Evidence That Runt Acts as a Counter-Repressor of Groucho During Drosophila melanogaster Primary Sex Determination

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ABSTRACT

Runx proteins are bifunctional transcription factors that both repress and activate transcription in animal cells. Typically, Runx proteins work in concert with other transcriptional regulators, including co-activators and co-repressors to mediate their biological effects. In Drosophila melanogaster, the archetypal Runx protein, Runt, functions in numerous processes including segmentation, neurogenesis, and sex determination. During primary sex determination, Runt acts as one of four X-linked signal element (XSE) proteins that direct female-specific activation of the establishment promoter (Pe) of the master regulatory gene Sex-lethal (Sxl). Successful activation of SxlPe requires that the XSE proteins overcome the repressive effects of maternally deposited Groucho (Gro), a potent co-repressor of the Gro/TLE family. Runx proteins, including Runt, contain a C-terminal peptide, VWRPY, known to bind to Gro/TLE proteins to mediate transcriptional repression. We show that Runt’s VWRPY co-repressor-interaction domain is needed for Runt to activate SxlPe. Deletion of the Gro-interaction domain eliminates Runt’s ability to activate SxlPe, whereas replacement with a higher affinity, WRPW, sequence promotes Runt-mediated transcription. This suggests that Runt may activate SxlPe by antagonizing Gro function, a conclusion consistent with earlier findings that Runt is needed for Sxl expression only in embryonic regions with high Gro activity. Surprisingly, we found that Runt is not required for the initial activation of SxlPe. Instead, Runt is needed to keep SxlPe active during the subsequent period of high-level Sxl transcription, suggesting that Runt helps amplify the difference between female and male XSE signals by counter-repressing Gro in female, but not in male, embryos.

KEYWORDS

X-chromosome counting, Genetic Switch, X-signal element, WRPW, WRPY, deadpan, X:A ratio, Genetics of Sex

Cell fate decisions are commonly made in response to small quantitative differences in signal molecules. Often such signals are rendered only for brief periods during early development but lead to distinct and permanent cell fates. Sex determination in Drosophila is a well-defined example of a cell fate decision where a transient twofold concentration difference in the proteins that define X-chromosome dose leads to the activation of the master regulatory gene Sex-lethal (Sxl). Successful activation of SxlPe requires that the XSE proteins overcome the repressive effects of maternally deposited Groucho (Gro), a potent co-repressor of the Gro/TLE family. Runx proteins, including Runt, contain a C-terminal peptide, WRPY, known to bind to Gro/TLE proteins to mediate transcriptional repression. We show that Runt’s VWRPY co-repressor-interaction domain is needed for Runt to activate SxlPe. Deletion of the Gro-interaction domain eliminates Runt’s ability to activate SxlPe, whereas replacement with a higher affinity, WRPW, sequence promotes Runt-mediated transcription. This suggests that Runt may activate SxlPe by antagonizing Gro function, a conclusion consistent with earlier findings that Runt is needed for Sxl expression only in embryonic regions with high Gro activity. Surprisingly, we found that Runt is not required for the initial activation of SxlPe. Instead, Runt is needed to keep SxlPe active during the subsequent period of high-level Sxl transcription, suggesting that Runt helps amplify the difference between female and male XSE signals by counter-repressing Gro in female, but not in male, embryos.
Nagengast et al. 2003; Gonzalez et al. 2008). In male embryos, the one-X dose of XSEs is insufficient to activate SxlPe. Consequently, the transcripts from SxlPm are spliced by default so as to produce nonfunctional truncated Sxl protein.

The four XSE elements are necessary for proper Sxl expression but differ in their sensitivities to gene dose and in their molecular effects on SxlPe (Cline 1993). The two “strong” XSEs, sc and sisA, encode transcriptional activators essential for SxlPe expression in all parts of the embryo (Torres and Sanchez 1991; Erickson and Cline 1993; Walker et al. 2000). The two “weak” XSEs upd and runt govern SxlPe expression in a broad region in the center of XX embryos, but neither gene is needed for expression at the embryonic poles (Duffy and Gergen 1991; Kramer et al. 1999; Avila and Erickson 2007). Changes in sc and sisA gene dose have dramatic effects on Sxl expression and consequently on viability (Cline 1988; Cline 1993). Loss of one copy of each of sc and sisA is strongly female lethal due to the failure to efficiently activate SxlPe. Reciprocally, simultaneous duplication of both genes is strongly male-lethal because SxlPe is activated in male embryos bearing an extra dose of sc* and sisA*.

In contrast to sc and sisA, both upd and runt are relatively insensitive to changes in gene dose (Duffy and Gergen 1991; Torres and Sanchez 1992; Cline and Meyer 1996; Kramer et al. 1999; Sefton et al. 2000). Double heterozygotes between upd or runt and either of the strong XSEs show comparatively modest effects on Sxl expression and on female viability. Duplications of upd* or runt* have even smaller effects on male viability as the various combinations lead to, at most, only low-level activation of Sxl in XY animals. In the case of runt, it was only possible to detect a strong effect of runt dose in males, after overexpression by microinjection of runt mRNA into embryos (Kramer et al. 1999).

The upd gene encodes a ligand for the JAK-STAT signaling pathway and its effects on SxlPe are mediated via the maternally supplied transcription factor Stat92E (Harrison et al. 1998; Jinks et al. 2000; Sefton et al. 2000). Interestingly, active Stat92E is not needed for the initial activation of SxlPe but is required instead to keep the promoter active during the period of maximum SxlPe expression (Avila and Erickson 2007). Stat92E binds to several defined DNA sites at SxlPe and is thought to be a conventional activator of SxlPe transcription that augments the functions of earlier acting XSE proteins but its actual mechanism of action is unknown (Jinks et al. 2000; Avila and Erickson 2007).

runt, encodes the archetypal member of the Runx (Runt-related transcription factor) family of proteins (Duffy and Gergen 1991; Torres and Sanchez 1992). Runx proteins are highly conserved in metazoans and act, depending on the promoter context, as either activators or repressors in a diverse array of biological processes (Walrad et al. 2010; Ito et al. 2015; Hughes and Woollard 2017; Mevel et al. 2019). Runx proteins are defined by the Run domain, a 128 amino acid conserved DNA binding domain that binds to the consensus binding site ‘GGYGGY’ (reviewed by (Tahirov and Bushweller 2017)), and by the presence of a conserved C-terminal peptide, VWRPY, that binds to co-repressors of the Groucho/TLE family (Aronson et al. 1997; Ito 1997; Jennings et al. 2006). Other conserved regions of Runx proteins mediate transcriptional activation and repression independent of the Gro-TLE family (Walrad et al. 2010). The runt gene is best known for its pair-rule function in embryonic patterning, but its initial role in the fly is as an XSE to establish female-specific expression of Sxl in somatic sex determination (Duffy and Gergen 1991; Kramer et al. 1999).

In this paper we address the mechanism by which runt functions to regulate SxlPe. We build on the experiments of Kramer et al. (Kramer et al. 1999) who demonstrated that Runt works directly on Sxl rather than through an intermediary gene. Kramer et al. (Kramer et al. 1999) considered three general mechanisms for how Runt might control SxlPe. First, Runt could act as a conventional direct activator, second; it could facilitate the binding of Sc and SisA transcription factor complexes, or third; Runt could act as a “quencher” of negative regulators. Several observations focused our attention on the third possibility, that Runt activates SxlPe by antagonizing Groucho-mediated repression of the promoter.

Maternally supplied Groucho (Gro) is a potent co-repressor of SxlPe that is recruited to the promoter by DNA binding repressors of the hairy/E(spl) (Hes)-family, including Deadpan (Paroush et al. 1994; Fisher et al. 1996; Jennings et al. 2006; Lu et al. 2008). Loss of Gro leads to ectopic activity of SxlPe in males and premature expression in females (Lu et al. 2008). The first connection between Runt and Gro was the correlation between the region-specific effects of runt on Sxl and the region-specific regulation of the co-repressor Gro by the Torso RTK-dependent pathway. In precellular embryos, Gro is phosphorylated directly by MAPK at the embryonic poles with phosphorylation reducing the ability of Gro to repress target genes (Cinnammon et al. 2008; Helman et al. 2011). Suggestively, the regions where Gro is phosphorylated correspond to the areas where SxlPe activity does not depend on runt (Duffy and Gergen 1991; Kramer et al. 1999). This raised the possibility that Runt is needed only in regions where Gro is highly active, a conjecture supported by early experiments showing that ubiquitous activation of Torso (which leads to ubiquitous phosphorylation of Gro (Cinnammon et al. 2008; Cinnammon and Paroush 2008; Helman et al. 2011) completely bypassed the requirement for runt in Sxl expression (Duffy and Gergen 1991). Reasoning that if Runt activates SxlPe by interfering with Gro, it would most likely do so via its C-terminal VWRPY peptide, we created runt transgenes with or without Gro-interacting motifs. We found that deletion of the WRPY sequence eliminated Runt’s ability to activate SxlPe, but that Runt’s transcriptional activation function was restored when the higher-affinity WRPY sequence was used. Since Runt’s ability to activate SxlPe depends both on the presence of a functional co-repressor-interacting motif, and an intact DNA binding domain, a straightforward interpretation is that Runt activates SxlPe by acting as a “counter-repressor” of Gro function (Pinto et al. 2015; Vincent et al. 2018). We also demonstrate that Runt is needed only after the onset of Sxl transcription, suggesting that runt, like upd and Stat92E (Avila and Erickson 2007), functions to maintain SxlPe in an active state. We propose a model suggesting how counter-repression by Runt could both explain Runt’s role in Sxl regulation and answer the paradoxical question of how a sparingly dose-sensitive XSE can play a central role in X-chromosome signal amplification.

MATERIALS AND METHODS

Fly culture
Flies were grown at 25° on a standard cornmeal and molasses medium. w1118 flies were received from Bloomington stock center. The w f run3/Binsinscy Dp(1;Y)y+ mal1080 (run+) stock was obtained from T.W. Cline. Null run mutant embryos were generated from the cross between w f run3/Binsinscy females and w+ Yy+ mal1080 (run+) males. All the transgene lines generated were maintained with two copies in w f run3/Binsinscy / Yy+ mal1080 (run+) background.

Plasmids, vectors and transformation
The runt-WRPY+ 10,050 bp genomic fragment, was amplified from w1118 fly genomic DNA using Expand Long Template PCR System.
(Roche) and cloned into pCR II-TOPO TA vector (Invitrogen). An AvrII site was introduced abutting the runt stop codon. The fragment ends were defined by primers: 5'-GGAAAGAATTCGAGCATTGGCAGG-3' and 5'-GCACCCAAATGTCCTTGTGAATGGA. The runt-WRPY+ construct was modified to runt-ΔWRPY+ and runt-WRPW using PCR to amplification to change the C-terminal amino acids. The entire runt coding sequences, including modifications, were introduced into the genomic clone using an Ascl site located in the runt 5' UTR and the introduced AvrII site and confirmed by DNA sequencing. All Runt domain mutations: Cys-127-Ser and Arg-177-Ala mutants were generated in pCR II-TOPO TA vector using QuikChange site directed mutagenesis kit (Agilent). The wild type and the respective modifications were confirmed by DNA sequencing. All constructs were cloned, using vector derived EcoRI sites, in the pattB transformation vector kindly provided by Johannes Bischof, Basler lab, Zurich. Transgenic injections were carried out by Genetic services Inc. MA. Constructs were inserted into fly genomic attP2 site on the third chromosome by targeted insertion (Venken et al. 2006).

In situ hybridization

Embryos were collected 0 to 3hr 30 min after the egg laying. Fixation of embryos and in situ hybridization with whole mount embryos was as described (Lu et al. 2008). Embryos are mounted in 70% glycerol in PBS for imaging. Stages of embryo were detected based on number of nuclei, shape of the nuclei, and cellular furrows as outlined (Lu et al. 2008). Templates for in vitro RNA transcription was made by PCR amplification with a forward primer and a reverse primer along with T3 promoter using genomic DNA from w1118 flies. A Digoxigenin labeled antisense RNA probe was synthesized using in vitro transcription kit (MAXSCRIPT T3 kit, Ambion). Probe was detected using anti-Digoxigenin antibody (Roche) that cross react with NBT-BCIP solution staining the embryos. Primers used to in vitro templates were: Sxl forward 5'-CCCTACGTCGACGGCATGCAGC-3', Sxl reverse 5'-TATACGACTCATATAGGG-GAATGACCCAATGGAAATCG-3', and runt forward 5'-AACGAGGAAACTACGTCGCGC-3', runt reverse 5'-AATTAACGCCCTACATACACGCTACCTTGATGGCTTTGC-3'.

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

RESULTS

Runt maintains but does not initiate SxlPe expression

Loss of runt function eliminates Sxl protein and SxlPe activity, as measured by SxlPe-lacZ transgenes, in a broad central region in early embryos but has no apparent effect on Sxl at the anterior and posterior poles (Duffy and Gergen 1991; Kramer et al. 1999). To define precisely when and where loss of runt affects SxlPe we analyzed the effects of the runt null mutation on the production of nascent transcripts from the endogenous Sxl locus. Nascent transcripts from SxlPe were visualized as nuclear dots by in situ hybridization using an RNA probe derived from the SxlPe-specific exon E1 and downstream intron sequences. Typical results are shown in Figure 1 with Figure 1A highlighting nascent transcripts in magnified surface views made from the centers of the whole embryos shown in Figure 1B. As previously reported, SxlPe transcripts appear in wild-type (w1118) females during nuclear cycle 12 (Erickson and Cline 1998; Avila and Erickson 2007; Erickson and Quintero 2007; Lu et al. 2008; Li et al. 2011). Initial expression during cycle 12 was mosaic with some nuclei expressing one or both Sxl alleles and other nuclei neither allele. By late cycle 12 nearly all nuclei express exhibit two nuclear dots showing that both copies of SxlPe are active. This pattern continued, with the dots becoming more intense through cycle 13 and the first 10-15 min of cycle 14 (Figure 1). SxlPe activity decreases thereafter with the nuclear dots disappearing by mid cycle 14. Neither wild-type male embryos, nor males carrying a duplication of runt+, express SxlPe.

To examine the effects of loss of runt function on SxlPe activity we examined embryos generated from crosses between runt+/+ females and runr/Yy+mal080(runt+) males. Stained progeny were runr/ runr and runr/+ female embryos in equal proportions. Male embryos with either one or two copies of runt+ do not express SxlPe and were unstained. We found over multiple experiments that female embryos completely lacking Runt (runr/runt+) had obvious defects in Sxl expression during cycles 13 and 14. In early cycle 13 runr/runt+ mutants, the defects were evident as a loss of nuclear dots, and thus of expressing nuclei, in the central portions of embryos with the non-expressing regions expanding as cycle 13 progressed (Figure 1). By early cycle 14, runr null mutants displayed the expression pattern characteristic of runt mutants carrying SxlPe-lacZ fusions (Duffy and Gergen 1991; Kramer et al. 1999): strong expression at the poles and no expression in the broad central regions of the embryos. We note that the SxlPe expression phenotype of runt+ mutant females was...
Transgenes providing early runt function

To further analyze how runt regulates SxlPe we needed to create transgenes that express runt at the proper time and at appropriate levels. The runt gene, however, has complex regulatory regions scattered over many kilobases (Butler et al. 1992; Klingler et al. 1996) and no transgenes have yet been isolated that complement runt null mutations. We chose instead to isolate transgenes that reproduced the early runt expression pattern needed for its XSE function without concern for all of runt’s later functions. Using the deletion analysis of Klingler et al. (Klingler et al. 1996) as a guide we generated a transgene carrying a 10,050 bp genomic fragment, spanning 5,284 bp upstream of the runt start codon and 2,824 bp downstream of the runt termination codon and integrated it into the 3rd chromosome using site-specific 4eC3I mediated integration. We named the resulting transgene runt-VWRPY+. (Figure 2A). We analyzed the transgenic runt expression pattern in the progeny of a cross between runt/Binsinscy females and runt/Yy+mal108 (run+), runt-VWRPY+ males. Equal numbers of run+ and run+/+ females and +/- Yy+mal108 (run+) and run+/Yy+mal108 (run+) males, each bearing one copy of runt-VWRPY+, were expected. The runt expression patterns could not be distinguished among the embryo types as all embryos appeared wt. (B) runt pair rule expression pattern. Wild type and runt mutant embryos at the indicated times during nuclear cycle 14 stained to detect runt mRNA.

Runt-VWRPY+ transgenes provide XSE function

To determine if the runt-VWRPY+ transgene can provide XSE function, we asked if the transgene could restore normal SxlPe expression in homozygous runt1 mutants. We found that a single
copy of the runt-WRPY⁺ transgene fully complemented the run3 defect as every stained embryo from crosses between run3/Binsinscy females and run3/Yy males exhibited a wild-type SxlPe staining pattern (Figure 3). Likewise, we could discern no differences in SxlPe activity between the run3 mutant and the heterozygous female progeny when the runt-WRPY⁺ transgene was introduced from the female parents as expected for a zygotically acting XSE (data not shown). Taken together, the complete rescue of SxlPe activity in runt null mutants and the normal transgenic runt expression pattern (Figure 2B) suggest the runt-WRPY⁺ transgene produces normal or near normal levels of runt protein during the time when X chromosome dose is assessed.

DNA binding is needed for Runt to activate SxlPe

A requirement for Runt DNA binding in Sxl activation was reported by Kramer et al. (Kramer et al. 1999) who found that a runt variant carrying two amino acid changes, C127S and K199A (CK), predicted to disrupt DNA binding without greatly perturbing Runt structure, was unable to activate Sxl when overexpressed after microinjection of runt mRNA into embryos. To confirm that this finding applied to more normal levels of Runt, and to guard against the possibility that the CK amino acid replacements might otherwise alter Runt structure, we introduced the same C127S and K199A changes, as well as five single amino changes (R80A, R139A, R142A, R174A, R177A) predicted to inhibit DNA binding without altering structure (Nagata and Werner 2001) into our runt-WRPY⁺ transgenes creating runt(DB⁺) transgenic lines (see Materials and Methods).

We found that each of the amino acid changes abolished the ability of the Run transgenes to activate SxlPe as the female progeny of crosses between run3/Binsinscy females and run3/Yy males exhibited either the characteristic run3 mutant SxlPe staining pattern or the fully wild-type pattern seen in run3/+ heterozygotes in the expected 1:1 ratio (data not shown). In no case did we observe evidence for partial complementation confirming that Runt’s DNA binding motif is needed for its XSE function.

Loss of Runt’s WRPY Gro-interaction motif abolishes SxlPe expression

To test the significance of Runt’s Gro interactive motif in SxlPe activation, the WRPY portion of the motif was precisely deleted from the transgene to produce a runt-ΔWRPY derivative. (Figure 4A). Using φC31-mediated integration, the runt-ΔWRPY transgene was inserted in the same genomic location as the wild type runt-WRPY⁺ transgene. We found that Runt lacking its WRPY motif failed to rescue SxlPe expression in run3 mutants (Figure 4A) as one half of the cycle 13 and 14 female progeny of crosses between run3/Binsinscy females and run3/Yy males exhibited defective SxlPe staining patterns indistinguishable from those of run3 mutants alone. Indeed, the SxlPe pattern in runt-ΔWRPY bearing run3 null mutants was indistinguishable from run3 mutants alone suggesting...
that the Gro-interacting WRPY motif is essential for Runt to function as a transcriptional activator at SxlPe.

To ensure that the failure of the runt-ΔWRPY transgene to provide sex determination reflected the loss of the WRPY motif, rather than a lack of runt protein, we sought a functional assay that would demonstrate the ability of the modified Runt to function in embryos in the absence of the WRPY motif. We chose to examine fushi tarazu (ftz) as previous work has shown that transcription of ftz is partially dependent upon runt activity in precellular embryos (Tsai and Gergen 1994; Aronson et al. 1997; Swantek and Gergen 2004; Vanderzwan-Butler et al. 2007). Most important, ftz is activated by Runt in a partially WRPY-independent manner, as overexpressed Runt lacking the C-terminal Gro interaction domain, shows a clear activation of ftz expression in regions between the normal ftz stripes (Aronson et al. 1997).

We first confirmed that expression of ftz stripes is reduced prior to gastrulation in runt null mutants (Figure 5). We then showed that wild type runt-WRPY+ transgene largely restored the endogenous ftz pattern. Critically, we found that the runt-ΔWRPY transgene also restored much of the normal ftz pattern in runt null mutants, showing that the runt-ΔWRPY transgene produces functional Runt protein (Figure 5). We note that wild type Runt was more effective at rescuing ftz expression than the ΔWRPY derivative. This observation, however, is entirely consistent with previous findings showing that a Runt variant lacking the C-terminal RPY residues was less effective at ftz activation than was the wild type when overexpressed (Aronson et al. 1997) as well as with the notion that runt likely regulates ftz expression by more than one mechanism (Aronson et al. 1997; Swantek and Gergen 2004; Vanderzwan-Butler et al. 2007).

The potent Gro-interacting motif ‘WRPW’ also provides activation function at SxlPe

Deletion of the WRPY tetrapeptide eliminates both Runt’s interactions with Groucho (Aronson et al. 1997) and with its ability to activate SxlPe (Figure 3B). We reasoned that if Runt normally employs its WRPY motif to antagonize Gro-mediated repression at SxlPe then it should be possible to substitute a different Gro interaction motif and retain Runt’s ability to activate transcription from SxlPe. We chose to test the well-known and potent “WRPW” Gro-interacting motif found in the dedicated repressor proteins of the hairy-E(spl) (HES) family. HES proteins bind Gro through their C-terminal ‘WRPW’ motif and recruit it to target gene promoters (Fisher et al. 1996; Fisher and Caudy 1998). The molecular interactions of Gro with WRPY and WRPW peptides are similar except that the WRPW peptide interacts with considerably higher affinity (-Aronson et al. 1997; Jennings et al. 2006). We created a runt-WRPW+ transgene by changing the C-terminal ‘Y’ residue into ‘W’ and inserted the transgene into the same genomic site as the other transgenes we tested. In situ hybridization experiments confirmed that the runt-WRPW+ transgene restored normal SxlPe expression to female runt embryos (Figure 4B). This confirms that Runt can act as transcriptional activator of SxlPe if its C terminus contains either a WRPY or VWRPW co-repressor interaction motif.

**DISCUSSION**

Drosophila primary sex determination is known for its sensitivity to the concentrations of XSEs and for the rapidity of its response to the sex determination signal. During a 30–40 min period from cycle 12 through early cycle 14, SxlPe is turned on, its expression ramped up, and then shut down in female embryos, all while being left inactive in male embryos (Barbash and Cline 1995; Avila and Erickson 2007; Gonzalez et al. 2008; Lu et al. 2008; Li et al. 2011). Despite the short time available, the XSEs appear to act in at least two mechanistic stages: an initiation phase in which X dose is first sensed and a second, maintenance phase, during which the SxlPe activity is reinforced (Avila and Erickson 2007). The highly dose-sensitive “strong” XSE proteins, Sc and SisA, appear to act in both stages as complete loss of either, or a twofold reduction in both, effectively eliminate SxlPe activity and the temperature-sensitive period for sc extends into cellularization (Erickson and Cline 1993; Walker et al. 2000; Wrischnik et al. 2003). Remarkably the two more weakly dose-sensitive XSE proteins, Runt and Upd, act at the second stage as both are dispensable for the initial activation of SxlPe but are critical for maintaining full promoter activity during cycles 13 and 14 (Figure 1, (Avila and Erickson 2007)). A two-stage model offers a possible explanation for the paradoxical notion that two critical players in this textbook example of a dose-sensitive genetic switch are themselves relatively dose-insensitive (Duffy and Gergen 1991; Torres and Sanchez 1992; Cline 1993; Sanchez et al. 1994; Kramer et al. 1999; Sefton et al. 2000). The exact gene dose of the weak XSE elements would not matter to male embryos if Runt and Upd, or the Stat92E transcription factor it activates, are only capable of enhancing transcription from an already active SxlPe. This could be the case if Runt or Stat92E are unable to bind to or function at SxlPe unless the promoter has already been activated by the strong XSE proteins. We note that male-specific viability is unaffected even with a total of four copies of wild-type runt, (one each on the X and Y; and mal+08 chro- mosomes, and two transgenic copies, unpublished data), a finding in stark contrast to what was seen with sc or sisA which are strongly male-lethal if either one is present in three copies (Erickson and Cline 1991; Erickson and Cline 1993; Cline and Meyer 1996; Wrischnik et al. 2003). In females, Runt plays a critical role in maintaining SxlPe in the state during nuclear cycles 13 and 14; however, females would be relatively insensitive to runt and upd dose if a single copy of each gene provided enough Runt or active Stat92E to effectively reinforce the actions of Sc and SisA. In contrast, if SxlPe activity were partially compromised by reductions in sc or sisA dose an additional reduction in runt dose might exacerbate the Sxl expression defect leading to the observed female-lethal effects (Duffy and Gergen 1991; Torres and Sanchez 1992).

Evaluating the validity of models of dose-sensitivity requires that the molecular functions of the XSEs be elucidated. The XSE protein Sc
and its maternally supplied partner, Daughterless, are bHLH transcriptional activators that bind as heterodimers to six or more sites at SxlPe known to be important for transcription (Yang et al. 2001). SisA remains an enigma but appears to be a non-canonical bZIP transcription factor (Erickson and Cline 1993; Fassler et al. 2002). The upd protein signals activation of Stat92E, a maternal transcription factor that binds sequences needed for full SxlPe activity (Jinks et al. 2000; Avila and Erickson 2007; Cline et al. 2010). Stat proteins, like Runx proteins, tend to be relatively weak activators that require interactions with other proteins to activate transcription (Horvath 2000; Goenka and Kaplan 2011). Intriguingly, Stat92E, has been shown to function as a positive regulator of the crumbs enhancer, crb518, via a counter-repression mechanism (Pinto et al. 2015), raising the possibility that Stat92E could function at SxlPe in a manner conceptually similar to what we propose here for Runt.

Runt is a bifunctional transcription factor that activates or represses a variety of cellular targets. A common mechanism of repression involves Runt’s C-terminal pentapeptide, VWRPY, which is needed to recruit the potent co-repressor Gro to targets including even-skipped, hairy, and engrailed (Aronson et al. 1997; Walrad et al. 2010). Still other targets of Runt and Runx proteins are repressed via gene that failed to activate transcription by interfering with Gro’s interactions with the Hes-family repressor, Dpn. While we cannot exclude the possibility that Runt’s VWRPY peptide could mediate transcriptional activation via unidentified co-activators, the idea that Runt might antagonize Gro fits well with both the central role of Gro-mediated repression in SxlPe regulation (Paroush et al. 1994; Lu et al. 2008) and with a variety of published data on Gro and Runt function.

Maternally supplied Gro is recruited to SxlPe by DNA binding proteins including the Hes protein, Dpn. Dpn binds to three sites within 160 bp of the start of SxlPe transcription (Lu et al. 2008). While Gro is often considered a long-range repressor, recent analyses have revealed that short-range repression, with Gro-binding near the promoter, as occurs at SxlPe, is more common (Kaul et al. 2014; Kaul et al. 2015). Loss of maternal Gro has several effects on SxlPe. It causes ectopic expression in male embryos and premature SxlPe activity in females. This suggests maternal Gro defines the initial threshold XSE concentrations needed to activate SxlPe and that it actively keeps the promoter off in males. In the absence of Gro, SxlPe appears to be expressed in direct proportion to X chromosome dose suggesting that Gro plays a central role in X-signal amplification (Lu et al. 2008). Antagonism of Gro function is thus a plausible means by which an XSE might regulate the SxlPe switch. The most suggestive prior indication that Runt might work by inhibiting Gro function was that Runt is needed for Sxl expression only in the broad central domain of the embryo where Gro-mediated repression is most effective. Runt is not required at the embryonic poles where Torso-signaling leads to the down regulation of Gro activity via phosphorylation (Cinnamon et al. 2008; Kaul et al. 2015). In this context, the then mysterious observation by Duffy and Gergen (Duffy and Gergen 1991), that a torso gain-of-function allele completely bypasses the need for runt in Sxl activation, is easily explained. Expression of constitutively active torso leads to uniform phosphorylation and inactivation of Gro (Cinnamon et al. 2008; Cinnamon and Paroush 2008; Helman et al. 2011). Absent active Gro, there is nothing for Runt to counter-repress at SxlPe.

How might Runt inhibit Gro function? Based on our findings and those of Kramer et al. (Kramer et al. 1999) it would appear that Runt must bind to DNA to activate SxlPe suggesting that Runt likely inhibits Gro at the promoter. This would rule out a titration scheme in which Runt binds Gro and prevents it from being recruited to SxlPe by DNA binding repressors. Plausible mechanisms of Gro inhibition could involve local phosphorylation of Gro at SxlPe if Runt could recruit a protein kinase to the promoter, or direct competition with the Hes-repressors, such as Dpn, for Gro-binding (Mclaren et al. 2000; Mclaren et al. 2001). It is also possible that changes in Gro structure induced by Runt binding to it at SxlPe might inactivate Gro. An intriguing possibility is that Runt’s interaction with Gro at SxlPe could be mediated by an XSE or an XSE-dependent co-factor. The ability of the Drosophila Runx protein, Lozenge, to stably associate with Gro in eye development depends on its interactions with the transcription factor Cut (Canon and Banerjee 2003). While the interaction with Cut regulates Lozenge’s function as a repressor, a similar mechanism could promote a counter-repressing interaction with Gro.

A remaining mystery is where Runt binds at SxlPe as no specific Runt DNA binding sites have been identified near the promoter. Kramer et al. (Kramer et al. 1999) reported that Runt, and its CBF-β DNA binding partner, Brother (Bro), bound several 200-300 bp DNA fragments from the SxlPe region; however, binding specificity was tested only by competitive challenge with high-affinity consensus
DNA binding sequences. Our laboratory also found that Runt, in combination with Bro (or the other CBF-β, Big-brother) bound a variety of SxlPe fragments, but we observed that binding was efficiently competed in every case by low concentrations of non-specific (poly dI-dC) competitor (unpublished data). Given the absence of obvious matches to the Runt binding site consensus at SxlPe and the inability to identify specific in vitro binding sites, it suggests that Runt may bind to SxlPe only in combination with other protein complexes.

The notion that Runt might target Gro function only after SxlPe has been activated offers a possible explanation for how the sparingly dose-sensitive, runt protein could play an important role in amplifying the twofold difference in male and female XSE doses into a reliable developmental signal. We previously proposed a model in which female-specific dampening of Gro-mediated repression was a central part of X-chromosome signal amplification (Lu et al. 2008; Salz and Erickson 2010). Our focus in the earlier paper was a hypothetical feedback mechanism by which active transcription of Sxl reduced Gro-mediated repression of SxlPe. In the modified version of the model (Figure 6), Runt, and potentially Stat92E, counteract Gro-mediated repression in female, but not in male, embryos. The central tenets of the model are that the 2X dose of the strong XSEs provides sufficient Sc and SisA to cross the threshold for SxlPe activation during cycle 12, but that their combined concentrations are insufficient to keep the promoter active in the face of increasing repression as the zygotic repressor Dpn accumulates and translation of maternal Gro mRNA continues. The “weak” XSEs function to counteract repression after Sxl transcription begins, either by further enhancing SxlPe, as may be the case if Stat92E functions as an activator, or by directly inhibiting Gro function by counter-repression as we propose for Runt. Signal amplification would occur because the increasing XSE protein concentrations in 2X embryos maintains the promoter in an active state, whereas the 1X dose of XSEs can never overcome the ever-increasing repression in males.

Might the kind of counter-repression mechanism we propose for Runt at Sxl exist for other genes? Interestingly, McLarren et al. (Mclarren et al. 2001) observed that mammalian Runx2 inhibited the ability of Hes1 and the mammalian Gro protein, TLE1, to repress an artificial promoter in cultured rat osteosarcoma cells. While the authors did not test if the Runx2 VWRPY residues were needed for relief of TLE1-mediated inhibition, they did note the apparent commonalities with Drosophila sex determination. Further analysis of genes co-regulated by Runx, Hes, and Gro/TLE family proteins should reveal whether it is common for Runx proteins to activate genes by interfering with repression.

**Figure 6** Model for regulation of SxlPe. In female embryos the two X dose of the XSE transcription factors, Sc and SisA, overcomes maternal Gro repression initiating expression from SxlPe in nuclear cycle 12. During cycles 13 and 14 increased levels of Sc and SisA, assisted by Runt and Upd, maintain SxlPe transcription. Runt counter-regresses Gro function via its VWRPY domain. Upd, acting through the STAT92E transcription factor, may activate SxlPe directly or counteract repression. In male embryos, the single X doses of Sc and SisA fail to overcome Gro-mediated repression and do not activate SxlPe. Without SxlPe activation, Runt and Upd/Stat92E do not function at SxlPe.

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