaries (second lane) and \textit{tj}\textsuperscript{+}chinmo\textsuperscript{RNAi} testes (third lane). All three samples contain a band for total \textit{tra} (first row, blue arrowhead). However, a \textit{tra}\textsuperscript{f} band is detected in ovaries, as expected, and in \textit{tj}\textsuperscript{+}chinmo\textsuperscript{RNAi} testes (second row, red arrowhead) but not in control \textit{tj}\textsuperscript{+} testes. \textit{rpl15} was used as a loading control (third row). Flies were aged 9–20 days prior to dissection. (B-C) qRT-PCR of total \textit{tra} (B) or \textit{tra}\textsuperscript{f} (C) in control \textit{tj}\textsuperscript{+} testes (white bars), control \textit{tj}\textsuperscript{+} ovaries (red bars) and \textit{tj}\textsuperscript{+}chinmo\textsuperscript{RNAi} testes (blue bars). (D-J) Immunofluorescence analysis of \textit{tj}\textsuperscript{+} UAS-\textit{tra}\textsuperscript{f} T2AGFP flies showing changes in \textit{Fas3} and \textit{Fsh} expression levels in testes and ovaries. (K-P) Immunofluorescence analysis of \textit{tj}\textsuperscript{+} UAS-chinmo\textsuperscript{RNAi} flies showing changes in \textit{Vasa} and \textit{Fas3} expression levels in testes and ovaries.
abundance (p<0.001) in tjTS>chinmo ovaries compared with tjTS> ovaries (S6A Fig). dxsF was also decreased 5.8-fold (p<0.001) in tjTS>chinmo ovaries compared with tjTS> ovaries, presumably as a result of reduced TraF (S6A Fig). Taken together, our results demonstrate that Chinmo is both necessary and sufficient to prevent somatic expression of the female sex determinants traF and dxsF.

These findings suggest that sex transformation in chinmo-deficient cyst cells is due to ectopic TraF. To test this, we concomitantly depleted both tra and chinmo in the somatic lineage of the testis. Whereas 98% of tj>chinmoRNAi testes contained Fas3-positive aggregates outside of the niche, only 48% of tj>traRNAi; chinmoRNAi testes had such aggregates, indicating a significant block in feminization (p<0.0001) (Fig 5I; Fig 5J, purple bar; S1 Table). In these rescued tj>traRNAi; chinmoRNAi testes, CySCs no longer expressed Fas3, and the germline appeared normal (Fig 5I). We also performed epistatic experiments with tra mutant alleles, similar to the dxsF/dxsF experiment. XX tra1/Df(3L)st-j7 animals develop male somatic structures due to loss of TraF (S4E–S4H Fig; [66]). Whereas 100% of chinmoST/chinmoST testes were feminized as assessed by Fas3-positive aggregates, only 61% of chinmoST/chinmoST; tra1/Df(3L)st-j7 testes were feminized (p<0.001) (Fig 4D, green bar; S1 Table). The phenotype was not sensitive to tra dose as chinmoST/chinmoST; tra1+ testes were still feminized (Fig 4D, yellow bars; S1 Table). These results demonstrate that Chinmo prevents both tra transcription and alternative splicing in CySCs and that feminization of male somatic cells in the absence of chinmo is due to ectopic traF.

**Ectopic TraF impairs somatic differentiation but is not sufficient for CySC feminization**

Global expression of TraF in XY flies during development causes female somatic differentiation [67]. To test whether TraF expression alone is sufficient to cause male-to-female sex transformation in adult CySCs, we over-expressed traF cDNA in tj-gal4 expressing cells and used gal80TS to restrict expression to only adult CySCs (tjTS). While we observed accumulation of somatic aggregates in tjTS>traF testes, they did not express Fas3 or Cas, in contrast to those in tj>chinmoRNAi testes (compare Fig 5K and 5L to Fig 2I for Fas3 and compare Fig 5N and 5O to Fig 2F for Cas). These data suggest that traF-misexpressing cyst cells have not fully acquired a follicle-like fate. However, we found on average 121.0±8.8 somatic cells expressing Zinc finger homeodomain 1 (Zfh1), which marks CySCs and their earliest differentiating daughters [68], in tjTS>traF testes compared with 40.1±1.6 cells in control tjTS> testes (p<0.0001) (S7A, S7B and S7I Fig). Upon somatic traF mis-expression, we also observed accumulation of
somatic cells expressing Tj, which marks a broader population of CySCs and early cyst cells [69] (S7C and S7D Fig). $tj^{TS}>tra^{F}$ testes contained 158.7±14.5 Tj-positive cells compared with 80.3±3.9 cells in $tj^{TS}>>$ testes ($p<0.001$) (S7J Fig). We interpret the accumulation of Zfh1-positive, Tj-positive cells in $tj^{TS}>tra^{F}$ testes as a delay in somatic differentiation. Because cyst cells must exit the cell cycle in order to support the developing male germline, there are no somatic cells located away from the niche in wild type testes that are positive for 5-ethynyl-2’-deoxyuridine (EdU), an S-phase marker. We previously showed that when somatic differentiation is delayed, EdU-positive cyst cells are observed several cell diameters away from the niche [36]. Consistent with our prior results, in control $tj^{TS}>>$ testes, only Tj-positive cells near the hub incorporated EdU (S7E Fig, arrowheads; n = 0/20 testes with EdU-positive cyst cells located away from the niche). By contrast, in $tj^{TS}>tra^{F}$ testes we detected EdU-positive somatic cells located many cell diameters away from the niche, suggesting that these cells had delayed differentiation (S7F Fig, arrows; n = 20/26 testes with EdU-positive cyst cells located away from the niche).

Consistent with a defect in somatic differentiation, cyst cells mis-expressing $tra^{F}$ were impaired in their ability to support the germline. In $tj^{TS}>tra^{F}$ testes, early germ cells accumulated (identified by dot- and dumbbell-shaped α-spectrin-positive fusomes) at the expense of more differentiated spermatagonia, as fewer germ cysts with branched fusomes were observed (S7G and S7H Fig). $tj^{TS}>tra^{F}$ testes also contained significantly fewer EdU-positive, 4- and 8-cell spermatogonial cysts than $tj^{TS}>>$ testes (S7E, S7F and S7K Fig). These results demonstrate that ectopic $Tra^{F}$ in CySCs is deleterious to their differentiation, but alone cannot drive CySCs to assume a follicle-like fate. Taken together with our previous finding that $tra$ is down-stream of chinmo in CySC feminization, we conclude while $Tra^{F}$ induction is important for CySC feminization upon loss of chinmo, it is not sufficient.

**Sxl is not required for feminization of chinmo-deficient CySCs**

Our finding that chinmo-deficient CySCs produce $tra^{F}$ (Fig 5A and 5C) reveals that they possess machinery to splice $tra$ pre-mRNA into the female isoform. We considered the possibility that wild type CySCs might be competent to alternatively splice $tra$. However, somatic mis-expression of UAS-$tra^{F}$T2AGFP in wild type somatic cells did not lead to $tra$ alternative splicing, since GFP was absent from the somatic lineage (Fig 5D). Thus, wild type CySCs are intrinsically unable to generate $tra^{F}$ mRNA, precluding this model.

It follows, then, that one or more factors are ectopically expressed upon loss of chinmo that alternatively splice $tra$ pre-mRNA into $tra^{F}$. Since Sxl is required for $tra^{F}$ production in wild type females (Fig 6A; [12]), we investigated whether Sxl is ectopically expressed in chinmo-mutant CySCs. As expected, Sxl protein was absent from wild type testes but was detectable in wild type ovaries (Fig 6B and 6C). Importantly, we did not observe Sxl in chinmo-mutant testes (Fig 6D). These results were validated by assessing Sxl mRNA isoform abundance in adult gonads. Semi-quantitative RT-PCR demonstrated that control $tj>>+$ testes express male-specific Sxl$^{M}$ (Fig 6E, left lane), which contains an early stop codon and encodes no functional protein, while control $tj>>+$ ovaries express female-specific Sxl$^{F}$ (Fig 6E, middle lane), which encodes functional Sxl. $tj>>+$chinmoRNA testes still express Sxl$^{M}$ (Fig 6E, right lane), consistent with the absence of Sxl protein in these testes (Fig 6D). We also tested whether mis-expression of chinmo in female follicle cells could prevent Sxl alternative splicing; however, both $tj^{TS}>>+$ and $tj^{TS}>>$chinmo ovaries expressed only the female-specific Sxl$^{F}$ isoform (S6B Fig).

Furthermore, unlike depletion of $tra$, depletion of Sxl in feminizing, chinmo-deficient somatic cells did not suppress Fas3 expression or the epithelial organization of somatic cells (Fig 5), light blue bar; S1 Table). [As expected, somatic depletion of Sxl in an otherwise wild
**Fig 6. Sxl is not required for feminization of chinmo-mutant CySCs.** (A) Schematic of tra pre-mRNA splicing. The poly(U) tract upstream of exon 2 is bound by the RRM domain of Sxl in females, causing skipping of exon 2. In wild type males, exons 1–4 comprise tra mRNA and translation terminates at the early stop codon in exon 2 (red star). Pink dashed lines indicate female-specific alternative splicing and blue dashed lines indicate non-sex-specific default splicing. (B-D) Sxl is not expressed in a control $tj^{>}$ testis (B’) but is expressed in follicle cells (C’, arrowheads) and in an early germ cell (C’, arrow) of a control $tj^{>}$ ovary. Sxl protein is not detected in a $tj^{>}$ chinmo RNAi testis (D’). (E) Semi-quantitative RT-PCR on Sxl in homogenized control $tj^{>}$ testes (left lane), control $tj^{>}$ ovaries (middle lane), and $tj^{>}$ chinmo RNAi testes (right lane). Control $tj^{>}$ testes express Sxl<sub>M</sub> transcripts (blue arrowhead), while control $tj^{>}$ ovaries express Sxl<sub>F</sub> transcripts (red arrowhead). Sxl<sup>M</sup> is still present and Sxl<sup>F</sup> is undetectable in $tj^{>}$ chinmo RNAi testes (right lane). Sxl<sup>YR</sup> primers were used to differentiate between Sxl<sub>M</sub> and Sxl<sub>F</sub> mRNA isoforms in this experiment. $tub\alpha$ (tub) was used as a loading control. (F) Quantification of CySC feminization in Sxl<sup>-/-</sup>; chinmo<sup>ST</sup> backgrounds. Sample sizes are indicated within bars. **** denotes p<0.0001 as determined by Fisher’s Exact Test (compared to
type background produced no testis phenotype (S1 Table). We confirmed that the UAS-Sxl-RNAi line was effective at knockdown because somatic depletion of Sxl in females led to only a rudimentary ovary with 100% penetrance, n = 23.] Consistent with this, none of three distinct mutant alleles of Sxl prevented feminization in chinmo$^{ST}$/chinmo$^{ST}$ tests (Fig 6F–6K; S1 Table). Taken together, these data support a model where the ectopic tra pre-mRNA in chinmo-mutant CySCs is alternatively spliced into tra$^F$ via a non-canonical, Sxl-independent mechanism.

**vir and fl(2)d are upregulated in chinmo-deficient CySCs and are required for sex transformation**

We next examined a potential role for other candidates with known roles in female-specific alternative splicing of tra. We found that vir, fl(2)d, and nito transcripts were 1.5-fold (p<0.05), 3.4-fold (p<0.001), and 5.7-fold (p<0.0001) higher in adult ovaries compared with adult testes, respectively, suggesting sex-biased expression in adult gonads (Fig 7A and 7B). This observation is consistent with ModEncode RNA-seq data demonstrating that adult testes, respectively, suggesting sex-biased expression in adult gonads (Fig 7A and 7B). We confirmed that the female reproductive structures and contain an oviduct, but lack ovaries (S8D and S8E Fig). Both tj$^{TS}$>vir$^{RNAi}$ and tj$^{TS}$>fl(2)d$^{RNAi}$ females failed to lay fertilized eggs. To test whether vir or fl(2)d are necessary for tra$^F$ splicing in adult ovaries, we depleted vir or fl(2)d in the female somatic gonad using tj$^{TS}$, rearing flies at the permissive temperature to prevent vir or fl(2)d knockdown during development. After eclosion, adult females were then reared at the restrictive temperature to allow for vir and fl(2)d depletion. While wild type follicle cells express GFP produced by UAS-traF$^{TA2AGFP}$ (Fig 7D), GFP is dramatically reduced in the follicle cells of tj$^{TS}$>vir$^{RNAi}$ and tj$^{TS}$>fl(2)d$^{RNAi}$ ovaries (Fig 7C, 7E and 7F). These results demonstrate that vir and fl(2)d are both female-biased in the adult gonad and are required for tra$^F$ alternative splicing in follicle cells.

To test whether vir or fl(2)d are required for sex transformation upon somatic loss of chinmo, we depleted each factor concomitantly with chinmo and monitored the frequency of CySC feminization. Depletion of vir or fl(2)d in tj$^{TS}$>chinmo$^{RNAi}$ testes significantly reduced the percentage of feminized testes (p<0.001 and p<0.0001, respectively) (Fig 7G and 7H; Fig 5J, green and yellow bars, respectively; S1 Table). In contrast, depletion of nito did not prevent feminization (Fig 7I; Fig 5J, red bar; S1 Table). As expected, somatic depletion of vir, fl(2)d or nito in an otherwise wild type testis had no effect (S1 Table). We also tested the sufficiency of fl(2)d for CySC sex transformation. We found that mis-expression of fl(2)d in the adult CySC lineage (tj$^{TS}$>fl(2)d) did not cause Fas3-positive aggregates to accumulate (Fig 5M). Furthermore, the follicle cell marker Cas was not induced in tj$^{TS}$>fl(2)d testes (Fig 5P). Due to the lack of a UAS-vir transgenic Drosophila line, we were unable to test the sufficiency of vir for CySC feminization. Based on these findings, we conclude that Vir and Fl(2)d are epistatic to chinmo and are required, but not sufficient, for feminization of chinmo-mutant CySCs. Taken together
with our previous results, this strongly implicates Vir and Fl(2)d in alternative splicing of the ectopic tra pre-mRNA observed in sex-transformed CySCs.

**Discussion**

Chinmo prevents female sex identity in adult male CySCs

Here, we show that one single factor, Chinmo, preserves the male identity of adult CySCs in the Drosophila testis by regulating the levels of canonical sex determinants. We demonstrate that CySCs lacking chinmo lose DsxM expression not by transcriptional loss but rather by alternative splicing of dsx pre-mRNA into dsxF. These chinmo-mutant CySCs ectopically express...
Tra^F and Dsx^F, and both factors are required for their feminization. Furthermore, our results demonstrate that *tra* alternative splicing in cyst cells lacking *chinmo* is achieved independently of Sxl. Instead, our work strongly suggests that *tra* production in the absence of *chinmo* is mediated by splicing factors Vir and Fl(2)d. We propose that male sex identity in CySCs is maintained by a two-step mechanism whereby *tra* is negatively regulated at both transcriptional and post-transcriptional levels by Chinmo (Fig 8). In this model, loss of *chinmo* from male somatic stem cells first leads to transcriptional upregulation of *tra* pre-mRNA as well as of *vir* and *fl(2)d*. Then the *tra* pre-mRNA in these cells is spliced into *tra*^F^ by the ectopic Vir and Fl(2)d proteins. The ectopic Tra^F^ in *chinmo*-deficient CySCs then splices the *dsx* pre-mRNA into *dsx*^F^, resulting in loss of Dsx^M^ and gain of Dsx^F^, and finally induction of target genes usually restricted to follicle cells in the ovary.

**Chinmo regulates levels of *tra*, *vir* and *fl(2)d***

Chinmo has motifs associated with transcriptional repression and its loss clonally is associated with ectopic transcription [54]. One interpretation of our data is that Chinmo directly represses *tra*, *vir*, and *fl(2)d* in male somatic gonadal cells. As the binding site and potential cofactors of Chinmo are not known, future work will be needed to determine whether Chinmo directly regulates expression of these genes. We also note that ~50% of *chinmo*-mutant testes still feminize in the genetic absence of *tra* or *dsx*^F^. These latter data indicate that Chinmo regulates male sex identity through another, presumably parallel, mechanism that does not involve canonical sex determinants. However, this *tra/dsx*-independent mode of sex maintenance downstream of Chinmo is not characterized and will require the identification of direct Chinmo target genes.

**Regulation of *chinmo* expression in adult CySCs**

We previously showed that JAK/STAT signaling promotes *chinmo* in several cell types, including CySCs [54]. Since JAK/STAT signaling is itself sex-biased and restricted to the embryonic male gonad, we presume that activated Stat92E establishes *chinmo* in male somatic gonadal precursors, perhaps as early as they are specified in the embryo [33, 71]. Because loss of Stat92E from CySCs does not result in an apparent sex transformation phenotype [29, 40, 60], we favor the interpretation that Stat92E induces expression of *chinmo* in CySCs but that other sexually biased factors maintain it. One potential candidate is Dsx^M^, which is expressed specifically in early somatic gonads and at the same time when Stat92E activation is occurring in these cells [72]. In fact, multiple Dsx^M^ ChIP-seq peaks were identified in the *chinmo* locus, suggesting potential regulation of *chinmo* by Dsx^M^ [26]. Taken together with our findings, this suggests a potential autoregulatory feedback loop whereby Dsx^M^ preserves its own expression in adult CySCs by maintaining Chinmo expression, which in turn prevents *tra*^F^ and *dsx*^F^ production.

**Non-canonical mechanisms of sex-specific cell fate and tissue homeostasis**

Recent studies on tissue-specific sex maintenance demonstrate that while the Sxl/Tra/Dsx hierarchy is an obligate and linear circuit during embryonic development, at later stages it is more modular than previously appreciated. For example, Sxl can regulate female-biased genes in a *tra*-independent manner [73, 74]. Additionally, Sxl and Tra^F^ regulate body size and gut plasticity independently of the only known Tra^F^ targets, *dsx* and *fru* [3, 4]. We find that negative regulation of the *Tra^F^-Dsx^F* arm of this cascade is required to preserve male sexual identity in CySCs but unexpectedly is independent of Sxl. Because depletion of Vir or Fl(2)d significantly blocks sex transformation and both are required for *tra* alternative splicing in the ovary, our
work reveals they can alternatively splice tra pre-mRNA even in the absence of Sxl. To the best of our knowledge, this is the first demonstration of Sxl-independent, Tra-dependent feminization. These results raise the broader question of whether other male somatic cells have to safeguard against this novel mechanism. Because recent work has determined that sex maintenance is important in systemic functions regulated by adipose tissue and intestinal stem cells [3, 4], it will be important to determine whether Chinmo represses tra in these settings.

Finally, since the transcriptional output of the sex determination pathway is conserved from Drosophila (Dsx) to mammals (DMRT1), it is possible that transcriptional regulation of sex determinants plays a similar role in adult tissue homeostasis and fertility in higher organisms.

Materials and methods
Fly stocks and husbandry
The following fly stocks were used and are described in FlyBase: Oregon^R; yw; tj-gal4; tub-gal80TS; dsx-gal4; dsx-gal^-; GMR40A05-gal4; dsx^M160950-GFST^-; dsx^D; chinmo^ST; tra^1; Df (3L)st-j7; UAS-GFP^alt; UAS-dcr2; UAS-chinmo^RNAi (HMS00036); UAS-tra^RNAi (HMS02830); UAS-tra^3; UAS-3xHAf(2)d; UAS-dx^3; UAS-5’UTR-chinmo-3’UTR; UAS-Sxl^RNAi (HMS0609); Sx^P; Sx^D; Sxl^1; UAS-3xHAf(2)d; UAS-tra^3; UAS-fl(2)d^RNAi (HMC03908); UAS-fl(2)d^RNAi (HMC03833); UAS-nito^RNAi (HMS00166).

For RNAi-mediated depletion of chinmo, Sxl, tra, vir, fl(2)d, and nito, flies were reared at an ambient temperature (21˚C). Adult males were collected twice a week and aged at 29˚C to increase Gal4 activity. For temporal control of gene expression, tj-gal4, tub-gal80TS virgins were crossed to UAS-tra^F or UAS-3xHAf(2)d males and progeny were reared at the permissive
temperature (18˚C) to prevent tra′ or fl(2)d mis-expression during embryonic, larval, and pupal development. Adult males of the correct genotype were collected twice a week and shifted to the restrictive temperature (29˚C) to inactivate Gal80.

**Generation of UAS-traFΔT2AGFP**

In this transgene, most of the third exon of tra is replaced by the coding sequences for self-cleaving T2A peptide and GFP. Specifically, the coding sequences of T2A and GFP were cloned in frame immediately downstream of the 26th nucleotide (nt) of tra exon 3 and immediately upstream of the last 18 nt of this exon. PCR was performed with Q5 high-fidelity polymerase from New England Biolabs (M0491S). The PCR product was digested with EcoRI and XhoI before cloning into the pUASTattb vector [75]. The construct was verified by sequencing, and a transgenic line was established through FlC-31 integrase mediated transformation (Bestgene, attP site VK05, BDSC#9725).

**Antibodies**

The following primary antibodies were used: rat anti-Chinmo (1:1000; gift of N. Sokol, Indiana University, IN, USA), goat anti-Vasa (1:50, dC-13, Santa Cruz), rabbit anti-Vasa (1:1500; gift of R. Lehmann, Skirball Institute/NYU School of Medicine, NY, USA), guinea pig anti-Tj (1:5000; gift of D. Godt, University of Toronto, ON, Canada), rabbit anti-Zfh1 (1:5000; gift of R. Lehmann), mouse anti-Fasciclin-3 (1:50; Developmental Studies Hybridoma Bank (DSHB)), mouse anti-Eya (1:20; DSHB), rat anti-DsxM (1:200; gift of B. Oliver, National Institutes of Health, MD, USA), rat anti-DsxC (1:50; gift of M. Arbeitman, Florida State University, FL, USA), rabbit anti-Castor (1:50; gift of W. Odenwald, National Institutes of Health, MD, USA), mouse anti-α-spectrin (1:20, DSHB), mouse anti-SxlM18 (1:5; DSHB), rabbit anti-GFP (1:500; Invitrogen). Secondary antisera used were all raised in donkey (Jackson ImmunoResearch).

**Immunofluorescence**

Testes and ovaries were dissected in 1x PBS and fixed in 4% paraformaldehyde in 1x PBS for 30 minutes at room temperature (RT). Fixed tissue was washed twice at RT in 0.5% PBST (1x PBS with 0.5% Triton X-100) and blocked in PBTB (1x PBS, 0.2% Triton X-100, 1% BSA) for 1 hour at RT or overnight at 4˚C. Primary antibodies were incubated overnight at 4˚C and washed off twice at RT in PBTB. Secondary antibodies were incubated for 2 hours at RT in the dark and washed off twice in 0.2% PBST (1x PBS with 0.2% Triton X-100). Tissue was mounted in Vectashield Medium (Vector Laboratories) prior to confocal analysis, and confocal images were captured using a Zeiss LSM 510 confocal microscope, 63x objective.

**DIC microscopy on whole ovaries**

DIC images of adult female reproductive structures (at 5x) were obtained using a Zeiss Axioplan microscope with a Retiga Evi (QImaging) digital camera and QCapture Pro 6.0 software.

**Immunofluorescence using anti-DsxC**

Testes and ovaries were dissected in 1x PBS and fixed in 20% EM-grade paraformaldehyde (Electron Microscopy Sciences) in 1x PBS for 20 minutes at RT. Fixed tissue was washed 3 times for 15 minutes each in TNT (0.1M Tris-Cl, 0.3M NaCl, 0.05% Tween-20) and blocked using Image-iT FX Signal Enhancer (ThermoFisher) for 30 minutes at RT, then washed 3 times for 15 minutes each in TNT. Primary anti-DsxC was incubated overnight at 4˚C. After
anti-Dsx\(^C\) incubation, tissue was blocked in PBTB for 1 hour and then treated with anti-Vasa and anti-Tj. Primary antibodies were washed twice for 15 minutes each in PBTB, then secondary antibodies were incubated overnight at 4˚C in PBTB. Finally, the Dsx\(^C\) signal was amplified by TSA (see below) and testes were mounted in Vectashield prior to analysis.

**Tyramide signal amplification (TSA)**

TSA (Perkin Elmer) was performed to amplify Dsx\(^M\) and Dsx\(^C\) signals. HRP anti-rat (Jackson ImmunoResearch) was used as a secondary antibody and the tertiary Cy3-conjugated tyramide reaction was performed per the manufacturer’s instructions.

**CySC purification by fluorescence-activated cell sorting (FACS)**

To purify CySCs and early cyst cells, the somatic cell lineage was labeled using \textit{tj-gal4} to drive \textit{UAS-GFP\(_{\text{nls}}\)} expression. Testes were dissociated in trypsin/collagenase for 15 minutes and the cell suspension was passed through 70μm filters (Falcon). GFP-expressing somatic cells were purified from the resulting filtrate by FACS using a Sony SY3200 highly automated parallel sorting (HAPS) cell sorter into TRIzol LS (ThermoFisher), and RNA was extracted according to the manufacturer’s instructions. Post-sort purity of samples was confirmed by immunocytochemistry and the absence of Vasa-positive germ cells.

**5-ethynyl-2’-deoxyuridine (EdU)-labeling of adult testes**

EdU-labeling of testes was performed using the Click-iT EdU Alexa Fluor 647 Imaging Kit (ThermoFisher). Testes were dissected in S2 cell culture medium (Life Technologies) then incubated in 10 μM EdU for 30 minutes. Testes were then fixed, washed, and stained as described above. The cycloaddition reaction was performed per the manufacturer’s instructions. Testes were mounted in Vectashield prior to confocal analysis.

**Quantitative and semi-quantitative RT-PCR**

To detect mRNA levels of canonical sex determinants by PCR, whole ovaries (\(n = 5–10\)) or whole testes (\(n = 55–200\)) were isolated and homogenized into TRIzol (ThermoFisher). RNA was extracted and DNase-treated (Ambion) per the manufacturer’s instructions. Reverse transcription was performed using Maxima reverse transcriptase (ThermoFisher) according to the manufacturer’s instructions and 1–2 μg of RNA as template. qRT-PCR was performed using SYBR Green PCR Master Mix (ThermoFisher) and a Biorad CFX96 Real-Time PCR Machine. Semi-quantitative RT-PCR was performed on a Biorad iCycler. Because the proportion of somatic cells is significantly increased in \(tj>\text{chinmo}^{\text{RNAi}}\) testes compared to \(tj>+\) controls, the qRT-PCR values were normalized first to \textit{tubulin} and second to \textit{zfh1}, an early somatic marker.

**Primers**

- \textit{total tra}: \texttt{fwd-GAGCCCGCATCGGTATAATC; rev-GACGTGTTAGCCTTTTGATC}
- \textit{tra\(^F\) }: \texttt{fwd-AAACCCAATCGAGATTTCC; rev-CGAACTCGTTGCAAGATGA}
- \textit{dsx\(^C\) }: \texttt{fwd-GAAAGAACGGCGCAAAT; rev-GGCGTCTGCTTATTATA}
- \textit{dsx\(^M\) }: \texttt{fwd-GAGCTGATGCCACTCATGTATAT; rev-CTGGGCTACAGTGCGATTAA}
- \textit{dsx\(^F\) }: \texttt{fwd-GAATGAGTACTCCCTCAACAT; rev-GGGCAAAGTAGTATTTAC}
- \textit{rpl15}: \texttt{fwd-AGGATGCACTTATGGCAAGC; rev-GCGCAATCCAATACGAGTT}
- \textit{α-tub84b}: \texttt{fwd-CAACCAGATGAGGAGATGCG; rev-ACGTTGCTGGCACAACATC}
- \textit{β-tub56d}: \texttt{fwd-CTCAAGTGCTGATGTGTTGCC; rev-CCGAAAGGAGTGTTGAGTT}
- \textit{Sxl\(^JYR\) }: \texttt{fwd-ACACAAGAGATTTGAACAGGG; rev-CATTCGATGGCAGGATGG}

Chinmo represses \textit{transformer} alternative splicing
**Fertility assays**

The fertility of adult males was assayed by mating individual males with two wild type (Oregon\(^B\)) virgin females (between 5–10 days old) for 48 hours at 25˚C. After a 2-day mating period, males were recovered and preserved for subsequent matings using fresh virgin Oregon\(^B\) females. Fertility was scored by counting the number of F1 offspring produced by each individual cross and reported as the average number of F1 offspring for each genotype.

**Statistical analysis**

Statistical parameters for each experiment are reported in the figure legends. Data were analyzed using Microsoft Excel and are reported to be statistically significant when \(p < 0.05\) by the appropriate statistical test. For qRT-PCR data, significance was determined by two-tailed Student’s t-test. For fertility assays and cyst cell quantifications, significance was determined using single-factor ANOVA. For rescue of CySC feminization (Fas3-positive aggregates), significance was determined using Fisher’s Exact Test.

**Supporting information**

**S1 Table.** Quantification of testes with Fas3-positive somatic aggregates (referred to as “feminized”). Data are presented as the percentage of testes with Fas-3-positive aggregates in testes of the indicated genotypes from the total number of testes examined.

(DOCX)

**S1 Fig.** Loss of chinmo in the CySC lineage causes sex transformation and loss of the germ-line. (A-C) A transcriptional reporter for slow border cells (slbo-GFP) is not expressed in a wild type testis (A’, arrowhead). slbo-GFP (green) is expressed in mature follicle cells (B’, arrowhead). slbo-GFP is ectopically expressed in the CySC lineage upon loss of chinmo (C’, arrowhead). Time point is 11 days post-eclosion. Tj (blue) marks cyst cells. Vasa (red) marks the germline.

(D) Semi-quantitative RT-PCR on Yp1 using RNA extracts from homogenized control \(tj^+\) testes (left lane), control \(tj^+\) ovaries (middle lane), and \(tj>\text{chinmo}^{\text{RNAi}}\) testes (right lane). Yp1 is expressed in control \(tj^+\) ovaries (middle lane), but not in control \(tj^+\) testes (left lane). In \(tj>\text{chinmo}^{\text{RNAi}}\) testes (right lane), Yp1 is expressed. \(\alpha\)-tubulin (tub) was used as a loading control. Timepoint is 9–14 days post-eclosion.

(E-F) Loss of chinmo in male niche cells using \(\text{upd-gal4}, \text{gal80}^{\text{TS}}\ (\text{upd}^{\text{TS}})\) causes no overt defects in testis development or spermatogenesis. Time point is 8 days post-eclosion. TOPRO (blue) marks DNA. Fas3 (green) marks niche cells.

(G-H) Representative images of agametic \(tj>\text{chinmo}^{\text{RNAi}}\) testes at 7 days post-eclosion. Fas3-positive somatic aggregates (green) fill the apex of the testis, which is devoid of Vasa-positive (red) germ cells. Zfh1 (blue) marks somatic cells.

(I-K) Expression of Chinmo in adult gonads. Chinmo is expressed in the CySC lineage of the adult testis (I’, arrowheads) but is absent from follicle cells in the adult ovary (J’, arrowheads). Upon chinmo depletion in the testis (\(tj>\text{chinmo}^{\text{RNAi}}\)), Chinmo protein is lost from feminizing cyst cells (K’, arrowheads). The remaining Chinmo protein observed in K’ represents Chinmo expression in the male germline. Vasa (red) marks germ cells and Tj (blue) marks somatic cells.
cells.
Scale bars = 20 μm.

(TIF)

S2 Fig. Three different dsx transcriptional reporters show variable expression in adult gonads. (A-B) Expression of dsx-gal4Δ2 in adult gonads. In the testis, dsx-gal4Δ2 is expressed in the entire CySC lineage (A). In the ovary, dsx-gal4Δ2 is expressed in escort cells, but not follicle cells (B).
(C-D) Expression of GMR40A05-gal4 in adult gonads. In the testis, GMR40A05-gal4 is expressed in escort cells, but not follicle cells (C). In the ovary, GMR40A05-gal4 is expressed in escort cells, but not follicle cells (D).
(E-F) Expression of dsxΔM103050-GFSTF.1 in adult gonads. In the testis, dsxΔM103050-GFSTF.1 is expressed weakly in the CySC lineage (E) and is undetectable in adult ovaries (F). Fas3 (red) marks testicular niche cells and ovarian follicle cells. Tj (blue) marks somatic cells in both gonads. Time point for all adults is 5 days post eclosion. Scale bars = 20 μm.

(TIF)

S3 Fig. DsxC antibody detects DsxF protein. Immunostaining of tj>dsxF ovaries reveals that DsxF protein is detectable by DsxC antibody (magenta). Tj (green) marks somatic cells. Scale bars = 20 μm.

(TIF)

S4 Fig. Blocking DsxF or TraF production genetically in females causes masculinization of the soma. (A-D) Blocking dsxF production using the dsxD/dsx1 heteroallelic combination masculinizes the soma of XX animals. Chromosomal sex of flies was determined based on inheritance of X-linked traits (eye color, w; cuticle color, y). Genotype for A (XX animal) is yw/y+w+; chinmoST/chinmoST; dsx1/TM2; for B (XX animal) is yw/y+w+; chinmoST/chinmoST; dsxF/dsx1; for C (XY animal) is yw/Y; chinmoST/chinmoST; dsx1/TM2; for D (XY animal) is yw/Y; chinmoST/chinmoST; dsxF/dsx1.
(E-H) Blocking traF production using tra1/Df(3L)st-j7, Ki1 masculinizes the soma of XX animals. Chromosomal sex of flies was determined based on inheritance of X-linked traits (eye color, w). Genotype for E (XX animal) is w/w+; chinmoST/chinmoST; tra1/TM6B, Tb; for F (XX animal) is w/w+; chinmoST/chinmoST; tra1/Df(3L)st-j7, Ki1; for G (XY animal) is w/Y; chinmoST/chinmoST; tra1/TM6B, Tb; for H (XY animal) is w/Y; chinmoST/chinmoST; tra1/Df(3L)st-j7, Ki1.

(TIF)

S5 Fig. Diagram of tra pre-mRNA and UAS-traFΔT2AGFP. In the transgene, most of the third exon of tra is replaced with self-cleaving T2A peptide and GFP, followed by a poly-adenylation signal (pA). Black shaded regions indicate exons. Red star indicates early stop codon in exon 2. Pink dashed lines indicate female-specific alternative splicing, and blue dashed lines indicate non-sex-specific default splicing.

(TIF)

S6 Fig. Chinmo mis-expression in ovaries leads to reduced traF and dsxF levels. (A) qRT-PCR analysis of homogenized ovaries demonstrates that mis-expression of chinmo in follicle cells leads to decreased levels of total tra, traF, and dsxF. Lower transcript levels were not due to a change in the relative abundance of somatic cells, as zfh1 levels were unaffected in tjTS>chinmo ovaries. The values were normalized to tubulin. Data are presented as the mean of three biological replicates. *** denotes p<0.001 as determined by two-tailed Student’s t-test. Error bars represent SEM.
(B) Semi-quantitative RT-PCR on RNA extracts from 5 male or 5 female larvae (first two
lanes), *tj*<sup>TS</sup>+ adult ovaries (third lane), and *tj*<sup>TS</sup>+*chinmo* adult ovaries (last lane). RNA from male larvae express *Sxl<sup>M</sup>* (first lane), while RNA from female larvae express *Sxl<sup>F</sup>* (second lane). Both *tj*<sup>TS</sup>+ (third lane) and *tj*<sup>TS</sup>+*chinmo* (last lane) ovaries express *Sxl<sup>F</sup>* exclusively. *Sxl*<sup>EM</sup> primers were used to differentiate between *Sxl<sup>M</sup>* and *Sxl<sup>F</sup>* mRNA isoforms in this experiment. α-tubulin (*tub*) was used as a loading control.

**S7 Fig. Tra<sup>F</sup> is necessary but not sufficient for CySC feminization.** (A-B) Zfh1 (blue) expression in *tj*<sup>TS</sup>+ (A) versus *tj*<sup>TS</sup>+*tra<sup>F</sup>* (B) testes. A and B represent single Z slices; A’ and B’ show maximal Z-max projections (Z-max) of Zfh1-expressing cells in the entire confocal stack. Fas3 (green) marks the niche.

(C-D) Tj (blue) expression in *tj*<sup>TS</sup>+ (C) versus *tj*<sup>TS</sup>+*tra<sup>F</sup>* (D) testes. C and D represent single Z slices; C’ and D’ show Z-max projections of Tj-expressing cells.

(E-F) EdU (blue)-labeled *tj*<sup>TS</sup>+ (E) and *tj*<sup>TS</sup>+*tra<sup>F</sup>* (F) testes. EdU-positive spermatogonial cysts are outlined. Tj (green) marks cyst cells. Arrowheads (E’) point to EdU-positive CySCs. Arrows (F’) point to EdU-positive differentiating cyst cells away from the niche. Asterisk marks the niche.

(G-H) Visualization of germ cell stages in *tj*<sup>TS</sup>+ (G) and *tj*<sup>TS</sup>+*tra<sup>F</sup>* (H) testes. α-spectrin (green) marks fusomes, which are dot- and dumbbell-shaped in early germ cells (G’, arrowheads) and become branched in later differentiating spermatogonia (G”, arrows). Note that the niche is not in the plane in G’. Tj (blue) marks cyst cells. Arrowheads in H’ indicate spermatogonia away from the niche that have dot and dumbbell shape fusomes in *tj*<sup>TS</sup>+*tra<sup>F</sup>* testes. Asterisk marks the niche.

(I-J) Quantification of Zfh1-expressing (I) and Tj-expressing (J) cells in *tj*<sup>TS</sup>+ (gray bars) versus *tj*<sup>TS</sup>+*tra<sup>F</sup>* (green bars) testes. *tj*<sup>TS</sup>+*tra<sup>F</sup>* testes contain significantly more Zfh1-expressing and Tj-expressing somatic cells than *tj*<sup>TS</sup>+ testes, as determined by single-factor ANOVA.

(K) Quantification of EdU-positive germ cells upon somatic *tra<sup>F</sup>* mis-expression. *tj*<sup>TS</sup>+*tra<sup>F</sup>* testes contain significantly fewer EdU-positive 4-cell and 8-cell spermatogonia than *tj*<sup>TS</sup>+ testes. For quantifications, * denotes p<0.05; ** denotes p<0.01; *** denotes p<0.001; **** denotes p<0.0001 as determined by single-factor ANOVA. Quantification data are presented as mean ± SEM.

Vasa (red) marks the germline in A-H. Scale bars = 20 μm.

**S8 Fig. Loss of vir and fl(2)d causes defects in the ovary but not the testis.** (A-C) *tj>*<sup>vir</sup><sup>RNAi</sup> (B) and *tj>*<sup>fl(2)d</sup><sup>RNAi</sup> (C) testes resemble control *tj>*+ (A) testes, showing no overt defects in testis development or spermatogenesis. Vasa (red) marks the germline, Tj (blue) marks somatic cells, and Fas3 (green) marks niche cells. Scale bars = 20 μm.

(D-E) Reproductive structures in adult *tj>*+ (D) and *tj>*<sup>vir</sup><sup>RNAi</sup> (E) females. Ovaries (D, brackets) and accessory structures like spermathecae (SP) (D, arrows) can be observed in *tj>*+ females. Ovaries, but not somatic accessory structures like SP and oviduct, fail to develop in females lacking *vir* in the somatic gonad (E, arrows).

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