Sex-specific Pattern Formation During Early
Drosophila Development

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The deleterious effects of different X chromosome dosage in males and females are buffered by a process called dosage compensation, which in *Drosophila* is achieved through a doubling of X-linked transcription in males. The male-specific lethal complex mediates this process, but is known to act only after gastrulation. Recent work has shown that the transcription of X-linked genes is also upregulated in males prior to gastrulation; whether it results in functional dosage compensation is not known. Absent or partial early dosage compensation raises the possibility of sex-biased expression of key developmental genes, such as the segmentation genes controlling anteroposterior patterning. We assess the functional output of early dosage compensation by measuring the expression of *even-skipped (eve)* with high spatiotemporal resolution in male and female embryos. We show that *eve* has a sexually-dimorphic pattern, suggesting an interaction with either X-chromosome dose or the sex determination system. By manipulating the gene copy number of an X-linked transcription factor, *giant (gt)*, we trace sex-biased *eve* patterning to *gt* dose, indicating that early dosage compensation is functionally incomplete. Despite sex-biased *eve* expression, the gene networks downstream of *eve* are able to produce sex-independent segmentation, a point we establish by measuring the proportions of segments in elongated germ-band embryos. Finally, we use a whole-locus *eve* transgene with modified *cis* regulation to demonstrate that segment proportions have a sex-dependent sensitivity to subtle changes in Eve expression. The sex-independence of downstream segmentation despite this sensitivity to Eve
expression implies that additional autosomal gene- or pathway-specific mechanisms are required to ameliorate the effects of partial early dosage compensation.
INTRODUCTION

In *Drosophila*, dosage compensation occurs by an upregulation of transcription from the X chromosome in males (Mukherjee and Beermann 1965; Belote and Lucchesi 1980; Straub *et al.* 2005). The best characterized mechanism of upregulation depends on the male-specific activity of the *male-specific lethal* (MSL) complex (Gelbart and Kuroda 2009) which binds to sites on the X chromosome and enhances the rate of transcript elongation (Larschan *et al.* 2011). MSL activity is inhibited in females by the translational repression of Msl2, a protein necessary for complex formation, by the protein product of the sex-determination gene *Sex-lethal* (*Sxl*) (Kelley *et al.* 1995). The canonical MSL-dependent mechanism does not appear to be active prior to gastrulation as *msl2* transcript is not detected until cleavage cycle 14 (Lott *et al.* 2011) and the earliest expression of Msl3 protein in male embryos is detected only at stage 6 (gastrulation) (Rastelli *et al.* 1995; Franke *et al.* 1996; Cline 2005).

Despite the lack of MSL-dependent dosage compensation during the blastoderm stage, the transcription of X-linked genes expressed before gastrulation is, in fact, elevated in males (Lott *et al.* 2011). With the exception of *runt* (*run*), which is known to be dosage compensated in an MSL-independent but *Sxl*-dependent manner (Gergen 1987), the mechanism of the upregulation or dosage compensation of early-expressed genes is not known.

Although the increased transcription of early expressed X-linked genes in males is suggestive of early dosage compensation, it has not yet been determined whether the observed upregulation results in functional dosage compensation. Two aspects of the pattern of
upregulation of X-linked genes lead us to suspect that early dosage compensation can be incomplete: first, the pattern of upregulation is not uniform over the X chromosome and many genes are upregulated less than 2-fold in males. Lott et al. (2011) found that 36 of 85 zygotically-expressed X-linked genes had an average female/male ratio greater than 1.5 and that X-linked gene expression was female biased. In general, the sex ratios of gene expression were smoothly distributed between 1 and 2, similar to later, MSL-mediated compensation. The second aspect of the pattern of upregulation suggesting incomplete compensation is that the female/male ratio of transcript abundance of X-linked genes varies over cycle 14 (Lott et al. 2011), implying that the efficacy early dosage compensation can also vary in time during early development.

Many early-expressed X-linked genes are transcriptional regulators of key developmental processes like anteroposterior (AP) (e.g. giant) and dorsoventral (DV) (e.g. brinker) patterning. Autosomal targets of these X-linked regulators, such as even-skipped (eve), are expressed as early as cycle 8 (Pritchard and Schubiger 1996), making it possible to test whether early dosage compensation is functional by comparing the expression of target genes in the two sexes. If the observed upregulation of the early expression of X-linked genes is still insufficient for functional dosage compensation, their autosomal targets are expected to be expressed in sexually-dimorphic patterns.

We investigate the possibility of incomplete functional dosage compensation and sex-biased pattern formation by characterizing the spatiotemporal expression of an autosomal AP patterning gene (Akam 1987; Surkova et al. 2008), eve, in male and female embryos. Pattern formation has been extensively studied in Drosophila but no attempt has been made in previous
studies to distinguish between the two sexes. Investigations of dosage compensation (Hamada et al. 2005; Lott et al. 2011), in contrast, have ignored potential spatial and/or temporal variation in sex-biased gene expression. *eve* is expressed in seven stripes during the latter half of cleavage cycle 14 of the *Drosophila* blastoderm (Macdonald et al. 1986; Frasch et al. 1987) (Fig. 1A). Each stripe is about 3-5 nuclei wide and is established by localized repression of early broad *eve* expression over a period of approximately 30 mins (Surkova et al. 2008; Ludwig et al. 2011). Eve expression levels can be measured to an accuracy of ~10% (Surkova et al. 2008; Ludwig et al. 2011) and provide a sensitive readout of potential sex differences over time and position. This level of precision is desirable since the magnitude of sex ratio variation of X-linked gene expression is relatively small – at most 2-fold. Here we demonstrate that Eve is expressed at different levels in male and female embryos in the region between stripes one and two (1-2 interstripe).

One advantage of querying functional dosage compensation with a segmentation gene is that it is relatively easy to trace any observed difference both upstream and downstream in the well-established regulatory network (Schroeder et al. 2004; Jaeger 2011). It is known that the second stripe of *eve*, for example, is activated by Bicoid (Bcd) and Hunchback (Hb) and repressed in the anterior by Giant (Gt) and the posterior by Krüppel (Kr) (Frasch and Levine 1987; Stanojevic et al. 1991; Small et al. 1992). We exploit this feature of the segmentation system to test the functional compensation of *gt*, an X-linked gene, by measuring the response of *eve* expression to *gt* dose.

Next, we determine whether the sex-biased expression of Eve also leads to sex-biased
segmentation during later development. Eve is a regulator of the segment-polarity gene *engrailed* (*en*) (Macdonald *et al.* 1986; Fujioka *et al.* 1995; Fujioka *et al.* 2002), which, along with *wingless* (*wg*) establishes the molecular prepattern of the segmented embryo (Ingham *et al.* 1985; Baker 1987). *en* patterning and the proportions of parasegments have not yet been critically examined in the sexes. Using En expression as a marker, we measure the ratio of parasegments in extended germ-band embryos of each sex to establish that segmentation at a later developmental stage is sex independent.

A potential explanation for the robustness of *en* patterning and parasegment proportions is that *en* expression is insensitive to Eve expression in the 1-2 interstripe region. In a last set of experiments, we test this hypothesis by characterizing the sensitivity of *en* expression to Eve expression in the 1-2 interstripe. For this purpose, we take advantage of an *eve* transgene that has a reduced level of Eve expression in the interstripe region. This transgene was derived from the endogenous *eve* locus by modifying the *cis* regulation of the stripe 2 enhancer (S2E) (Ludwig *et al.* 2011). S2E is ~800bp long and contains 17 binding sites, identified by *in vitro* DNase protection assays, for Bcd, Hb, Gt, and Kr (Fig. 1B). A 480bp fragment, called the minimal stripe 2 element, is however necessary and sufficient for stripe 2 expression (Small *et al.* 1992; Ludwig *et al.* 2005). The transgene, “MSE”, was derived from the endogenous locus by deleting 244bp of sequence flanking the minimal stripe 2 element and tagging the C-terminus of Eve with YFP. We had also constructed a control YFP-tagged transgene, “WT”, that has wild type *eve cis*-regulatory sequences. Both WT and MSE provided healthy rescue of the lethality of *eve^R13*, a null allele, Df(2R)*eve*, a small deletion covering *eve*, and *eve^AMSE*, a synthetic allele of *eve* in which the
minimal stripe 2 element has been replaced by \( w^+ \) sequence. Nevertheless, MSE drove defective stripe 2 formation: Eve expression was lower in both stripe 2 and the 1-2 interstripe region.

We measure Eve and En expression driven by the MSE transgene in each sex to show that 1) 1-2 interstripe Eve expression is lowered specifically in males and becomes indistinguishable from the female level and 2) En expression and parasegment proportions are perturbed in males to give sex-biased segmentation in MSE. Based on these results we argue that \( en \) regulation is sensitive to the level of Eve expression in the 1-2 interstripe and postulate that additional mechanisms must act on the segmentation genes to ensure sex-independent larval segmentation in the wild type.
MATERIALS AND METHODS

**Stocks and Transgenic Fly Lines:** The following laboratory stocks were used: \( w^{1118}, y^{1}, sc^{1}, gt^{X11}/FM6 \) (Bloomington 1529), and \( Df(1)JA27/FM7c,P[\text{GAL4-Kr.C}],P[\text{UAS-GFP.S65T}] \) (Bloomington 5193). The latter two stocks were crossed to produce \( y^{1}, sc^{1}, gt^{X11}/FM7c,P[\text{GAL4-Kr.C}],P[\text{UAS-GFP.S65T}] \) flies. \( eve^\text{AMSE} \) is a recessive lethal mutant of \( eve \) created by replacing the 480bp fragment corresponding to the minimal stripe 2 element from the endogenous \( eve \) locus with the \( \text{white}^{+} \) gene using ends-out homologous recombination (Ludwig et al. 2011).

The construction of the transgenic strains is reported in detail elsewhere (Ludwig et al. 2011). Briefly, WT is a 16.4kb fragment from the \( eve \) locus created by recombineering with Red/ET counter-selection BAC Modification Kit (Gene Bridges GmBH, Heidelberg). A superfolding variant of YFP, SYFP2 (Ben Glick, University of Chicago), was added to the C-terminus of the Eve peptide, allowing us to visualize transgenic expression independently of the endogenous locus. MSE was derived from WT by deleting 33bp and 211bp 5’ and 3’ of the minimal stripe 2 element, respectively (Fig. 2A). Both constructs were integrated into the same site on the third chromosome (attP2) using phiC31 site-specific integration (Markstein et al. 2008). The \( gt^{YFP} \) construct was made similarly, using a 26.2kb region of the \( gt \) locus, tagged with SYFP2 employing *Drosophila* codon usage (File S1), and provides healthy rescue of a null allele of \( gt, gt^{X11} \) (Table S2).

**Genetic Crosses, Staining, and Genotyping for En and Eve patterning:** Wild type Eve patterning was assayed in \( w^{1118} \) embryos (Fig. 1). Eve patterning driven by the WT and MSE
transgenes (Fig. 2) was assayed in embryos from a cross between eve^{R13}/CyO,P[hb-LacZ] females and eve^{R13};WT(MSE) males, yielding the eve^{R13};WT(MSE)/+ or eve^{R13}/CyO,P[hb-LacZ];WT(MSE)/+ genotypes. The gt dosage series (Fig. 3) was constructed in two separate crosses. Genotypes with 0-2 doses were the offspring of a cross between gt^{Ts1}/FM7c,P[GAL4-Kr.C],P[UAS-GFP.S65T] females and w^{1118} (w) males while the 3-4 dose genotypes were the offspring of the cross between gt^{YFP} males and females. To measure the effects of the rescue transgenes on En pattern and segmentation (Fig. 4), we crossed eve^{AMSE}/CyO,P[hb-LacZ] and eve^{R13};WT(MSE) flies and collected embryos for further analysis.

Embryos were collected, fixed, and immunostained with antibodies against Eve (Figs. 1E and 3), GFP (Fig. 2), or En (Fig. 4) as described (Ludwig et al. 2005; Ludwig et al. 2011). Embryos stained fluorescently for GFP and Eve were imaged with a confocal microscope (see below). The lengths of both parasegments 3 and 4 in En pattern were measured at the ventral site of early stage 11 embryos. The reported numbers are the mean of 3 repeated measurements by hand using ImageJ (NIH). After imaging, individual embryos were genotyped by PCR with primers for the Y chromosome, P[hb-LacZ], and P[UAS-GFP.S65T] (File S1) to determine sex and eve or gt dosage. The protocol is available on request.

Confocal Imaging and Feature Detection: Immunofluorescent-stained embryos were imaged with a Vti Infinity 3 confocal (Visitech International, Sunderland) and a Zeiss AxioPlan 2 microscope (Carl Zeiss, Inc.) using a 16x PLAN-NEOFLUAR objective. The 491nm, 561nm, 642nm lasers, a 488/565/643 dichroic, 535/605/700 emission filter, and an EMCCD camera (Hamamatsu Photonics UK Ltd, Hertfordshire) were employed to acquire images at a resolution
of 512x512. To maximize dynamic range, camera gain and exposure were chosen so that the brightest embryos in an experiment had a few saturated pixels. All the embryos in an experiment, irrespective of genotype, were imaged with the same settings. Laterally-oriented embryos were imaged in three planes 1 micron apart transecting the nuclei at the embryo surface flattened against the coverslip and the images were averaged. Embryos were also imaged at the mid-sagittal plane to determine their length.

Embryos were staged according to a standard scheme (Surkova et al. 2008) using only membrane invagination as a marker; “Early” cycle 14 corresponds to time classes T3-T4, the earliest time at which a nascent stripe 2 is detectable, “Middle” is T5-T6, and “Late” is T7-T8, ~9 min prior to gastrulation. The images were segmented using described methods (Janssens et al. 2005) and the location of and the mean fluorescence intensity in each nucleus were saved for further processing.

The positions of and expression values at the extrema were estimated using a smoothing cubic spline (CSAPS function of MATLAB, MathWorks Inc.) fit to data extracted from the nuclei lying in a 10%-wide dorsoventral strip along the anteroposterior axis. The level of smoothing was chosen to minimize the error made by the spline approximation without detecting spurious extrema (Ludwig et al. 2011). The same level of smoothing was applied to all embryos in the same time class irrespective of experiment or genotype. The error made by the spline approximation at the extrema was ~5%.
RESULTS

**Sex-specific eve expression in wild type:** In order to investigate early dosage compensation, we measured the spatiotemporal dynamics of eve expression separately in male and female embryos. We fluorescently immunostained embryos of a standard laboratory stock, *w^{1118}*, with anti-Eve antibody. These embryos were mounted one per slide and imaged with a confocal microscope. After imaging, the embryo was removed and genotyped for sex using PCR. The images were staged into three classes according to the scheme of Surkova *et al.* (Surkova *et al.* 2008), using only nuclear morphology and membrane invagination as markers. Each class is approximately 12 minutes long, spanning the period from early cycle 14 when Eve is expressed in a tri-band pattern, to just before gastrulation, when Eve is expressed in seven fully mature saw-tooth shaped stripes (Frasch *et al.* 1987; Surkova *et al.* 2008). We processed the confocal images using an image processing algorithm (Janssens *et al.* 2005) to estimate Eve expression in individual nuclei. Finally, we extracted data from a narrow strip lying along the AP axis of the embryo and fit a cubic spline to these data to measure several features of the pattern (Fig. 1C).

In all, we measured 43 features of the Eve pattern. We estimated the expression level at the peak of each stripe and at the trough between two stripe peaks, henceforth referred to as the interstripe. These measurements were normalized to the mean of Eve expression in all nuclei of the embryo to correct for experimental image intensity variation. We also computed the positions of the stripe peaks, interstripe troughs, and the stripe borders along the AP axis. Since Eve stripes form by localized repression in the interstripe nuclei (Stanojevic *et al.* 1991; Small *et al.* 1992),
we also define a measure for the strength of repression in an interstripe. Let the height of a stripe border be the difference in the expression level of the stripe peak and the interstripe trough (for example, 2A in Fig. 1D). We take the ratio of the border heights of the two borders of a stripe, for example 2A and 2P (Fig. 1D), to obtain a normalized measure of the repression of a border, which we refer to as “relative repression”.

We found evidence for sex-specific patterning differences in the relative repression of three of the seven Eve stripes: stripe 2 (Fig. 1E; $p=0.0538$ mid-cycle 14 and $p=0.0096$ late; the Wilcoxon ranksum test was used here and in all subsequent statistical tests), stripe 3 (Fig. S1C, $p=0.0312$ late-cycle 14), and stripe 4 (Fig. S1C, $p=0.0345$ late cycle 14). No sex bias was detectable in any of the other features (Fig. S1 and S2).

As we show below, the relative repression of stripe 2 (Fig. 1E) is the most consistent sex-biased patterning feature and we focused our subsequent analysis on this feature. There is no detectable difference in early cycle 14, possibly due to the large variability of relative repression during the early stages of stripe 2 formation (Ludwig et al. 2011) (Fig. 1E). A measurable difference of ~7% appears in middle cycle 14 (Fig. 1E), with the male having a lower value of relative repression than the female. The difference reduces to ~4% in late cycle 14, but is still statistically significant due to the precise measurement allowed by the low variability of the late Eve pattern – the standard deviation of relative repression is ~3%.

Lower values of relative repression imply that the male embryo has a shorter — that is derepressed — stripe 2 anterior border compared to the female, which is shown schematically in the inset of Fig. 1E. We could detect greater Eve expression in the 1-2 interstripe in males during
late cycle 14 (Fig. S3A, $p=0.0604$), whereas no sex difference was detectable in Eve expression in the 2-3 interstripe (Fig. S3B, $p>0.2$) – suggesting that sex-biased relative repression is attributable to higher Eve expression in the 1-2 interstripe.

Sex-specific features of Eve patterning were rechecked in independent experiments using the “WT” eve transgene, a 16.4 kb region from the eve locus, tagged with YFP and integrated on the third chromosome (Table S1). We crossed eve$^{R13}$/CyO,$P[hb-LacZ]$ females with eve$^{R13}$;WT males. Embryos collected from the cross were mounted, imaged, and genotyped in the same manner as the w$^{1118}$ embryos, with two differences. First, we detected the CyO balancer by co-staining with β-galactosidase antibody to identify embryos with 1 (eve$^{R13}$;WT/) or 2 (eve$^{R13}$/CyO,$P[hb-LacZ]$;WT/) eve doses. Second, we focused on middle cycle 14 in order to maximize sample size since this cross produced many more genotypes than the w$^{1118}$ experiment. This experiment confirmed the sex-dependent patterning of stripe 2. Males have a derepressed anterior border compared to females in WT (Fig. 2B; $p=0.0012$, 1 eve dose and $p=0.0346$, 2 dose). Relative repression and 1-2 interstripe expression were the only sex-biased stripe 2 features in WT (Fig. S4); Eve expression in the 1-2 interstripe was elevated in males relative to females (Fig. S5; $p=0.0021$, 1 eve dose and $p=0.2395$, 2 eve dose), replicating w$^{1118}$ results. The derepression of the 1-2 interstripe is eve-dose dependent, since relative repression differs between the sexes by ~22% and ~8% in the one and two dose rescues respectively (Fig. 2B). The other sex differences, in the relative repression of stripes 3 and 4 in w$^{1118}$ (Fig. S1C), and a new one found in this experiment, in the relative repression of stripe 5 (Fig. S6C), were inconsistent between the two experiments.
Incomplete dosage compensation of \textit{gt}: The sex-specific features of \textit{eve} expression in wild type implies that there is an as yet unappreciated interaction between the sex determination or dosage compensation systems and the segmentation genes. One possibility is that sex determination genes, such as \textit{Sxl}, regulate \textit{eve}’s expression in the 1-2 interstripe. Another possibility is that one or more X-linked genes regulating \textit{eve} are not dosage compensated and have different levels of expression in the two sexes, resulting in \textit{eve}’s sex-dependent features. In order to regulate \textit{eve} specifically in the 1-2 interstripe, the sex determination genes would need to be expressed in an AP position-dependent manner, but are, in fact, expressed uniformly throughout the embryo (Erickson and Cline 1993). Although uniform expression does not completely preclude an interaction with \textit{eve}, we first checked the latter possibility — that \textit{eve}’s sex-specific expression originates in the lack of dosage compensation for one or more X-linked loci — by varying the dosage of a candidate X-linked locus, \textit{gt}, and measuring the response of the \textit{eve} interstripe phenotype.

\textit{gt} is a promising candidate as a source of dimorphic \textit{eve} expression in the 1-2 interstripe because Gt protein is known to repress \textit{eve} expression in that region. The minimal stripe 2 enhancer contains three footprinted binding sites for Gt (Stanojevic \textit{et al.} 1991; Small \textit{et al.} 1992). Deletion of these sites leads to a derepression of the anterior border in a reporter assay (Stanojevic \textit{et al.} 1991; Small \textit{et al.} 1992). Stripes 1 and 2 are fused in \textit{gt} embryos (Frasch \textit{et al.} 1987), suggesting that the phenotype is responsive to \textit{gt} dose. Given the known role of Gt as a repressor of \textit{eve}, the observed derepression of \textit{eve} expression in the 1-2 interstripe is consistent with a lower level of Gt protein expression in male embryos.
Our strategy was to vary the gene dose of \textit{gt} in the two sexes and to measure the response of the \textit{eve} 1-2 interstripe phenotype. The reasons to measure the response of \textit{eve} instead of \textit{Gt} expression levels directly are twofold. First, the difference between male and female \textit{gt} expression is potentially quite small (Lott \textit{et al.} 2011) and the precise and dynamic spatiotemporal expression of \textit{eve} allows for much greater sensitivity of detection. Second, this experiment would not only test whether \textit{gt} is dosage compensated but also whether it drives the observed sex-specific features of \textit{eve} expression.

We crossed \textit{gt}^{X11}/FM7c flies (a null allele; Table S1) to \textit{w1118} to generate \textit{gt} male embryos (0M; the number is \textit{gt} dose and the letter signifies sex), 1 \textit{gt} dose female embryos (1F), 1 \textit{gt} dose male embryos (1M), and 2 \textit{gt} dose female embryos (2F) (Fig. 3A). Using the same experimental procedures used to establish the sex-specific expression of Eve, we imaged Eve expression and measured its features quantitatively in these genotypes. In agreement with our earlier results, the 1M genotype has a derepressed 1-2 interstripe relative to 2F in middle cycle 14 (Fig. 3B, \textit{p}=0.0011).

The 1F genotype is hemizygous for \textit{gt} but homozygous for all other X-linked loci. 1F also produces a derepressed interstripe (Fig. 3B, \textit{p}=0.0002), showing that lower \textit{gt} dose leads to a derepression of the 1-2 interstripe in female embryos. In fact, 1F and 1M are indistinguishable from each other (\textit{p}=0.3864), strongly suggesting that \textit{gt} accounts for all of the effect.

Because \textit{gt} regulates the expression of \textit{eve} 5-6 and 6-7 interstripes (Frasch and Levine 1987) in addition to that of interstripe 1-2, their expression should have a similar response to \textit{gt} dose. Fig. S7 shows the \textit{p}-values of the ranksum test for relative interstripe expression between
2F and 0M, 1F, or 1M. 0M (gt) differs from 2F in the interstripes 1-2, 5-6, and 6-7. Both 1M and 1F differ from 2F in the 1-2 and 6-7 interstripes but not elsewhere.

We also used a whole-locus transgene for gt, gt-YFP (Table S1), that provides healthy rescue of gt- (Table S2) to boost gt dose to 3 in males (3M) and 4 in females (4F) (Fig. 3A). As expected, repression of the 1-2 interstripe is an increasing function of gt dose (Fig. 3B). The dose-response curve saturates after 2 doses, however, suggesting that two gt doses are sufficient to completely turn off eve transcription in 1-2 interstripe nuclei.

**Late segmentation is sex independent:** Do the sex-dependent features of Eve expression lead to sexually dimorphic segmentation of the embryo? Although no gross differences in the segmentation of embryos between males and females have been observed to date, we conducted a quantitative analysis to detect more subtle differences in the proportions of segments. We measured the proportions of parasegments 3 and 4 in extended germ band stage embryos using En expression as a marker. en is expressed in the anterior part of parasegments and its anterior margin marks parasegmental grooves (Ingham et al. 1985). Parasegment 3 is under the control of Eve expression in stripe 2 since embryos homozygous for eve^{AMSE} (Table S1), the endogenous eve locus in which the stripe 2 enhancer has been replaced by w^+ sequence, lack eve stripe 2 expression and have defective third parasegments (Ludwig et al. 2005).

We crossed eve^{AMSE}/CyO,P[hb-LacZ] and eve^{R13};WT flies to specifically rescue the lethality of stripe 2 ablation with one dose of the WT eve transgene. Stage 11 embryos were costained with β-galactosidase, to identify the eve^{AMSE}/eve^{R13} genotype, and En antibodies. We
measured the number of pixels between the anterior margins of En stripes 3-4 and 4-5 on the ventral side to estimate of the length of parasegments 3 and 4 respectively (Fig. 4A). The length of parasegment 3 was normalized to that of the total length of parasegments 3 and 4 to correct for variation in embryo size. This comparison between male and female embryos revealed no evidence for a difference in the median ratio of the parasegmental length of 3 to 3+4 (Fig. 4B, \( p=0.2937 \)). The sex-specific features of Eve stripe 2 expression do not, therefore, appear to be carried forward developmentally to segmentation in extended germ band stage embryos.

**Sex-independent segmentation in WT requires compensatory mechanisms:** The sex-dependent features of *eve* expression pose potential challenges for its downstream targets, such as the pair rule genes *fushi-tarazu, runt, hairy*, and the segment polarity genes *en* and *wg*. For late segmentation to be the same in the two sexes, are downstream genes indifferent to sex-specific *eve* expression, or are there mechanisms interceding to attenuate the sex-dependent differences in *eve* expression?

The relative repression in males differs by a small amount, \(~10\%\), from the female level. One possible explanation for the apparent robustness is simply that *en* or other *eve* targets are insensitive to the relative repression of stripe 2. In this section, we test the sensitivity of *en* regulation to sex-specific Eve expression by assaying Eve and En expression in male and female embryos of a transgenic line having altered levels of relative repression.

In earlier work investigating the robustness of the *cis* regulation of *eve* stripe 2 (Ludwig et al. 2011), we modified the WT *eve* transgene to create another transgene, MSE (Table S1). MSE
was derived from the WT transgene by deleting 244 bp of regulatory sequence flanking the minimal stripe 2 element (Fig. 2A). Stripe 2 expression driven by the MSE transgene is weaker than WT expression, has greater variability than WT, and lacks temperature compensation. Of more direct interest to us, MSE also has a different level of interstripe repression than WT, which offers an opportunity to test the potential functional impact of interstripe derepression in males. Time series data of live Eve-YFP expression showed that the relative repression of the 1-2 interstripe was greater in MSE relative to WT (Ludwig et al. 2011). If the ~10% difference between males and females is functionally relevant, modulation of the level of repression should result in downstream segmentation phenotypes.

The live-imaging experiments (Ludwig et al. 2011) had not distinguished between the sexes; male or female embryos, or both, might have greater relative repression than their WT counterparts. We therefore measured the features of Eve expression driven by the MSE transgene separately in fixed male and female embryos that had either one (eveR13;MSE/+) or two (eveR13/CyO,P[hb-Lacz];MSE+/) doses of eve. Confocal imaging and image processing of MSE were carried out with settings identical to WT.

MSE males lose the derepression of the 1-2 interstripe and have a level of relative repression indistinguishable from MSE females (Fig. 2C; \( p=0.3145 \), 1 dose and \( p=0.5686 \), 2 dose). The increase in relative repression in MSE relative to WT is restricted to males (Fig. 2B,C; \( p=0.3711 \), 1 dose and \( p=0.0002 \), 2 dose) and relative repression is not altered in females (Fig. 2B,C; \( p=0.7609 \), 1 dose and \( p=0.2423 \), 2 dose); the sequences removed in MSE appear to affect eve expression specifically in males. One possible explanation for the insensitivity of relative
repression in females is that 2 gt copies are sufficient to completely turn transcription off in the interstripe (Fig. 3B). We were not able to detect sex-specific expression in any other stripe 2 feature in MSE (Fig. S4). In particular, though peak stripe 2 expression is lower in MSE relative to WT (Fig. 2D,E; \( p(\text{female}) = 0.0126 \) and \( p(\text{male}) = 0.0950 \) in 1 dose, \( p(\text{female}) = 4.9548 \times 10^{-6} \) and \( p(\text{male}) = 0.0001 \) in 2 dose), peak expression does not differ between males and females in either line (\( p>0.4 \), WT and \( p>0.6 \), MSE). The loss of 1-2 interstripe derepression in MSE males allows us to test its functional impact on downstream segmentation.

We assayed the proportions of parasegments 3 and 4 in eve\(^{\text{MSE}}\) embryos rescued by one dose of the MSE transgene in the same manner as WT. We found that eve misregulation by MSE induces sex-specific en patterning. In contrast to WT (Fig. 4B), the median ratio of the parasegmental length of 3 to 3+4 differs significantly between MSE males and females (Fig. 4C; \( p=0.0571 \)). Assuming that each parasegment is 20 cells long (Ingham et al. 1985), the difference in MSE males and females (3.7%) corresponds to a shift of about one cell. This result indicates that the proportions of parasegments 3 and 4 are sensitive to subtle alteration of Eve expression. Even though both 1-2 interstripe and peak stripe 2 expression are altered in MSE, only the former is sex dependent (Fig. 2). For this reason, we tentatively attribute the sex-bias in En patterning to the sensitivity of en regulation specifically to 1-2 interstripe repression. It is not the case that sexually dimorphic eve expression is simply below the detection limit of downstream gene regulation. This in turn implies that as yet unknown mechanisms must compensate for sexually-dimorphic eve expression in WT to produce sex-independent segmentation.
DISCUSSION

Genomic transcriptional profiling of individual embryos established that transcription from the X chromosome is upregulated in males prior to gastrulation (Lott et al. 2011). It was not known whether the upregulation of transcription in males leads to functional dosage compensation. We suspected that early dosage compensation might be incomplete since the female/male ratio of gene expression is not uniform but rather varies between 1 (perfect compensation) and 2 (no compensation) (Lott et al. 2011). Incomplete early dosage compensation could lead to sex-biased pattern formation in the blastoderm.

We confirmed this suspicion by measuring the spatiotemporal expression of eve in wild type male and female embryos. Whereas studies of spatiotemporally-resolved patterning have ignored sex (Fowlkes et al. 2008; Surkova et al. 2008), genome-wide studies of dosage compensation (Hamada et al. 2005; Lott et al. 2011) have relied on whole-embryo extracts and hence have ignored differential spatial regulation between the two sexes. Eve expression differs between males and females in the 1-2 interstripe (Fig. 1E and 2B) and perhaps in the 5-6 and 6-7 interstripes (Fig. S7) – which altogether comprise only ~15% of embryo length. Dimorphic patterning of eve is remarkable since the robustness of eve expression has been extensively documented (Lucchetta et al. 2005; Holloway et al. 2006; Lott et al. 2007; Surkova et al. 2008; Ludwig et al. 2011); this is perhaps the first example of the lack of robustness among the segmentation genes.
Genetic analysis showed that the derepression phenotype is under the specific control of 
gt dose (Fig. 3B). Despite empirical evidence for the upregulation of gt transcription in males (Lott et al. 2011), our results suggest that gt dosage compensation is partial and that incomplete compensation has functional consequences. It is notable that 1 gt dose males and females are indistinguishable with respect to 1-2 interstripe repression (Fig. 3B). The equivalence of gt functional output in males and hemizygous females implies that the mechanism compensating for gt dose operates independently of sex. run was found to rely on Sxl for its dosage compensation (Gergen 1987) and its mRNA contains Sxl binding sites (Kelley et al. 1995). Consistent with a non-sex-dependent mode of compensation, gt mRNA lacks Sxl binding sites (Lott et al. 2011).

Despite eve’s sex-biased expression, we could not detect any sex bias in the expression of En in extended germ-band eve\textsuperscript{AMSE}/eve\textsuperscript{R13},WT/+ embryos (Fig. 4B). It is possible that the shift in the placement of parasegments is smaller than our measurement precision. However, given that we can detect a sex-specific shift of about 1 cell in eve\textsuperscript{AMSE}/eve\textsuperscript{R13},MSE/+ embryos, the shift in WT, if any, is likely too small to be biologically meaningful.

The differential expression of Eve in WT and MSE suggests two alternative hypotheses for the sex-specific shift in parasegment 3 En expression in MSE. Stripe 2 expression differs between WT and MSE in two aspects (Ludwig et al. 2011): 1) 1-2 interstripe repression is greater in MSE (Fig. 2B,C) and 2) peak expression is lower in MSE (Fig. 2D,E). According to the first hypothesis, the shift of parasegment 3 En expression in MSE males is specifically attributable to the first Eve phenotype – greater repression of the 1-2 interstripe. The alternative hypothesis proposes that the shift in En expression is due to lowered Eve stripe 2 expression in MSE,
whereas its sex dependence arises for unrelated and as yet undefined reasons. We favor the first explanation for two reasons: 1) the increased repression of the 1-2 interstripe is, in fact, a sex-dependent Eve phenotype (Fig. 2 and S4) and 2) it is unlikely that the sex dependence of the En phenotype is unrelated to Eve expression because WT and MSE are co-isogenic strains, differing only in their eve transgene sequence. Nevertheless, experiments selectively disrupting either 1-2 interstripe expression or peak stripe 2 expression will be required to conclusively distinguish between these two hypotheses.

We propose that additional mechanisms are required to compensate for the effects of dimorphic Eve expression and to produce sex-independent En patterning. Our results allow us to infer some general properties of such buffering. First, it must be independent of known dosage compensation mechanisms since it would have to act on autosomal gene expression in a tissue-specific manner. Second, the mechanism appears to be specifically targeted to the derepression of the 1-2 interstripe in wild type males, since the MSE line, which has lost the derepression, has defective En patterning. One hypothesis for a downstream buffering mechanism is eve autoregulation (Harding et al. 1989), since we observe a smaller median shift of relative repression between males and females with two eve doses (Fig. 2B, 8%) than one eve dose (22%). Alternatively, the differential expression of gt may induce sex-specific expression in other pair-rule genes, which in turn could compensate for sex-specific eve expression in the regulation of the segment polarity genes. This might explain why en expression becomes sex-biased (Fig. 4C) when eve is misregulated in MSE to have sex-independent expression (Fig. 2C). The MSE transgene also shows that a functional stripe 2 enhancer architecture that suppresses sex-specific
gt expression is possible even though the WT enhancer itself transduces differential gt expression. Cis-regulatory architecture, therefore, may be playing an integral role in fine-tuning sex-biased gene expression and perhaps has evolutionarily conserved features.

In previous work (Ludwig et al. 2011), we noted that the modifications to eve’s cis-regulation in MSE sensitize the strain to X dosage perturbations. Even though both WT and MSE rescue eve^R13 lethality with high efficiency, male/female ratio is much lower in rescue by MSE (p=0.01, homozygote, p=2.3E-23, hemizygote; Figs. 1B and S1 of Ludwig et al. (2011) respectively). Sensitivity to X dosage appears to specifically originate in stripe 2 regulatory sequences: while WT and MSE both rescue the lethality of eve^AMSE (Ludwig et al. 2005; Ludwig et al. 2011), an eve locus lacking the minimal stripe 2 element, only the MSE rescue genotype exhibits a reduced male/female ratio (p=2.1E-5; Fig. S3 of Ludwig et al. (2011)).

Based on available evidence, we argue that the modulation of the repression of the 1-2 interstripe by gt dosage and stripe 2 cis-regulatory interactions underlies the sensitivity of the MSE strain to X dosage, although a definitive proof of causality will have to await further experiments. First, the loss of adult male viability in MSE is not peculiar to any mutant chromosome – the rescue of all tested chromosomes, eve^R13/eve^R13, eve^R13/Df(2R)eve, and eve^AMSE/eve^R13, show loss of male viability (Ludwig et al. 2011). Second, in hemizygous rescue, MSE has significantly greater lethality than WT during embryogenesis, a point established in our earlier study (Ludwig et al. 2011). Third, as we noted above, the loss of male viability originates specifically in stripe 2 sequences; the WT and MSE transgenes are co-isogenic except for 244bp of sequence flanking the minimal stripe 2 element. Notwithstanding these arguments, it is
possible that the mutated stripe 2 sequences cause lethality not via parasegment 3 en misregulation, but by their pleiotropic effects on eve regulation in other tissues, such as the CNS (Fujioka et al. 1999). Although enhancers have been regarded as largely autonomous so far, long-range interactions between enhancers have been reported recently (Dunipace et al. 2011; Perry et al. 2011). Inspecting eve’s neuronal expression for sex bias might help to affirm or eliminate this possibility.

The incomplete dosage compensation of gt induces differences that are able to propagate through the segmentation hierarchy to the pair-rule genes in a sex-specific manner, but are attenuated before reaching the segment-polarity genes. We speculate that the propagation of these differences and mechanisms for their correction must be widespread since incomplete dosage compensation itself is common in other organisms and in evolution. In Drosophila, MSL-mediated compensation, like early dosage compensation, is also non-uniform (Hamada et al. 2005). In humans, a total of 25% of X-linked genes escape or show variable patterns of inactivation (Carrel and Willard 2005), and as in Drosophila, canonical dosage compensation is inactive early in embryonic development (Steele 1970). Incomplete dosage compensation also arises during the evolution of neo-X chromosomes, and in ZW sex-determination systems, such as birds, global dosage compensation is replaced by a variable system of tissue-specific compensation of individual genes (Mank and Ellegren 2009). Phenotypic robustness is achieved not only through intrinsic or emergent mechanisms acting in gene networks (Manu et al. 2009; Acar et al. 2010) but also through specific adaptations involving individual genes, as exemplified by the ubiquity of microRNAs and redundant enhancers (Li et al. 2009; Frankel et al. 2010; Perry
et al. 2010). Buffering of incomplete dosage compensation is likewise expected to be target-
specific and therefore common, but subject to continual adaptive pressure to track changes in the
efficacy of dosage compensation of continually evolving X-linked genes.

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FIGURE LEGENDS

Figure 1. Sexually-dimorphic Eve expression in \textit{w}^{1118}. \textbf{A}, The Eve expression pattern in middle-late cleavage cycle 14. Anterior is to the left and dorsal is above. \textbf{B}, The 800bp long 5' regulatory region that drives stripe 2 expression (Ludwig \textit{et al.} 2005). Distance is relative to the transcription start site. The box in the middle is the minimal stripe 2 element. Footprinted binding sites (Stanojevic \textit{et al.} 1989; Small \textit{et al.} 1991) for the activators, Bicoid (Bcd) and Hunchback (Hb), and the repressors, Giant (Gt) and Krüppel (Kr), are shown above and below respectively. \textbf{C}, Measurement of the features of the Eve expression pattern. The red points are average fluorescence intensities of Eve staining in nuclei lying in a strip that extends 10\% dorsoventrally around the AP axis. The blue line is a smoothing cubic spline fit to data. Black circles are local maxima and minima of the spline. \textbf{D}, Schematic of the measurement of stripe 2 border height. \textbf{E}, Boxplot of relative repression of the 1-2 interstripe in \textit{w}^{1118} embryos. In all boxplots shown, the box lines are the first quartile, median, and the third quartile. The whiskers extend to the most extreme values lying within 1.5 times the interquartile range and any datapoints outside the whiskers are shown as circles. Embryos were classified into three 12 min long bins, Early, Middle, and Late (see Materials and Methods). Relative repression was measured as the ratio of the height of the anterior border to that of the posterior border (panel \textbf{D}). \textit{N}=14,13,25,31,16,15. The inset is a schematic illustration of the sex-specific expression of Eve.

Figure 2. Stripe 2 expression is sexually dimorphic in WT but lacks sex bias in MSE. Embryos had 1 copy of the transgene and were either null (\textit{eve}^{R13};\textit{WT(MSE)}/+) or hemizygous (\textit{eve}^{R13}/\textit{CyO,P[hb-Lacz];WT(MSE)}/+) for endogenous eve, giving a total eve dose of either 1 or 2 respectively. Data are from embryos in mid-cycle 14 stained for Eve-YFP with anti-GFP. \textbf{A}, The stripe 2 regulatory region in the WT
and MSE eve transgenes. Yellow regions were deleted in MSE. Binding sites are shown as in Fig. 1B.

**B,D**, WT. **C,E**, MSE. **B,C**, Boxplots of relative repression of the 1-2 interstripe. Relative repression was measured as the ratio of the height of the anterior border to that of the posterior border (Fig. 1D). **B**, WT, \( N=20,15,18,20 \). **C**, MSE, \( N=25,20,23,9 \). **D,E**, Boxplots of expression at stripe 2 peak. Peak expression was normalized to mean Eve fluorescence in each embryo. Sample sizes are the same as panels **B** and **C**.

**Figure 3.** The response of stripe 2 relative repression to \( gt \) dose. **A**, The genotypes used to make a dosage series for \( gt \). **B**, Response of the relative repression of the 1-2 interstripe to \( gt \) dose. Relative repression was measured as before (Fig. 1D,E). Embryos were mid-cycle 14.

**Figure 4.** Parasegmental proportions in the rescue of \( eve^{\Delta MSE}/eve^{R13} \) lethality by the WT or MSE transgenes. The genotype of the rescued embryos is \( eve^{\Delta MSE}/eve^{R13};WT(MSE)^+/+ \). **A**, Ventral view of a stage 11 embryo stained for En. Red lines show the measurement of the length of parasegments 3 (left) and 4 (right). **B,C**, Boxplot of the ratio of parasegment 3 length to the total length of 3 and 4. **B**, WT; from top to bottom, \( N=8,13 \) embryos. **C**, MSE; \( N=14,7 \) embryos.
LITERATURE CITED


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Figure 1
Figure 2
Figure 3
Figure 4