PPS, a Large Multidomain Protein, Functions with Sex-Lethal to Regulate Alternative Splicing in Drosophila

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Abstract

Alternative splicing controls the expression of many genes, including the Drosophila sex determination gene Sex-lethal (Sxl). Sxl expression is controlled via a negative regulatory mechanism where inclusion of the translation-terminating male exon is blocked in females. Previous studies have shown that the mechanism leading to exon skipping is autoregulatory and requires the SXL protein to antagonize exon inclusion by interacting with core spliceosomal proteins, including the U1 snRNP protein Sans-fille (SNF). In studies begun by screening for proteins that interact with SNF, we identified PPS, a previously uncharacterized protein, as a novel component of the machinery required for Sxl male exon skipping. PPS encodes a large protein with four signature motifs, PHD, BRK, TFS2M, and SPOC, typically found in proteins involved in transcription. We demonstrate that PPS has a direct role in Sxl male exon skipping by showing first that loss of function mutations have phenotypes indicative of Sxl misregulation and second that the PPS protein forms a complex with SXL and the unspliced Sxl RNA. In addition, we mapped the recruitment of PPS, SXL, and SNF along the Sxl gene using chromatin immunoprecipitation (ChIP), which revealed that, like many other splicing factors, these proteins bind their RNA targets while in close proximity to the DNA. Interestingly, while SNF and SXL are specifically recruited to their predicted binding sites, PPS has a distinct pattern of accumulation along the Sxl gene, associating with a region that includes, but is not limited to, the SxlPm promoter. Together, these data indicate that PPS is different from other splicing factors involved in male-exon skipping and suggest, for the first time, a functional link between transcription and SXL–mediated alternative splicing. Loss of zygotic PPS function, however, is lethal to both sexes, indicating that its role may be of broad significance.

Introduction

Understanding tissue- and stage-specific gene regulation remains one of the central issues in developmental biology. Studies of developmentally important genes, such as those that specify and maintain cell fate, have revealed that many genes are regulated post-transcriptionally. The Drosophila sex-determination gene Sex-lethal (Sxl) is a prime example of a developmental switch gene regulated by alternative splicing. Throughout most of development and in adult tissues, Sxl is controlled by sex-specific alternative splicing to produce mRNAs with different coding potentials [1]. In males, all transcripts include the translation-terminating third exon leading to the production of mRNAs that encode truncated, inactive proteins. In females, the third exon is always skipped to generate protein encoding mRNAs. The mechanism leading to exon skipping is autoregulatory and depends on the SXL protein binding to multiple intronic sites located both upstream and downstream of the regulated exon. Current models, based on both biochemical and genetic studies, suggest that SXL forces the male exon to be skipped by interacting with and antagonizing a set of general splicing factors, including the U1 snRNP, the U2AF heterodimer, FL2/2 and SPF45 [2–4]. Because Sxl controls both its own expression and the expression of a set of downstream target genes, this autoregulatory splicing loop serves as a heritable and irreversible molecular switch for the developmental pathways controlling both somatic sex determination and X-chromosome dosage compensation.

Initiation and stable engagement of the Sxl autoregulatory splicing loop requires the coordinated use of two alternative promoters [5–7]. Throughout most of development, Sxl is first expressed during the maternal to zygotic transition, but prior to that time Sxl is transiently expressed from the female-specific “establishment” promoter, SxlPm. The SxlPm-derived transcripts, unlike the transcripts produced from SxlPm, are spliced by default to produce SXL protein. Thus the SXL protein present in XX embryos when SxlPm is first activated serves to drive the initiating round of exon skipping which leads to a self-sustaining splicing loop. In XY animals, on the other hand, SxlPm is not activated, there is no SXL protein, and all SxlPm-derived transcripts are spliced in the male mode. While coordinated promoter switching is critical for successful establishment of the Sxl autoregulatory splicing loop in early embryogenesis, it has been generally assumed that transcription plays little, if any, role in sex-specific regulation after this point.
Here we report the identification and analysis of a previously uncharacterized protein, named Protein Partner of Sans-fille (PPS, CG6525), as a novel component of the machinery that controls Sxl alternative splicing. PPS, a large multidomain protein classified as a transcription regulator based on the presence of 4 distinct and conserved sequence motifs, was identified in a yeast two hybrid screen for proteins that interact with Sans-fille (SNF), the Drosophila homolog of the U1 snRNP protein, U1A. We provide compelling evidence that PPS has a direct role in Sxl male exon skipping by showing first that the loss of pps function interferes with Sxl function, and second that PPS can form a complex with the U1 snRNP, SXL and the Sxl pre-mRNA. In addition, we mapped the association of PPS, SXL and SNF along the Sxl gene by chromatin immunoprecipitation (ChIP), providing evidence that these proteins, like many other splicing factors, bind their RNA targets while in close proximity to the DNA. While we found that SXL and SNF associate with their predicted binding sites, PPS has a distinct pattern of accumulation along the Sxl gene which suggests that PPS is loaded onto the RNA at the promoter. Finally, we show that PPS function is not restricted to Sxl splicing regulation, indicating that PPS is likely to be more broadly involved in development.

**Results**

**Identification of PPS, a SNF–interacting protein**

CG6525 was identified in a yeast two hybrid screen for SNF-interacting proteins, giving the gene its name protein partner of sans-fille (pps; Figure 1A). To demonstrate that the PPS/SNF interaction also occurs in Drosophila cell extracts, we assayed for complex formation by pull-down experiments in which a GST fusion protein containing the C-terminal end of PPS (amino acids 1370–2016) was expressed in E. coli, bound to glutathione sepharose beads, and incubated with protein extracts made from Drosophila cells. Together with the observation that the PPS protein contains four signature motifs typically found in proteins that function in transcriptional regulation, our data suggest that linking transcription to splicing regulation is important for controlling Sxl expression. This idea is especially intriguing because it indicates that the coupling of transcription and splicing seen in vitro and in cell culture studies is likely to be pertinent to developmentally controlled patterns of gene expression in the living animal.
embryos. The presence or absence of SNF in the complex formed on the beads was assayed by Western blot analysis (Figure 1B). In control studies, we used a GST:SXL fusion protein since it is known to form a complex with SNF [2]. As predicted by the two hybrid data, we found that GST::PPS, but not GST alone, was capable of selecting SNF out of extracts as efficiently as GST::SXL. These data therefore confirm that PPS and SNF associate in vivo.

PPS is located on the 3rd chromosome (87B) and, in agreement with the predicted gene structure, we found that the pps transcription unit extends over 6.7 kb, and the 11 constitutively spliced exons are predicted to encode an uncharacterized 2016 amino acid protein (Figure 1C and 1D). The pps open reading frame contains 4 conserved motifs: PHD finger (plant homeodomain), BRK (Brahma and Kismet), TFSS2M (transcription elongation factor SII middle) and SPOC (Spen paralogue and orthologue C-terminal). According to the Gene Ontology Database, which assigns functions to uncharacterized proteins based the presence of sequence motifs, PPS is likely to function in transcriptional regulation (see discussion).

**pps is an essential gene**

To gain insight into the biological role of PPS, we generated a molecular null allele using an FRT-based targeted deletion strategy [8,9]. Briefly, we induced recombination in animals heterozygous for two FRT-bearing piggyBac insertions with controlled expression of the FLP recombinase and identified a deletion with the desired endpoints using a PCR based strategy. The resulting deletion, depicted in Figure 1D, removes the entire coding sequence of pps as well as the adjacent gene, Scg-β. Animals homozygous for this two gene deletion die during the third instar larval stage. Two critical experiments demonstrate that the lethality is due to the loss of pps and not Scg-β. First, lethality was fully rescued by one or two copies of P(pps+), a genomic transgene that carries just the pps gene (90%, n = 554). Second, all aspects of the mutant phenotype remained unchanged by the addition of multiple copies of the adjacent P(Scg-β+) genomic transgene (see Materials and Methods for details). Thus, these data provide strong evidence that disruption of PPS is responsible for the larval lethal phenotype and the two gene deletion we have isolated behaves as a pps null allele. Based on these genetic data, we have named this deletion *pps*{sup}1{}/sup.

Homozygous *pps*{sup}1{}/sup mutant animals fail to survive to adulthood, although all animals reach the third instar larval stage. Consistent with the failure to pupate, mutant third instar larvae were found to have a number of defects, including small, undeveloped imaginal discs, abnormal polytene chromosome morphology and melanized patches of tissue that resemble melanotic tumors (data not shown).

Although *pps* null mutants complete embryogenesis without any apparent defects, we cannot rule out an earlier function in embryogenesis. PPS is a maternally provided protein and the extended stability common to many maternally provided proteins typically result in the rescue of homozygous mutant animals into the larval stages. Thus, *pps* mutant animals may survive until the maternal stores of protein are depleted, masking a potential requirement in embryogenesis.

**Incomplete rescue of *pps*{sup}1{}/sup reveals a role in Sxl regulation**

During the course of this analysis, we noted that, while either one or two copies of the *P(pps+)* transgene was sufficient to rescue the lethality of *pps*{sup}1{}/sup homozygous mutant females, two copies were necessary to rescue the females to fertility. An examination of the ovaries isolated from these sterile mutant females revealed that the ovaries contained tumors (Figure 2A). Ovarian tumor phenotypes are also observed in partial loss of function *snf* mutant backgrounds, where the phenotype is caused by defects in Sxl splicing regulation [2,10]. To investigate the possibility that the *pps* tumor phenotype is also correlated with *Sxl* misregulation, we used RT-PCR to assay the Sxl RNA products present in isolated ovarian tissue. Using a single primer pair capable of detecting the female and the larger male spliced products, we found that in ovarian tissue isolated from sterile mutant females, a significant proportion of the spliced products contained the male-specific exon (Figure 2B and 2C). Thus, based on these partial loss of function mutant phenotypes, we conclude that *pps*, like *snf*, is required to achieve stable Sxl activity in the female germline.

**pp*{sup}1{}/sup is a maternal effect modifier of Sxl**

Activation of Sxl in the embryo is a multi-step process, starting with the coordinated use of two promoters and culminating with successful engagement of the autoregulatory splicing loop. Thus, perturbation of any single step in the process can lead to a defect in alternative splicing. As a consequence, embryos homozygous for the normally recessive null allele of Sxl (*Sxl{sup}f1{/sup}+/+) are particularly sensitive to the supply of specific splicing and transcription factors deposited into the egg by the mother (e.g. [2–4]). We therefore reasoned that if maternally provided PPS protein is important for any aspect of Sxl regulation, we might expect the viability of *Sxl{sup}f1{/sup}+/+ females to be affected if their mothers were heterozygous for *pps* (*pps*{sup}+/+). However, we found that these *Sxl{sup}f1{/sup}+/+ females were as viable as their control siblings (data not shown). To increase the sensitivity of this assay, we introduced a mutant allele of *Sxl* (*Sxlf1{sup}/+*) into the genetic background. *da* encodes a maternally supplied transcription factor required to activate Sxl [11,12]. We chose *da* to sensitize the genetic background because we have previously shown that the genetic interaction between *saff* and *da* is particularly strong [13]. In control crosses, we found that 57% of the expected *Sxl{sup}f1{/sup}/+ daughters from *da*{sup}+{/sup}/+ mothers survived to adulthood (n = 275; Figure 5). However, when the mothers were heterozygous for both *pps*{sup}1{}/sup and *da*{sup}1{}/sup, there was a significant reduction in viability with only 7% of the expected *Sxl{sup}f1{/sup}/+ daughters surviving to adulthood.

![Figure 2](https://example.com/figure2.png)

**Figure 2. Sxl splicing is disrupted in the ovaries of incompletely rescued *pps*{sup}1{}/sup females.** (A) DAPI-stained ovariole from a wild female (WT) and a *P(pps+)*/+; *pps*{sup}/+; *Sxl{sup}f1{/sup}/+; *Df(3R)Exel7316* female. (B) Diagram of the alternative splicing event that produces sex-specific Sxl transcripts. The arrows below the diagram indicate the position of the PCR primer pairs used for RT-PCR. (C) The tumor phenotype is correlated with Sxl splicing defects. Splicing was assayed by RT-PCR using RNA isolated from ovaries dissected from *P(pps+)*/+; *pps*{sup}/+; *Sxl{sup}f1{/sup}/+; *Df(3R)Exel7316* females (pps tumors). Controls include splicing in ovaries isolated from wild type (WT) females and splicing in adult males.

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Female progeny scored. On the assumption that an equal number of male and female progeny will be generated from each cross, the percent female viability was calculated by comparing the number of females recovered with the number of males recovered.

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To confirm the genetic relationship between \( pps \) and \( Sxl \), we looked for synergistic interactions with mutant alleles of \( r(2)A4 \), \( U2af38 \) and \( spf45 \). Mutations in these three genes were picked because they encode core spliceosomal proteins known to play an important role in \( Sxl \) autoregulation [2–4]. These data show that \( pps \) in combination with mutations in each of these spliceosomal genes exerts a detrimental synergistic effect on the viability of \( Sxl^{+/+} \) females (Table 1). Together, these data indicate that the maternally provided PPS protein contributes, in some way, to \( Sxl \) regulation.

Table 1. Lethal interactions between \( Sxl^{+/+} \), \( pps^{+/+} \), and mutations in core spliceosomal proteins.

| Maternal Genotype | Viability of \( Sxl^{+/+} \) female progeny |
|-------------------|---------------------------------|
| \( r(2)A4^{+/+} \) \( pps^{+/+} \) | 29% \((n = 140)\) |
| \( U2af38^{+/+} \) \( pps^{+/+} \) | 12% \((n = 154)\) |
| \( spf45^{+/+} \) \( pps^{+/+} \) | 18% \((n = 140)\) |

(Female progeny scored. On the assumption that an equal number of male and female progeny will be generated from each cross, the percent female viability was calculated by comparing the number of females recovered with the number of males recovered.)

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PPS associates with the U1 snRNP and the SXL protein

Previous studies have shown that \( Sxl \) interacts with SNF in the context of the U1 snRNP [2]. We reasoned, therefore, that if \( pps \) has a direct role in \( Sxl \) splicing autoregulation, then we might be able to detect physical interactions between PPS, the U1 snRNP, and SXL. To test this, we generated an antibody against the C-terminal end of PPS (amino acids 1370–2016) for co-immunoprecipitation assays. PPS is predicted to encode a single polypeptide of 222 Kd, and as predicted, we found that PPS in combination with mutations in each of these spliceosomal genes exerts a detrimental synergistic effect on the viability of \( Sxl^{+/+} \) females (Table 1). Together, these data indicate that the maternally provided PPS protein contributes, in some way, to \( Sxl \) regulation.

To determine whether the SXL protein is required for the association between PPS and the U1 pre-mRNA, we carried out RIP assays in nuclear extracts made from embryos collected from mothers homozygous for a viable allele of \( da^{+/+} \), \( da/dol \), \( da^{+/+} \) mutant mothers produce eggs that lack SXL protein because \( Sxl^{Pm} \) is not activated [12]. \( Sxl^{Pm} \), however, is activated, and the resulting transcripts are therefore spliced in the male mode. As illustrated in Figure 4C, PPS was able to co-immunoprecipitate unspliced \( Sxl \) RNA in these \( Sxl^{-} \)-deficient mutant extracts. In control reactions, we found that \( Sxl \) RNA was detected in \( Snf^{Pm} \) immunoprecipitates, but not in extracts treated with antibodies against the chromatin binding protein Polycomb (PC) or in pre-immune serum.

Recruitment of PPS, SNF, and SXL during transcription

To gain a better understanding of the functional relationship between PPS, SXL and SNF, we compared the dynamics of their recruitment to the nascent \( Sxl \) transcript by combining genetic analysis with chromatin immunoprecipitation (ChiP) assays (Figure 6). Splicing factor-ChiP assays, which have been used in both yeast and mammalian cells, are possible because many splicing factors are recruited to their RNA targets while still in close contact with template DNA [15–17].

To validate this approach, ChiP analysis was first carried out with antibodies against SNF in a sexually mixed population of wild type 8–12 hour embryos. ChiP studies in mammalian cells have shown that U1 snRNP proteins specifically target regions of genes
that include 5′ splice sites of recognized exons [17]. This predicts that SNF will accumulate on a region that includes the male-specific third exon (Ex3), but not on the SXL binding site which is located ~250 bp away in the third intron (In3). As a specificity control, we assayed for SNF accumulation on the first exon of the SxlPe transcripts (E1) because in 8–12 hour embryos E1 is treated as an intron, and thus should not be recognized by the splicing machinery. In agreement with our expectations, we found that SNF was present at the third exon (Ex3), but not at the other two locations. Additional controls for specificity include our demonstration that these three regions of the Sxl gene were not precipitated in controls or in ChIP assays carried out with the DNA binding Heat Shock Factor (HSF). As a final control for specificity, ChIPs were also carried out with the 8WG16 antibody against the hypophosphorylated form of RNA polymerase II (Pol IIa), because previous studies have shown that Pol IIa does not accumulate within the body of actively transcribed genes [18,19].

Having shown that recruitment of SNF to the Sxl gene can be detected by ChIP, we next asked whether we could use this methodology to view SXL and PPS recruitment. In agreement with in vitro RNA binding assays [20], we found that SXL was present at the intronic SXL binding site, In3. PPS, on the other hand, was not only present on the third exon (Ex3) but also localized to the intronic E1 and In3 regions. Together these results argue that PPS, in contrast to both SNF and SXL, is uniformly distributed across the Sxl transcription unit.

Figure 4. PPS associates with SNF and U1-70K in embryonic extracts. (A) pps1 is a protein-null allele. Western blot of extracts made from wild type and pps1 mutant animals probed with antibodies against PPS. SNF is used here as a loading control. (B) PPS interacts with SNF in a RNA-independent manner. Western blots of PPS and U2A' immunoprecipitations (Co-IP) in nuclear extracts made from embryos probed with an antibody against SNF. The RNase sensitivity of this association was tested by pretreating the extract with a combination of RNase A and RNase T1. Controls include the previously described RNase sensitive SNF/U2A' association. (C) PPS associates with U1-70K. Western blot of PPS and SNF immunoprecipitations (Co-IP) in nuclear extracts made from embryos probed with an antibody against U1-70K. Controls include the previously described SNF/U1-70K association. The lanes marked 2.5% input are controls in which the amount of extract corresponds to 2.5% of the material used in each Co-IP experiment. doi:10.1371/journal.pgen.1000872.g004

Figure 5. PPS associates with the SXL protein and the Sxl pre-mRNA. (A) PPS interacts with SXL in a RNA-dependent manner. Western blots of PPS immunoprecipitations (Co-IP) in nuclear extracts made from embryos probed with an antibody against SXL. The RNase sensitivity of this association was tested by pretreating the extract with a combination of RNase A and RNase T1. (B,C) PPS associates with the unspliced Sxl pre-mRNA in a SXL-independent manner. RNA-immunoprecipitation assays (RIP) were carried out in nuclear extracts made from wild type embryos (WT), or embryos from da1/da1 mothers. The presence of unspliced Sxl RNA in the IP pellet was detected by RT-PCR using an intron 3/exon 4 primer pair. Immunoprecipitations with SXL or SNF were included as positive controls. Negative controls included precipitations with no antibody, pre-immune serum and Polycomb (PC). The lanes marked input are controls in which the amount of extract corresponds to a percentage of the material used in each Co-IP experiment. doi:10.1371/journal.pgen.1000872.g005
Next we asked whether the pattern of recruitment is different on nascent transcripts destined to be spliced in the female or male mode. Males do not express SXL protein; therefore, SXL-ChIP of chromatin isolated from a mixed sex population of embryos resulted in the analysis of only female embryos. PPS and SNF, on the other hand, are expressed in both male and female embryos, thus the analysis of chromatin from wild type embryos would mask any sex-specific differences, should they exist. To circumvent this issue, we repeated the SNF and PPS ChIP experiments in two mutant populations of embryos. To exclusively assay $S_xl$ transcripts destined to be spliced in the female mode, chromatin was prepared from embryos collected from a stable stock in which all females carry an attached X chromosome and all males carry $S_xl^{7BO}$, an X-linked deletion allele of $S_xl$. As there is no $S_xl$ DNA present in the male embryos, this analysis is limited to $S_xl$ chromatin isolated from female embryos. To generate a population of embryos where all nascent $S_xl$ transcripts are destined to be spliced in the male mode, we prepared chromatin from embryos from $da^1$ mothers. As described above, maternal DA protein is required to initiate $S_xl^{Pe}$ transcription early in embryonic development, therefore all eggs laid by homozygous mutant females fail to produce SXL protein. As shown in Figure 6, we found that the pattern of recruitment is not sex-specific. Taken together, these results suggest that PPS associates with the $S_xl$ promoter.

PPS also targets the $S_xl$-regulated transformer (tra) pre-mRNA

In addition to its autoregulatory function, the $S_xl$ protein also binds the tra pre-mRNA to regulate its sex-specific expression [21]. To determine whether PPS is involved in tra pre-mRNA splicing, we first carried out RIP assays and found that tra pre-mRNA is detectable in PPS immunoprecipitates, as well as in control SXL and SNF immunoprecipitates (Figure 5A). We then carried out ChIP experiments to determine whether PPS is recruited to the tra promoter region (Figure 8B). To demonstrate that we had targeted the promoter region, ChIP experiments with antibodies against the hypophosphorylated form of RNA polymerase II (Pol IIa), detected by the 8WG16 antibody, is highly concentrated at the start of actively transcribed genes [18,19]. In agreement with these studies, we found that Pol IIa specifically accumulates at P1, P2 and P3 (Figure 7). SNF, as expected, only accumulates on P3, the region that overlaps with the first exon. As shown in Figure 7, we found that PPS accumulates on P1, P2 and P3 and that this distribution is not sex-specific. Taken together, these results suggest that PPS associates with the $S_xl$ promoter.

Recruitment of PPS to the $S_xl^{Pm}$ promoter region

The uniform distribution of PPS on the $S_xl$ transcription unit, together with its classification in the Gene Ontology Database as a protein involved in transcription, suggested to us that PPS might initially be recruited near $S_xl^{Pm}$. We therefore repeated the ChIP experiments using two different primer sets targeting sequences upstream of the $S_xl^{Pm}$ transcription start site (P1 and P2) and one that includes the first exon (P3). ChIP studies in Drosophila and mammalian cells have shown that the hypophosphorylated form of RNA polymerase II (Pol IIa), detected by the 8WG16 antibody, is highly concentrated at the start of actively transcribed genes [18,19]. In agreement with these studies, we found that Pol IIa specifically accumulates at P1, P2 and P3 (Figure 7). SNF, as expected, only accumulates on P3, the region that overlaps with the first exon. As shown in Figure 7, we found that PPS accumulates on P1, P2 and P3 and that this distribution is not sex-specific. Taken together, these results suggest that PPS associates with the $S_xl$ promoter.
While these studies clearly suggest that PPS has an additional role in tra splicing regulation, it is unlikely that PPS is globally associated with all actively transcribed genes, as we fail to detect associations with the intronless U2A gene and the intron containing snf gene (Figure 8A and 8B). On the other hand, PPS is clearly not limited to SXL-mediated splicing events because loss of PPS function is lethal to both sexes. What these additional functions are remains to be determined.

Discussion

Genetic studies have established that SXL protein is both necessary and sufficient to engage the Sxl autoregulatory splicing loop [22]. Mechanistically, however, SXL does not act alone and collaborates with components of the general splicing machinery, including the U1 snRNP, to block inclusion of the male exon [2]. In this study, ChIP assays showed that SNF and SXL are specifically recruited to their predicted binding sites on the nascent transcript: SNF to 5’ splice sites and SXL to its intronic binding sites. These data, together with our observation that the recruitment of SNF is not influenced by the presence or absence of SXL, support the current model in which SXL blocks male exon inclusion by interacting with general splicing factors bound to authentic splice sites (Figure 9). Splicing could be blocked immediately, or spliceosome assembly could continue, stalling only later in the pathway. The U1 snRNP, however, is only transiently associated with the spliceosome as it assembles on the splicing substrate and is released before the spliceosome is catalytically active [23]. Therefore it is likely that SXL acts by interrupting spliceosome assembly at some point after splice site recognition by the U1 snRNP, but before catalysis begins.

In studies begun by screening for SNF-interacting proteins, we identified PPS, a conserved and previously uncharacterized Drosophila protein, as a novel component of the machinery required for skipping the Sxl male exon. We were able to establish this connection by demonstrating that (1) animals carrying loss of function pps mutations are compromised in their ability to regulate Sxl splicing, (2) PPS associates with the U1 snRNP via a direct interaction with SNF and (3) PPS associates with the SXL protein and the unspliced Sxl RNA.

Although physically associated with the U1 snRNP, PPS does not appear to be a general splicing factor because it does not associate with all spliced transcripts (this study), it is not found in affinity-purified Drosophila spliceosomal complexes [23] and it is not a homolog of a previously identified human splicing protein [24]. Thus, PPS stands apart from the other proteins known to facilitate proper Sxl splicing, all of which are known to be components of the splicing machinery.

The results of our ChIP analysis also distinguishes PPS from known splicing factors, as it reveals a strikingly distinct pattern of accumulation along the Sxl gene, including occupancy at the SxlPm promoter region. This pattern of accumulation suggests that PPS is loaded onto the RNA at the promoter and/or that it has a role in its recognition by the spliceosome.

Figure 7. Accumulation of PPS near the SxlPm promoter in embryos. ChIP assays using SNF– and PPS–specific antibodies were carried out using the same population of embryos as described in Figure 6. After ChIP the extracted DNA was analyzed by PCR using primer pairs positioned around the SxlPm promoter as diagramed. The 8WG16 antibody, which detects the hypophosphorylated Pol II (Pol IIa), is used here to mark the promoter. Consistent with published studies, Pol IIa was largely detected at the promoter whereas SNF was only detected by a primer set designed to detect the beginning of the transcription unit.
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Figure 8. tra is a PPS target gene. (A) RIP assays demonstrating that PPS associates with the tra pre-mRNA, but not the snf pre-mRNA or the intronless U2A transcript. The presence of unspliced RNA in the IP pellet was detected by RT–PCR. (B) ChIP assays demonstrating that PPS is detected at the tra promoter (identified by Pol IIa accumulation), but not at the snf or the U2A promoter. The exact position of the primers used in the RIP and ChIP assays are described in the Materials and Methods section.
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transcription. Numerous studies have documented physical interactions between the transcriptional machinery and splicing factors [25]. Thus, PPS may well act in concert with the transcription machinery to promote SXL-mediated exon skipping (Figure 9). For example, PPS could serve as a bridging protein to accelerate recruitment of SXL to the nascent transcript, or it could facilitate the formation of the inhibitory SXL/U1 snRNP interaction.

Whether PPS is physically coupled to the transcription machinery and/or has a role in controlling transcription will require additional studies. However, the fact that PPS contains 4 signature motifs typically found in proteins with known functions in transcription adds credence to this idea. Of these 4 motifs, the PHD finger is the most extensively studied. Numerous studies have shown that PHD fingers have histone methylation binding activity. Indeed, PPS is likely to have histone binding activity, as the PHD domains of both the S. cerevisiae (BYE1) and mammalian (DIDO) PPS homologs preferentially bind to tri-methylated H3K4 (H3K4me3) in vitro [26,27]. The possibility of a PPS–histone link is further strengthened by the presence of the metazoan specific BRK motif, a domain that is found in only two other Drosophila proteins–Brahma and Kismet–both of which are known to be chromatin binding proteins [28,29].

A connection to transcription is also suggested by the presence of the TFS2M motif. This motif is named after its founding member located in the center of the transcription elongation factor S-II, where it is essential for binding Pol II [30]. Finally, SPOC domains have been identified in a variety of proteins linked to transcription, the best characterized of which is the human SHARP nuclear hormone co-repressor [31,32]. A conserved function in transcription is particularly compelling in light of the current view that transcription and splicing are mechanistically coupled. In this regard, there are a few well-documented examples of mammalian chromatin binding proteins that affect alternative splicing [33]. For example the H3K4me3 binding protein, CHD1, associates with the spliceosome and is required for efficient splicing [34]. In another example the BRK domain containing chromatin remodeling protein, BRAHMA/BRG1, influences the alternative splicing of several transcripts [35].

Although still speculative, a mechanism linking transcription to splicing regulation is likely to be of major importance in early embryogenesis. Engagement of the autoregulatory splicing loop requires that the initiating source of SXL protein, produced from the transiently expressed SxlPe derived transcripts, be present when SxlPm is activated so that its transcripts can be alternatively spliced to produce more SXL protein. The changeover from SxlPe to SxlPm is tightly coordinated and uncoupling these events leads to disruptions in Sxl regulation [6,7]. While these studies suggest that transcriptional regulation of SxlPm is important for the switch to autoregulation, our studies lead us to propose that PPS contributes to the success of this switch by concurrently facilitating SxlPm transcription and promoting male-exon skipping.

PPS function is not restricted to Sxl splicing regulation. In studies designed to test for specificity, we discovered that PPS also associates with the SXL-regulated tra pre-mRNA. In addition, we found that pps function is essential for viability of both sexes, indicating that pps function is not limited to SXL-mediated splicing events and is involved in other developmental pathways. In humans, the PPS homolog DIDO has been linked to a blood disorder called myeloproliferative disease (MPD) [36]. The relevance of this connection is suggested by our finding that homozygous pps mutant larvae contain melanotic tumors, tumors that often result from over-proliferation and aggregation of blood

Figure 9. Co-transcriptional model for Sxl splicing autoregulation. PPS associates with Pol II during transcription (Pol II, oval) to help recruit the U1 snRNP (U1, blue circle) and SXL (grey circle) to the appropriate locations on the nascent transcript. In addition, PPS may help nucleate the interaction between the U1 snRNP and SXL. Splicing could be blocked immediately (insert) or spliceosome assembly could continue, stalling only later in the pathway. The end result is a dead-end complex that guarantees that the male exon will be skipped, and that exon 2 is spliced to exon 4. In males, where there is no SXL protein, the U1 snRNP is free to assemble into a functional spliceosome and exon 3 is included in the mature transcript (not shown).

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cells [37]. Thus, the discovery of PPS’ role in controlling alternative splicing may be of significance to additional developmental pathways.

Materials and Methods

Yeast two hybrid screen

Using the entire SNF protein as bait, we screened 9.8x10^7 clones from Drosophila embryonic and adult cDNA libraries and identified 78 positive clones, all of which included the C-terminal end of the pps (CG6525) gene. PPS was also reported to be a binding partner of CDK7 (CG3319) [38]. However, we have not been able to verify the authenticity of this interaction (data not shown), and suspect that this interaction is based on an annotation error because the spf and cd7 genes partially overlap [39].

Drosophila genetics

Mutant alleles and deficiencies used in this study include: Sxl[P1], Sxl[P3] da; da2, b(2)[f]; U2af38[43] Dr(2)H[4] (designated as spf/43 in Table 1), Df[3R]/E[1731], PBac[WH]/Dip-C[00706] and PBac[WH]/CG17292[10799] [2,8,12,40]. We generated pps by FRT-mediated recombination between PBac[WH]/Dip-C[00706] and PBac[WH]/CG17292[10799] using the conditions described previously [9,10]. Throughout this analysis we found that the phenotypes of pps and pps animals to be identical, indicating the absence of confounding background mutations on the pps mutant chromosome. The P[spfP1] and P[Sgf-fht, CG17292] genomic rescue constructs were generated by standard methods in the pCpaSpeR4 transformation vector and transgenic flies were produced at Genetic Services (http://www.geneticeservices.com). Functional P[Sgf-fht, CG17292] transgenes (abbreviated as P[Sgf-fht] in the text) were selected based on their ability to complement a known point mutation in CG17292. Each transgenic line was then tested for its ability to rescue the different mutant phenotypes, including the lethality of pps /Df[3R]/E[1731] and pps / pps animals. The data presented in this paper are obtained with P[spfP1] line # 10. Additional marker mutations and balancers used in this study are described on Flybase (http://www.flybase.org).

Antibodies, GST-pull downs, co-immunoprecipitations, and western Blots

The antibody against PPS was raised in guinea pig by Covance (http://www.covance.com) against a glutathione S-transferase (GST) tagged C-terminal domain PPS fragment (amino acids 1370-2016) purified from bacteria. We note here that this PPS antibody has not proven to be useful for immunohistochemistry. The other antibodies used in this study include mouse anti-SNF-4G3 [43,44], guinea pig anti-U2AF [45], rabbit anti-U170K-151 [2], mouse anti-SXL-M114 [46], guinea pig anti-HSF [47], rabbit anti-PIC [48], and mouse anti-RNA Pol II (531). Crude extracts for GST-pull down experiments (Figure 1) and Western blots (Figure 4) were prepared from 3–18 hour old embryos, sexed and genotyped third instar larvae or sexed adults in NET buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA) supplemented with 0.05% NP-40 and Complete Mini Protease Inhibitor Cocktail Tablets (Roche). Nuclear extracts for co-immunoprecipitation experiments were prepared from 3–18 hour old embryos as described previously [49] using NET buffer supplemented with 0.05% NP-40 and 0.05% NP-40 for the co-IPs in Figure 4 and 0.05% NP-40 for the co-IPs in Figure 5. For experiments in which the extracts were pretreated with RNase, 1/10 volume of RNase A (10 mg/ml) and 1/20 volume of RNase T1 (100,000 units/ml) were added directly to the extract and incubated overnight at 4°C. Co-immunoprecipitations, Western blot analysis and GST pull down assays were carried out according to standard protocols, using the conditions described previously [2,4,50].

RT–PCR analysis

Total RNA was isolated from ovaries, adults or embryos using TRIzol (Invitrogen) as directed by the manufacturer. To analyze the endogenous Sxl splicing products, the first strand synthesis was carried out with 1 μg of RNA, 500 ng/ml random hexamers with the SuperScript II Reverse Transcriptase System (Invitrogen). The PCR reactions, using the High Fidelity Taq system (Roche), were performed in 50 μl volume with 2 μl of the RT reaction with the following primers: GTGGTTATCCCACATATGGC and GATGCAGAGTGGGAC. The PCR conditions were as follows: 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a single final step at 72°C extension for 10 min. Products were detected on a 2% agarose gel by staining with ethidium bromide.

RNA immunoprecipitation (RIP)

RNA/protein complexes were immunoprecipitated from nuclear extracts and diluted to 5 μg/μl in NET buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA), supplemented with 0.05% NP-40, Complete Mini Protease Inhibitor Cocktail Tablets (Roche) and RNase inhibitor (100 U/ml) using the conditions described previously [50]. RNA was isolated from the RNA/protein complexes using TRIzol (Invitrogen) as directed by the manufacturer. RNA was resuspended in 20 μl RNase-free water and DNase-treated. cDNA was synthesized with the SuperScript II Reverse Transcriptase System (Invitrogen) using 4 μl of the eluted RNA with a Sxl gene specific primer to exon 4 (GATGGCGAGAATGGGAC; Figure 6) or random hexamers (Figure 8). The PCR reactions, using the High Fidelity Taq system (Roche), were performed in 50 μl volume with 2 μl of the RT reaction with the following primers—Sxl: GAGGGCTACGTTAAGTTATATCCG and GATGGCGAGAATGGGAC; srf: GGGATGTGCGAATTAGTAG and GACTGGAGTTGCGTTCAC; tau: GATGGCAGACGATGGAAG and GGCTGGTCACACGTTCATT; U2A: GGTGAACACTACCGCCGAGC and CTAGCCTCTCGAGCTTGGTGTTG. PCR conditions were as follows: 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a single final step at 72°C extension for 10 min. 2 μl of the first-round PCR amplification was subjected to a second round of PCR. Products were detected on a 2% agarose gel by staining with ethidium bromide.

Chromatin immunoprecipitation (ChIP)

Live embryos were dechorionated with 50% bleach and fixed for 15 min in a 1.8% paraformaldehyde/heptane fixative solution. Chromatin was prepared from 1–2 gram of fixed 8–12 hour old embryos using the conditions described previously [51] and sonicated for a total of 80 seconds (20 sec pulses with a 1 min rest on ice) to produce sheared products of 300 to 400 bp. ChIP assays were performed with a commercially available ChIP assay kit (#17–295; Millipore). Antibodies used for the IP step were diluted 1:40 (Pol II, HSF, PC and P) and 1:20 (SNF and SXL). After purification, the ChIPed DNA samples were resuspended in 30 μl water. Enrichment of specific DNA fragments was analyzed by PCR on 2 μl ChIP material with the following primer sets: For Sxl-P1: CGGGGGCTCCTCAAAAGAATTAAAA and GCGTTGAATTACGCTCAC; P2: CGGGTACGAGAGGAAG and GGGTGGTGACAGTGTTTCACT; P3: CAGCGGAGGCTCGTGAAGAAGAAA and ACTGTCTCGTCTGGCGCAAGCA; E1: CAGGAGGTGGTTCGTTTCAGCA and TCGAGAGGAGATCGAT; Ex3: CGAAAGGCAAAGACGCTC.
and GTG CCCGCTCAAACAA; for In3: TGGTTCTG; and for In3 and U2:4 GGGGAGGGAGTTTCTTCTG and GATTC- TTTGACCGCGCAAA. For In3/TGCTCTC; for snf/AAN: CACCGGTGCAATAC and CGTTTGGTTGGGTAG- CATCT. The PCR conditions for Sxl primers P1, P2, P3, E1 and Ex3, for and snf were as follows: 94°C for 2 min, followed by 25 cycles of 94°C for 30 sec, 53°C for 30 sec, and 72°C for 1 min. The PCR conditions for In3 and U2: were as follows: 94°C for 2 min, followed by 25 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. Products were detected on a 3% agarose gel by staining with ethidium bromide.

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Author Contributions

Conceived and designed the experiments: MLJ AAN HKS. Performed the experiments: MLJ AAN HKS. Analyzed the data: MLJ AAN HKS. Wrote the paper: MLJ HKS.
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