The Translation Initiation Factor eIF4E Regulates the Sex-Specific Expression of the Master Switch Gene Sxl in Drosophila melanogaster

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Abstract

In female fruit flies, Sex-lethal (Sxl) turns off the X chromosome dosage compensation system by a mechanism involving a combination of alternative splicing and translational repression of the male specific lethal-2 (msl-2) mRNA. A genetic screen identified the translation initiation factor elf4e as a gene that acts together with Sxl to repress expression of the Msl-2 protein. However, elf4e is not required for Sxl mediated repression of msl-2 mRNA translation. Instead, elf4e functions as a co-factor in Sxl-dependent female-specific alternative splicing of msl-2 and also Sxl pre-mRNAs. elf4e shows maternal-effect female-lethal interactions with Sxl. This female lethality can be enhanced by mutations in other co-factors that promote female-specific splicing and is caused by a failure to properly activate the Sxl-positive autoregulatory feedback loop in early embryos. In this feedback loop Sxl proteins promote their own synthesis by directing the female-specific alternative splicing of Sxl-Pm pre-mRNAs. Analysis of pre-mRNA splicing when elf4e activity is compromised demonstrates that Sxl-dependent female-specific splicing of both Sxl-Pm and msl-2 pre-mRNAs requires elf4e activity. Consistent with a direct involvement in Sxl-dependent alternative splicing, elf4E is associated with unspliced Sxl-Pm pre-mRNAs and is found in complexes that contain early acting splicing factors—the U1/U2 snRNP protein Sans-fils (Snf), the U1 snRNP protein U1-70k, U2AF38, U2AF50, and the Wilms’ Tumor 1 Associated Protein Fl(2)d—that have been directly implicated in Sxl splicing regulation.

Introduction

Translation initiation is mediated by the binding of a pre-initiation complex to the 5’ cap of the mRNA (reviewed in [1,2]) that in turn recruits the small subunit of the 40S ribosome to the mRNA. The pre-initiation complex consists of the cap binding protein, elf4E, and a scaffolding protein, elf4G, which mediates interactions with various components of the 40S initiation complex. In many organisms there is also a third protein in the complex, elf4A, an ATP dependent RNA helicase. Modulating elf4E activity appears to be a key control point for regulating translation. One of the most common mechanisms of regulation is by controlling the association elf4E with elf4G. Factors such as poly-A binding protein that promote the association between elf4E and elf4G activate translation initiation, while factors such as the 4E-binding proteins (4E-BPs) that block their association, inhibit initiation [3,4].

Although elf4E’s primary function in the cell is in regulating translation initiation, studies over the past decade have revealed unexpected activities for elf4E at steps prior to translation. Among the more surprising findings is that there are substantial amounts of elf4E in eukaryotic nuclei [5–9]. One role for elf4E in the nucleus is the transport of specific mRNAs, like cyclin D1, to the cytoplasm [10]. This elf4E activity is distinct from translation initiation since an elf4E mutation that prevents it from forming an active translation complex still allows cyclin D1 mRNA transport [8]. The transport function of elf4E is modulated by at least two other proteins, PML and PRH [11,12]. While PML seems to be ubiquitously expressed, PRH is found only in specific tissues [13]. In addition, the intracellular distribution of elf4E exhibits dynamic changes during Xenopus development [9]. These observation raise the possibility that elf4E might have additional functions in the nucleus during development. Consistent with this idea, we show here that elf4E plays a novel role in the process of sex determination in Drosophila melanogaster.

Sex determination in the fly is controlled by the master regulatory switch gene Sex-lethal (Sxl) (reviewed in [14–16]). The activity state of the Sxl gene is selected early in development by an X chromosome counting system. The target for the X/A signaling system is the Sxl establishment promoter, Sxl-Pe [17]. When there are two X chromosomes, Sxl-Pe is turned on, while it remains off when there is a single X chromosome. Sxl-Pe mRNAs encode
RRM type RNA binding proteins which mediate the transition from the initiation to the maintenance mode of Sxl regulation by directing the female-specific splicing of the first pre-mRNAs produced from a second, upstream promoter, the maintenance promoter, Sxl-Pm [18,19]. Sxl-Pm is turned on before the blastoderm cellularizes, just as Sxl-Pe is being shut off. In the presence of Sxl-Pe proteins, the first Sxl-Pm transcripts are spliced in the female-specific pattern in which exon 2 is joined to exon 4 (see Figure 1A). The resulting Sxl-Pm mRNAs encode Sxl proteins that direct the female specific splicing of new Sxl-Pm pre-mRNAs, and this establishes a positive autoregulatory feedback loop that maintains the Sxl gene in the “on” state for the remainder of development. In male embryos, which lack the Sxl-Pe proteins, the Sxl-Pm pre-mRNAs are spliced in the default pattern, incorporating the male specific exon 3 (Figure 1A). This exon has several in-frame stop codons that prematurely truncate the open reading frame so that male specific Sxl-Pm mRNAs produce only small non-functional polypeptides. As a consequence the Sxl gene remains off throughout development in males.

In females, Sxl orchestrates sexual development by regulating the alternative splicing of transformer (tra) pre-mRNAs [20–23]. Like Sxl, functional Tra protein is only produced by female-specific tra.
mRNAs, while mRNAs spliced in the default, male pattern encode non-functional polypeptides. Sxl also negatively regulates the dosage compensation system, which is responsible for hyperactivating X-linked transcription in males, by repressing male-specific lethal-2 (msl-2). Sxl represses msl-2 by first blocking the splicing of an intron in the 5' UTR of the msl-2 pre-mRNA (see Figure 1B), and then by inhibiting the translation of the mature mRNA [24–31]. In addition, there are two other known targets for Sxl translational repression. One is the Sxl mRNA itself. Sxl binds to target sequences in the Sxl 5' and 3' UTRs and downregulates translation. It is thought that this negative autoregulatory activity provides a critical homeostasis mechanism that prevents the accumulation of excess Sxl protein. This is important as too much Sxl can disrupt development and have female lethal effects [32]. The other known target is the Notch (N) mRNA [33]. Sxl-dependent repression of N mRNA translation is important for the elaboration of sexually dimorphic traits in females. Like msl-2 and Sxl, translational repression appears to be mediated by Sxl binding to sites in the N UTRs.

Translational repression of msl-2 mRNA by Sxl is thought to involve two separate mechanisms acting coordinately. Binding sites for Sxl in the unspliced intron in the 5' UTR and in the 3' UTR of msl-2 are required for complete repression [25,26]. Sxl binding to the 5' UTR blocks recruitment of the 40S pre-initiation complex [31,34]. While factors that act with Sxl at the 5' UTR of msl-2 have yet to be identified, repression by the 3' UTR requires Sxl, PABP and a co-repressor UNR [35–37]. Somewhat unexpectedly, this complex does not affect recruitment of eIF4E or eIF4G to the 5' end. Instead it prevents ribosomes that do manage to attach to the msl-2 mRNA from scanning [31,38].

Although eIF4E does not appear to be a key player in the translational repression of msl-2 mRNAs, we report here that it has an important role in the process of sex determination in Drosophila. We find that eIF4E activity is required in females to stably activate and maintain the Sxl positive autoregulatory feedback loop and to efficiently repress msl-2. Surprisingly, this requirement for eIF4E activity in fly sex determination is in promoting the female-specific splicing of the Sxl and msl-2 transcripts, not in translational regulation.

**Results**

**Mutations in elf4e rescue males expressing a Sxl transgene**

In previous studies we examined the biological properties of a truncated Sxl protein, Sx-N, that contains both RRM RNA binding domains, but is missing 40 amino acids from the N-terminus [39]. We found that the splicing activity of Sx-N is impaired; it can not direct the female-specific splicing of tra and has substantially reduced autoregulatory activity. However, the truncated protein is able to inhibit the translation of msl-2 mRNA and kills males even in the absence of a wild type Sxl gene. As would be expected if the male lethal effects of Sx-N are due to repression of msl-2 mRNA translation, hsp83:Sx-N;M males can be fully rescued by an hsp83:msl-2 transgene that lacks the Sxl binding sites in the 5' and 3' UTRs.

With the aim of discovering factors important for Sxl dependent repression of msl-2 we screened for deletions that dominantly suppress the male lethal effects (in a Sxl background) of a transgene, hsp83:Sx-N;M, that constitutively expresses the truncated Sx-N protein. We then identified the interacting locus by testing mutations mapping to the suppressing deletion. We anticipated that genes recovered in this screen would fall into two general classes. In the first would be genes required for efficient expression of Sx-N by the transgene. Consistent with this expectation, one of the suppressing mutations was the heat shock factor, hsf. Genes in the second class would be required for efficient repression of msl-2 by the truncated Sx-N protein. In this group we expected to find factors required by Sxl to inhibit msl-2 translation; however, since the Sxl binding sites in the msl-2 5' UTR intron are needed to completely repress translation, we anticipated that we might also recover genes that collaborate with Sxl to block the removal of this intron [25,26,29,31].

One of the candidate translation factors recovered in the screen was the elf4e gene, which encodes the cap binding protein. Three independent alleles of elf4e were tested. In an otherwise wild type background less than one in $10^5$ Sxl males carrying the hsp83:Sx-N;M transgene survive. By contrast, when the hsp83:Sx-N;M;Sxl males were also heterozygous for an elf4e mutation, between 2% and 9% of the transgenic males survived depending upon the allele.

**elf4e mutations do not impair the negative autoregulatory activity of the Sx-N protein**

Since Sxl-dependent repression of msl-2 translation *in vitro* is independent of the cap and does not seem to be mediated through interactions with eIF4E [34,38], it was surprising that elf4e was recovered in our screen. However, it seemed possible that an *in vivo* requirement for elf4e activity might be bypassed in *in vitro* translation systems. In this case, the levels of Msl-2 should increase in hsp83:Sx-N;M transgene males when they are heterozygous for one of the elf4e mutations. However, testing whether elf4e mutations perturb Sx-N dependent translational repression of msl-2 mRNA in adults or at earlier stages of development is complicated by the male-lethal effects of the truncated Sxl protein.

To circumvent this complication, we tested the effects elf4e on Sxl negative autoregulation as this can be done in females where Sx-N doesn’t have such deleterious consequences. The endogenous Sxl-Pm mRNAs have one Sxl binding site in the 5' UTR, while there can be eight or more in the 3' UTR. Sxl binds to these sites and downregulates translation. Though the truncated Sx-N protein can also repress translation of Sxl-Pm mRNAs, its inhibitory effects are somewhat weaker than the full-length protein [39]. However, it is possible to detect Sx-N repression of endogenous Sxl mRNAs using the hsp83:Sx-N;M transgene. This transgene expresses Sx-N mRNAs that lack the 5' Sxl binding site and most of the 3' UTR binding sites, and as a consequence are less sensitive to repression than the endogenous mRNAs [39]. For this reason, Sx-N protein produced by the transgene preferentially represses translation of the endogenous mRNAs and in hsp83:Sx-N;M transgenic females the amount of Sx-N is typically greater than the two major endogenous Sxl proteins.

We compared the repression of the endogenous Sxl in hsp83:Sx-N;M transgenic females either wild type or heterozygous for elf4e. Figure 1C shows that in transgenic, wild type females the level of endogenous Sxl is less than Sx-N. Consistent with the results of the *in vitro* translation experiments, reducing elf4e activity does not have an obvious effect on repression of Sxl-Pm mRNAs by Sx-N and the ratio of the endogenous protein to Sx-N in elf4e/+ females remains similar to that in wild type females. With the caveat that Sxl may require a different set of accessory proteins to repress the translation of each of its target mRNAs, this finding does not support the idea that eIF4E functions as a co-factor in Sxl inhibition of msl-2 translation *in vivo*.

**msl-2 mRNA splicing in elf4e/+ hsp83:Sx-N;M transgene males**

The alternative possibility is that elf4e rescues the male lethal effects of Sx-N because Sxl requires elf4e activity to effectively
prevent the splicing of the intron in the 5' UTR of msl-2 pre-RNA. To test this idea, we examined the splicing pattern of msl-2 mRNA in three surviving Sxl eif4e+/w; hsp83:Sx-NΔ males. In wild type females, Sxl efficiently blocks the splicing of the msl-2 5' UTR intron and in most female mRNAs the intron is unspliced. In wild type males the 5' intron is spliced out of most msl-2 mRNAs. As expected, we found that ectopically expressed Sx-N protein blocks the splicing of the 5' intron and as shown for one of the surviving Sxl- eif4e+/w; hsp83:Sx-NΔ males in Figure S1, msl-2 mRNA spliced in the female pattern is readily detected. However, we found that Sx-N wasn’t able to fully inhibit the splicing of the 5' intron, and roughly similar quantities of male spliced msl-2 mRNAs were also found in both of the other Sxl- eif4e+/w; hsp83:Sx-NΔ males. Since the Sxl binding sites in the 5' UTR are essential for efficient translational repression, Sx-N would not be able to completely block the translation of these male spliced msl-2 mRNAs.

*eif4e* is required for the stable activation of the Sxl positive autoregulatory feedback loop in early embryos

Though the results described in the previous section could explain why a small percentage of eif4e/+ males escape the lethal effects of Sx-NΔ, it is not possible to determine if the relative amount of male spliced msl-2 mRNA is increased compared to eif4e- males because the controls don’t survive. However, as it seemed possible that the effects of eif4e on Sxl dependent splicing might not be limited to msl-2, we took advantage of a simple genetic test for genes involved in Sxl positive autoregulation. The initial activation of the positive Sxl autoregulatory loop in female embryos is sensitive to alterations in the dose of gene products that play a critical role in promoting the female specific splicing of Sxl-Pm pre-mRNAs. Because of this sensitivity, mutations in splicing factors like the U1A/U2B* snRNP protein Snf often show effects of eif4e is needed to help repress the translation of Sxl target mRNAs, then reducing eif4e activity should increase the translation of Sxl isoforms and would be expected to suppress rather than enhance any female specific lethality. The results in Table 1 show that eif4e homozygous females with the known predictions. All three of the eif4e alleles we tested, eif4e560, eif4e587/11, and eif4e715, showed dominant female lethal interactions with the null mutation SxlΔ (Table 1) [47]. These eif4e alleles are P-element insertions and are thought to be hypomorphic mutations [48–49]. The weakest allele, eif4e560, reduces female viability by a quarter, while female viability is reduced by a third to nearly a half for the two stronger alleles eif4e587/11 and eif4e715. Although the reductions in female viability seen for the three eif4e mutations are not as great as that observed for the msl-2 null allele J210 or the dominant negative allele 1621, they are roughly equivalent to that seen for the hypomorphic allele J42 (Table 1).

In the experiments described above the eif4e/+ females were crossed to SxlΔ males giving two classes of SxlΔ progeny, those carrying the eif4e mutation and those with the wild type chromosome. We noticed that the viability of both classes of SxlΔ progeny were affected equally (data not shown) suggesting that the lethality is predominantly the result of a lowered maternal contribution of eIF4E rather than a reduction in zygotic eIF4E. Consistent with this conclusion, when we did the reciprocal cross in which the eif4e mutation was introduced from the father and the Sxl mutation introduced from the mother, we found that the viability of Sxl-/+ females was close to that of wild type females (not shown).

To confirm that the female lethal interactions are due to a reduction in eif4e activity, we tested whether they can be rescued by an eif4e transgene. Two isoforms of eIF4E are expressed Drosophila. We introduced transgenes expressing each isoform into eif4e715/+ females and mated them to SxlΔ males. We found that both could suppress the maternal effect lethal interactions between eIF4E and Sxl (data not shown). We also tested a second independent Sxl allele, Sxl7BO [50]. Like SxlΔ, Sxl7BO exhibited dominant female lethal interactions with eif4e (Table 1).

*eif4e* mutations do not show dominant female lethal interactions with a mutation, SxlΔ, that only eliminates Sxl-Pe activity

The null mutations SxlΔ and Sxl7BO discussed above eliminate both early Sxl initiation functions provided by Sxl-Pe mRNAs and late Sxl sex determination functions (maintenance, sexual differentiation, and dosage compensation) provided by the Sxl-Pm mRNAs [47,50]. While there are no known mutations that specifically eliminate only the late Sxl functions, the SxlΔ mutation disrupts the initiation function of the Sxl-Pe transcripts [51–52]. If the reduction in eif4e activity impairs the female-specific splicing of Sxl-Pm pre-mRNAs, then eif4e mutations should have a smaller

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**Table 1. eif4e and snf interactions with Sxl.**

| Maternal Genotype | Female Viability x Sxl/
|-------------------|--------------------------|
| snf587/11/w       | 75                       |
| snf587/11/w       | 66                       |
| snf587/11/w       | 54                       |
| snf587/11/w       | 10                       |
| snf587/11/w       | 2                        |
| snf587/11/w       | 14                       |
| snf587/11/w       | 14                       |
| snf587/11/w       | 34                       |
| snf587/11/w       | 0.3                      |
| snf587/11/w       | 99                       |
| snf587/11/w       | 84                       |

Females heterozygous for the indicated mutation(s) were crossed to SxlΔ, Sxl7BO or SxlΔ males at 29°C. Female viability was calculated as ((# females)/(# males))/100. Except w, a minimum of 700 progeny were scored for each cross. doi:10.1371/journal.pgen.1002185.t001
effect on the viability of flies carrying a Sxl mutation that only affects the Sxl-Pe pre-mRNAs as these transcripts do not require Sxl for proper splicing [53–54]. As can be seen in Table 1, Sxl(f9) differs from Sxl(f1) and Sxl(7B0) in that it shows only a weak female lethal interaction with eif4e mutations. It also interacts much less strongly with snf(1621) than either of the Sxl null alleles (data not shown).

Sxl protein expression is disrupted in progeny of snf and eif4e mothers

The female lethal interactions between Sxl and co-factors like snf that are critical for the female splicing of Sxl-Pm pre-mRNAs arise because the positive autoregulatory feedback loop is not properly set in motion [43–45]. However, there are no special requirements for these co-factors in the activation of Sxl-Pm by the X chromosome counting system or the splicing and translation of Sxl-Pe transcripts [53–54]. For these reasons, defects in Sxl accumulation are not observed in blastoderm stage embryos compromised for a sex-specific splicing co-factor. However, later in development, when protein expression depends upon female spliced Sxl-Pm mRNAs, the pattern of Sxl accumulation becomes abnormal. To determine if this is true for eif4e as well, we examined the expression of Sxl in blastoderm and post-blastoderm stage embryos.

Consistent with the idea that eif4e functions downstream of Sxl-Pe, eif4e mutations have no apparent effect on the expression of Sxl from the Sxl-Pe mRNAs. As shown in Figure 2 and Table S1, blastoderm stage progeny from eif4e/+/ and snf/+/ mothers crossed to Sxl(f1) fathers resemble wild type in that about 50% of the embryos (females) express Sxl protein (compare panels A & B with C & D). While reducing eif4e activity does not perturb activation of Sxl by the X chromosome counting system, it does have a significant effect on the expression of Sxl in older embryos. In the wild type controls (either w x w or w x Sxl(f1)), high uniform levels of Sxl protein are observed in about 50% of the embryos, while a equal number show no staining (panels E & F). For the dominant negative snf(1621) allele only 11% of the embryos show the expected high uniform level of Sxl while Sxl expression in the remaining female embryos is either irregular or quite low (Table S1). As would be expected from the relative severity of the synthetic lethal interactions, the effects of the hypomorphic eif4e alleles on Sxl expression in post-cellular blastoderm embryos are not as strong as snf(1621). For both eif4e(587/11) and eif4e(713) about one third of the embryos (or about two thirds of the females) show a high uniform level of Sxl accumulation (Table S1). The remaining female embryos show either a patchy pattern of Sxl protein accumulation or only low levels of protein (Figure 2G and 2H). These defects in Sxl expression in post-blastoderm embryos

Figure 2. eif4e mutations alter expression of Sxl from the late, but not the early, promoter. Embryos from wild type (A, B, E, F) and eif4e/+(C, D, G, H) mothers crossed to Sxl(f1)/Y fathers were stained with antibody to Sxl. Male embryos from either cross do not express Sxl protein (A, C, E). Female embryos from wild type mothers express Sxl evenly throughout the embryo both early (B) and late (F). Female embryos from eif4e/++ mothers express Sxl normally early (D), but often display patchy expression late (G,H). doi:10.1371/journal.pgen.1002185.g002
indicate that the Sxl autoregulatory feedback loop is not properly established in the female progeny of eif4e−/+ mothers.

The constitutively active SxlM mutations suppress the dominant female lethal interactions between eif4e and Sxl

To confirm that the female lethal effects of eif4e are due to a failure to activate the Sxl positive autoregulatory loop we tested whether Sxl+/+ female progeny of eif4e−/+ mothers can be rescued by three different gain-of-function Sxl alleles, SxlM1, SxlM4, and SxlM6, that constitutively splice Sxl-Pm transcripts in the female mode [55]. As a positive control we generated an equivalent combination of SxlM1 and snf1621. Females trans-heterozygous for each combination were mated with Sxl+/ males. As can be seen in Table S2 for the positive control, SxlM1 suppresses the maternal effect female lethal interactions between snf and Sxl+. Similarly, SxlM4 and both of the other gain-of-function alleles also suppress the maternal effect lethal interactions between eif4e+/+ and Sxl+. In these crosses only half of the female progeny inherit the Sxl gain-of-function allele. As expected, most of the surviving females are the ones that carry the gain-of-function allele.

Female embryos from eif4e−/+ mothers produce male Sxl transcripts

If the positive autoregulatory loop is not properly activated when eif4e is compromised, we would expect to find male spliced Sxl transcripts in female blastoderm/early gastrula embryos. To examine the splicing pattern of Sxl-Pm transcripts specifically in female embryos during this period we took advantage of an X-linked Sxl splicing reporter. The splicing reporter has a Sxl genomic fragment extending across the regulated splice sites from exon 2 to exon 4 while exon 4 is fused to β-galactosidase sequences (see Figure 3A: [56]). Expression of the fusion gene is driven by the hsp83 promoter. This promoter is activated in the zygote during the late syncytial blastoderm stage around the time when Sxl-Pm transcription commences [57]. Figure 3B shows that the transcripts spanning the regulated exon2-exon3-exon4 splicing cassette are spliced in the appropriate sex-specific pattern in control adult flies collected from a stock homozygous for the transgene: exon 2–4 in females and exons 2–3–4 in male.

Sxl+/ or Sxl− males carrying the splicing reporter were crossed to eif4e+ or control wild type females. To visualize the splicing of the regulated exon2-exon3-exon4 cassette when the autoregulatory feedback loop is first activated, we isolated RNA from 1–3 hr embryos and analyzed the structure of the transcripts expressed from the reporter by RT-PCR. When the mother is wild type we find that transcripts spanning the exon2-exon3-exon4 cassette are spliced exclusively in the female pattern (Figure 3B). This is true not only for female embryos that have two wild type copies of Sxl (fathers are Sxl+/Y), but also for female embryos that are heterozygous for the SxlM mutation (fathers are SxlM+/Y). A different result is obtained when the mother is heterozygous for eif4e+ (Figure 3B). In this case, we detect not only female but also male spliced reporter RNAs. With this allele, male spliced RNAs are observed in both Sxl+/+ embryos and in embryos that are wild type for Sxl. Similar results were obtained for snf1621 (not shown). We also observed male spliced reporter RNAs in the female progeny of mothers heterozygous for two other eif4e alleles. However, for both of these eif4e alleles the male transcripts were only present when the female embryos were heterozygous for the Sxl mutation (not shown).

Does eIF4E function in Sxl-dependent splicing regulation?

Two general mechanisms, one direct and the other indirect, could potentially account for the effects of eif4e on Sxl activation. In the direct mechanism, eif4e would function as a Sxl co-factor in the female specific processing of Sxl-Pm pre-mRNAs. In this case,
reducing eif4e activity would compromise the female specific splicing of Sxl-Pm pre-mRNAs and prevent full activation of the positive autoregulatory feedback loop when the loop is first being initiated. In the second, eif4e would be required at a point subsequent to the splicing of the Sxl-Pm pre-mRNAs. For example, it may be needed in the cytoplasm for the efficient translation of Sxl-Pm mRNAs, or it might function in their nuclear export. In this scenario, the expression of Sxl proteins from the newly synthesized Sxl-Pm mRNAs would be impaired and sub-optimal levels of Sxl-Pm proteins would be produced. As a consequence, when the Sxl-Pm proteins decay, there would be an insufficient amount of Sxl remaining to stably maintain the positive autoregulatory feedback loop, and splicing would gradually switch from the female to the male pattern. Though our experiments with the splicing reporter suggest an immediate rather than a gradual effect on splicing of the Sxl-Pm transcripts, we cannot rule out the possibility that there is some disruption in the export or translation of Sxl-Pm mRNAs during the initial activation of the positive autoregulatory feedback loop. Moreover, consistent with the possible importance of post-splicing steps in Sxl activation, Sitzinger et al [58] found female lethal interactions with Sxl when mothers are simultaneously heterozygous for mutations in aspartyl tRNA synthetase and sfl. Although the aspartyl tRNA synthetase mutants differ from eif4e in that they do not show female lethal interactions with Sxl on their own, the fact that reductions in the maternal dose of this synthetase can affect the activation of the autoregulatory loop lends credence to a post-splicing function. For these reasons we sought experimental paradigms in which we could assay for eif4e induced perturbations in Sxl dependent female-specific splicing under conditions in which the autoregulatory loop had already been “fully” activated and Sxl proteins were present at wild type levels.

Effects of eif4e mutations on Sxl pre-mRNA splicing in a sensitized background

In previous studies on snf we found that though there is substantial female lethality when snf421/++ mothers are mated to Sxl− fathers, the surviving snf421/Sxl− trans-heterozygous females are morphologically normal, fertile, and express wild type levels of Sxl protein. When we examined the splicing of the Sxl-Pm mRNAs in these surviving females using RT-PCR primer sets that give products spanning the regulated exon2-exon3-exon4 cassette, we found that unlike wild type females (which give only female spliced mRNAs), we observed male spliced Sxl-Pm mRNAs. This allows us to directly compare the relative ratio of female to male spliced mRNAs in each genetic background. Figure 4B shows that the very modest defects in the female specific splicing of Sxl-Pm pre-mRNAs evident in snf421Sxl1/++ females are clearly exacerbated when eif4e activity is reduced. For both eif4e alleles there is a marked increase in the amount of male-spliced Sxl-Pm mRNAs compared to the snf421Sxl1/++ control.

Effects of eif4e mutations on msl-2 pre-mRNA splicing in a sensitized background

We used this same sensitized background to examine the effects of reducing eif4e activity on the splicing of the intron in the 5′ UTR of msl-2 mRNAs. As illustrated in Figure 4C, Sxl blocks the splicing of the 5′ UTR intron so that it is retained in most msl-2 mRNAs in females, while this intron is spliced out efficiently in males. In control snf421Sxl1/++ females the female-specific splicing of the msl-2 mRNA is partially compromised and, we observe a nearly equal mixture of female and male spliced transcripts. As observed for Sxl-Pm splicing, reducing eif4e activity in this sensitized background further disrupts the female specific splicing of msl-2 mRNAs. In addition to demonstrating a role for eif4e in the splicing of a second Sxl target pre-mRNA, these findings provide additional evidence that the male lethal effects of the khp33;Sx-NA transgene are suppressed because eif4e mutations perturb the female specific splicing of msl-2 mRNAs.

Male spliced Sxl mRNAs are also observed in eif4e/+ females

The results in the previous sections demonstrate that the modest defects in Sxl and msl-2 pre-mRNA splicing evident in a sensitized snf421Sxl1/++ background are significantly enhanced by reducing eif4e activity. We wondered whether splicing defects are also observed in eif4e/+ females that are wild type for both snf and Sxl. To test this possibility, we examined the splicing of transcripts from the endogenous Sxl gene and the Sxl splicing reporter in females heterozygous for two different eif4e alleles. When we used primers that allow us to visualize simultaneously both the male and female spliced Sxl mRNAs from either the endogenous gene (Figure 4D) or from the splicing reporter (not shown), only female spliced Sxl mRNAs were observed in wild type females. In contrast, a very small amount of Sxl mRNA was detectable from the endogenous gene (Figure 4D) and also from the splicing reporter (not shown) in females heterozygous for eif4e568 or eif4e587. To confirm that male spliced Sxl mRNAs from the endogenous gene are present in these eif4e/+ females we used RT primers from exon 5 and then PCR amplified using a primer from the male exon and a primer from exon 4. Figure 4E shows that male spliced Sxl mRNAs from the endogenous gene are readily evident in both eif4e568/++ and eif4e587/++ females, but not in wild type. Figure 4F shows that male spliced Sxl mRNAs from the reporter are also present in these eif4e heterozygous females, while there is little male spliced splicing reporter mRNAs in control wild type females.

Mutations in eif4E do not affect the alternative splicing of ddx mRNA

To determine whether the effects of eif4e on sex-specific splicing are general or only restricted to Sxl dependent
Alternative splicing was examined by sequencing the mRNA of doublesex (dsx) mRNAs. The dsx gene is downstream of Sxl and like Sxl its transcripts are sex-specifically spliced. However, female-specific splicing of dsx mRNA is dependent upon tra and tra-2, not Sxl (reviewed in [14–16]). We used primer sets that would RT-PCR amplify either female or male spliced dsx mRNAs isolated from either wild type or eif4e+/+ females. As expected, wild type females produce only female, not male products (Figure S2). Significantly, females heterozygous for eif4e also produce only female dsx mRNAs.

Could eIF4E play a direct role in Sxl-dependent alternative splicing?

The results described in the previous sections show that eIF4E is required for Sxl splicing. Since eIF4E is known to function in translation initiation, it might be needed for the synthesis of some limiting Sxl co-factor. In this scenario, the amount of this splicing co-factor would drop below some critical threshold when eIF4E activity is reduced, and this would impair the ability of Sxl to regulate splicing. Alternatively, eIF4E itself could be the Sxl splicing co-factor. This latter model makes several predictions that we have tested below.

**eif4e mutations enhance the female specific lethality of dominant negative snf1621 and fl(2)C14.** If eIF4E functions in Sxl-dependent alternative splicing, we might expect genetic interactions between eIF4E and genes like snf that are required for female specific splicing of Sxl pre-mRNAs. To test this possibility females trans-heterozygous for different eIF4E alleles and snf were mated to Sxl+/+ or Sxl males. When combined with the Sxl allele, the weaker eIF4E allele reduces the viability of female progeny of snf mothers two-fold, while the stronger eIF4E allele reduces female viability ten-fold (see Table 1). An equivalent synergistic maternal effect female lethality is observed in progeny of snf mothers mated to fathers carrying the deletion allele Sxl. We also observed weak, female lethal interactions when eIF4E was combined with a mutation in another Sxl splicing co-factor fl(2)C14, which encodes the fly Wilm’s Tumor 1 Associated Protein (WTAP) [31,59–61].

**eIF4E is localized in the nucleus of somatic cells but not germ cells.** A splicing function requires that some eIF4E co-factor. In this scenario, the amount of this splicing co-factor would drop below some critical threshold when eIF4E activity is reduced, and this would impair the ability of Sxl to regulate splicing. Alternatively, eIF4E itself could be the Sxl splicing co-factor. This latter model makes several predictions that we have tested below.

**eIF4E regulates Sxl.** Endogenous Sxl expression in male embryos is transcriptionally silent. We observed weak, female lethal interactions when eIF4E was combined with a mutation in another Sxl splicing co-factor fl(2)C14, which encodes the fly Wilm’s Tumor 1 Associated Protein (WTAP) [31,59–61].

**eIF4E is localized in the nucleus of somatic cells but not germ cells.** A splicing function requires that some eIF4E protein be present in the nucleus. To test this we probed late pre-cellular and cellular blastoderm embryos with antibodies against eIF4E and the germ line marker Vasa. This is the stage in development when the first Sxl transcripts are expressed and the positive autoregulatory feedback loop must be set in motion in females [62]. There are also marked differences in RNA polymerase activity between the soma and germline. In the soma, transcription is substantially upregulated following the midblastula transition. By contrast, newly formed germ cells are transcriptionally quiescent and genes specifying somatic development, including Sxl, are off. Figure 5 shows that as expected for a translation factor, most of the eIF4E in soma is not altogether surprising as females can tolerate an hyp-83 transgene that expresses an msl-2 mRNA lacking not only the 5’ but also the 3’ Sxl binding sites (26). Panels D, E and F show that male spliced Sxl and Sxl reporter mRNAs are present in female heterozygous for two different hypomorphic eIF4E alleles while they absent in wild type females (WT-F). In D, primers in exon2 and exon4 that amplify both male and female spliced mRNAs were used for the PCR. In E we used primers in exon3 and exon4 that amplify male spliced Sxl mRNAs. For the splicing reporter in F, we did two PCR reactions using nested primers in LacZ. eIF4E alleles: 4e-87: eIF4E; 4e-87: eIF4E.

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**Figure 4.** eIF4E mutations shift Sxl regulated splicing toward male mode although Sxl protein levels are normal. A) Western blot of control snf homozygous females and of snf homozygous males when assayed with primers that amplify only the male transcript (E,F) or primers that amplify both the male and female mRNAs (D,F). D) Wild-type females (WT-F) express no male Sxl mRNA. Females heterozygous for mutations in snf and Sxl express a small amount of male Sxl mRNA (B). E) Wild-type females (+) express significantly more male Sxl mRNA. Similarly, addition of an eIF4E mutation increases the amount of male (intron removed) msl-2 mRNA (C). Though all of the msl-2 mRNA in these triply heterozygous females appears to be spliced in the male pattern, there is not an obvious effect on their viability. This is likely not altogether surprising as females can tolerate an hyp-83 transgene that expresses an msl-2 mRNA lacking not only the 5’ but also the 3’ Sxl binding sites (26). B) D) Primers in exon2 and exon4 that amplify both male and female spliced mRNAs were used for the PCR. In E we used primers in exon3 and exon4 that amplify male spliced Sxl mRNAs. For the splicing reporter in F, we did two PCR reactions using nested primers in LacZ. eIF4E alleles: 4e-87: eIF4E; 4e-87: eIF4E.

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localized in the cytoplasm. However, as has been reported for Drosophila S2 tissue culture cells [8], there is a small but readily detectable amount of eIF4E in somatic nuclei. Interestingly, the transcriptionally quiescent germ cells differ from the somatic cells in that eIF4E is exclusively cytoplasmic and is not observed in their nuclei.

eIF4E is bound to Sxl pre-mRNAs. To function in Sxl dependent alternative splicing, eIF4E has to be bound to incompletely spliced Sxl transcripts. We first tested for the binding of Sxl and eIF4E to nuclear Sxl RNAs that had undergone the first splice of exon 1 to exon 2. As shown in the top panel of Figure 6, exon 1–2 spliced Sxl RNAs are found associated with both Sxl and eIF4E in nuclear extracts. Since splicing of the regulated sex-specific exons in the Sxl-Pm pre-mRNA is known to occur more slowly than the splicing of the non-regulated exons in the transcript [63], we next assayed the immunoprecipitates for Sxl-Pm RNAs in which exon1 has been spliced to exon2, but the Sxl regulated splice between exon2 and either exon3 or exon4 has not yet occurred (see 2nd panel in Figure 6). Consistent with previous studies which have shown that Sxl binds to partially spliced RNAs [43], exon1-exon2-intron2 Sxl-Pm RNAs are found in Sxl immunoprecipitates. Consistent with a function in the sex-specific splicing of Sxl-Pm pre-mRNAs, exon1-exon2-intron2 Sxl-Pm RNAs are also found in eIF4E immunoprecipitates, but not in control Scute immunoprecipitates. To exclude the possibility that Sxl and eIF4E associate non-specifically with any pre-mRNA in nuclear extracts, we assayed for the presence of incompletely processed tango transcripts; however, unspliced tango RNAs were not detected in either Sxl or eIF4E immunoprecipitates (data not shown). Since we were able to detect tango pre-mRNAs in U2UF50

Figure 5. Some eIF4E protein is located in the nucleus. Wild type pre-cellular and cellular blastoderm stage embryos were stained with eIF4E (green) or Vasa (red) antibodies and hoechst (blue) to label the DNA. The embryo shown here is a late pre-cellular blastoderm embryo. A, D: All three channels. B, E: eIF4E only. C, F: Vasa only. Note the high levels of cytoplasmic eIF4E in the soma and in the Vasa positive germline pole cells. eIF4E can also be readily detected in the somatic nuclei, though the levels are less than in the somatic cytoplasm (see panel E). By contrast, there is only little eIF4E in the pole cell nuclei (Vasa plus cells at posterior in panel B).

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Figure 6. eIF4E co-immunoprecipitates with Sxl pre-mRNAs. Nuclear extracts were incubated with antibodies to Scute (Sc), Sxl or eIF4E (4E). RNA was isolated from the immunoprecipitates and used for RT-PCR reactions. Top: Diagram of the 5' region of the Sxl-Pm transcription unit showing the exon-intron structure and the position of primers used for PCR. Bottom: Southern blots of RT-PCR products that are amplified from the immunoprecipitates using the indicated primers. Next to the blots is a diagram of the amplification product. Antibodies to eIF4E and Sxl immunoprecipitate both spliced and partially spliced Sxl-Pm mRNAs. Antibodies to Sc do not immunoprecipitate any Sxl-Pm mRNAs.

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immunoprecipitates, it would appear that eIF4E does not bind to all pre-mRNAs.

**eIF4E is associated with Sxl and Snf in nuclear extracts.** If eIF4E participates in Sxl dependent splicing regulation, it should be associated not only with Sxl but also with the U1/U2 snRNP protein that has been implicated in Sxl splicing regulation. As can be seen in Figure 7A, eIF4E is present in Sxl, but not control immunoprecipitates of nuclear extracts. Similarly, a small but readily detectable amount of eIF4E is found in the Snf immunoprecipitates (Figure 7B). Though recombinant Sxl and Snf are able to interact directly with each other in vitro, the complex between these two proteins in nuclear extracts are disrupted by RNase digestion [43]. Figure 7A and 7B show that like nuclear Sxl:Snf complexes, both the eIF4E:Sxl and eIF4E:Snf complexes are also RNase sensitive.

eIF4E is associated with splicing factors that function in the assembly of the spliceosome complexes E and A.** We [43,61] and Nagengast et al [45] have presented genetic and biochemical evidence that Sxl autoregulation depends upon interactions between Sxl and components of the splicing machinery that are involved in the initial assembly of the U1 snRNP on the 5’ splice sites of the Sxl-Pre mRNAs and the U2

![Figure 7](image_url)

**Figure 7. eIF4E co-immunoprecipitates with several splicing factors.** Western blots of immunoprecipitates isolated using antibodies to splicing factors (Sxl, Snf, U1-70K, U2AF50, U2AF38, and Fl(2)d) or negative controls (β-Galactosidase or Scute (Sc)) were probed with antibodies against eIF4E. Nuclear extract (lane 1 all blots) contains substantial amounts of eIF4E. Two isoforms are usually observed in nuclear extracts; however, the lower isoform is often obscured by the immunoglobulin light chain in the IPs. A, B) eIF4E is present in Sxl and Snf immunoprecipitates (2nd lane from left in panels A and B as indicated), but is released from the Sxl and Snf complexes by pre-treatment with RNase (3rd lane from left as indicated). eIF4E is not immunoprecipitated by antibodies to β-galactosidase (lane 4 from left). Note that though Snf and Sxl interact directly with each other in vitro and in vivo, Sxl protein is typically not detected in Snf immunoprecipitates of total nuclear extracts (37) whereas Snf is readily detected in Sxl IPs. The reason for this discrepancy is that only a small amount of the Snf protein is associated with Sxl. As we are able to recover only a fraction of the total Snf in the IPs, there is probably too little Sxl to be detected. On the other hand, Sxl can be readily detected in the Snf IPs when Snf:Snl complexes are first partially purified away from bulk Snf protein on sucrose gradients and then immunoprecipitated. (C) eIF4E is not present in Scute (Sc) immunoprecipitates (2nd lane), but is present in the U1-70K, U2AF50 and U2AF38 immunoprecipitates (lanes 3–5 from left as indicated). (D) eIF4E is not present in β-galactosidase immunoprecipitates (2nd lane, but is present in the U1-70K (3rd lane from left) and Fl(2)d (4th lane from left) immunoprecipitates. Band visible at very bottom of the IP lanes in panels A, B and also C is the immunoglobulin light chain.

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snRNP on the 3′ splice sites. If eIF4E plays a role in Sxl autoregulation, it should also be present in RNP complexes that contain factors that function at these early steps in the splicing reaction. Both U1-70K, which is a component of the U1 snRNP and the U2AF proteins, U2AF38 and U2AF50 play important roles in Sxl autoregulation and are found associated with Sxl protein in nuclear extracts [45]. U1-70K/U1 snRNP and the U2AF heterodimer function in one of the first steps in the splicing reaction, the formation of the E complex. This complex is formed by the binding of the U1-70K/U1 snRNP to the 5′ splice site and the interaction of the U2AF heterodimer with the polypyrimidine tract at the 3′ splice junction. U2AF at the 3′ splice site then recruits the U2 snRNP which becomes loosely associated with the pre-mRNA [64–69]. The E spliceosome complex then undergoes an ATP dependent rearrangement that stabilizes the pairing interactions between the U2 snRNP and the pre-mRNA to form the A complex [70–73]. U1-70K/U1 snRNP and U2AF remain associated with the splicing complex when the three other snRNPs, U4/U6 and U5, are recruited to give spliceosomal complex B [74]; however, when the B complex rearranges during formation of the activated complex B* both U1-70K and U2AF dissociate from the spliceosome along with the U1 and U4 snRNPs [75–77]. To determine if eIF4E is associated with these early acting splicing factors, we immunoprecipitated nuclear extracts with antibodies against U1-70K and the two U2AF subunits dU2AF38 and dU2AF50. Figure 7C and 7D show that eIF4E is in complexes in the nucleus with the U1-70K protein and with both of the U2AF subunits.

Another factor required for Sxl regulated splicing is the fly WTAP protein Fl(2)d [59–60,77]. The interaction of Fl(2)d with the spliceosomal apparatus more closely parallels that seen for Sxl than U1-70K, U2AF or Sfn. Like Sxl, Fl(2)d is found associated with splicing factors that are present during the formation of the spliceosomal E and A complexes which define the 5′ and 3′ exon–intron junctions and position the U2 snRNP, but appears to dissociate from the spliceosome before the tripartite snRNPs, U4/U6 and U5, are recruited to the pre-mRNA to form pre-catalytic complex B. The available evidence indicates that Sxl/Fl(2)d interactions may facilitate the incorporation of Sxl into pre-mRNA spliceosome complexes and perhaps mediate its interactions with Sfn [61]. If eIF4E is important for Sxl dependent alternative splicing, we would expect to find it associated not only with Sxl but also with Fl(2)d in nuclear extracts. Figure 7D shows that this prediction is correct: eIF4E can be co-immunoprecipitated with Fl(2)d.

Discussion

The RNA binding protein Sxl orchestrates sexual development by controlling gene expression post-transcriptionally at the level of splicing and translation. To exert its different regulatory functions Sxl must collaborate with sex-non-specific components of the general splicing and translational machinery. In the studies reported here we present evidence that one of the splicing co-factors is the cap binding protein eIF4E. We initially identified eIF4E in a screen for mutations that dominantly suppress the male lethal effects induced by ectopic expression of a mutant Sxl protein, Sx-N, which lacks part of the N-terminal domain. The Sx-N protein is substantially compromised in its splicing activity, but appears to have closer to wild type function with inhibition of Msl-2 expression [39] we anticipated that general translation factors needed to help Sxl repress msl-2 mRNA would be recovered as suppressors in our screen. Indeed, one of the suppressors identified was eIF4E. However, consistent with in vitro experiments, which have shown that Sxl dependent repression of msl-2 mRNA translation is cap independent [54], we found that eIF4E does not function in Sxl mediated translational repression of at least one target mRNA in vivo. Instead, our results indicate that eIF4E is needed for Sxl dependent alternative splicing and argue that it is this splicing activity that accounts for the suppression of male lethality by eIF4E mutations. In wild type females, Sxl protein blocks the splicing of a small intron in the 5′ UTR of the msl-2 pre-mRNA. This is an important step in msl-2 regulation because the intron contains two Sxl binding sites that are needed by Sxl to efficiently repress translation of the processed msl-2 mRNA. When this intron is removed repression of msl-2 translation by Sxl is incomplete [25–28] and this would enable eIF4E+/+ males to escape the lethal effects of the Sx-N transgene.

Several lines of evidence support the conclusion that eIF4E is required for Sxl dependent alternative splicing. One comes from our analysis of the dominant maternal effect female lethal interactions between eIF4E and Sxl. The initial activation of the Sxl positive autoregulatory feedback loop in early embryos can be compromised by a reduction in the activity of splicing factors like Sfn, Fl(2)d, and U1-70K, and mutations in genes encoding these proteins often show dose sensitive maternal effect, female lethal interactions with Sxl. Like these splicing factors, maternal effect female lethal interactions with Sxl are observed for several eIF4E alleles. Moreover, these female lethal interactions can be exacerbated when the mothers are trans-heterozygous for mutations in eIF4E and the splicing factors sfn or fl(2)d. Genetic and molecular experiments indicate that female lethality is due to a failure in the female specific splicing of Sxl-Pm mRNAs. First, female lethality can be rescued by gain-of-function Sxl mutations that are constitutively spliced in the female mode. Second, transcripts expressed from a Sxl-Pm splicing reporter in the female Sxl+/+ progeny of eIF4E/+ mothers are inappropriately spliced in a male pattern at the time when the Sxl positive autoregulatory loop is being activated by the Sxl-Pe proteins. While splicing defects are evident in these embryos at the blastoderm/early gastrula stage, obvious abnormalities in expression of Sxl protein are not observed until several hours later in development.

Though this difference in timing would favor the idea that eIF4E is required for splicing of Sxl-Pm transcripts rather than for the export or translation of the processed Sxl-Pm mRNAs, we can not exclude the possibility that there are subtle defects in the expression of Sxl protein at the blastoderm/early gastrula stage that are sufficient to disrupt splicing regulation during the critical activation phase yet aren’t detectable in our antibody staining experiments. However, evidence from two different experimental paradigms using adult females indicates that this is likely not the case. In the first, we found that reducing eIF4E activity in a sensitized sfn221 Sxl+/+/ females background can compromise Sxl dependent alternative splicing even though there is no apparent reduction in Sxl protein accumulation. In this experiment we took advantage of the fact that once the positive autoregulatory feedback loop is fully activated a homeostasis mechanism (in which Sxl negatively regulates the translation of Sxl-Pm mRNAs) ensures that Sxl protein is maintained at the same level even if there are fluctuations in the amount of female spliced mRNA. While only a small amount of male spliced Sxl-Pm mRNAs can be detected in sfn221 Sxl+/+/ females, the level increases substantially when eIF4E activity is reduced. Since these synergistic effects occur even though Sxl levels in the triply heterozygous mutant females are the same as in the control sfn221 Sxl+/+ females, we
conclude that the disruption in Sxl dependent alternative splicing of Sxl-Pm transcripts in this context (and presumably also in early embryos) can not be due to a requirement for eIF4E in either the export of Sxl mRNAs or in their translation. Instead, eIF4E activity must be needed specifically for Sxl dependent alternative splicing of Sxl-Pm pre-mRNAs. Consistent with a more general role in Sxl dependent alternative splicing, there is a substantial increase in msd-2 mRNAs lacking the first intron when eIF4E activity is reduced in af1627 Sxl(+/+) females. In the second experiment we examined the splicing of pre-mRNAs from the endogenous Sxl gene and from a Sxl splicing reporter in females heterozygous for two hypomorphic eIF4E alleles. Male spliced mRNAs from the endogenous gene and from the splicing reporter are detected the eIF4E/+ females, but not in wild type females. Moreover, the effects on sex-specific alternative splicing seem to be specific for transcripts regulated by Sxl as we didn’t observe any male spliced dsx mRNAs in eIF4E/+ females.

Two models could potentially explain why eIF4E is needed for Sxl dependent alternative splicing. In the first, eIF4E would be required for the translation of some critical and limiting splicing co-factor. When eIF4E activity is reduced, insufficient quantities of this splicing factor would be produced and this, in turn, would compromise the fidelity of Sxl dependent alternative splicing. In the second, the critical splicing co-factor would be eIF4E itself. It is not possible to conclusively test whether there is a dose sensitive requirement for eIF4E in the synthesis of a limiting splicing co-factor. Besides the fact that the reduction in the level of this co-factor in flies heterozygous for hypomorphic eIF4E alleles is likely to be rather small, only a subset of the Sxl co-factors have as yet been identified (unpublished data). For these reasons, the first model must remain a viable, but in our view, unlikely possibility. As for the second model, the involvement of a translation factor like eIF4E in alternative splicing is unexpected if not unprecedented. For this to be a viable model, a direct role for eIF4E must be consistent with what is known about the dynamics of Sxl pre-mRNA splicing and the functioning of the Sxl protein. The evidence that the second model is plausible is detailed below.

Critical to the second model is both the nuclear localization of eIF4E and an association with incompletely spliced Sxl pre-mRNAs. Nuclear eIF4E has been observed in other systems, and we have confirmed this for Drosophila embryos. We also found that eIF4E is bound to Sxl transcripts in which the regulated exon2-exon3-exon4 cassette has not yet been spliced. In contrast, it is not associated with incompletely processed transcripts from the tango gene, which are constitutively spliced. With the caveat that we have only one negative control, it is not surprising that Sxl transcripts might be unusual in this respect. There is growing body of evidence that splicing of constitutively spliced introns is co-transcriptional [78–83]. However, recent in vivo imaging experiments have shown that the splicing of the regulated Sxl exon2-exon3-exon4 cassette is delayed until after the Sxl transcript is released from the gene locus in female, but not in male cells [84]. These in vivo imaging studies also show that, like bulk pre-mRNAs, the 1st Sxl intron is spliced co-transcriptionally in both sexes. Consistent with a delay in the splicing of the regulated cassette, we’ve previously reported that polyadenylated Sxl RNAs containing introns 2 and 3 can be readily detected by RNase protection, whereas other Sxl intron sequences are not observed [19]. The delay in the splicing of the regulated Sxl cassette until after transcription is complete and the RNA polyadenylated could provide a window for exchanging eIF4E for the nuclear cap binding protein.

To function as an Sxl co-factor, eIF4E would have to be associated with the pre-mRNA-spliceosomal complex before or at the time of the Sxl dependent regulatory step. There is still a controversy as to exactly which step in the splicing pathway Sxl exerts its regulatory effects on Sxl-Pm pre-mRNAs and two very different scenarios have been suggested. The first is based on an in vitro analysis of Sxl-Pm splicing using a small hybrid substrate consisting of an Adenovirus 5’ exon-intron fused to a short Sxl-Pm sequence spanning the male exon 3’ splice site [85]. These in vitro studies suggest that Sxl acts very late in the splicing pathway after the 1st catalytic step, which is the formation of the lariat intermediate in the intron between exon 2 and the male exon. According to these experiments Sxl blocks the 3rd catalytic step, the joining of the free exon 2 5’ splice site (or Adeno 5’ splice site) to the male exon 3’ splice site (see Figure 1A). It is postulated that this forces the splicing machinery to skip the male exon altogether and instead join the free 5’ splice site of exon 2 to the downstream 3’ splice site of exon 4. Since we have shown that eIF4E binds to Sxl-Pm pre-mRNAs that have not yet undergone the 1st catalytic step (Figure 6), it would be in place to influence the splicing reaction if this scenario were correct.

The second scenario is more demanding in that it proposes that Sxl acts during the initial assembly of the spliceosome. Evidence for Sxl regulation early in the pathway comes from the finding that Sxl and the co-factor Fl(2)d show physical and genetic interactions with spliceosomal proteins like U1-70K, Snf, U2AF38 and U2AF50 that are present in the early E and A complexes and are important for selecting the 5’ and 3’ splice sites [45,61,64–71]. In addition to these proteins, Sxl can also be specifically cross-linked in nuclear extracts to the U1 and U2 snRNAs [43]. Formation of the E complex depends upon interactions of the U1 snRNP with the 5’ splice site, and this is thought to be one of the first steps in splicing. The other end of the intron is recognized by U2AF, which recruits the U2 snRNP to the 3’ splice site. After the base pairing of the U2 snRNP with the branch-point to generate the A complex the next step is the addition of the U4/U5/U6 snRNPs to form the B complex. However, Sxl and Fl(2)d are not found associated with components of the splicing apparatus like U5-40K, U5-116K or SKIP that are specific for complexes B and B*, or the catalytic C complex [70–71,74–75,86–88]. Nor can Sxl be cross-linked to the U4, U5 or U6 snRNAs [43]. If Sxl and Fl(2)d dissociated from the spliceosome before U4/U5/U6 are incorporated into the B complex, then they must influence splice site selection during the formation/functioning of the E and/or A complex. (Since the transition from the E to the A complex has been shown to coincide with an irreversible commitment to a specific 5’—3’ splice site pairing, Sxl would likely exerts its effects in the E complex when splice site pairing interactions are known to still be dynamic [89].) If this scenario is correct, eIF4E would have to be associated with factors present in the earlier complexes in order to be able to promote Sxl regulation. This is the case. Thus, eIF4E is found in complexes containing the U1 snRNP protein U1-70K, the U1/U2 snRNP protein Snf, and the two U2AF proteins, U2AF38 and U2AF50. With the exception of the Snf protein bound to the U2 snRNP, all of these eIF4E associated factors are present in the early E or A complexes, but are displaced from the spliceosome together with the U1 and U4 snRNPs when the B complex is rearranged to form the activated B* complex. This would imply that eIF4E is already in place either before or at the time of B complex assembly. Arguing that eIF4E associates with these E/A components prior to the assembly of the B complex is the finding that eIF4E is also in complexes with both Sxl and Fl(2)d. Thus, even in this more demanding scenario for Sxl dependent splicing, eIF4E would be present at a time when it could directly impact the regulatory activities of Sxl and its co-factor Fl(2)d.
Taken together these observations would be consistent with a Sxl co-factor model. While further studies will be required to explain how eIF4E helps promote female specific processing, an intriguing possibility is suggested by the fact that hastening the nuclear export of msl-2 in females would favor the female splice (which is no splicing at all). Hence, one idea is that eIF4E binding to the pre-mRNA provides a mechanism for preventing the Sxl regulated splice sites from re-entering the splicing pathway, perhaps by constituting a “signal” that blocks the assembly of new E/A complexes. A similar post-transcriptional mechanism could apply to female-specific splicing of the regulated Sxl exon2-exon3-exon4 cassette. The binding of eIF4E (and PABP) to incompletely processed Sxl transcripts after transcription has terminated in females would prevent the re-assembly of E/A complexes on the two male exon splice sites, and thus promote the formation of an A complex linking splicing factors assembled on the 5’ splice sites of exons 2 and on the 3’ splice site of exon 4.

Materials and Methods

Fly culture

Crosses were performed at 29°C unless otherwise indicated with 3–7 females and 2–4 males per vial. Crosses were transferred to new vials every 2–3 days. Similar crosses were performed at 25°C, but the effects were significantly weaker.

Stocks

Unless otherwise noted stocks are referenced by Lindsley & Zimm [89]; w; eif4e^{S50T/111}/TM3Sb (eif4e^{S50T}), FBal0129763, w; eif4e^{KPS85}/TM3Sb (eif4e^{KPS85}), FBal0129764, w; eif4e^{S50T115}/TM3Sb (eif4e^{S50T115}), FBal0175609, y^{2}c^{71,72}, w; cm Sxl^{1} ct/Bincinscy, y w (FBal0016680), Sx^{FBio}/Bincinscy (FBal0016694), y pn Sxl^{111}/Bincinscy (FBal0016703), y pn Sxl^{MT4}/Bincinscy (FBal0016710), y pn Sxl^{MT4}/Bincinscy (FBal010394), cm Sxl^{1}/Bincinscy (FBal0016686), y w snf1621 ct/Bincinscy, y w snf1621 ct/Bincinscy.

Screen for suppressors of hsp83/Sx-N/Ja transgene

To identify suppressors of the dominant male lethality conferred by Sx-N, we crossed w Sx^{FBio}/Bio; hsp83/Sx-N/Ja transgene mothers to Deficiency/Balancer fathers and scored for viable, non-Balancer males containing the transgene. The 67A8-A9 region was one of the chromosomal intervals that was found to contain a suppressor. The eif4e gene mapped to this region and was a strong candidate gene for the dominant suppressor. Four independent eif4e alleles suppressed the male lethal effects of hsp83/Sx-N/Ja transgene as indicated in the text. All crosses for both screens were conducted in vials with five females and three males of the appropriate genotype. Matings were allowed to occur for three days at 25°C, at which time the parents were transferred to new vials to ensure that larvae were not crowded.

Immunohistochemical staining

Embryos were collected on apple juice plates sprinkled with yeast at 29°C. They were dechorionated in bleach and fixed in 4% paraformaldehyde:heptane for 20–25 minutes. The fix was removed and embryos devitilinized and stored in methanol at −20°C. To stain, embryos were stepped into PBS, incubated for 1 hour in PAT (PBS with 1% BSA, 1% Triton-X100) and blocked for 30 minutes in PBT (PBS with 5% BSA). Embryos were incubated overnight at 4°C with primary antibody at the appropriate concentration in PBT. The next day the embryos were washed with PBS-T (PBS with 1% Triton-X100) then, incubated for 2 hours at room temperature with secondary antibody at the appropriate concentration in PBT. Embryos were washed with PBS-T, then with PBS. For embryos with fluorescently tagged secondary antibodies, the embryos were incubated for 5 minutes with a 1:1000 dilution of Hoescht, rinsed twice with PBS, then mounted in Aquamount (Polysciences, Inc.). For embryos with HRP conjugated secondary antibodies, embryos were incubated with 400 ul of 0.4 mg/ml DAB in PBS, 1 ul of 3% hydrogen peroxide and 0.6 ul of 1 M NiCl2 until the embryos appeared fully stained. To prepare for mounting embryos were stepped into 100% ethanol, then incubated overnight in methyl salicylate. The following morning, embryos were mounted in Permount (Fisher Scientific). Primary antibodies used were: anti-Sx18 monoclonal at 1:10, anti-snf 9G3 monoclonal at 1:10 and anti-eIF4E polyclonal at 1:500 (gift from Paul Lasko). Secondary antibodies used were: HRP conjugated goat anti-mouse (Jackson ImmunoResearch) at 1:500, rhodamine conjugated goat anti-rabbit (Alexa) at 1:500, fluorescence conjugated goat anti-mouse (Alexa) at 1:500.

RT-PCR analysis and Southern blotting

Embryonic RNA was prepared as described by Bell et al [90]. Adult RNA from 33 flies was prepared using GE Healthcare mini-spin columns. Reverse transcription was performed according to the procedure of Frohman et al. [91]. 1.5–3% of the cDNA was used as template. PCR cycles for embryonic cDNAs were 1X 95°C 4 minutes, 30X 95°C 1 minute, 60–65°C 45 seconds, 72°C 30 seconds, 1X 72°C 10 minutes. If re-amplification was needed, only 10 cycles were performed in the first PCR. Up to 40% of the first PCR was used as template for the second PCR. Primers and temperatures were the same for the second reaction as in the first and 10–30 cycles were performed as needed. Number of cycles needed was evaluated by examining 10 ul samples with EtBr. For adult cDNAs PCR cycles were as follows: 2X 95°C 1 minute, 70–72°C 45 seconds, 2X 95°C 1 minute, 2–4X 95°C 1 minute, 68–70°C 45 seconds, 72°C 1 minute, 2–4X 95°C 1 minute, 66–67°C 45 seconds, 72°C 1 minute, 2–4X 95°C 1 minute, 65–66°C 45 seconds, 72°C 1 minute, 10X (first PCR) or 3–30X (second PCR) 95°C 1 minute, 65–67°C 45 seconds, 72°C 1 minute. 5 ul of the first PCR diluted 1/100 was used as template for the second PCR. For Southern blotting DNA was run on 1–1.2% agarose gels, and Southern blotted to Zeta-Probe membrane or nitrocellulose. For Sxl reactions blots were hybridized with randomly primed Sxl 3BIa cDNA [39]. For msl-2 mRNAs the membrane was hybridized to randomly primed msl-2 5’UTR PCR product. Primers used are described in Figure 6 and listed in Table 2.

Nuclear extraction

Nuclear extract was prepared by collecting embryos laid by w^{1} stock overnight (<24 hours). Embryos were washed with distilled water and 0.12 M NaCl, 0.04% Triton-X 100, then dechorionated in 100% bleach for 3 minutes. Dechorionated embryos were rinsed with NaCl, Triton, then NaCl, blotted dry and collected. Embryos were homogenized at 4°C in buffer 1 (15 mM HEPES-KOH pH 7.6, 10 mM KCl, 5 mM MgCl2, 0.1 mMEDTA, 0.5 mM EGTA, 0.35 M sucrose, with 1 mM DTT, 1 mMNa2SO4, protease inhibitors, benzamidine and 1mM PMSF), using 4 ml buffer/ml lightly packed embryos. The homogenate was filtered through three layers of Mira-cloth, then centrifuged at 2000 xg for 10 minutes at 4°C. Supernatant was removed with a pipet. The pellet was re-suspended in 4 ml buffer/ml embryos, and overlaid onto an equal volume of buffer 2 (same as buffer 1 except 0.8 M sucrose), then spun 10 minutes at 2000 xg, at 4°C. The supernatant was removed. The pellet was resuspended in TEN (10 mM Tris-HCl pH 7.8–8, 1.5 mM EDTA, 100 mM
Table 2. Primers used for RT-PCR experiments.

| Experiment    | RT primer | PCR primers | Sequence                           |
|---------------|-----------|--------------|------------------------------------|
| Splice reporter Figure 3 | lacZ1       | CGCATCGTAAACCGTGACATCTGC |
| Sxl in adult females Figure 4B | lacZ2, EX2   | CGCCATTAGCGTTCGACACTTG, GTGGTTATCCCCCATATGCC |
| Sxl in adult females Figure 4C | T41-3       | CGGTGCAAGCTGACGTGTC |
| msl-2 in adult females Figure 4D | T41-3, mes17, BelA | CGGTGCAAGCTGACGTGTC |
| IP-RT-PCR Figure 6B | T41-3       | CGGTGCAAGCTGACGTGTC |

| Experiment | RT primer   | PCR primers | Sequence                          |
|------------|-------------|-------------|-----------------------------------|
| Sxl        | Sxl1, Sxl2, Sxl3, Sxl4 | GGGCCGAGGAAAGCTGCC | |

with primers as indicated in Figure 6 and Table 2. Southern blotting conditions were as described above using randomly primed Sxl 3B1A cDNA [39] as the probe. Antibodies used for immunoprecipitation were; anti-scute SA10, anti-Sxl 104 and 114 mixed 1:1, and anti-eIF4E.

Western blotting

2–5 flies of each genotype were collected and frozen at −80°C. 10 ul of 2x Laemmli sample buffer per fly was added to the flies, which were then homogenized with a hand held Dounce homogenizer. Samples were boiled for 5 minutes and spun for three minutes at 14,000 rpm. Samples were diluted as needed with 2x Laemmli sample buffer and up to 10 ul of sample were loaded onto sodium dodecyl sulfate (SDS)-12% acrylamide gels, run out and transferred to Immobilon-P or nitrocellulose. Blots were incubated for 60 minutes in PBST (PBS with 1% Triton-X100), with 10% dry milk then, incubated overnight at 4°C with primary antibody at the appropriate concentration in PBST with 10% dry milk. The next day the blots were washed with PBST for at least an hour, then incubated for 2–4 hours at room temperature with secondary antibody at the appropriate concentration in PBST with 10 mg/ml BSA. Blots were washed with PBST then, developed with ECL Plus (Amersham). Primary antibodies used were: a 1:1 mixture of anti-SXL104 and 114 at 1/10–1/1000, anti-eIF4E 1739 at 1/1000, anti-u2af50 at 1/5000, and anti-dFMR J11 at 1/1000. HRP conjugated goat anti-mouse and goat anti-rabbit (Jackson ImmunoResearch) secondary anti-bodies were used at 1/2500 or 1/5000.

Supporting Information

Figure S1 Male and female spliced msl-2 mRNA in surviving Sxl+/eif4e/+; hsp83:Sx-N+ males. RNA isolated from one of the 3 surviving Sxl+/eif4e/+; hsp83:Sx-N+ males was reversed transcribed with a primer complementary to sequences in exon 3 (downstream NaCl), 2 ml TEN/ml embryos, and sonicated. 20 ul–40 ul of 50% antibody linked protein AG beads, 350 ul co-immunoprecipitation buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 250 mM sucrose, 0.05% (w/v) Tergitol NP-40, 0.5% (v/v) Triton-X 100 plus 1 mM DTT, 1 mM Na2S2O5, protease inhibitors, benzamidine and 1 mM PMSF) and 12.5 ul RNAsin were added to a 150 ul aliquot of sonicate. The mixture was rocked at 4°C overnight, then washed 5 times with co-IP buffer. The beads were boiled for 5–10 minutes with 20 ul protein sample buffer, then spun for 5–10 minutes. 5–10 ul of sample was loaded onto a 12% polyacrylimide gel. The proteins were transferred to Immobilon-P or nitrocellulose. Blots were prehybridized in PBS-5% nonfat dry milk and hybridized with horseradish peroxidase-conjugated antibody at the appropriate concentration in PBS-5% milk. The next day the blots were washed with PBST for at least an hour, then incubated for 2–4 hours at room temperature with secondary antibody at the appropriate concentration in PBST with 10% dry milk. The next day the blots were washed with PBST for at least an hour, then incubated for 2–4 hours at room temperature with secondary antibody at the appropriate concentration in PBST with 10 mg/ml BSA. Blots were washed with PBST then, developed with ECL Plus (Amersham). Primary antibodies used were: a 1:1 mixture of anti-SXL104 and 114 at 1/10–1/1000, anti-eIF4E 1739 at 1/1000, anti-U2AF50 at 1/5000, and anti-dFMR J11 at 1/1000. HRP conjugated goat anti-mouse and goat anti-rabbit (Jackson ImmunoResearch) secondary anti-bodies were used at 1/2500 or 1/5000.

Immunoprecipitation, RT-PCR

Nuclear extract was prepared essentially as above except, after the first centrifugation, the pellet was resuspended in 1 ml buffer/ml embryos and sonicated. 20 ul of 50% antibody linked protein A beads were added to a 150 ul aliquot of sonicate. The mixture was allowed to rock 1 hour at room temperature, then washed as above. RNA was isolated using TRIreagent (Molecular Research Center, Inc.) then, treated with DNase 1. Reverse transcription, PCR and Southern blotting conditions were as described above.
of primer 3). We then used two different primer combinations, P1–P2 and P1–P3 to amplify female and male ml-2 transcripts as indicated. Similar results were obtained from the two other surviving Sxl+/eif4e++; hop83;Sx-M females.

(Fig. 2) Female specific splicing of dsx mRNA is unaffected by reducing eif4e activity. RNA from wild type males and females and females heterozygous for either eif4e−/− (4E560) or eif4e−/−− (4E567) were reverse transcribed with primers specific for the female spliced or male spliced 3′ UTRs. The resulting cDNAs were then PCR amplified using primers complementary to the common exon 3 and the female exon 4 (dsx female), or to the common exon 3 and the first male exon 5 (dsx male). Female specific, but not male specific amplification products are detected in wild type females and in females heterozygous for the two eif4e mutations. To ensure that the amplification products we are seeing are specific dsxF and dsxM, we used nested primers in the common exon for two rounds of PCR amplification.

(Fig. 2) Figure S2 Female specific splicing of dsx mRNA is unaffected by reducing eif4e activity. RNA from wild type males and females and females heterozygous for either eif4e−/− (4E560) or eif4e−/−− (4E567) were reverse transcribed with primers specific for the female spliced or male spliced 3′ UTRs. The resulting cDNAs were then PCR amplified using primers complementary to the common exon 3 and the female exon 4 (dsx female), or to the common exon 3 and the first male exon 5 (dsx male). Female specific, but not male specific amplification products are detected in wild type females and in females heterozygous for the two eif4e mutations. To ensure that the amplification products we are seeing are specific dsxF and dsxM, we used nested primers in the common exon for two rounds of PCR amplification.

Table S1 Sex-lethal staining patterns in older embryos. Unless otherwise indicated females were crossed to Sxl+/ males at 29°C. Progeny were collected as embryos and stained with antibody to Sxl. Embryos at the cellular blastoderm stage or those past nuclear cycle 13 were examined and placed into one of the indicated categories. The number scored (a) is the total number of male and female embryos.

| Sex   | Male | Female |
|-------|------|--------|
| Wild type | 50 | 50 |
| Sxl−/− | 50 | 0 |

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Table S2 Male lethal mutations of Sxl suppress the synthetic female lethality. All females were crossed with Sxl+/ males at 29°C. Female viability was calculated as (# females)/(# females)x100 except in crosses with Sxl− mutations that affected male viability. In those crosses female viability was calculated as (# females)/(2-non-mutant males). %Sxl surviving is calculated as (% Sxl− females/# Sxl− females expected)x100. % Sxl− surviving is calculated as (% Sxl− females/# Sxl− females expected)x100. Number scored = total number counted. NA = not applicable.

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Author Contributions
Conceived and designed the experiments: PLG JLY JKMP GD. Performed the experiments: PLG JLY JKMP GD. Analyzed the data: PLG JLY JKMP GD. Contributed reagents/materials/analysis tools: PLG JLY JKMP GD. Wrote the paper: PLG JLY PS.
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