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sisterless A is required for activation of *Sex lethal* in the *Drosophila* germline

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Abstract

Both somatic cells and germ cells must establish their correct sexual identity for proper gametogenesis. In *Drosophila,* sex determination in somatic cells is controlled by the switch gene *Sex lethal* (*Sxl*), which is activated in females by the presence of two X chromosomes. Though germline sex determination is much less well understood, *Sxl* is also essential for the female identity in germ cells. Loss of *Sxl* function in the germline results in ovarian germline tumors, a characteristic of male germ cells developing in a female soma. Further, *Sxl* expression is sufficient for XY (male) germ cells to produce eggs when transplanted into XX (female) somatic gonads. As in the soma, the presence of two X chromosomes activates *Sxl* in the germline, but the mechanism for "counting" X chromosomes in the germline is thought to be different from the soma. Here we have explored this mechanism at both *cis*- and *trans*-levels. Our data support the model that the *Sxl* "establishment" promoter (*SxlPE*) is activated in a female-specific manner in the germline, as in the soma, but that the timing of *SxlPE* activation, and the DNA elements that regulate *SxlPE,* are different in the germline. Nevertheless, we find that the X chromosome gene *sisterless A (sisA),* which helps activate *Sxl* in the soma, is also essential for *Sxl* activation in the germline. Loss of *sisA* leads causes of Sxl expression in the germline, and to ovarian tumors and germline loss. These defects can be rescued by Sxl expression, demonstrating that *sisA* lies upstream of *Sxl* in germline sex determination. We conclude that *sisA* acts as an X chromosome counting element in both the soma and the germline, but that additional factors regulating female-specific expression of *Sxl* in the germline remain to be discovered.

Author summary

The production of sperm and eggs requires proper sexual identity to be established in both somatic cells and the germ cells, which ultimately produce the gametes. While somatic sex determination has been well studied in a number of organisms, how germ cells establish their sexual identity is much less well understood. In Drosophila, the RNA binding protein Sex lethal (Sxl) is essential for female sexual identity in both the soma and the germline, but its regulation in the germline is thought to be different than in the soma. Here we explore how *Sxl* is activated in the germline. We find that the germline uses a different set of DNA elements to control activation of the key sex-specific *Sxl* promoter. Nonetheless, one of the activators of *Sxl* in the soma, the transcription factor Sisterless A (SisA), also acts to activate *Sxl* in the germline. Our data indicate that, while SisA acts as a common activator in both the soma and germline, additional, germline-specific *Sxl* activators remain to be discovered.

eLife assessment

This **useful** study reports that the Drosophila transcription factor sisterless A (sisA) regulates the expression of Sex-lethal (Sxl) in female germ cells. The data supporting claims regarding the genetic requirement of sisA are **convincing**, but the characterization of the cis-regulatory elements controlling Sxl expression in the female germline is viewed as **incomplete**. The work will be of significant interest to colleagues studying reproductive biology and sex determination.

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Introduction

Sex determination influences the development of many different tissues, but is particularly important in the germline to control the production of either sperm or eggs. While in some species somatic sex is sufficient to determine germline sex via inductive signaling, in flies and humans, the sex chromosome karyotype also plays a role intrinsically in the germline. Since a failure to match germline and somatic sex leads to defects in gametogenesis, understanding how intrinsic sex determination is regulated in the germline is important for our understanding of gonad development, reproductive biology, and human health.

Sex determination in *Drosophila* is under the control of the switch gene *Sex lethal* (*Sxl*) which is activated in females by the presence of two X chromosomes [1–11]. *Sxl* is both necessary and sufficient for the female sexual identity of somatic cells [3,6,12–19]. Expression of *Sxl* in the soma is regulated by two different promoters [20,21]. The *Sxl* "establishment promoter" (*SxlPE*) is sex-specific and is activated by the presence of two X chromosomes [2]. Default splicing of the *SxlPE* transcript produces a pulse of 'Early' Sxl protein starting at embryonic nuclear cycle 12. Later, at nuclear cycle 14, dosage compensation equalizes X chromosome expression in males and females and so X chromosomes can no longer be counted. Thus, there is a transition to the "maintenance promoter," *SxlPM*, which is activated in both sexes [20,21]. The transcript from *SxlPM* requires alternative splicing regulated by the early Sxl protein (only made in females) in order to produce more 'Late' Sxl protein, creating an auto-regulatory loop that maintains Sxl expression in females [3]. The initial, female-specific activation of *SxlPE* in the soma is in direct response to a diploid dose of X-linked Signaling Elements (XSEs) – genes on the X chromosome that activate *SxlPE* when present in two copies, but not one. The somatic XSEs include the transcription factors Sisterless A (SisA), Sisterless B (SisB) and Runt, along with the JAK/STAT ligand Unpaired (Upd) [9,22–30].

Unlike primary sex determination in the soma, which is autonomously dependent on a cell's sex chromosome genotype, sex determination in the *Drosophila* germline is regulated by non-autonomous signals from the soma in addition to the sex chromosome make-up of the germline (reviewed in [31]). In order for proper gametogenesis to occur, the "sex" of the germ cells has to match the sex of the soma. For example, XX germ cells developing in a male soma result in a testis with a severely atrophic germline [32–45], while XY germ cells developing in a female soma produce an ovary with a tumorous germline ("ovarian tumor") and a complete failure to produce eggs [32,46–48]. Similar to the soma, *Sxl* is both necessary and sufficient for determining the autonomous component of germline sexual identity. *Sxl* loss-of-function in the germline results in ovarian germline tumors, similar to male germ cells developing in a female soma [32,33,37,39,42,46,48,49]. Further, XY (male) germ cells expressing *Sxl* are able to produce eggs when transplanted into XX (female) somatic gonads, demonstrating that *Sxl* is

sufficient for female sexual identity in the germline as long as the surrounding soma is female [50]. Female-specific Sxl expression in the germline is dependent on the X chromosome dose: XX germ cells express Sxl while XY germ cells do not [32,39]. Evidence also indicates that *SxlPE* is important for female-specific expression of Sxl in the germline. Transcript data from early germ cells show a *Sxl* RNA that matches the *SxlPE* transcript in the soma [50], and data indicate that the positive auto-regulation of *Sxl* expression that occurs in the soma also acts in the germline [49]. However, the mechanism for activating *Sxl* expression in the germline appears to be different from the soma [33,46,50–54]. When germ cells simultaneously heterozygous for *Sxl* and the somatic XSEs *sisA*, *sisB*, and *runt*, were transplanted into wildtype female embryos, they were still able to undergo oogenesis indicating that the germ cells were not masculinized, even though this same genotype is sufficient to masculinize somatic cells [52]. Further, germ cells homozygous for mutations in *sisB*, or maternally deficient for the *sisB* co-factor *daughterless*, can also make normal eggs [33,46]. Thus, different XSEs, or at least a different combination of XSEs, is required to activate Sxl expression in the germline.

Here we utilize a combination of approaches to investigate the female-specific activation of *Sxl* in the germline. Using RNA FISH and CRISPR-tagging of specific Sxl isoforms, we show that the timing of *SxlPE* activation relative to *SxlPM* is different in the germline than in the soma. Using promoter/enhancer reporter constructs, we demonstrate that the regulatory sequences required for female-specific activation of *SxlPE* in the germline are different from those required in the soma. Lastly, we show that the somatic XSE, *sisA*, is also required for female-specific expression of Sxl in the germline. Together our data support a model in which *sisA* acts as a germline XSE, but that additional factors, different from the somatic XSEs, are also important for *Sxl* activation in the female germline.

Results

Analysis of *Sex lethal* transcription in PGCs

Since the activation of *SxlPE* appears to be the key sex-specific decision in germline sex determination, similar to what is observed in the soma, we first decided to examine when and how *SxlPE* is activated in the germline. We hypothesized that, as in the soma, *SxlPE* would be activated prior to *SxlPM*. To study this, we performed RNA fluorescent *in situ* hybridization (FISH) using oligopaints to examine nascent RNA (nRNA) being transcribed from *SxlPE* and *SxlPM* at the *Sxl* locus. As *SxlPM* is upstream of *SxlPE*, we generated one set of probes that exclusively targets *SxlPM*-derived transcripts (*PM*-probe, Cy5, Green), and another that targets the common transcript from both *SxlPE* and *SxlPM* (*Com*-probe, Cy3, Red) (**Fig. S1A**). Thus, if *SxlPE* alone is active, we should observe signal from the *Com*-probe (Red) but not the *PM*-probe (Green), while if *SxlPM* is active we should observe signal from both probes (and cannot make a conclusion about *SxlPE*).

In the soma, female-specific activation of *SxlPE* occurs prior to expression of *SxlPM*. Consistent with this, in the female soma we initially observed RNA FISH signals as fluorescent nuclear foci with the *Com*-probe but not the *PM*-probe, indicating that only *SxlPE* was activated (**Fig. S1C**-**S1C''**). Subsequently, we observed signals from both probes, indicating that *SxlPM* had been activated (Data not shown). Conversely, in the male soma, we always observed a signal from both the *PM*- and *Com*-probes simultaneously (**Fig. S1B**-**S1B''**), indicating that that *SxlPE* is not expressed (or does not precede *SxlPM* activation). We observed two *Sxl* foci per nucleus in females and only one focus in males, which was expected as *Sxl* is an X chromosome locus. These data are consistent with the previous understanding of *Sxl* activation in the soma - that *SxlPE* is on initially only in females followed by *SxlPM* activation in both sexes.

In contrast, in both female and male germ cells, we only observed RNA FISH signals from both the *PM*-probes and the *Com*-probes simultaneously, and never from the *Com*-probe alone (**Fig. 1A**-**1B''**). This indicates that *SxlPE* is not activated before *SxlPM* in the germline. (Note: we only observed a single focus in both XX and XY germ cells suggesting that the X chromosomes may be paired in the germline.) The simultaneous expression of *PM*-probes and *Com*-probes occurred in a subset of female germ cells at stage 5 (11.1%), and increased to 100 % of germ cells by stage 11 (**Fig. 1C**). Similarly, both probe sets were expressed simultaneously in a subset of male germ cells, starting at stage 5 (7%) and increased to 100% of germ cells by stage 11 (**Fig. 1D**). The gradual increase in the number of cells expressing *SxlPM* transcripts is similar to what is observed in the soma [21]. We conclude that, unlike in female somatic cells, *SxlPE* does not precede *SxlPM* activity in the germline, but instead is likely activated either simultaneously with, or later than, *SxlPM*. This is still consistent with a model where 'Early Sxl' protein produced from default splicing of the *SxlPE*-derived transcript is required for productive RNA splicing of the *SxlPM*-derived transcript, and thus for the subsequent production of 'Late Sxl' protein; even if *SxlPM* is active prior to *SxlPE* activation, these transcripts would not be able to produce Sxl protein until *SxlPE* is activated.

SxlPE activity in the germline requires different *cis*-regulatory elements than in the soma

We next wanted to identify if and when *SxlPE* is activated in the germline, and also to compare the *cis*-regulatory logic of *SxlPE* activation in the germline to that in the soma. To do this we used transcriptional reporter constructs specific for *SxlPE.* We first tested a *SxlPE*-EGFP transcriptional reporter that contains the 1.5kb somatic enhancer immediately upstream of *SxlPE* (*SxlPE*-1.5kb), which had previously been reported to recapitulate both somatic and germline *SxlPE* activity [50,74]. Contrary to previous reports, we did not observe EGFP expression from *SxlPE*-1.5kb in developing PGCs, even though *SxlPE*-1.5kb showed sex-specific EGFP expression in somatic cells (Data not shown). Thus, we conclude that the *cis*-regulatory region sufficient for sex-specific expression of *SxlPE* in the soma is insufficient for *SxlPE* expression in the germline. We next extended the *SxlPE* transcriptional reporter to contain the entire 5.2kb genomic sequence upstream of *SxlPE* (but excluding *SxlPM*) (*SxlPE*-5.2kb, **Fig. S2A**). Once again, even though we observed female-specific nuclear EGFP expression from the *SxlPE*-5.2kb reporter in somatic cells, we found no evidence of EGFP expression in the germline of either male or female embryos up to stage 15 (**Fig. S2B**, **S2F**). To accommodate the possibility of a delay between the activation of the *SxlPE*-5.2kb transgene and our ability to detect EGFP expression (as is observed in the somatic cells), we also characterized its expression at later stages of development. However, we failed to observe any germline EGFP expression at the first, second, or third larval instar (L1, L2, or L3 respectively) stages (**Fig. S2B**-**S2I**). Thus, *SxlPE*-5.2kb also does not contain the *cis*-regulatory elements sufficient for *SxlPE* activity in the germline. Interestingly, *SxlPE*-5.2kb exhibited sex-specific EGFP expression generally in the soma until L1, and this expression persisted in the somatic gonad until L2 (**Fig. S2F**-**S2H**). This was unexpected as *SxlPE* has been reported to shut off in the soma following activation of dosage compensation in the early embryo. Our observations could be due to the stability of the EGFP reporter used or may point at a possible mechanism that keeps *SxlPE* active even after X chromosomes can no longer be counted.

We next generated a *SxlPE*-EGFP reporter that includes the 5.2kb sequence discussed above, as well as an additional 5kb downstream of *SxlPE* up to the start of Exon 4 (**Fig. S2A**) (*SxlPE*-10.2kb). Flies carrying this transgene showed sex-specific nuclear EGFP expression in the soma by stage 15 of embryogenesis (**Fig. S2J**-**S2K**). Excitingly, we also observed sex-specific nuclear EGFP expression in the female germline during the first larval instar (L1) stage (GFP immunostaining **Fig. 2A**-**2B'**, endogenous GFP and quantification, **Fig. 6F,G,I**). Together, these data suggest that *SxlPE* activity in the germline is sex-specific as it is in the soma, and that it requires additional *cis*-regulatory elements that lie downstream of *SxlPE*. Further, germline *SxlPE*

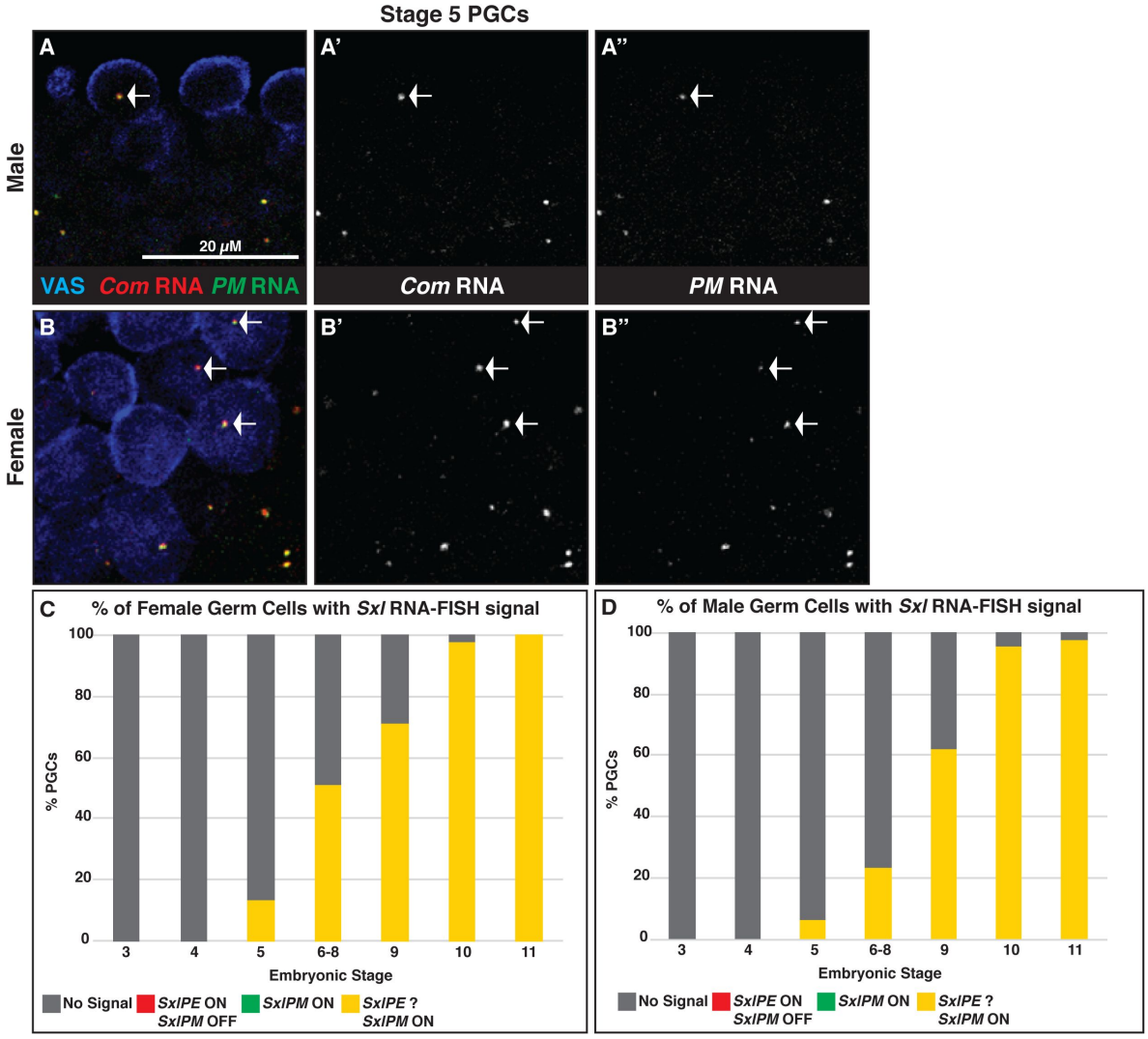

Figure 1

SxlPE activity does not precede *SxlPM* in the germline

A-B) RNA-FISH (+ Immunofluorescence) against the transcript from *SxlPM* only (*PM* RNA) versus the common transcript from *SxlPE* and *SxlPM* (*PE+PM* RNA) in embryonic stage 5 primordial germ cells (PGCs). A) Male PGCs with both *PE+PM* and *PM*-probe signals. B) Female PGCs with both *PE+PM* and *PM*-probe signals. Arrows mark fluorescent nuclear foci of RNA-FISH signals. Note that a single focus is observed in female PGCs suggesting that X chromosomes may be paired in the germ cells. VAS stains germ cells. C-D) Graphs showing percentage of PGCs with observable RNA-FISH probe signals between stages 3 and 11 of embryogenesis.

activation appears to occur later in development than previously thought (L1), although it is possible that there is a delay between when *SxlPE* is actually activated and when we are first able to detect EGFP expression.

Late Sex lethal protein is expressed only after *SxlPE* is activated in the germline

Since *SxlPE* is sex-specifically activated in the germline, we hypothesized that, like in the soma, Early Sxl protein derived from *SxlPE* would be required to produce Late Sxl protein derived from *SxlPM*. To study expression of the Early and Late Sxl proteins in the germline, we used CRISPR-Cas9 genome editing to separately tag Sxl's Early and Late protein products. We inserted a 2x human influenza hemagglutinin (2xHA) epitope at the N-terminus of the coding sequence of exon E1, which is specific to the mRNA produced by *SxlPE*, to generate a HA:SxlE1 'Early (E) Sxl' tag (**Fig. S3A**). Separately, we inserted a 3xFLAG epitope at the N-terminus of the coding sequence of exon L2, which is specific to the mRNA produced by *SxlPM*, to generate a FLAG:SxlL2 'Late (L) Sxl' tag (**Fig. S3A**) (Note: these tags are in separate stocks). Both of these tagged alleles of *Sxl* appear wildtype for *Sxl* function as homozygous females are viable and fertile.

Using anti-HA antibody, we were able to observe female-specific Sxl Early protein in somatic cells beginning at stage 9, and this expression was primarily nuclear, consistent with Sxl's role in alternative splicing. Early Sxl expression in the soma persisted until the L1 stage (**Fig. S3B**-**S3G**), and no expression was observed after this time. In addition to the somatic gonadal tissue, we confirmed Early Sxl expression in the L1 stage in another somatic tissue, the developing gut (**Fig. S3H**-**S3I**). The continued presence of Early Sxl protein at the L1 stage is consistent with what we observed with the *SxlPE*-10.2kb reporter and suggests that Early Sxl protein is expressed, or at the very least maintained, much later in development than previously thought, and well after dosage compensation has been initiated. To our surprise, we were unable to detect anti-HA immunolabeling in the developing germline at any stage examined (embryo, and L1-L2). A low level of signal was observed in the cytoplasm of the germline (arrows in **Fig. 2C-D'**), but this was not clearly different between males and females, and may represent background staining. Detection of Early Sxl in the soma required tyramide amplification of the antibody signal, and so Early Sxl protein may be expressed at very low levels, which was undetectable in the germline even after tyramide amplification. Regardless, this prevents us from making a conclusion about Early Sxl protein expression in the germline.

Using the FLAG-tagged Late Sxl allele (FLAG:SxlL2), we observed anti-FLAG immunolabeling in female germ cells starting at the second larval instar (L2) stage (**Fig. 2E**-**2F'**). Female germ cells remained positive for anti-FLAG labeling through to adulthood and expression patterns were consistent with anti-Sxl antibody labeling (**Fig. S3J**-**S3M'**), which can be used to visualize Sxl in the germline starting at the third larval instar stage and onwards (Data not shown). No anti-FLAG immunoreactivity was observed in male germ cells. Though our RNA FISH analysis indicates that expression of *SxlPM* begins in the germline at stage 5, and is active in most germ cells by stage 10 (**Fig. 1C**), the *SxlPE* reporter (*SxlPE*-10.2kb) is only detectable in L1 germ cells (**Fig. 2B**), indicating that *SxlPE* expression begins much later in the germ cells than the soma. This is consistent with our ability to detect Late Sxl protein only in L2 germ cells; if Early Sxl protein is required to splice *SxlPM* transcripts to produce Late Sxl protein, then we would expect to detect Late Sxl protein only after *SxlPE* is active in the germline.

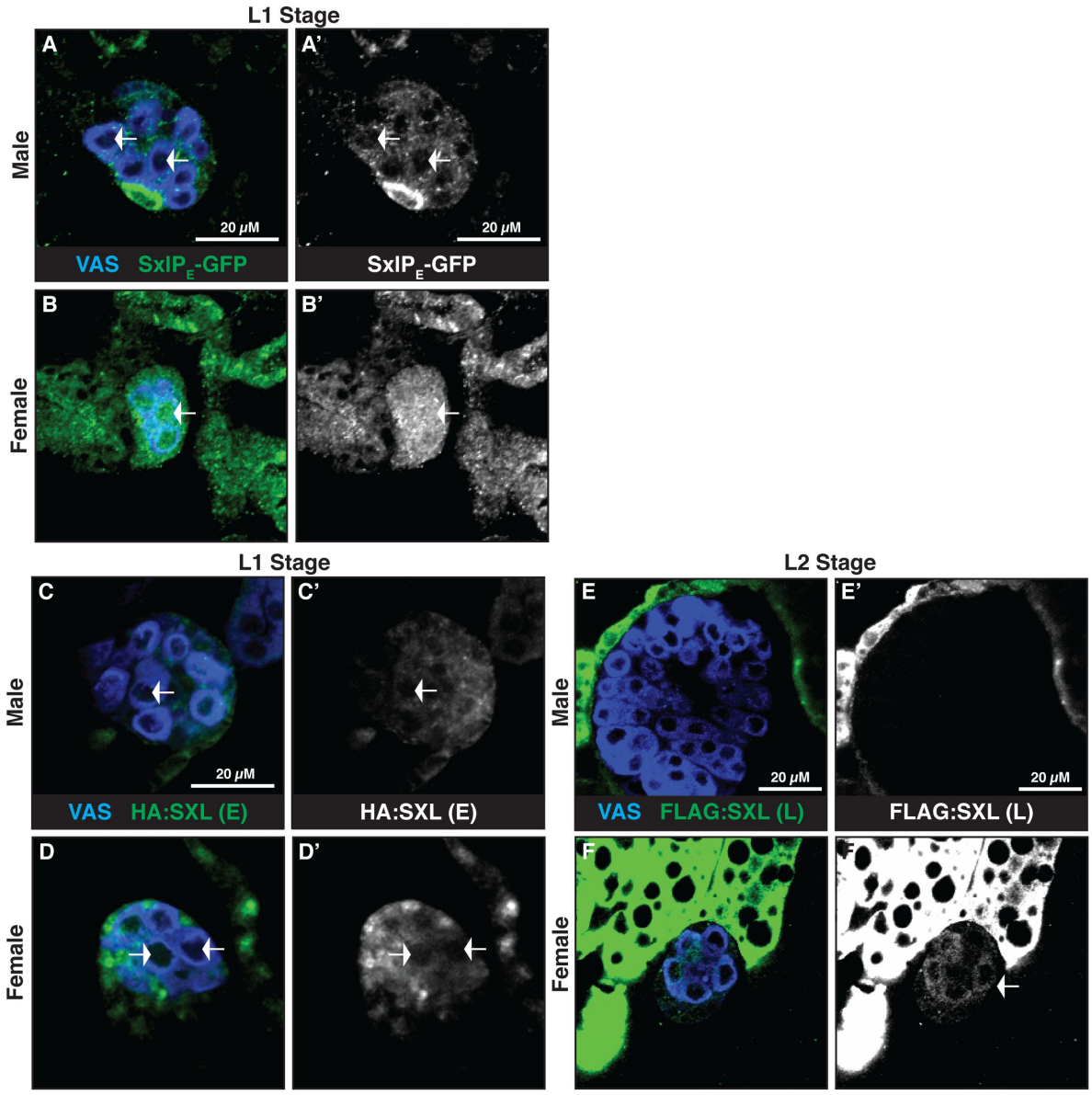

Figure 2

SxlPE is sex-specifically activated in the germline

A-B ') Immunofluorescence of first instar (L1) gonads from flies bearing *SxlPE*-10.2kb reporter transgene. Note the presence of sex-specific nuclear GFP expression in female germ cells only. Arrows mark nuclei of germ cells. C-D') Immunofluorescence of first instar (L1) gonads from flies bearing the HA:SxlE1 (Early Sxl) tag. Arrows mark cytoplasmic HA-background staining. E-F') Immunofluorescence of second instar (L2) gonads from flies bearing the FLAG:SxlL2 (Late Sxl) tag. Arrows mark FLAG-positive germ cells. Note the presence of FLAG (Late Sxl) expression in female germ cells only. The anti-FLAG immunoreactivity in the fat body of both sexes is also present in wild-type stocks with no FLAG-tagged proteins, indicating it is antibody background. VAS stains germ cells.

sisterless A loss-of-function in the female germline results in ovarian tumors and germline loss

We next wanted to investigate the *trans*-acting factors that regulate *Sxl* in the germline. As discussed above, previous work indicates that germ cells use a different combination of XSEs than the soma. However, it remains possible that some of the individual somatic XSEs contribute to the X chromosome counting mechanism in the germline, perhaps along with unknown germline-specific XSEs. To investigate the potential role of the individual somatic XSEs in germline *Sxl* activation, we knocked down their expression specifically in the germline using RNAi (*nanos*-GAL4; UAS-*sisA* RNAi, UAS-*sisB* RNAi, UAS-*sisC* RNAi, or UAS-*runt* RNAi). We then immunolabeled adult gonads to look for phenotypes resembling *Sxl* loss of function, such as the formation of ovarian tumors. We found that RNAi knockdown of *sisB*, *sisC*, or *runt* in the germline did not result in any aberrant ovarian phenotypes (**Fig. S4A**-**S4D'**). Germ cells in these ovaries appear to differentiate properly as indicated by the progressive increase in the size of their nuclei, characteristic of polyploid nurse cells during oogenesis.

In contrast, *sisA* germline RNAi (*sisA* RNAi 1) resulted in ovaries that exhibited ovarian germ cell tumors (**Fig. 3C**-**3C'**, **S5C**, **S5D**) similar to those observed in *Sxl* LOF (**Fig. 3B**-**3B'**, **S5C**) or when XY germ cells develop in an XX soma [32,46–48]. While a high percentage of ovaries exhibited germline tumors (**Fig. S5C**), not all ovarioles in each ovary were tumorous. To confirm that this phenotype was not due to off-target effects sometimes seen with RNAi, we generated an additional UAS-*sisA* RNAi transgene (*sisA* RNAi 2) (**Fig. S5D**). Expression of this RNAi line in the germline also produced ovarian germ cell tumors (**Fig. S5B**-**S5B'**, **S5D**), but these tumors were less severe and occurred with lower frequency (**Fig. S5C**). An additional UAS-*sisA* RNAi transgene did not result in any observable phenotypes (**Fig. S5D**, Data not shown). When 2 copies of *nanos*-GAL4 were used to drive increased expression of UAS-*sisA* RNAi 1, we observed severe germline loss resulting in germ cell-less ovaries (**Fig. 3D**-**3D'**, **S5C**) and subsequent infertility. This is similar to what is observed in strong alleles of other sex determination genes such as *ovo* and *ovarian tumor (otu)*, as well as when XY germ cells develop in an XX soma [40,75–79].

Next we wanted to investigate the null phenotype of *sisA* in the germline. Since homozygous *sisA* mutant females and hemizygous *sisA* mutant males are embryonic lethal [57], we generated *sisA* germline-specific loss of function mutations using tissue-specific CRISPR-Cas9 genome editing ("G0 CRISPR", [69–71]). Male flies that ubiquitously express either 2 or 4 different guide RNAs (gRNAs) targeting the *sisA* gene region (**Fig. S5D**) were mated to females expressing Cas9 in the germline under the *nanos* promoter. F1 females from this cross also exhibited ovarian tumors as well as germ cell-less ovaries (**Fig. 3E**-**3G'**, **S5C**), similar to what was observed using RNAi (**Fig. 3C-D'**). Taken together, these data indicate that *sisA* is required for female germline differentiation and maintenance. Additionally, germline *sisA* loss of function is similar to *Sxl* loss of function, suggesting *sisA* is a candidate XSE for *SxlPE* activation in the germline.

sisterless A expression in the embryonic germline precedes *Sex lethal* activation

In order for *sisA* to act as an XSE for activating *Sxl* in the germline, it should be expressed zygotically in germ cells prior to sex-specific activation of *SxlPE* in the germline. It has been reported that *sisA* mRNA is excluded from the nuclei that bud off to form pole cells [28]. However, reported microarray expression data demonstrates that *sisA* transcripts are enriched in PGCs at the 1-to-3 hour time point [80]. To observe zygotic transcription of *sisA* in the germline we utilized RNA FISH against nRNA being transcribed from the *sisA* locus. As a validation of our approach, we observed signals from *sisA* RNA FISH probes as fluorescent nuclear foci in somatic nuclei starting at nuclear cycle 8 (**Fig. S6A**-**S6A'**) and also at later stages in yolk cell nuclei,

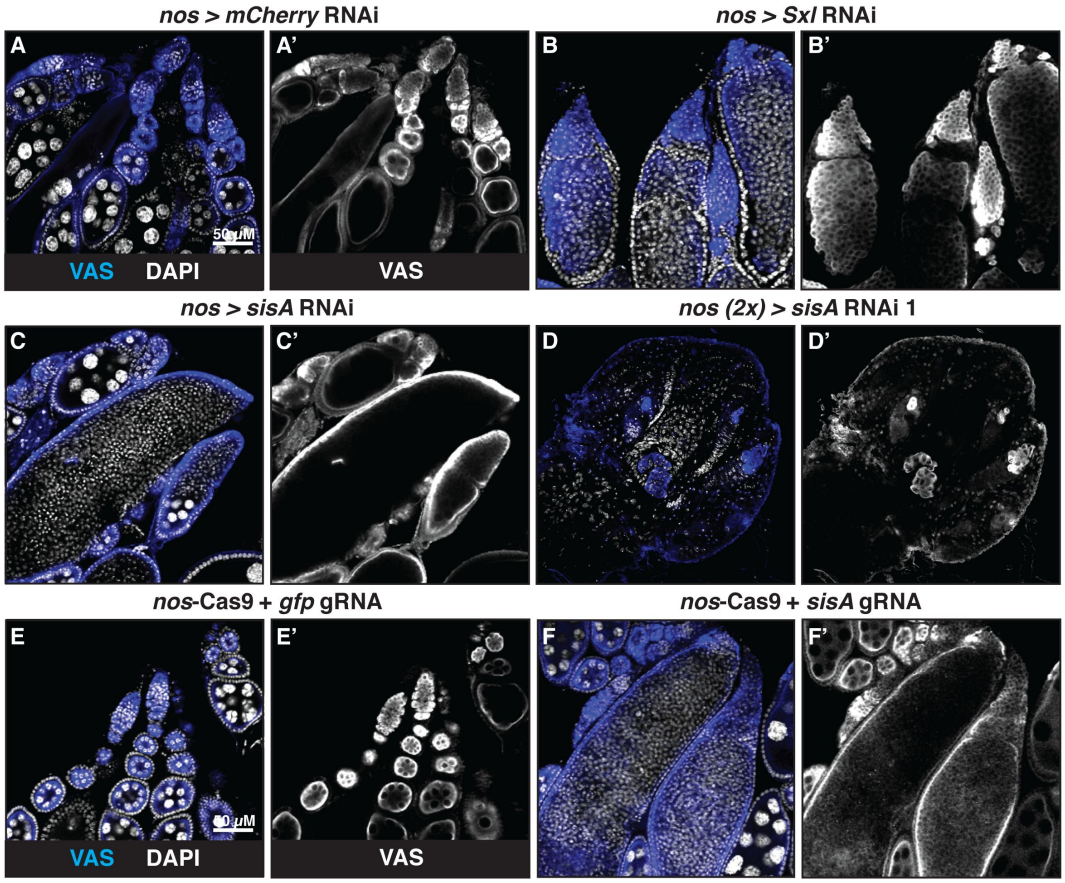

Figure 3

sisA loss of function in the female germline results in ovarian tumors and germ cell loss

A-F') Immunofluorescence of adult ovaries to characterize germ cell phenotypes. A-A') Wildtype ovary from fly with germline-specific *mCherry* RNAi as control. B-B') Ovary with germ cell tumors from fly with germline-specific *Sxl* RNAi. Knockdown of *Sxl* causes a germline tumor phenotype. C-C') Ovary with germ cell tumors from fly with germline-specific *sisA* RNAi (*sisA* RNAi 1). D-D') Ovary with severe germ cell loss from fly with strong germline-specific *sisA* RNAi (2x GAL4). E-G) Animals expressing Cas9 in the germline along with guide RNAs for control (*gfp*) or *sisA* to create *de novo* "G0" mutations in the germline. *E*-E') Wildtype ovary from fly with control guide RNAs for *gfp*. F-F') Ovary with germ cell tumors from fly with guide RNAs generating mutations in *sisA*. G-G') Germ cell-less ovary from fly with guide RNAs generating mutations in *sisA*. VAS stains germ cells. DAPI stains DNA (nucleus).

consistent with previous reports (**Fig. S6B**-**S6B'**) [28,57]. Interestingly, we were able to detect *sisA* RNA FISH signals in PGCs at stages 3-6 (3-5 shown in **Fig. 4A**-**4C'**). At later stages, the strong expression levels of *sisA* RNA in yolk cell nuclei made it difficult to distinguish fluorescent nuclear foci in neighboring PGCs. This suggests that *sisA* is indeed zygotically transcribed in the early embryonic germline. We quantified this expression in sexed embryos and found that *sisA* is expressed in the PGCs of both sexes (**Fig. 4D**).

We next wanted to observe SisA protein in the germline. Since no antibodies against SisA have been generated to date, we used CRISPR-Cas9 genome editing to insert a super-folder Green Fluorescent Protein (sfGFP) epitope tag at the N-terminus of the SisA protein (sfGFP:SisA) (**Fig. S6C**). Importantly, the epitope tag does not appear to affect SisA function as homozygous females and hemizygous males were viable and had no observable gonad phenotypes (**Fig. S6D**-**S6E'**). Furthermore, anti-GFP immunolabeling is restricted to the nucleus, which is consistent with the predicted function of SisA as a bZIP transcription factor. In somatic cells, we observed expression of sfGFP:SisA in pre-blastoderm syncytial nuclei (**Fig. S6F**-**S6F'**) and in yolk cell nuclei (**Fig. S6G**-**S6G'**), consistent with somatic *sisA* RNA expression.

Excitingly, similar to what we observed using RNA FISH, sfGFP:SisA expression was also observed in PGCs at stage 3, 4, and 5 (**Fig. 4E**-**4G'**). Additionally, this expression persisted in PGCs until stage 10 (**Fig. S6H**-**S6H'**). This late expression of SisA is consistent with its potential role in acting as an XSE for *SxlPE* activation in the germline. Like with *sisA* RNA, we were only able to detect sfGFP:SisA in a subset of PGCs in any one embryo. sfGFP:SisA expression was not observed later in first, second, or third larval instar stages, in either the germline or the soma (Data not shown). We conclude that SisA is expressed in the embryonic germline and this expression precedes the activation of *SxlPE*, which is consistent with a role as a germline XSE for the activation of *Sxl*.

sisterless A is required for expression of Sxl in the female germline

If *sisA* acts as an activator of *Sxl* in the female germline, we would expect a loss of Sxl expression upon loss of *sisA* function. Strikingly, using anti-Sxl immunolabeling, we observed that the germ cell tumors in *sisA* RNAi ovaries had a strong reduction in Sxl expression when compared with controls (**Fig. 5A**-**5C'**, **S7A**-**S7B'**). Tumorous germ cells that were mutant for *sisA* using G0 CRISPR also had lower levels of Sxl expression (**Fig. S7C**-**S7D'**). Somatic Sxl expression remained unaffected in both cases. To examine Sxl expression specifically in those germ cells that express highest levels of Sxl (i.e. the early, undifferentiated germline), we performed *sisA* RNAi in the germline of *bag of marbles* (*bam*) mutants, which are enriched for germ cells robustly expressing Sxl (**Fig 5D**-**5D'**). We found that knocking down *sisA* using RNAi led to a dramatic reduction of Sxl antibody labeling in the germ cells of *bam* mutant ovaries (**Fig. 5E**-**5E'**), demonstrating that knocking down *sisA* leads to a loss of Sxl expression in the germline. To test whether *sisA* acts as a regulator of the Sxl PE promoter, we examined the effects of knocking down *sisA* function in the germline (*nos>sisARNAi)* on the expression of the Sxl PE-GFP reporter (*SxlPE*-10.2kb). Indeed, loss of *sisA* caused a significant reduction of germline GFP expression from the Sxl PE-GFP reporter, but did not reduce expression down to levels observed in males (**Fig. 6F-I**). This is consistent with SisA being one of, but not the only, regulator of Sxl PE expression in the germline.

These data show that *sisA* is necessary for Sxl expression in the germline, and we next wanted to test whether ectopic expression of *sisA* would be sufficient to promote Sxl expression. Expression of UAS-*Sxl* in the male germline leads to inhibition of the JAK/STAT pathway and loss of germline stem cells ([86] and **Fig. 6B**-**6B'**). Expression of UAS-*sisA* did not lead to detectible expression of Sxl in the testis (**Fig. 6C**-**6C'**). This indicates that the overexpression of *sisA* alone is not sufficient to activate *Sxl* in the germline and that, like in the soma, additional XSEs are required to activate *SxlPE* in the germline.

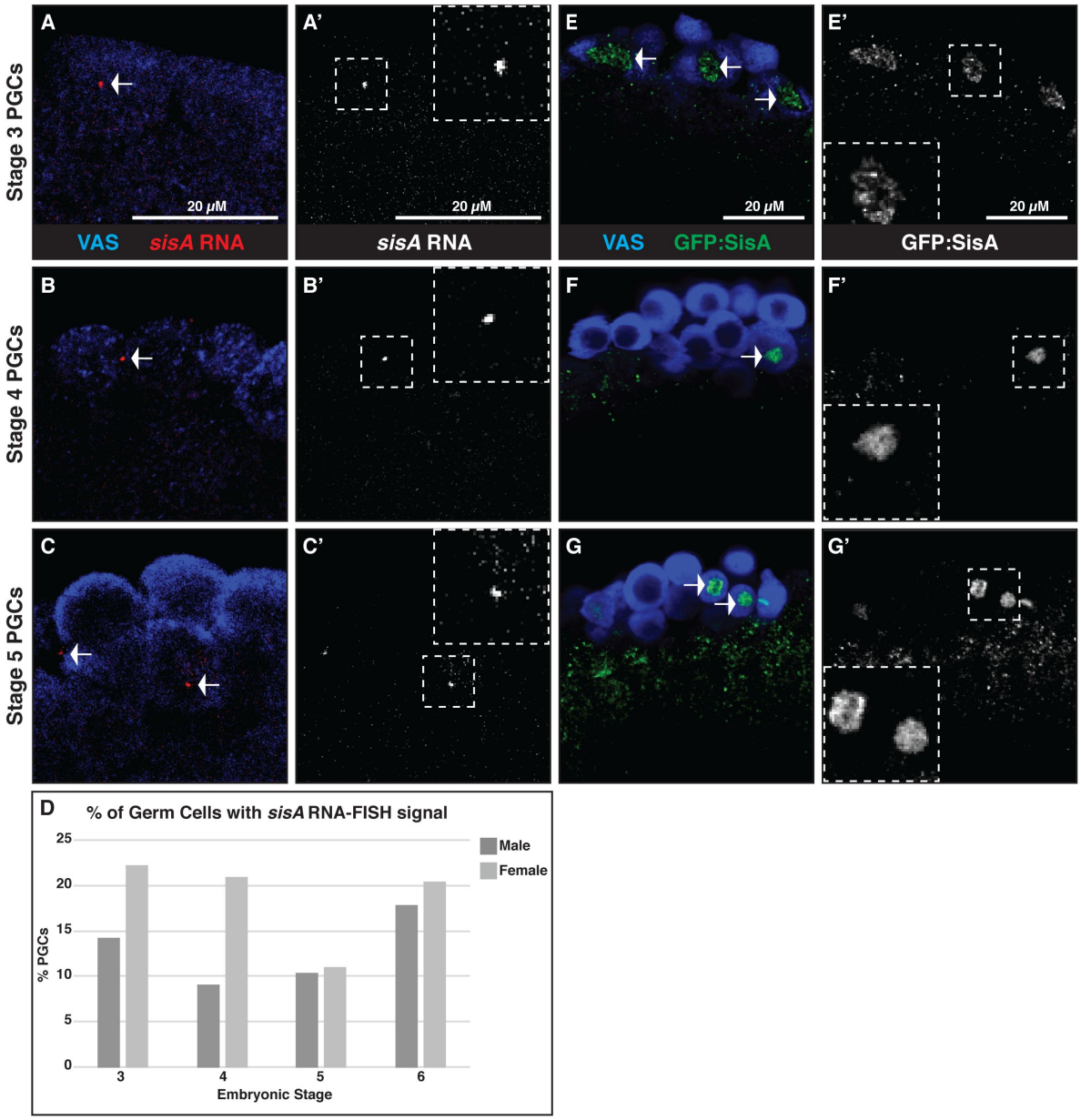

Figure 4

sisA is zygotically expressed in the germline

A-C ') RNA-FISH (+ Immunofluorescence) against *sisA* in embryonic PGCs (Stages 3-5 shown). Smaller white dashed boxes specify the region zoomed in on, and presented in larger white dashed boxes. Arrows mark fluorescent nuclear foci of RNA-FISH signals. Note that a single focus is observed in female PGCs suggesting that X chromosomes may be paired in the germ cells. VAS stains germ cells. D) Graph showing percentage of PGCs with observable RNA-FISH probe signals between stages 3 and 11 of embryogenesis. *sisA* is zygotically expressed in PGCs of both sexes. E-G') Immunofluorescence of embryonic PGCs (Stages 3-5 shown) from flies bearing sfGFP:SisA (SisA tag). Note that this expression is nuclear, consistent with SisA's characterization as a bZIP transcription factor. Arrows mark GFP-positive germ cell nuclei. VAS stains germ cells.

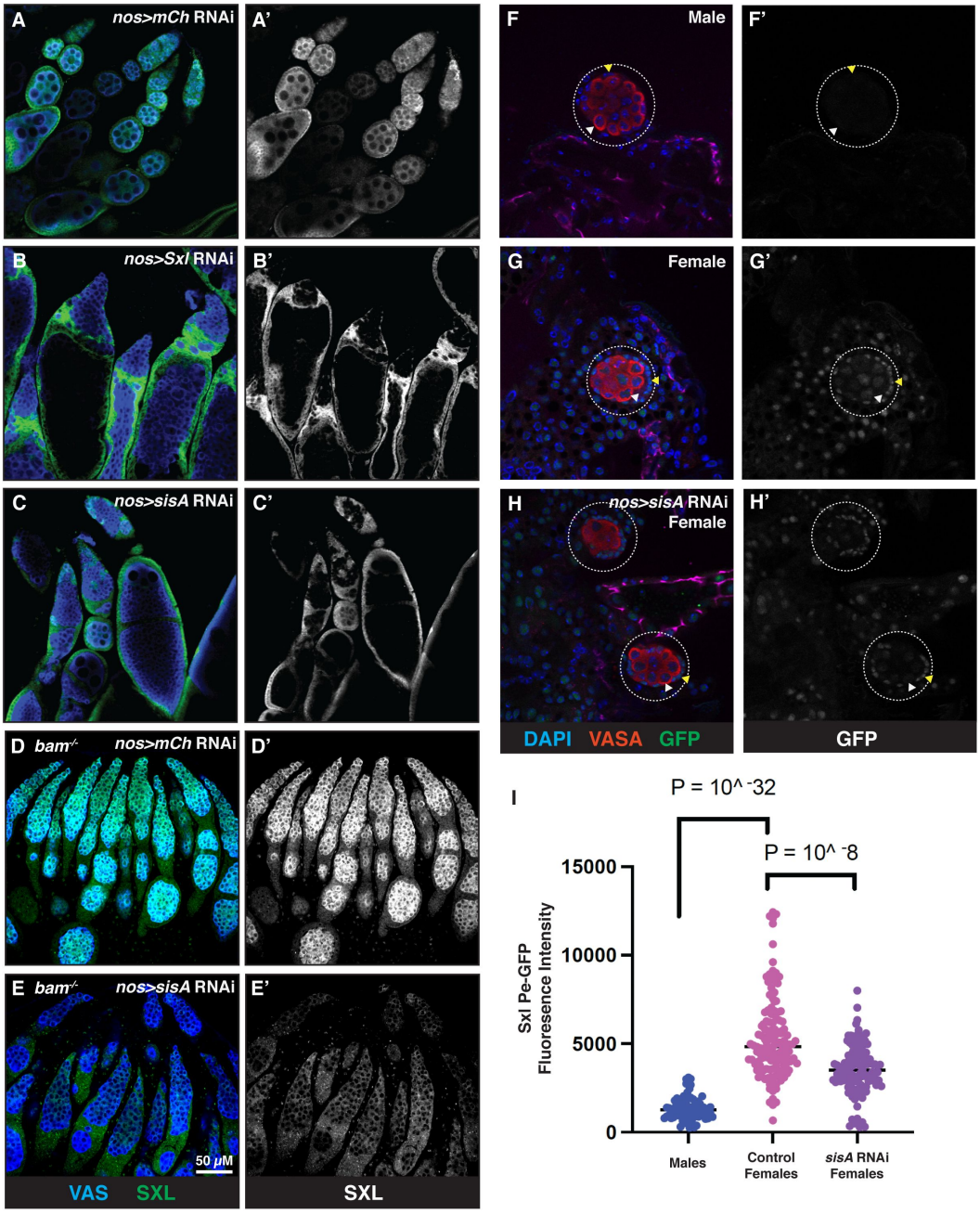

Figure 5

Sxl expression in the female germline depends on *sisA*

A-E') Immunofluorescence of adult ovaries using anti-Sxl antibody (green). Anti-Vas stains the germ cells. A-A') Wildtype ovary from fly with germline-specific *mCherry* RNAi. Sxl staining is highest in the early germ cells and decreases in differentiating germ cells. B-B') Ovary with germ cell tumors from fly with germline-specific *Sxl* RNAi. Knockdown of *Sxl* causes a germline tumor phenotype and germ cells lack Sxl staining. C-C') Ovary with germ cell tumors from fly with germline-specific *sisA* RNAi. Note that tumorous germ cells lack Sxl staining. Somatic Sxl remains unaffected. D-D') *bam* mutant ovary with germline-specific *mCherry* RNAi. *bam* mutations cause a germline tumor phenotype and an expansion of germ cells that highly express Sxl. E-E') *bam* mutant ovary with germline-specific *sisA* RNAi. Sxl staining is dramatically reduced in the germline. F-G') Visualization of endogenous GFP expression (Green) from the *SxlPE*-10.2kb reporter in L1 stage males (F, F'), females (G, G') and females expressing *sisA RNAi* in the germline (*nos>sisARNAi)*. White arrowheads indicate examples of germ cells and yellow arrowheads indicate somatic gonadal cells. H) Quantification of GFP fluorescence intensity in germ cells from samples as in F-G.

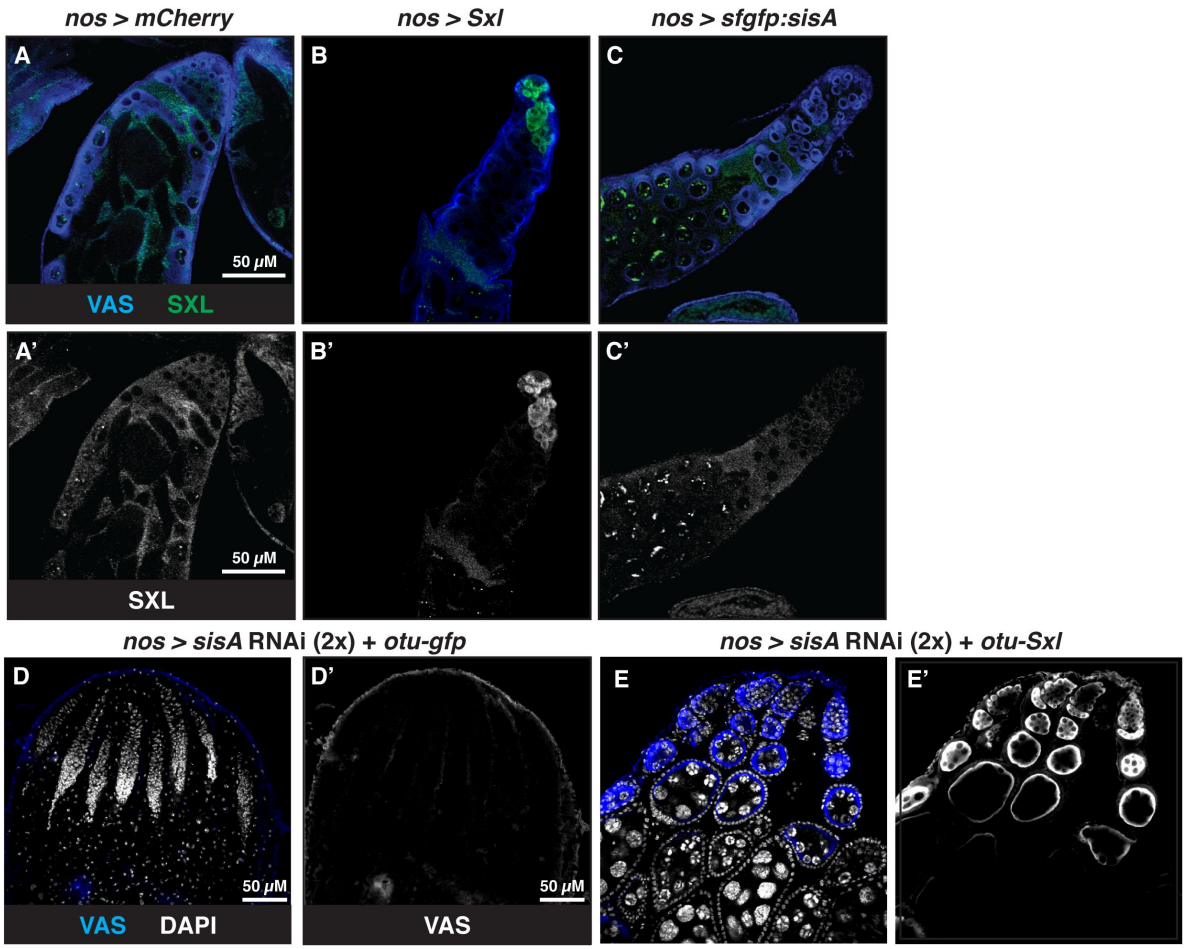

Figure 6

sisA is necessary but not sufficient to feminize the germline

A-C ') Immunofluorescence of adult testes to characterize anti-Sxl staining. A-A') Wildtype testis from fly with germline-specific overexpression of *gfp*. B-B') Testis from fly with germline-specific overexpression of *Sxl*. Sxl staining is observed in the early germ cells. The anterior tip of the testis looks mildly atrophied. C-C') Testis from fly with germline-specific overexpression of *sisA*. No Sxl staining is observed in the germ cells. The testis resembles the wildtype control. D-E) Immunofluorescence of adult ovaries to characterize rescue of *sisA* loss-of-function by *Sxl* expression. All animals strongly express UAS-*sisA* RNAi using two copies of *nos*-Gal4. D-D') Germ cell-less ovary from *sisA*-RNAi flies with ectopic expression of a control protein (GFP) using the *otu* promoter. Flies of this genotype are sterile. E-E') Ovary from *sisA*-RNAi flies with ectopic expression of *Sxl* under the control of the *otu* promoter. Note the rescue of the germline and the wildtype appearance of the ovary, indicating normal oogenesis. VAS stains germ cells. SXL stains Sxl. DAPI stains DNA (nucleus).

If *sisA* is required for *Sxl* expression, and this is its primary role in the germline, then expression of *Sxl* should bypass the need for *sisA* in the germline and rescue the germline defects observed with *sisA* LOF. To test this we expressed *Sxl* under the control of a female germline-specific promoter (*otu*) in a *sisA* LOF background. We used the strongest *sisA* loss of function condition (2x *nos*-GAL4 > *sisA* RNAi 1) which results in severe germline loss, tumor formation, and infertility (**Fig. 6D**-**6D'**, **S7E**). Excitingly, 50% of *sisA* RNAi + *otu* > *Sxl* ovaries exhibited a wildtype germline morphology (**Fig. 6E**-**6E'**, **S7E**) and were fertile, suggesting *Sxl* was able to provide a robust rescue. Another 20% of these ovaries showed a partial rescue of the germline, but contained pervasive germline tumors (**Fig. S7E**). Taken together, these data indicate that *sisA* is necessary, but not sufficient, for *Sxl* expression in the female germline and that activation of *Sxl* is the primary germline role for *sisA*.

Discussion

The sex-specific activation of *Sxl* in the germline

Previous evidence indicated that sex-specific activation of *SxlPE* is important for *Sxl* expression in germline, as it is in the soma. Transcripts from the *SxlPE* promoter were detected in early germ cells [50] and positive autoregulation of Sxl was observed in the germline [49], suggesting that the same mechanism of Early Sxl protein from *SxlPE* being required for splicing of *SxlPM* transcripts occurs in germ cells and soma. Our data also support this model. Using a *SxlPE* transcriptional reporter, we found that *SxlPE* is active in female germ cells by L1 (**Fig. 2B**-**2B'**). Further, while we observed activation the *SxlPM* promoter in embryonic germ cells (**Fig. 1B**-**1B''**, **1C**), we only observed expression of Late Sxl protein in the germ cells at L2 (**Fig. 2F**-**2F'**), after we observed activation of *SxlPE*. These data are consistent with *SxlPM* transcripts being unable to produce Sxl protein until after sex-specific activation of *SxlPE*. One difference between the germline and the soma appears to be the timing of *SxlPE* activation relative to that of *SxlPM*. In the soma, *SxlPE* is activated prior to *SxlPM*, while in the germline, there is no time at which we detect *SxlPE* alone by RNA FISH (**Fig. 1C**), and *SxlPE* is activated later in development, as judged by the *SxlPE* transcriptional reporter. This is still consistent with the autoregulatory model for *Sxl* activation, but suggests that the control of *SxlPE* activation is different in the germline than the soma.

We also found that the *cis*-regulatory elements that regulate female-specific *SxlPE* expression in the germ cells are different from those in the soma. Previously, it was reported that a 1.5kb genomic fragment just upstream of *SxlPE* was sufficient to regulate female-specific expression in both the soma [20,74] and the germline [50]. However, we were unable to observe germline expression from this *SxlPE* reporter or even a larger *SxlPE* reporter containing more of the upstream DNA (**Fig. S2F-I**). We only observed activation of *SxlPE* when we included an even larger genomic region, including sequences both upstream and downstream of *SxlPE* (**Fig. S2A**, **2B**-**2B'**). These data do not exclude sequences within the 1.5 kb region upstream of *SxlPE* being important for its activation in the germline, but indicate that these sequences are not sufficient for activation, as they are in the soma. Similarly, sequences outside this 1.5 kb region may also augment expression in the soma. The fact that the *cis*-regulatory logic of *SxlPE* is different in the germline than the soma is consistent with previous work demonstrating that the combination of *trans*-acting factors, the XSEs, that activate *SxlPE* in the germline are different from the soma [33,52,81]. Future work will involve identifying the specific enhancers that regulate *SxlPE* expression in the germline, which could in turn help identify additional germline *trans*-regulators of *Sxl*. In summary, we have shown that the *cis*-regulatory logic of *Sxl* activation is different between the germline and the soma, however, the key step in determining the sex of both these cell types is the female-specific activation of *SxlPE*.

Finally, a surprising observation from our study was the presence of sex-specific *SxlPE* transcriptional reporter activity, as well as Early Sxl protein, in somatic cells as late as the L1 larval stage (**Fig. S2F**-**S2G**, **S3E**-**S3F**, **S3I**). In order for XSEs to act, they need to be expressed at higher levels in XX cells than XY cells. Once X chromosome dosage compensation is initiated at the late blastoderm stage, there should no longer be a difference in XSE expression between XX and XY cells. This is the entire logic behind the autoregulatory model for Sxl expression; *SxlPE* is activated by XSEs only in females, and is then turned off prior to the time when dosage compensation equalizes XSE expression. Why then is Early Sxl protein and *SxlPE* activity detected at the L1 stage? One possibility is that both the GFP reporter and the Early Sxl protein are stable and perdure to the L1 stage. An alternative possibility, however, is that some other mechanism, such as feedback regulation within the sex determination system, maintains *SxlPE* activity much longer than previously thought. Interestingly, *Sxl* contains a highly-conserved binding site for the Doublesex transcription factor which could facilitate such an XSE-independent feedback regulation of *SxlPE* [82].

sisterless A as an activator of *Sxl*

While the combination of XSEs at work in the germline may be different than in the soma, our data also indicate that at least one XSE, SisA, is shared between them. Loss of *sisA* in the germline leads to the formation of germline tumors (and germline loss) (**Fig. 3C**-**3D'**, **3F**-**3G'**, **S5B**-**S5C**) as well as a loss of Sxl expression in the female germline (**Fig. 5C**-**5E'**, **S7B**-**S7D'**). Additionally, we report that *sisA* is expressed in the germline prior to *SxlPE* (**Fig. 4**) and that the expression of Sxl is able to rescue the *sisA* loss-of-function phenotype in the germline (**Fig. 6D**-**6E'**, **S7E**), indicating that *sisA* lies upstream of *Sxl* in the germline sex determination cascade. Taken together, these data indicate that SisA is a germline activator of *Sxl* and may act as a germline XSE.

It remains to be seen whether SisA acts directly on *Sxl* in the germline or acts on other *Sxl* regulators, such as *ovo* or *otu*. Current thinking is that SisA is a transcriptional activator for *SxlPE* in the soma [9,28], and the simplest model would be that it acts similarly in the germline. However, the gap in timing we observe between *sisA* expression in the germline (Stage 3-10, **Fig. 4E**-**4G'**, **S6H**-**S6H'**) and our ability to detect *SxlPE* activation (L1 larvae, **Fig. 2B**-**2B'**) leaves open the possibility that there are intermediate steps between *sisA* and *SxlPE.* SisA is a somewhat unusual leucine zipper protein and its DNA binding activity and specificity have never been determined. Thus, there is no evidence that it regulates *SxlPE* directly in the soma or germline. The transient nature of SisA expression during embryogenesis makes it difficult to conduct chromatin immunoprecipitation (ChIP) experiments under native conditions. Instead, we expressed GFP:SisA in *Drosophila* S2 tissue culture cells for ChIP analysis, but this experiment did not identify SisA genomic binding sites. Lastly, yeast two-hybrid analysis has indicated that SisA might physically interact with the basic-helix-loop-helix protein Daughterless (Da) [83], but co-expressing GFP:SisA and Da in S2 cells also did not result in an identifiable ChIP signal. Thus, while our data clearly indicate that *sisA* is required for activation of *Sxl* in the germline, and that this is its primary role in the germline, evidence that SisA is a direct, transcriptional activator of *SxlPE* will require identification and study of a SisA-binding enhancer within the *Sxl* locus.

It is puzzling that *Sxl* is a critical regulator of both germline and somatic sex determination, and is regulated by a two-X dose in both cell types, yet its mechanism of activation and its role in these cell types is so different. It is somewhat simplifying that at least one factor, *sisA*, is important for activation of *Sxl* in both cell types. Yet evidence still suggests that other aspects of *Sxl* activation in these cells will be different. Further, the most significant Sxl targets in the soma, *transformer* for sexual identity and *msl-2* for dosage compensation, do not play a role in the germline [45,47]. Instead, distinct factors such as *Tdrd5l* and the Jak/Stat pathway, which controls *Phf7* expression, are regulated by *Sxl* in the germline [67,84-86]. It will be interesting to examine how these independent roles for *Sxl* came to be, and how conserved each role is in different species.

Materials and methods

Fly stocks

The following fly stocks were used: *SxlPE-EGFP* (BDSC# 24105), *SxlPE-EGFP* (BDSC# 32565), *vas*-Cas9 (BDSC# 51324), *vas*-Cas9 (BDSC# 56552), alphatub-piggyBac (BDSC# 32070), *nos*-Gal4 ([55]), *nos*-Cas9 (BDSC# 54591), *nos*-Cas9 (BDSC# 78782), UAS-*sisA*-RNAi 1 (TRiP.HMC03864, BDSC# 55181), UAS-*Sxl*-RNAi (TRiP.HMS00609, BDSC# 34393), UAS-*sisB*-RNAi (TRiP.GL01130, BDSC# 41594), UAS-*sisC*-RNAi (TRiP.HMS00545, BDSC# 33680), UAS-*run*-RNAi (TRiP.HMS01186, BDSC# 34707), UAS-*mCherry*-RNAi control (BDSC# 35785), *bam[1]* (Gift from A. Spradling, [56]), *bam[delta86]* (BDSC# 5427), *sisA[5]* (Gift from J. Erickson, [57]), UAS-*Sxl* (BDSC# 58484), *otu-GFP*.K10 (BDSC# 29727), *otu-GFP*.SV40 (BDSC# 29729), *otu*-Sxl (BDSC# 58491). Oregon R flies were used as wildtype flies, w[1118];Sco/CyO;MKRS/TM6B,Tb,Hu double balancers (Gift from X. Chen) were used for 2nd and 3rd chromosome balancing. FM7c was used for X chromosome balancing.

Antibody staining and Tyramide Signal Amplification (TSA)

Adult testes were dissected in 1X PBS and fixed at room temperature for 20 minutes in 4.625% formaldehyde in PBS containing 0.1% Triton X-100 (PBTx). Adult ovaries, and larval gonads (first, second, and third instar) were dissected in 1X PBS and fixed at room temperature for 20 minutes in 5.25% formaldehyde in PBTx. Blocking and immunostaining was performed as previously described [58], and samples were mounted in 2.5% DABCO. Embryo collection, fixing, blocking and staining was performed as described previously [59]. All images were taken with a Zeiss LSM 700 confocal microscope. The following primary antibodies (sources) and concentrations were used: chicken anti-Vas 1:10,000 (K. Howard); rabbit anti-Vas 1:10,000 (R. Lehmann); mouse anti-Sxl 3:100 (M18, DSHB); mouse anti-Fas3 1:50 (7G10, DSHB); rat anti-NCad 3:100 (DN-Ex #8, DSHB); mouse anti-Lamin B 1:100 (ADL67.10, DSHB); mouse anti-Pros 1:10 (MR1A, DSHB); guinea pig anti-TJ 1:1,000 (J. Jemc); rabbit anti-GFP 1:1,000 (ab290, Abcam); mouse anti-FLAG 1:50 (F3165, Millipore Sigma); rat anti-HA 1:100 (ROAHAHA Clone 3F10, Roche); rabbit anti-HA 1:800 (C29F4 #3724, CST). DSHB: Developmental Studies Hybridoma Bank; CST: Cell Signaling Technologies. Secondary antibodies were used at 1:500 (Alexa Fluor, Host:Goat, Invitrogen).

TSA was performed using the manufacturer's protocol (Tyramide SuperBoost Kits with Alexa Fluor Tyramides, Invitrogen). Larval gonads and embryos were fixed as previously described (See above). Samples were blocked in 10% Goat Serum for 1 hour at room temperature followed by incubation with a single primary antibody (target of signal amplification) as previously described [58,59] overnight at 4°C with nutation. Primary antibody was washed off using 1X PBS and samples were incubated with poly-HRP-conjugated secondary antibody for 1 hour at room temperature. Secondary antibody was washed off using 1X PBS and samples were incubated in tyramide working solution for 10 minutes at room temperature before stopping the reaction. Samples were washed with 1X PBS and immunolabeling with TSA was multiplexed with other antibodies following the standard immunolabeling protocol.

Developmental Staging and Sexing

To obtain stage-specific embryos, embryos were pre-collected overnight on apple juice plates with fresh yeast paste and discarded to synchronize egg-laying. Embryos were then collected for 2 hours at 25°C after which the flies were removed and the embryos were aged as required. To obtain first (L1) and second (L2) instar larvae, embryos were aged 20 hours. The plates were cleared of any larvae that had hatched early. L1 larvae were collected after 4-8 hours. For L2 larvae, the L1 larvae were transferred to fresh plates with yeast paste and aged for an additional 24 hours. Third instar (L3) larvae were collected directly from vials. Sex of embryos was determined by counting the number of *Sex lethal* or *sisterless A* RNA FISH signals corresponding to

number of X chromosomes or by somatic EGFP expression using the transcriptional *SxlP$_E$-EGFP* reporters. Stage of the embryos was approximately determined by the position of the primordial germ cells (PGCs). Sex of the first and second larval instar gonads was determined by looking for presence or absence of a hub using anti-FasIII or anti-NCad immunolabeling. Sex of the third larval instar gonads was determined by morphology.

Oligopaints Probe Design and Synthesis

Oligopaint probes for RNA FISH were designed using Oligopaints [60–62] by Dr. Kayla Viets from Dr. Robert Johnston's laboratory, Department of Biology at Johns Hopkins University. Gene target sequences (*Sex lethal* and *sisterless A*) were run through an open-source bioinformatics pipeline made available by the Wu lab at Harvard Medical School (*http://genetics.med.harvard.edu/oligopaints/*) to identify sets of 50-bp (*Sex lethal*) or 30-bp (*sisterless A*) optimized probe sequences (libraries) to tile the nascent RNA transcript [63]. Library of Cy3-conjugated oligos against *sisterless A* RNA was ordered individually. Library of oligos against *Sex lethal* and its sub-libraries (*PM* vs. *PE+PM* probes) were ordered as part of a 90k oligopool including oligos for other gene targets (Gift from R. Johnston, Johns Hopkins University). To isolate target-specific probes (*Sex lethal*), five 19-bp barcording primers, target F and R; universal (univ) F and R; and sublibrary (sub) F were appended to the 5' and 3' ends of each probe.

PCR using target F and R primers allowed amplification of libraries of probes against target RNA from oligopool. PCR using sub F and target R primers allowed amplification of sub-libraries from whole target libraries. PCR using univ F and R primers allowed the conjugation of fluorophores (Cy3, Cy5), generation of single-stranded DNA (ssDNA) probes as well as addition of secondary sequences to allow amplification of RNA FISH signal using secondary probes (conjugated with fluorophores) that bind to primary probes. RNA FISH probes were generated as previously described [60–62]). Total number of probes per transcript are as follows: *sisA* RNA – 12; *SxlPM* RNA – 19; *SxlPE+PM* RNA – 36.

RNA fluorescent in situ hybridization (FISH) and immunofluorescence

RNA FISH was performed using modified versions of the protocols described previously [60–63]. Oregon R embryos were collected (after a pre-collection was discarded) on apple juice plates with fresh yeast paste for 2 hours at 25°C and aged as needed. Embryos were collected, rinsed with 1X PBTx, and dechorionated using 50% bleach for 90 seconds and washed with 1X PBTx. Fixing was performed in scintillation vials in 50µL 10X PBS, 100µL 0.25M EGTA, 125µL fresh 16% formaldehyde, 225µL Milli-Q H_2O, 1µL NP-40 (Tergitol solution), and 500µL heptane. Embryos were shaken vigorously by hand for 1 minute, then fixed for 20 minutes at room temperature with gentle agitation. The aqueous phase was removed and the embryos were devitellinized in by adding 500µL methanol and vigorous shaking for 2 minutes. Devitellinized embryos were collected washed three times in methanol. Embryos were rehydrated via 5-minute serial single washes in 75%, 50%, and 25% methanol in 1X PBTx. This was followed by three 5-minute washes in 1X PBTx and two 15-minute washes in 1X PBTx with 0.2U/µL RNase inhibitor. Embryos were blocked for 1 hour at room temperature in blocking buffer (1X PBTx + Western Blocking Reagent, 1:1) with nutation. They were then incubated in primary antibody diluted in 1X PBTx with 3% normal goat serum and 0.2U/µL RNase inhibitor overnight at 4°C with nutation. Primary antibody was rinsed off followed by three 20-minute washes with 1X PBTx. Embryos were then incubated with secondary antibody diluted in 1X PBTx with 3% normal goat serum and 0.2U/µL RNase inhibitor for two hours at room temperature. Secondary antibody was rinsed off followed by two 20-minute washes in 1X PBTx and one 20-minute wash in 1X PBS. Embryos were then washed as follows: four 5-minute washes in 2X SSCT, one 10-minute wash in 20% formamide in 2X SSCT, one 10-minute wash in 50% formamide in 2X SSCT, one 4-hour wash in 50% formamide in 2X SSCT at 37°C with shaking. Embryos were incubated with primary probe at a concentration of ≥5 pmol fluorophore/µL in hybridization buffer (50% formamide in 2X SSCT with 10% dextran sulfate (w/v)

+ 0.2U/μL RNase inhibitor) for 16-20 hours at 37°C with shaking. Primary probe was rinsed and followed by a 1-hour wash in 50% formamide in 2X SSCT at 37°C with shaking. Secondary probes were hybridized for 1 hour at 37°C with shaking at a concentration of ≥5 pmol fluorophore/μL in 50% formamide in 2X SSCT and 0.2U/μL RNase inhibitor. This was followed by two 30-minute washes in 50% formamide in 2X SSCT at 37°C, one 10-minute wash in 20% formamide in 2X SSCT, two 10-minute washes in 2X SSCT at room temperature, and one 10-minute wash in 2X SSC at room temperature. Embryos were washed for 10 minutes in 1X PBTx containing DAPI and incubated at room temperature for 10 minutes in SlowFade Diamond with DAPI, followed by mounting. All images were taken with a Zeiss LSM 700 confocal microscope.

SxlPE reporter transgenes and constructs

SxlPE-10.2kb, a 10.2kb genomic sequence from a *Sxl* genomic clone BAC# CH321-74P19 (BACPAC Resources Center) cloned into the pJR16 vector in two steps (Gift from R. Johnston, Johns Hopkins University) using HiFi DNA Assembly (New England Biolabs). pJR16 uses an EGFP reporter with a nuclear localization sequence (nls). Fragment 1 extended from 116 bp downstream of *SxlPE* TSS to 5,116 bp upstream of *SxlPE* TSS and was assembled into pJR16 digested with AgeI and AscI to generate *SxlPE*-5.2kb. Fragment 2 extended from 117 bp downstream of *SxlPE* TSS to 5,108 bp downstream of *SxlPE* TSS and was assembled into Fragment 1 + pJR16 digested with AgeI.

- Fragment 1 *SxlPE*-10.2kb Fw (5'-3') – CCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATGGTGGCGACCGGTAA TGGGATAATCACAAAGTT
- Fragment 1 *SxlPE*-10.2kb Rv (5'-3') – CATGCTGCAGCAGATCTGGTCTAGAGCCCGGGCGAATTCGCCGGCGCGCCGT AATTTTTCTTTGCTCCTCCTG
- Fragment 2 *SxlPE*-10.2kb Fw (5'-3') – CAGCTCCTCGCCCTTGCTCACCATGCTGTACGATGAATCGA
- Fragment 2 *SxlPE*-10.2kb Rv (5'-3') – GAAAAACGTAACTTTGTGATTATCCCATTATGGATTTCAATTTTGATAC

The primers ensured that EGFP remained in frame with Exon E1's coding sequence (CDS). Constructs were injected into embryos and integrated via PhiC31 integrase-mediated transgenesis (done at BestGene Inc.) into the same genomic location at P{CaryP}attP40 on Chromosome II.

Generation of sisA RNAi 2 and 3

Transcript-specific knockdown was achieved using the VALIUM vector system (PMID: 21460824). A 21-nucleotide (nt) sequence without any off-targets greater than 16nt was selected based on the algorithm of [64]. The following sequences were selected:

- sisA-RNAi 2 Sense – CGCCGACGAGGAGCAACGCUA
- sisA-RNAi 2 Antisense – UAGCGUUGCUCCUCGUCGGCG
- sisA-RNAi 3 Sense – CCGGUUCUGGUUCGGAUGUCA
- sisA-RNAi 3 Antisense – UGACAUCCGAACCAGAACCGG

The top and bottom strands for the short hairpin were as follows:

- sisA-RNAi 2 Top Strand – CTAGCAGTCGCCGACGAGGAGCAACGCTATAGTTATATTCAAGCATATAGCG TTGCTCCTCGTCGGCGGCG
- sisA-RNAi 2 Bottom Strand – AATTCGCCGCCGACGAGGAGCAACGCTATATGCTTGAATATAACTATAGCGT TGCTCCTCGTCGGCGACTG

- sisA-RNAi 3 Top Strand – CTAGCAGTCCGGTTCTGGTTCGGATGTCATAGTTATATTCAAGCATA-TGACATCCGAACCAGAACCGGGCG
- sisA-RNAi 3 Bottom Strand – AATTCGCCCGGTTCTGGTTCGGATGTCATATGCTTGAATATAACTATGACATC CGAACCAGAACCGGACTG

The top and bottom strands were annealed and cloned into the VALIUM20 vector #1467 (Drosophila Genomics Resource Center, DGRC). Constructs were injected into embryos and integrated via PhiC31 integrase-mediated transgenesis (done at BestGene Inc.) into the same genomic location at P{CaryP}attP40 on Chromosome II. For RNAi-mediated germline-specific knockdown of *sisA*, *sisB*, *sisC*, *runt*, *Sxl*, *Ovo*, and *mCherry*, male flies carrying shRNA transgenes were mated with *nanos*-GAL4:VP16 virgin females [55] and the crosses were maintained at 29°C. The progeny were reared at 29°C until 3-5 days post-eclosion (unless otherwise specified).

CRISPR-tagging

sfGFP:sisA, 2xHA:SxlE1, and 3xFLAG:SxlL2 were generated using scarless genome editing described in [65] to generate N-terminal tags. Guide RNA (gRNA) sequences were selected using the FlyCRISPR algorithm (*http://flycrispr.molbio.wisc.edu*) [66], contain 20 nucleotides each and have no predicted off-targets. The following gRNAs were selected:

- sfGFP:sisA gRNA Target (5'-3') – GTCCAATGGCAAGCTACCTG
- 2xHA:SxlE1 gRNA Target (5'-3') – GCCTCCTTCGATCTTCTACC
- 3xFLAG:SxlL2 gRNA Target (5'-3') – GACTTGTTGTTGTAGCCATA

The guides were cloned into the pU6-2-BbsI-gRNA vector #1363 (DGRC) using the listed primers that were phosphorylated using T4 polynucleotide kinase (PNK).

- sfGFP:sisA gRNA Target sense (5'-3') – CTTCGTCGTTGGCCAATCCGGATGC
- sfGFP:sisA gRNA Target antisense (5'-3') – AAACGCATCCGGATTGGCCAACGAC
- 2xHA:SxlE1 gRNA Target sense (5'-3') – CTTCGCCTCCTTCGATCTTCTACC
- 2xHA:SxlE1 gRNA Target antisense (5'-3') – AAACGGTAGAAGATCGAAGGAGGC
- 3xFLAG:SxlL2 gRNA Target sense (5'-3') – CTTCGACTTGTTGTTGTAGCCATA
- 3xFLAG:SxlL2 gRNA Target antisense (5'-3') – AAACTATGGCTACAACAACAAGTC

Donor plasmids to facilitate homology dependent repair were generated. The coding sequences of the different tags are cloned from genomic DNA of *vas*-Cas9 expressing flies (BDSC# 51324, 56552) adjacent to a piggyBac transposon that contained a DsRed expression construct resulting in a selectable-tagging-cassette in vectors pHD-sfGFP-ScarlessDsRed #1365, pHD-2xHA-ScarlessDsRed #1366, pHD-3xFLAG-ScarlessDsRed #1367 (DGRC). Approximately 1kb 5' and 3' Homology arms were assembled using HiFi DNA Assembly (New England Biolabs) upstream and downstream of this cassette with the following primers (5'-3'):

- sfGFP:sisA 5' Arm Fw (5'-3') – GAATTCGCCAAAGGGATTTC
- sfGFP:sisA 5' Arm Rv (5'-3') – CCGGAACCTCCAGATCCACCGGTGATTTTTTTCGATGTGTG
- sfGFP:sisA cassette Fw (5'-3') – ACACATCGAAAAAAATCACCGGTGGATCTGGAGGTTCC
- sfGFP:sisA cassette Rv (5'-3') – AAGTAAAGATGACTCCGTTCGGAACCTCCTGAACCACC
- sfGFP:sisA 3' Arm Fw (5'-3') – CTGGTGGTTCAGGAGGTTCCGAACGGAGTCATCTTTACTTGCC
- sfGFP:sisA 3' Arm Rv (5'-3') – GGTACCGCATTGGCCCAATTC
- 2xHA:SxlE1 5' Arm Fw (5'-3') – GCAATCTGTGTTCTTGGTATTTTG
- 2xHA:SxlE1 5' Arm Rv (5'-3') – GGAACATCGTATGGGTACATAATGGGATAATCACAAAGTTAC
- 2xHA:SxlE1 cassette Fw (5'-3') – AACTTTGTGATTATCCCATTATGTACCCATACGATGTTCC
- 2xHA:SxlE1 cassette Rv (5'-3') – ACAGTATCAAAATTGAAATCGGAACCTCCTGAACCACC
- 2xHA:SxlE1 3' Arm Fw (5'-3') – CTGGTGGTTCAGGAGGTTCCGATTTCAATTTTGATACTGTGAC

- 2xHA:SxlE1 3' Arm Rv (5'-3') – CGATCGAAGGTGAGTTTC
- 3xFLAG:SxlL2 5' Arm Fw (5'-3') – CGACCATGTCGTCCTACTATAAC
- 3xFLAG:SxlL2 5' Arm Rv (5'-3') – TCATGGTCTTTGTAGTCCATATCCTGAGAGTTGGGAGTG
- 3xFLAG:SxlL2 cassette Fw (5'-3') – ACACTCCCAACTCTCAGGATATGGACTACAAAGACCATGAC
- 3xFLAG:SxlL2 cassette Rv (5'-3') – CCCGGATTATTGTTGCCGTAGGAACCTCCTGAACCACC
- 3xFLAG:SxlL2 3' Arm Fw (5'-3') – CTGGTGGTTCAGGAGGTTCCTACGGCAACAATAATCCG
- 3xFLAG:SxlL2 3' Arm Rv (5'-3') – GCTAATGAGGGGATTCCTATG

Assembled donor constructs were cloned into pCR2.1-TOPO (TOPO TA Cloning Kit, ThermoFisher). Site-specific mutagenesis was also used to mutate the PAM sites in each donor plasmid. For sfGFP:sisA, the included linker sequence between 3' UTR and the start of sfGFP was removed using site-specific mutagenesis (QuikChange, Agilent). The gRNA expression plasmid and donor plasmid were injected by BestGene into embryos of Vas-Cas9 flies (#56552, #51324, BDSC). DsRed positive flies were then crossed to a piggyBac transposase expressing line (#32070, #32073, BDSC) to excise DsRed resulting in an in-frame fusion of the tags' and the respective genes' coding sequences. Successful generation of CRISPR-tags was confirmed by sequencing.

Generation of overexpression constructs

UAS-*sfGFP*, UAS-*sisA*, and UAS-*da* were generated by cloning the ORFs of *sfGFP*, *sisA*, and *da-PD* into pUASpB [67] using the listed primers. pUASpB is a modified version of pUASP [68] including an attB site for phiC31-mediated integration. UAS-*sfGFP:sisA* was generated by cloning a flexible linker sequence followed by the ORF of *sisA* (lacking a start codon) using the listed primers into UAS-*sfGFP* that had been linearized with SpeI. Constructs were assembled using HiFi DNA Assembly (New England Biolabs).

- UAS-*sfGFP* Fw (5'-3') – TACCCGCCCGGGGATCAGATCCGCGGCCGCATGGTGTCCAAGGGCGAG
- UAS-*sfGFP* Rv (5'-3') – GACTCTAGAGGATCCAGATCCACTAGTTCACTTGTACAGCTCATCCATGC
- UAS-*sfGFP:sisA* Fw (5'-3') – GCCGGCATCACCCTGGGCATGGATGAGCTGTACAAGATTAAGGCCGGCGGGT CG
- UAS-*sfGFP:sisA* Rv (5'-3') – ACGTTAACGTTCGAGGTCGACTCTAGAGGATCCAGATCCATCACTGCTCCATT TCCAGGC
- UAS-*sisA* Fw (5'-3') – CTGTTCATTGGTACCCGCCCGGGGATCAGATCCGCATGGAACGGAGTCATCTT TACTTGC
- UAS-*sisA* Rv (5'-3') – ACGTTAACGTTCGAGGTCGACTCTAGAGGATCCAGATCCATCACTGCTCCATT TCCAGGC
- UAS-*da-PD* Fw (5'-3') – AGGTCCTGTTCATTGGTACCCGCCCGGGGATCAGATCCGCATGGCGACCAGT GACGATG
- UAS-*da-PD* Rv (5'-3') – ACGTTAACGTTCGAGGTCGACTCTAGAGGATCCAGATCCATTAAAAGTGTTG TACATTTTGTAGGGG

Constructs flies were injected into embryos and integrated via PhiC31 integrase-mediated transgenesis (done at BestGene Inc.) into the same genomic location at P{CaryP}attP40 on Chromosome II. For germline-specific overexpression, male flies carrying UAS transgenes were mated with *nanos*-GAL4:VP16 virgin females [55] and the crosses were maintained at 29°C. The progeny were reared at 29°C until 3-5 days post-eclosion (unless otherwise specified).

G0 CRISPR (Tissue-specific CRISPR)

Guide RNA (gRNA) sequences were selected using the FlyCRISPR algorithm (*http://flycrispr.molbio.wisc.edu*) [66], contain 20 nucleotides each and have no predicted off-targets. For *sisA*, four different gRNA sequences were selected, one within the coding region (Target 4) and one within

the 3' UTR (Target 3), and two upstream of the gene locus (Targets 1 and 2). As a control, four different gRNA sequences were selected against GFP. The following gRNAs were selected:

- sisA-G0 gRNA Target 1 (5'-3') – TCGTTGGCCAATCCGGATGCAGG
- sisA-G0 gRNA Target 2 (5'-3') – CACTGAGTCTACCTGATAATTGG
- sisA-G0 gRNA Target 3 (5'-3') – ATAGTGTAGCTATGTGTCGCAGG
- sisA-G0 gRNA Target 4 (5'-3') – GCTGAAAACGGAGCTTGCTATGG
- gfp-G0 gRNA Target 1 (5'-3') – CAGGGTCAGCTTGCCGTAGG
- gfp-G0 gRNA Target 2 (5'-3') – AGCACTGCACGCCGTAGGTC
- gfp-G0 gRNA Target 3 (5'-3') – CGGCCATGATATAGACGTTG
- gfp-G0 gRNA Target 4 (5'-3') – CATGCCGAGAGTGATCCCGG

Four guides (or two) were cloned into the pCFD5 vector #73914 (Addgene) as previously described [69] using the listed primers.

- sisA-G0-4-PCR1-Fw (5'-3') – GCGGCCCGGGTTCGATTCCCGGCCGATGCATCGTTGGCCAATCCGGATGCGT TTTAGAGCTAGAAATAGCAAG
- sisA-G0-4-PCR1-Rv (5'-3') – ATTATCAGGTAGACTCAGTGTGCACCAGCCGGGAATCGAACCC
- sisA-G0-4-PCR2-Fw (5'-3') – CACTGAGTCTACCTGATAATGTTTTAGAGCTAGAAATAGCAAG
- sisA-G0-4-PCR2-Rv (5'-3') – GCGACACATAGCTACACTATTGCACCAGCCGGGAATCGAACCC
- sisA-G0-4-PCR3-Fw (5'-3') – ATAGTGTAGCTATGTGTCGCGTTTTAGAGCTAGAAATAGCAAG
- sisA-G0-4-PCR3-Rv (5'-3') – ATTTTAACTTGCTATTTCTAGCTCTAAAACTAGCAAGCTCCGTTTTCAGCTGC ACCAGCCGGGAATCGAACCC
- GFP-G0-4-PCR1-Fw (5'-3') – GCGGCCCGGGTTCGATTCCCGGCCGATGCACAGGGTCAGCTTGCCGTAGGGT TTTAGAGCTAGAAATAGCAAG
- GFP-G0-4-PCR1-Rv (5'-3') – GACCTACGGCGTGCAGTGCTTGCACCAGCCGGGAATCGAACCC
- GFP-G0-4-PCR2-Fw (5'-3') – AGCACTGCACGCCGTAGGTCGTTTTAGAGCTAGAAATAGCAAG
- GFP-G0-4-PCR2-Rv (5'-3') – CAACGTCTATATCATGGCCGTGCACCAGCCGGGAATCGAACCC
- GFP-G0-4-PCR3-Fw (5'-3') – CGGCCATGATATAGACGTTGGTTTTAGAGCTAGAAATAGCAAG
- GFP-G0-4-PCR3-Rv (5'-3') – ATTTTAACTTGCTATTTCTAGCTCTAAAACCCGGGATCACTCTCGGCATGTGC ACCAGCCGGGAATCGAACCC

Constructs were injected into embryos and integrated via PhiC31 integrase-mediated transgenesis (done at BestGene Inc.) into the same genomic location at P{CaryP}attP40 on Chromosome II. For germline-specific CRISPR-mediated knockout of *sisA* and *gfp*, male flies carrying gRNA expressing transgenes were mated with *nanos*-Cas9 virgin females ([70], BDSC# 54591 or [71], BDSC# 78782) and the crosses were maintained at 29°C. The progeny were reared at 29°C until 3-5 days post-eclosion (unless otherwise specified).

Generating sisA mutant

CRISPR-Cas9 was used as previously described to generate a *sisA* deletion mutant [72,73]. 2 guide RNA (gRNA) sequences were selected flanking the *sisA* gene using the FlyCRISPR algorithm (*http://flycrispr.molbio.wisc.edu*) [66], contain 20 nucleotides each, and have no predicted off-targets. The following gRNAs were selected:

- sisA-del gRNA Target 1 (5'-3') – GTCCAATGGCAAGCTACCTG
- sisA-del gRNA Target 2 (5'-3') – GATTACCTTCGGCCAGGCGA
- The guides were cloned into the pU6-2-BbsI-gRNA vector #1363 (DGRC) using the listed primers that were phosphorylated using T4 PNK.

- sisA-del gRNA Target 1 sense (5’-3’) – CTTCGTCGTTGGCCAATCCGGATGC
- sisA-del gRNA Target 1 antisense (5’-3’) – AAACGCATCCGGATTGGCCAACGAC
- sisA-del gRNA Target 2 sense (5’-3’) – CTTCGCACTGAGTCTACCTGATAAT
- sisA-del gRNA Target 2 antisense (5’-3’) – AAACATTATCAGGTAGACTCAGTGC

A donor plasmid to facilitate homology dependent repair was generated. A 5’ homology arm and a 3’ homology arm were PCR-amplified from genomic DNA of *vas*-Cas9 expressing flies (BDSC# 51324, 56552) and cloned into multiple cloning sites found upstream and downstream of a removable 3xP3-DsRed marker in the pHD-DsRed-attP vector #1361 (DGRC).

- sisA-del 5’ Arm Fw (5’-3’) – AGCACACCTGCACGACCGATGAAAATGGAGCAAGTGGAAAGCACACCTGCA CGACCGATGAAAATGGAGCAAGTGGAA
- sisA-del 5’ Arm Rv (5’-3’) – AGCACACCTGCACGATTAACCTTAGGCAATATGTCAGCCAGCACACCTGCAC GA TTAACCTTAGGCAATATGTCAGCC
- sisA-del 3’ Arm Fw (5’-3’) – GGCGGCTCTTCCTAAAAAACGAATGCTTTCTTATTGGCGGCTCTTCCTAA AAAACGAATGCTTTCTTATT
- sisA-del 3’ Arm Rv (5’-3’) – GGCGGCTCTTCCCGGTTTAATATACATATATTTGTGGCGGCTCTTCCCGG TTTAATATACATATATTTGT

The gRNA expression plasmid and donor plasmid were injected by BestGene into embryos of Vas-Cas9 flies (#56552, #51324, BDSC). DsRed-positive flies were balanced without DsRed removal to maintain a selectable marker. Successful deletion of the *sisA* gene and replacement with the DsRed marker was confirmed by sequencing.

Author contributions

Raghav Goyal: Conceptualization, Investigation, Formal Analysis, Writing (original draft and editing), Funding Acquisition. Ellen Baxter: Investigation, Formal Analysis, Project Administration. Mark Van Doren: Conceptualization, Writing (review and editing), Supervision, Project Administration, Funding Acquisition.

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Figure legends

Cartoons in **Figures S1A**, **S2A**, **S3A**, **S5D**, and **S6C** were created with *Biorender.com*

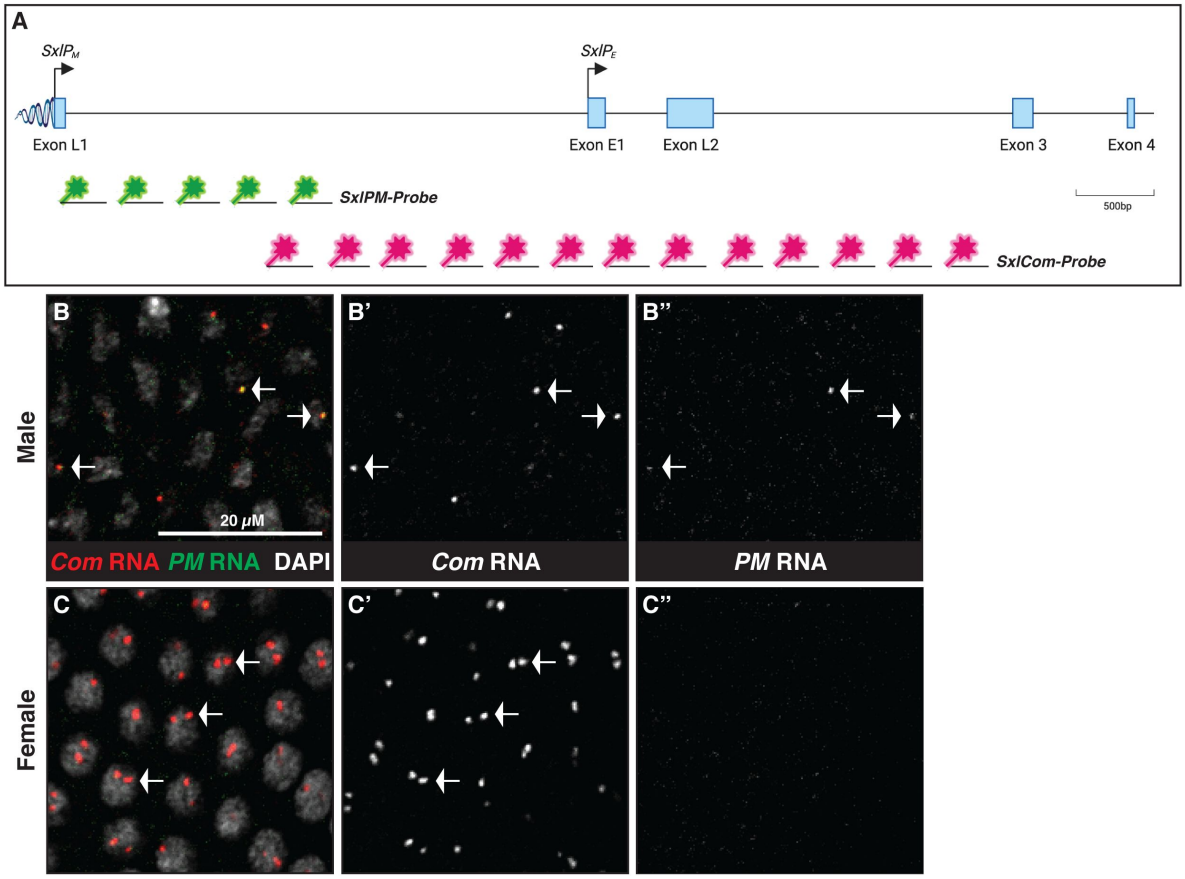

Figure S1

RNA-FISH against nascent RNA can be used to study *Sxl* promoter initiation

A) Cartoon showing relevant portion of the *Sxl* gene locus and span of *SxlPM* and *SxlPE+SxlPM* transcripts probed by *PM*-probes (Cy5, false colored Green) and *PE+PM*-probes (Cy3, Red) respectively, using RNA-FISH. Locus is drawn to scale. Size of probes is not drawn to scale. CDS: Coding sequences. UTR: Untranslated Region. B-C'') RNA-FISH (+ Immunofluorescence) against the transcript from *SxlPM* only (*PM* RNA) versus the common transcript from *SxlPE* and *SxlPM* (*PE+PM* RNA) in embryonic stage 3 somatic cells. B-B'') Male somatic cells always show both *PE+PM* and *PM*-probe signals. Note that a single focus is observed indicating a single X chromosome. C-C'') Female somatic cells first have *PE+PM*-probe signals, indicative of *SxlPE* activity alone. Note that two foci are observed per nucleus indicating the presence of two X chromosomes that are unpaired. Arrows mark fluorescent nuclear foci of RNA-FISH signals except in C'' which has no RNA-FISH signals. *PE+PM* RNA is probed by *PE+PM*-probes. *PM* RNA is probed by *PM*-probes. DAPI stains DNA (nucleus).

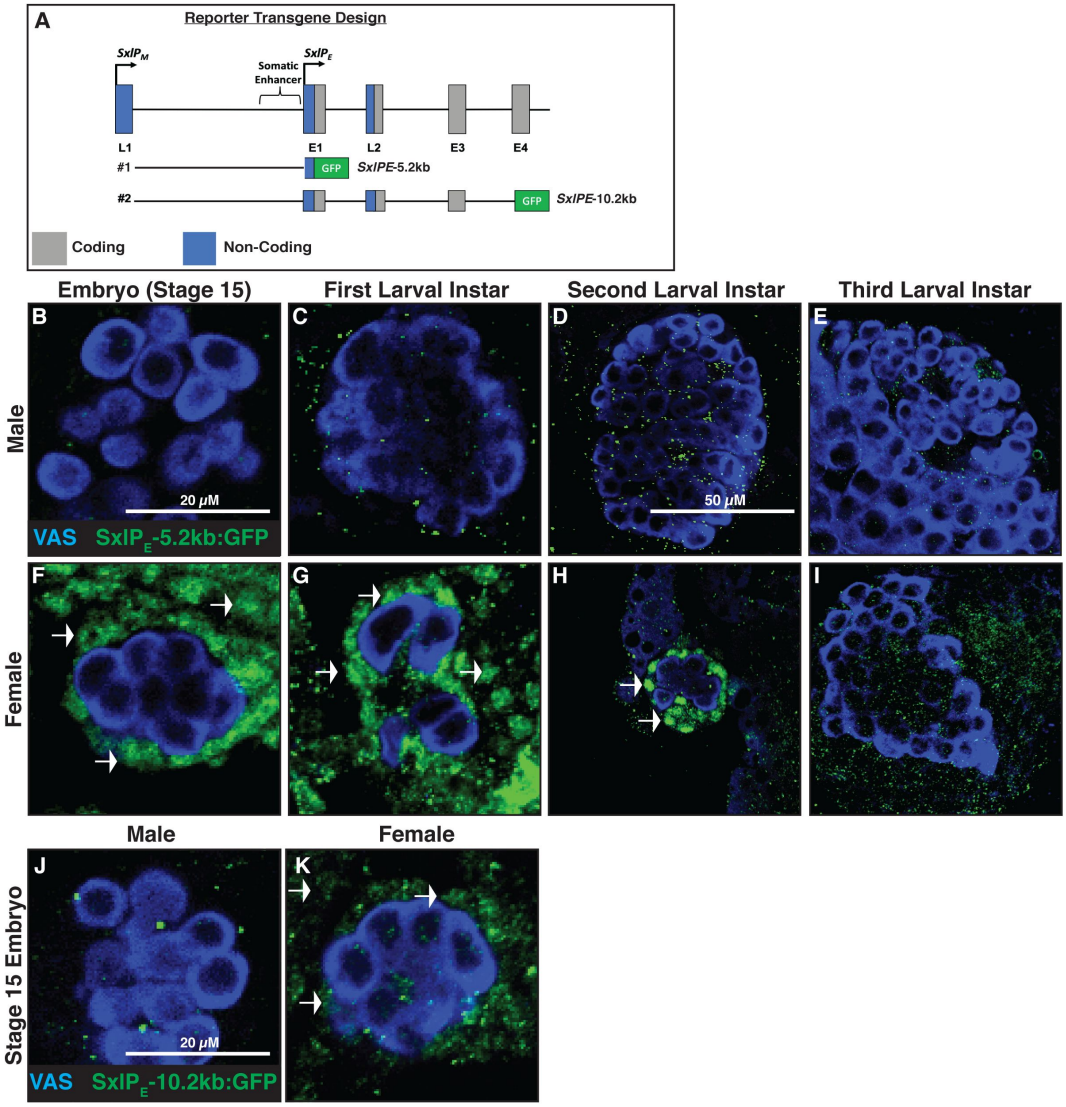

Figure S2

SxlPE requires different *cis*-regulatory elements in the soma and the germline

A) Cartoon showing relevant portion of the *Sxl* gene locus and design of transcriptional reporter constructs specific for *SxlPE*. EGFP reporters include a nuclear localization sequence (nls) to aid in visualization of expression. The 1.5kb somatic enhancer is illustrated. In *SxlPE*-5.2kb, EGFPnls replaces the CDS of Exon E1. In *SxlPE*-10.2kb, EGFPnls replaces the CDS of Exon 4. Locus is drawn to scale. EGFPnls is not drawn to scale. CDS: Coding sequences. UTR: Untranslated Region. B-I) Immunofluorescence of developing gonads to characterize *SxlPE*-5.2kb expression from stage 15 of embryogenesis to the third larval instar stage (L3). Note the presence of sex-specific nuclear GFP expression in female somatic cells. No GFP expression is observed in the germ cells at any stage. Arrows mark GFP-positive somatic cells. J-K) Immunofluorescence of embryonic stage 15 gonads to characterize *SxlPE*-10.2kb expression. Note the absence of GFP expression in female germ cells but presence of GFP expression in female somatic cells. Arrows mark GFP-positive somatic cells. VAS stains germ cells.

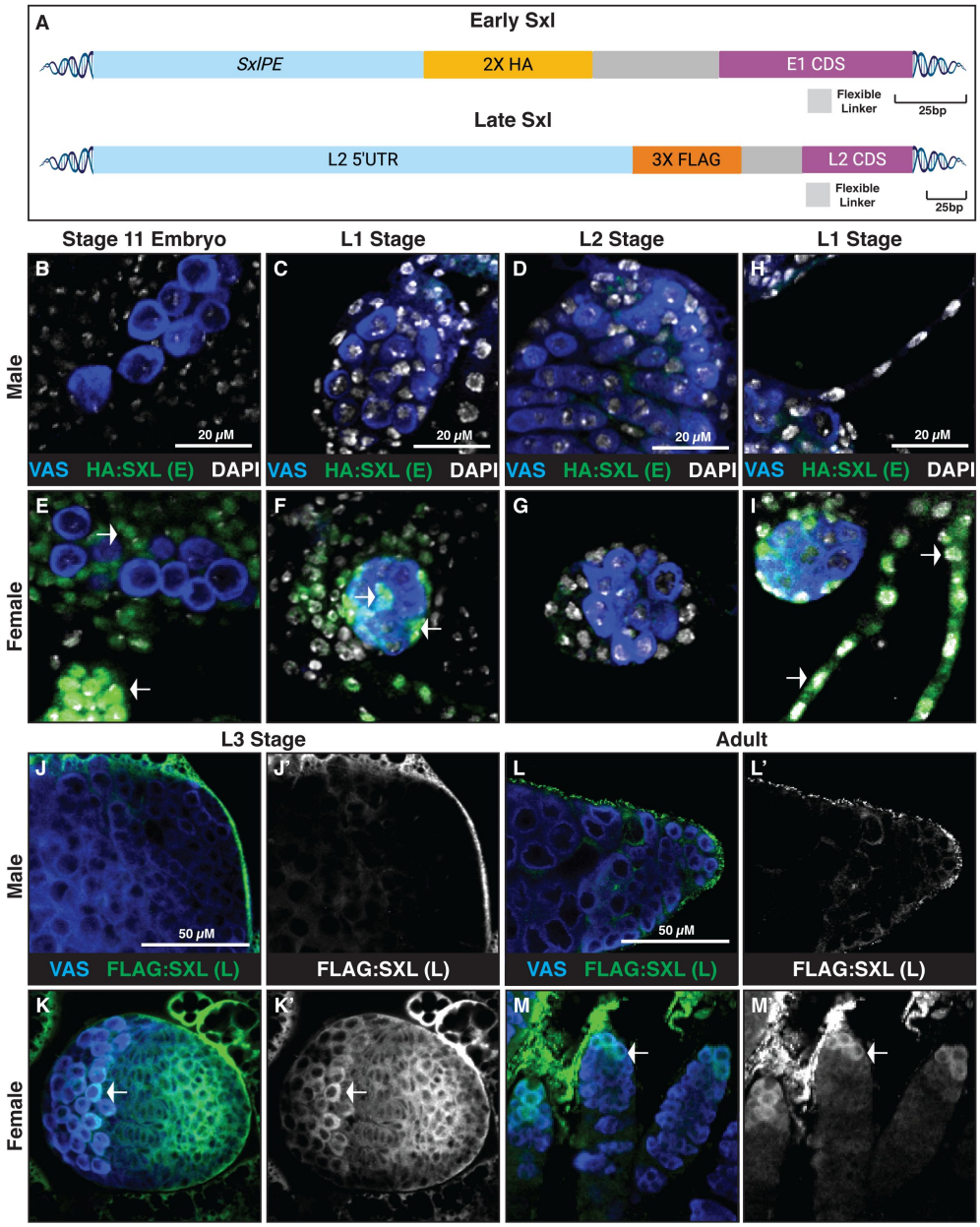

Figure S3

Endogenous tagging of Early and Late Sxl isoforms

A) Cartoon showing relevant portion of the *Sxl* gene locus and location of endogenous tags for Early and Late Sxl isoforms. A 2X HA tag is inserted at the N-terminus of Exon E1's CDS to generate the HA:SxlE1 'Early (E) Sxl' tag. A 3X FLAG tag is inserted at the N-terminus of Exon L2's CDS to generate the FLAG:SxlL2 'Late (L) Sxl' tag. Locus is drawn to scale. Tags are not drawn to scale. CDS: Coding sequences. UTR: Untranslated Region. B-G) Immunofluorescence of developing gonads to characterize HA:SxlE1 expression from stage 11 of embryogenesis to the second larval instar stage (L2). Note the presence of sex-specific Early Sxl expression in the nuclei of female somatic cells. Arrows mark HA-positive somatic cells. H-I) Immunofluorescence of developing guts to character HA:SxlE1 expression at the first larval instar stage (L1). Note the presence of sex-specific Early Sxl expression in the nuclei of female gut cells. Arrows mark HA-positive gut cells. J-M') Immunofluorescence of developing gonads to characterize FLAG:SxlL2 expression from the third larval instar stage (L3) to adult stage. Note the presence of sex-specific Late Sxl expression in the cytoplasm of female germ cells. Arrows mark FLAG-positive germ cells. VAS stains germ cells. DAPI stains DNA (nucleus).

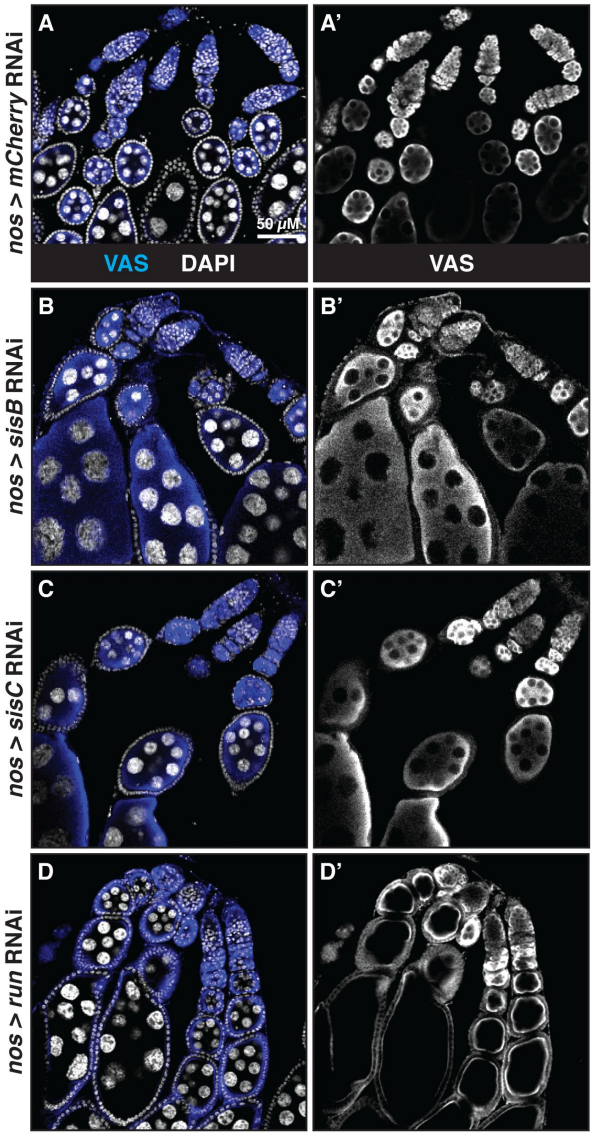

Figure S4

RNAi against most somatic XSEs does not affect the female germline

A-D ') Immunofluorescence of adult ovaries to characterize germ cell phenotypes resulting from RNAi against somatic XSEs. A-A') Wildtype ovary from fly with germline-specific *mCherry* RNAi. B-B') Ovary from fly with germline-specific *sisB* RNAi. C-C') Ovary from fly with germline-specific *sisC* RNAi. D-D') Ovary from fly with germline-specific *run* RNAi. All ovaries resemble the wildtype control. VAS stains germ cells. DAPI stains DNA (nucleus).

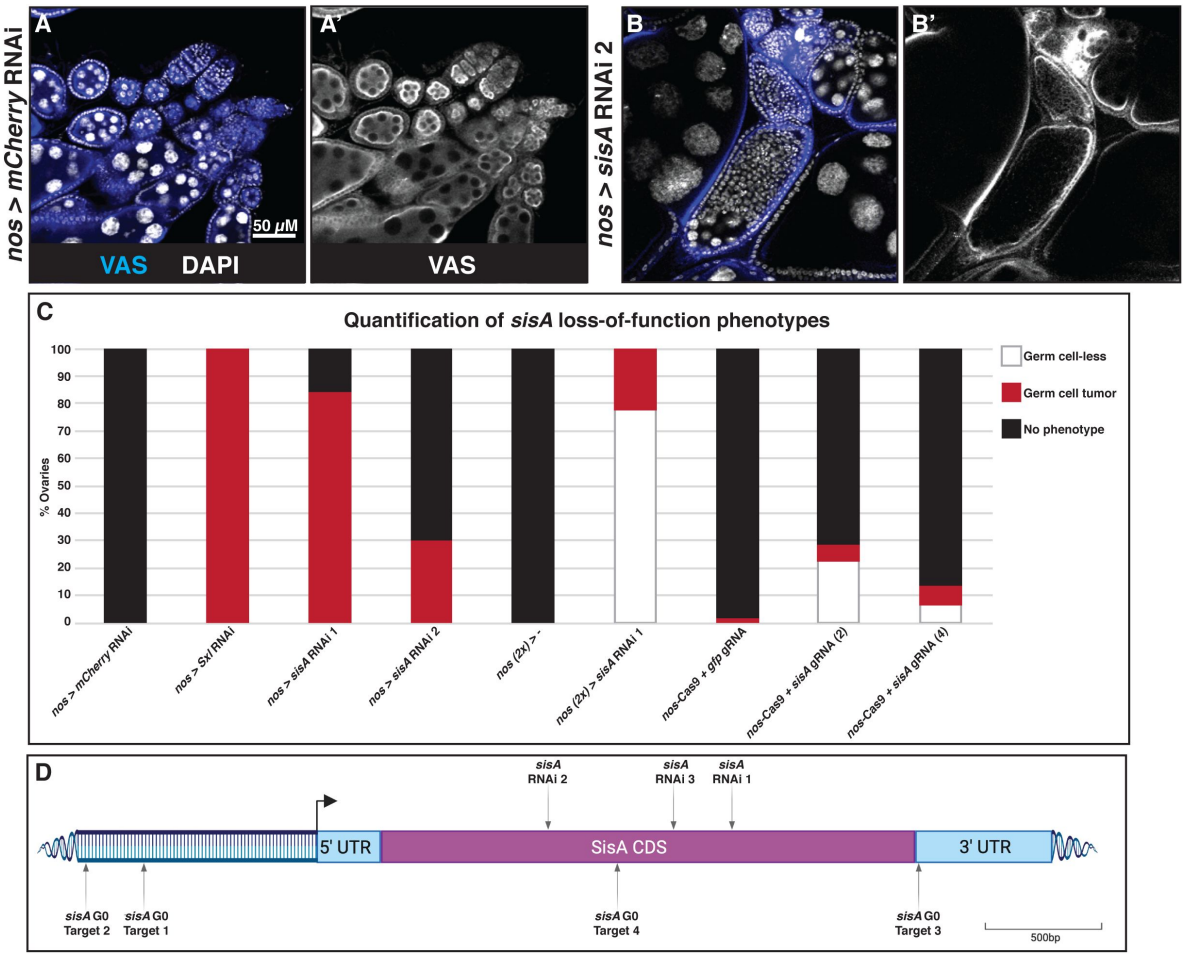

Figure S5

sisA loss of function in the female germline

A-B') Immunofluorescence of adult ovaries to characterize germ cell phenotypes. A-A') Wildtype ovary from fly with germline-specific *mCherry* RNAi. B-B') Ovary with germ cell tumors from fly with germline-specific *sisA* RNAi (*sisA* RNAi 2). Note that the severity of tumors is less than those observed with *sisA* RNAi 1. VAS stains germ cells. DAPI stains DNA (nucleus). C) Graph showing percentage of ovaries exhibiting either wildtype, germ cell tumor, or germ cell-less phenotypes resultant from different loss of function conditions. D) Cartoon showing extended *sisA* locus with target sites for RNAi and guide RNA targets for G0 CRISPR. Locus is drawn to scale. CDS: Coding sequences. UTR: Untranslated Region.

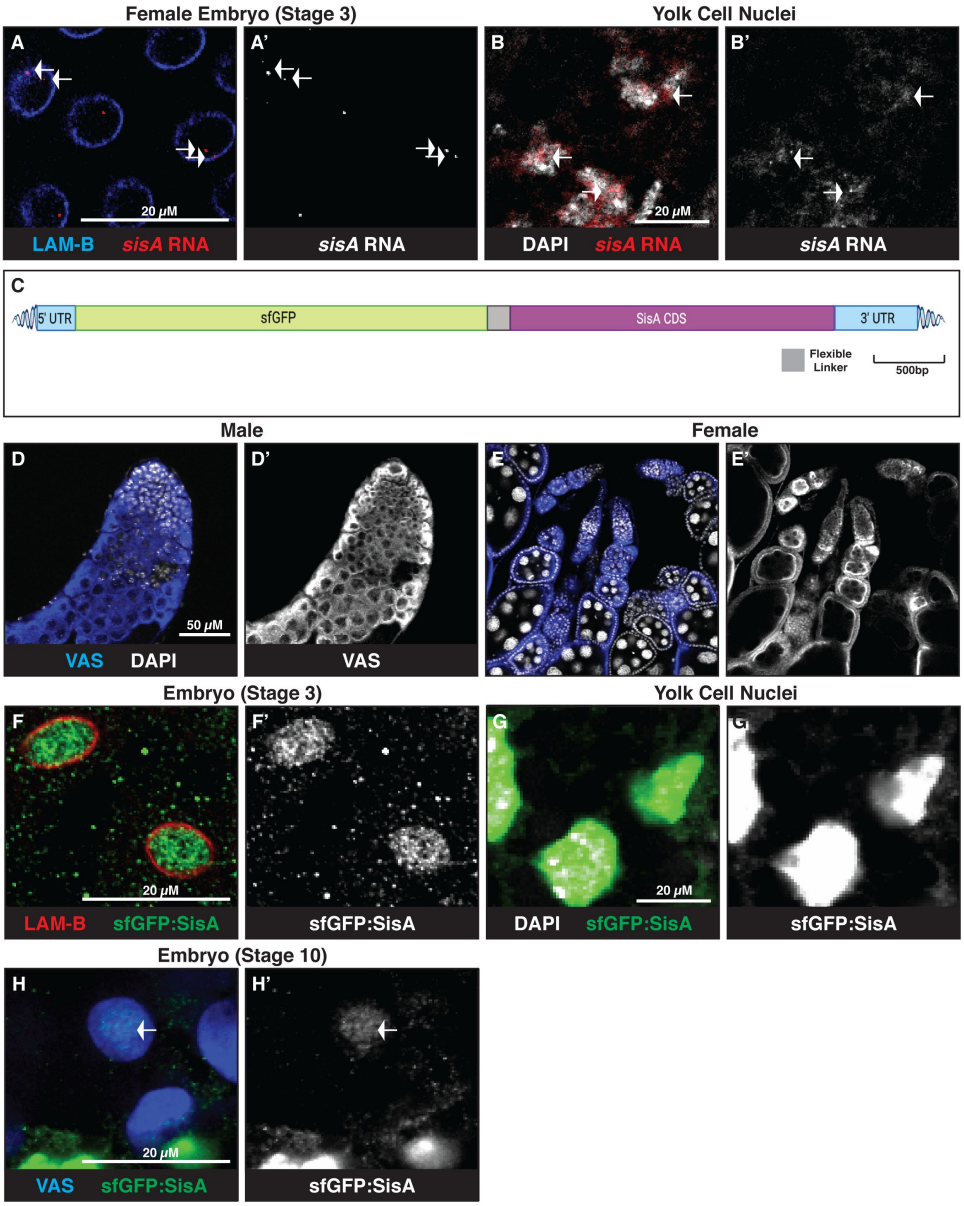

Figure S6

Characterizing *sisA* expression

A-B') RNA-FISH (+ Immunofluorescence) against *sisA* in embryonic somatic cells. A-A') Female somatic cells at stage 3 with *sisA* RNA-FISH signals. Note that two foci are observed per nucleus indicating the presence of two X chromosomes that are unpaired. Arrows mark fluorescent nuclear foci of RNA-FISH signals. B-B') Yolk cell nuclei with *sisA* RNA-FISH signals. Arrows mark fluorescent nuclear foci of RNA-FISH signals. DAPI stains DNA (nucleus). C) Cartoon showing the *sisA* gene locus and location of endogenous sfGFP tag. An sfGFP tag is inserted at the N-terminus of *sisA*'s CDS to generate the sfGFP:SisA tag. Locus is drawn to scale. Tag is not drawn to scale. CDS: Coding sequences. UTR: Untranslated Region. D-E') Immunofluorescence of adult gonads to characterize germ cell phenotypes caused by the N-terminal sfGFP tag on SisA. The gonads resemble wildtype gonads, suggesting that the tag does not impair SisA function. F-F') Immunofluorescence of female somatic cells at stage 3 expressing sfGFP:SisA. G-G') Immunofluorescence of yolk cell nuclei expressing sfGFP:SisA. Note that this expression is nuclear, consistent with SisA's characterization as a bZIP transcription factor. H-H') Immunofluorescence of embryonic stage 10 PGCs from flies bearing sfGFP:SisA (SisA tag). LAM-B stains nuclear lamina. VAS stains germ cells. DAPI stains DNA (nucleus).

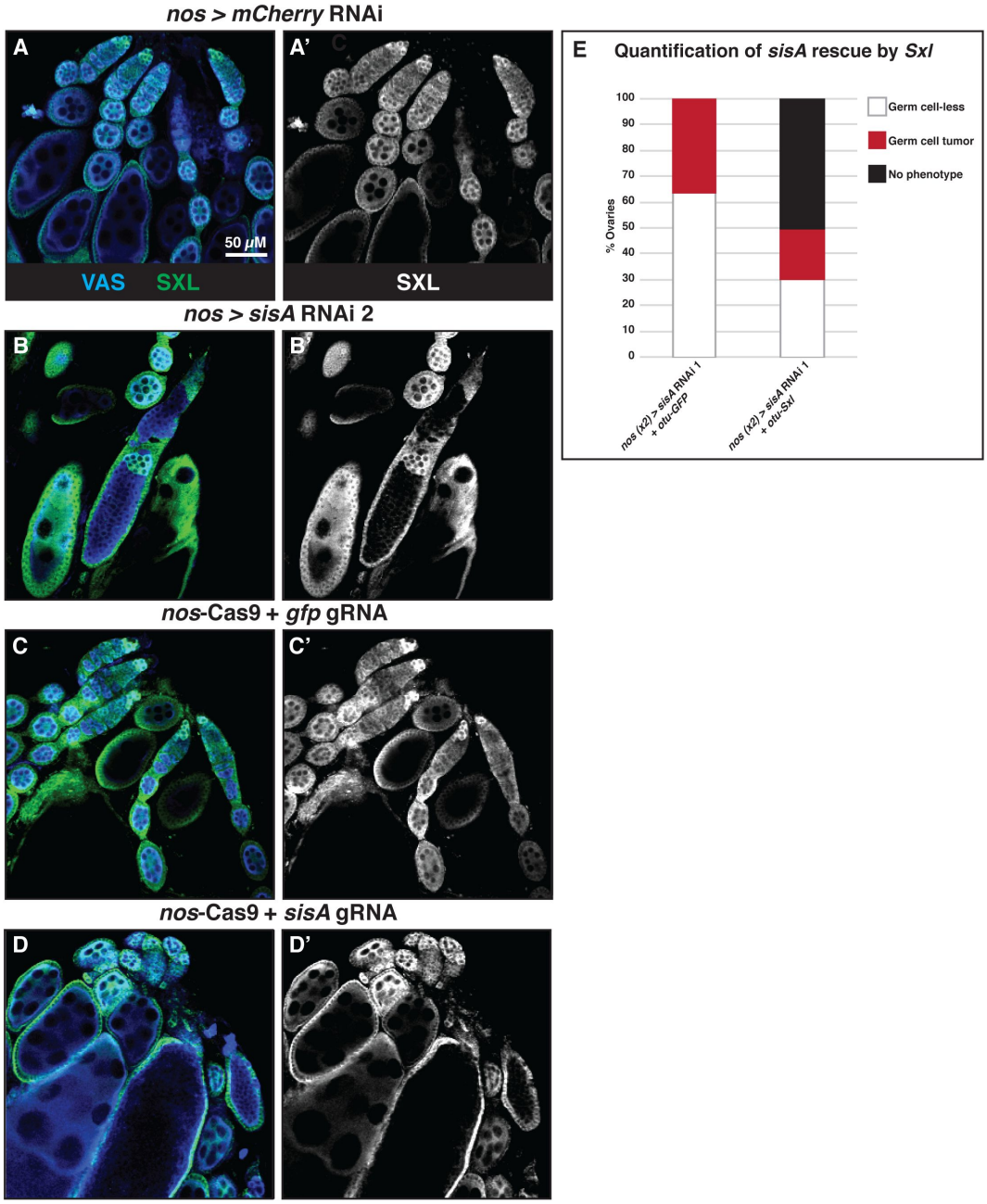

Figure S7

sisA lies upstream of *Sxl* in the female germline

A-D ') Immunofluorescence of adult ovaries to characterize Sxl expression. A-A') Wildtype ovary from fly with germline-specific *mCherry* RNAi. Sxl expression is highest in the early germ cells and decreases in differentiating germ cells. B-B') Ovary with germ cell tumors from fly with germline-specific *sisA* RNAi (*sisA* RNAi 2). Note that tumorous germ cells lack Sxl expression. Somatic Sxl remains unaffected. C-C') Wildtype ovary from fly with mutations in the germline against *gfp*. Sxl expression is wildtype. D-D') Ovary with germ cell tumors from fly with mutations in the germline against *sisA*. Note that tumorous germ cells lack Sxl expression. VAS stains germ cells. SXL stains Sxl. E) Graph showing percentage of ovaries exhibiting either wildtype, germ cell tumor, or germ cell-less phenotypes, with or without rescue of *sisA* loss of function by *Sxl*.

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Reviewer #1 (Public Review):

Summary:

In Drosophila melanogaster, expression of Sex-lethal (Sxl) protein determines sexual identity and drives female development. Functional Sxl protein is absent from males where splicing includes a termination codon-containing "poison" exon. Early during development, in the soma of female individuals, Sxl expression is initiated by an X chromosome counting mechanism that activates the Sxl establishment promoter (SxlPE) to produce an initial amount of Sxl protein. This then suppresses the inclusion of the "poison" exon, directing the constructive splicing of Sxl transcripts emerging from the Sxl maintenance promotor (SxlPM) which is activated at a later stage during development irrespective of sex. This autoregulatory loop maintains Sxl expression and commits to female development.

Sxl also determines the sexual identity of the germline. Here Sxl expression generally follows the same principles as in somatic tissues, but the way expression is initiated differs from the soma. This regulation has so far remained elusive.

In the presented manuscript, Goyal et al. show that activation of Sxl expression in the germline depends on additional regulatory DNA sequences, or sequences different from the ones driving initial Sxl expression in the soma. They further demonstrate that sisterless A (sisA), a transcription factor that is required for activation of Sxl expression in the soma, is also necessary, but not sufficient, to initiate the expression of functional Sxl protein in female germ cells. sisA expression precedes Sxl induction in the germline and its ablation by RNAi results in impaired expression of Sxl, formation of ovarian tumors, and germline loss, phenocopying the loss of Sxl. Intriguingly, this phenotype can be rescued by the forced expression of Sxl, demonstrating that the primary function of sisA in the germline is the induction of Sxl expression.

Strengths:

The clever design of probes (for RNA FISH) and reporters allowed the authors to dissect Sxl expression from different promoters to get novel insight into sex-specific gene regulation in the germline. All experiments are carefully controlled. Since Sxl regulation differs between the soma and the germline, somatic tissues provide elegant internal controls in many experiments, ensuring e.g. functionality of the reporters. Similarly, animals carrying newly generated alleles (e.g. genomic tagging of the Sxl locus) are fertile and viable, demonstrating that the genetic manipulation does not interfere with protein function. The conclusions drawn from the experimental data are sound and advance our understanding of how Sxl expression is induced in the female germline.

Weaknesses:

The assays employed by the authors provide valuable information on when Sxl promoters become active. However, since no information on the stability of the gene products (i.e. RNA

and protein) is available, it remains unclear when the SxlPE promoter is switched off in the germline (conceptually it only needs to be active for a short time period to initiate production of functional Sxl protein). As correctly stated by the authors, the persisting signals observed in the germline might therefore not reflect the continuous activity of the SxlPE promoter.

Mapping of regulatory elements and their function: SxlPE with 1.5 kb of flanking upstream sequence is sufficient to recapitulate early Sxl expression in the soma. The authors now provide evidence that beyond that, additional DNA sequences flanking the SxlPE promoter are required for germline expression. However, a more precise mapping was not performed. Also, due to technical limitations, the authors could not precisely map the sisA binding sites. Since this protein is also involved in the somatic induction of Sxl, its binding sites likely reside in the region 1.5kb upstream of the SxlPE promoter, which has been reported to be sufficient for somatic regulation. The regulatory role of the sequences beyond SxlPE-1.5kb therefore remains unaddressed and it remains to be investigated which trans-acting factor(s) exert(s) its/their function(s) via this region.

The central question of how Sxl expression is initiated and controlled in the germline still remains unanswered. Since sisA is zygotically expressed in both the male and the female germline (Figure 4D), it is unlikely the factor that restricts Sxl expression to the female germline.

How does weak expression of Sxl in male tissues or expression above background after knockdown of sisA reconcile with the model that an autoregulatory feedback loop enforces constant and clonally inheritable Sxl expression once Sxl is induced? Is the current model for Sxl expression too simple or are we missing additional factors that modulate Sxl expression (such as e.g. Sister of Sex-lethal)? While I do not expect the authors to answer these questions, I would expect them to appropriately address these intriguing aspects in the discussion.

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Reviewer #2 (Public Review):

Summary:

The authors wanted to determine whether cis-acting factors of Sxl - two different Sxl promoters in somatic cells - regulate Sxl in a similar way in germ cells. They also wanted to determine whether trans-acting factors known to regulate Sxl in the soma also regulate Sxl in the germline.

Regarding the cis-acting factors, they examine the Sxl "establishment promoter" (SxlPE) that is activated in female somatic cells by the presence of two X chromosomes. Slightly later in development, dosage compensation equalizes X chromosome expression in males and females and so X chromosomes can no longer be counted. The second Sxl promoter is the "maintenance promoter," (SxlPM), which is activated in both sexes. The mRNA produced from the maintenance promoter has to be alternatively splicing from early Sxl protein generated earlier in development by the PE. This leads to an autoregulatory loop that maintains Sxl expression in female somatic cells. The authors used fluorescent in situ hybridization (FISH) with oligopaints to determine the temporal activation of the PE or PM promoters. They find that - unlike the soma - the PE does not precede the PM and instead is activated contemporaneously or later than the PM - this is confusing with the later results (see below). Next, they generated transcriptional reporter constructs containing large segments of the Sxl locus, the 1.5 kb used in somatic studies, a 5.2 kb reporter, and a 10.2 kb. Interestingly the 1.5 kb reporter that was reported to recapitulate Sxl expression in soma and germline was not observed by the authors. The 5.2 kb reporter was observed in female somatic cells but not in germ cells. Only when they include an additional 5 kb downstream of

the 5.2 kb reporter (here the 10.2 kb reporter) they did see expression in germ cells but this occurred at the L1 stages. Their data indicate that Sxl activity in the germ requires different cis-regulation than the soma and that the PE is activated later in germ cells than in somatic cells. The authors next use gene editing to insert epitope tags in two distinct strains in the hopes of creating an early Sxl and a later Sxl protein derived from the PE and PM, respectively. The HA-tagged protein from the PE was seen in somatic cells but never in the germline, possibly due to very low expression. The FLAG-tagged late Sxl protein is observed in L2 germ cells. Because the early HA-Sxl protein is not perceptible in germ cells, it is not possible to conclude its role in the germline. However, because late FLAG-Sxl was only observed in L2 germ cells and the PE was detected in L1, this leaves open the possibility that PE produces early HA-Sxl (which currently cannot be detected), which then alternatively splices the transcript from the PM. In other words, the soma and germline could have a similar temporal relationship between the two Sxl promoters. While I agree with the authors about this conclusion, the earlier work with the oligopaints leads to the conclusion that SE is active after PM. This is confusing.

Next, the authors wanted to turn their attention to the trans-acting factors that regulate Sxl in the soma, including Sisterless A (SisA), SisB, Runt, and the JAK/STAT ligand Unpaired. Using germline RNAi, the authors found that only knockdown of SisA causes ovarian tumors, similar to the loss of Sxl, suggesting that SisA regulates Sxl (ie the PE) in both the soma and the germline. They generated a SisA null allele using CRISPR/Cas9 and these animals had ovarian tumors and germ cell-less ovaries. FISH revealed that sisA is activated in primordial germ cells in stages 3-6 before the activation of Sxl. They used CRISPR-Cas9 to generate an endogenously-tagged SisA and found that tagged SisA was expressed in stage 3-6 PCGs, which is consistent with activating PE in the germline. They showed that sisA is upstream of Sxl as germline depletion of sisA led to a significant decrease in expression from the 10.2 kb PE reporter and in SXL protein. The authors could rescue the ovarian tumors and loss of Sxl protein upon germline depletion of sisA by supplying Sxl from another protein (the otu promoter). These data indicate that sisA is necessary for Sxl activation in the germline. However, ectopic sisA in germ cells in the testis did not lead to ectopic Sxl, suggesting that sisA is not sufficient to activate Sxl in the germline.

Strengths:

(1) The genetic and genomic approaches in this study are top-notch and they have generated reagents that will be very useful for the field.

(2) Excellent use of powerful approaches (oligo paint, reporter constructs, CRISPR-Cas9 alleles).

(3) The combination of state of art approaches and quantification of phenotypes allows the authors to make important conclusions.

Weaknesses:

(1) Confusion in line 127 (this indicates that SxlPE is not activated before SxlPM in the germline) about PE not being activated before the PM in the germline when later figures show that PE is activated in L1 and late Sxl protein is seen in L2. It would be helpful to the readers if the authors edited the text to avoid this confusion. Perhaps more explanation of the results at specific points would be helpful.

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Reviewer #3 (Public Review):

Summary:

The mechanisms governing the initial female-specific activation of Sex-lethal (Sxl) in the soma, the subsequent maintenance of female-specific expression and the various functions of Sxl in somatic sex determination and dosage compensation are well documented. While Sxl is also expressed in the female germline where it plays a critical role during oogenesis, the pathway that is responsible for turning Sxl on in germ cells has been a long-standing mystery. This manuscript from Goyal et al describes studies aimed at elucidating the mechanism(s) for the sex-specific activation of the Sex-lethal (Sxl) gene in the female germline of Drosophila.

In the soma, the Sxl establishment promoter, Sxl-Pe, is regulated in pre-cellular blastoderm embryos in somatic cells by several X-linked transcription factors (sis-a, sis-b, sis-c and runt). At this stage of development, the expression of these transcription factors is proportional to gene dose, 2x females and 1x in males. The cumulative two-fold difference in the expression of these transcription factors is sufficient to turn Sxl-Pe on in female embryos. Transcripts from the Sxl-Pe promoter encode an "early" version of the female Sxl protein, and they function to activate a splicing positive autoregulatory loop by promoting the female-specific splicing of the initial pre-mRNAs derived from the Sxl maintenance promoter, Sxl-Pm (which is located upstream of Sxl-Pm). These female Sxl-Pm mRNAs encode a Sxl protein with a different N-terminus from the Sxl-Pe mRNAs, and they function to maintain female-specific splicing in the soma during the remainder of development.

In this manuscript, the authors are trying to understand how the Sxl-Pm positive autoregulatory loop is established in germ cells. If Sxl-Pe is used and its activation precedes Sxl-Pm as is true in the soma, they should be able to detect Sxl-Pe transcripts in germ cells before Sxl-Pm transcripts appear. To test this possibility, they generated RNA FISH probes complementary to the Sxl-Pe first exon (which is part of an intron sequence in the Sxl-Pm transcript) and to a "common sequence" that labels both Sxl-Pe and Sxl-Pm transcripts. Transcripts labeled by both probes were detected in germ cells beginning at stage 5 (and reaching a peak at stage 10), so either the Sxl-Pm and Sxl-Pe promoters turn on simultaneously, or Sxl-Pe is not active.

They next switched to Sxl-Pe reporters. The first Sxl-Pe:gfp reporter they used has a 1.5 kb upstream region which in other studies was found to be sufficient to drive sex-specific expression in the soma of blastoderm embryos. Also like the endogenous Sxl gene it is not expressed in germ cells at this early stage. In 2011, Hashiyama et al reported that this 1.5 kb promoter fragment was able to drive gfp expression in Vasa-positive germ cells later in development in stage 9/10 embryos. However, because of the high background of gfp in the nearby soma, their result wasn't especially convincing. Though they don't show the data, Goyal et al indicated that unlike Hashiyama et al they were unable to detect gfp expressed from this reporter in germ cells. Goyal et al extended the upstream sequences in the reporter to 5 kb, but they were still unable to detect germline expression of gfp.

Goyal et al then generated a more complicated reporter which extends 5 kb upstream of the Sxl-Pe start site and 5 kb downstream-ending at or near 4th exon of the Sxl-Pm transcript (the Sxl-Pe10 kb reporter). (The authors were not explicit as to whether the 5 kb downstream sequence extended beyond the 4th exon splice junction-in which case splicing could potentially occur with an upstream exon(s)-or terminated prior to the splice junction as seems to be indicated in their diagram.) With this reporter, they were able to detect sex-specific gfp expression in the germline beginning in L1 (first instar larva). With the caveat that gfp detection might be delayed compared to the onset of reporter activation, these findings indicated that the sequences in the reporter are able to drive sex-specific transcription in the germline at least as early as L1.

The authors next tagged the N-terminal end of the Sxl-Pe protein with HA (using Crispr/Cas9) and the N-terminal end of Sxl-Pm protein with Flag. They report that the HA-Sxl-Pe protein is first detected in the soma at stage 9 of embryogenesis. Somatic HA-Sxl-Pe protein persists into

L1, but is no longer detected in L2. However, while somatic HA-Sxl-Pe protein is detected, they were unable to detect HA-Sxl-Pe protein in germ cells. In the case of FLAG-Sxl-Pm, it could first be detected in L2 germ cells indicating that at this juncture the Sxl-positive autoregulatory loop has been activated. This contrasts with Sxl-Pm transcripts which are observed in a few germ cells at stage 5 of embryogenesis, and in most germ cells by stage 10. The authors propose (based on the expression pattern of the Sxl-Pe10kb reporter and the appearance of Flag-Sxl-Pm protein) that Sxl-Pe comes on in germ cells in L1, and that the Sxl-Pe protein activates the female splicing of Sxl-Pm transcripts, giving detectable Flag-Sxl-Pm proteins beginning in L2.

To investigate the signals that activate Sxl-Pe in germ cells, the authors tested four of the X-linked genes (sis-a, sis-b, sis-c, and runt) that function to activate Sxl-Pe in the soma in early embryos. RNAi knockdown of sis-b, sis-c, and runt had no apparent effect on oogenesis. In contrast, knockdown of sis-a resulted in tumorous ovaries, a phenotype associated with Sxl mutations. (Three different RNAi transgenes were tested-two gave this phenotype, the third did not.) Sxl-Pe10kb reporter activity in L1 female germ cells is also dependent on sis-A.

Several approaches were used to confirm a role for sis-a in a) oogenesis and b) the activation of the Sxl-Pm autoregulatory loop. They showed that sis-a germline clones (using tissue-specific Crispr/Cas9 editing) resulted in the tumorous ovary phenotype and reduced the expression of Sxl protein in these ovaries. They found that sis-a transcripts and GFP-tagged Sis-A protein are present in germ cells. Finally, they showed tumorous ovary phenotype induced by germline RNAi knockdown of sis-a can be partially rescued by expressing Sxl in the germ cells.

Critique:

While this manuscript addresses a longstanding puzzle - the mechanism activating the Sxl autoregulatory loop in female germ cells-and likely identified an important germline transcriptional activator of Sxl, sis-a, the data that they've generated doesn't make a compelling story. At every step, there are puzzle pieces that don't fit the narrative. In addition, some of their findings are inconsistent with many previous studies.

(1) The authors used RNA FISH to time the expression of Sxl-Pe and Sxl-Pm transcripts in germ cells. Transcripts complementary to Sxl-Pe and Sxl-Pm were detected at the same time in embryos beginning at stage 5. This is not a definitive experiment as it could mean a) that Sxl-Pe and Sxl-Pm turn on at the same time, b) that Sxl-Pe comes on after Sxl-Pm (as suggested by the Sxl-Pe10kb reporter) or c) Sxl-Pe never comes on.

(2) Hashiyama et al reported that they detected gfp expression in stage 9/10 germ cells from a 1.5 kb Sxl-Pe-gfp. As noted above, this result wasn't entirely convincing and thus it isn't surprising that Goyal et al were unable to reproduce it. Extending the upstream sequences to just before the 1st exon of Sxl-Pm transcripts also didn't give gfp expression in germ cells. Only when they added 5 kb downstream did they detect gfp expression. However, from this result, it isn't possible to conclude that the Sxl-Pe promoter is actually driving gfp expression in L1 germ cells. Instead, the Sxl promoter active in the germ line could be anywhere in their 10 kb reporter.

(3) At least one experiment suggests that Sxl-Pe never comes on in germ cells. The authors tagged the N-terminus of the Sxl-Pe protein with HA and the N-terminus of the Sxl-Pm protein with Flag. Though they could detect HA-Sxl-Pe protein in the soma, they didn't detect it in germ cells. On the other hand, the Flag-Sxl-Pm protein was detected in L2 germ cells (but not earlier). These results would more or less fit with those obtained for the 10 kb reporter and would support the following model: Prior to L1, Sxl-Pm transcripts are expressed and spliced in the male pattern in both male and female germ cells. During L1, Sxl protein expressed via a mechanism that depends upon a 10 kb region spanning Sxl-Pe (but not on Sxl-Pe) is

produced and by L2 there are sufficient amounts of this protein to switch the splicing of Sxl-Pm transcripts from a male to a female pattern-generating Flag-tagged Sxl-Pm protein.

(4) The 10kb reporter is sex-specific, but not germline-specific. The levels of gfp in female L1 somatic cells are equal to if not greater than those in L1 female germ cells. That the Sxl-Pe10kb reporter is active in the soma complicates the conclusion that it represents a germ line-specific promoter. Germline activity is, however, sensitive to sis-A knockdowns which is plus. Presumably, somatic expression of the reporter wouldn't be sensitive to a (late) sis-A knockdown- but this wasn't shown.

(5) Their results with the HA-Sxl-Pe protein don't fit with many previous studies-assuming that the authors have explained their results properly. They report that HA-Sxl-Pe protein is first detected in the soma at stage 9 of embryogenesis and that it then persists till L2. However, previous studies have shown that Sxl-Pe transcripts and then Sxl-Pe proteins are first detected in ~NC11-NC12 embryos. In RNase protection experiments, the Sxl-Pe exon is observed in 2-4 hr embryos, but not detected in 5-8 hr, 14-12 hr, L1, L2, L3, or pupae. Northerns give pretty much the same picture. Western blots also show that Sxl-Pe proteins are first detectable around the blastoderm stage. So it is not at all clear why HA-Sxl-Pe proteins are first observed at stage 9 which, of course, is well after the time that the Sxl-Pm autoregulatory loop is established.

Given the obvious problems with the initial timing of somatic expression described here, it is hard to know what to make of the fact that HA-tagged Sxl-Pe proteins aren't observed in germ cells.

As for the presence of HA-Sxl-Pe proteins later than expected: While RNase protection/Northern experiments showed that Sxl-Pe mRNAs are expressed in 2-4 hr embryos and disappear thereafter, one could argue from the published Western experiments that the Sxl-PE proteins expressed at the blastoderm stage persist at least until the end embryogenesis, though perhaps at somewhat lower levels than at earlier points in development. So the fact that Goyal et al were able to detect HA-Sxl-Pe proteins in stage 9 embryos and later on in L1 larva probably isn't completely unexpected. What is unexpected is that the HA-Sxl-Pe proteins weren't present earlier.

(6) The authors use RNAi and germline clones to demonstrate that sis-A is required for proper oogenesis: when sis-A activity is compromised in germ cells, i) tumorous ovary phenotypes are observed and ii) there is a reduction in the expression of Sxl-Pm protein. They are also able to rescue the phenotypic effects of sis-a knockdown by expressing a Sxl-Pm protein. While the experiments indicating sis-a is important for normal oogenesis and that at least one of its functions is to ensure that sufficient Sxl is present in the germline stem cells seem convincing, other findings would make the reader wonder whether Sis-A is actually functioning (directly) to activate Sxl transcription from promoter X.

The authors show that sis-a mRNAs and proteins are expressed in stage 3-5 germ cells (PGCs). This is not unexpected as the X-linked transcription factors that turn Sxl-Pe on are expressed prior to nuclear migration, so their protein products should be present in early PGCs. The available evidence suggests that their transcription is shut down in PGCs by the factors responsible for transcriptional quiescence (e.g., nos and pgc) in which case transcripts might be detected in only one or two PGC-which fits with their images. However, it is hard to believe that expression of Sis-A protein in pre-blastoderm embryos is relevant to the observed activation of the Sxl-Pm autoregulatory loop hours later in L2 larva.

It is also not clear how the very low level of gfp-Sis-A seen in only a small subset of migrating germ cells in stage 10 embryos (Figure S6) would be responsible for activating the Sxl-Pe10kb reporter in L1. It seems likely that the small amount of protein seen in stage 10 embryos is left over from the pre-cellular blastoderm stage. In this case, it would not be surprising to

discover that the residual protein is present in both female and male stage 10 germ cells. This would raise further doubts about the relevance of the gfp-Sis-A at these early stages.

In fact, given the evidence presented implicating sis-a in activating Sxl, (the germline activation of the Sxl-Pe10kb reporter, the RNAi knockdowns, and the germ cell-specific sis-a clones) it is clear that the sis-A RNAs and proteins seen in pre-cellular blastoderm PGCs aren't relevant. The germline clone experiment (and also the RNAi knockdowns) indicates that sis-A must be transcribed in germ cells after Cas9 editing has taken place. Presumably, this would be after transcription is reactivated in the germline (~stage 10) and after the formation of the embryonic gonad (stage 14) so that the somatic gonadal cells can signal to the germ cells. With respect to the reporter, the relevant time frame for showing that sis-A is present in germ cells would be even later in L1.

(7) As noted above, the data in this manuscript do not support the idea that Sxl-Pe proteins activate the Sxl-Pm female splicing in the germline. Flybase indicates that there is at least one other Sxl promoter that could potentially generate a transcript that includes the male exon but still could encode a Sxl protein. This promoter "Sxl-Px" is located downstream of Sxl-Pm and from its position it could have been included in the authors' 10 kb reporter. The reported splicing pattern of the endogenous transcript skips exon2, and instead links an exon just downstream of Sxl-Px to the male exon. The male exon is then spliced to exon4. If the translation doesn't start and end at one of the small upstream orfs in the exons close to Sxl-Px and the male exon, a translation could begin with an AUG codon in exon4 that is in frame with the Sxl protein coding sequence. This would produce a Sxl protein that lacks aa sequences from N-terminus, but still retains some function.

Another possible explanation for how gfp is expressed from the 10 kb reporter is that the transcript includes the "z" exon described by Cline et al., 2010.

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Author response:

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *In Drosophila melanogaster, expression of Sex-lethal (Sxl) protein determines sexual identity and drives female development. Functional Sxl protein is absent from males where splicing includes a termination codon-containing "poison" exon. Early during development, in the soma of female individuals, Sxl expression is initiated by an X chromosome counting mechanism that activates the Sxl establishment promoter (SxlPE) to produce an initial amount of Sxl protein. This then suppresses the inclusion of the "poison" exon, directing the constructive splicing of Sxl transcripts emerging from the Sxl maintenance promotor (SxlPM) which is activated at a later stage during development irrespective of sex. This autoregulatory loop maintains Sxl expression and commits to female development.*
>
> *Sxl also determines the sexual identity of the germline. Here Sxl expression generally follows the same principles as in somatic tissues, but the way expression is initiated differs from the soma. This regulation has so far remained elusive.*
>
> *In the presented manuscript, Goyal et al. show that activation of Sxl expression in the germline depends on additional regulatory DNA sequences, or sequences different from the ones driving initial Sxl expression in the soma. They further demonstrate that*

> *sisterless A (sisA), a transcription factor that is required for activation of Sxl expression in the soma, is also necessary, but not sufficient, to initiate the expression of functional Sxl protein in female germ cells. sisA expression precedes Sxl induction in the germline and its ablation by RNAi results in impaired expression of Sxl, formation of ovarian tumors, and germline loss, phenocopying the loss of Sxl. Intriguingly, this phenotype can be rescued by the forced expression of Sxl, demonstrating that the primary function of sisA in the germline is the induction of Sxl expression.*
>
> *Strengths:*
>
> *The clever design of probes (for RNA FISH) and reporters allowed the authors to dissect Sxl expression from different promoters to get novel insight into sex-specific gene regulation in the germline. All experiments are carefully controlled. Since Sxl regulation differs between the soma and the germline, somatic tissues provide elegant internal controls in many experiments, ensuring e.g. functionality of the reporters. Similarly, animals carrying newly generated alleles (e.g. genomic tagging of the Sxl locus) are fertile and viable, demonstrating that the genetic manipulation does not interfere with protein function. The conclusions drawn from the experimental data are sound and advance our understanding of how Sxl expression is induced in the female germline.*
>
> *Weaknesses:*
>
> *The assays employed by the authors provide valuable information on when Sxl promoters become active. However, since no information on the stability of the gene products (i.e. RNA and protein) is available, it remains unclear when the SxlPE promoter is switched off in the germline (conceptually it only needs to be active for a short time period to initiate production of functional Sxl protein). As correctly stated by the authors, the persisting signals observed in the germline might therefore not reflect the continuous activity of the SxlPE promoter.*
>
> *Mapping of regulatory elements and their function: SxlPE with 1.5 kb of flanking upstream sequence is sufficient to recapitulate early Sxl expression in the soma. The authors now provide evidence that beyond that, additional DNA sequences flanking the SxlPE promoter are required for germline expression. However, a more precise mapping was not performed. Also, due to technical limitations, the authors could not precisely map the sisA binding sites. Since this protein is also involved in the somatic induction of Sxl, its binding sites likely reside in the region 1.5kb upstream of the SxlPE promoter, which has been reported to be sufficient for somatic regulation. The regulatory role of the sequences beyond SxlPE-1.5kb therefore remains unaddressed and it remains to be investigated which trans-acting factor(s) exert(s) its/their function(s) via this region.*

We agree that a more precise mapping of the essential elements within the 10.2 kb reporter is an important direction in which to proceed. Unfortunately, this is out of the scope of the current manuscript given current lab personnel. In regard to the 1.5 kb promoter that activates SxlPE in the soma, we do not feel that the Sisa binding sites are necessarily in this region. It is important to note that, while the 1.5 kb promoter is sufficient for female-specific expression in the soma, it may not contain all of the regulatory elements that normally regulate PE from the endogenous locus. Activation of PE in the soma is thought to be regulated by a combination of positive-acting factors (SisA, SisB, etc.) and repressive factors (e.g. Dpn) that set a threshold for PE activation. Much more work would need to be done to determine whether all of these factors bind to the 1.5 kb promoter, or whether additional sequences are also involved to control the proper timing and robustness of normal Sxl PE activation in the soma.

> *The central question of how Sxl expression is initiated and controlled in the germline still remains unanswered. Since sisA is zygotically expressed in both the male and the female*

> *germline (Figure 4D), it is unlikely the factor that restricts Sxl expression to the female germline.*

X chromosome "counting" elements like SisA are always expressed in both males and females, but it is thought that the 2X does of them in females activates PE, while the 1X does in males does not. Thus, we do expect SisA to be expressed in both males and females as we observed.

> *How does weak expression of Sxl in male tissues or expression above background after knockdown of sisA reconcile with the model that an autoregulatory feedback loop enforces constant and clonally inheritable Sxl expression once Sxl is induced? Is the current model for Sxl expression too simple or are we missing additional factors that modulate Sxl expression (such as e.g. Sister of Sex-lethal)? While I do not expect the authors to answer these questions, I would expect them to appropriately address these intriguing aspects in the discussion.*

It is difficult to know what is "background" and what is actual weak Sxl expression in males. We agree that, if it is real, then why it doesn't activate autoregulation of the Sxl PM transcript is mysterious. And yes, the current model for female-specific expression of Sxl in the soma may well be incomplete. Sxl PM transcript is present in the testis based on community RNA-seq data and our own analysis of male vs. female bam-mutant gonads (PMID 31329582), but it is at lower levels. Whether the lower level in the testis is due to tissue differences or sex-specific regulation of RNA levels is unknown. Our observations that the HA-tagged Sxl Early protein remains present in somatic cells in L1 larvae, and that GFP expression from the 10.2 kb Sxl PE-GFP can be detected in the soma until L2 could either be due to perdurance of the protein products, or continued sex-specific expression of PE long after the time that it was thought to shut off. This is also long after dosage compensation should have equalized the expression of X chromosome gene expression, meaning that X chromosomes can no longer be "counted" by factors like SisA and SisB. Thus, sex-specific expression of PE at this time would require another mechanism besides the current model (such as feedback regulation of Sxl PE transcription from downstream factors).

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *The authors wanted to determine whether cis-acting factors of Sxl - two different Sxl promoters in somatic cells - regulate Sxl in a similar way in germ cells. They also wanted to determine whether trans-acting factors known to regulate Sxl in the soma also regulate Sxl in the germline.*
>
> *Regarding the cis-acting factors, they examine the Sxl "establishment promoter" (SxlPE) that is activated in female somatic cells by the presence of two X chromosomes. Slightly later in development, dosage compensation equalizes X chromosome expression in males and females and so X chromosomes can no longer be counted. The second Sxl promoter is the "maintenance promoter," (SxlPM), which is activated in both sexes. The mRNA produced from the maintenance promoter has to be alternatively splicing from early Sxl protein generated earlier in development by the PE. This leads to an autoregulatory loop that maintains Sxl expression in female somatic cells. The authors used fluorescent in situ hybridization (FISH) with oligopaints to determine the temporal activation of the PE or PM promoters. They find that - unlike the soma - the PE does not precede the PM and instead is activated contemporaneously or later than the PM - this is confusing with the later results (see below). Next, they generated transcriptional reporter constructs containing large segments of the Sxl locus, the 1.5 kb used in somatic studies, a 5.2 kb reporter, and a 10.2 kb. Interestingly the 1.5 kb reporter that was reported to*

> *recapitulate Sxl expression in soma and germline was not observed by the authors. The 5.2 kb reporter was observed in female somatic cells but not in germ cells. Only when they include an additional 5 kb downstream of the 5.2 kb reporter (here the 10.2 kb reporter) they did see expression in germ cells but this occurred at the L1 stages. Their data indicate that Sxl activity in the germ requires different cis-regulation than the soma and that the PE is activated later in germ cells than in somatic cells. The authors next use gene editing to insert epitope tags in two distinct strains in the hopes of creating an early Sxl and a later Sxl protein derived from the PE and PM, respectively. The HA-tagged protein from the PE was seen in somatic cells but never in the germline, possibly due to very low expression. The FLAG-tagged late Sxl protein is observed in L2 germ cells. Because the early HA-Sxl protein is not perceptible in germ cells, it is not possible to conclude its role in the germline. However, because late FLAG-Sxl was only observed in L2 germ cells and the PE was detected in L1, this leaves open the possibility that PE produces early HA-Sxl (which currently cannot be detected), which then alternatively splices the transcript from the PM. In other words, the soma and germline could have a similar temporal relationship between the two Sxl promoters. While I agree with the authors about this conclusion, the earlier work with the oligopaints leads to the conclusion that SE is active after PM. This is confusing.*

The temporal relationship between Sxl PE and Sxl PM in the germline is indeed confusing. One source of confusion comes from whether one is discussing Sxl protein production or promoter activity. As the reviewer nicely summarizes, our transcription analysis with oligopaints indicates that, unlike in the soma, Sxl PE is NOT on in the germline prior to PM. Our other data indicate that PE is instead likely only active well after transcription from PM has begun. However, this still means that the temporal order of the EARLY and LATE Sxl proteins can be the same as the soma. Even if PM is active well before PE in the germline, the PE transcript cannot produce any functional protein in the absence of being alternatively spliced by the Sxl protein (Sxl autoregulation). Thus, even if PM is active before PE in the germline, we would not expect to observe any LATE Sxl protein until the PE promoter comes on, and produces a pulse of EARLY Sxl protein. The fact that we observe LATE Sxl protein at L2 is consistent with our observation that the 10.2 kb Sxl PE reporter is active at L1. We will attempt to explain all of this better in a revised manuscript.

> *Next, the authors wanted to turn their attention to the trans-acting factors that regulate Sxl in the soma, including Sisterless A (SisA), SisB, Runt, and the JAK/STAT ligand Unpaired. Using germline RNAi, the authors found that only knockdown of SisA causes ovarian tumors, similar to the loss of Sxl, suggesting that SisA regulates Sxl (ie the PE) in both the soma and the germline. They generated a SisA null allele using CRISPR/Cas9 and these animals had ovarian tumors and germ cell-less ovaries. FISH revealed that sisA is activated in primordial germ cells in stages 3-6 before the activation of Sxl. They used CRISPR-Cas9 to generate an endogenously-tagged SisA and found that tagged SisA was expressed in stage 3-6 PCGs, which is consistent with activating PE in the germline. They showed that sisA is upstream of Sxl as germline depletion of sisA led to a significant decrease in expression from the 10.2 kb PE reporter and in SXL protein. The authors could rescue the ovarian tumors and loss of Sxl protein upon germline depletion of sisA by supplying Sxl from another protein (the otu promoter). These data indicate that sisA is necessary for Sxl activation in the germline. However, ectopic sisA in germ cells in the testis did not lead to ectopic Sxl, suggesting that sisA is not sufficient to activate Sxl in the germline.*
>
> *Strengths:*
>
> *(1) The genetic and genomic approaches in this study are top-notch and they have generated reagents that will be very useful for the field.*

> *(2) Excellent use of powerful approaches (oligo paint, reporter constructs, CRISPR-Cas9 alleles).*
>
> *(3) The combination of state of art approaches and quantification of phenotypes allows the authors to make important conclusions.*
>
> *Weaknesses:*
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> *(1) Confusion in line 127 (this indicates that SxlPE is not activated before SxlPM in the germline) about PE not being activated before the PM in the germline when later figures show that PE is activated in L1 and late Sxl protein is seen in L2. It would be helpful to the readers if the authors edited the text to avoid this confusion. Perhaps more explanation of the results at specific points would be helpful.*

We agree--see response above.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
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> *The mechanisms governing the initial female-specific activation of Sex-lethal (Sxl) in the soma, the subsequent maintenance of female-specific expression and the various functions of Sxl in somatic sex determination and dosage compensation are well documented. While Sxl is also expressed in the female germline where it plays a critical role during oogenesis, the pathway that is responsible for turning Sxl on in germ cells has been a long-standing mystery. This manuscript from Goyal et al describes studies aimed at elucidating the mechanism(s) for the sex-specific activation of the Sex-lethal (Sxl) gene in the female germline of Drosophila.*
>
> *In the soma, the Sxl establishment promoter, Sxl-Pe, is regulated in pre-cellular blastoderm embryos in somatic cells by several X-linked transcription factors (sis-a, sis-b, sis-c and runt). At this stage of development, the expression of these transcription factors is proportional to gene dose, 2x females and 1x in males. The cumulative two-fold difference in the expression of these transcription factors is sufficient to turn Sxl-Pe on in female embryos. Transcripts from the Sxl-Pe promoter encode an "early" version of the female Sxl protein, and they function to activate a splicing positive autoregulatory loop by promoting the female-specific splicing of the initial pre-mRNAs derived from the Sxl maintenance promoter, Sxl-Pm (which is located upstream of Sxl-Pm). These female Sxl-Pm mRNAs encode a Sxl protein with a different N-terminus from the Sxl-Pe mRNAs, and they function to maintain female-specific splicing in the soma during the remainder of development.*
>
> *In this manuscript, the authors are trying to understand how the Sxl-Pm positive autoregulatory loop is established in germ cells. If Sxl-Pe is used and its activation precedes Sxl-Pm as is true in the soma, they should be able to detect Sxl-Pe transcripts in germ cells before Sxl-Pm transcripts appear. To test this possibility, they generated RNA FISH probes complementary to the Sxl-Pe first exon (which is part of an intron sequence in the Sxl-Pm transcript) and to a "common sequence" that labels both Sxl-Pe and Sxl-Pm transcripts. Transcripts labeled by both probes were detected in germ cells beginning at stage 5 (and reaching a peak at stage 10), so either the Sxl-Pm and Sxl-Pe promoters turn on simultaneously, or Sxl-Pe is not active.*
>
> *They next switched to Sxl-Pe reporters. The first Sxl-Pe:gfp reporter they used has a 1.5 kb upstream region which in other studies was found to be sufficient to drive sex-specific expression in the soma of blastoderm embryos. Also like the endogenous Sxl gene it is not expressed in germ cells at this early stage. In 2011, Hashiyama et al reported that*

this 1.5 kb promoter fragment was able to drive gfp expression in Vasa-positive germ cells later in development in stage 9/10 embryos. However, because of the high background of gfp in the nearby soma, their result wasn't especially convincing. Though they don't show the data, Goyal et al indicated that unlike Hashiyama et al they were unable to detect gfp expressed from this reporter in germ cells. Goyal et al extended the upstream sequences in the reporter to 5 kb, but they were still unable to detect germline expression of gfp.

Goyal et al then generated a more complicated reporter which extends 5 kb upstream of the Sxl-Pe start site and 5 kb downstream-ending at or near 4th exon of the Sxl-Pm transcript (the Sxl-Pe10 kb reporter). (The authors were not explicit as to whether the 5 kb downstream sequence extended beyond the 4th exon splice junction-in which case splicing could potentially occur with an upstream exon(s)-or terminated prior to the splice junction as seems to be indicated in their diagram.) With this reporter, they were able to detect sex-specific gfp expression in the germline beginning in L1 (first instar larva). With the caveat that gfp detection might be delayed compared to the onset of reporter activation, these findings indicated that the sequences in the reporter are able to drive sex-specific transcription in the germline at least as early as L1.

The authors next tagged the N-terminal end of the Sxl-Pe protein with HA (using Crispr/Cas9) and the N-terminal end of Sxl-Pm protein with Flag. They report that the HA-Sxl-Pe protein is first detected in the soma at stage 9 of embryogenesis. Somatic HA-Sxl-Pe protein persists into L1, but is no longer detected in L2. However, while somatic HA-Sxl-Pe protein is detected, they were unable to detect HA-Sxl-Pe protein in germ cells. In the case of FLAG-Sxl-Pm, it could first be detected in L2 germ cells indicating that at this juncture the Sxl-positive autoregulatory loop has been activated. This contrasts with Sxl-Pm transcripts which are observed in a few germ cells at stage 5 of embryogenesis, and in most germ cells by stage 10. The authors propose (based on the expression pattern of the Sxl-Pe10kb reporter and the appearance of Flag-Sxl-Pm protein) that Sxl-Pe comes on in germ cells in L1, and that the Sxl-Pe protein activates the female splicing of Sxl-Pm transcripts, giving detectable Flag-Sxl-Pm proteins beginning in L2.

To investigate the signals that activate Sxl-Pe in germ cells, the authors tested four of the X-linked genes (sis-a, sis-b, sis-c, and runt) that function to activate Sxl-Pe in the soma in early embryos. RNAi knockdown of sis-b, sis-c, and runt had no apparent effect on oogenesis. In contrast, knockdown of sis-a resulted in tumorous ovaries, a phenotype associated with Sxl mutations. (Three different RNAi transgenes were tested-two gave this phenotype, the third did not.) Sxl-Pe10kb reporter activity in L1 female germ cells is also dependent on sis-A.

Several approaches were used to confirm a role for sis-a in a) oogenesis and b) the activation of the Sxl-Pm autoregulatory loop. They showed that sis-a germline clones (using tissue-specific Crispr/Cas9 editing) resulted in the tumorous ovary phenotype and reduced the expression of Sxl protein in these ovaries. They found that sis-a transcripts and GFP-tagged Sis-A protein are present in germ cells. Finally, they showed tumorous ovary phenotype induced by germline RNAi knockdown of sis-a can be partially rescued by expressing Sxl in the germ cells.

Critique:

While this manuscript addresses a longstanding puzzle - the mechanism activating the Sxl autoregulatory loop in female germ cells-and likely identified an important germline transcriptional activator of Sxl, sis-a, the data that they've generated doesn't make a compelling story. At every step, there are puzzle pieces that don't fit the narrative. In addition, some of their findings are inconsistent with many previous studies.

We respect and appreciate this reviewer for the detailed comments. However, we feel that the claim that our work doesn't "make a compelling story" and that many "pieces…don't fit the narrative" is incorrect. The main issue that this reviewer raises is that we do not know if Sxl "early" transcription in the germline initiates from the Pe promoter. This is true, which we fully acknowledge, but the detail of whether "germline early" transcription of Sxl initiates from Pe or from other, as yet undefined, germline promoter does not affect the main conclusions of the paper. These conclusions are that a) regulation of Sxl in the germline is fundamentally different from in the soma and 2) despite point (1), sisA acts as an activator of Sxl in both the soma and the germline. Neither of these main points is disputed by this reviewer.

> *(1) The authors used RNA FISH to time the expression of Sxl-Pe and Sxl-Pm transcripts in germ cells. Transcripts complementary to Sxl-Pe and Sxl-Pm were detected at the same time in embryos beginning at stage 5. This is not a definitive experiment as it could mean a) that Sxl-Pe and Sxl-Pm turn on at the same time, b) that Sxl-Pe comes on after Sxl-Pm (as suggested by the Sxl-Pe10kb reporter) or c) Sxl-Pe never comes on.*

When designing this experiment, we wanted to test whether the "soma model" of Pe activation before Pm was also true in the germ cells. Our data clearly demonstrate that transcripts beginning downstream of Pe are not expressed prior to transcripts beginning downstream of Pm. Thus, we can state that the "soma model" of Pe first and then Pm does not occur in the germline, which is very interesting. However, we cannot make any other conclusions about Pe in the germline from these data, as the reviewer indicates.

> *(2) Hashiyama et al reported that they detected gfp expression in stage 9/10 germ cells from a 1.5 kb Sxl-Pe-gfp. As noted above, this result wasn't entirely convincing and thus it isn't surprising that Goyal et al were unable to reproduce it. Extending the upstream sequences to just before the 1st exon of Sxl-Pm transcripts also didn't give gfp expression in germ cells. Only when they added 5 kb downstream did they detect gfp expression. However, from this result, it isn't possible to conclude that the Sxl-Pe promoter is actually driving gfp expression in L1 germ cells. Instead, the Sxl promoter active in the germ line could be anywhere in their 10 kb reporter.*

We agree that we have not determined the transcriptional start sites for Sxl in the germline and it is possible that the 10.2 kb reporter uses a different promoter than Pe, as long as that transcript can also be spliced into exon 4 where the GFP tag has been placed. The three types of experiments conducted—FISH to regions of the nascent transcripts, tagged versions of the different predicted ORFs, and promoter-GFP constructs—are extensive, but all have different limitations. Indeed, it would be challenging to determine the transcription start sites in the germline, as it would require obtaining enough L1 larvae to be able to dissociate the animals, or isolated gonads, into single cells in order to FACS purify the germ cells for RACE or long-read sequencing (I'm not sure that L1 larval single-nucleus seq would be enough for calling start sites). Otherwise, there would be no way to determine if expected or unexpected transcripts came from the soma or the germline. We can consider these experiments in the future.

Fortunately, the main conclusions from this paper do not require knowing whether the germline uses Pe or some other "germline early" promoter that can produce Sxl protein in the absence of autoregulation by existing Sxl protein. The observations that a nascent transcript including the region downstream of Pm is observed in embryonic germ cells, but that the tagged LATE protein is not observed until L2, suggest that the transcript produced in early germ cells cannot produce a functional protein. This is consistent with the need for Sxl autoregulation of the Pm transcript in the germline as in the soma, as was previously thought. This is further supported by the observations that activity of the 10.2 kb reporter is

only observed in L1 germ cells, and that the LATE Sxl protein is only observed in germ cells after this point. Thus, we can conclude that either Pe, or another "germline early" promoter, acts to produce female-specific Sxl protein to initiate autoregulation of Sxl splicing and protein production in the germline. We feel that this is a significant advance for the field, and we will make it more clear in the text that the initial expression of Sxl in the germline may not be from the Pe promoter.

Other conclusions of the manuscript are unaffected by the start site for "germline early" Sxl transcription, including that the germline activates Sxl protein expression much later than the soma, which calls into question previous work indicating an early role for Sxl in the germline. Also unaffected is our conclusion that different enhancer sequences are required for activation of Sxl expression in the germline than in the soma, consistent with previous work demonstrating that the genetics of Sxl activation in the germline are different than in the soma. Lastly, our conclusions that sisA acts upstream of Sxl, and is required for Sxl germline expression, either directly or indirectly, are also unaffected by the nature of the Sxl "germline early" start site.

> *(3) At least one experiment suggests that Sxl-Pe never comes on in germ cells. The authors tagged the N-terminus of the Sxl-Pe protein with HA and the N-terminus of the Sxl-Pm protein with Flag. Though they could detect HA-Sxl-Pe protein in the soma, they didn't detect it in germ cells. On the other hand, the Flag-Sxl-Pm protein was detected in L2 germ cells (but not earlier). These results would more or less fit with those obtained for the 10 kb reporter and would support the following model: Prior to L1, Sxl-Pm transcripts are expressed and spliced in the male pattern in both male and female germ cells. During L1, Sxl protein expressed via a mechanism that depends upon a 10 kb region spanning Sxl-Pe (but not on Sxl-Pe) is produced and by L2 there are sufficient amounts of this protein to switch the splicing of Sxl-Pm transcripts from a male to a female pattern-generating Flag-tagged Sxl-Pm protein.*

As described above, it is indeed possible that another promoter besides Pe is active as the "germline early" promoter. We will make this more clear in a revised version, but the major conclusions of the manuscript are unaffected.

> *(4) The 10kb reporter is sex-specific, but not germline-specific. The levels of gfp in female L1 somatic cells are equal to if not greater than those in L1 female germ cells. That the Sxl-Pe10kb reporter is active in the soma complicates the conclusion that it represents a germ line-specific promoter. Germline activity is, however, sensitive to sis-A knockdowns which is plus. Presumably, somatic expression of the reporter wouldn't be sensitive to a (late) sis-A knockdown- but this wasn't shown.*

We are confused by this comment because we do not conclude that the Pe is a germline-specific promoter. Pe is known to be expressed in the soma, from considerable previous work cited by this reviewer, and the simplest model is that Pe is used in both the soma and the germline, as reflected by our 10.2 kb reporter. It is actually quite interesting how late this promoter seems active in the soma, contrary to current dogma, but we did not study somatic activation of Sxl in this work.

> *(5) Their results with the HA-Sxl-Pe protein don't fit with many previous studies-assuming that the authors have explained their results properly. They report that HA-Sxl-Pe protein is first detected in the soma at stage 9 of embryogenesis and that it then persists till L2. However, previous studies have shown that Sxl-Pe transcripts and then Sxl-Pe proteins are first detected in ~NC11-NC12 embryos. In RNase protection experiments, the Sxl-Pe exon is observed in 2-4 hr embryos, but not detected in 5-8 hr, 14-12 hr, L1, L2, L3, or pupae. Northerns give pretty much the same picture. Western blots also show that Sxl-Pe*

> *proteins are first detectable around the blastoderm stage. So it is not at all clear why HA-Sxl-Pe proteins are first observed at stage 9 which, of course, is well after the time that the Sxl-Pm autoregulatory loop is established.*
>
> *Given the obvious problems with the initial timing of somatic expression described here, it is hard to know what to make of the fact that HA-tagged Sxl-Pe proteins aren't observed in germ cells.*
>
> *As for the presence of HA-Sxl-Pe proteins later than expected: While RNase protection/Northern experiments showed that Sxl-Pe mRNAs are expressed in 2-4 hr embryos and disappear thereafter, one could argue from the published Western experiments that the Sxl-PE proteins expressed at the blastoderm stage persist at least until the end embryogenesis, though perhaps at somewhat lower levels than at earlier points in development. So the fact that Goyal et al were able to detect HA-Sxl-Pe proteins in stage 9 embryos and later on in L1 larva probably isn't completely unexpected. What is unexpected is that the HA-Sxl-Pe proteins weren't present earlier.*

We thank the reviewer for this detailed analysis. Since we were not focused on somatic expression of Sxl in this work, it is possible that stage 9 was the earliest stage we observed in our experiments, rather than the earliest stage in which it is ever observed. We will repeat these experiments to verify when the HA-tagged early Sxl protein is first observed. However, these comments have no bearing on our conclusions about Sxl expression in the germline, which is the focus of this manuscript.

> *(6) The authors use RNAi and germline clones to demonstrate that sis-A is required for proper oogenesis: when sis-A activity is compromised in germ cells, i) tumorous ovary phenotypes are observed and ii) there is a reduction in the expression of Sxl-Pm protein. They are also able to rescue the phenotypic effects of sis-a knockdown by expressing a Sxl-Pm protein. While the experiments indicating sis-a is important for normal oogenesis and that at least one of its functions is to ensure that sufficient Sxl is present in the germline stem cells seem convincing, other findings would make the reader wonder whether Sis-A is actually functioning (directly) to activate Sxl transcription from promoter X.*

It is true that we do not know the binding specificity for SisA, which is why we have made no claims about the directness of SisA regulation of Sxl. This does not change our conclusions that sisA is upstream of Sxl activation, since loss of sisA function has a similar phenotype to loss of Sxl, loss of sisA blocks Sxl protein expression, and expression of Sxl rescues the sisA mutant phenotype.

> *The authors show that sis-a mRNAs and proteins are expressed in stage 3-5 germ cells (PGCs). This is not unexpected as the X-linked transcription factors that turn Sxl-Pe on are expressed prior to nuclear migration, so their protein products should be present in early PGCs. The available evidence suggests that their transcription is shut down in PGCs by the factors responsible for transcriptional quiescence (e.g., nos and pgc) in which case transcripts might be detected in only one or two PGC-which fits with their images. However, it is hard to believe that expression of Sis-A protein in pre-blastoderm embryos is relevant to the observed activation of the Sxl-Pm autoregulatory loop hours later in L2 larva.*
>
> *It is also not clear how the very low level of gfp-Sis-A seen in only a small subset of migrating germ cells in stage 10 embryos (Figure S6) would be responsible for activating the Sxl-Pe10kb reporter in L1. It seems likely that the small amount of protein seen in stage 10 embryos is left over from the pre-cellular blastoderm stage. In this case, it would not be surprising to discover that the residual protein is present in both female*

> *and male stage 10 germ cells. This would raise further doubts about the relevance of the gfp-Sis-A at these early stages.*
>
> *In fact, given the evidence presented implicating sis-a in activating Sxl, (the germline activation of the Sxl-Pe10kb reporter, the RNAi knockdowns, and the germ cell-specific sis-a clones) it is clear that the sis-A RNAs and proteins seen in pre-cellular blastoderm PGCs aren't relevant. The germline clone experiment (and also the RNAi knockdowns) indicates that sis-A must be transcribed in germ cells after Cas9 editing has taken place. Presumably, this would be after transcription is reactivated in the germline (~stage 10) and after the formation of the embryonic gonad (stage 14) so that the somatic gonadal cells can signal to the germ cells. With respect to the reporter, the relevant time frame for showing that sis-A is present in germ cells would be even later in L1.*

The reviewer is correct in wondering how early sisA transcription can affect late Sxl activation, and we are clear about this conundrum in our manuscript. However, they are incorrect about the early sisA expression. Our experiments examining nascent sisA transcripts indicate that sisA is zygotically expressed in the formed germ cells rather than being leftover from expression in early nuclei. The fact that only a portion of germ cells express sisA at any time may well be due to a timing issue, where not all germ cells express sisA at the same time. They are also incorrect about the timing of Cas9 editing in the germline —the guide RNAs are expressed from a general promoter that is active both maternally and in the early embryo, and the Cas9 RNA from the nos promoter is deposited in the germ plasm where it is translated long before cellularization, meaning that sisA CRISPR knockout can begin at the earliest stages of germ cell formation or before.

> *(7) As noted above, the data in this manuscript do not support the idea that Sxl-Pe proteins activate the Sxl-Pm female splicing in the germline. Flybase indicates that there is at least one other Sxl promoter that could potentially generate a transcript that includes the male exon but still could encode a Sxl protein. This promoter "Sxl-Px" is located downstream of Sxl-Pm and from its position it could have been included in the authors' 10 kb reporter. The reported splicing pattern of the endogenous transcript skips exon2, and instead links an exon just downstream of Sxl-Px to the male exon. The male exon is then spliced to exon4. If the translation doesn't start and end at one of the small upstream orfs in the exons close to Sxl-Px and the male exon, a translation could begin with an AUG codon in exon4 that is in frame with the Sxl protein coding sequence. This would produce a Sxl protein that lacks aa sequences from N-terminus, but still retains some function.*
>
> *Another possible explanation for how gfp is expressed from the 10 kb reporter is that the transcript includes the "z" exon described by Cline et al., 2010.*

As discussed above, the exact location of the start site for the Sxl transcript in the germline remains to be determined, but does not affect the main conclusions of the paper.

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