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Sex determination in Drosophila
The view from the top

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One of the most important decisions in development is whether to be male or female. In *Drosophila melanogaster*, most cells make this choice independent of their neighbors such that diploid cells with one X chromosome (XY) are male and those with two X chromosomes (XX) are female. X-chromosome number is relayed through regulatory proteins that act together to activate *Sex-lethal (Sxl)* in XX animals. The resulting SXL female specific RNA binding protein modulates the expression of a set of downstream genes, ultimately leading to sexually dimorphic structures and behaviors. Despite the apparent simplicity of this mechanism, *Sxl* activity is controlled by a host of transcriptional and posttranscriptional mechanisms that tailor its function to specific developmental scenarios. This review describes recent advances in our understanding of *Sxl* regulation and function, highlighting work that challenges some of the textbook views about this classical (often cited, yet poorly understood) binary switch gene.

Introduction

The observation that sex in Drosophila is under genetic control was published over 90 years ago.[1] In these studies, Calvin Bridges observed that in diploid cells sex is determined by the number of X chromosomes and that the Y chromosome played no part in this process. We now know that *Sex-lethal (Sxl)* is the immediate downstream target of a chromosome counting mechanism that distinguishes one X chromosome from two. Simply stated, *Sxl* is the female or male switch of fly sex determination (**Fig. 1**). In XX animals, *Sxl* is ON and its expression directs all aspects of female development. *Sxl* expression in females also prevents the activation of the male-specific dosage compensation system. In XY animals, *Sxl* remains OFF, dosage compensation is activated, and male development ensues. By virtue of sitting at the top of a regulatory cascade that includes dosage compensation, loss of *Sxl* function in XX animals results in female-specific lethality, and inappropriate *Sxl* expression in XY animals leads to male-specific lethality.

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The purpose of this review is to summarize our current understanding of *Sxl* regulation and function, highlighting recent studies that illustrate the precision of *Sxl* activation and the versatility of the SXL RNA binding protein. We begin with an overview of the *Sxl* gene and its products. We then examine what is known about how *Sxl* is turned ON in response to X-chromosome number early in embryogenesis, how *Sxl* serves as a heritable and irreversible molecular switch by controlling its own expression, how *Sxl* activity is controlled at the posttranscriptional level to tailor its function to specific developmental scenarios and its subsequent control of a set of downstream genes that direct cells to adopt the appropriate fate. Lastly, because there are substantial differences in *Sxl* regulation and function in the soma versus the germline, we consider these two lineages separately.

The *Sxl* Gene: Two Promoters, Alternative Splicing and Multiple Polyadenylation Sites Generate Sex-, and Stage-Specific Products

FlyBase release 5.4 indicates that *Sxl* encodes 21 different transcription products.[2] Building on earlier studies,[3-5] these 21 *Sxl* products can be divided into three groups: late female-specific, late male-specific and early female-specific (**Fig. 2**). The late female-specific and male-specific mRNAs are expressed from the "maintenance" promoter, *SxlPm*, from the cellular blastoderm stage through adulthood. Although these transcripts all have a common 5' exon (exon L1), they are sex-specifically spliced to produce mRNAs with different coding potentials. In males, all transcripts include the translation-terminating third exon and encode truncated, inactive proteins. In females, the third exon is always skipped to generate protein encoding mRNAs. Additional structural differences arise from alternative internal splicing, and 3' end variations, including added or alternative terminal exons and/or alternative polyadenylation. These structural variants, which are evolutionarily conserved, encode slightly different proteins. However, because it is not yet possible to tie specific protein forms with particular functions we will simplify our discussion by referring to these products collectively as "the" SXL protein.

The "early" female-specific *Sxl* mRNAs are transiently expressed in the precellular embryo from a 2nd promoter, the female-specific "establishment" promoter, *SxlPe*. Like the late mRNAs, the assorted early RNAs differ from each other by variations in their 3' ends, while having a common 5' exon (exon

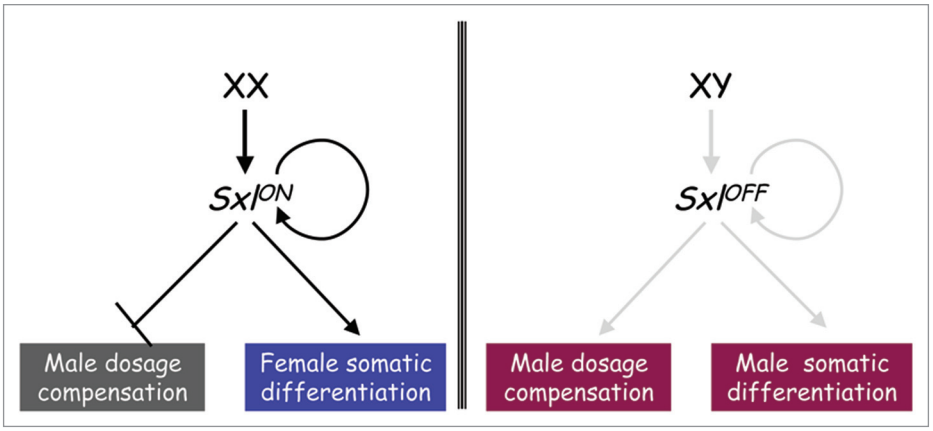

Figure 1. *Sxl* is a sexually-dimorphic genetic switch. *Sxl* is expressed in XX but not in XY animals. Once expressed, an autoregulatory feedback loop ensures continued expression throughout the remainder of development. The presence or absence of *Sxl* modulates expression of a set of downstream genes whose products are required for control of somatic sex determination/sexual behavior and dosage compensation.

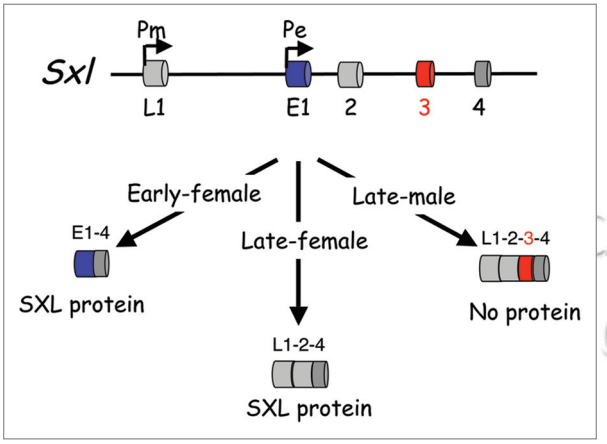

Figure 2. *Sxl* gene structure & products. Schematic illustrating the portion of the 11 exon ~20 kb *Sxl* gene that gives rise to the three major classes of sex-specific transcripts through the differential use of two promoters and alternative splicing. The embryo-specific exon (E1) is blue. The translation-terminating male-specific third exon is red. Other exons are gray. The mechanism that drives splicing of the *SxlPe* pre-mRNAs such that exon E1 is joined directly to exon 4 is not understood.[105,106]

E1) that is joined directly to exon 4 via skipping of exons 2 and 3. Thus, the early mRNAs encode the same *Sxl* proteins as the late female-specific products, aside from a 25 amino acid difference in their N termini. Whether this N-terminal domain confers unique properties to this transiently expressed form of SXL is unknown.

All of SXL's biological functions are believed to be a result of its ability to recognize and selectively bind to its target RNAs. SXL contains two highly conserved RRM-type RNA binding domains at its core,[6] and, as described in detail in the following sections, regulates different aspects of RNA metabolism both in the nucleus and in the cytoplasm. In vitro analysis using SELEX indicates that SXL binds preferentially to targets with long poly(U) stretches interrupted by guanosines.[7] Surprisingly, this consensus sequence—UUU UGU U(G/U) U(G/U) UUU (G/U)UU—is relatively common, with thousands of copies identified in the genome.[8] Given the number of putative binding sites, it seems unlikely that a single consensus binding sequence is sufficient for specific, efficient and/or functional recruitment of SXL to its targets.

How then is specificity achieved? Analysis of biologically validated targets of SXL, described in the following sections, suggest that: (1) Context is key: when SXL binding sites are moved they fail to function as efficiently as in their endogenous locations; (2) SXL binding sites are rarely found alone: multiple sites can be both clustered together and at distant locations; and (3) SXL does not act alone: SXL function depends on cross talk or communication between proteins bound at different sites. Together, these observations suggest that in addition to its RNA binding activity SXL requires protein-protein interactions to achieve selective and specific binding to its target RNAs. Nevertheless, the defining characteristics of a biologically relevant SXL target cluster remains obscure and as a consequence, the task of identifying authentic targets from genomic sequence alone is fraught with difficulty.

Turning *Sxl* ON in Early Embryogenesis: Counting X Chromosomes and Promoter Choice

Sxl regulation in somatic cells can be divided into two phases: initiation, and maintenance (**Fig. 3**). Initiation is primarily a transcriptional response by *SxlPe* to X chromosome dose. The window of opportunity for initiation is a brief period ending at the cellular blastoderm stage, when the *SxlPe* promoter is shut down and *Sxl* begins to be transcribed from *SxlPm*. Maintenance relies on positive autoregulatory splicing control of the "late" transcripts produced from *SxlPm*. Once splicing control is established, *Sxl* is locked into the ON mode for the reminder of the fly's life span.

Transcriptional activation in XX embryos. The decision of whether or not to activate *Sxl* depends on the expression levels of four X-encoded proteins, collectively called X-linked signal elements (XSE). These four proteins, encoded by the *scute*, *sisA*, *runt and unpaired* genes serve as the primary determinants of X dose.[9-13] The XSE *scute* encodes a bHLH class transcription factor

that, when bound to its heterodimeric partner, Daughterless (Da), directly activates *SxlPe*.[14] *sisA* and *runt,* which encode bZIP and Runx family members, are also thought to bind to and activate *SxlPe* as heterodimers, although their sex partners have not been identified.[15,16] The XSE *unpaired* encodes the activating ligand for the Jak-Stat pathway and exerts its effects on *SxlPe* via activating the maternally supplied *Stat92E* transcription factor.[11,13,17] Consideration of the kinetics of XSE product accumulation and the timing of *SxlPe* expression suggests that *Pe* responds directly to threshold concentrations of XSE proteins. The XSE threshold is first reached in females during syncytial cycle 12 and then exceeded or maintained for some 30–40 min until *SxlPe* shuts off early in cycle 14.[17-20] This leads to a brief burst of early *Sxl* mRNA (exon E1 to 4 splice forms) and SXL protein.[21,22] In males, XSE proteins never exceed threshold levels and *Pe* remains inactive.

The central question with respect to the initiation of sex determination is how does *SxlPe* reliably distinguish between one X chromosome and two. Presumably, some form of signal amplification converts the two-fold female/male difference in XSE protein concentrations into an all-or-nothing response at *SxlPe*. Recent work has identified the corepressor Groucho (Gro) as the key mediator of XSE signal amplification because when maternal *gro* is mutated, or when it can not be recruited to *Sxl* DNA, *SxlPe* is expressed in both sexes in direct proportion to XSE dose[20] (Mahadevarju and

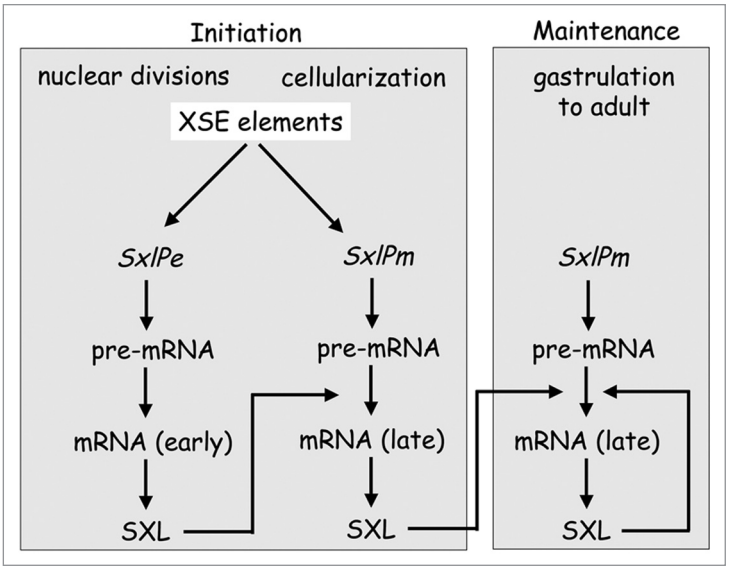

Figure 3. Overview of the regulatory logic that guarantees *Sxl* protein expression in XX animals. During the initiation phase, which takes place during syncytial blastoderm, *SxlPe* transcription is activated in response to two X-chromosomes worth of XSE products. The *Sxl* protein produced from the *SxlPe* transcripts directs the splicing of the newly transcribed RNA from the *SxlPm* promoter. During the maintenance phase, after *SxlPe* is shut down and *SxlPm* is expressed in both males and females, the autoregulatory splicing loop converts the decision to activate *Sxl* into an irreversible commitment.

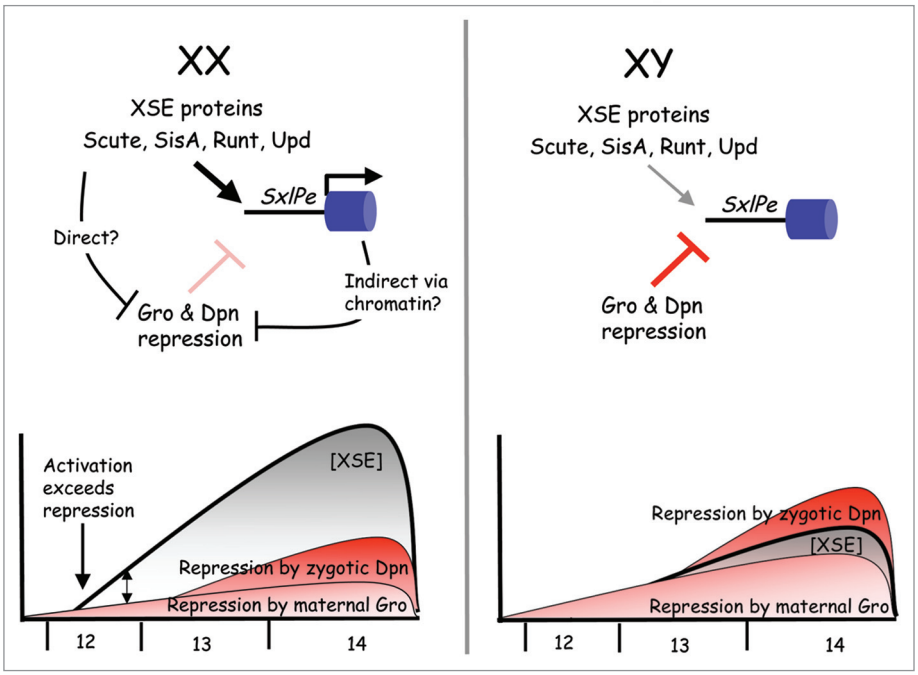

Figure 4. Threshold response model. The maternally provided Gro corepressor establishes the initial threshold against which the dose of XSE elements is measured. In XX embryos the levels of the XSE proteins exceeds this threshold in cycle 12. Once *SxlPe* transcription is initiated, repression is dampened to allow the XSE proteins to more efficiently stimulate *SxlPe* transcription during cycles 13 and 14. This might occur directly, if Gro activity is antagonized by an XSE, or indirectly, if Gro binding is reduced in the face of transcription-induced changes in chromatin architecture. In XY embryos, *gro*-mediated repression is sufficient to keep *SxlPe* silent in the face of XY levels of XSE proteins until cycle 13. Thereafter, zygotic expression of Dpn, combined with Gro, increases the threshold, thereby insuring that *SxlPe* will remain silent through cellular blastoderm.

Erickson JW, unpublished). In other words, when maternal Gro is absent, or can not associate with *SxlPe*, there is no XSE signal amplification.

Gro is the founding member of the widely distributed Gro/TLE family of corepressors, noted for their ability to effectively repress transcription.[23-26] How might Gro amplify the XSE signal and ensure proper operation of the *SxlPe* switch? Gro lacks DNA-binding activity but functions via interactions with a variety of DNA-binding proteins including Deadpan (Dpn), another known negative regulator of *SxlPe*.[18,24,27-29] Lu et al.[20] posit that amplification occurs because the actions of the XSE proteins interfere with Gro-mediated repression in XX, but not in XY, embryos (**Fig. 4**). The key features of this model are: First, that XX embryos accumulate sufficient XSE proteins by cycle 12 to overcome Gro-mediated repression and activate *SxlPe*, while XY embryos do not. Second, once *SxlPe* is active, the XSEs continue to counteract Gro-mediated repression to stimulate still higher levels transcription from *SxlPe* ensuring sufficient SXL is present to modulate the subsequent switch to maintenance control. This could occur directly, if an XSE antagonizes Gro function; or indirectly, via transcription-associated changes in chromatin structure that reduce the ability of Gro to associate with *SxlPe*. Third, although XSE proteins continue to accumulate during cycles 13 and 14, *SxlPe* remains silent in XY embryos because Gro-mediated repression is augmented by expression of the zygotic *dpn* repressor. In this scenario, Dpn serves to make this system "leak-proof" by adjusting the *SxlPe* activation threshold upward so that it compensates for the XSE proteins that accumulate during the later cycles. The net effect of the sex-specific antagonism of Gro-mediated repression is that the two-fold difference in XSE dose is converted into a robust all-or-nothing response at *SxlPe*.

The X:A ratio model is dead, long live the X-counting model. Readers familiar with textbook descriptions of Drosophila sex determination may find it surprising that the X:A ratio first appears several pages into this review, as the governing paradigm has, since the 1920s, been that it is the value of the X chromosome to autosome ratio that signals sex. The answer, as alluded to above, is that X:A hypothesis does not fit with our molecular understanding of *Sxl* regulation.

The notion that the X:A ratio rather than the number of X chromosomes signals sex originated in Calvin Bridges' classic experiments showing that animals with two X chromosomes and three sets of autosomes (XX;AAA—ratio of 0.67) develop as intersexes rather than females, and that haploid cells (X;A—ratio of 1) develop as females rather than males.[30-32] In molecular terms, what distinguishes the X:A ratio model from X chromosome-counting schemes is the prediction that the activity of XSEs is measured against a background of zygotically-acting, autosomally-encoded, factors that antagonize XSE function. However, only one genetically identifiable autosomal element, the relatively weak and late-acting *dpn* locus, appears to exist.[18,33] In striking contrast, an abundance of maternally provided factors that participate in *Sxl* regulation have been identified, suggesting that maternal components, rather than autosomal elements, could be the key reference by which XSE dose is assessed.[18,33,34] A correct inference, as we now know that maternal *gro* is a key factor in signal amplification.[20]

Despite these doubts, the X:A ratio model persisted, in part, because it provided an explanation for why haploid cells develop as females and XX;AAA triploid animals develop as sexual mosaics, findings seemingly at odds with a simple X-counting model. A recent molecular examination of the dynamics of *Sxl* activation, however, shows that sex in haploids and triploids is entirely consistent with our molecular understanding of *SxlPe* activation and its dependence on reaching threshold concentrations of XSE gene products.[19] The key here is that the window of opportunity for *SxlPe* activation is limited and ends abruptly at cellular blastoderm. In haploids, cellular blastoderm formation is delayed by a single cell division cycle and occurs during nuclear cycle 15.[35] Following up on this observation, Erickson and Quintero[19] show that *SxlPe* is activated in haploid embryos because this extra nuclear division is just enough time to allow the build up of XSE products to reach the same level as in XX cells before cellularization and the permanent shut-off of *SxlPe*. In a reciprocal manner, the sexual mosaic phenotype of XX;AAA triploids was found to be caused, at least in part, by the premature onset of cellularization, during cycle 13, that brings the X-counting process to a halt before sufficient SXL is produced to ensure that all cells can successfully engage autoregulatory splicing.

Together these data suggest that sex is not assigned by a static evaluation of the X:A ratio, but rather by sensing if a threshold concentration of XSE gene products has been reached during the short time between the onset of zygotic transcription and the beginning of cellularization. Formation of the cellular blastoderm marks the completion of the maternal to zygotic transition, a series of reprogramming events that lead to the elimination of numerous transcripts and proteins, and activation of the majority of the zygotic genome.[36] It would not be surprising if the machinery that controls the timing of the maternal to zygotic transition also controls the timing of *SxlPe* shutdown.

X Counting Continued: Activation of *SxlPm* and the Transition to Splicing Control

Although *SxlPe* is clearly the central focus of the X-counting system, it is not its only target, as the "maintenance" promoter *SxlPm* is also regulated by X chromosome dose.[37] Throughout most of life, starting before gastrulation, and lasting through adulthood, *SxlPm* appears to be expressed in all somatic cells of both sexes. The view that *SxlPm* was a boring "housekeeping" promoter made the finding that *SxlPm* is both activated earlier, and initially expressed more strongly in females than in males, something of a surprise.[37] The early onset in females, which causes *SxlPm* activity to overlap with that of *SxlPe* at the beginning of cycle 14, is controlled in part by the XSE elements, encoded by the *scute and runt* genes (as well as the maternally provided *da* protein) acting through an enhancer common to both promoters. Remarkably, this sex-differential response, which amounts to a 10–15 minute lag in onset, and a somewhat longer period of lower expression of *SxlPm* in males, is evolutionarily conserved across the breadth of the Drosophila radiation.

Table 1. Core spliceosomal proteins required for *Sxl* male-exon skipping

Drosophila gene name	Human protein name	Biochemical role(s)	Representative reference(s)
sans-fille (snf)	U1A/U2B"	U1 snRNP & U2 snRNP component	44, 45
U1-70K	U1-70K	U1 snRNP component	45, 107
SPF45	SPF45	3' splice site recognition & recruitment of the U2 snRNP	47, 48
U2AF-50	U2AF-65	3' splice site recognition & recruitment of the U2 snRNP	45, 47
U2AF-38	U2AF-35	3' splice site recognition & recruitment of the U2 snRNP	45, 47
fl(2)d	WTAP	unknown/present in purified spliceosome	62, 108
virilizer (vir)	fSAP121	unknown/present in purified spliceosome	62, 109

Why would such a subtle regulatory difference between males and females be conserved? For females, an overlap between *SxlPe* and *SxlPm* makes sense. It would ensure that sufficient amounts of SXL and its pre-mRNA substrates are present together to efficiently engage splicing control. (In this context, it is important to note that while there is sometimes a tendency to view the *Sxl* autoregulatory splicing reactions as almost infinitely sensitive, stable engagement is likely to require substantial amounts of SXL[38,39]). For males, however, there would seem no need either to delay activating *SxlPm,* or express it at a lower level, as their failure to activate *SxlPe* would make the issue moot. The answer may be that a system that actively facilitates the transition from sex signal assessment to maintenance regulation in XX cells, should also work to prevent mistakes in XY cells. For example, even if random fluctuations in XSE levels lead to *SxlPe* activation, the mistakenly expressed SXL would be deprived of pre-mRNA substrate, and the splicing loop would not be engaged.

In summary, the initiation phase of sex determination is sometimes viewed as being poised on a knife's edge, where small shifts in concentration are rapidly converted into dramatically different outcomes. We suggest that a better idea is that the dramatically different outcomes arise as a consequence of subtle reinforcement of correct decisions.

Keeping *Sxl* ON: The Autoregulatory Splicing Loop

During the maintenance phase, *Sxl* converts the transient X-chromosome dose signal into long-term cellular memory by regulating its own expression at the level of splicing.[40,41] Without *Sxl* protein, as in XY embryos, the transcripts expressed from the *SxlPm* promoter are non-functional because they contain the translation-terminating third exon. In XX embryos, the presence of *Sxl* protein forces the third, male-specific, exon to be skipped, thereby generating only protein-encoding mRNAs. Successful engagement of this autoregulatory splicing mechanism converts the sex-fate decision made earlier in development into an irreversible commitment.

How does SXL promote *Sxl* male exon skipping? In vivo studies, using large transgenic reporters containing the entire exon 2-3-4 region, have revealed that SXL-mediated splicing regulation depends primarily on binding sites located >200 nucleotides downstream, and >200 nucleotides upstream of the male exon.[42,43] Although recognition of the appropriate binding site by SXL is essential for exon skipping, SXL does not act alone.

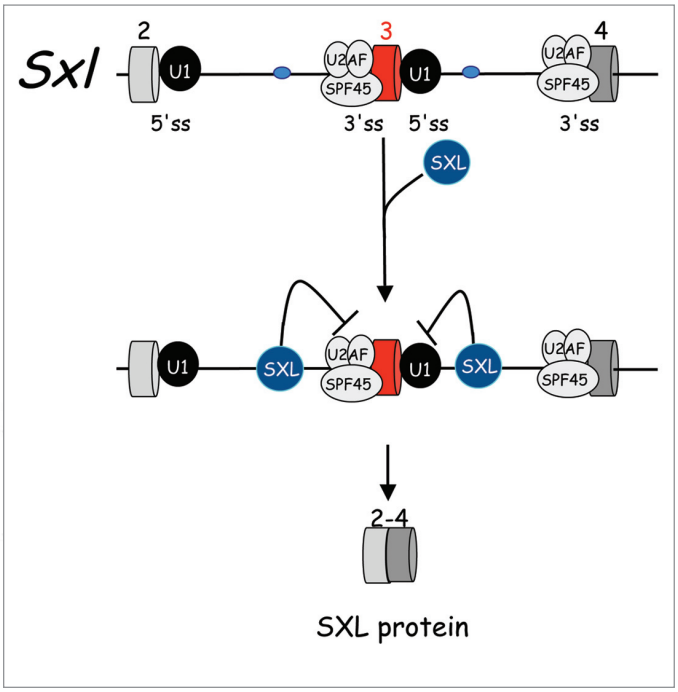

Figure 5. *Sxl* splicing autoregulation via SXL-mediated exon skipping. In both male and females, the spliceosome begins to assemble on the male specific exon 3 (red), with the binding of the U1 snRNP to the 5' splice site (ss) and the binding of the U2AF/SPF45 proteins near the 3' splice site. In females, SXL forces the exon 3 to be skipped by binding to sequences (blue ovals) in the flanking introns and antagonizing the function of general splicing factors, including the U1 snRNP, the U2AF complex, SPF45, FL(2)d (not shown) and Vir (not shown).

Current models, supported by both genetic and biochemical studies, suggest that SXL interacts with and antagonizes the functions of several general splicing factors (**Table 1**). A version of this model was first suggested by genetic studies in which Sans-fille (SNF), a protein component of the U1 and U2 snRNPs, was shown to be important for *Sxl* splicing autoregulation.[44] That an association between the U1 snRNP and SXL is particularly important for autoregulation was demonstrated by showing that SXL forms a stable complex with the integral U1 snRNP components, SNF and U1-70K, and by showing that the loss of U1-70K, or SNF, interferes with *Sxl* splicing regulation in vivo.[45] Interestingly, when ChIP analysis was used to visualize the co-transcriptional recruitment of SXL and SNF along the *Sxl* gene

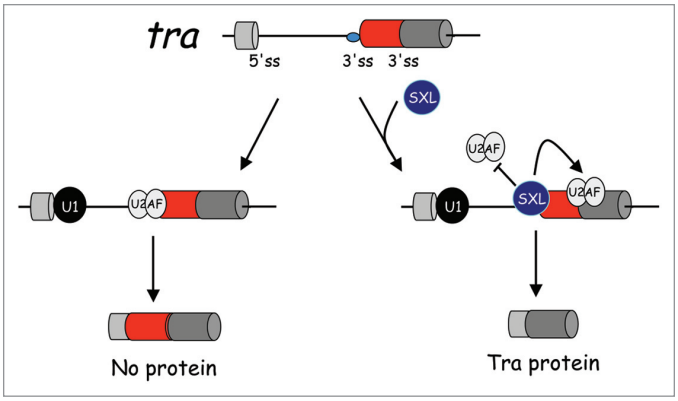

Figure 6. *Sxl* controls *tra* expression by regulating 3' splice site selection. In the absence of SXL, the U2AF complex binds preferentially to the proximal 3' splice site (ss) and a non-coding mRNA is produced. The SXL binding site (blue oval) overlaps with the proximal U2AF binding site. SXL out competes U2AF for binding to this site, thereby allowing U2AF to bind the weaker distal 3' splice site. The arrow indicates that SXL may promote U2AF binding to this alternate 3' splice site.

in female embryos, it was found that SXL does not interfere with the deposition of the U1 snRNP at the male exon 5' splice site.[46] These data, together with studies showing that SXL requires interactions with several other general splicing factors, including the U2AF heterodimer and SPF45,[45,47,48] support a model in which SXL blocks splicing by interacting with general splicing factors bound to their authentic splice sites (**Fig. 5**). Spliceosome assembly starts with the deposition of the U1 snRNP at the 5' splice site and U2AF near the 3' splice site,[49] thus SXL could block assembly immediately, or splicing could continue, stalling only later in the pathway. Interestingly, biochemical studies have shown that the U1 snRNP, U2AF and SPF45 are only transiently associated with the growing spliceosome as it assembles on the splicing substrate and are released before formation of the B*/C catalytically active complex.[50] Thus it is likely that SXL acts before catalysis begins. We note that the conclusions drawn from these in vivo data are difficult to reconcile with data from in vitro splicing assays which show SXL blocking splicing of a chimeric substrate during the process of intron removal.[47] The 48 base pairs of intronic *Sxl* sequence included in this substrate contains the male exon 3' splice sites and the adjoining SXL binding site, which earlier studies had shown to be dispensable in vivo.[42,43] Thus, while these studies clearly show SXL is capable of blocking the 2nd step of splicing, the relevance of this finding to *Sxl* autoregulation remains an open question.

Are there other, as yet unidentified proteins necessary to drive *Sxl* male exon skipping? Probably. Recent genetic studies suggest that *Sxl* expression is subject to positive reinforcement from its downstream target gene *transformer (tra)*.[51] Whether this effect is direct or indirect has not been tested, but TRA binds RNA and the presence of a tandem pair of TRA binding sites in the intron upstream of the male exon is suggestive, especially given that the TRA consensus binding site occurs only 42 times in the Drosophila genome. Biochemical studies should clarify how *tra* might augment or reinforce the decision to skip exon 3 in females.

Another protein recently identified as part of the machinery required for skipping the male exon is Protein Partner of Sansfille (PPS), the Drosophila protein most closely related to the yeast histone H3K4me3 binding protein BYE1.[46] Identified as a protein that interacts with the U1 snRNP, SXL and the *Sxl* pre-mRNA, PPS is co-transcriptionally loaded onto the RNA at *SxlPm*. Although suggestive of a connection between *Sxl* regulation and chromatin structure, it is not yet clear whether PPS has chromatin binding activity and if so, whether this activity is necessary for its role in regulating *Sxl* splicing. Nevertheless, there is precedent for a role of chromatin binding proteins in alternative splicing,[52] thus one might imagine that PPS acts in concert with the transcription machinery to promote male-exon skipping. For example, PPS could serve as a bridging protein to accelerate recruitment of SXL to the nascent transcript, or it might facilitate the formation of the inhibitory SXL/U1 snRNP interaction.

Sxl Regulation: Beyond Transcription and Splicing

A number of studies have established that even moderate changes in RNA binding protein stoichiometry can have a large impact on target specificity,[53] therefore it is perhaps not surprising (in retrospect) to find that the subcellular distribution of *Sxl* protein is tightly regulated. The surprise came when it was discovered that in some tissues, such as the wing disc, the nuclear/cytoplasmic distribution is controlled by the Hedgehog (Hh) signaling pathway.[54,55] Because the redistribution of SXL from the cytoplasm to the nucleus is likely to lead to changes in *Sxl* target gene expression, this mechanism could be exploited by the cell to generate sex-specific features that are also cell- and tissue-specific. For example, the intersection of these two pathways might tailor Hh's control over body size[56] and regulate the size difference between the sexes—a phenomenon under the control of *Sxl*, but independent of *tra*.[40] While still speculative, this hypothesis is particularly appealing because it explains how body size can be sexually dimorphic without disrupting pattern formation.

Uncontrolled accumulation of SXL protein can be lethal to females,[56] indicating that there may be a mechanism to limit SXL protein levels. Studies have shown that removal of a set of SXL binding sites in the 3' untranslated region (UTR) of the *Sxl* transcript results in excessive protein accumulation.[57,58] Thus, it is possible that SXL downregulates its own expression by interfering with translation in much the same way that it negatively regulates *msl-2* translation (see below). This ying/yang approach to autoregulation might enable *Sxl* to perpetuate its own activity while simultaneously guarding against potential adverse effects that might occur if expression went unchecked.

Sxl Target Genes: Imposing a Female Perspective on Development

Sxl activity orchestrates sex-specific development and behavior by modulating the expression of a set of downstream genes. In the following section we focus on how SXL activates *tra*, which regulates most sexually dimorphic characteristics and behaviors, and how SXL represses the activity of *male-specific-lethal-2 (msl-2),*

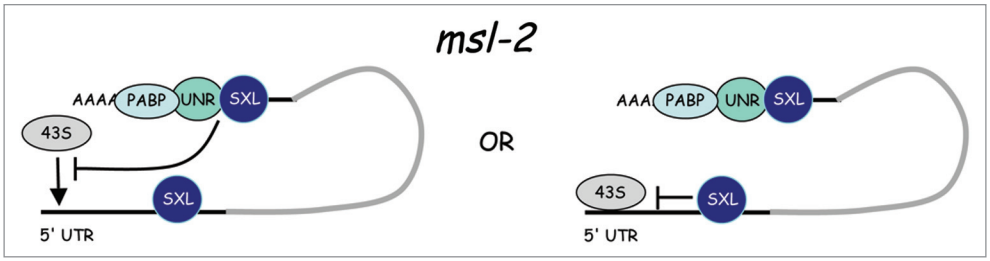

Figure 7. SXL-mediated *msl-2* translational repression. SXL associates with the 5' and 3' UTR of *msl-2* mRNA. SXL protein recruits UNR to the 3' UTR where it interacts with PABP. The SXL/UNR/PABP complex then represses translation initiation by blocking the association of the 43S ribosomal preinitiation complex with the 5' end of the *msl-2* mRNA. SXL can also inhibit scanning of any 43S subunits that escape the SXL/UNR/PABP blockage.

a key component of the male-specific dosage compensation complex. We will then discuss the evidence that SXL also functions as a sex-specific modulator of *Notch* activity. Lastly, we will briefly discuss the evidence that additional biologically important targets are yet to be found.

transformer (tra). Many (but not all) aspects of sexual dimorphism and behavior are controlled through a cascade of sex-specific events that begins with SXL regulating the splicing of *tra* transcripts. *Sxl* controls the production of the female-specific *tra* protein by controlling the use of a pair of alternative 3' splice sites at the end of the first intron (**Fig. 6**). In the absence of SXL, the proximal splice site is always used and an mRNA with no long open reading frame is produced. In the presence of SXL, 50% of the pre-mRNA is processed using the downstream 3' splice site thereby producing protein-encoding mRNAs.[59] The *tra* pre-mRNA contains a single SXL binding site located just upstream of the proximal 3' splice site. Biochemical studies have shown that SXL antagonizes the use of the proximal 3' splice site by competing with the largest subunit of U2AF, U2AF,[50] for binding to their overlapping and mutually exclusive binding sites.[60] Several studies, however, suggest that the mechanism by which SXL antagonizes the use of the proximal 3' splice site may be more complicated than a simple competition with U2AF.[61,62] One intriguing possibility, suggested by the observation that SXL is capable of associating with the U2AF complex,[45] is that SXL redirects U2AF[50] to bind to and activate the downstream 3' splice site.

male-specific-lethal-2 (msl-2). In females, the male-specific dosage compensation complex is left unassembled because SXL represses the production of the *msl-2* protein. SXL's role in this process is multifaceted. SXL functions in the nucleus, where it prevents the first intron, located in the 5' UTR, from being spliced out (intron retention), and in the cytoplasm, where it inhibits translation.[63-65] Intron retention is thought to require two sets of intronic SXL binding sites: one binding site is located adjacent to the 5' splice site and the other is located just upstream of the 3' splice site. The proximity of the binding sites to the splice sites, together with data from in vitro splicing assays, suggests a mechanism in which SXL prevents recognition of the intron by displacing U2AF at the 3' splice site and the U1 snRNP at the 5' splice site.[66,67] Remarkably, the purpose of this precise sex-specific splicing event appears to be to ensure that the two SXL-binding sites are retained in the mature *msl-2* mRNA so that SXL can subsequently repress *msl-2* translation. This conclusion is supported by the findings that transgenic variants that block splicing, but retain the intron, do not interfere with *msl-2* regulation or function.[63,64]

While the splicing process per se is not necessary for *msl-2* regulation, the SXL binding sites retained within this intron, combined with four additional SXL binding sites located in the 3' UTR are important for inhibiting translation.[63,64] How does SXL interfere with translation? By examining the 5' and 3' bound SXL complexes independently, studies in cell free systems and tissue culture cells show that SXL can block two consecutive steps in translation initiation.[68] 3' bound SXL blocks recruitment of the 43S ribosomal pre-initiation complex to the 5' end of the mRNA, whereas 5' bound SXL does not interfere with 43S recruitment but instead prevents the scanning 43S complex from reaching the initiator AUG codon. Why use a two-stage strategy? In the animal, elimination of either of the two SXL binding sites results in some MSL-2 protein production, but complete derepression accompanied by ectopic activation of the male-specific dosage compensation system requires the elimination of both the 5' and 3' SXL binding sites.[63,64] These in vivo data suggest that while neither mechanism is able to effect a complete blockade on its own, together they constitute a "leak-proof" method of translational inhibition.

While the mechanism by which SXL inhibits translation is still poorly understood, we do known that SXL requires at least one additional co-repressor encoded by *Unr* (*Upstream of n-ras,* also known as CSDE-1).[69-71] UNR is an RNA binding protein that, in mammalian cells, is involved in translational control of several cellular and viral mRNAs.[72,73] As in human cells, UNR can form a complex with the polyA-binding protein (PABP), but interestingly in vitro studies show that the SXL/UNR/PABP complex does not inhibit PABP-mediated eIF4E/eIF4G recruitment to the 5' UTR,[74,75] suggesting that SXL interferes with translation only after formation of the PABP-mediated closed-loop mRNP structure (**Fig. 7**). This model is reminiscent of *Sxl* autoregulation and evokes a mechanistic theme in which SXL acts by interacting with and antagonizing the function of key RNA metabolic proteins.

Notch. Although *tra* is clearly the primary effector through which *Sxl* controls sexual differentiation, *tra* does not control all phenotypic differences between the two sexes. As noted above, adult size dimorphism can be affected by *Sxl*, but not by *tra* mutations.[40] A second example where a male-female difference is

independent of the *tra* regulatory cascade is neurosensory bristle number on the A5 abdominal sternite.[76,77] As it turns out, *Sxl* controls this morphological difference by negatively regulating *Notch*, who's activity has long been known to control bristle number on the adult cuticle.[77,78] Recent work has shown that the presence of *Sxl* protein increases the number of bristles on A5 by reducing Notch accumulation.[77] SXL's effect on *Notch* protein accumulation seems likely to be direct, as *Notch* mRNA contains a set of SXL binding sites in its 5' and 3' UTRs and SXL is capable of binding *Notch* mRNA. Thus, SXL might downregulate *Notch* protein accumulation by interfering with translation in much the same way as it negatively regulates *msl-2* translation.

Notch signaling is used reiteratively during development in numerous cell-fate specification events. However, the majority of cell-fate specification events under *Notch*-control are not sexually dimorphic, raising the intriguing possibility that *Sxl-Notch* regulatory interactions are tissue-specific and/or used for purposes other than establishing sexual identity. An example of such a mechanism takes place in the follicle cells of the ovary, where *Sxl* modulation of *Notch* activity is important for controlling how many cells adopt a polar cell fate.[77] Adoption requires a high level of *Notch* activity and the cells with the highest level of *Notch* protein accumulation have the lowest levels of cytoplasmic SXL. The remaining follicle cells show the reciprocal expression pattern of high cytoplasmic SXL and lower levels of Notch. Given that SXL's effect on *Notch* protein accumulation is likely to be direct,[77] it is thought that specification of polar cell fate involves the clearing of SXL from the cytoplasm, which in turn releases *Notch* mRNAs from SXL-mediated translational repression. The mechanism that controls the subcellular localization of SXL in these particular cells remains to be discovered, but in other cell types the turnover of cytoplasmic SXL and/or its relocalization to the nucleus is mediated by the Hh signaling pathway.[54,55]

Other biologically relevant targets. Although it is generally assumed that SXL has only a few biologically relevant target genes, exactly how many is unknown. Recent bioinformatic approaches have already identified two plausible targets,[8,79] and it seems likely that more targets remain to be discovered. For example, it has been proposed that SXL downregulates the expression of a group of X-linked genes, all which contain multiple SXL binding sites in their 3' UTRs.[80] This, still untested, proposal stemmed from earlier studies indicating that a second, *Sxl*-dependent and *msl*-independent, dosage compensation system must exist.[40,81-83] How many X-linked genes are subject to SXL-dependent dosage compensation in females, whether this system is limited to early embryogenesis or continues to operate throughout development, and how *Sxl* regulates the process are questions that remain to be explored.

Sxl in the Germline

The observation that neither the loss of *Sxl* function in XX germ cells nor the gain of *Sxl* function in XY germ cells leads to sex reversal has been used to argue that *Sxl* does not control sexual identity in the germline.[84,85] The expectation of complete sex-reversal, however, even for mutations in a "master switch gene", is perhaps unrealistic given that germ cell differentiation is not cell autonomous and requires interactions with the surrounding somatic gonadal cells.[86] In fact, during embryogenesis the germ cells' sex-specific behavior mirrors the sexual phenotype of the surrounding somatic gonadal cells rather than the chromosomal sex of the germ cells themselves: XY germ cells initiate a female-specific program when in a female embryonic gonad and XX germ cells initiate a male-specific program when in a male embryonic gonad.[87-90] Interestingly, the female-specific program activated in XY germ cells residing in a female gonad includes *Sxl*.[88,91,92] Thus even the decision to activate *Sxl* can be made by the surrounding somatic environment, regardless of the intrinsic sex chromosome constitution. The timing of *Sxl* expression in the primordial germ cells (pole cells) further suggests that activation depends on contact with the gonadal mesoderm, as *Sxl* protein is not detectable in the pole cells until after they have migrated to the interior of the embryo and colonized the presumptive gonad.[93,94] While these studies suggest that *Sxl* expression in the germline is governed by extrinsic factors, there is little definitive information about the mechanism that transmits this information. Once initiated, however, *Sxl* expression is maintained by a positive autoregulatory splicing loop that appears, by all criteria, to be similar to that used in the soma.[85]

What does *Sxl* do in the germline? The answer, perhaps not surprisingly considering *Sxl's* multiple roles in the soma, is that it has several functions. The latest acting, is a little understood role in meiosis.[95-97] When *Sxl* germline function is compromised, meiotic recombination rates are dramatically decreased, while non-disjunction increases. Curiously, recombination on the X is more sensitive to reductions in SXL levels than autosomal recombination, but both can be eliminated by severe reductions in *Sxl* germline function.[97]

Most studies, however, have focused on its effect prior to the onset of meiosis. These studies show that *Sxl* is required in the adult ovary for both germ cell differentiation and for maintaining aspects of female identity, as the loss of *Sxl* in XX germ cells leads to the formation of germ cell tumors that ectopically express a select group of testis-enriched markers.[85,87,94,96,98,99] In the adult, each ovariole contains 2 to 3 germline stem cells (GSC) located at the tip of the germarium. When a germline stem cell divides, one daughter cell remains at the tip and retains its stem cell identity. The other daughter cell, called a cystoblast (CB), differentiates, beginning with exactly four rounds of synchronous mitotic divisions prior to entering meiosis. Germ cells that lack *Sxl* fail to initiate this differentiation program, continue to proliferate while expressing a set of molecular markers indicating a fate that is intermediate between a GSC and a CB.[99] The reason that *Sxl*-deficient germ cells fail to progress beyond this intermediate stage is that the differentiation-promoting *bag-of-marbles* (*bam*) protein, although present, appears to be non-functional. In females *bam* is thought to antagonize the function of the stem cell maintenance factor *nanos (nos)* by repressing translation.[100] Given that there are several putative SXL-binding sites in the 5' and 3' UTR of the *nos* mRNA, it is conceivable that SXL and BAM function together to repress *nanos* translation in much the same way as SXL represses *msl-2* translation. Although this hypothesis is

untested, it is consistent with the observation that SXL and BAM are coexpressed in the cytoplasm of CB cells where cytoplasmic *nos* protein is low, and with the reappearance of NOS in mature cysts just as SXL is cleared from the cytoplasm (Chau and Salz HK, unpublished).

The rapid clearance of SXL protein from the cytoplasm of the dividing cysts is also important for oocyte differentiation.[58] Several lines of evidence indicate that the translational repressor Bruno (BRU) has a role in clearing SXL protein from the cytoplasm at this time, including the finding that BRU binds to the 3' UTR of the *Sxl* pre-mRNA, and that the absence of BRU leads to persistent and unregulated SXL protein accumulation.[58,101] Interestingly, *bru* mutant cells attempt to enter meiosis but fail to progress, returning to the mitotic cycle to generate a tumor that resembles the *Sxl* overexpression phenotype.[58,102] While these studies suggest that the redistribution of SXL protein is necessary for the mitotic/meiotic transition, attribution of this function to *Sxl* is not easily done as BRU is known to regulate at least one other target gene in the germarium.[102] Nevertheless, a case can be made for *Sxl* because some *Sxl* mutations exhibit defects in meiotic chromosome segregation and recombination, two of the many meiotic processes that differ in males and females.[95-97]

Conclusion and Evolutionary Perspectives

Over the past few years, we have come to understand the key principles that govern how X-chromosome number is transmitted to *Sxl* to control the choice between male and female development. Nonetheless, it remains to be discovered how this complex, self-reinforcing, system is converted into a robust all-or-nothing response. In addition, although we now know that the window of opportunity for *SxlPe* activation is correlated with the events leading to the maternal to zygotic transition and cellularization, the mechanism that brings *SxlPe* responsiveness to an end remains a mystery. Achieving a deeper understanding of how *Sxl* regulation is connected to the more general regulatory events occurring during this dynamic period of development will be a challenge for the field in the coming years.

Given the diversity of mechanisms that animals use for determining sex, it is not surprising that SXL's sex-specific function extends only to its sibling species within the genus Drosophila.[103] While SXL is a recent addition to the regulatory cascade that controls sex determination, a comparison of the molecular strategies used by SXL to the strategies used by other RRM-domain containing RNA binding proteins in other developmental contexts has revealed striking parallels. For example the mammalian Hu proteins resemble SXL in that they have diverse molecular functions ranging from splicing to translational regulation. In addition, like SXL, they function mainly by counteracting, or redirecting the activity of other regulatory proteins.[104] We expect that future studies focused on understanding SXL-regulated processes, especially the cell and tissue-specific features that allow SXL to operate in different developmental contexts, will expand our understanding of how RNA binding proteins have evolved to recognize their target RNAs with the affinity and selectivity needed to exert tissue-, sex- and/or temporal-specific post-transcriptional control.

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