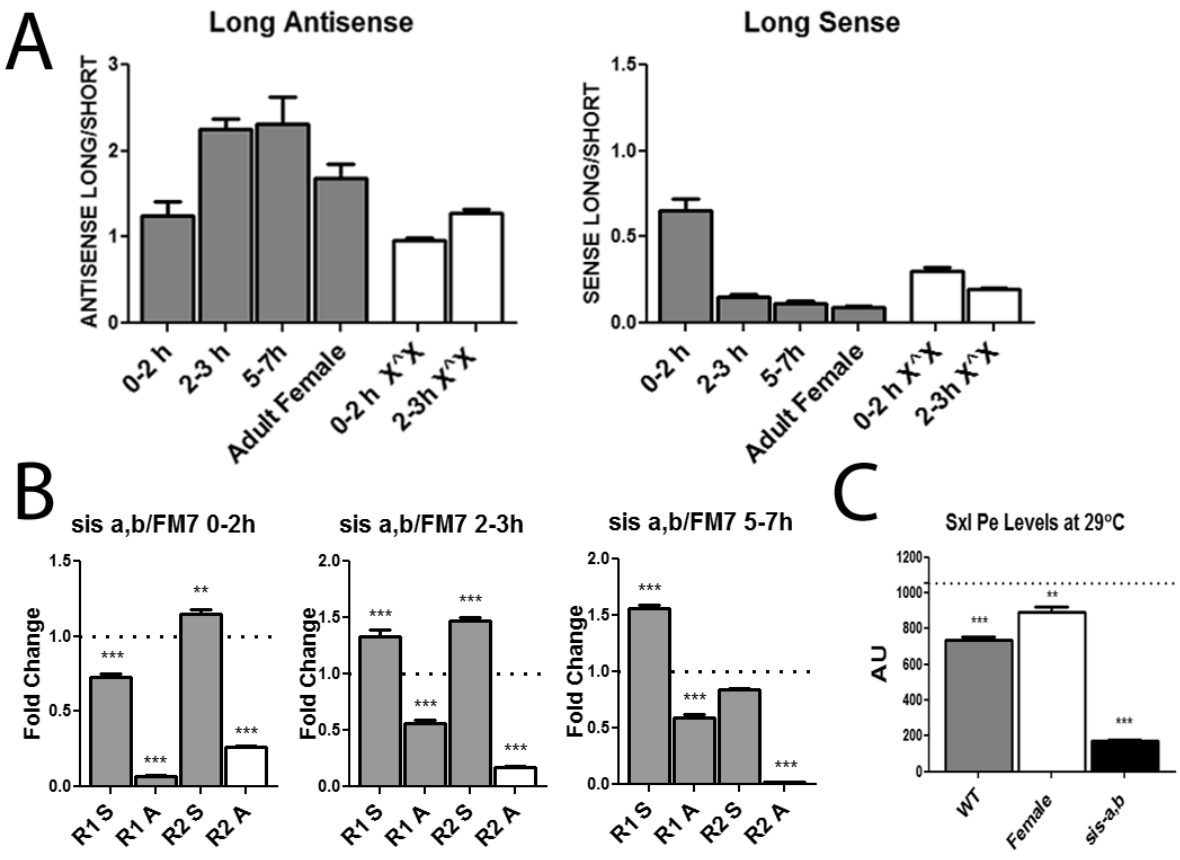
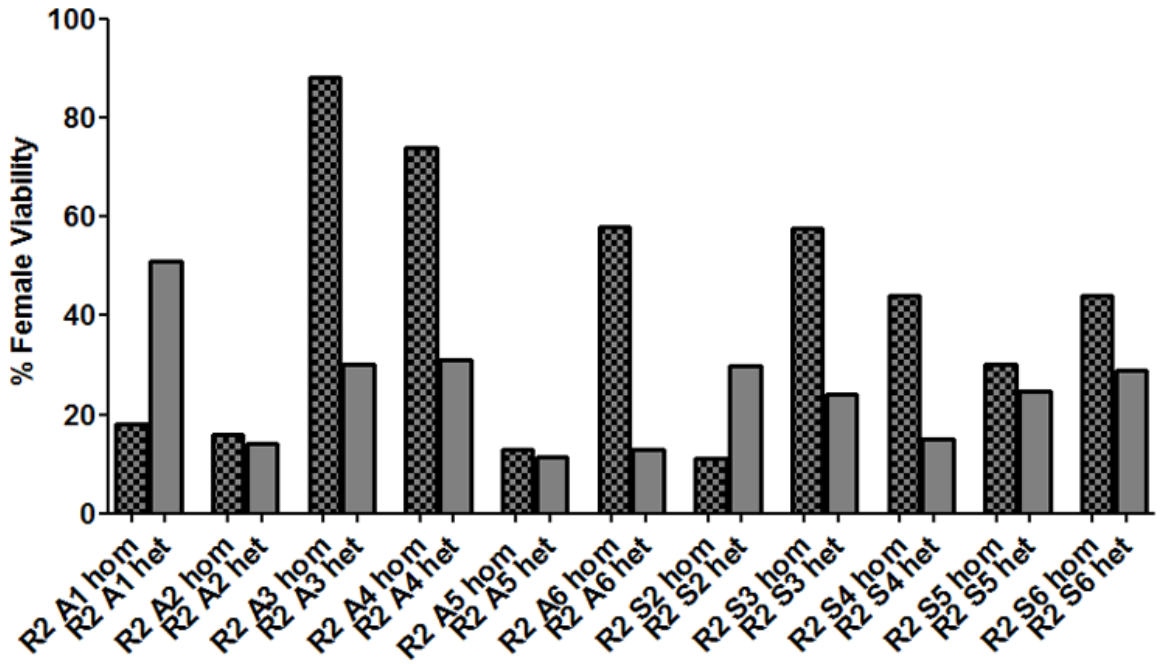
Supplementary Figures, Tables and Discussion



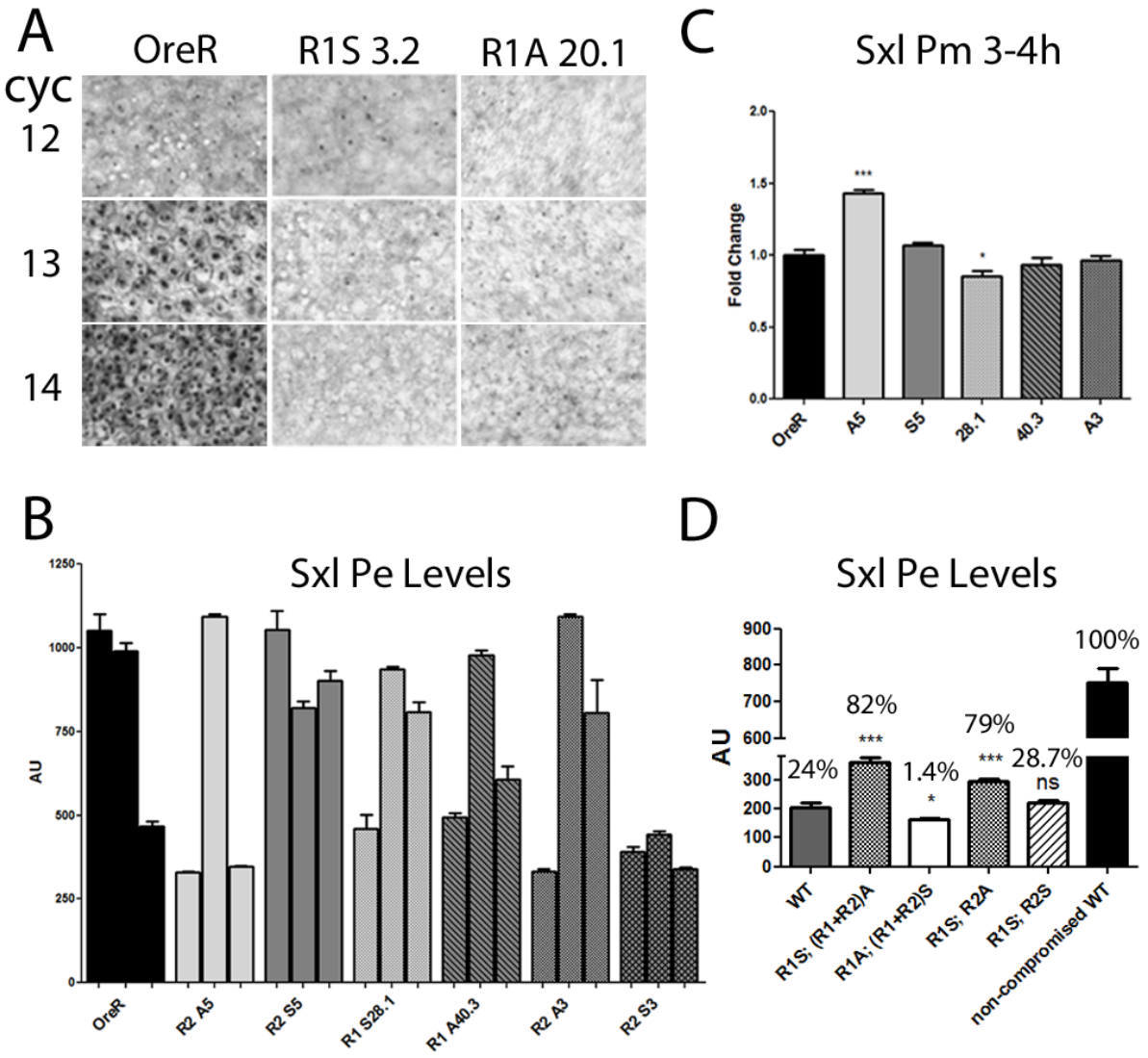
Supplemental Figure 1: lncRNA transcripts can span both R1 and R2, changes in profile of lncRNAs when dose of X chromosome counting genes is reduced, and Sxl_{Pe} mRNA levels at 29°C, Related to Figure 1.

(A) lncRNAs spanning both the R1 and R2 regions in Ore R and X^X; *Sxl^{fP7B0}/Y* quantified at the stages indicated, compared to its respective short transcript at the 5'end. For the short transcripts the RT primer is near the 3' end of the short transcript, whereas for the long transcript it is 3' of both R1 and R2. Amounts for the two RTs were normalized using *tubulin* levels. (B) Relative levels of the lncRNAs in 0-2, 2-3 and 5-7h in *sis-a^{1}, sis- b$^{sc3\text{-}1}$/FM7* flies at the non-permissive temperature of 29°C. This is the same data in Fig. 1D plotted relative to wild-type also at 29°C, which is set to 1 (dotted line). It shows the change from wild-type produced by reducing the dose of X chromosome counting genes. (C) Levels of Sxl_{Pe} mRNA for 2-3h embryos at 29°C for Ore R (wild-type, WT), X^X; *Sxl^{fP7B0}/Y* (female) and *sis-a^{1}, sis- b$^{sc3\text{-}1}$/FM7* (sis-a,b). Dotted line shows the mRNA levels from wild-type 2-3h embryos at 25°C in the same arbitrary units (AU), suggesting the higher temperature produces a small but significant drop even in the wild-type and X^X stocks. As expected, the *sis-a^{1}, sis- b$^{sc3\text{-}1}$/FM7* stock shows a much larger decrease. Average of 2 biological replicates shown, AU are from normalizing Sxl_{Pe} to *tubulin* mRNA levels. Error bars represent mean +/- SEM. ** P-value <0.005, *** P-value <0.0005.



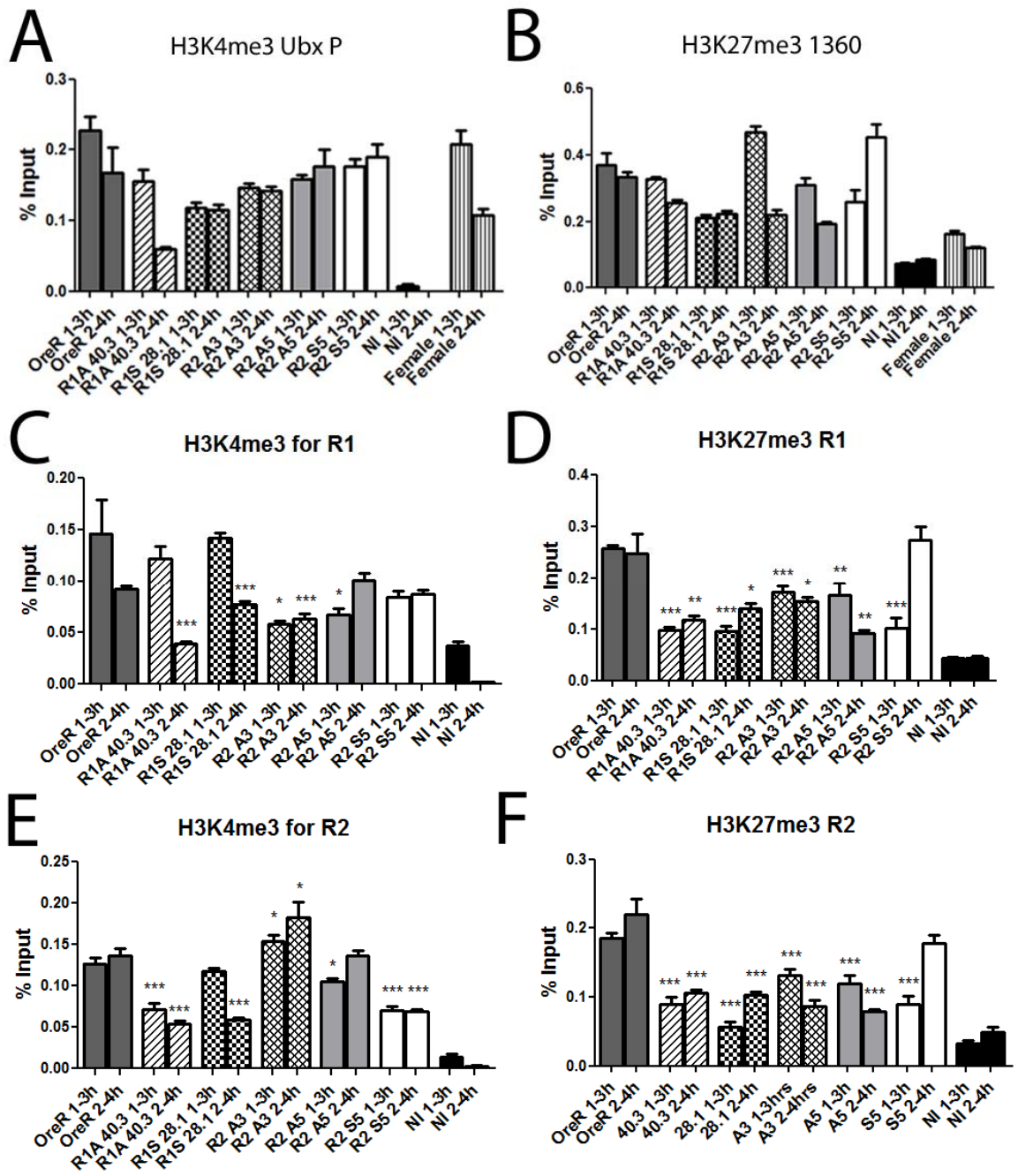
Supplemental Figure 2: effect of one versus two copies of transgenes from R2 region

Relative to the control *white*$^{-}$ background which showed female viability at ~24% under the same conditions (Fig. 2), lines which improved female viability as homozygotes showed a decrease in their positive effect when their copy number was reduced. Conversely, lines which worsened or had slightly below control female viability as homozygotes, either improved or stayed unchanged as heterozygotes. For the 2 negative lines which did not show much of a change, presumably reducing their dose by half does not reduce their levels enough to uncover an effect. Mothers homozygous or heterozygous for the indicated lncRNA line were crossed to males with decreased *sis-a ,b* numerator dose. Viability of eclosed females relative to males expressed as a percent. For heterozygotes, viability of progeny which received the transgene is shown. Minimum number of reference males 125, for each of the crosses.

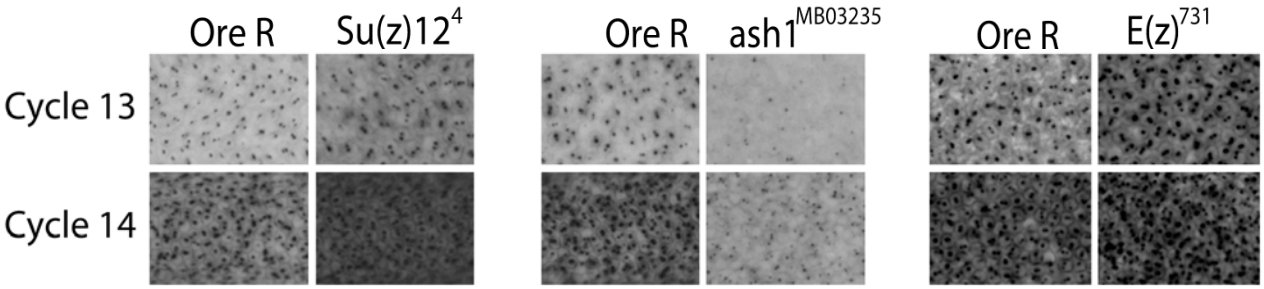


Supplemental Figure 3: Transgenic lines alter Sxl_{Pe} levels, Related to Figures 2 and 4.

(A) *in situ* hybridization of Sxl_{Pe} transcripts in additional R1 lines (B) Quantitation of Sxl_{Pe} mRNA in Ore R and transgenic lines, normalized to *tubulin* levels. The bars represent levels in 2-3h, 2.5-3.5h, and 3-4h embryos, respectively. Except for a few, most time points were done with at least two biological sample replicates. Replicates for genotypes with poor egg-laying were not collected as the quantitation matched the *in situ* results in Fig. 2. (C) Quantitation of Sxl_{Pm} mRNA relative to Ore R in 3-4h embryos. (D) Quantitation of Sxl_{Pe} mRNA in 2-3.5hr embryos from WT or females with the lncRNA transgenes shown, crossed to males with decreased *sis-a ,b* numerator dose. The non-compromised WT bar shows the Sxl_{Pe} mRNA levels with the normal *sis-a ,b* numerator dose (100% female viability relative to males). Reduction in *sis-a,b* significantly reduces the Sxl_{Pe} mRNA levels in an otherwise normal background. Improvement or decline in female viability by the presence of the transgenes correlates with changes in Sxl_{Pe} mRNA levels. Female viability (%) for each lncRNA transgene combination is from Figure 4, shown above each genotype. Note, Sxl_{Pe} mRNA only needs to be compromised in some cells for lethality, as *Sxl* expression is required in all cells. Conversely, for rescue all cells must express adequate levels of the mRNA to successfully activate the splicing feedback loop for females to survive. ns, not significant. * P-value <0.05, *** P-value <0.0005. Error bars represent mean +/- SEM.



Supplemental Figure 4: H3K4me3 and H3K27me3 ChIPs scored at R1 and R2 regions.
(A) H3K4me3 ChIPs scored for the *Ubx* promoter which is known to contain this histone mark, as a positive control for the ChIPs. (B) H3K27me3 ChIPs scored for 1360, a repetitive element known to have this histone mark, as a positive control. (C-F) H3K4me3 and H3K27me3 ChIPs scored at R1 and R2 respectively (* P-value <0.05, ** P-value <0.005, *** P-value <0.0005 compared to Ore R. Largest P-value compared to NI = 0.0186). Error bars represent mean +/- SEM.



Supplemental Figure 5. *in situ* hybridization for Sxl_{Pe} specific transcripts in embryos from wild-type (*Ore R*), *Su(z)12^4/TM6*, *ash1^{MB03235}/TM6*, and *E(z)^{731}/TM6* parents. Same sized areas shown of images taken at 40x. Mutant embryos displayed with matching *Ore R* embryos that were simultaneously processed. Nuclear cycle of embryos (designated by nuclear divisions) shown on left. *Sxl* is on the X chromosome so females have 2 dots. In wild-type embryos cycle 14 has the strongest expression levels, accumulation of the message in the cytoplasm gives the appearance of a grayer background. The dark background in *Su(z)12^4* is from elevated mRNA levels. The *E(z)^{731}* signal is consistently a little stronger than wild-type during both cycles 13 and 14.

Table S1. Primers used in this study, Related to all Figures

Primer Name	Primer Sequence
Transgene construction primers	
R1 F NotI	5'- ATAGCGGCCGCCCTGCAATCTGTGTTCTTGG -3'
R1 R NotI	5'- ATAGCGGCCGCTCCAATAACCATGAGGGC -3'
R2 F NotI	5'-ATAGCGGCC GCT GACCGTACCACCCCATTCC -3'
R2 R NotI	5'- ACATGCGGCCGCAAGACAGTCAAGGTGTTTGG -3'
RT and qPCR primers	
R1 Sense RT	5’-AAG ATT CAA TCT GAA CTT GGG C-3’
R1 Antisense RT	5’-AAG CGG GGA GGT AAA TCT GC-3’
R1 F	5’-ATG GGT TAT TTA TGG CGG GG-3’
R1 R	5’-AAC GTT ATC CGT TTC TGA AGC-3’
R2 Sense RT	5'-CAC TCT AAT CGC ACG TTT GG-3'
R2 Antisense RT	5’-CCA ATG GAT TTG CTT CCT GC-3’
R2 F	5’-CTA GGG CTT AGG GCT CAC TGG-3’
R2 R	5’-CTA ATC GAA CGC CAA GAA CAG-3’
HSP83 RT Near	5'- AAA GCT TGG GCT GCA GGT CG -3’
Sxl 3’ F	5’-CGC TTA AGG GTT GCC ATA CCA-3’
Sxl 3’R	5’-CGG CAC CCC TCT GTC GAT CC-3’
Ubx P 5’	5’-CCA TGA TGA ATT TCC CGC GGC-3’
Ubx P 3’	5’-AGC GGT AAA GCG CTG AGG GC-3’
1360 F	5’-GAG AGC GAG AGA GCG AAG AGC GC-3’
1360 R	5’-GCG GAC ACA AGC ACT CAA CAA TCA TTG C-3’
tub56D 5’	5’-ACG AGC AGA TGC TGA ACA TCC AGA-3’
tub56D 3’	5’-CGG TGT ACC AAT GCA AGA AAG CCT-3’

Table S2. Fraction of lncRNA with or without polyA tail in wild-type embryos as assessed by oligo dT versus specific RT primer

lncRNA strand	Fraction of lncRNA with polyA tail 0-2h embryos	Fraction of lncRNA with polyA tail 2-3h embryos
R2S	1.8%	2.4%
R2A	4.9%	5.4%

Table S2 legend. Fraction of signal from equal amount of RNA that was RT with either an R2-specific primer or oligo dT primer and quantified by qPCR. Amounts of oligos and qRT-PCRS were as described in the Materials and Methods.

Table S3. Fraction of each lncRNA expressed from its corresponding Transgene

Transgenic Line	Fraction of lncRNA from transgene (Near RT)	Fraction of lncRNA from transgene (Far RT (Oligo dT))
R1A 40.3	0.83	0.86
R1A 20.1	1.1	1.2
R1S 28.1	0.075	0.0094
R1S 3.2	0.027	0.0092
R2 A3	0.095	0.19
R2 A5	0.18	0.30
R2 S3	0.077	0.013
R2 S5	0.065	0.014

Table S3 Legend. RNA from dissected adult ovaries scored for total lncRNA using RT primer in the lncRNA transgene fragment (includes both endogenous and transgene produced lncRNAs); lncRNA from the transgene only was detected by HSP83 vector-specific RT primer or oligo dT primer. The vector has a polyA addition signal ~60 bases downstream of the cloning site. The fraction represents transgene signal divided by the total lncRNA signal. For the sense lines, these fractions were lower as the RT primer was moved farther from the lncRNA fragment, consistent with the instability of these transcripts. The antisense lines had fractions which were similar for both RT primers. Note, that both the R1 and R2 antisense levels are normally quite low in wild-type adults, and the transgenic lines elevate the antisense signal. This data represents the fraction of the lncRNA within the lines, which for R1 anti-sense suggests practically all the lncRNA comes from the transgene. One biological sample scored. qRT-PCRS were performed as described in the Materials and Methods.

Supplementary Discussion

Nearly all of the R1 antisense lncRNAs progress through the R2 region as long antisense transcripts. For the sense strand, R2 sense is transcribed through the R1 region as a long transcript ~65% of the time in the 0-2h window, and below ~15% in later time windows (Fig. S1A). Levels of the short R1 antisense region actually appeared higher when measured in the form of long transcripts. As the R1 region is

transcribed at very low levels, small differences can give a large apparent fold change. Alternatively, splicing of the RNA in the region of the primer(s) might explain the apparent higher levels of the longer lncRNA.

In the 0-2h window prior to Sxl_{Pe} activation, males appear to contribute disproportionately more of the long sense transcript. Males may be utilizing this transcript to shut down the promoter as constructs expressing the long sense transcript show reduced female viability in crosses with reduced X-linked numerator genes. Additionally, in the attached X-chromosome stock which represents only females, the amount of this long sense transcript is significantly lower in this early window suggesting it is primarily male derived.

Both the R1 sense and R2 sense lines (Fig. 3) show a reduction in the total levels (endogenous and ectopic) of their own respective transcript. All R2 expression lines show a dramatic increase in the R2 antisense strand in marked contrast to the R1 lines which show little or no increase. The latter shows a relative increase in the R1 antisense transcript.