Regulation of translation and alternative splicing by the Drosophila RNA-binding protein Sister-of-Sex-lethal

DISSERTATION

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Vorgelegt von
Rebecca Moschall

aus Würzburg

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„*Supercalifra*
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Mary Poppins

Abstract

In *Drosophila melanogaster*, the protein Sxl Lethal (Sxl) is the master regulator of female development, controlling sex-specific differences in morphology, behaviour and dosage compensation. The female-specific protein is involved in a variety of posttranscriptional regulatory pathways, which have been intensively studied during the past decades. In contrast, the molecular function of its closely related paralog Sister-of-Sex-Lethal (Ssx) had not been elucidated. Despite their high sequence similarity, the two proteins exhibit surprising functional differences. Here we show that, unlike Sxl, Ssx is expressed in both sexes. Applying iCLIP analyses, we further determine the RNA targets of Ssx and demonstrate that the protein binds to an RNA sequence motif that is highly similar to the motif recognized by Sxl. Both proteins can recognize and bind to sequence elements previously shown to function in the regulation of alternative splicing and translational control. Using two model substrates, the *male-specific lethal-2* (*msl-2*) and *Sxl* RNAs, we addressed the role of Ssx in post-transcriptional regulation of gene expression and its functional interplay with Sxl.

To establish and to maintain its continuous expression in female flies, Sxl establishes an auto-regulatory, positive feedback loop by promoting constitutive splicing of its own primary transcript. Binding to several intronic RNA sequences, Sxl acts as an inhibitor of splicing that prevents inclusion of an exon with a premature termination codon in the mature mRNA. We demonstrate that Ssx can compete with Sxl for binding to these regulatory RNA elements. By this, Ssx can function as a competitive inhibitor of the Sxl auto-regulatory feedback loop in cultured cells. In line with this finding, male flies that lack Ssx protein exhibit aberrant Sxl expression.

In contrast to alternative splicing in which Ssx functions as an antagonist of Sxl function, both proteins can act as inhibitors of translation. For translational control of *msl-2* mRNA, Sxl employs two previously characterized pathways that operate via binding sites in either the 5' or 3' UTR of the RNA. Ssx recapitulates 5'UTR-mediated translational repression, however, it does not exhibit regulatory activity when operating via the 3'UTR-mediated regulation. A thorough mutagenesis reveals that this difference in activity is based on three amino acid substitutions, allowing deep insights into the recent evolutionary history and functional diversification of the two proteins.

In sum, our studies reveal the molecular function of Ssx and unravel a surprisingly complex interplay between the two closely-related proteins in sex-specific development of *Drosophila*.

Zusammenfassung

Das RNA Bindeprotein Sex Lethal (Sxl) ist der Hauptregulator der weiblichen Fliegenentwicklung in *Drosophila melanogaster*, indem es die geschlechtsabhängige Morphologie und das Verhalten reguliert und die Dosiskompensation kontrolliert. Das Weibchen-spezifische Protein wirkt in einer Vielzahl von post-transkriptionellen Mechanismen, welche über die letzten Jahrzehnte intensiv untersucht worden sind. Im Gegensatz zu Sxl, ist die Funktion des nah verwandten Paralogs Sister-of-Sex-Lethal (Ssx) weitgehend unbekannt. Trotz ihrer hohen Sequenzähnlichkeit weisen beide Proteine große funktionelle Unterschiede auf. In dieser Arbeit zeigen wir, dass Ssx, im Gegensatz zu Sxl, in beiden Geschlechtern exprimiert wird. ICLIP Analysen identifizierten von Ssx gebundene RNAs und bestätigten, dass Ssx RNA Sequenzen bindet, die den Sxl Bindemotiven stark ähneln. Beide Proteine erkennen und binden Sequenzelemente, durch welche sie in der Regulation des alternativen Spleißens und der translationalen Kontrolle von Bedeutung sind. Unter Verwendung von zwei Modelsubstraten, *male-specific lethal-2* (*msl-2*) und *Sxl* mRNA, analysierten wir die Rolle von Ssx innerhalb der post-transkriptionellen Regulation der Genexpression und dessen funktionelles Zusammenspiel mit Sxl.

Um eine kontinuierliche Expression in weiblichen Fliegen zu garantieren, etabliert Sxl eine autoregulatorische positive Rückkopplung, welche ein konstitutives Spleißmuster des eigenen primären Transkripts gewährleistet. Dabei bindet Sxl an mehrere intronische RNA Sequenzen und verhindert als Spleißinhibitor die Inklusion eines Exons in die reife *Sxl* mRNA, welche sonst für ein verfrühtes Terminationskodon kodieren würde. In diesem Zusammenhang konnten wir zeigen, dass Ssx mit Sxl um die Bindung an diese regulatorischen RNA Elemente kompetitiert. Somit kann Ssx in männlichen Zellen als kompetitiver Inhibitor der Sxl autoregulatorischen Rückkopplung funktionieren. Im Einklang mit diesen Ergebnissen konnten wir ebenfalls beweisen, dass der Verlust von Ssx in männlichen Fliegen zu einer aberranten Sxl Expression führt.

Im Gegensatz zum alternativen Spleißen, in welchem Ssx als Antagonist der Sxl Funktion agiert, können beide Proteine Translation inhibieren. Sxl verfügt über zwei gut charakterisierte Mechanismen, um die Translationskontrolle von *msl-2* zu gewährleisten. Diese Regulation beruht auf Bindestellen innerhalb der 5'UTR oder innerhalb der 3'UTR der *msl-2* mRNA. Ssx reprimiert die Translation innerhalb des 5'UTR vermittelten Mechanismus, zeigt jedoch keine regulatorische Aktivität innerhalb der 3'UTR vermittelten Regulation. Detaillierte Mutageneseanalysen des Proteins ergaben, dass der Aktivitätsunterschied auf drei Aminosäuren zurückzuführen ist. Diese Erkenntnis erlaubte tiefe Einsichten in die evolutionäre Beziehung und die funktionelle Diversifikation der zwei Proteine.

Zusammengefasst konnten wir die molekulare Funktion von Ssx aufzeigen und das überraschend komplexe Zusammenspiel der zwei nah verwandten Paraloge in der geschlechtsabhängen Fliegenentwicklung darlegen.

Contents

1 Introduction

1.1 Sex determination, dosage compensation and female development in humans, *Caenorhabditis elegans* and *Drosophila melanogaster*

At the time of early developmental stages in most organisms from flies to humans an important decision has to be made: whether to be male or female. This decision has to be tightly regulated and evolution achieved a colorful spectrum of mechanisms to solve this task. In general, three main ways of sex determination are found in nature: hermaphroditism, sex determination by environmental influences and genotypic sex determination (Bachtrog et al. 2014). While e.g. clownfish start life being a male and exclusively the most dominant animal in the hierarchy develop into a female over time (sequential hermaphrodites) (Munday et al. 2006) many flowering plants harbour both, female and male organs (simultaneous hermaphrodites) (Renner and Ricklefs 1995). In other organisms, such as e.g. turtles and crocodiles, sex determination is governed by environmental factors such as temperature during embryonic development with higher temperatures producing more females and lower temperatures more males. Both are genetically identical without the necessity of sex specific chromosomes and dosage compensation (reviewed in Crews 2003; Lance 2009).

In contrast, higher organisms mostly employ genetic sex determination which coincides with different sex chromosomes counts (XX vs. XY) which consequently leads to gene dose imbalances. To overcome this problem, different organisms have evolved a variety of strategies to adjust the gene dose between the sexes. Humans, *Caenorhabditis elegans (C. elegans)* and *Drosophila melanogaster (D. melanogaster)* employ different strategies. Sex determination in humans makes use of the Y-chromosome encoded regulator SRY: this protein promotes the development of male testis, whereas its absence results in the formation of ovaries (Sinclair et al. 1990). The resulting gene dose imbalance between males and females is compensated in females by random inactivation of one of the two X chromosomes (Okamoto et al. 2011). X-inactivation is mediated by the long non-coding RNA Xist which is exclusively expressed from the inactive X chromosome. The Xist RNA acts in *cis* and coats the major part of the inactive X chromosome to promote hypoacetylation of histones and methylation of promoters, which finally results in the formation of heterochromatin and a highly condensed chromosome, the Barr body (reviewed in Payer and Lee 2008).

Analogous to humans, the roundworm *C. elegans* expresses a male specific protein (Xol-1) to define the male state and to ensure dosage compensation. In *C. elegans* sex determination relies on a X chromosome counting system, with XX animals developing into hermaphrodites and XO into males. In XX animals, dosage compensation is achieved by reducing the gene expression from each X chromosome by one half. This “female” specific down regulation of gene expression is mediated by the dosage compensation complex (DCC). DCC expression in males is prevented through repression of its limiting component *sdc-2* by Xol-1 (reviewed in Lucchesi et al. 2005; Ercan and Lieb 2009).

In contrast to *C. elegans*, dosage compensation in *D. melanogaster* is achieved by the hyper-transcription of the single male X chromosome which has to be prevented in females. Again, sex specific regulation is achieved by the sex-specific expression of a dosage compensation complex active in chromatin remodeling. In female flies, DCC formation is repressed by the multifunctional protein Sex lethal (Sxl) that is produced by a mechanism that is sensitive to the number of X chromosomes. Functional protein is expressed in females, while it remains unexpressed in males.

1.2 The X-chromosome counting system in *D. melanogaster* and early female-specific *Sxl* expression

The role of Sxl as the master regulator is unique for the species of *D. melanogaster* (Sawanth et al. 2016). In other flies, the protein Transformer (Tra) usually is at the top of the developmental cascade (Traut et al. 2006; Salz 2011). In *D. melanogaster* instead, the developmental cascade is initiated by Sxl which regulates a variety of downstream targets, committing to female development. In females, alternative splicing of *tra* is under control of Sxl. Functional Tra protein is only expressed in females where it dimerizes with the ubiquitously expressed Tra2 protein and this heterodimer controls the alternative splicing of its target mRNAs *doublesex* (*dsx*) and *fruitless* (*fru*), leading to female specific splice patterns. The proteins Dsx and Fru represent two important key regulators involved in the development of the central nervous system as well as the somatic sexual differentiation (Fig. 1.1 B). Depending on their isoforms, they promote the establishment of female or male morphology and behavior (reviewed in Penalva and Sanchez 2003). But how is sex-specific expression of functional Sxl protein achieved?

The *Sxl* gene harbours two promoters, *SxlPe* and *SxlPm*, which are strictly regulated in time. The establishment promoter *SxlPe* functions as the initiation promoter acting in a short time window ending at the cellular blastoderm stage and is switched on exclusively in females (Parkhurst et al. 1990; Keyes et al. 1992). When entering the next developmental stage, the promoter *SxlPe* is inactivated and *Sxl* is transcribed from the maintenance promoter *SxlPm* releasing a slightly different transcript (Bell et al. 1988; Salz et al. 1989). While the *SxlPe*

promoter is switched on exclusively in females, the maintenance promoter *SxlPm* is constitutively active in both sexes (Fig. 1.1 A). Within this chapter, we will first focus on the transcription from the *SxlPe* promoter, while the transcription from *SxlPm* and the processing of its transcripts is described in chapter 1.3 and chapter 1.6.

The activation of the *SxlPe* promoter and thus the decision of “becoming a female” is dependent on the expression levels of four X-linked signal elements (XSE), which are encoded on the X chromosome. These proteins, namely scute (SisB), runt, sisA and unpaired (SisC*)* are directly translating the two-fold X chromosome dose in females into the transcriptional activation of Sxl from *SxlPe* promoter (Cline 1988; Duffy and Gergen 1991; Keyes et al. 1992; Sanchez et al. 1994; Jinks et al. 2000; Sefton et al. 2000). While the X chromosomes encode transcriptional activators for Sxl expression, autosomes harbour negative regulators of Sxl transcription; Groucho, extramachrochetae (Emc) and deadpan (dpn) (maternally or zygotically provided) (Youngershepherd et al. 1992; Paroush et al. 1994; Barbash and Cline 1995). The final ratio with a two-fold difference of activator vs. repressor protein levels, which is determined by the X chromosomal dose (XX=1:1; XY=0.5:1), translates this fine-tuned balance of regulators into an ON/OFF signal for Sxl expression from the *SxlPe* promoter (Figure 1.1 B). When the dose of transcriptional activators in females overcomes the autosomal expression of repressors, *SxlPe* is activated and Sxl expression is initiated. This later on leads to the establishment of a positive auto-regulatory feedback loop of Sxl and finally ends in the development of female flies (reviewed in Salz and Erickson 2010).

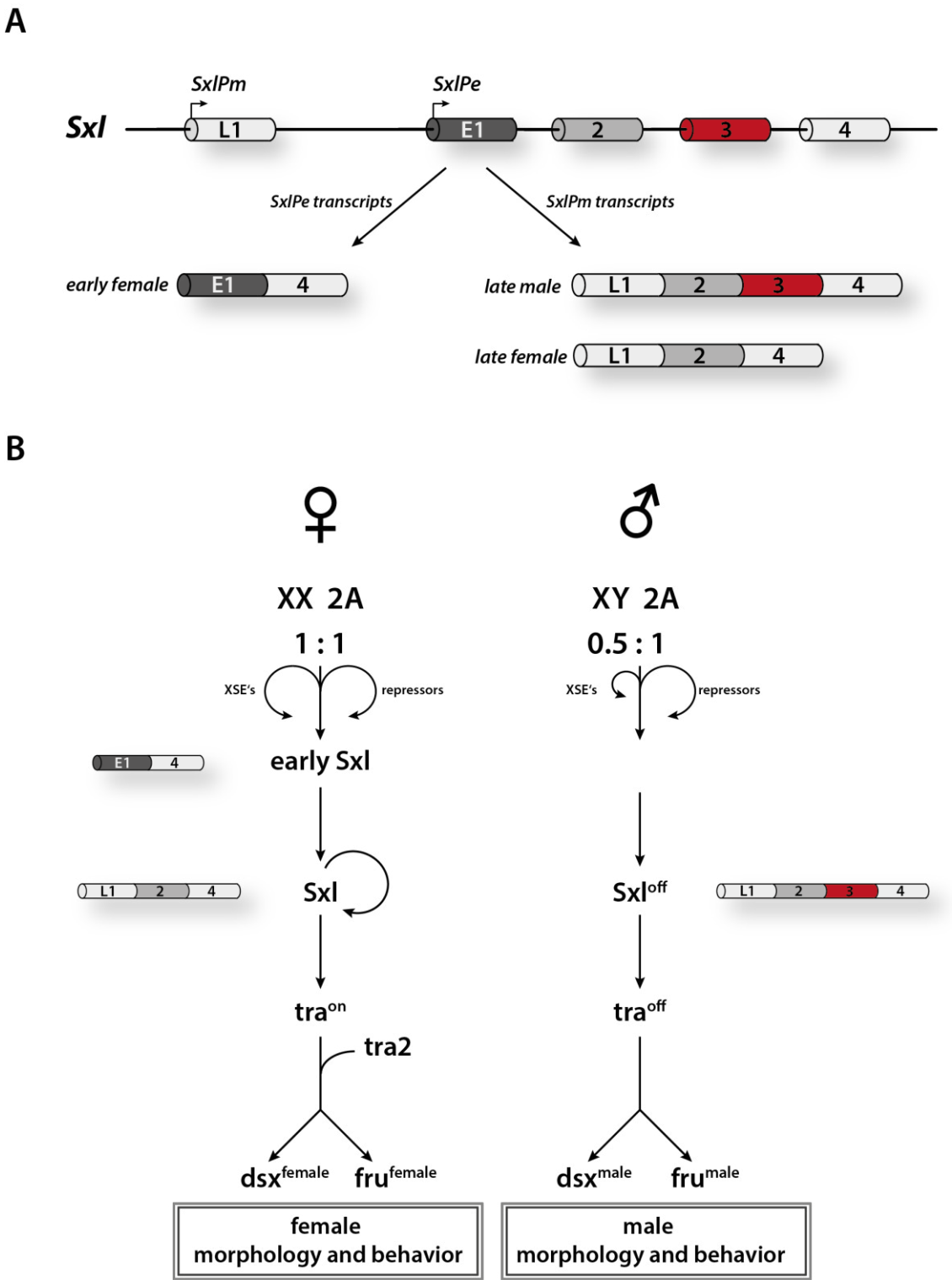

Figure 1.1: Overview of the sex determination cascade within the development of *D. melanogaster*.
A) Sxl transcription from the Sxl early promoter *SxlPe* and Sxl maintenance promoter *SxlPm* results in different transcripts. Sxl "early female" transcripts are transcribed from *SxlPe* promoter while Sxl "late female" and Sxl "late male" transcripts are generated from the promoter *SxlPm.* **B)** Expression of functional Sxl protein is regulated by an X chromosome counting mechanism. In females, XSE proteins activate the early Sxl transcription by competing with autosomal supplied repressor proteins, boosting the developmental cascade. Expression of early Sxl protein in females is followed by the expression of Sxl from *SxlPm* transcripts. Tra protein translated from functional spliced *tra* mRNA in females induces female-specific splicing of *dsx* and *fru*. In males, autosomal repressors overcome the amount of XSE lacking early Sxl expression. This triggers male-specific splicing of the effector targets *dsx* and *fru,* inducing to male traits.

With the exception of the protein Unpaired which acts as a ligand in the Jak/Stat pathway (Sefton et al. 2000), all XSE proteins belong to a family of transcriptional regulators. Unpaired influences *Sxl* transcription by activating the maternally supplied transcriptional activator *Stat92E* (Jinks et al. 2000; Sefton et al. 2000; Avila and Erickson 2007). Furthermore, a heterodimer consisting of the bZip transcription factor SisA and the AML1-like transcription factor Runt is known to interact and directly activate *SxlPe* (Erickson and Cline 1993; Kramer et al. 1999). In addition, Scute (a bHLH-transcription factor) together with the maternally supplied Daughterless (Da) protein recognize non-canonical binding sites in *SxlPe* and further trigger *Sxl* transcription (Yang et al. 2001). In order to translate the proper XSE dose in an all-or-nothing response to *SxlPe*, autosomally supplied (equally expressed in males and females) transcriptional repressors interfere with the activity of XSE proteins.
The maternally supplied repressor Emc is a member of the HLH proteins and preferentially forms heterodimers with bHLH activators like Scute or Daughterless, preventing their binding to DNA promoter sequences (Massari and Murre 2000; Campuzano 2001). Since the influence of Emc on dosage compensation is rather limited (Youngershepherd et al. 1992), Groucho is considered to be the major repressor of early Sxl transcription. It was demonstrated that Groucho translates the dose of XSE into an all-or-nothing response since the lack of maternally supplied Groucho leads to an expression of *SxlPe* in both sexes proportionally to their XSE dose (Lu et al. 2008). The amplification of the XSE dose by Groucho is implemented by its interaction with Dpn, a DNA binding protein which is also able to negatively regulate *SxlPe*. While in females XSE proteins overcome the negative regulation by the Groucho and Dpn complex ending in an overall amplification of *Sxl* transcripts from *SxlPe*, in males the Groucho-Dpn heterodimer successfully competes with XSEs for binding to the promoter regions and prevents *Sxl* transcription (Paroush et al. 1994; Lu et al. 2008).

1.3 The transition of *Sxl* promoters and the determination of the sexual fate

The transition from the early *SxlPe* promoter to the “housekeeping” promoter of *Sxl*, *SxlPm* is tightly regulated and far more complex than initially thought. It was demonstrated that transcription from *SxlPm* is activated within cycle 13 in female embryos and is overlapping for a short period with the remaining activity of *SxlPe*. Male embryos instead are delayed in the onset of *SxlPm* transcription which is first detected approximately 10 minutes later in the early cycle 14 (Gonzalez et al. 2008). This female-first pattern can be explained by the differing dose of the XSEs Runt and Scute and the maternally provided Daughterless which are not exclusively involved in activating *SxlPe* but also in directly promoting the initial *SxlPm* response. *SxlPe* and *SxlPm* share a common enhancer element and since females express twice the dose of XSE, this forces earlier transcription from the *SxlPm* promoter in females

compared to males (Gonzalez et al. 2008). As a result, the early female accumulation of *SxlPm* transcripts together with early Sxl protein guarantee efficient amplification of functionally spliced *Sxl* transcripts and forces the female development. Furthermore, in rare cases due to fluctuations in XSE amounts, low levels of Sxl protein could be mistakenly produced in males. This robust and temporary controlled fail-safe mechanism ensures that erroneously expressed early Sxl protein would not regulate splicing of *SxlPm* transcripts and thus preventing accumulation of Sxl proteins in male embryos (Gonzalez et al. 2008).
Entering the maintenance phase, the initial Sxl burst in females is amplified to a robust and life-lasting expression of Sxl protein. With that, the transcriptional regulation of *SxlPe* promoter is changed to a control at splicing level from *SxlPm* transcripts, in which the positive auto-regulatory feedback loop of Sxl protein triggers the alternative splicing of its own transcript (Cline 1984; Bell et al. 1991).

1.4 Molecular dissection of the RNA-binding protein Sxl

Despite its variety of functions, Sxl surprises with a rather simple protein architecture. The central domain of the protein comprises of two RNA recognition motifs (RRM) that are flanked by a N-terminal glycine-rich region and a C-terminal proline-rich region. The RRMs mediate binding to U-rich or UG-rich RNA sequences of at least seven nucleotides in length (Sosnowski et al. 1989; Kanaar et al. 1995). Oligomerization through the glycine-rich N-terminal region stabilises RNA binding. In addition, the N-terminal domain of Sxl participates in numerous interactions with different binding partners. In contrast, the function of the C-terminal part remains elusive (Wang et al. 1997). Sxl has versatile functions within the fly, since it is able to bind efficiently to RNA and interacts with specific protein partners. These skills enable Sxl to adapt to a respective mechanism, in which the protein can then act as promoter, inhibitor or fine-tuner. In females, Sxl is ubiquitously expressed and predominantly localized in the nucleus but due to its shuttling ability the protein can be exported. The localization pattern of Sxl within the germline is more dynamic. First, Sxl is mainly localized within the cytoplasm, peaking in cystoblasts and 2-cell stage of the germarium. Afterwards, a diffuse and low-level Sxl expression is detected in 4-16 cell cysts, while elevated and nucleic levels of Sxl protein were detected again at later stages (Bopp et al. 1991; Bopp et al. 1993). Moreover, the RNA binding protein Sxl is involved in a great number of posttranscriptional regulation pathways, ranging from splicing control to translational control. The following chapters will focus on the role of Sxl within each step of posttranscriptional regulation of its various target mRNAs.

1.5 Splicing of eukaryotic pre-mRNAs by the spliceosome

Splicing is a fundamental step in posttranscriptional gene regulation, in which exons are joined together. The splicing mechanism is highly dynamic and connects the accuracy of splice site recognition with the flexibility of splice site choice. Intronic regions are normally defined by three core splicing signals; the 5'splice site (5'ss), 3'splice site (3'ss) and a branchpoint which is located upstream of the 3'ss in its close proximity (18-40nt) and is next to a polypyrimidine tract (PPT) (Burge et al. 1999). The spliceosome assembles on the intron (Fox-Walsh et al. 2005) which is excised from the pre-mRNA by two constitutive transesterification reactions. Here, the free 2'OH group of the branchpoint adenosine attacks the phosphate group of the 5'ss guanosine (in rare cases also other than G are recognized), generating a 2'-5' phosphodiester bond. This is followed by a second transesterification step, during which the free hydroxyl group of the 5'ss attacks the phosphodiester bond of the 3'ss. This joins the two exons and releases the intron in form of a lariat structure (reviewed in Will and Lührmann 2011). Pre-mRNA splicing is (with few exceptions) catalyzed by the spliceosome, a multi-megadalton ribonucleoprotein complex, consisting of in total four small nucleolar ribonucleoprotein (snRNP) complexes (U1, U2, U5 and U4/U6 snRNPs) in case of the major spliceosome and several non-snRNP proteins. Each snRNP itself is composed of one snRNA (two in the case of U4/U6 snRNP) complexed with seven Sm proteins (B/B', D3, D2, D1, E, F and G) or Sm-like proteins (reviewed in Will and Lührmann 2011) and other additional proteins. For assembly of the spliceosome, the 5'ss and the branch point sequence are recognized by the U1 and U2snRNPs and their associated proteins. Initially, U1snRNP recognizes the 5'ss while the splice factor 1 (SF1) binds the branch point sequence (BPS) and the U2 auxiliary factor (U2AF, composed of the two subunits U2AF65 and U2AF35) is recruited to the PPT, generating the E-complex. Furthermore, the small subunit U2AF35 recognizes the AG of the 3'ss and is tightly bound with U2AF65 in a heterodimer. This reaction is followed by the base-pairing of U2snRNA with the BPS (A-complex), which is promoted and stabilized by other components of U2snRNP (SF3a and SF3b and the RS-rich domain of U2AF65). After reorganization of the A-complex, including the displacement of SF1 from the BPS, the trisnRNP, consisting of the preassembled U5 with U4/U6 snRNP, is guided to the A-complex, generating the pre-catalytic B-complex. Extensive remodeling of RNA-protein and RNA-RNA interactions results in destabilization and release of the U1 and U4snRNP (B^{act}-complex). Subsequent activation by the RNA helicase Prp2 generates the B*-complex. Prp2 stimulates the first transesterification step leading to additional rearrangements (C-complex) promoting the second nucleophilic attack. Finally, the spliceosome disassembles and the snRNPs are recycled (reviewed in Wahl et al. 2009; Will and Lührmann 2011).

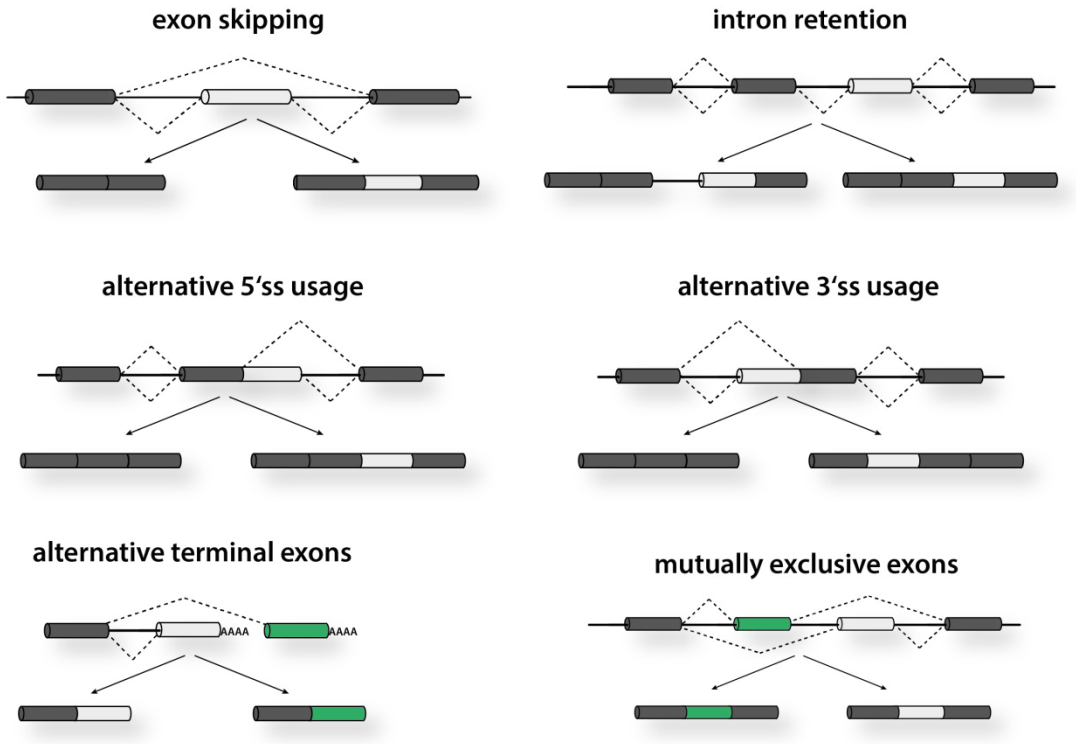

Figure 1.2: Overview of alternative splicing patterns. Alternative splicing events can occur individually, while also a combination of two alternative splicing events is possible.

Besides the canonical, constitutive splicing of mRNA, the majority of mRNAs is alternatively processed (Johnson et al. 2003; Modrek and Lee 2002). Constitutive splicing generates a single mature mRNA species. In contrast, alternative splicing generates a great variety of different splicing products. This can result in a large number of protein isoforms which can originate from one precursor transcript (Nilsen and Graveley 2010). Alternative splicing events are grouped in several major forms shown in Fig. 1.2: exon skipping, intron retention, alternative 5’ splice site and alternative 3’ splice site, alternative terminal exons and mutually exclusive exons (Kan et al. 2002; Wang and Burge 2008). The recognition of splice sites is regulated by enhancer and silencer sequences, located within the intron or the exon sequence. They help to define the exon intron border in alternative spliced transcripts but also in constitutively spliced transcripts. The principal aim of exonic splice enhancers (ESE) is the recruitment of proteins usually of the SR family. Hence, the interactions of SR proteins with respective spliceosomal components promote an efficient spliceosome assembly, even in the case of rather weak splice site sequences. Exonic and intronic splicing silencer (ESS and ISS) are often recognized by members of heterogeneous nuclear ribonucleoprotein family (hnRNPs), which act as splicing repressors and interfere with splicing by various mechanisms. For instance, the protein hnRNP I competes with U2AF for binding. In contrast, the silencing activity of hnRNP A1 is varying depending on the sequence context. The protein is able to interfere with the binding of SR proteins but also with the binding of U1 and

U2snRNP to the splice sites (reviewed in Wang and Burge 2008; Matlin et al. 2005). In general, the definition of hnRNPs is rather broad. They are defined by containing one or more RNA binding domains (RRM-like, KH-like or RGG-like). In addition, the majority of hnRNPs contain auxiliary domains, such as proline-, glycine- or acid-rich domains which are often linked to splicing inhibitory activities (Geuens et al. 2016). In this view, the domain architecture of Sxl, which contains an N-terminal glycine-rich region and two RRM domains strongly resembles the members of the hnRNP family (Matlin et al. 2005). The activity of Sxl as a splice silencer requires a defined sequence context within the regulated mRNAs. In addition, Sxl mediates a fine-tuned regulation of alternative splicing by acting in concert with different co-factors and competitors. The following paragraphs will describe the great variety of interactions in which the protein Sxl is involved to fulfill its function as the major regulator of female development.

1.6 Female-specific, alternative splicing of the *Sxl* pre-mRNA

To establish its continuous expression in female flies, Sxl regulates alternative splicing of its own primary transcript. It interferes with the inclusion of the male-specific exon 3, referred to as the “poison exon” because it harbours a premature stop codon. In males, exon 3 is included in the *Sxl* mRNA, resulting in the expression of a truncated, nonfunctional Sxl protein (2-3-4). In contrast, in females, skipping of exon 3 and the joining of exon 2 with exon 4 results in production of a functional full-length Sxl protein (2-4). The control of *Sxl* mRNA splicing is far more complex than previously expected, and many protein interaction partners support Sxl in regulation. The “hotspots” within the *Sxl* mRNA which are bound by several Sxl proteins are located approximately 200 nucleotides upstream and downstream of the poison exon (Horabin and Schedl 1993a; Horabin and Schedl 1993b). Once the protein has recognized its binding sites, Sxl is able to interact with components of the U1snRNP and U2snRNP. However, Sxl does not act by simply displacing the U1snRNP from the 5’ss splice and its impact on splicing is based on a variety of interactions with several other splicing components (Johnson et al. 2010). One of these factors is Sans fille (Snf), which shares a high degree of homology with the mammalian proteins U1A and U2B`` (Samules et al. 1998; Flickinger and Salz 1994; Harper et al. 1992). Both proteins, Sxl and Snf, directly interact with their RRM domains independently of wether Snf is complexed with U1snRNP or not (Nagengast 2003). Interestingly, some mutations within Snf lead to the disruption of the *Sxl* auto-regulatory splicing cascade (Bopp et al. 1993; Albrecht and Salz 1993; Salz and Flickinger 1996). Besides Snf, also another protein component of the U1snRNP, namely U1-70K, is known to become essential for *Sxl* splicing in a sensitized background of low Sxl and Snf levels (Nagengast 2003). In addition to the stable complex formation between U1snRNP, Sxl and Snf, the transcription factor homolog protein partner of sans fille (PPS) was also

found to be associated with the *Sxl* mRNA, whereas its function remains to be elusive (Johnson et al. 2010). This example nicely demonstrates the robustness of the *Sxl* splicing cascade in which redundant interactions ensure exon skipping and therefore guarantee female-specific Sxl expression (Fig. 1.3 B). Moreover, Snf does not contribute to splicing only by its interaction with the U1snRNP, but it has been also described as a component of the U2snRNP (Harper 1992). By that, a similar interaction of Sxl and Snf in the context of the U2snRNP could be possible as well.

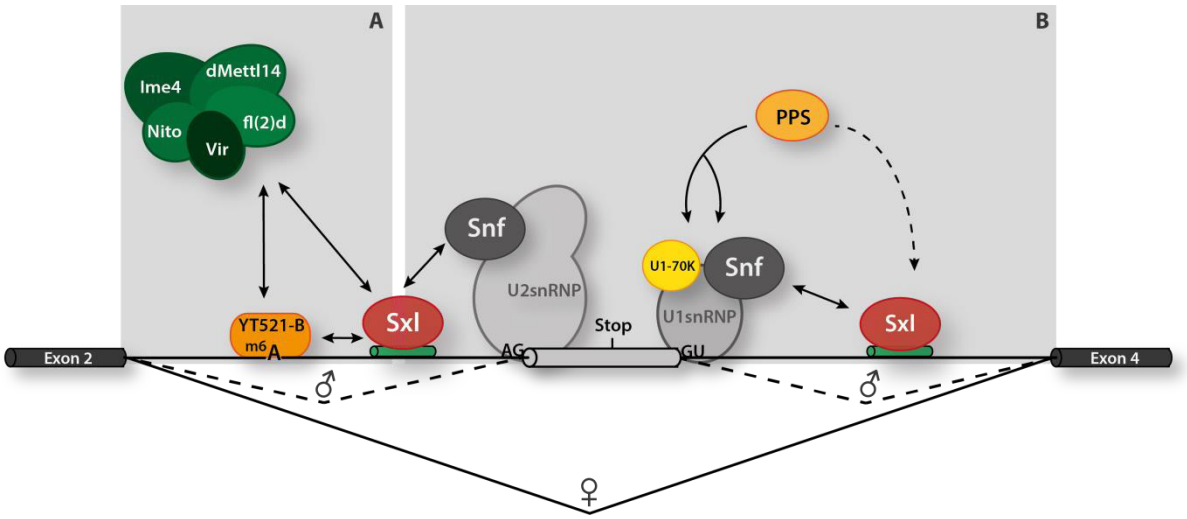

Figure 1.3: Alternative splicing of *Sxl* pre-mRNA is regulated by the Sxl protein. (A) Female-specific splicing of *Sxl* pre-mRNA is promoted by methylation marks in close proximity to Sxl binding sites. Methylation sites are modified by the METTL complex consisting of Ime4, dMettl14, Nito, Virilizer (Vir) and Fl(2)d (green). The modification is recognized by the reader protein YT521-B (orange) which is also directly interacting with Vir (dark green) and Sxl (red). This interaction network enforces the female-specific splicing pattern. **(B)** *Sxl* splicing is regulated by the Sxl protein which directly interferes with spliceosomal components of U1snRNP (grey) and U2snRNP (grey) and finally promotes female-specific exon skipping.

1.7 The interplay of alternative splicing and RNA methylation on the *Sxl* pre-mRNA

Sxl autoregulatory splicing also depends on several proteins that are involved in RNA methylation. The protein Vir was first described in 1995 to promote the sex-specific splicing of *Sxl*. The absence of Vir was shown to lead to female-specific lethality in flies (Hilfiker et al. 1995). A second protein, namely Fl(2)d was also described to interact directly with Sxl protein and to promote *Sxl* sex-specific splicing: a loss of function mutation led to a male-specific splice pattern (Granadino et al. 1996). After two decades of research, these two proteins were identified as important components of the methyltransferase complex in *D. melanogaster*. The methylation marks introduced by this RNA m^6A methyltransferase complex were shown to be necessary for the sex-specific splicing of *Sxl* mRNA and of other transcripts (Lence et al. 2016; Haussmann et al. 2016). In general, the complex methylates

adenosines (embedded in “RRACH” motifs; A methylated A; R=purine; H=A, C or U) at the N6 position. By that, RNAs are marked for subsequent steps of post-transcriptional RNA processing, like pre-mRNA splicing or decay (Meyer et al. 2012). In mammals, recent findings indicate that mRNA modifications are associated with enhanced cytoplasmic mRNA turnover rather than pre-mRNA splicing (Ke et al. 2017). However, in *Drosophila* m^6A RNA methylation is mainly linked to alternative splicing. This finding is supported by the fact that the absence of m^6A results in an overall change in alternative splice patterns in 2% of all transcripts. Among them, a bias of 75% was observed for alternative splicing events occurring within 5’UTRs (Lence et al. 2016; Haussmann et al. 2016).

The composition of the methyltransferase complex strongly resembles the orthologs of the human complex, which is composed of METTL3, METTL14, and WTAP which correspond in D. melanogaster to Ime4, dMETTL14 and Fl(2)d, respectively. Other associated proteins are KIAA1429, the ortholog of Vir and RNA-binding motif protein 15 (RBM15), which is known in flies as Spenito (Fig. 1.3 A) (Lence et al. 2016; Haussmann et al. 2016, Liu et al. 2015; Horiuchi et al. 2013; Wang et al. 2016a; Wang et al. 2016b). In general, the effects of a single knockout of individual components of the METTL complex are rather mild in flies compared to mammals. Although flies are still viable, they show defects in locomotion, in their behavior and their lifespan is reduced. The fact that the m^6A methylation of the *Sxl* mRNA and its splicing are tightly linked becomes apparent when looking at *Sxl* splice patterns in fly strains depleted of components of METTL complex. The knockout of Ime4 shifts the *Sxl* splicing pattern towards the male-specific transcript isoforms in females, but the flies are viable. In contrast, an Ime4 knock out in a heterozygous *Sxl* background leads to female-specific lethality, whereas males remain unaffected. This supports the hypothesis that the splicing reaction is dependent on Sxl binding and also depends on methylation marks in close proximity to the respective Sxl binding sites (Lence et al. 2016; Haussmann et al. 2016). M^6A modifications on the mRNAs are recognized by specialized reader proteins. While in mammals five members of two subfamilies have been characterized (YTHDC1/2, YTHDF1/2/3) (Roignant and Soller 2017), only two reader proteins, YT521-B and CG6422, were identified in *D. melanogaster*. Of note, the impact of the m^6A methylation on dosage compensation and locomotion is mainly linked to the activity of the nuclear reader protein YT521-B, while the influence of CG6422 is rather limited in this context (Lence et al. 2016). Intriguingly, YT521-B is involved in *Sxl* splicing, as its knock out induces male-specific splicing pattern in females. Moreover, forced overexpression of YT521-B was found to be associated with male lethality but can be rescued by the deletion of Ime4 (Lence et al. 2016; Haussmann et al. 2016). Until today, it remains to be determined how exactly YTH proteins behave when they recognize the m^6A-modified mRNA and if a celltype- and transcript-

specific recognition is facilitated by interactions with specific RNA binding proteins, such as Sxl or Ssx (Lence et al. 2016) or other Hu-related proteins (Lence et al. 2017).

1.8 Alternative splicing of the *tra* pre-mRNA

The development of female morphology and behavior is mostly followed by the expression of tra which is under the control of Sxl. Female Tra protein in here pairs up with the ubiquitous expressed Tra2 protein and promotes the production of a female-specific isoform of the transcription factor *dsx* and *fru* that drive female development.

Once female Dsx and Fru protein versions are expressed, these transcription factors guide fundamental steps in fly development defining the female identity characterized by the appropriate morphology and behavior (Lopez 1998; Graveley 2002; Penalva and Sanchez 2003; Salz and Erickson 2010; Salz 2011). Similarly to *Sxl*, also *tra* transcripts are produced in both sexes, whereby a functional transcript variant is exclusively processed in females. The production of Tra protein in female flies is regulated by Sxl on the level of splicing, suppressing the proximal 3'ss and directing splicing to a distal site further downstream. In the *tra* mRNA, intron 1 harbors two alternative 3' splice sites, while the PPT of the proximal 3'ss has an approximately 100-fold higher affinity for the heterodimeric splicing factor U2AF. Usage of the proximal 3'ss results in the inclusion of a premature termination codon which subsequently leads to the translation of a truncated, non-functional protein in males (Valcarcel et al. 1993). In females, Sxl has a high binding affinity exclusively for the proximal 3'ss, while U2AF is, in theory, able to interact with both PPTs to a similar extent. This results in the competition between Sxl and U2AF at the favored proximal 3'ss and ultimately shifts the binding of U2AF towards the weaker distal 3'ss. Consequently, the U2snRNP is recruited to the distal 3'ss which gives rise to a spliced mRNA that encodes for the functional, full-length Tra protein (Fig. 1.4) (Sosnowaki et al. 1989; Inoue et al. 1990; Valcarcel et al. 1993).

A deletion of the first 40aa of the Sxl protein was shown to severely impair its ability to regulate *Sxl* and *tra* splicing (Wang and Bell 1994; Yanowitz et al. 1999). Sxl binds RNA motifs with its central region, hence RNA binding is not affected by the deletion of the N-terminus. Therefore, a simple competition reaction between Sxl and U2AF could be excluded. Moreover, an overexpression of the Sxl N-terminus in males showed a splicing shift towards the distal 3'ss by a yet unidentified mechanism (Desphande et al. 1999). This further demonstrates the importance of N-terminal protein-protein interactions of Sxl with not yet characterized protein partners to ensure proper *tra* splicing in females to trigger the female development.

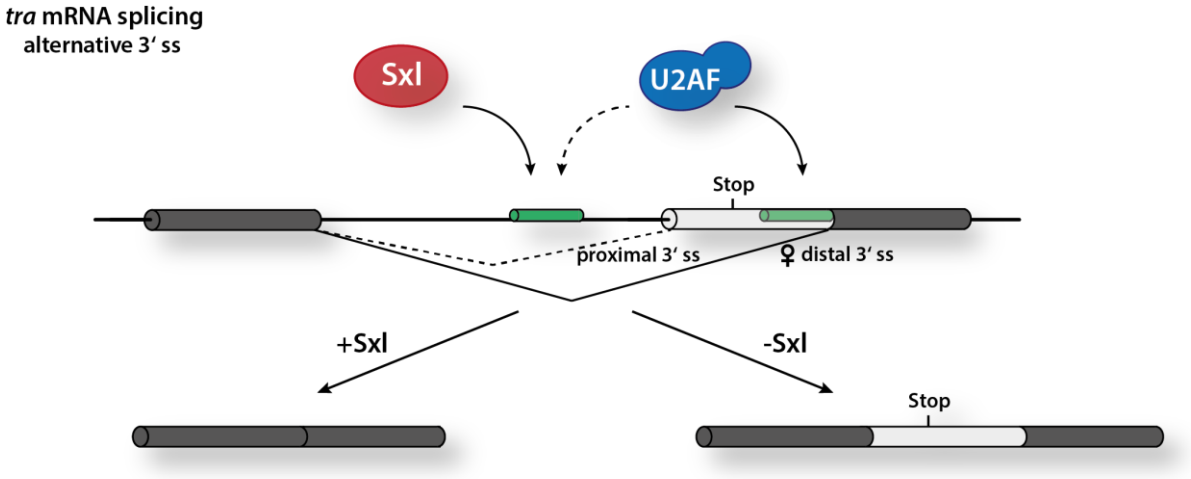

Figure 1.4: Sxl regulates the alternative 3'ss usage in the *tra* pre-mRNA. Here, Sxl (red) competes with U2AF (blue) for binding to the proximal PPT (green) and forces binding of U2AF to the distal PPT (light green) resulting in the female-specific splicing of the *tra* mRNA.

1.9 Alternative splicing of the *msl-2* pre-mRNA

Dosage compensation in *D. melanogaster* is achieved by the hypertranscription of the single male X chromosome. In females, hyperactivation of the X-chromosomes has to be repressed in female flies to ensure equal gene doses between sexes (reviewed in Conrad and Akhtar 2012). The hypertranscription is mediated by the DCC, with Msl-2 protein as the limiting component which is not expressed in female flies. This strict control of *msl-2* expression is achieved by several mechanisms acting at different post-transcriptional levels (Bashaw and Baker 1997; Gebauer et al. 1998; Kelley et al. 1997). In contrast to the fail-safe regulation of *Sxl* expression, which is exclusively based on alternative splicing, robustness in the regulation of *msl-2* expression is achieved by the interplay of several processes. These comprise the regulation of alternative splicing, mRNA export and translational control. The central function of Sxl in these pathways will be discussed separately in the following chapters.

First of all, sex-specific, alternative splicing of *msl-2* is necessary in females to include the facultative 5'UTR intron which harbours Sxl binding sites for translational regulation. Alternative splicing regulation of *msl-2* is based on the competition between the Sxl and the heterodimeric splicing factor U2AF resulting in intron retention. Consequently, female-specific transcripts harbor additional 133nt within their 5'UTRs compared to their male counterparts (Fig. 1.5) (Zhou et al. 1995; Bashaw and Baker 1997; Merendino et al. 1999; Yanowitz et al. 1999). Similarly to the *tra* transcripts (see chapter 1.8), *msl-2* transcripts contain two U-stretches in the intronic region in close proximity of the respective 5' and 3'ss (Gebauer et al. 1998; Forch et al. 2001). One of these U-stretches is embedded in the PPT and is recognized by U2AF65. However, due to the relative long distance between the 3'ss

and the intronic PPT, the interaction between U2AF35 and the 3'ss cannot be established. This weakens the binding affinity of U2AF65 to the RNA. This weakness is exploited by Sxl which efficiently competes with U2AF for binding and thereby stimulates intron retention in the *msl-2* mRNA (Merendino et al. 1999). In addition to the competition with U2AF, Sxl interferes with TIA-1 (Rox8 in *D. melanogaster*) for binding to the corresponding U-stretch at the 5'ss within the 5'UTR of *msl-2* (Fig. 1.5). Rox8/TIA-1 normally promotes binding of the U1snRNP to the 5'ss (Del Gatto-Konczak et al. 2000; Forch et al. 2000). Thus, competition between Sxl and TIA-1/Rox8 prevents recruitment of the U1snRNP and hinders the removal of the 5'UTR intron (Förch et al. 2001). In sum, together with different co-factors Sxl promotes the inclusion of the 5'UTR intron in the *msl-2* mRNA to ensure efficient downregulation of *msl-2* expression.

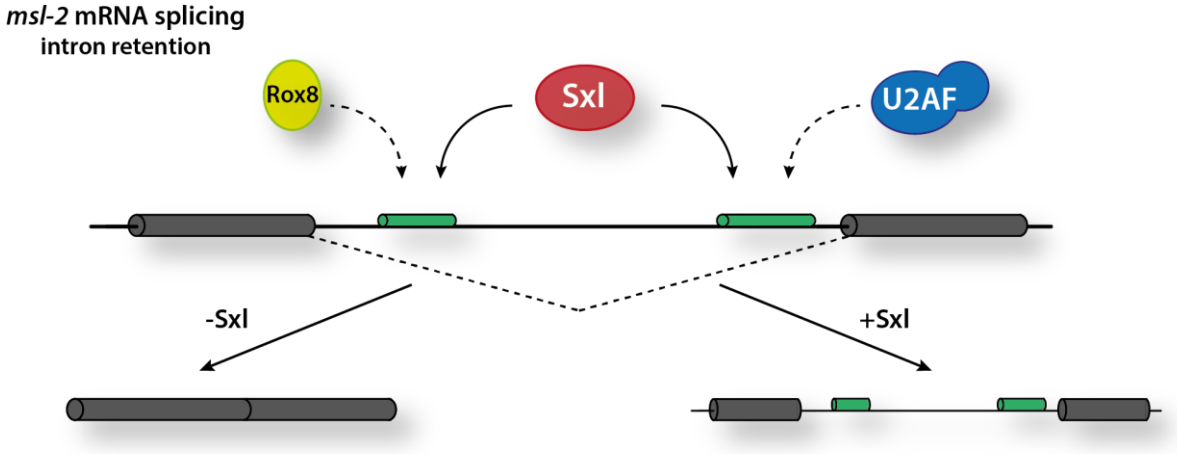

Figure 1.5: Intron retention in the 5'UTR of the *msl-2* mRNA is promoted by Sxl. Sxl (red) competes with Rox8/TIA (yellow) and U2AF (blue) for binding to U-rich sequences (green) at the 5'ss and 3 ss, respectively. By that, Sxl forces alternative splicing of *msl-2* leading to intron retention in female flies.

1.10 Sxl regulates alternative polyadenylation and interferes with the nuclear export of specific mRNAs

Besides splicing, the alternative polyadenylation of mRNAs is an additional regulatory mechanism that determines the fate of mRNAs. In general, poly(A) tails are an important feature to be later on recognized by the nuclear export machinery and they can further influence the translational efficiency. Importantly, Sxl was shown to influence the poly(A) site choice of the *enhancer of rudimentary* (*e(r)*) mRNA in the female germline. Here, alternative poly(A) site choice of *e(r)* mRNA is established by a competition between Sxl and CstF-64 for binding to the proximal, male-specific poly(A) signal. This competition and the switch in poly(A) site choice results in the production of an extended, female-specific transcript wich exhibits a reduced translation efficiency (Gawande et al. 2006). The impact of Sxl on co- and

post-transcriptional processing events is not limited to alternative polyadenylation. In addition, Sxl prevents together with the protein Held out wings (How) the nuclear export of the *msl-2* mRNA.

Alternative splicing of *msl-2* is followed by the nuclear retention of *msl-2* transcripts. Here, the retained 5'UTR intron harbors two U-stretches which are bound by Sxl, while additional flanking sequences were shown to be bound by the How protein. Sxl and How interact directly with each other and can bind independently to the *msl-2* mRNA. Together, they retain the transcript in the nucleus, while the detailed role of Sxl in the *msl-2* mRNA export needs further investigation. Moreover, the molecular details of this mechanism and the potential involvement of further interactors still remain to be clarified (Graindorge et al. 2013).

1.11 Translation of mature mRNAs in eukaryotes

In the cytoplasm, mRNAs serve as templates for the translation of proteins. Compared to the post-transcriptional control of gene expression, translational regulation enables the most rapid changes in protein abundance, allowing an efficient adaption of the protein levels to specific requirements. Basically, the translation of an mRNA depends on the molecular features which were added during the maturation of pre-mRNAs: A 3' poly(A) tail and a 5' 7-methylguanosine (m^7G) cap. The majority of eukaryotic mRNAs is translated by the cap-dependent mechanism. In general, translation initiation is the rate limiting step during protein expression and starts with the recruitment of the PIC to the AUG start codon at the 5'end of the mRNA. The PIC itself is a ternary complex, composed of the initiator methionyl tRNA (Met-$tRNA_i$) and the GTP-bound version of eukaryotic initiation factor 2 (eIF2), attached to the 40S small ribosomal subunit. Furthermore, the eIFs 1, 1A, 5 and eIF3 are also participating in this interaction. PIC recruitment takes place in a so-called "closed loop" formation of the mRNA, which is characterized by the functional interaction of the 5' to 3' end of the mRNA. In this conformation, the 5' cap with the bound cap binding complex interacts with 3' poly(A) tail and its bound Poly(A) binding protein C1 (PABP-C1), enabling an efficient translation of the mRNA. The 5' cap structure is recognized by the eIF4F complex, which is comprised of the cap binding protein eIF4E, the scaffolding protein eIF4G and the RNA helicase eIF4A. EIF4G also directly interacts with PABP resulting in the "circularization" of the linear mRNA. This "closed loop" model could explain how 3'UTR-bound regulatory factors can interfere with and regulate translation initiation which occurs at the 5'end of the mRNA (reviewed in Graindorge et al. 2011; Hinnebusch and Lorsch 2012). Once the PIC assembles on the mRNA, it scans for an appropriate AUG start codon. Specific sequences in proximity of the start codon have been shown to contribute to initiation codon selection (Kozak 1987). Finally, base pairing between the anticodon of the Met-$tRNA_i$ and the AUG occurs, triggering the joining of the large (60S) ribosomal subunit and, by that, the formation

of the elongation-competent 80S ribosome (reviewed in Jackson et al. 2010; Hinnebusch and Lorsch 2012).

Scanning by the PIC takes place in 5' to 3' direction, which determines the order of AUG recognition. If the first AUG triplet is embedded in an unfavorable sequence context, it can be skipped and the AUG further downstream is selected (a mechanism called leaky scanning) (Ingolia 2016). In contrast, the recognition of upstream open reading frames (uORFs) preceding the downstream main open reading frame (ORF) may influence the translation efficiency by various mechanisms (Hinnebusch et al. 2016). Indeed, Sxl has been shown to interfere with the translation of *msl-2* via two different pathways acting in the context of translational initiation. First, Sxl impedes the recruitment of PIC in the closed loop conformation (Gebauer et al. 2003) and second, Sxl cooperates with an uORF hindering the scanning PIC to reach the main ORF (Beckmann et al. 2005; Medenbach et al. 2011).

1.12 Cytoplasmic function of Sxl and translational control of the *msl-2* mRNA

The regulatory activity of Sxl on translation is best studied for translation of *msl-2*. As already mentioned in previous chapters, the strict inhibition of Msl-2 expression is essential for female survival. The Msl-2 protein is the limiting component of the DCC which enables the hypertranscription from the single male X chromosome, while this has to be prevented in female flies to guarantee equal gene doses (reviewed in Conrad and Akhtar 2012). Sxl successfully prevents expression of *msl-2* by several pathways (splicing, mRNA export and translational regulation). The *msl-2* transcript itself harbors six binding sites for Sxl within its UTR's, with two located in the facultative intron of the 5'UTR (A site and B site), which is spliced out in males, and four in the 3'UTR (C-F site) (Bashaw and Baker 1997; Gebauer et al. 1998; Kelley et al.1997). Although the general Sxl binding site is represented by an U-stretch which can be interrupted by single guanosines, the composition of the six binding sites within *msl-2* differs. In general, the 5'UTR sites are longer than their 3'UTR counterparts (U11/U16 versus U9/U7). Importantly, the 3'UTR binding sites (Esite and Fsite) are adjacent to the binding sites for the cold-shock domain protein Upstream of N-ras (UNR) (Bashaw and Baker 1997; Gebauer et al. 1998; Duncan et al. 2006). Not only the binding site composition between 5'UTR and 3'UTR differs, also the mechanisms of translational regulation are distinct and interfere with different steps of translational initiation (Beckmann et al. 2005).

Translational repression mediated by the 3'UTR Sxl-binding sites takes place in the early steps of translational initiation and prevents the recruitment of the small ribosomal subunit to the *msl-2* mRNA (Gebauer et al. 2003). As already demonstrated for other regulatory pathways, Sxl often exerts its function together with additional co-factors. In fact, for translational repression via the *msl-2* 3'UTR, binding of the co-repressor UNR is of critical importance. Although UNR is an RNA-binding protein, in the case of *msl-2*, UNR is not able

to bind in the absence of Sxl, which is required to recruit UNR to the mRNA (Grskovic et al. 2003; Abaza et al. 2006; Duncan et al. 2006). Recently, a crystal structure gave detailed insights into the cooperative binding of Sxl and of the cold shock domain 1 (CSD1) of UNR to the *msl-2* mRNA. The binding of Sxl to the proximal part of the mRNA resembles the RNA binding behavior observed for the binding to the *tra* mRNA (Handa et al. 1999). Here, the region downstream of the Sxl binding site gets enclosed by Sxl and UNR and folds around the RRM1 of Sxl, forming additional stabilizing contacts. Of note, the repressive complex consisting of Sxl and UNR-CSD1 bound to the *msl-2* mRNA forms a molecular zipper, underlining the strong interaction between each single component, which together enable the downregulation of *msl-2* translation (Hennig et al. 2014).

The question whether Sxl acts as a direct translational repressor or as an assembly factor requiring the activity of other interaction partners was addressed by tethering studies. These experiments suggested that Sxl recruits other components needed for translational repression via the *msl-2* 3'UTR mechanism, since forced binding of Sxl to the mRNA did not lead to translational repression. In contrast, directly tethered UNR leads to efficient down regulation of *msl-2* expression even in the absence of Sxl (Grskovic et al. 2003). This further demonstrates the mutual dependency between Sxl and UNR for translational repression of *msl-2* in *D. melanogaster*. Sxl confers a sex-specific function to UNR, since UNR is present in both sexes but depends on female-specific Sxl protein to be recruited to the mRNA. Besides the interaction with Sxl, UNR directly interferes with PABP (Fig. 1.6 B) (Duncan et al. 2009). As already mentioned, the contact of the poly(A) tail-bound PABP with the cap binding complex component eIF4G forms a stable closed loop, which leads to efficient translation initiation. Since this conformation was detected via a GRNA approach in *msl-2* particles which were associated with UNR, the interaction of UNR and PABP was proposed to take place after the formation of the closed loop. Nevertheless, further studies are required to uncover the exact mechanism (Duncan et al. 2009). In theory, the closed loop promotes translation initiation, but the repressive complex was shown to inhibit the translation of non-adenylated mRNAs as well. Thus, additional and yet unidentified repressor components might be targeted to the repressed transcripts after the closed loop formation occurred (Gebauer et al. 1999; Gebauer et al. 2003; Duncan et al. 2009).

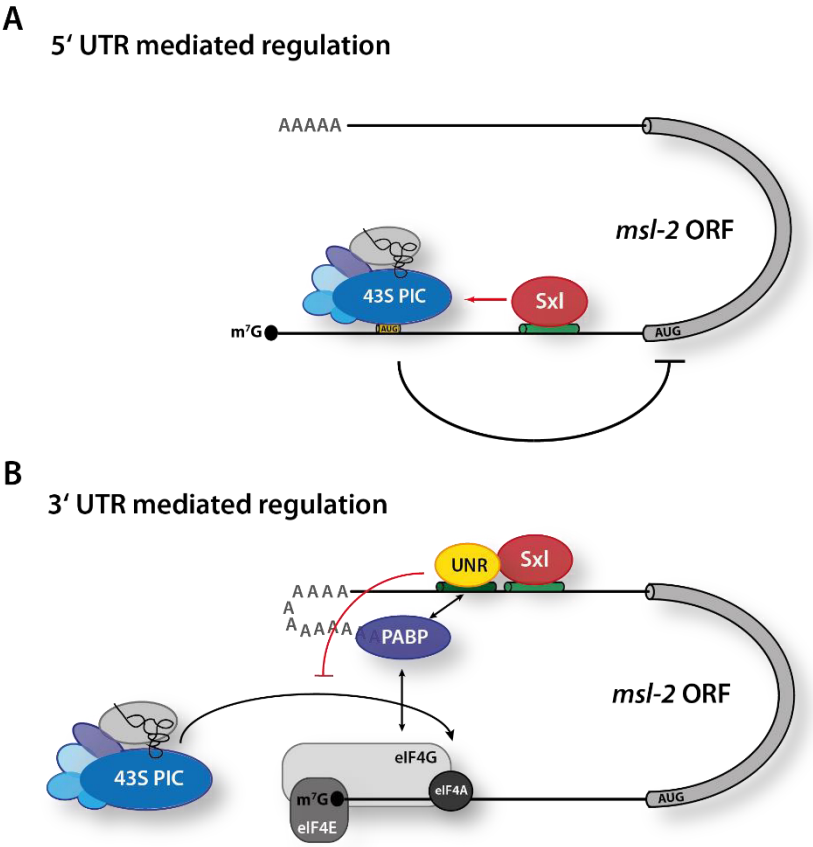

Figure 1.6: Sxl-dependent regulation of *msl-2* translation by a dual mechanism. A) Within the 5' UTR mediated mechanism, Sxl (red) is bound to its binding site (green) and interferes with a scanning ribosome (blue) and inhibits the recognition of the main open reading frame. **B)** The 3'UTR mediated mechanism is based on the binding of Sxl (red) with UNR (yellow) to the binding sites within the closed loop. UNR directly interacts with the PABP (purple). The inhibition of the recruitment of the 43S PIC (blue) to the mRNA is promoted.

MRNAs which escaped the 3'UTR translational repression are regulated by the 5'UTR mechanism, intervening in the second step of translational initiation. Sxl attached to its binding site activates an upstream open reading frame and interferes with the scanning of the small ribosomal subunit on the mRNA. This functional interaction prevents the recognition of the main *msl-2* ORF AUG by the 43S ribosomal complex (Fig. 1.6 A). Importantly, this scenario cannot be explained by a simple roadblock model. Replacing of Sxl with another high affinity RNA binding protein does not recapitulate the full translational repression. Still, the molecular details of the 5'UTR repressive mechanism remain to be identified (Medenbach et al. 2011). Why are two independent but synergistic strategies required for the complete translational repression? In the fly, isolation of one mechanism (either 3' or 5') does not lead to a complete translational inhibition, allowing the expression of some Msl-2 protein (Bashaw and Baker 1997; Kelley et al. 1997). To prevent dosage compensation in female flies and to inactivate the DCC, the complete leak-proof shutdown of *msl-2* expression is guaranteed by the synergism of 5' and 3' Sxl binding sites.

1.13 A well-balanced Sxl expression is mediated by its 3'UTR

Sxl's formula for success lies within the ability to adjust its concentration at a specific developmental stage. Too much or too little Sxl protein expression comes along with sex-specific lethality, sterility and sexual transformations (reviewed in Cline and Meyer 1996). For instance, removal of the Sxl locus in combination with heterozygous Sxl overexpression enables the study of male escaper flies. These males demonstrate the deleterious effects of inappropriate Sxl expression, suffering from intersexuality and sterility having rotated genitalia, reduced sex combs and a lighter abdomen color compared to wildtype flies (Yanowitz et al. 1999). Male identity is established by a default splicing pattern of the *Sxl* mRNA, generating an entirely nonfunctional protein and thus avoiding the repression of dosage compensation. As already described, Sxl defines the female identity by the establishment of an auto-regulatory positive feedback loop to ensure a sustained expression of Sxl (Bell et al. 1991; Horabin and Schedl 1993b). In females, Sxl also interferes with the translation of X-linked factors and promotes the reduced expression of X chromosomal encoded mRNAs (Horabin 2005; Penn and Schedl 2007). While the feedback splicing loop in females guarantees the expression of Sxl throughout the female life, an additional mechanism has to hinder an uncontrolled snowballing of Sxl expression. If not, this could end in a toxic accumulation of Sxl protein with devastating consequences. For instance, Notch is a protein necessary for development of proper female morphology and its expression is fine-tuned by Sxl. A deregulation of *Notch* mRNA by too little or too much Sxl protein is associated with phenotypically abnormalities in the wing, bristles and others (Penn and Schedl 2007; Suissa et al. 2010).

This raises the question how females are keeping the balance to express Sxl in adequate and physiological levels. The 3'UTR of *Sxl* mRNA harbours several Sxl binding sites, while transcripts of early embryonic stages differ in their length compared to dominant Sxl transcripts at later developmental stages. While the majority of the early transcripts have short 3'UTRs with fewer Sxl binding sites, the 3'UTR length and number of binding sites increases over development. Thus, Sxl, which is stably expressed at high levels in adult females, binds to a great number of U-stretches within the longer 3'UTRs of the *Sxl* mRNA, and, by that, limits the further production of Sxl protein probably via translational downregulation (Yanowitz et al. 1999). However, the molecular details on how this is achieved remain uncharacterized in particular whether Sxl exerts this regulation in combination with other co-repressors. Interestingly, also the hnRNP protein Hrp48 was postulated to be a repressor of Sxl expression. Here, Hrp48 restricts the expression of Sxl to specific tissues, which, in turn, ensures the fine-tuned Sxl-mediated regulation of *Notch* expression (Suissa et al. 2010).

1.14 Sxl-related proteins

Sxl has similarities to the conserved family of the *embryonic lethal abnormal visual system* (ELAV)/Hu proteins, but although Sxl is evolutionary related, the protein is not a member of this protein family. In *D. melanogaster* ELAV proteins encompass three members, namely embryonic lethal abnormal visual system (ELAV), RNA binding protein 9 (RBP9) and found in neurons (FNE) (Campos et al. 1985; Kim and Baker 1993; Samson and Chalvet 2003). In mammals, the homologous family of Hu-proteins comprises the four members HuB, HuC, HuD and HuR (reviewed in Hinman and Lou 2008, Colombrita et al. 2013). All ELAV/Hu family members are characterized by three RNA recognition motifs (RRM), whereby RRM1 and RRM2 are required for binding to RNA targets harboring AU-rich elements (ARE) (Yannoni and White 1999). Also GU-rich or U-rich stretches have been identified as high affinity targets of this protein class (Colombrita et al. 2013). RRM1 and RRM2 are separated from RRM3 by a highly variable hinge region. The latter domain was shown to interact with poly(A) sequences and to stabilize RNA-protein interactions. The hinge region itself shows the lowest sequence conservation in ELAV proteins and in addition to mediate protein-protein interactions it also encodes a nuclear localization signal which enables nuclear shuttling (Fan and Steitz 1998; Colombrita et al. 2013). Sxl is the closest related protein to ELAV proteins, albeit it lacks RRM3 (Birney et al. 1993). The close relation of Sxl to the ELAV family members becomes especially apparent when comparing their binding properties and their partially redundant functions.

Members of the ELAV and Hu family are predominantly expressed within the neuronal system (HuR is ubiquitously expressed, FNE and RBP9 are also found in ovary and testis). They are multi-functional proteins, which interfere with various processes e.g. alternative splicing, polyadenylation, regulation of mRNA stability and transport and translational regulation. Similar to Sxl, the majority of the ELAV family members in *Drosophila* (ELAV and RBP9) is primary localized in the nucleus and participates in the regulation of alternative splicing and polyadenylation. In contrast, mammalian members are mainly found in the cytoplasm (all but HuR) and are predominantly involved in RNA transport, regulation of mRNA stability and translation but they also contribute to alternative splicing and polyadenylation. Like Sxl, ELAV functions as a splice regulator and modulates the neuronal-specific alternative splicing of selected transcripts. For instance, in *Drosophila* ELAV promotes exon skipping in the *armadillo* mRNA and enhances intron splicing in *ewg* transcripts, ensuring correct isoform expression which contributes to a functional nervous system (Koushika et al. 1996; Koushika et al. 2000). Moreover, a nice example of the regulation of alternative splicing by ELAV/Hu proteins is represented by the mammalian calcitonin/CGRP pre-mRNA. Here, nuclear Hu proteins promote exon skipping of the non-

neural exon nr.4, enabling the expression of the neurotransmitter CGRP. In contrast, the transcript version with the included exon results in the expression of calcitonin. This was achieved by the competition of Hu proteins and TIA-1/TIAR for binding to an U-rich sequence (Zhu et al. 2006). The competition with TIA1 and TIAR is not restricted to the calcitonin/CGRP mRNA, since a similar mechanism has been proposed to occur for neurofibromatosis type 1 pre-mRNA, Ikaros pre-mRNA and others (Bellavia et al. 2007; Zhu et al. 2008). The primary and most prominent task of ELAV-like proteins is the binding to AU-rich RNA elements (ARE) within 3'UTRs and, by that, enhancing or reducing the stability of mRNAs. While in *Drosophila*, FNE and RBP9 lead to mRNA destabilization, in mammals each Hu protein, besides HuR, ensures a prolonged mRNA half-life. For instance, by binding to AU-rich elements within the 3'UTR of the neuroserpin mRNA and Gap43 mRNA, HuD enhances the mRNAs half-life (Mobarak et al. 2000; Cuadrado et al. 2002). This is thought to be achieved by the competition between Hu members and other ARE interacting proteins, which would normally trigger exosomal degradation (Colombrita et al. 2013). Furthermore, the binding of ELAV-like proteins to AREs resembles the binding of Sxl to its targets. ELAV, FNE and also HuR were shown to restrict their own expression by similar negative auto-regulatory feedback loops. Once a certain threshold is reached, the proteins bind AREs and directly interfere with the polyadenylation factors Cpsf160 and CstF64. Consequently, an alternative poly(A) site choice is promoted, resulting in altered or destabilized transcripts which contain an elevated number of ARE or miRNA binding sites. These sequence elements prevent an excessive protein accumulation and therefore, they are required to ensure viability (Samson 1998; Samson and Chalvet 2003; Borgeson and Samson 2005; Dai et al. 2012). A prominent target of ELAV family members and the main target of Sxl is a U-stretch or a UG-stretch (Sosnowski et al. 1989). To some extent, ELAV members and Sxl can partially overlap in their cellular regulatory tasks, since it was postulated that RBP9 can also interfere with the expression of *msl-2* and, by that, regulates the formation of the DCC (Zarahieva et al. 2015). Taken together, the main difference between Sxl and ELAV/Hu proteins only partially lies within their molecular structure but is rather based on their cellular localization, distribution and expression level.

1.15 How Sxl became the master regulator of sex determination

The fact that Sxl determines the sexual identity is restricted to the clade of *Drosophila*, whereas in other flies the Tra protein is the master regulator in females (reviewed in Pane et al. 2002; Penalva and Sanchez 2003; Cline et al. 2010; Hediger et al. 2010; Verhulst et al. 2010). In other flies, for instance in the housefly (*Musca domestica*) and the medfly (*Ceratitis capitata*) Tra is at the top of the sex determination cascade and directly triggers female-specific splicing of *dsx* which, in turn establishes the female developmental program. The

maternally provided Tra protein establishes an auto-regulatory feedback loop to sustain the expression of Tra protein, which a similar situation is occurring in *D.melanogaster* with Sxl. In males, this enrichment of functional Tra protein is prevented by the paternal-transmitted factor M, which interferes with the auto-regulatory feedback loop (Pane et al. 2002; Hediger et al. 2010). Intriguingly, other Drosophilids and flies outside the *Drosophila* clade also express an Sxl ortholog, which lacks the key structural components necessary for female-specific functions, and which is also expressed in both sexes (Traut et al. 2006; Siera and Cline 2008). The functional role of the “non-sex-specific” Sxl protein remains enigmatic. It is assumed from evolutionary analyses that Sxl gained its new function approximately 100 million years ago, when the ancestors of the medfly and the fruitfly separated their ways (Cline et al. 2010). At the same time, a gene duplication event generated the closely related paralog of Sxl, which has been named Sister-of-Sex-lethal (Ssx) (Traut et al. 2006). The uncharacterized protein Ssx was initially thought to execute the function of the ancestral non-sex-specific Sxl protein. More recent evolutionary analyses argue against the hypothesis that Ssx fulfills the task of the ancestral protein, while Sxl underwent a neo-functionalization acquiring its female-specific function. Instead, these findings supported the idea that the proteins underwent a sub-functionalization and gained novel functions unrelated to the function of their common ancestor (Mullon et al. 2012). While the role of Sxl is characterized in great molecular detail, that of Ssx remains largely elusive. The two proteins share a high degree of sequence similarity in their central region, consisting of two RRMs, but they differ within their N-terminal and C-terminal domains. Another difference is found in the knockout phenotype of the two paralogs. While the depletion of Sxl results in female lethality, Ssx was found to be as a nonessential gene showing no phenotype under standard laboratory conditions (Cline 1984; Cline et al. 2010). Interestingly, the knockdown of Ssx was found to be associated with an increased sensibility towards infections with gram-positive bacteria indicating a possible function in the innate immune system (Ayres et al. 2008). Since these are the only data published so far, molecular function of Ssx needs to be elucidated in more detail. For that, the molecular dissection and direct comparison of Sxl and Ssx will help to unravel so far unknown functions of the closely related paralog Ssx.

2 Results

2.1 Molecular dissection of the Sxl paralog Ssx

The molecular function of Sxl acting as the master regulator in female development has been described in great detail and many pathways and interactions have been discovered during the past decades of research (reviewed in Salz and Erickson 2010; Moschall et al. 2017). Nevertheless, the molecular role of its closely related paralog Ssx stays enigmatic. So far, only a link between Ssx and innate immunity has been described, in which the knock down of Ssx was associated with an increased sensibility towards infection with gram positive bacteria (Ayres et al. 2008). Under standard laboratory conditions, however, no phenotypic consequences have been observed when Ssx is missing (Cline et al. 2010).

2.1.1 Ssx is a non essential protein

In order to dissect the molecular function of the uncharacterized *D. melanogaster* protein Ssx, two different fly strains were generated. For the first fly strain (Δssx), the endogenous *ssx* locus was knocked out and replaced by the RFP/dsRed coding sequence using the CRISPR-Cas9 method. The second fly strain (UASt::ssx;da::GAL4) constitutively overexpresses a N-terminal FlagHA-tagged Ssx protein under the control of a UASt-GAL4 promoter. Here, the integrated driver daughterless (da)::GAL4 enables the expression of the transcription factor GAL4 in da specific tissues. Since the ubiquitous expression of da starts early within the fly development, GAL4 protein is supplied in great amounts within the fly which in turn ensures a constant overexpression from the responder transgene encoding FlagHA-Ssx. Homozygous flies carrying two transgenes for the forced overexpression of the FlagHA-Ssx construct are not viable, independently of the temperature (18°C, 20°C or 25°C). Animals that are heterozygous for the transgene and presumably have a lower FlagHA-Ssx expression level and are partially viable at a lower temperature (20°C). In addition, we monitored survival of the different fly strains to test for effects on viability. Here, control flies were compared to flies overexpressing FlagHA-Ssx protein, to a Ssx knock out strain and to the fly strain $ssx^{EY14203}$, which carries a transposable element within the first intron of the *ssx* locus, therefore disturbing Ssx expression.

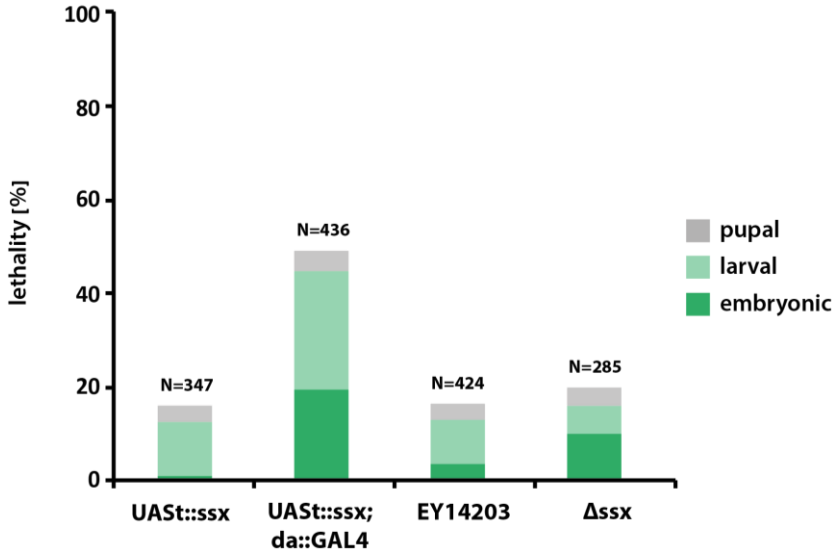

Figure 2.1: Ssx expression levels impact on the viability of flies. The survival of developing flies was monitored for embryonic (dark green), larval (light green) and pupal (grey) fly stages. Constitutive overexpression of FlagHA-tagged Ssx increased lethality by 34% compared to the control strain UASt::ssx. The overall lethality was not affected in fly strains lacking Ssx expression.

Compared to the control UASt::ssx strain (15% lethality), the overall lethality of heterozygously overexpressed Ssx, UASt::ssx;da::GAL4 (49% lethality), is drastically increased by 34%. Death occurred mainly during the embryonal and larval developmental stages. Moreover, neither the depletion of Ssx (Δssx, 20% lethality) nor a strong reduction of Ssx expression levels ($ssx^{EY14203}$, 16% lethality) has an impact on the overall fly survival rate (Fig. 2.1). Of note, the unaffected viability of the Δssx fly strain generated in this study confirms previous findings of Cline, Dorsett et al. 2010, who did not observe any phenotype under standard laboratory conditions.

2.1.2 Ssx is expressed in females and males

As described in previous sections, the *Sxl* pre-mRNAs is alternatively spliced in a sex-dependent manner resulting in a female-specific expression of the functional full-length Sxl protein. Overall, 25 transcript variants of Sxl have been reported, which are subsequently translated into six different protein isoforms (Flybase; FB2017_05; Gramates et al. 2017). In contrast, *ssx* mRNA was previously reported to be expressed in both sexes showing a tendency towards higher levels in males (Lebo et al. 2009). In total, four *ssx* transcript variants (Ssx-RB/RC/RD/RE) have been annotated (Flybase; FB2017_05; Gramates et al. 2017). The 2945nt long *ssx*-RD transcript encodes for the full-length protein Ssx-PD of 53kDa. An alternative splicing event within the last exon of the *ssx* pre-mRNA results in the *ssx*-RB and *ssx*-RE isoforms (3055 and 3635nt) which differ in the length of their 3'UTRs. Both transcript variants encode an identical Ssx protein: Ssx-PB/-PE (49kDa). The alternative splicing event results in a shortened open reading frame and a protein slightly shorter than the protein Ssx-PD. Finally, the *ssx*-RC transcript variant (3096nt) encodes a truncated Ssx-PC protein isoform (18kDa) (Fig. 2.2).

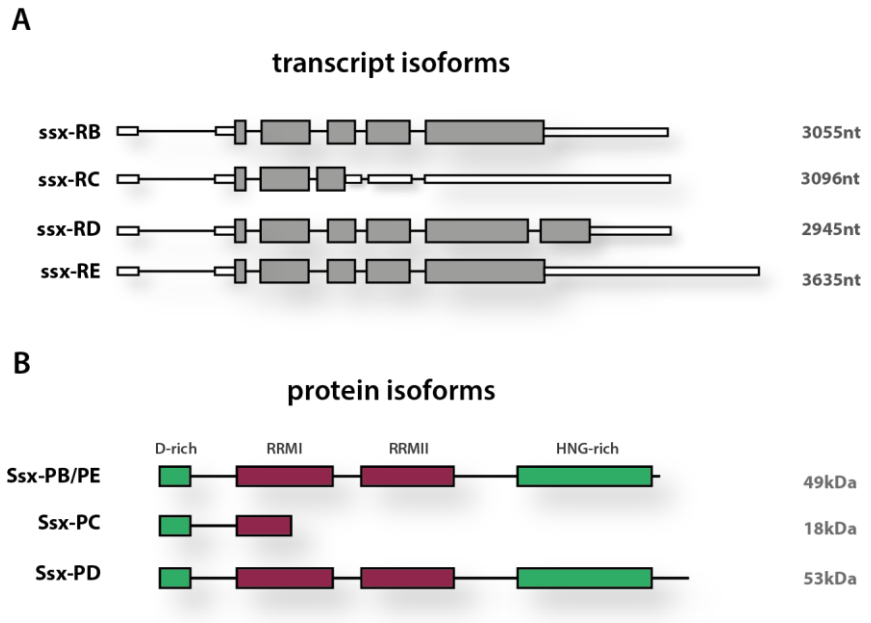

Figure 2.2: Annotated transcript and protein isoforms of Ssx. A) Schematic representation the four *ssx* transcript isoforms (RB, RC, RD, RE). Protein-coding regions are colored in grey, untranslated regions are shown in white and intron are depicted as lines. Nucleotide sizes are indicated on the right **B).** In total, three protein isoforms of Ssx are annotated (Ssx-PB/PE, Ssx-PC and Ssx-PD). Expression of transcript isoforms *ssx*-RB and -RE results in an identical protein isoform Ssx-PB/PE. The central region of the protein containing two RRM motifs is shown in magenta, the C-terminal part and aspartic acid-rich region is depicted in green. The N-terminal part which is rich in amino acids histidine, aspargine and glycine is colored in green. Protein sizes are indicated on the right.

We examined the endogenous transcript isoforms of *ssx* expressed in male and female flies to gain insight into the expression of functional *ssx* transcripts within the fly. Primers indicated in Fig. 2.3 A were used for the detection of *ssx* transcripts encoding the full-length protein. In contrast to *Sxl*, full length *ssx* transcripts are detected in both sexes (Fig. 2.3 B). A comparison of the control RT-PCR for *Sxl* confirms the successful separation of male and female flies, by revealing the expected sex-specific transcript isoforms (Fig. 2.3 C). Of note, *ssx* is also expressed within the embryonal stage.

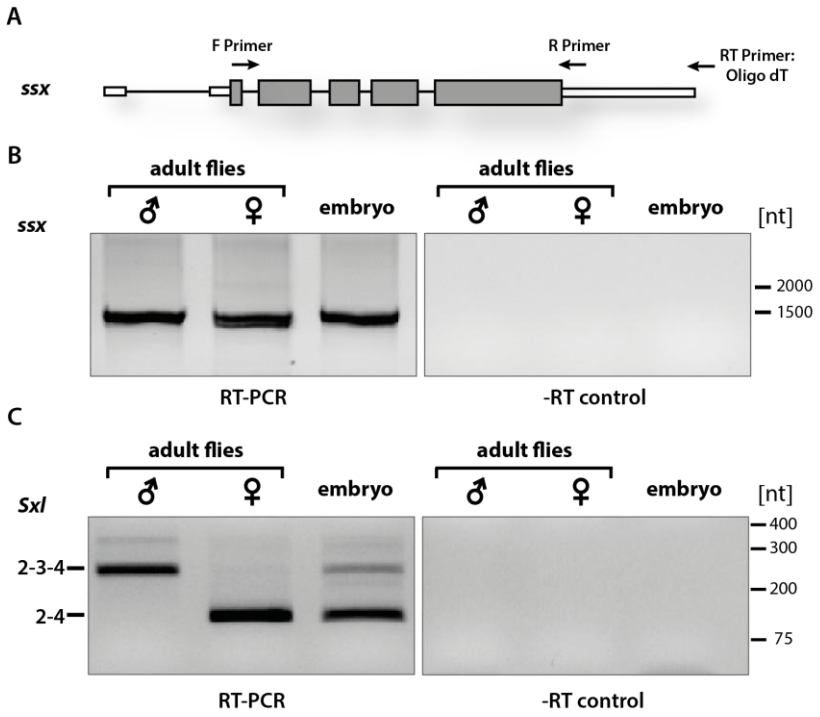

Figure 2.3: Ssx is expressed in male and female flies A) Schematic representation of primers used for the detection of full length *ssx* transcript isoforms. Reverse transcription reactions were performed using oligo dT primers. **B)** Ssx transcripts are expressed in both sexes and are already detectable at embryonic stages. **C)** Control RT-PCR using Sxl-specific primers confirmed correct sex separation of flies. -RT control reactions lacking reverse transcriptase enzyme confirmed specificity of the reactions. Molecular size marker is indicated on the right.

A different primer set was used to determine the presence of the *ssx*-RC transcript variant, which encodes for a truncated *ssx*-PC protein (Fig. 2.4 A). In this case, the *ssx* RC-transcript was exclusively detected as a faint band in female animals and was therefore excluded from further analyses. We next determined by RT-PCR analysis the relative abundance of the *ssx*-RB/RE and *ssx*-RD transcripts. Fig. 2.4 B and C indicate that the predominant transcript isoform having a relative abundance of 99% in embryos, 66% in adult females and 52% in males is the isoform RB/RE encoding for a 443aa long Ssx protein. In adult animals, elevated proportions of the alternatively spliced isoform RD are detected (Fig 2.4 B and C) differing slightly between sexes with 48% in males and 34% in females. In sum, this further confirms the data from the modENCODE project, demonstrating that no sex-specific expression of the *ssx* transcripts can be observed (Celniker et al. 2009).

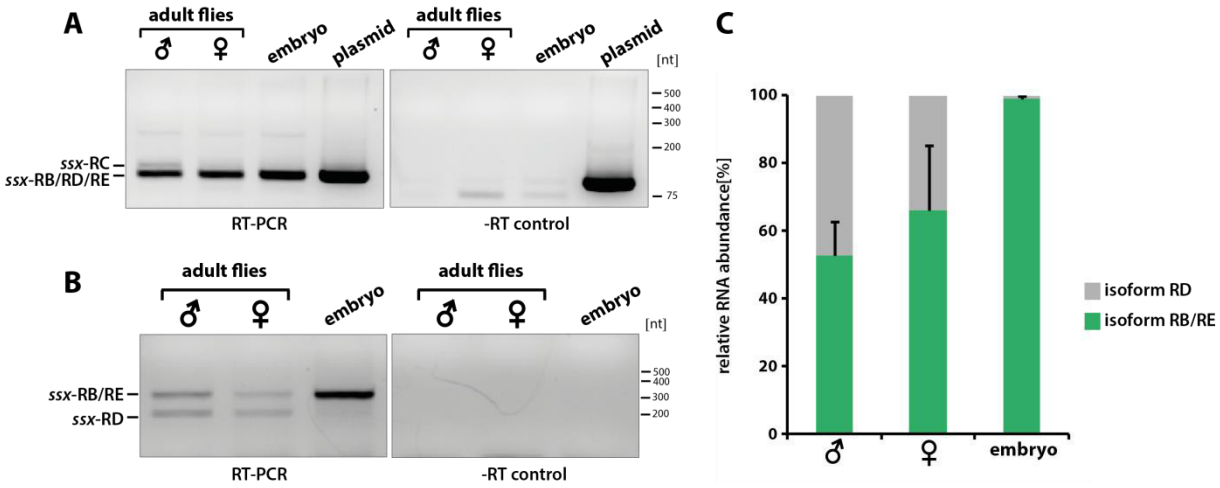

Figure 2.4: *Ssx* transcript isoforms are expressed in a sex-independent manner. A) Detection of the short transcript isoform *ssx*-RC in sex-selected adult animals and embryos. A positive control plasmid detected the *ssx*-RB/RD/RE transcript (indicated on the left). **B)** Distribution of isoforms RB/RE and RD (indicated on the left) in males and females, whereas embryos express predominantly isoform *ssx*-RB/RE. –RT reactions lacking the reverse transcriptase demonstrated no DNA contamination. A molecular size marker is indicated on the right. **C)** Quantification of the relative abundance of *ssx*-RB/RE (shown in green) and RD transcript (shown in grey) levels in males, females and embryos calculated out of three biological replicates using the software ImageJ.

2.1.3 Analysis of Ssx protein expression

To gain a deeper insight into the biological function of Ssx, we generated several molecular tools. First of all, we raised four specific antibodies against Ssx in order to confirm the sex-independent expression pattern. Therefore, we recombinantly expressed and purified the Ssx-RBD4 protein, which was then injected into two different rabbits (SY6158 and SY6159). Injection of rabbits and preparation of sera was performed by the company Eurogentec. Ssx-RBD4 encompasses the central region of the protein and consists of two RRMs. In order to diminish the possibility of cross reactivity with Sxl protein, a N-terminal Ssx-specific peptide (aa19-34; h-DIEGSGDNVGRDDGTD-nh2) was injected into two additional rabbits (SY6156 and SY6157). Different sera containing antibodies were tested by Western Blot analysis, using lysates of male-derived Dmel-2 cells and female-derived Kc167 cells. Since Dmel-2 cells were generated from late male embryos, they do not express detectable amounts of the female-specific protein Sxl, whereas the female cell line Kc167 stably expresses the functional Sxl protein. Lysates of the two cell lines were separated by SDS-PAGE and the different antibodies were used for the specific detection of endogenous Ssx, as well as overexpressed FlagHA-Ssx protein. Moreover, antibodies were tested for a possible cross-reactivity with endogenous female-specific Sxl protein or with overexpressed FlagHA-Sxl protein.

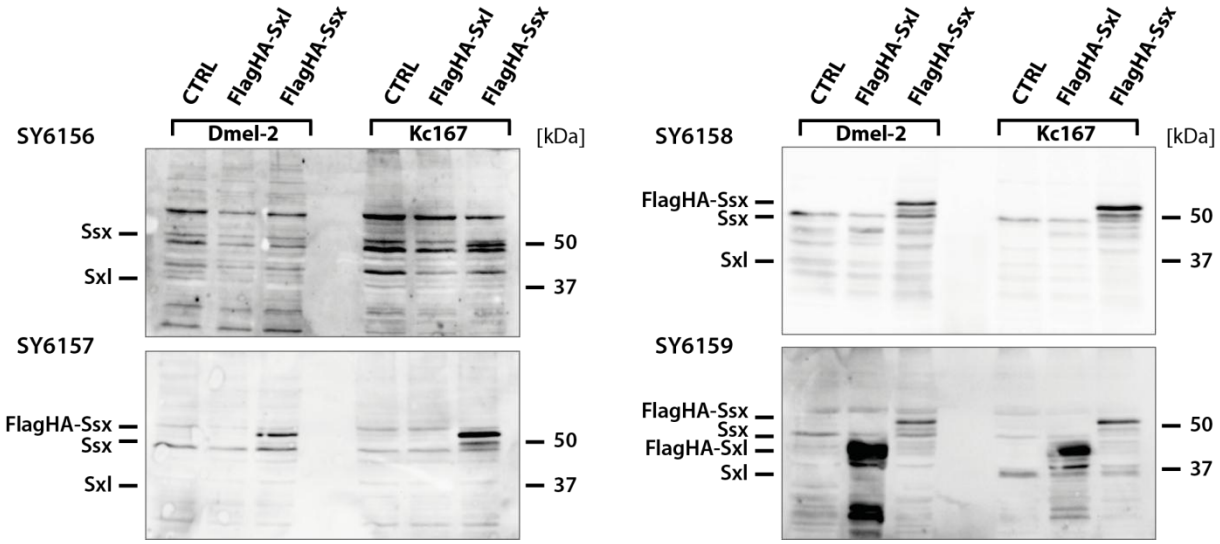

Figure 2.5: Testing raised anti-Ssx antibodies SY6156, SY6157, SY6158 and SY6159 by Western blot analysis. Each antibody was tested for its ability to recognize endogenous Ssx and Sxl, overexpressed FlagHA-Sxl and FlagHA-tagged Ssx in male Dmel-2 cells and female Kc167 cells (depicted above each lane). Cross-reactivity with endogenous Sxl in females and overexpressed FlagHA-Sxl protein in Dmel-2 and Kc167 was examined (indicated on the left). Molecular weight marker sizes are indicated on the right.

Western Blot analysis (Fig. 2.5) revealed that the peptide-specific antibody, SY6157 specifically recognized the endogenous and overexpressed Ssx protein (50kDa and 54kDa) in both sexes, while Sxl was not detected. Several unspecific bands appear with the antibody SY6156, which was therefore omitted in further analyses. The antibodies SY6158 and -59 were generated by injection of purified, recombinant Ssx-RBD4 protein into rabbits and both specifically recognized endogenous Ssx as well as overexpressed FlagHA-Ssx in female and male cell lysates. The antibody SY6159 showed cross reactivity with endogenous Sxl (37kDa) and overexpressed FlagHA-Sxl protein (41kDa). To further validate the specificity of the selected antibody SY6158, endogenous Ssx was depleted by RNAi in Dmel-2 cells and knock down of Sxl protein served as control.

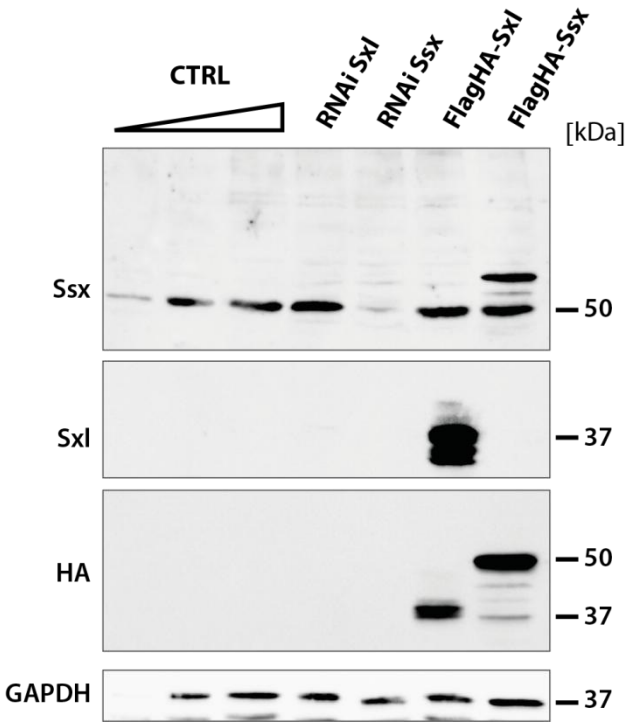

Figure 2.6: Antibody SY6158 specifically recognizes endogenous Ssx protein. The presence of endogenous Ssx protein in Dmel-2 cells was observed by Western Blot analysis using the antibody SY6158. Titration of *Drosophila* whole lysate (lane 1-3, CTRL) detects endogenous Ssx protein by the antibody SY6158. Treatment with dsRNA targeting either Sxl (lane 4) or Ssx (lane 5) validates specificity of the antibody. Overexpression of FlagHA-Sxl (lane 6) and FlagHA-Ssx (lane 7) was confirmed using an anti-HA antibody. Correct loading of the samples was assayed by GAPDH probing. Molecular weight marker sizes are indicated on the right.

Knock down of Ssx confirmed the specificity of the antibody (Fig. 2.6), whereas the control knock down of the female specific protein Sxl verified specificity of the RNAi treatment. Endogenous Ssx protein was monitored at 50kDa, while FlagHA-tagged Ssx was detected with a shift in size at around 55kDa. Confirming the results shown in Figure 2.5, antibody SY6158 did not cross react with overexpressed FlagHA-tagged Sxl protein. The correct expression of both FlagHA-tagged proteins was demonstrated by HA-probing. Equal loading of the samples was assayed by the detection of GAPDH. Additionally these data demonstrate that Ssx is expressed in male cells (Fig. 2.5 and 2.6). Next, the relative abundance of Ssx protein levels between the sexes was observed by Western Blot analysis. For this, male and female cell lysates were probed with antibodies specific for Sxl or Ssx. Figure 2.7 A shows that Ssx is expressed in both sexes with slightly higher protein levels in males, while it's closely related paralog Sxl is clearly a female-specific protein. A similar result was obtained for adult animals (Fig. 2.7 B lanes 5 and 6) showing equal expression of Ssx protein in both sexes as well as in embryos (Fig 2.7 C lane 3). This result further confirmed RT-PCR analysis shown in Fig. 2.3. Moreover, probing of embryonic lysates and adult fly lysates for Ssx demonstrated the absence of Ssx protein in the Δssx fly stain (Fig. 2.7 C lane 1 and Fig. 2.7 B lane 2 and 3) and a strong reduction of Ssx protein (close to knock out levels) in the fly strain $ssx^{EY14203}$, which is carrying an insertion in the *ssx* locus. Finally, forced expression of Ssx in the fly strain UASt::ssx; da::GAL4 resulted in a higher migrating band which corresponds to the FlagHA-tagged Ssx protein (Fig. 2.7 B lane 7 and 8 and Fig. 2.7 C lane 4).

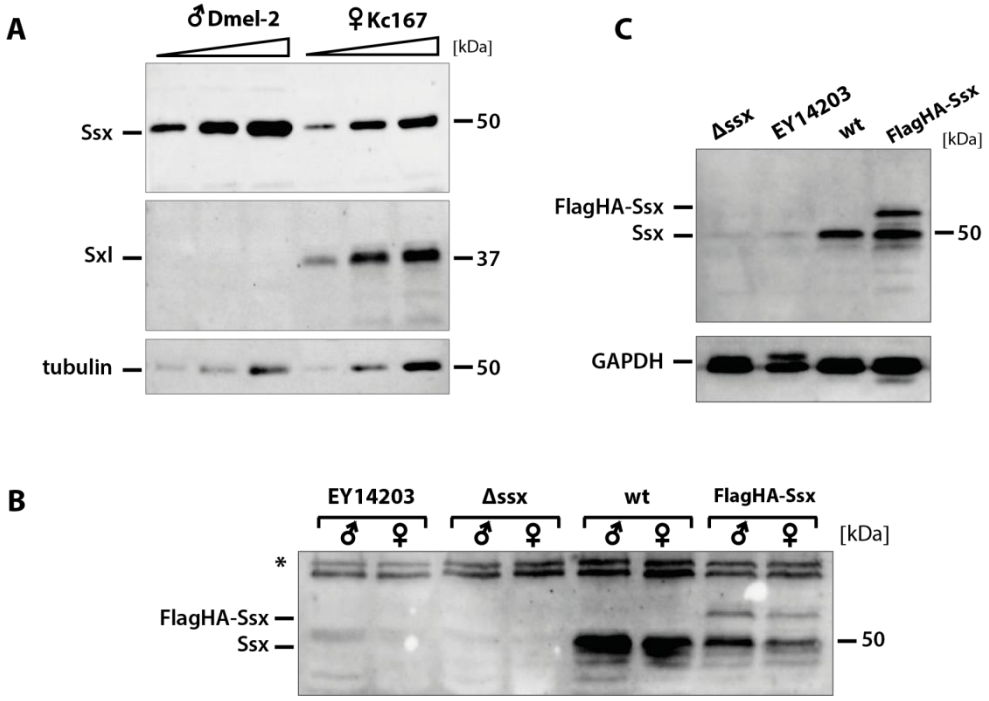

Figure 2.7: Analysis of Ssx expression in cultured cells and different fly strains. A) Western Blot analysis of titrated cell lysates (10, 20, 40µg) demonstrates the expression of Ssx in male and female cultured cells (lane 1-3 and lane 4-6), while expression of the female-specific Sxl protein served as internal reference. **B)** Expression pattern of Ssx in sex-selected adult animals (lane 4-5) is almost identical as observed in C) for embryos (lane 3). Different fly lysates were subjected to Western Blot analysis. Ssx protein is absent from the Δssx (lane 3-4), or strongly reduced in the $ssx^{EY14203}$ fly strains (lane 1-2). Overexpressed, tagged Ssx protein can be detected in UASt::ssx;da::GAL4 fly lysates (lane 7-8) while wt flies served as control (lane 5-6). Unspecific bands marked with an asterisk confirm equal loading. **C)** Expression of endogenous Ssx protein in embryos of different fly strains described in B). GAPDH probing confirmed equal loading. Molecular weight marker sizes are indicated on the right.

To gain further insight into the molecular function of the Ssx protein we next sought to identify its RNA targets and interacting protein partners. Therefore, the conditions for endogenous immunoprecipitation experiments with the anti-Ssx antibody SY6158 were optimized. We first tested the ability of the antibody to immunoprecipitate endogenous Ssx protein under increasing salt concentrations of the buffer used. The protein Ssx was efficiently immunoprecipitated and detected by Western blotting even upon washing with high salt concentrations up to 1000 mM (Fig. 2.8 lane 1-4). No signals for Ssx were detected in a control immunoprecipitation performed under similar conditions with an unrelated control serum, demonstrating the specificity of the immunoprecipitation (Fig. 2.8 lane 5).

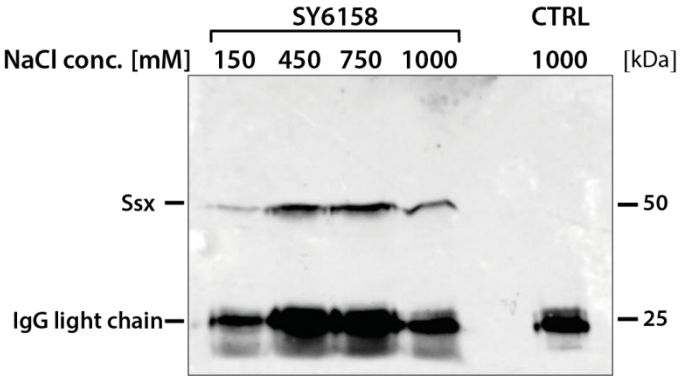

Figure 2.8: The Ssx-specific antibody SY6158 precipitates endogenous Ssx protein efficiently. Antibody SY6158 precipitates endogenous Ssx protein (indicated on the left) up to salt concentrations of 1M NaCl. A pull down experiment using an unrelated serum served as control (lane CTRL). Molecular weight marker sizes are indicated on the right. Detection of IgG light chain is displayed on the left.

2.2 Binding properties of Sxl and Ssx to RNA

2.2.1 The similarities: Sxl and Ssx bind related RNA binding motifs

The two RNA-binding proteins Sxl and Ssx share a high degree of homology within their central region, which consists of two RRMs. While the RNA binding region of the two proteins is 80% identical on amino acid level, the N- and C-terminal sequences are highly divergent (Fig. 2.9).

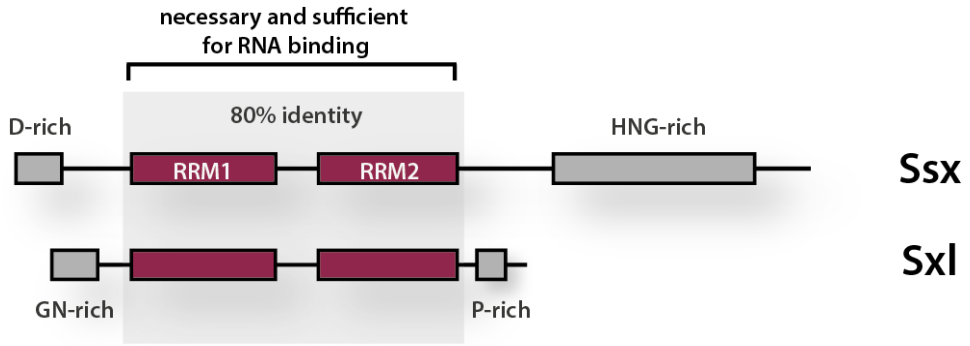

Figure 2.9: Schematic representation of the domain organization of Sxl and Ssx. Both proteins share a high degree of homology within their central region which consists of two RRMs (RRM1 and RRM2, shown in magenta). Both RRMs are necessary and sufficient for RNA binding (highlighted in grey). However, Sxl and Ssx differ within their N-terminal and C-terminal regions (depicted in dark grey).

In contrast to Ssx, Sxl is a well characterized RNA-binding protein with many experimentally validated binding sites in numerous different RNAs. Structural information clarified in great detail how Sxl exerts its function as a RNA binder on its target mRNAs *tra* and *msl-2* (Handa et al. 1999; Hennig et al. 2014). Evolutionary related proteins often share similarities concerning their binding behavior (Ray 2013). Strikingly, the RNA-binding interface of Sxl is largely identical in Ssx as depicted in Fig. 2.10.

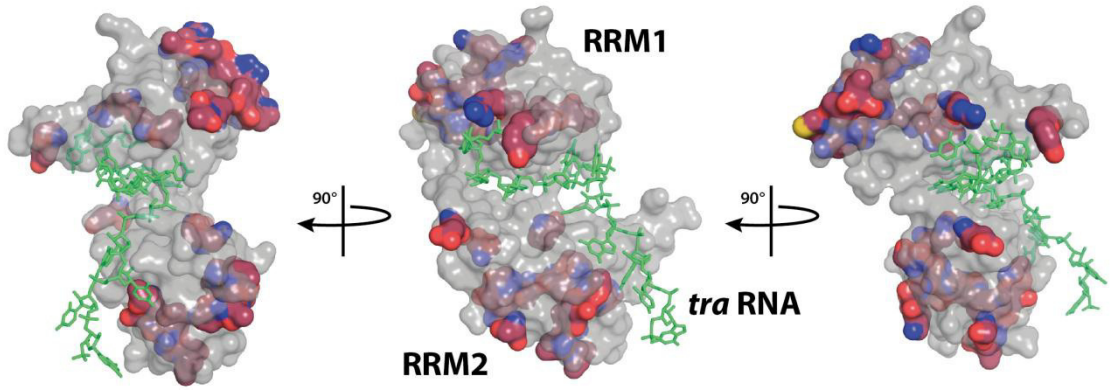

Figure 2.10: The RNA binding surface of Sxl is largely conserved in Ssx. 270° rotation of a Sxl structure bound to a fragment of *tra*-mRNA (shown in green) (based on the structure from Handa et al. 1999 (PDB 1B7F). Amino acids that are identical between Sxl-RBD4 and Ssx-RBD4 are shown in grey, whereas substitutions between the two proteins are highlighted in colors.

Due to the high conservation of amino acid residues located within the RNA binding surface, we hypothesized that Ssx is able to bind U-rich sequences similar to its paralog Sxl. To prove this hypothesis, recombinant proteins comprising the central region of Sxl (Sxl-RBD4: aa122-294 (Gebauer et al. 2003) or Ssx (Ssx-RBD4: aa93-269) harboring two RRMs were recombinantly expressed and purified (Fig. 2.31 B). The well characterized Sxl binding site on the *msl-2* mRNA termed Esite (5'-UUUUUUUGAGCACGUGAA-3') was selected as a synthetic RNA target for binding analysis. To test for binding specificity every second U was converted to C (Esite mut: 5'-UCUCUCUGAGCACGUGAA-3') (Fig. 2.11). Electro mobility shift assays (EMSAs) clearly confirmed that Sxl and Ssx bind the same RNA target with a similar binding affinity, while binding to the control RNA cannot be detected.

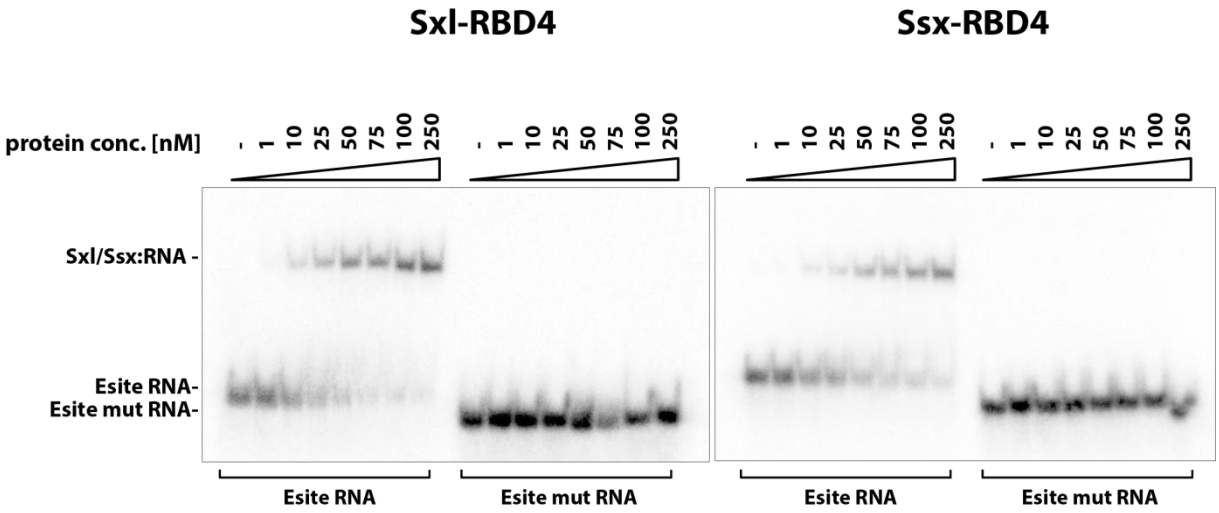

Figure 2.11: Sxl-RBD4 and Ssx-RBD4 interact with the *msl-2* Esite. EMSA with Sxl-RBD4 (left panel) or Ssx-RBD4 (right panel) proteins titrated in increasing amounts (1-250nM) to the *msl-2* Esite display the binding affinity of both proteins. Titration to a mutated binding site confirms specificity of the binding reaction. Running height of free RNA or protein-bound RNA is indicated on the left.

A similar binding behaviour of both proteins is not restricted to the *msl-2* Esite, as Sxl and Ssx also recognize the well-known *tra* binding site to a similar extent (Fig. 2.12). Therefore, we performed a binding assay using a radioactively labelled *tra* RNA fragment (5'-UUUUUGUUGUUUUUUUU-3'), which was already demonstrated to tightly interact with Sxl-RBD4 in the crystal structure from Handa et al. 1999 (PDB: 1B7F).

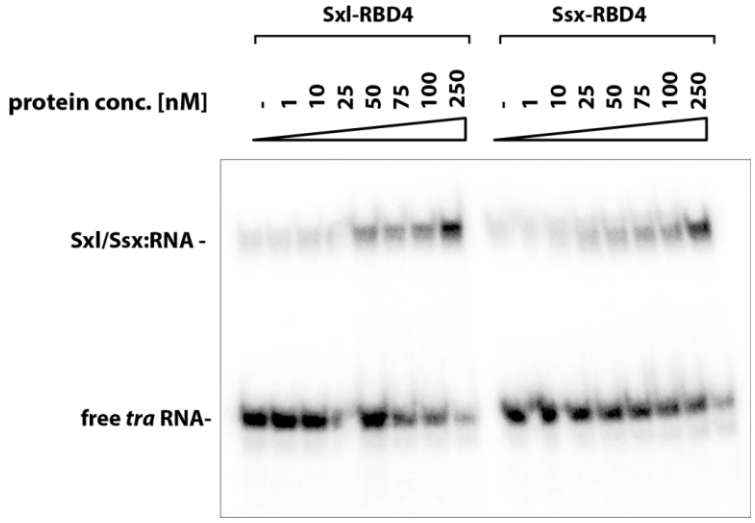

Figure 2.12: Sxl and Ssx bind to the *tra* RNA motif. EMSA of Sxl-RBD4 and Ssx-RBD4 proteins which were titrated in increasing amounts (indicated above each lane) to the radioactively labeled *tra* RNA fragment. Running height of free RNA substrate and Sxl/Ssx:UNR dimer is indicated on the left.

To broaden the knowledge about the uncharacterized protein Ssx and to gain a deeper insight into its biological function, we aimed to determine the mRNAs bound by Ssx. Therefore, full length constructs of Sxl, Ssx and GFP were FlagHA-tagged and overexpressed in male Dmel-2 cells. Afterwards, the proteins were immunoprecipitated and the bound RNAs were identified by high throughput sequencing. Bioinformatic analysis revealed that, relative to the GFP control, the Ssx immunoprecipitation significantly enriched approximately 940 mRNAs, while Sxl was found to be associated with approximately 200 mRNAs (Gene Expression Omnibus: GEO Series GSE98189). The overlap of the two datasets was very high with 95% (Fig. 2.14 A). Nevertheless, the total amount of immunoprecipitated mRNA targets for Ssx was very high and was presumably caused by massive protein overexpression and consequent binding also to low affinity targets which is likely not to be physiological. To minimize the risk of detecting potentially false positive interactors, we investigated, together with Dr. Oliver Rossbach from the University of Giessen (Germany), individual-nucleotide resolution crosslinking-immunoprecipitation (iCLIP) of endogenous Ssx protein in male Dmel-2 cells. Here, proteins were first crosslinked to their target RNAs with UV-light and endogenous Ssx was precipitated with the SY6158 antibody. After a mild RNase digestion, protein bound RNA fragments were radioactively labeled and RNA-protein complexes were separated by SDS PAGE followed by blotting on a membrane

(Fig. 2.13). Ssx-bound RNA complexes were subsequently excised from the membrane and, after digestion of the proteins, small RNA libraries were generated for high throughput sequencing. An experiment performed with a non-specific antibody served as negative control. All steps following crosslinking were performed by Dr. Oliver Rossbach.

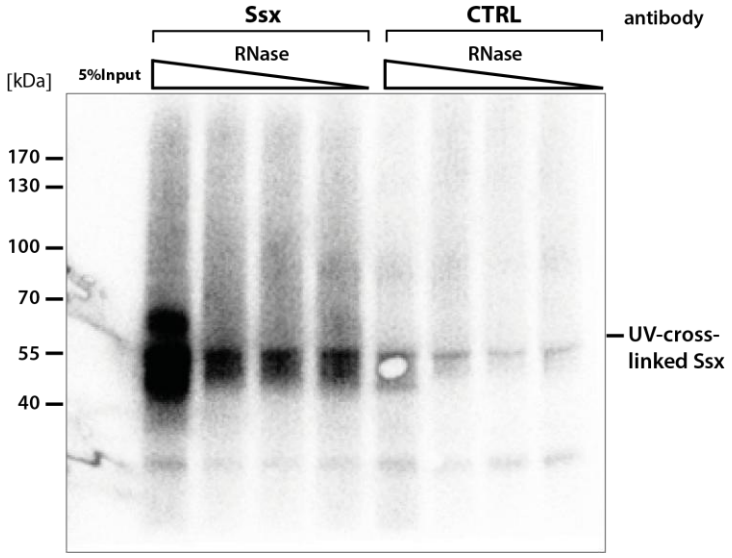

Figure 2.13: Individual-nucleotide resolution crosslinking-immunoprecipitation in male Dmel-2 cells of endogenous Ssx protein using the antibody SY6158. Autoradiogram of radiolabeled RNA fragments bound to endogenous Ssx protein (indicated on the right; lane 2-5). A control reaction in which an unrelated antibody was used confirms specificity (lane 6-9). Different RNase concentrations were tested. Molecular weights marker sizes are indicated on the left.

Sequencing of libraries was performed by Norbert Eichner and analysis of the sequencing data were performed by Gerhard Lehmann (Department of Biochemistry I, working group of Prof. Dr. Gunter Meister, University of Regensburg, Germany) and revealed that endogenous Ssx protein in male Dmel-2 cells was bound to 92 mRNA targets relative to control samples (Fig. 2.14 A, Table A.1). In addition, the overlap between the results of the RIP analysis and the iCLIP analysis highlighted approximately 60 mRNAs (Fig. 2.14 A).

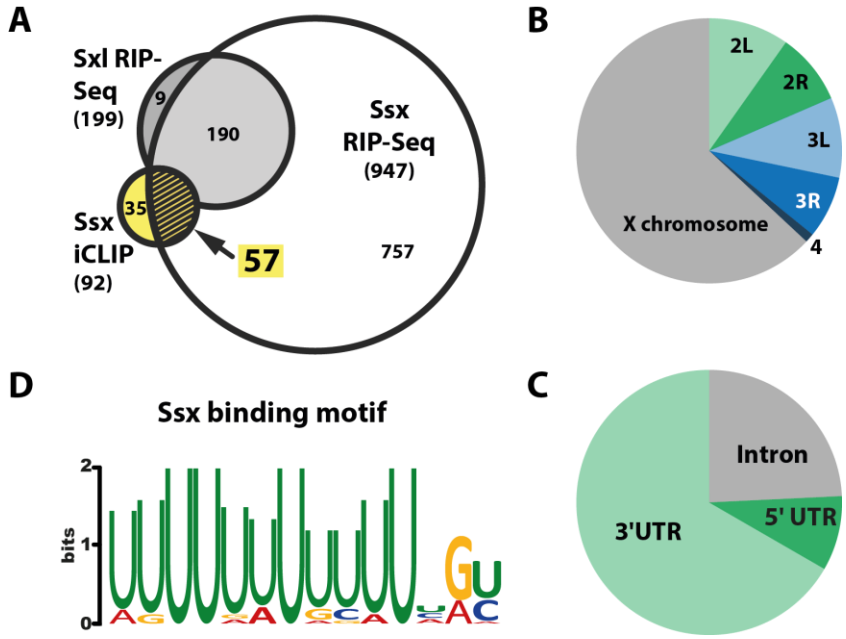

Figure 2.14: CLIP analysis revealed new insights into the binding properties of Ssx. A) Venn-diagram showing the overlay of RIP and iCLIP data sets which further lead to the determination of high fidelity targets for Ssx. **B)** Pie chart of chromosomal Ssx target distribution. **C)** Pie chart of Ssx-crosslink site distribution within intronic regions, 3' and 5' UTRs. **D)** Motif-enrichment analysis was performed with MEME and identified a U-stretch motif as putative Ssx target site.

Focusing on the distribution of identified Ssx targets along *Drosophila* chromosomes, a significant clustering on the X chromosome is striking (Fig. 2.14 B). Interestingly a similar enrichment of targets encoded on the X chromosome was already postulated for the female-specific Sxl protein (Kelley et al. 1995). In general, the coding capacity and gene density of the X chromosome is comparable to the other chromosome sets (excluding chromosome 4). Moreover, Ssx was found to be bound predominantly within the 3'UTR of its targets, whereas also one fourth was associated with intronic regions (Fig. 2.14 C). Intronic regions are normally barely detected in total RNA, due to their efficient removal during splicing. Therefore, the enrichment of bound intronic regions suggests that the intronic sites are highly occupied by Ssx. Furthermore, motif-enrichment analysis of the crosslinked positions of the Ssx iCLIP data set revealed that the identified binding motif for Ssx is very similar to the previously characterized motif bound by Sxl (Fig. 2.14 D). This finding supports the hypothesis that the two paralogs share same RNA binding characteristics. Having a deeper look at the iCLIP data, a highly interesting candidate bound by Ssx was identified. Ssx was found to be significantly enriched on the *Sxl* pre-mRNA at sites known to be bound by Sxl itself (Fig. 2.15). In females, these binding sites are normally occupied by Sxl protein, driving the auto-regulatory, positive feedback loop of *Sxl* alternative splicing (described in chapter 1.6). Since Sxl is absent in males, these binding sites might be available for Ssx binding.

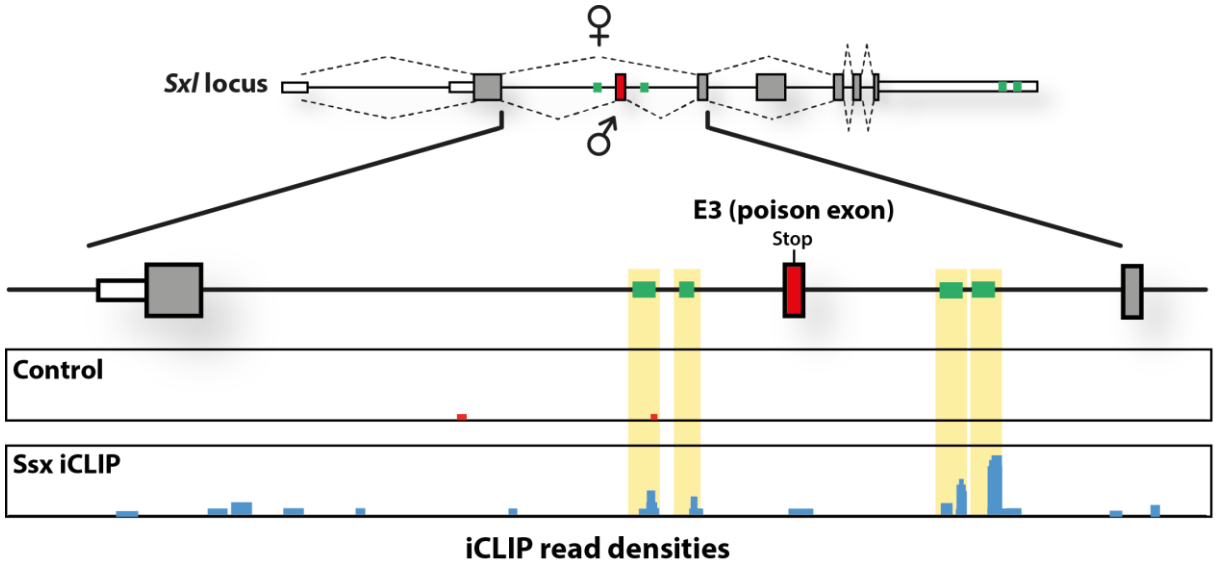

Figure 2.15: In the absence of Sxl endogenous Ssx is enriched on Sxl binding sites within the *Sxl* mRNA. Schematic representation of the Ssx iCLIP data analysis on *Sxl* mRNA (*Sxl* locus shown on top). Sxl mRNA introns are shown as black lines, exons are depicted as boxes, non-coding regions are colored in white. Sex-dependent splicing of *Sxl* mRNA is indicated as dashed lines (male-specific transcript isoform RF; FBtr0331249 and female-specific isoform RD; FBtr0331262). Sxl binding sites flanking the poison exon (colored in red) are depicted in green and binding events of endogenous Ssx are shown in blue and highlighted as yellow areas. Ssx iCLIP binding events were compared to binding events of an unrelated control (red boxes) and confirmed specificity.

2.2.2 Sxl and Ssx compete for binding to the same target RNAs

As shown by EMSA and iCLIP, both, Ssx and Sxl, associate with similar, if not identical sequence motifs. We therefore wanted to understand if the two proteins can compete for binding to the same RNA element. To answer this question, we performed competitive EMSAs. For this purpose, recombinant Sxl-RBD4 and Ssx-RBD4 proteins were expressed, purified and titrated onto radioactively labeled target RNA (U16). To discriminate between Sxl-RBD4 (approx. 20kDa) and Ssx-RBD4 (approx. 20kDa) bound to the RNA, we additionally used GST-tagged versions of the proteins (GST-Sxl-RBD4 previously described in Gebauer et al. 2003). Due to the slower migration of the GST-tagged protein constructs (approx. 45kDa) and the resulting higher shift of the bound RNA, we were able to discriminate the different RNA-protein complexes. Titration of increasing protein concentrations confirmed that RNA binding affinity of both proteins is not influenced by addition of the GST tag (Fig. 2.16 A, very left and very right panel). Using a defined amount of Ssx-RBD4 protein and titrating increasing amounts of GST-Sxl-RBD4 highlighted that Sxl competes with Ssx for binding to the RNA substrate and that Ssx is displaced from the RNA at higher Sxl concentrations (Fig. 2.16 A second left panel). The titration using a defined amount of GST-Sxl-RBD4 and titrating increasing amounts of Ssx-RBD4 showed the same competition pattern.

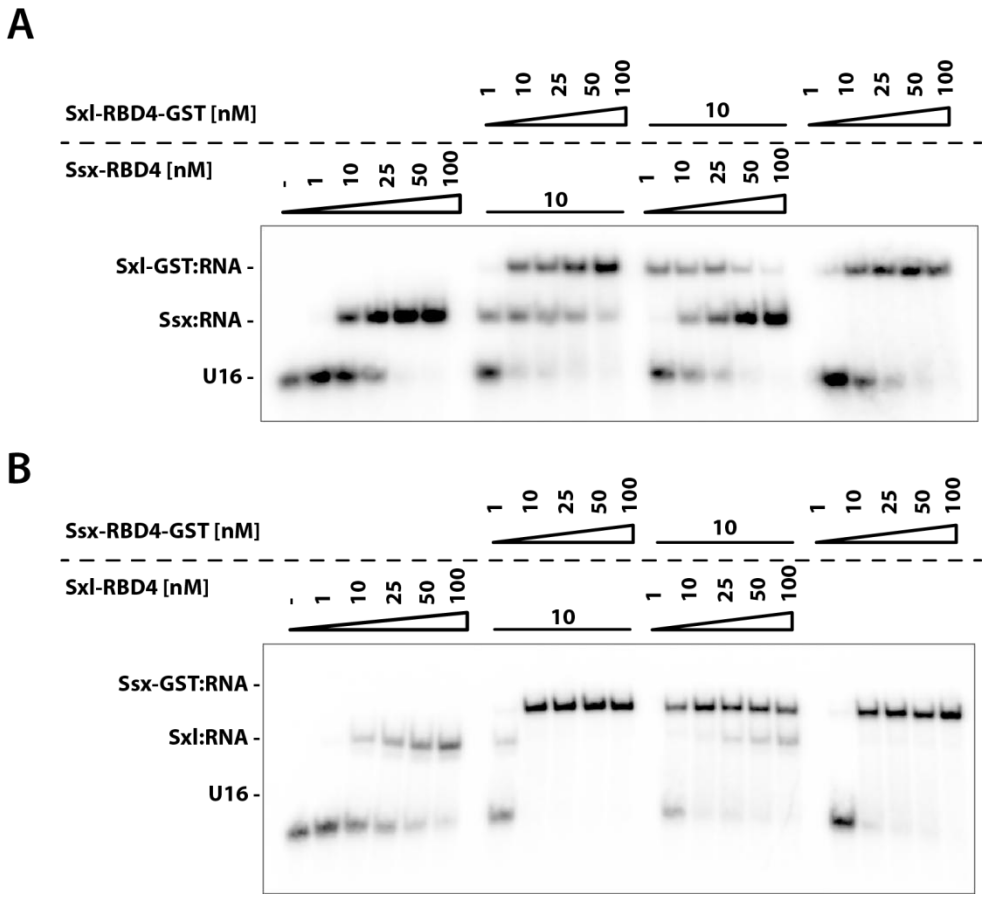

Figure 2.16: Sxl and Ssx compete for their binding to their RNA substrate. A) A similar binding affinity of proteins Sxl-RBD4-GST and Ssx-RBD4 independent of the GST-tag was employed by EMSA (very left and very right panel). Increasing amounts of one protein were titrated to a given amount of the other protein and shows a competitive binding in both directions (second and third panel). Used protein concentrations of Sxl-RBD4-GST and Ssx-RBD4 are indicated on top. Running height of the labeled free RNA substrate or RNA:protein complexes is depicted on the left. **B)** *Vice versa* experiment of A) demonstrating a competition of Sxl and Ssx independent of the GST-tag employing Sxl-RBD4 and Ssx-RBD4-GST.

The same effects were shown for the *vice versa* experiment with Sxl-RBD4 and GST-Ssx-RBD4 (Fig. 2.16 B) ruling out an influence of the GST-tag on individual protein binding behaviours. Specificity of the protein binding to the RNA was demonstrated using a mutated RNA target, in which every second U was converted to a C $(UC)_8$. This completely abolished binding to any of the titrated protein. Surprisingly, the $(UC)_8$ substrate run as two distinct bands. This is presumably reflecting different RNA structures in the native gel (Fig. 2.17).

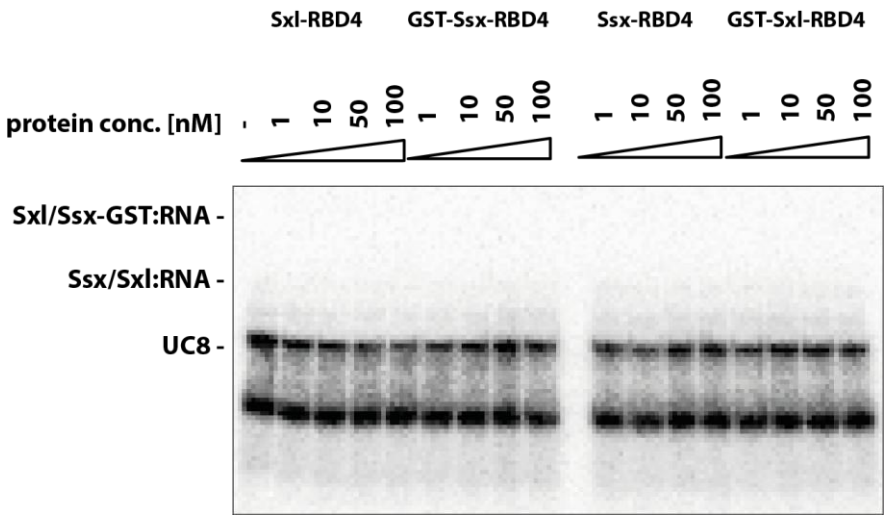

Figure 2.17: EMSA control experiment demonstrating specific binding of Sxl/Ssx proteins to RNA substrates. Control experiment employing a labeled RNA substrate with the mutated sequence $(UC)_8$ confirmed binding specificity of used recombinant proteins (depicted on top). Migrating free RNA or RNA:protein complexes are indicated on the left.

2.2.3 Overexpressed Ssx does not interfere with splicing of known Sxl targets

Since we found Ssx to recognize Sxl binding sites within *Sxl* pre-mRNA in male cultured cells, we wanted to clarify whether Ssx can also impact on alternative splicing of Sxl targets. Besides the homology within the central region of both proteins which is necessary for RNA binding, the C- and N-terminal regions of Sxl and Ssx differ drastically. In particular, the N-terminus of Sxl was shown to play a role in the regulation of alternative splicing and there is experimental evidence indicating that it might participate in protein-protein interactions and in homo-dimerization (Samuels et al. 1998, Deshpande et al. 1999, Yanowitz et al. 1999). Thus, we conducted RT-PCR experiments with RNA isolated from male and female flies, which constitutively overexpress a FlagHA-tagged version of Ssx (UASt::Ssx;da::GAL4) (Fig. 2.7 B). We chose to analyse several mRNAs which are known to show sex-specific and Sxl-sensitive splice patterns. Compared to the respective wt control, overexpression of FlagHA-Ssx did not alter the splice patterns of *msl-2*, *tra* and *Sxl* mRNAs in adult flies. Normally, Sxl promotes intron retention within the *msl-2* 5'UTR (Fig. 2.18 first panel, lane 1 and 2) resulting in a higher migrating RT-PCR product.

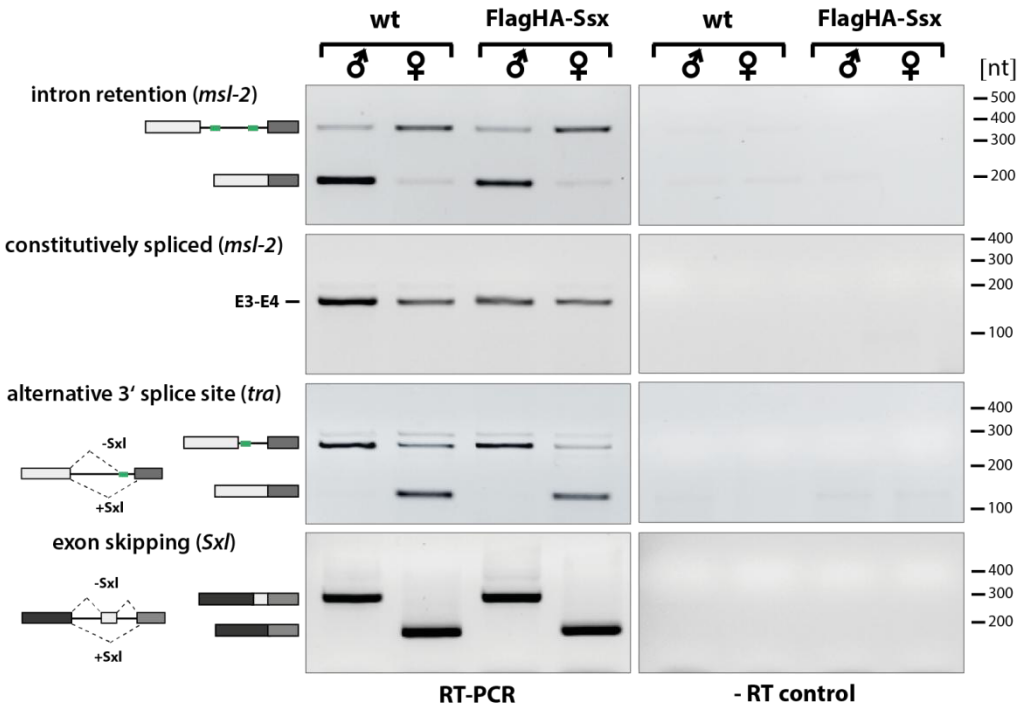

Figure 2.18: Overexpression of Ssx does not change alternative splicing of *msl-2*, *tra* and *Sxl* in adult flies. RT-PCR analysis of adult flies sorted by sex revealed that overexpressed FlagHA-tagged Ssx in the fly strain UASt::ssx;da::GAL4 does not interfere with sex-specific, alternative splicing of *msl-2*, *transformer* and *Sxl* pre-mRNA. Negative controls were performed without reverse transcriptase enzyme ruling out DNA contamination. Sex-dependent splice patterns of mRNAs are shown on the left, nucleotide size markers height is depicted on the right.

In contrast, elevated levels of Ssx protein did not correlate with increased intron retention in males and females (Fig. 2.18 first panel, lane 3 and 4). A constitutively spliced intron in *msl-2* was used as control (Fig. 2.18 second panel). In addition, also alternative splicing of the *tra* mRNA, the direct downstream target of Sxl in the developmental cascade, was not affected. (Fig. 2.18 panel 3). Moreover, the abundance of sex-specific *Sxl* transcripts was not altered by increasing the concentration of Ssx (Fig. 2.18 panel 4). In sum, alternative splicing patterns of Sxl target mRNAs are not affected by a moderate overexpression of FlagHA-Ssx.

2.2.4 Loss of Ssx in male flies induces female-specific splicing of *Sxl* pre-mRNA

Since the forced overexpression of Ssx reduces viability (Fig. 2.1) without affecting alternative splicing of selected Sxl target mRNAs, we investigated the effect of Ssx depletion on alternative splicing. Therefore, the fly strain $ssx^{EY14203}$ was used, which carries a transposable element within the *ssx* locus leading to a strong reduction of Ssx protein levels (Fig. 2.7 B). It was already highlighted in previous studies (Cline et al. 2010) and in Fig. 2.1 that the viability of flies is not affected upon loss of Ssx protein. Again, adult animals were sorted by sex and isolated RNA was analysed by RT-PCR for changes in alternative splicing patterns. As expected, female flies of the mutant and wildtype strains were indistinguishable

from each other in their *Sxl* and *tra* specific splicing pattern, showing female-specific shorter isoforms in a comparable intensity (Fig. 2.19, lanes 2, 4 and 6).

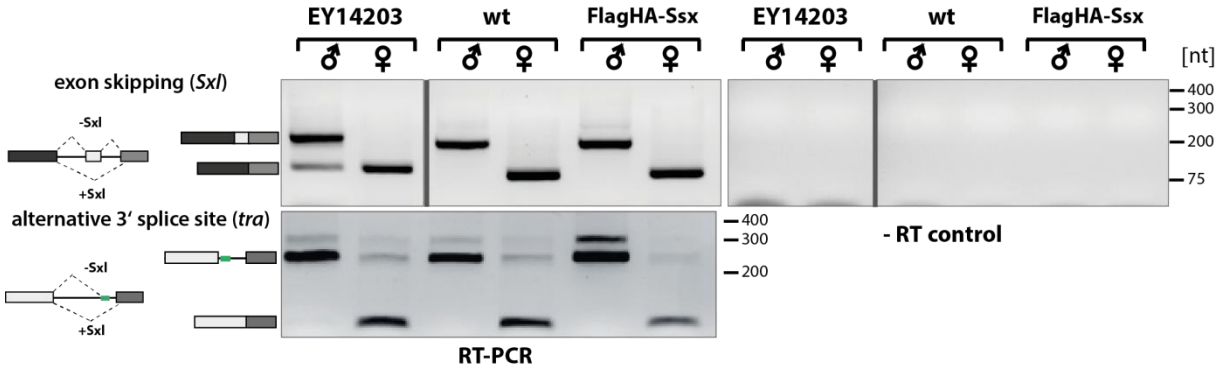

Figure 2.19: Loss of endogenous Ssx forces female-specific *Sxl* splicing in male flies. RT-PCR analysis of flies which harbor a disrupted Ssx locus due to a transposable element insertion ($ssx^{EY14203}$). Wild type and FlagHA-Ssx overexpression flies were used for comparison. Males of the $ssx^{EY14203}$ strain showed an altered splicing pattern of the *Sxl* mRNA and detectable levels of the female-specific isoform (lane 1 and 2, upper panel). The splicing pattern of *tra* mRNA was not affected (lane 1 and 2, lower panel). –RT reactions were performed without reverse transcriptase proofing no DNA contamination.

However, hemizygous males of $ssx^{EY14203}$ strain displayed an exceptional splicing of *Sxl* mRNA. Here, in approximately 50% of all samples, *Sxl* mRNA was spliced to a moderate extent in the female-specific pattern, which is coding for a functional full-length Sxl protein (Fig. 2.19 upper panel, lane 1). However, the splicing of the downstream target *tra* remained unaffected (Fig. 2.18 lower panel, lane 1). To rule out that the observed effects on *Sxl* alternative splicing are caused by a rare, second site mutation, we further analysed females and males of the Δssx strain. We previously confirmed the complete loss of Ssx protein by Western blot analysis (Fig. 2.7 B and C). RT-PCR analyses of these animals revealed a genetic phenotype similar to the $ssx^{EY14203}$ strain: Δssx males exhibited a partially female-specific splicing of *Sxl* mRNA. Once again, in approximately 50% of all samples this aberrant splicing pattern was detected (Fig. 2.20 upper panel).

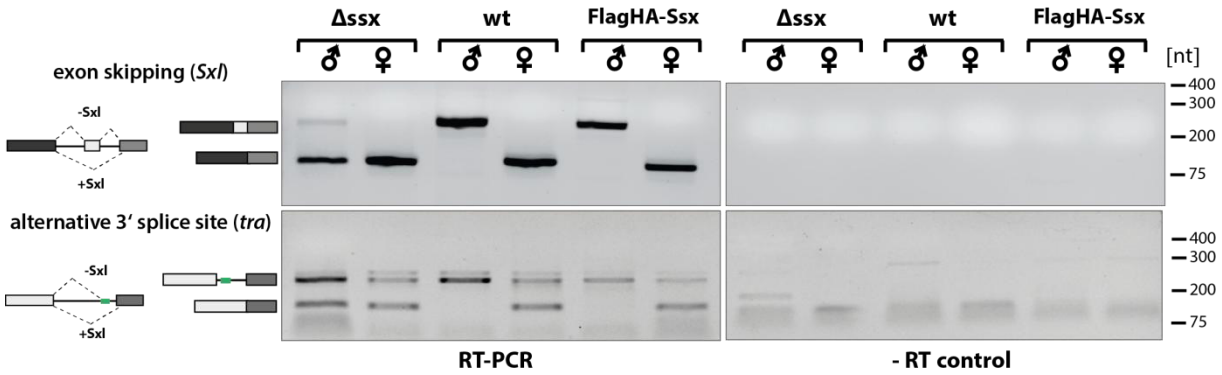

Figure 2.20: Knockout of Ssx promotes female-specific splicing of *Sxl* mRNA in male flies. Splicing analysis by RT-PCR of *Sxl* mRNA demonstrated an alteration in male vs. female isoform abundance. Knock out of Ssx resulted in detectable levels of the female-spliced isoform of *Sxl* in male flies, while flies with overexpressed FlagHA Ssx remained unaffected. Furthermore, a shift in *tra* alternative splicing was detected. Negative controls performed without reverse transcriptase excluded a genomic DNA or PCR product contamination. Alternative splice patterns are depicted on the left, a nucleotide size marker is shown on the right.

Furthermore, this altered ratio of *Sxl* transcript isoforms changed the splicing pattern of *tra* mRNA, promoting female-specific splicing.

2.2.5 Ssx can act as a splicing regulator of *Sxl* pre-mRNA

To confirm that Ssx can act as a splicing regulator of *Sxl* pre-mRNA, additional studies in male Dmel-2 cells and female Kc167 cells were performed. First, we overexpressed Ssx in female cells, transfecting increasing amounts of FlagHA-tagged Ssx, whereas FlagHA-tagged GFP served as control. Expression levels were detected by Western Blot analysis using a monoclonal anti-HA antibody for detection of overexpressed proteins, whereas probing with a polyclonal anti-Sxl antibody allowed the detection of both, endogenous Sxl and Ssx protein due to cross reactivity (Fig. 2.21 A).

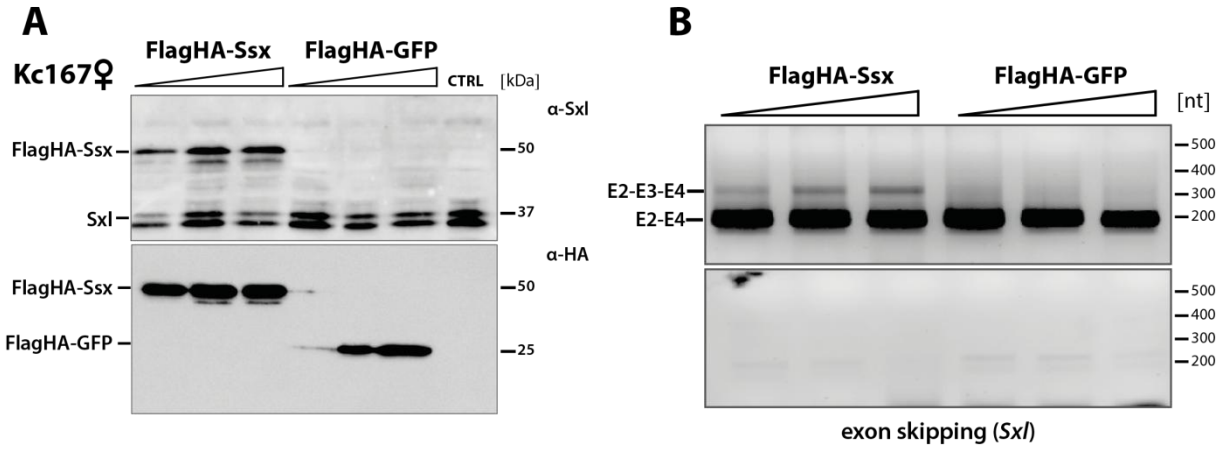

Figure 2.21: Ssx promotes poison exon inclusion of *Sxl* pre-mRNA in female cells. A) Anti-Sxl and anti-HA Western Blotting to detect expression levels of transfected protein variants and endogenous Sxl protein (indicated above each lane) in cultured Kc167 cells. Detected proteins are depicted on the left. **B)** RT-PCR analysis of alternative splicing of the endogenous *Sxl* mRNA in female Kc167 cells. Increasing amounts of FlagHA-Ssx and -GFP constructs were transfected (indicated above each lane). –RT reaction confirmed no DNA contamination. Spliced transcript isoforms are depicted on the left, molecular height marker is shown on the right.

Analysis of RT-PCR data clearly demonstrated that the increase of Ssx concentrations resulted in altered splicing patterns and reduces exon skipping of the poison exon within the *Sxl* transcript in female cells. Transcripts that contain the poison exon were exclusively detected in the Ssx overexpression sample, whereas forced GFP overexpression did not change female-specific *Sxl* splicing patterns (Fig. 2.21 B).

To further validate this result we moved from the female cultured cells that constitutively express functional Sxl protein, to male cultured cells in which functional Sxl protein is absent and the poison exon is constitutively included in the mature mRNA. We expressed Sxl protein only to demonstrate its ability to influence alternative splicing of the endogenous *Sxl* mRNA (Fig. 2.22 lanes 1 and 2). In parallel, FlagHA-Ssx was overexpressed under identical conditions but in this case, no altered *Sxl* splice pattern was observed (Fig. 2.22 lanes 3 and 4). Next, we forced expression of both Sxl and Ssx creating a situation where both proteins compete for binding to the endogenous Sxl primary transcripts.

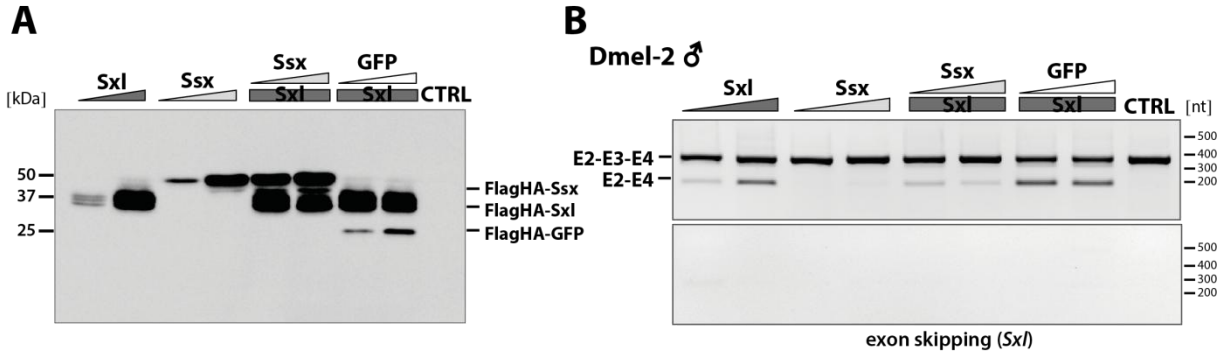

Figure 2.22: Ssx interferes with the Sxl-induced female splice pattern in male cells. A) Western Blotting against the HA-tag detected the expression of FlagHA-tagged Sxl, Ssx and GFP protein (indicated above each lane). **B)** RT-PCR analysis of sex-specific *Sxl* alternative splicing patterns in Dmel-2 cells transfected with FlagHA Sxl, FlagHA Ssx and co-transfections of FlagHA-Sxl in combination with -Ssx or -GFP (indicated above each lane). Minus RT control confirmed no DNA contamination. Alternatively-spliced *Sxl* transcripts are depicted on the left, molecular size marker is shown on the right.

Here, Ssx interfered with the Sxl-induced changes in *Sxl* mRNA alternative splice patterns, forcing it back to the male-specific transcript isoforms (Fig. 2.22, lanes 5 and 6). In contrast, this effect was not observed when a control vector coding for FlagHA GFP was used instead of FlagHA-Ssx (Fig. 2.22, lanes 7 and 8). In sum, Ssx competes with Sxl for binding to the same RNA binding elements and inhibits its regulatory effect on Sxl pre-mRNA alternative splicing to promote the inclusion of exon three in the *Sxl* pre-mRNA.

2.2.6 The N-terminus of Sxl is essential for the alternative splicing of *Sxl* mRNA

The effect of Sxl on alternative splicing of its own transcript does not only require on RNA binding, but also the N-terminus of Sxl which in this context plays a fundamental role by

mediating various interactions with spliceosomal components (Wang and Bell 1994). In previous studies it was shown that the Sxl N-terminus fused to a ß-galactosidase protein was sufficient to induce female-specific lethality. The authors proposed that the fusion construct is able to compete with the endogenous Sxl protein thereby interfering with correct splicing of *Sxl* in female flies. Furthermore, it was found that the chimeric protein containing the Sxl N-terminus interacted with Snf, a homologue of the mammalian U1A and U2B" splicing factors. In addition, also the female-spliced isoform of *tra* mRNA was found to be weakly enriched in male flies expressing the Sxl N-terminus (Deshpande et al. 1999; Yanowitz et al. 1999). To further validate the requirement of the Sxl N-terminus for the alternative splicing of *Sxl* in a more physiological setting, we generated chimeric proteins consisting of the Sxl N-terminus fused to the central and C-terminal regions of Ssx (SxlSsx), or *vice versa* (SsxSxl). All constructs were tested by RT-PCR for their ability to induce female-specific splicing in male Dmel-2 cells (Fig. 2.23 A). Expression of the proteins was confirmed by Western Blot analysis (Fig. 2.23 B).

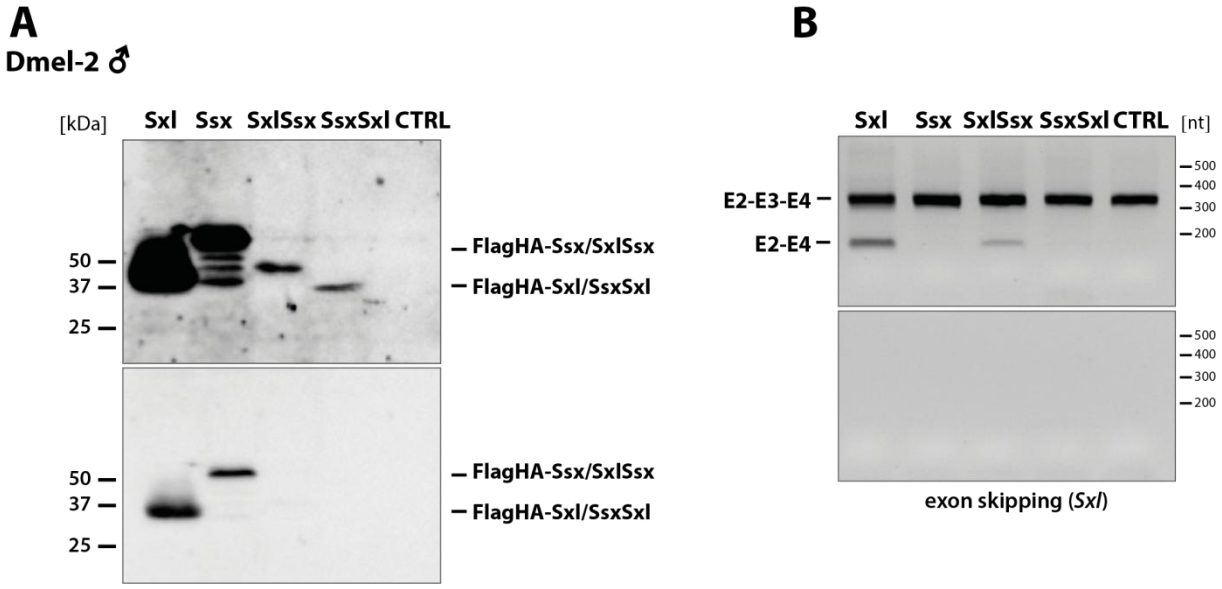

Figure 2.23: Splicing-regulatory activity of Sxl requires the N-terminal protein domain. A) Anti-HA Western Blotting to display the expression levels of transfected FlagHA-tagged constructs in cultured Dmel-2 cells (indicated above each lane). A long exposure is shown on top, a weak exposure of the same blot is shown below. Molecular weight marker sizes are depicted on the left. **B)** RT-PCR analysis to monitor sex-dependent splicing of endogenous Sxl-mRNA in Dmel-2 cells. Cells were transfected with FlagHA-tagged proteins Sxl, Ssx and chimeric proteins thereof and compared to a control sample (depicted above each lane). Transcript variants of *Sxl* mRNA are marked on the left, nucleotide weight marker is shown on the right.

The result of the RT-PCR of sex-specific *Sxl* transcript isoforms clearly demonstrated the requirement of the Sxl N-terminus for skipping of the alternative exon. Similar to wt Sxl, a protein that contains the N-terminal domain of Sxl followed by elements derived from Ssx (denoted as SxlSsx) promoted skipping of the alternative exon upon transfection into Dmel-2 cells. Differences in transcript ratios were mainly linked to unequal protein expression

(signals for wt proteins vs. chimeric proteins in Figure 2.23 B strong and weak exposure of the blot is shown in the upper and lower panel, respectively). In contrast to that, the chimeric protein SsxSxl does not have the ability to induce female-specific splicing of *Sxl* mRNA in the male cell line. Since we assume that the RNA binding properties of all four analysed proteins are comparable (see Fig. 2.11), sex-specific splicing of *Sxl* mRNA clearly depends on Sxl's N-terminus, confirming previous findings (Deshpande et al. 1999; Yanowitz et al. 1999). Moreover, these results further indicate that the N-termini of Sxl and Ssx are functionally distinct.

2.3 A new identified Ssx binding site is located in *msl-2* mRNA

2.3.1 Comparable binding affinities of Sxl and Ssx to the *msl-2* binding sites

According to our data (Fig. 2.14) *Sxl* was not the only target RNA found to be highly enriched in Ssx iCLIP experiments, but also another well-studied Sxl target RNA was identified: *msl-2*. Here, in the absence of Sxl, Ssx occupied several U-stretches of varying length. These U-stretches represent the six binding sites for Sxl within the msl-2 5' UTR (A and Bsite) as well as the 3' UTR (C, D, E and Fsite) (Bashaw and Baker 1997; Kelley et al. 1997; Gebauer et al. 1998). To further confirm the association of Ssx with *msl-2* RNA, RNA immunoprecipitation experiments were performed. Here, overexpressed FlagHA-tagged Ssx or FlagHA-tagged GFP as control were immunoprecipitated with anti-Flag antibodies and co-precipitated RNAs were isolated. QRT-PCR revealed a nearly 50-fold enrichment of *msl-2* mRNA bound by Ssx relative to the GFP control, whereas the levels of co-precipitated alpha-tubulin, lacking predicted binding sites, remained unaffected (Fig. 2.24). Furthermore, these findings are in agreement with the recently published results of Rogell et al. 2017, who showed by a specific RNP capture method that Ssx interacts with the *msl-2* RNA in *Drosophila* embryonic extracts.

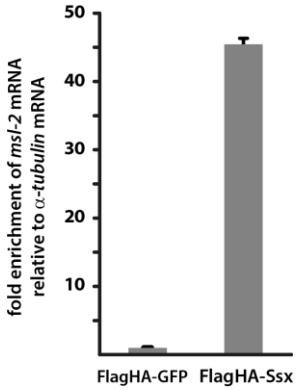

Figure 2.24: Immunoprecipitated Ssx protein is bound to endogenous *msl-2* mRNA. Immunoprecipitation of FlagHA-tagged Ssx protein and FlagHA-tagged GFP was followed by qRT-PCR analysis for the enrichment of *msl-2*. Results were normalized to *alpha-tubulin* levels.

To unravel the biological significance of this finding, the binding affinities of both proteins to the binding sites within *msl-2* were compared in EMSAs. For this, the central domains of Sxl (Sxl-RBD4 aa122-301) and Ssx (Ssx-RBD4 aa93-269) were recombinantly expressed and

purified (compare to Fig. 2.9 and Fig. 2.31 B). Binding affinities to short RNA oligonucleotides were measured by titration of increasing protein concentrations to a constant amount of radiolabeled RNA substrate. As already shown in Fig. 2.11 the binding affinities of Sxl and Ssx to the Esite within the 3'UTR of *msl-2* are comparable, whereas binding was completely abolished upon mutation of every second Uracil to a Cytosine. In a similar experiment we employed the well characterized 5'UTR binding site of *msl-2* consisting of an U16 stretch, denoted as Bsite. Here, both proteins show similar binding affinities to the RNA within the nanomolar range (Fig. 2.25). With higher Sxl protein concentrations, an additional shift is observed, which is likely to represent two Sxl molecules bound to a single target mRNA. This increase in stoichiometry was exclusively detected for Sxl-RBD4, and is in agreement with previous reports showing that Sxl is able to dimerize upon mRNA binding (Samuels et al. 1998). Instead, Ssx did not display such a binding behavior. The specificity of the interactions was confirmed by using the Bsite mut RNA substrate, in which every second uracil was converted to a cytosine abolishing protein binding (Fig. 2.25).

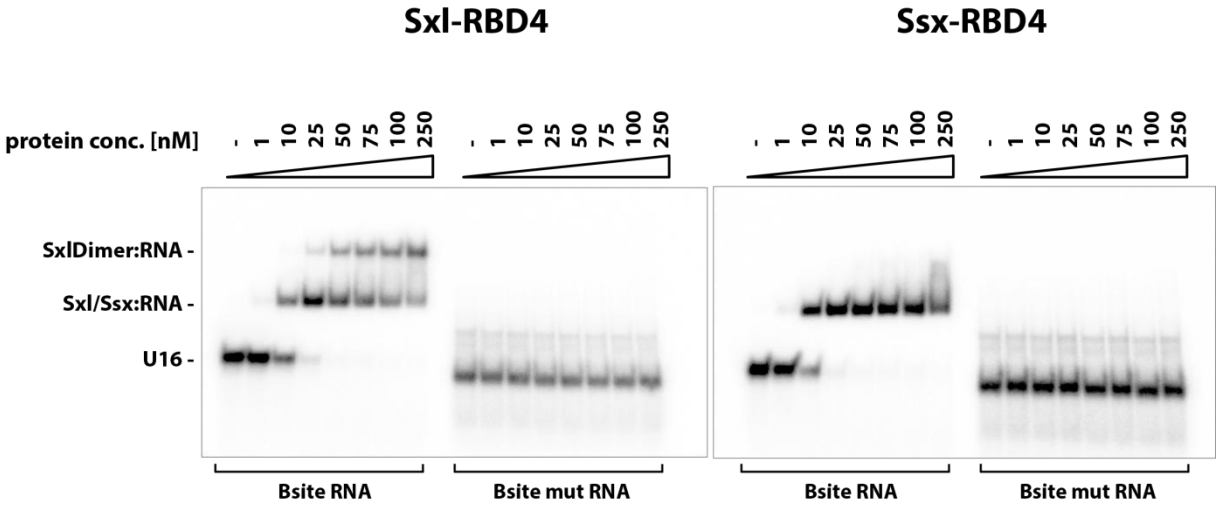

Figure 2.25: Sxl-RBD4 and Ssx-RBD4 bind with similar affinities to the *msl-2* Bsite. Binding affinities of Sxl-RBD4 and Ssx-RBD4 to radioactively labeled RNA substrates (Bsite: U16; Bsite mut $(UC)_8$) were monitored by EMSA. Amount of protein were indicated above each individual lane. Running height of free RNA and RNA:protein complexes were indicated on the left.

2.3.2 Ssx is able to regulate *msl-2* translational repression via the 5'UTR mechanism

Both proteins, Sxl and Ssx, can bind to the 5' and 3' regulatory sequences of *msl-2* mRNA. The functional consequence and the biological relevance of Ssx binding to *msl-2* however, remain unanswered. As described in previous sections, Sxl is able to promote retention of a facultative intron in the 5'UTR of *msl-2* as well as for its role as translational repressor of *msl-2*. The latter function is realized by a dual block to translation initiation mediated by binding sites in the 5' and 3' UTRs of *msl-2*. We already presented evidence suggesting that Ssx lack of ability to promote intron retention in the *msl-2* transcript (Fig. 2.18). However, it remains

unanswered if Ssx can act as translational regulator. In contrast to the function in splicing regulation, for which the N-terminal region of Sxl is required, the central region of Sxl is sufficient to mediate translational repression of *msl-2* (Grskovic et al. 2003). Since both proteins are differing within their N-terminal parts but are ~80% identical in the central protein domain (Fig. 2.9), we hypothesized that Ssx might also regulate *msl-2* translation similar to Sxl. We thus performed *in vitro* and *in vivo* translation assays to assess this activity (Fig. 2.26). Since the central region of Sxl is necessary and fully sufficient for translational regulation, *in vitro* translation assays were performed using Sxl-RBD4 and Ssx-RBD4 constructs. The proteins were incubated in *Drosophila* embryo extracts together with reporter RNA which bears a firefly luciferase open reading frame flanked by *msl-2* derived regulatory sequences. A control reporter RNA with encoding a renilla luciferase was included for normalization purposes (Gebauer et al. 1998; Gebauer et al. 2003; Grskovic et al. 2003; Beckmann et al. 2005; Duncan et al. 2006; Duncan et al. 2009; Medenbach et al. 2011). To distinguish between 5'UTR mediated and 3'UTR mediated translational repression, reporters with either inactivated 3'UTR or the 5'UTR Sxl-binding sites were employed (Beckmann et al. 2005; Medenbach et al. 2011).

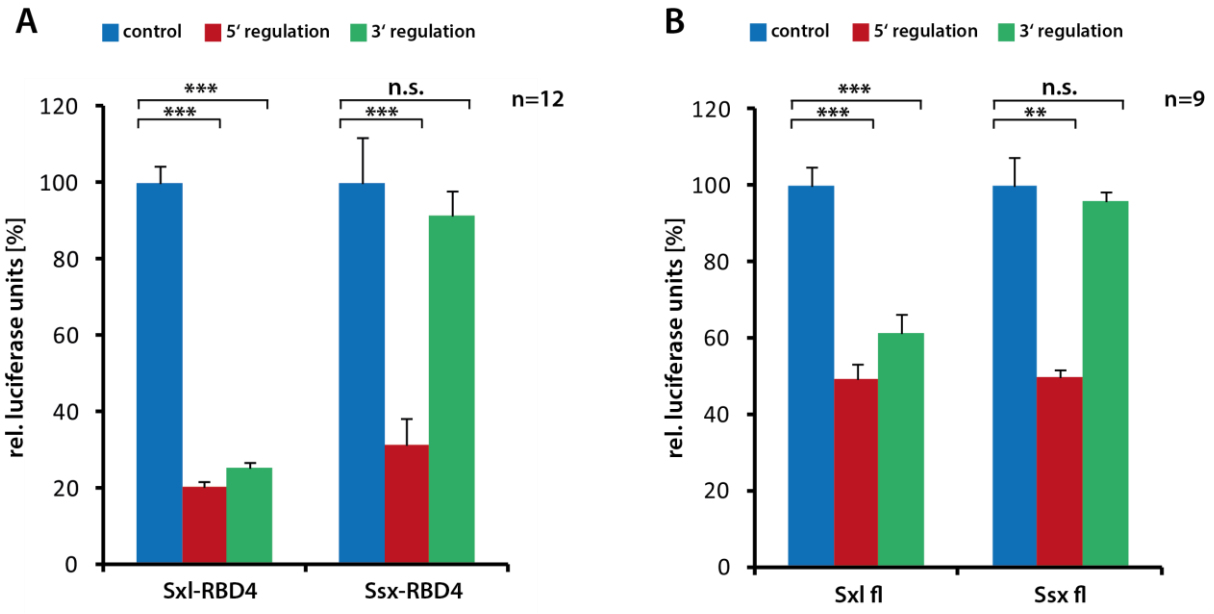

Figure 2.26: Ssx is a translational repressor regulating via the 5'UTR of *msl-2* but fails to repress translation via the 3'UTR mechanism. A) *In vitro* translation assays monitoring the translational repression of recombinant proteins Sxl-RBD4 and Ssx-RBD4 (Fig. 2.31 B) on *msl-2* 5'UTR (represented as red bars) and 3'UTR (shown as green bars) reporter constructs relative to a non-regulated reporter control (blue bars). Firefly luciferase counts were normalized to a co-translated renilla-luciferase control RNA. Experiments were performed in triplicates and four biological replicates and mean values were plotted with standard deviations. P-values were generated with student's t-test. **B)** *In vivo* translation assay of transfected FlagHA-tagged Sxl and Ssx full length proteins (for expression levels see Fig. 2.31 C) on *msl-2* 5'UTR (red bars) and *msl-2* 3'UTR (green bars) reporter plasmids relative to a unregulated reporter plasmid (blue bars) in male Dmel-2 cells. As described in A), renilla reporter luciferase counts were normalized to a co-transfected, non-regulated control firefly luciferase and mean values with standard deviation of triplicates performed in three or four biological replicates were plotted. P-values: ***<0.001: **<0.01: n.s. not significant.

As previously published (Beckmann et al. 2005), Sxl-RBD4 is able to interfere with *msl-2* translation via the 5'UTR as well as the 3'UTR mechanism (Fig. 2.26 A). Interestingly, Ssx-RBD4 downregulated translation to a similar extent compared to Sxl-RBD4 on the *msl-2* 5'UTR, but was inactive in translational repression via the 3'UTR (Fig. 2.26 A). In sum, although the binding affinities of the two proteins are comparable on both binding sites, they differ drastically in their ability to repress translation acting via the 3'UTR. In order to verify these findings in a more physiological setting, we performed *in vivo* luciferase assays. For this, Dmel-2 cells were transfected with FlagHA-tagged Sxl or Ssx full-length (fl) expression constructs together with the respective *msl-2* reporter plasmids. The translational repression mediated by the 5'UTR or 3'UTR of *msl-2* was determined by normalizing the firefly luciferase activity to renilla luciferase. Subsequently, the relative activities were compared to the control reporter. As expected and previously published, Sxlfl represses translation via both UTR pathways (Medenbach et al. 2011), whereas Ssxfl nicely recapitulated the *in vitro* situation (Fig. 2.26 B). A comparable expression level of the tagged proteins was confirmed by Western Blotting (Fig. 2.31 C). In sum, although Ssx is a closely related paralog of Sxl, it only interferes with translation via the 5'UTR dependent mechanism but failed to down-regulate translation via the 3'UTR of *msl-2* (Fig. 2.26).

2.3.3 Dissection of domains necessary for 3' translational regulation

The two closely related paralogs Sxl and Ssx in *D. melanogaster* share a high degree of identity between their central RNA binding domains, whereas their N- and C-terminal domains are divergent. Not surprisingly, the RNA binding behavior of the two proteins is highly similar and also their ability to down-regulate translation of *msl-2* mRNA via the 5'UTR mechanism is almost identical. However, they differ in their ability to regulate translation via the 3'UTR of *msl-2*. To uncover the reason for this functional difference, chimeric proteins were generated, allowing the identification of regions important in 3'UTR dependent translational repression. The activity of the chimeric proteins was assayed by *in vitro* luciferase assays. Here, the proteins Sxl-Ssx-RBD4 (containing the RRM1 of Sxl and the RRM2 of Ssx) and the protein Ssx-Sxl-RBD4 (containing the RRM1 of Ssx and the RRM2 of Sxl) were analysed for their functionality in translational repression and were compared to Sxl-RBD4 and Ssx-RBD4 (Fig. 2.27 A). First, all four proteins were able to interfere with 5'UTR mediated translation, confirming the correct folding and functionality of the chimeric proteins (Fig. 2.27 B red bars). Concerning the 3' mechanism of translational control, the Ssx-Sxl recombinant protein resembled the translational ability of Ssx-RBD4, whereas the chimeric protein consisting of Sxl RRM1 in combination with RRM2 of Ssx behaved exactly as Sxl-RBD4 (Fig. 2.27 B, green bars). Therefore, the functional elements required for 3'UTR mediated translational repression are located in RRM1 of Sxl.

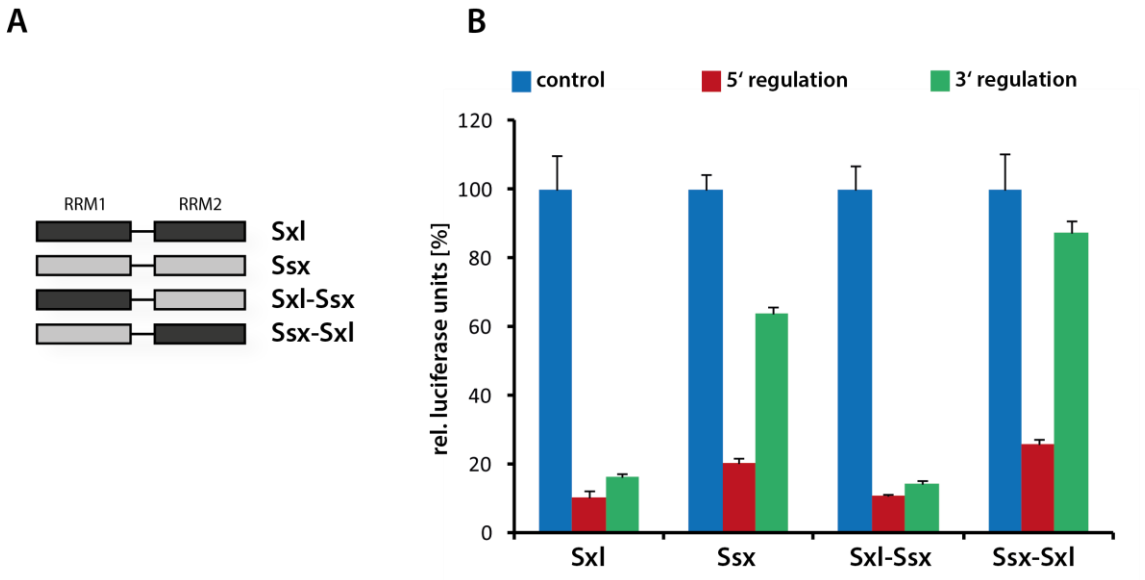

Figure 2.27: RRM1 of Sxl is necessary for msl-2 3'UTR mediated translational repression. A) Schematic overview of recombinant proteins Sxl-RBD4 (colored in dark grey), Ssx-RBD4 (depicted in light grey) and chimeric proteins SxlRRM1 fused to SsxRRM2 (Sxl-Ssx) and the *vice versa* protein SsxRRM1-SxlRRM2 (Ssx-Sxl) (see Fig. 2.31 A and B). **B)** *In vitro* translation assay monitoring translational repression of recombinant proteins shown in A) on *msl-2*-derived firefly reporter RNAs performed and analysed as described in Fig. 2.26A.

To unravel the difference in activity between Sxl and Ssx, we performed a detailed mutational analysis of RRM1. The RRM1 sequences of Sxl and Ssx differ in 18 amino acids (Fig. 2.29). These amino acid differences were divided into four clusters and each cluster (C1 to C4; Fig. 2.28 A) in Ssx RRM1 was individually converted to the corresponding Sxl sequence. Recombinant proteins were expressed, purified and analysed for differences in *in vitro* luciferase assays (Fig. 2.28 B). Again, correct folding and functionality of each mutant protein was confirmed by analysis of the *msl-2* 5'UTR translational repression (Fig. 2.28 B red bars). These experiments suggested that cluster 1 (containing amino acid substitutions N111A, S114R, G115A and C116I) was important for activity (Fig. 2.28 B, green bars). However, compared to wild type Sxl protein, Ssx C1 was still approximately 20% less efficient in translational repression, suggesting that additional amino acids contribute to function.

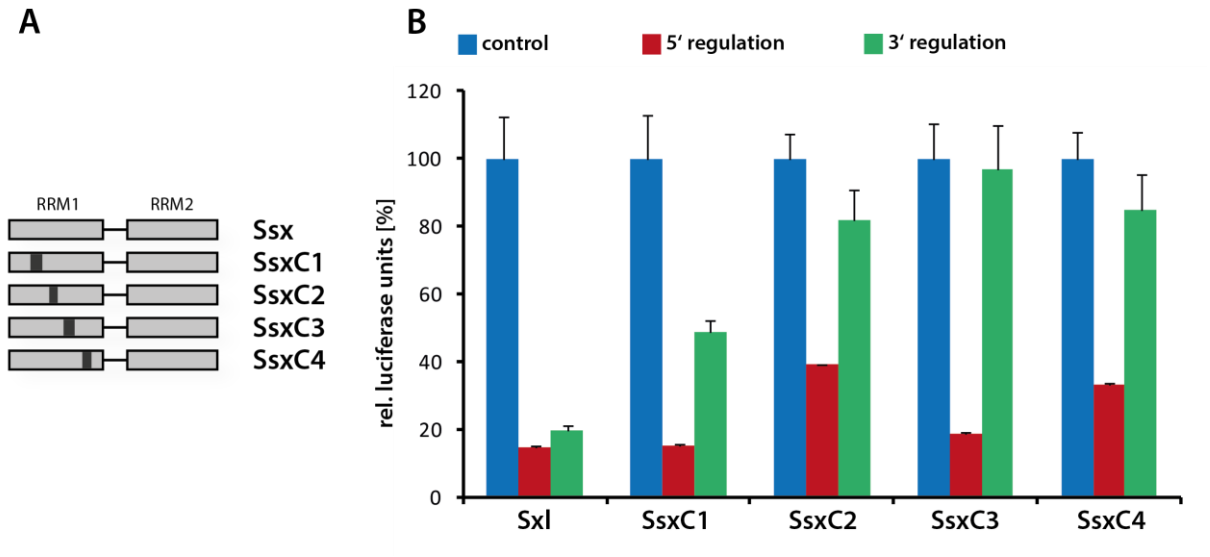

Figure 2.28: Conversion of cluster 1 in Ssx results in a 3'UTR-dependent translational repression of *msl-2* RNA. A) Schematic overview of Ssx-RBD4 proteins with individual mutated clusters 1 to 4 (shown as dark grey boxes). Following conversions were made within each respective cluster: Cluster 1:N111A, S114R, G115A and C116I; Cluster 2: Y141F, K142T, T143S; Cluster 3: S145M, E148Q, D149R, Q152K, K153V; Cluster 4: F157I, Y158T **B)** *In vitro* luciferase assay showing translational repression of each recombinant protein described in A). Translation assay conditions and calculations as described in Fig. 2.26 A.

In order to identify the minimal set of mutations necessary to artificially generate 3'UTR dependent translational repression of *msl-2*, we produced additional constructs comprising the mutation of cluster 1, or single residues within cluster 1, in combination with mutations of amino acids in close proximity (Fig. 2.29 A). This revealed that conversion of N111A, K123R and F128Y in Ssx resulted in a gain-of-function (Ssx GOF) and an efficient repression of *msl-2* translation via the 3'UTR.

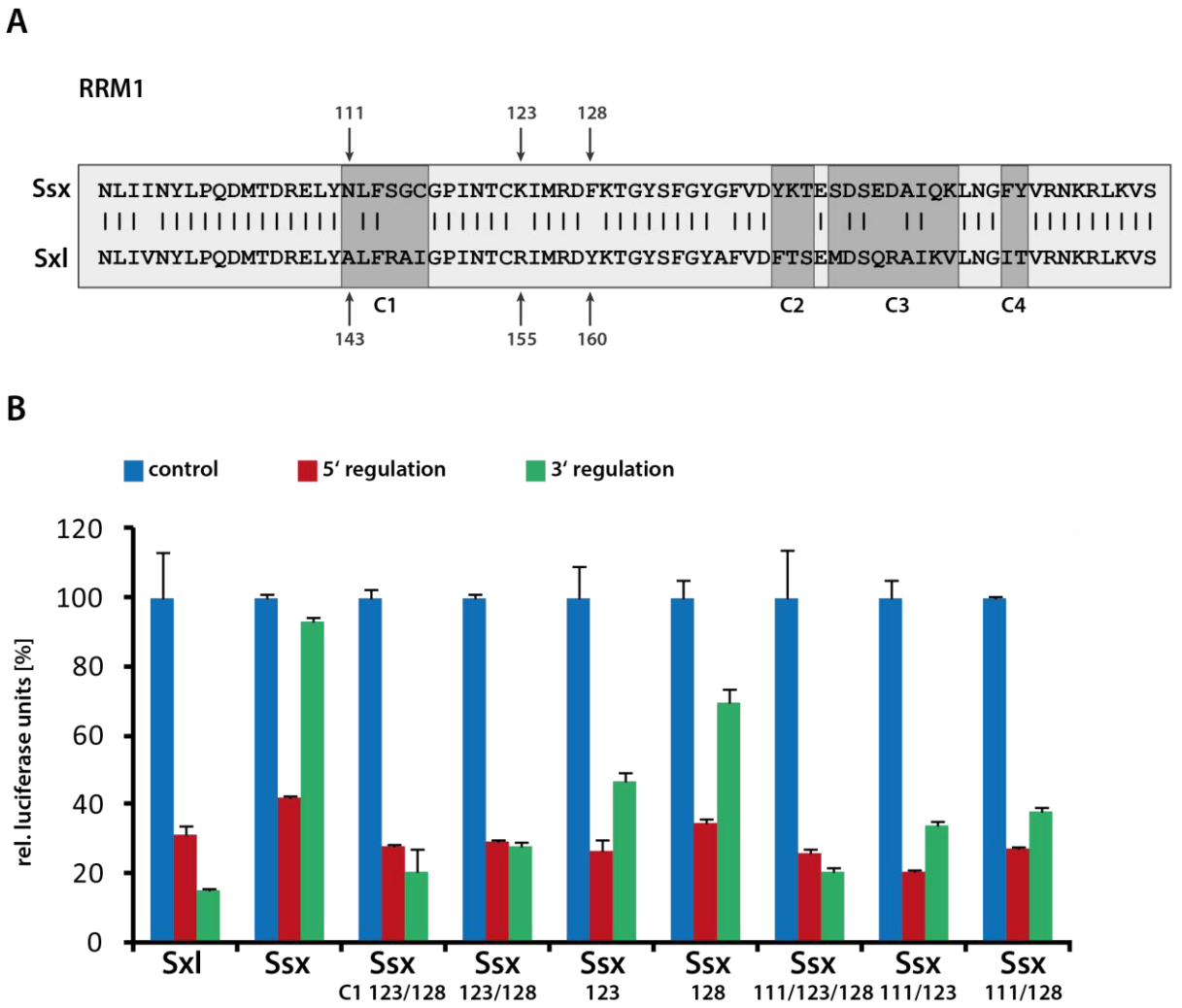

Figure 2.29: The Ssx GOF protein represses *msl-2* translation via both UTR mechanisms. A) Alignment of amino acid sequences of Sxl RRM1 and Ssx RRM1. Substitutions are indicated, cluster 1-4 (C1-C4) are highlighted as dark grey shadows, amino acid substitutions critical for Ssx GOF are marked by arrows (N111A; K123R and F128Y). **B)** *In vitro* luciferase assay of Ssx proteins containing single or combined amino acid substitutions (as indicated below each sample). The assay was performed and analysed as described in Fig. 2.26 A.

Again, *in vivo* luciferase assays were performed to confirm this finding under more physiological conditions employing full length proteins were used instead of the RBD4 constructs. In addition, the necessity of the amino acid combination was validated by engineering a Sxl derivative in which the critical amino acids were converted to their Ssx counterpart.

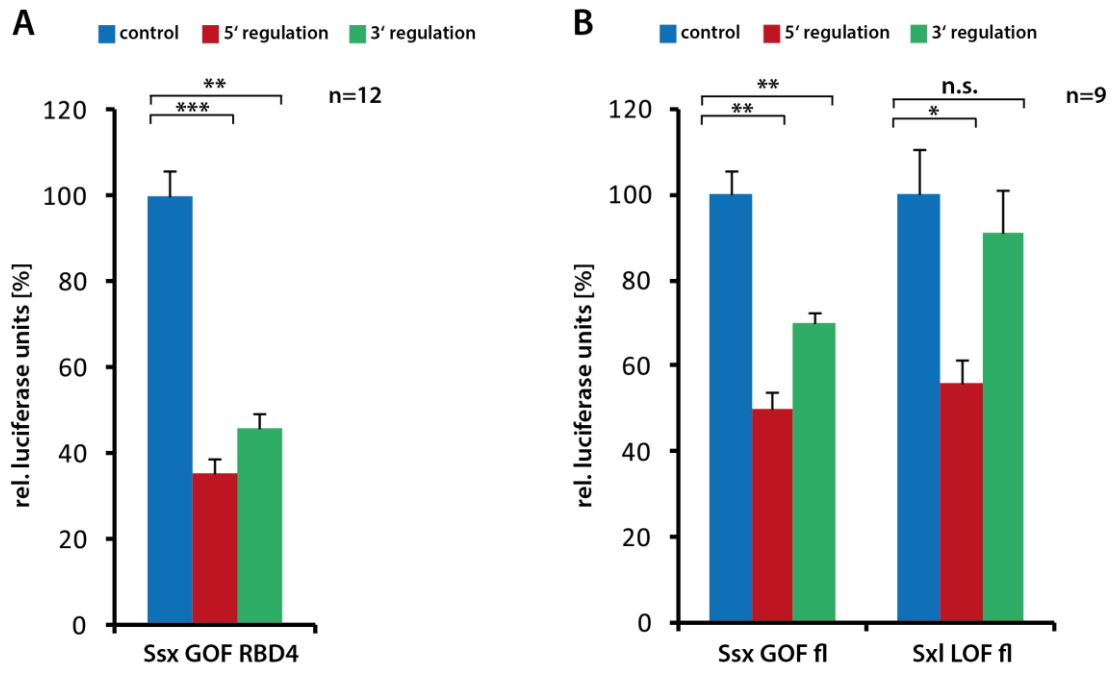

Figure 2.30: Ssx GOF is able to repress translation via the *msl-2* 5'UTR and 3'UTR. A) *In vitro* translation assay of recombinant protein Ssx GOF. Luciferase assay was performed as described previously in Fig. 2.25 A. **B)** *In vivo* translation assay of transfected FlagHA-tagged Ssx GOF (N111A; K123R and F128Y) and Sxl LOF (A143N; R155K and Y160F) full length proteins in Dmel-2 cells was performed as described in Fig. 2.26B. P-values: ***<0.001: **<0.01: *<0.05: n.s. not significant.

As expected, mutation of A143N, R155K and Y160F in Sxl abolished 3'UTR dependent translational repression of the *msl-2* reporter. A direct comparison of the decrease in repressor activity for Sxl LOF and the gain in activity for Ssx GOF is shown in Figure 2.30. Comparable expression of both RNA binding proteins was again confirmed by Western blotting (Fig. 2.31 B).

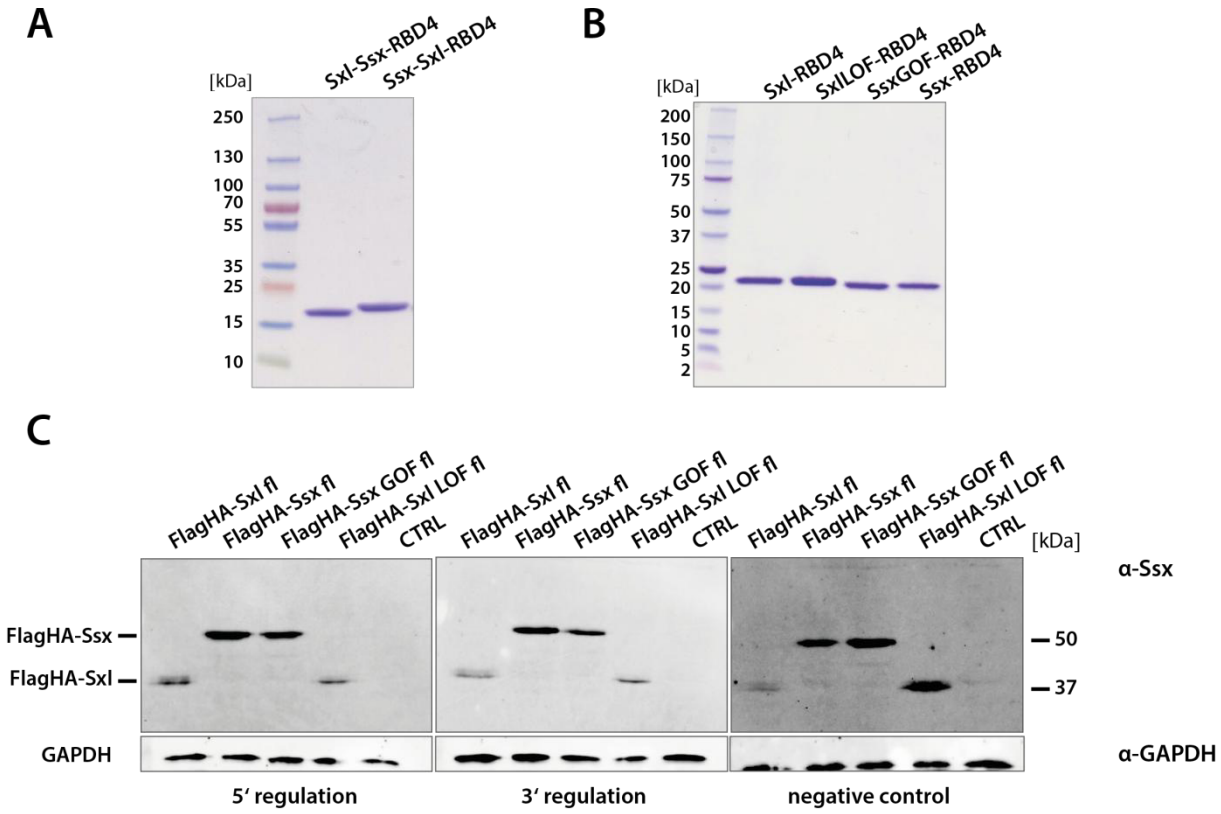

Figure 2.31: Coomassie staining of purified recombinant proteins and Western Blot analysis of overexpressed, FlagHA-tagged protein constructs. A) Coomassie staining of recombinant chimeric proteins Sxl-Ssx-RBD4 and Ssx-Sxl-RBD4. **B)** Coomassie staining of recombinant Sxl- and Ssx-RBD4 proteins and mutations thereof (indicated above each lane). **C)** Ha-probing in Western Blotting of FlagHA-tagged proteins (indicated above each lane) used in *in vivo* translation assays of samples for 5' and 3' regulation as well as for the negative control. GAPDH-probing confirmed equal loading amounts.

To further confirm that the binding behavior of Ssx GOF and Sxl LOF did not change upon mutation, EMSAs were performed according to previous EMSAs and compared to those of Fig. 2.11 and 2.25, in which the binding affinities of Sxl and Ssx were measured. In sum, Ssx GOF, Sxl LOF, Ssx and Sxl show similar binding behaviours to the 5' and 3' binding motif in *msl-2*, while amino acid substitutions in Ssx GOF and Sxl LOF did not influence the RNA:protein interaction (Fig. 2.32).

Taken together, we could unravel that by conversion of the three amino acids (N111A, K123R and F128Y) in Ssx RRM1 to their Sxl counterpart, the protein gains in function and is able to downregulate *msl-2* translation via the 3'UTR dependent pathway. In line with this, the substitution of these three amino acids in Sxl to their Ssx counterpart was associated with a loss of function in Sxl concerning the 3'UTR dependent translational repression of *msl-2* expression.

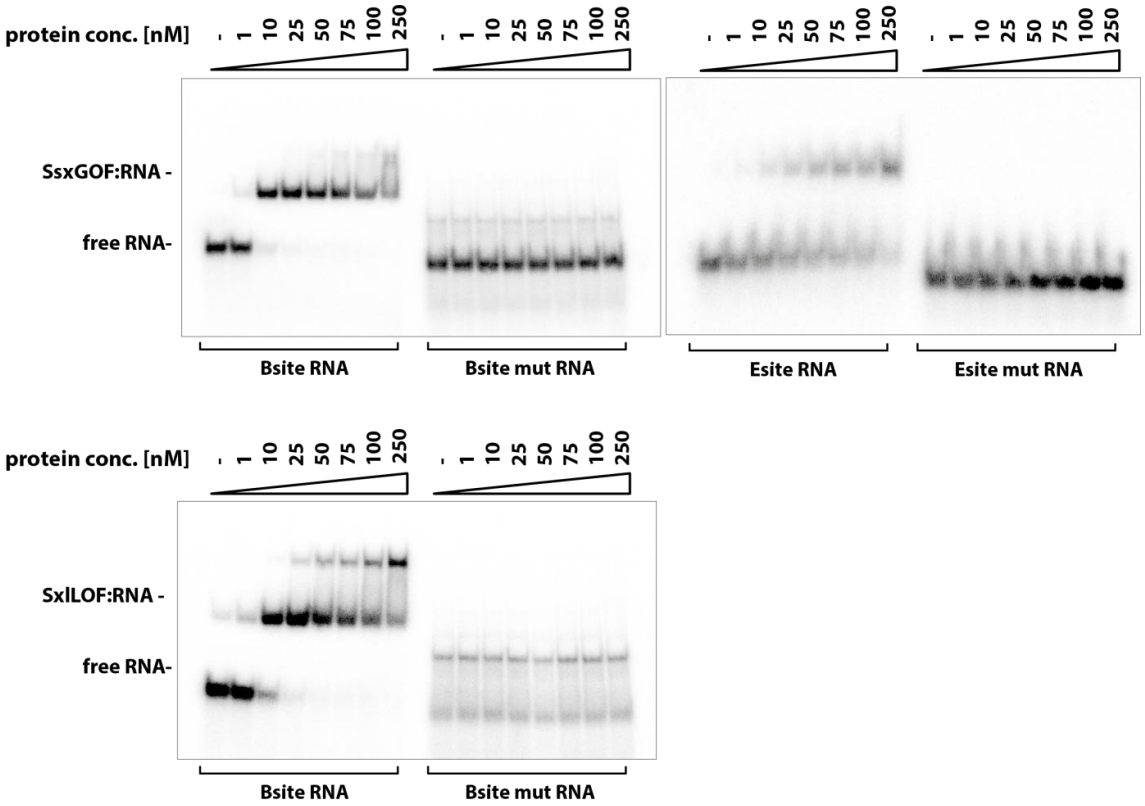

Figure 2.32: Ssx GOF and Sxl LOF exhibit a similar binding behaviour to the *msl-2* binding sites. A radioactively labeled RNA fragment derived from the *msl-2* 5'UTR (Bsite; left panel) or 3'UTR (Esite; right panel) and the respective mutant derivate were used for EMSAs. The RNA was incubated with indicated concentrations of SsxGOF or SxlLOF protein. RNA-protein complexes were resolved by native PAGE and are indicated on the left.

2.3.4 Differences in translational repressor activity correlate with the recruitment of the co-repressor UNR

Sxl-mediated translation control acting via the 5'UTR or 3'UTRs of *msl-2* differs in the requirement for co-factors. While the 5'UTR-mediated regulation requires an upstream open reading frame (Beckmann et al. 2005; Medenbach et al. 2011), the 3'UTR mechanism critically requires the cofactor UNR (Grskovic et al. 2003; Abaza et al. 2006; Duncan et al. 2006). In previous studies, it was shown that the cold shock domain 1 (CSD1) of UNR is necessary and sufficient to form a trimeric complex with Sxl-RBD4 and the *msl-2* mRNA, which harbors binding sites for both proteins (Abaza et al. 2006, Duncan et al. 2006, Hennig et al. 2014). Even though UNR itself is an RNA binding, it requires Sxl to be recruited at the *msl-2* 3'UTR binding site (Abaza et al. 2006, Duncan et al. 2006; Abaza and Gebauer 2006; Fig. 2.33). We assayed for trimeric (Sxl/Ssx:UNR-CSD1:RNA) complex formation using recombinant UNR protein and Sxl/Ssx proteins (or variants thereof) and a variety of RNA substrates. First, recombinant UNR (CSD1) protein was expressed, purified and analysed for binding to the *msl-2* 3'UTR (5'-UUUUUUUGAGCACGUGAA-3'). As expected, no Sxl-independent RNA binding of UNR-CSD1 was observed by EMSA (Fig. 2.33).

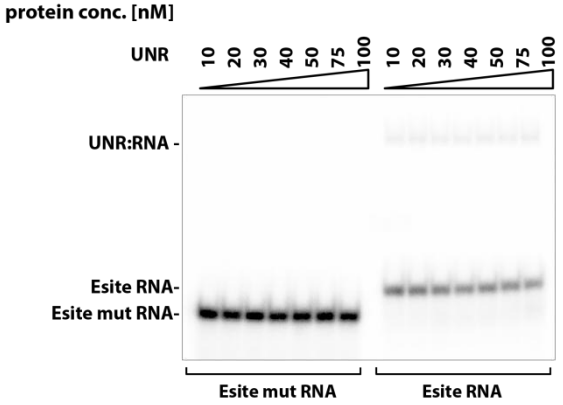

Figure 2.33: UNR-CSD1 does not bind to the *msl-2* 3'UTR binding site. EMSA of UNR-CSD1 and the radioactively labeled *msl-2* 3'UTR binding site (Esite: 5'-UUUUUUUGAGCACGUGAA-3', underlined sequence represents the UNR binding site) or a mutation thereof (Esite mut: 5'-UCUCUCUGAGCACGUGAA-3'). Protein concentrations are indicated above each lane. Free RNA substrate and RNA:protein complexes are indicated on the left.

To monitor the trimeric complex formation, we next performed binding experiments with Sxl/Ssx either in the presence or absence of recombinant UNR-CSD1. As expected, we did not observe the trimeric complex on the *msl-2* 5'UTR-derived RNA fragment that lacks the UNR binding site (Fig. 2.34).

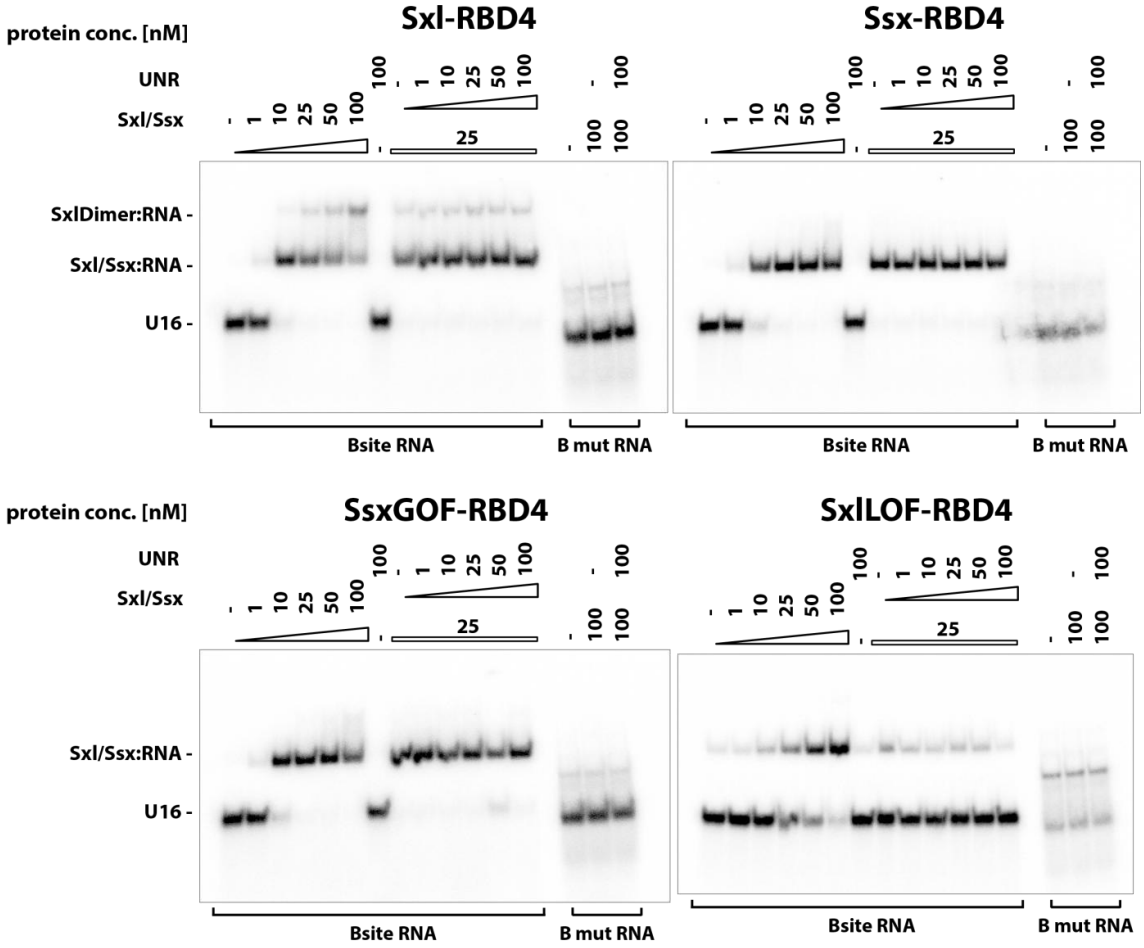

Figure 2.34: UNR is not recruited to the 5'UTR *msl-2* motif. EMSAs of proteins Sxl-, Ssx-RBD4 or derivatives thereof titrated to the *msl-2* Bsite or incubated with increasing concentrations of UNR-CSD1 (indicated above each lane). Conversion of every second U to C confirmed specificity. Free RNA or RNA:protein dimers are indicated on the left.

In contrast, when employing a 3'UTR-derived RNA fragment that contains both, a Sxl and a UNR binding site, robust complex formation could be observed for Sxl and UNR (Fig. 2.35, upper left panel). Both proteins exhibit synergistic binding to the *msl-2* 3'UTR binding motif. In contrast, Ssx shows a comparable binding affinity to the 3'UTR motif but differs in its ability to form a stable, trimeric complex which could only be observed at high protein concentrations (Fig. 2.35, upper right panel).

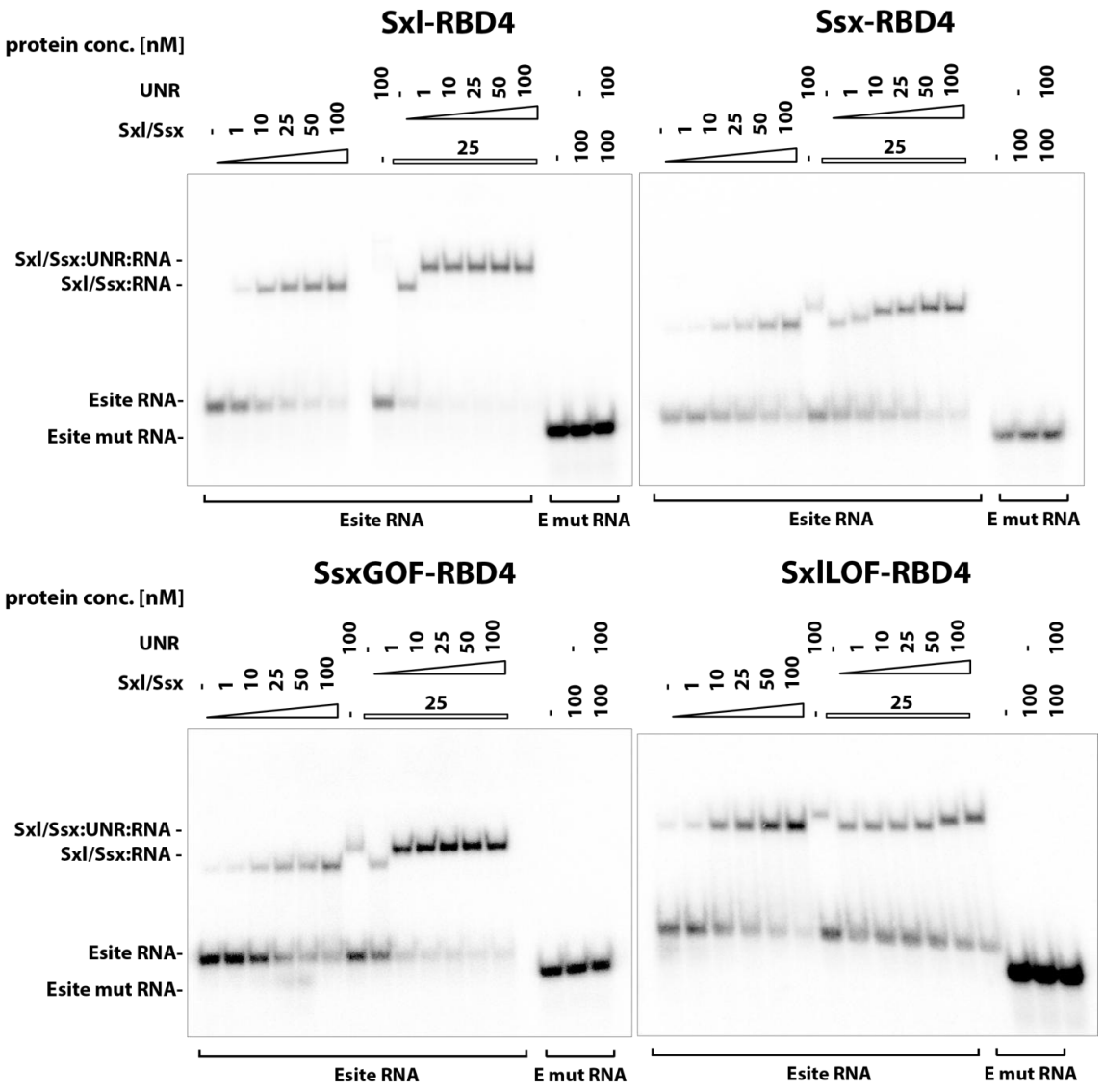

Figure 2.35: Ssx GOF recruits UNR-CSD1 to the *msl-2* Esite and forms a trimeric complex. EMSA performed as in Fig. 2.34 but using the radioactively labeled *msl-2* Esite. Sxl-, Ssx-RBD4, Ssx GOF and Sxl LOF were tested for their ability to recruit UNR-CSD1 to the *msl-2* Esite. Mutation of every second U to a C abolished binding and confirmed specificity. Running height of free RNA and RNA:protein dimers/trimers is depicted on the left.

Next, we assayed the Ssx GOF protein for binding to the *msl-2* 3'UTR either alone or in combination with UNR-CSD1. Its binding affinity was comparable to Sxl and Ssx (Fig. 2.35 lower left panel, lanes 1-6) and Ssx GOF could efficiently recruit UNR-CSD1 to the *msl-2*

RNA, assembling into a robust trimeric complex (Fig. 2.35 lower left panel, lanes 8-13). The opposite effect was observed for the Sxl LOF protein, which lost the synergistic binding to UNR due to the conversion of the three single amino acids A143N R155K Y160F. This resulted in an impaired Sxl-UNR-RNA complex formation (Fig. 2.35 lower right panel).

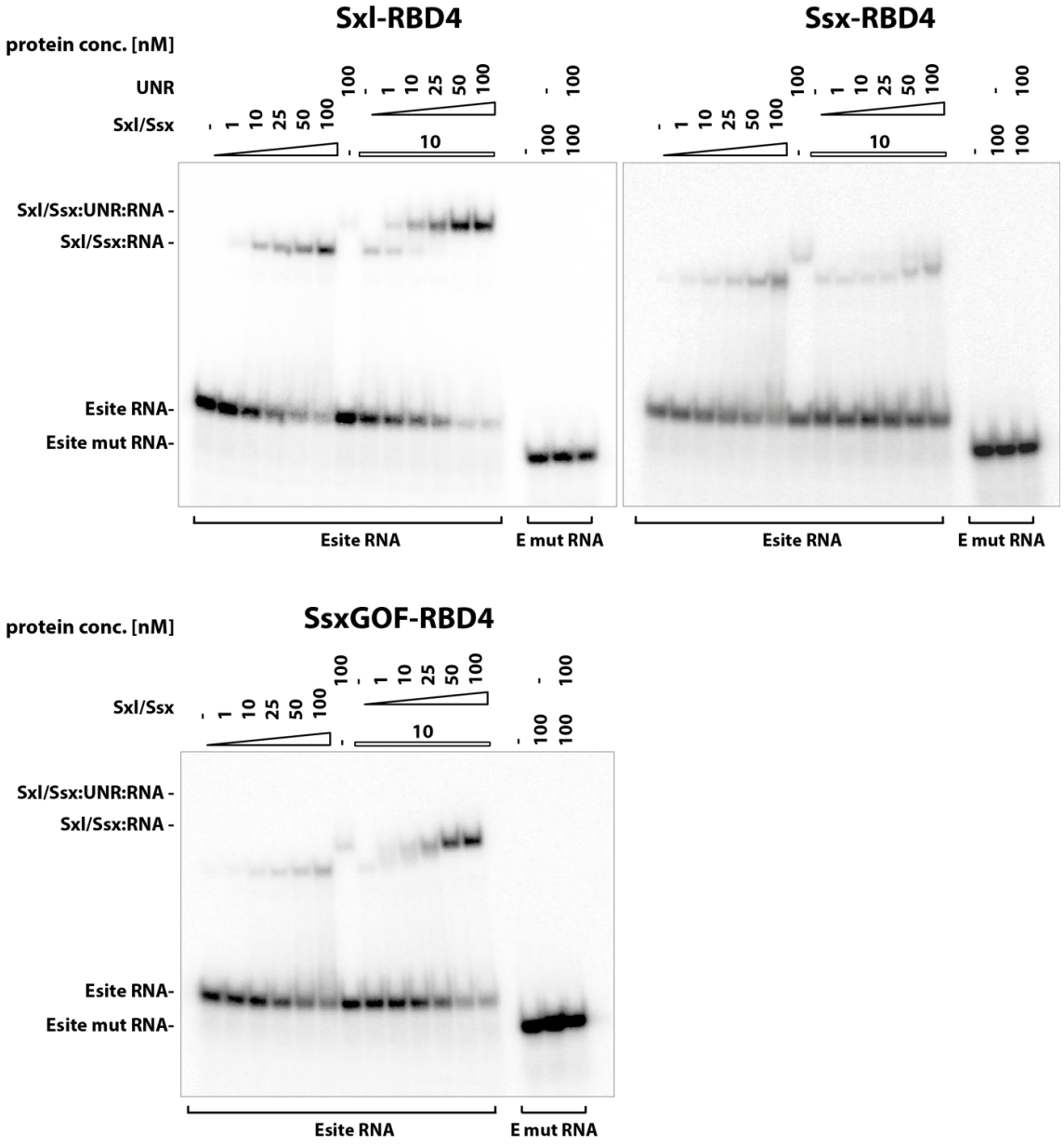

Figure 2.36: Ssx GOF interacts with UNR-CSD1 and recruits the protein to the *msl-2* RNA. EMSA performed as described in Fig. 2.35 using an amount of 10nM protein instead of 25nM Sxl-, Ssx-RBD4 or Ssx GOF protein**.** Running height of free RNA and RNA:protein dimers/trimers is depicted on the left.

Lastly, we aimed to dissect complex formation on the *msl-2* 3'UTR motif using 10nM Sxl/Ssx/Ssx GOF protein instead of 25nM. We observed that at a 1:10 molecular ratio of UNR-CSD1 to Sxl-RBD4 approximately 50% of the RNA-bound Sxl proteins shifted into the trimeric complex, while the other 50% formed a Sxl:RNA complex (Fig. 2.36 upper left panel, lane 9). A similar stoichiometry was observed for Ssx GOF (Fig. 2.36 lower left panel), while

for Ssx, even 100-fold higher UNR concentrations were not sufficient to form the trimeric Ssx:UNR-CSD1:RNA complex (Fig. 2.36 upper right panel). In sum, we were able to demonstrate that the conversion of the three amino acids N111A, K123R and F128Y in Ssx results in a gain-of-function. The interaction of Ssx GOF with UNR-CSD1 and the *msl-2* Esite results in a robust trimeric complex necessary for translational repression of *msl-2*.

3 Discussion

3.1 The complex relationship of Sxl and Ssx

In *D. melanogaster*, sexual development is controlled by the protein Sxl which controls sex determination and dosage compensation. The function of Sxl as master regulator of female development is unique for Drosophilids. In contrast, other fly and insect species employ the protein Tra as master regulator of sex determination (reviewed in Pane et al. 2002; Penalva and Sanchez 2003; Cline et al. 2010; Hediger et al. 2010; Verhulst et al. 2010). Sxl gained these skills approximately 100 million years ago. This coincides with a gene duplication event that gave birth to its closely related paralog Ssx (Cline 1984; Traut et al. 2006; Cline et al. 2010). Recent evolutionary analyses fuelled the hypothesis that both proteins underwent a subfunctionalization and developed functions unrelated to their ancestral task (Mullon et al. 2012). Even though Sxl is one of the best studied proteins in *Drosophila*, the molecular function of its paralog Ssx remains enigmatic. By comparing Sxl and Ssx on a molecular level, we observed several functional similarities (Fig. 2.11; 2.12; 2.25; 2.26). However, also differences between the two proteins became apparent (Fig. 2.18; 2.20-23; 2.26). The following chapters will compare and discuss the similarities and differences of Sxl and Ssx. The first part will focus on their role in the regulation of alternative splicing of *Sxl* mRNA and other sex-dependent mRNAs, while the second part discusses the impact of Sxl and Ssx on the translational regulation of the *msl-2* mRNA.

3.2 The complex interplay of Sxl and Ssx in *Sxl* mRNA splicing

3.2.1 Sxl promotes female-specific alternative splicing - Ssx stabilizes the male-specific splicing pattern

In contrast to other systems, flies do not encode a Y-specific protein like SRY in humans (Sinclair et al. 1990) or Xol-1 in *C. elegans.* These male-specific factors promote development of male individuals by initiating the production of male hormones by establishing male sexual characteristics (reviewed in Lucchesi et al. 2005; Ercan and Lieb 2009). In *Drosophila*, however, male hormones do not govern sexual development and the Y chromosome does not participate in sex determination. The sex decision in the early embryo is made by a syncytium, which is guided exclusively by the ratio of X-chromosomes to autosomes (X:A) (Gonzalez et al. 2008). This elementary decision is implemented by the

auto-regulatory positive feedback loop of the female-specific protein Sxl. This positive feedback loop converts a transient and weak signal into an all-or-nothing response that is continuously maintained in female flies, committing to female development. In contrast, Sxl protein production remains shut-off in males (reviewed in Salz and Erickson 2010). The role of Sxl in alternative splicing has long been appreciated, although numerous molecular details are still unclear. However, the question whether the paralog Ssx participates in this pathway remained unanswered. The analysis of the role of Ssx in alternative splicing was initiated due to the detection of binding events between Ssx and the Sxl pre-mRNA by iCLIP experiments in *Drosophila* male cells (Fig. 2.14). The expression of the Ssx protein was analysed in both sexes (Fig. 2.7) and our data excluded that Ssx promotes exon skipping similarly to Sxl in males, since this would cause deleterious consequences in sex determination and dosage compensation. The erroneously exon skipping event would enable the expression of functional Sxl protein in male animals. Functional Sxl protein in males would lead 1) to alternative splicing of sex-specific mRNAs triggering the female development cascade and 2) an inactivation of the dosage compensation complex by translational downregulation of *msl-2*, which would in turn trigger the repression of the hypertranscription of the single X chromosome and would result in a reduced gene dose in males.

Intriguingly, both proteins bind similar sequences and exhibit comparable affinities to the investigated binding sites (Fig. 2.11; 2.16; 2.25). Moreover, both proteins compete with each other for binding to a shared binding motif. In every competition set-up, the highest concentrated protein was binding the RNA the most efficient, displacing the lower concentrated protein from the RNA (Fig. 2.16). Irrespective of similar binding motifs (Fig. 2.14) and affinities, overexpression of FlagHA-Ssx in flies showed no impact on the splicing patterns of several well-characterized and sex-dependent Sxl targets (Fig. 2.18).

Since the overexpression of Ssx was rather mild (Fig. 2.7 B and C), we hypothesized that the amount of FlagHA-Ssx protein was not sufficient to interfere with sex-dependent splicing. Moreover, the increased lethality which we observed in heterozygous flies of the UASt::ssx;da::GAL4 strain, might be due to translational miss-regulation of several mRNA targets by the overexpressed protein. In addition, flies homozygous for the constitutive FlagHA-Ssx expression were not viable at all (Fig. 2.1).

Surprisingly, splicing analysis of the fly strain Δssx indicated amounts of the female-specific *Sxl* splice variant in male flies (Fig. 2.20). Unlike its closely related paralog, Ssx does not promote the skipping of the poison exon within *Sxl* mRNA in females. Instead, Ssx rather promotes functional exon 3 splicing in males by antagonizing the regulatory function of Sxl in this context (Fig. 2.21 and 2.22). For the establishment and the maintenance of *Sxl* female-specific splicing, numerous factors have been identified which act together with the Sxl protein to ensure correct pattern formation and female development. However, the question

of how male flies can protect themselves against low-level expression of Sxl protein which could inadvertently initiate the feedback loop, remains enigmatic. Presumably, Sxl expression in male flies occurs at a non-zero rate which could accidentally trigger the Sxl expression cascade resulting in erroneous activation of Sxl protein expression (Figure 3.1 A). We have identified the protein Ssx as an antagonist of the Sxl auto-regulatory feedback loop. It competes with Sxl for binding to the regulatory elements in the Sxl primary transcript and thus prevents it from exerting its auto-regulatory function in splicing. By this, Ssx establishes a threshold that prevents small amounts of Sxl protein from initiating the splicing cascade, protecting male flies from a runaway protein production (Fig. 3.1 A blue dashed line). Conversely, loss of Ssx sensitizes male flies to the auto-regulatory activity of Sxl resulting in production of detectable amounts of female-specific Sxl transcripts (Fig 3.1 A magenta dashed line).

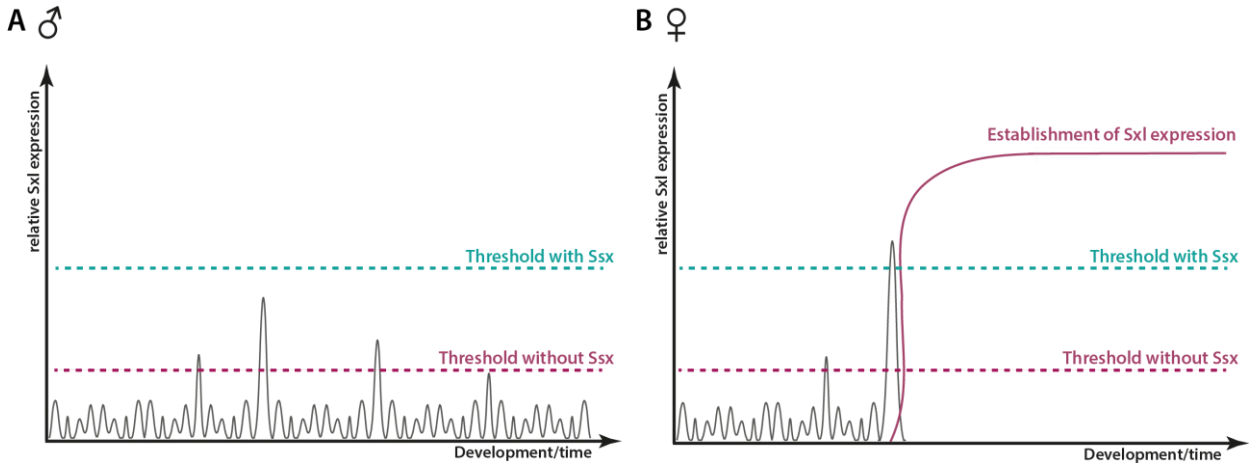

Figure 3.1: Ssx ensures the male-specific splice pattern of *Sxl* transcripts in male flies. A) Model for the protection in male flies from the accidental activation of the developmental cascade regulated by Sxl. Fluctuations of the non-zero background expression of Sxl are shown as a black curve. The threshold for the activation of the *Sxl* splicing cascade depicted as blue dashed line. Loss of Ssx is associated with a lowered threshold and an elevated risk of productive Sxl splicing (shown as dashed line in magenta). **B)** In females, a burst of early Sxl expression activates female-specific *Sxl* splicing and a continuous productive Sxl protein expression (depicted as a curve in magenta).

In sum, Ssx acts as a safeguard in male flies by preventing small amounts of aberrantly produced Sxl protein from initiating the auto-regulatory, positive feedback loop. It therefore contributes to the development of male individuals by stabilization of a male-specific gene expression pattern. Robustness in development and in cell fate decisions was initially described by Waddington in 1957 who established the term channelling. He described the robustness of biological systems which are buffered against external influences and fluctuations to propagate and ensure stable pathways throughout the development (Waddington 1957).

3.2.2 Sxl auto-regulatory splicing requires the N-terminal protein domain

As already described in previous sections, Sxl does not act alone to fulfil its function as post-transcriptional regulator of gene expression, but instead, it orchestrates an ensemble of co-factors to promote the female development. Previous studies already reported that several important protein-protein interactions occur between Sxl and major components of U1snRNP and U2snRNP. Furthermore, it was also shown that the N-terminal domain of Sxl participates in these interactions (Wang and Bell 1994; Deshpande et al. 1999; Yanowitz et al. 1999). The N-terminus of Sxl harbours glycine-rich repeats, often found in splicing regulators (Rogelj et al. 2011). Despite the fact that Sxl and Ssx share a high degree of similarity between their central regions, their N- and C-terminal parts differ substantially. We speculated that the apparent inability of Ssx to regulate splicing compared to Sxl is caused by the differences in the N-terminal domain. To test this hypothesis we generated chimeric proteins: When the N-terminus of Ssx was replaced by that of Sxl, the alternative, female-specific splice pattern of the *Sxl* pre-mRNA could be induced in male cultured cells upon expression of the chimeric protein. Here, the N-terminus of Sxl fused to Ssx functions as splice inhibitor and forces the skipping of the poison exon. In contrast, a chimeric Sxl protein that carries the N-terminus of Ssx lost its function as splicing silencer (Fig. 2.23). Since we demonstrate that the binding behaviour of both proteins to the *Sxl* pre-mRNA is comparable, the regulatory difference between these chimeras resides in their N-termini.

To date it is not fully understood how Ssx exerts function and controls the alternative splicing of *Sxl*. Does it simply interfere with RNA binding of Sxl (does Ssx occupy and block the *Sxl* binding site as a non-functional protein)? Or is Ssx a splicing enhancer, which actively promotes inclusion of the poison exon? Further experiments are necessary to address this question. In addition, it should be tested whether the unstructured C-terminus of Ssx is important for function.

Auto-regulatory loops are a widely used mechanism to modulate the expression levels of proteins, especially of RNA binding proteins and splicing regulators. Beside the positive autoregulation of Sxl, which is the only one known so far, many other textbook examples of negative autoregulation are found within the literature, e.g. hnRNP-L, hnRNP-A1, and PTB (Nadler et al. 1991; Blanchette and Chabot 1999; Wollerton et al. 2004; Rossbach et al. 2009; Suzuki and Matsuoka 2017). Exemplarily, PTB, which is also known as hnRNP-I, can adjust its own protein level according to physiological demand. This is achieved by a negative feedback loop, in which elevated levels of PTB regulate alternative splicing of its own transcript. At high concentrations, PTB associates with binding sites flanking exon number 11 and promotes exon skipping. The alternatively spliced and shortened transcript is subsequently subjected to nonsense-mediated decay, effectively reducing PTB protein synthesis (Wollerton et al. 2004).

Of note, the occurrence of glycine-rich repeats embedded in unstructured regions within splicing regulators acting in auto-regulatory loops is not restricted to Sxl. For instance, autoregulation of hnRNP-A1 alternative splicing depends on its C-terminal part harbouring such repeats. Interestingly, RNA binding was retained upon deletion of its C-terminal part, while autoregulation of hnRNP-A1 was clearly impaired (Blanchette and Chabot 1999). The glycine-rich regions of hnRNPA1 act as splicing silencers by mediating co-operative mRNA binding of several hnRNPA1 molecules along the hnRNA1 mRNA (Nadler et al. 1991; Blanchette and Chabot 1999; Zhu et al. 2001).

3.2.3 The role of Ssx within the fly

In this study we addressed the role of Ssx within *Sxl* alternative splicing and shed light onto to date unknown molecular mechanisms. At the same time, however, they are raising new questions. The positive auto-regulatory feedback loop of *Sxl* in *D. melanogaster* exemplifies a well-studied example of alternative self-regulatory splicing events throughout literature. The mechanism of the *Sxl* alternative splicing is rather simple when compared to other more sophisticated splicing events like in the case of the cell-surface protein Dscam which has up to 38,016 differentially spliced transcript isoforms (Schmucker and Flanagan 2004). Nonetheless, even in the case of *Sxl* splicing, several mechanistic aspects are still unknown, for example regarding the involvement of additional factors. Concerning the function of Ssx in *Sxl* splicing, the rather weak phenotype of the knockout fly demonstrates that Ssx is not essential for male survival, arguing that Ssx acts as a non-essential safeguard. Of note, it has been shown in the past that loss of function mutations of other splicing components often result in sex-specific lethality in flies as it has been shown for example for fl(2)d. Here, a point-mutation in the gene of fl(2)d (the *fl(2d)[1]* allele) leads to female-specific and temperature-dependent lethality. Females are sterile but viable at 18°C, whereas homozygous females are not viable at 29°C (Penalva et al. 2000). Male flies are unaffected by this mutation. Another example is the protein Vir which is, together with Ime4 (Mettl3) and Mettl14, part of the m^6A methylation complex in flies (Lence et al. 2016). Mutation of *vir* results in female-specific lethality (Hilfiker et al. 1995). In contrast to this severe phenotype, knockout or partial deletion of other components of the m^6A pathway, which also affects alternative splicing of *Sxl*, display rather mild effects. Here, Ime4 null flies are viable until adulthood and are fertile but suffer from behavioural and locomotion defects (Lence et al. 2016). The same mild phenotype was observed in flies lacking functional Mettl14 or YT521-B/YTHDC1 proteins (Lence et al. 2016). The latter is also involved in the m^6A pathway as a reader protein. Importantly, the feedback loop of *Sxl* seems to include several redundant pathways to ensure correct splicing and by that guarantee the proper expression of Sxl in female flies. These redundant pathways can probably take over, at least in part, each other's

function. Therefore, phenotypic consequences in knockout flies are rather mild or are completely missing. The same explanation could hold true in the case of the Ssx knockout.
Another aspect from the works performed on the methylation complex might be applied to Ssx as well. Strong genetic interactions are observed: while individual knockouts are aphenotypic, their combination results in strong phenotypes or even lethality. In more detail, this synergism was observed when homozygous Ime4 null females were crossed with Sxl null males. In Sxl null (SxlfP7BO) flies, the entire transcription unit of *Sxl* is missing, therefore only males are viable. The crossing of Sxl null males to Ime4 null females results in female flies having half doses of Ime4 and Sxl (Ime4 null/+;Sxl null/+). These animals exhibit increased female-specific lethality (of approximately 30%; Lence et al. 2016). In contrast, flies which are heterozygous for only Ime4 (Ime4 null/+) or Sxl (Sxl null/+) are fully viable. Moreover, this effect is phenocopied in female flies bearing from a heterozygous deletion of YT521-B/YTHDC1 and Sxl (YT521-B null/+;Sxl null/+) (Haussmann et al. 2016). In this context, reduction of the gene doses of two protein results in 1) reduction of the methylation marks (Ime4) or reduction their reading (YT521-B) which normally promotes alternative splicing of *Sxl* and 2) half dose of *Sxl* expression. In combination, these mutations result in a failure to establish and maintain Sxl expression in females. In addition, this refers again to the robustness of biological systems and the channelling of developmental decisions (chapter 3.2.1). The loss of one protein can be buffered, while two knocked out interactors destabilize the biological system. For this reason it would be interesting to cross our Ssx knockout fly with flies having deletions of specific splice factors or of factors of the m^6A complex, or with flies showing altered Sxl expression levels to analyse for genetic interactions that might yield additional clues about Ssx function.
A second point that is relevant to explain the viability of the Ssx knockout flies is to determine in which developmental context the previously described buffering function of Ssx might play a role. The initial expression of Sxl protein is primed in the female embryo during early, syncycial blastoderm stages (Parkhurst et al. 1990; Keyes et al. 1992) while it remains off in male embryos. This initial burst of early Sxl protein expression at a later stage regulates *Sxl* pre-mRNA splicing which is transcribed by the Sxl maintenance promoter. Compared to that, the spontaneous exon skipping events in males counteracted by Ssx are likely to be rare and to occur in a rather stochastic fashion at later stages. We thus speculate that even in the absence of the Ssx fail-safe mechanism, male flies can still develop normally. In addition, the erroneous expression of Sxl might be eventually triggered only in a minimal subset of male cells in the absence of Ssx. The given Sxl concentrations are likely to determine whether these cells survive or whether they die. Thus, it is unlikely that the whole organism will be affected. In addition, the fixation of sexual characteristics (sex combs, genitalia, colour of

abdominal pigmentation) might happen before the accidental expression of Sxl protein reaches critical concentration in Ssx knockout males.

In sum, although positive feedback loops are unstable in the “off” state, safe-guarding mechanisms such as the activity of Ssx prevent accidental triggering and hence guarantee robust pattern formation e.g. the male development in flies.

3.2.4 The influence of Ssx on sex-specific alternative splicing of other mRNAs

The role of Sxl as a RNA binding protein is, as already discussed in previous sections, not limited to the *Sxl* mRNA but comprises a variety of mRNAs. In particular, alternative splicing of the *msl-2* and *tra* mRNA but also alternative polyadenylation of the *e(r)* mRNA are under the control of Sxl. Often, Sxl appears to regulate gene expression by out-competing other RNA-binding proteins (Sosnowski et al. 1989; Inoue et al. 1990; Valcarcel et al. 1993; Merendino et al. 1999; Del Gatto-Konczak et al. 2000; Forch et al. 2000). This raises the question if also Ssx is able to influence other aspects of post-transcriptional regulation of gene expression. Sex-specific, alternative splicing of the *tra* mRNA is caused by the competition between Sxl and U2AF for binding to the PPT adjacent to the proximal 3’ss. In the presence of Sxl, U2AF binding to a distal PPT is favoured and forces alternative 3’ss selection (Sosnowski et al. 1989; Inoue et al. 1990; Valcarcel et al. 1993). Subsequent studies however challenged the simple road block model as an explanation and proposed that the N-terminus of Sxl might actively regulate 3’ss selection by interacting with several spliceosomal components (Wang and Bell 1994; Deshpande et al. 1999; Yanowitz et al. 1999). Ssx however appears to lack this activity on the *tra* mRNA and our data indicate that the N-terminus of Ssx functions on a different way (Fig. 2.23). In addition we did not detect any binding events of endogenous Ssx on the *tra* mRNA by our iCLIP analysis (Table A.1). However, EMSA experiments showed comparable binding affinities of Sxl- and Ssx-RBD4 to the *tra* binding site motif (Fig. 2.12). Of note, the expression profile of *tra* mRNA is rather low in Dmel-2 cells, which were used for the iCLIP (Flybase, FB2017_05; Gramates et al. 2017). This could also explain why we could not detect any *tra* binding for Ssx in the iCLIP experiments. Of note, we were not able to obtain reliable results from *tra* splice assays performed in cultured cells, therefore the analysis of the impact of Ssx protein was restricted to whole flies (data not shown).

Another well-studied and mRNA regulated by Sxl is *msl-2.* This mRNA bears two Sxl binding motifs (Asite: U11 and Bsite: U16) in a facultative intron in its 5’UTR which is retained in a Sxl-dependent manner. Similar to the regulation of the *tra* mRNA, the direct competition between U2AF and Sxl for binding to the PPT at the Bsite (U16) forces the usage of an alternative 3’ss. On the Asite Sxl additionally competes with the splicing regulator Rox8/TIA-1 for binding (Merendino et al. 1999; Del Gatto-Konczak et al. 2000; Forch et al. 2000). We

demonstrated by several approaches (iCLIP, EMSA, translation assays) that Ssx efficiently binds to the Bsite (U16) within the *msl-2* intron.

Of note, studies which addressed the mechanism for the intron retention, performed competition experiments with U2AF and the full length Sxl protein (Merendino et al. 1999; Forch et al. 2001). So far, it has not been addressed whether the N-terminus of Sxl is required for the splicing regulation of *msl-2*. Owing to our previous observations, we speculate that also in this case the N-terminus of Sxl might play an important role, and consequently, it is unlikely that Ssx promotes intron retention. In addition, our iCLIP studies demonstrate that Ssx exclusively interacts with the Bsite, while no crosslink event was monitored on the Asite (data not shown). However, the Asite was shown to be essential for intron retention in *msl-2* transcripts as well (Forch et al. 2001). This further suggests that the binding of Ssx to the Bsite of *msl-2* does not influence of intron retention.

3.3 The influence of Ssx on other post-transcriptional events

The influence of Sxl on post-transcriptional events is not limited to alternative splicing, Sxl also contributes to alternative polyadenylation of the *e(r)* mRNA in the germline and cooperates with the protein How to prevent nuclear export of *msl-2* mRNA (Gawande et al. 2006; Graindorge et al. 2013). At this point we asked whether Ssx could act similar to Sxl in these processes. Unfortunately, no data regarding the expression of Ssx in the female germline are available (Flybase; FB2017_05; Gramates et al. 2017). Therefore, we cannot predict if local concentrations of *e(r)* mRNA and Ssx are sufficient to establish a functional interaction to influence the alternative poly(A) site selection.

In addition to that, we investigated the nuclear retention of the *msl-2* mRNA. In contrast to *Sxl*, the regulation of *msl-2* occurs on several levels during its post-transcriptional processing. Here, nuclear retention is just one single component of the whole network which ensures the repression of *msl-2* in females. It has been shown that Sxl as well as How can promote the nuclear retention of the *msl-2* mRNA on their own. However, the effect is much stronger if both proteins bind together to the RNA (Graindorge et al. 2013). Since Ssx and How are expressed in both sexes, it seems rather unlikely that they might cooperate to efficiently inhibit the nuclear export of *msl-2*, which is required to be differentially expressed between females and males. Nonetheless, we cannot exclude that the binding of Ssx to the *msl-2* mRNA might, at least moderately, influence its export from the nucleus.

3.4 The action spectrum of Ssx is not limited to alternative splicing

3.4.1 Evolutionary development of Ssx

The role of Sxl as master regulator of female development is limited to the clade of Drosophilids (Sawanth et al. 2016), while other flies employ the protein Tra as the main controller of sex determination (reviewed in Pane et al. 2002; Penalva and Sanchez 2003; Cline et al. 2010; Hediger et al. 2010; Verhulst et al. 2010). This observation supports the hypothesis that Sxl acquired its new regulatory function rather recently during evolution (Cline et al. 2010). Coincidentally at the same time, a gene duplication event of the ancestral Sxl protein gave rise to the current Sxl protein and its closely related paralog Ssx. It has been hypothesized that having two gene copies originating from the ancestral Sxl gene has lowered the selective pressure on one of them and gave the opportunity to Sxl to rapidly evolve and gain its new, sex-specific function as master-regulator of sex determination (Traut et al. 2006; Cline et al. 2010). Since Sxl acquired a new role within Drosophilids, several questions still remain open. What was the function of the ancestral Sxl protein? Did Ssx continue to fulfil the ancestral tasks of Sxl while the novel Sxl protein gained new features during evolution (neo-functionalization)? Or did the gene duplication event promote the loss the original and non sex-specific function (sub-functionalization), while the two paralogs Sxl and Ssx gained new and unrelated tasks (Mullon et al. 2012)?

To answer these questions, the two proteins were analysed and compared to other Sxl and Ssx-related proteins from different insect species. Afterwards, several hypotheses were raised. The first hypothesis claimed that Ssx retained the ancestral non sex-specific function, whereas Sxl evolved its new female-specific master-regulatory function (neo-functionalization) (Traut et al. 2006). However, Cline and colleagues postulated that Sxl and Ssx underwent a sub-functionalization process (Cline et al. 2010). Moreover, the neo-functionalization theory was further supported by the concept that upon gene duplication, Sxl followed a purifying selection, in which several adaptive amino acid mutations occurred to enable its newly gained function. In contrast, the evolutionary pressure on Ssx was lowered and the protein was free to evolve leading to a rampant positive selection for the actual Ssx protein. In sum, the co-evolution of Sxl and Ssx most probably resulted in a sub-functionalization of both proteins (Mullon et al. 2012).

3.4.2 Ssx acts as a translational repressor of *msl-2*

Even if the two proteins are closely related throughout evolution, their biological difference became visible when each protein was individually knocked out in the fly. While the knockout of Sxl causes female-specific lethality, Ssx knockout in flies does not show any phenotype under standard laboratory conditions (Cline 1978; Cline et al. 2010; Fig. 2.1). Dissecting the similarities and differences of Sxl and Ssx in a biochemical approach, we investigated the

role of Ssx in the translational repression of *msl-2* mRNA. We demonstrated that both proteins share similarities and both can regulate translation via the 5'UTR mechanism (Fig. 2.26). Since Ssx is expressed in both sexes, we would argue that Ssx downregulates *msl-2* translation in both sexes. In male flies, a decreased amount of Msl-2 protein would normally cause a reduced hyper-transcription from the X-chromosome. However, splicing of the facultative 5'UTR intron removes the binding site from the majority of *msl-2* transcripts (Zhou et al. 1995). Therefore, the impact of Ssx on *msl-2* translational regulation is limited to the small subset of mRNAs, which still harbour the 5'UTR intron in males. This implies that translational repression by Ssx rather serves as a fine-tuning event, adjusting the Msl-2 protein levels in male flies.

Interestingly, the differences between Sxl and Ssx became obvious when we addressed the 3'UTR dependent regulation of *msl-2* translation. Translation assays show that Ssx fails to downregulate the translation of *msl-2* mRNA via the 3'UTR-mediated mechanism (Fig. 2.23). Importantly, the difference between the two paralogs is based on their ability to recruit the co-repressor UNR to the mRNA, while the general RNA binding properties remained unaffected (Fig. 2.11 and 2.32). Three amino acid substitutions in the RRM1 of Ssx (N111A, K123R and F128Y) resulted in a gain of function protein, which is able to efficiently recruit UNR to the *msl-2* mRNA, forming a trimeric complex (Fig. 2.35 and 2.36) which in turn represses *msl-2* translation (Fig. 2.30). The analysis of a Sxl loss of function mutant (conversion of amino acids A143N R155K and Y160F) further supports this finding (Fig. 2.30, 2.35 and 2.36). A sequence comparison of the Ssx GOF protein and the published crystal structure of Sxl-RBD4 and UNR-CSD1 bound to the *msl-2* mRNA (Hennig et al. 2014) revealed that of the three mutated amino acids in Ssx GOF only Y160 (F128 in Ssx) makes a direct contact to UNR-CSD1. Here, the interaction of Sxl Y160, UNR H213 and *msl-2* A9 together with the stacking of Sxl Y164, UNR R239 and *msl-2* C11 establish a triple zipper conformation which is based on electrostatic interactions as well as base/aromatic side chain stacking interactions (Hennig et al. 2014). in this triple zipper conformation, Y160 is additionally sandwiched between A9 in *msl-2* and S215 of UNR. The functional importance of the Y160 residue of Sxl compared to F128 of Ssx becomes apparent when looking at amino acids H213, F214 and S215 of UNR which form a pocket with their peptide backbones and thus stabilize the interaction of Y160 and S215. Here, the hydroxyl-group of the aromatic ring of Y160 establishes contacts with UNR, while F128 of Ssx presumably fails to do so, since the additional hydroxyl-group of the tyrosine is missing in the amino acid phenylalanine. Other amino acids identified by Hennig and colleagues to be required for the formation of the trimeric complex, namely D138, R139, Y142, Y164, are conserved within Ssx. Of note, the mutation of F128 in Ssx alone is not sufficient to enable the recruitment of UNR to the *msl-2* RNA (Fig. 2.29).

Two closely located amino acids, A143 and R155 in Sxl, were shown to contribute to complex formation as well. These are converted respectively to N111 and K123 in Ssx. Upon assembly of the trimeric complex, the *msl-2* RNA is sandwiched between Sxl and UNR-CSD1 and wraps around the RRM1 of Sxl (Fig. 3.2). This almost 180° turn of the RNA is supported by several RNA-Sxl contacts which are normally not established during canonical Sxl binding (Hennig et al. 2014). Among them, amino acids 143 and 155 in Sxl were shown to contribute to complex formation. Amino acid 155 contacts the RNA backbone and facilitates the correct orientation of the RNA while it is wrapped around RRM1. Moreover, amino acid A143 is rather small and thus does not interfere with complex formation. In contrast, when alanine 143 is replaced by asparagine, this interferes sterically with a stable Sxl-RNA interaction, in the context of the repressor complex.

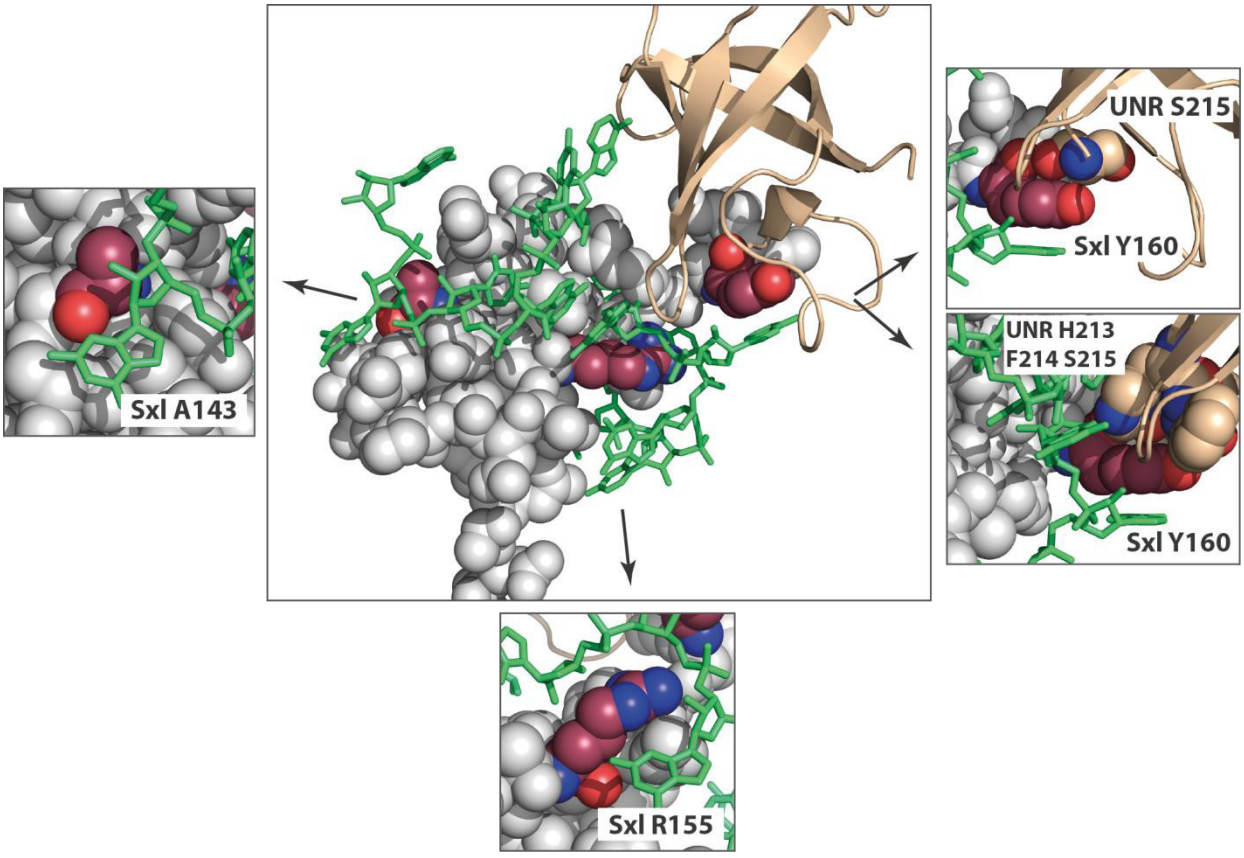

Figure 3.2: Amino acids A143, R155 and Y160 in Sxl RRM1 are critical for the trimeric complex formation with UNR-CSD1 and the *msl-2* RNA. Crystal structure adapted from Hennig et al. 2014 (PDB: 4QQB) showing the triple zipper formation of the *msl-2* mRNA (green), Sxl-RBD4-RRM1 (grey) and UNR-CSD1 (light brown). Amino acids converted in Ssx to gain function are highlighted in magenta. Critical interactions of the respective amino acids are depicted in separate boxes.

Besides Ssx, also the *Musca domestica* ortholog of Sxl, mSxl, is expressed in both sexes (Meise et al. 1998). Moreover, forced expression of mSxl in *Drosophila* does not have any feminizing effects (Meise et al. 1998). Protein sequence alignment of Ssx and mSxl showed that the three amino acids critical for the gain of function in Ssx are conserved in mSxl (A94,

R106 and Y111 in mSxl and A143, R155 and Y160 in Sxl). Interestingly, the essential amino acids for the trimeric complex formation characterized by Hennig and colleagues are also conserved between Sxl and mSxl. Nevertheless, similar to Ssx, mSxl efficiently represses translation of *msl-2* via the 5'UTR mechanism, while the protein fails to repress translation via the 3'UTR dependent pathway. Since mSxl still binds to the regulatory motif within the *msl-2* mRNA, the reason for this functional difference probably also reflects the inability of the protein to recruit UNR to the mRNA (Grskovic et al. 2003). This strongly suggests that, additional, not yet characterized features contribute to a stable Sxl-UNR-*msl-2* interaction.
Taken together, the molecular mode of action of mSxl and Ssx on the *msl-2* mRNA is similar. They downregulate translation via the 5'UTR mechanism but both proteins fail to assemble the trimeric complex on the 3'UTR binding site. Therefore, from an evolutionary point of view, it is more likely that Sxl has acquired new features over time rather than mSxl and Ssx both lost an ancestral ability to recruit UNR. This suggests a neo-functionalization event of Sxl in agreement with the theory of Traut et al. 2006. Again, the translational regulation of *msl-2* expression exemplifies the complex interplay between Sxl and its closely related paralog Ssx. While both proteins differ drastically in their ability to recruit UNR to the 3'UTR binding motif, they are functionally equivalent in regulation via the 5'UTR. Here, both proteins likely compete for binding to the same motif and Ssx serves as fine-tuning and desensitizing protein ensuring proper Msl-2 expression in males. General analysis of the already published binding sites of Sxl revealed that many of them are located within 5'UTRs, while only a small subset of Sxl-target sites was identified within 3'UTRs (Beckmann et al. 2005; Abaza et al. 2006; Duncan et al. 2006; Penn and Schedl 2007; Medenbach et al. 2011; Chau et al. 2012). Here, an analysis of the Sxl *in vivo* binding sites would shed further light onto so far unknown targets and, by that, onto the regulatory abilities of this protein.

3.5 Comparison of Sxl and Ssx to ELAV proteins

Even if the *Drosophila* proteins Sxl and Ssx are no official members of the ELAV/Hu protein family, they share many striking similarities with each other. Therefore they are referred as closely related proteins of this protein clade. As already addressed in previous sections, ELAV/Hu family members exhibit great regulatory versatility which involves the regulation of alternative splicing, mRNA stability, export and translational control (reviewed in Hinman and Lou 2008; Colombrita et al. 2013). Here, their action spectrum is mainly defined by their cellular distribution and local tissue concentration. For instance, most ELAV/Hu proteins are predominantly localized to the cytoplasm and are expressed in neuronal tissues (HuB, HuC and HuD), whereas others act in the nucleus and are ubiquitously expressed (HuR). Thus, the expression pattern which is limited in space and time, restricts the molecular function of these RNA binding proteins. This also applies for the *Drosophila* RNA-binding protein Sxl (Bopp et al. 1991; Keyes et al. 1992; Bopp et al. 1993). For Ssx instead, little is known about

the expression pattern in the fly. Therefore, studying the molecular distribution of Ssx in different fly tissues, its cellular localization and the expression pattern throughout development would further help to unravel the biological role of the protein.

In addition, another shared feature of Sxl and ELAV/Hu proteins is that the main function of these proteins is the binding to mRNA. Here, the role of Sxl within a certain pathway is then specified by its interaction with other protein partners. Consequently, Sxl serves as binding platform and mediator, while the interaction partners of Sxl execute specialized functions. This characteristic allows Sxl to regulate multiple biological processes, e.g., executing the role of a potent competitor in one mechanism, while acting as a strong enhancer in another regulatory pathway.

The discovery of the closely related paralog Ssx resembles the evolutionary fine-tuning occurring between ELAV/Hu protein family members. Selective mutations within their sequences allowed them to adapt and to become specialized for certain molecular contexts. This is exemplified by the complex interplay of the two paralogs on the *msl-2* mRNA. While they demonstrate a redundant behaviour within the 5'UTR dependent mechanism, they competing for binding to the 3'UTR since Ssx fails to establish the interaction with UNR. Moreover, this competitive binding is further shown on the *Sxl* mRNA.

3.6 The advantage of Ssx

In particular, the comparison of *Drosophila* Sxl and Ssx could help to further unravel unknown mechanisms in which Sxl might be also involved. Here, Sxl-dependent pathways might have remained undiscovered so far due to the lethal phenotype associated with Sxl depletion in female flies. The loss of proper sex determination and dosage compensation and the resulting lethality are therefore likely to mask any other effect related to novel Sxl functions. In such a scenario it would be helpful for future studies to characterize the function of Ssx and then investigate whether other and novel functions are shared with Sxl as well. For instance, the missing phenotype of Ssx could hint to a sex-unrelated function within the immune system of the fly (Ayres et al. 2008). Moreover, when studying the molecular details of the action mode of Sxl within a certain pathway, it might be perfectly suited to use Ssx to generate chimeric constructs, as we did in our experiments. This will contribute to further dissect and unravel the interaction platforms used by Sxl to fulfil its function as a multifaceted RNA binding protein.

4 Material

4.1 Chemicals and enzymes

All routinely used chemicals were purchased by the companies Biorad (Hercules, USA), Merck (Darmstadt, Germany), Roche (Basel, Switzerland), Roth (Karlsruhe, Germany), Sigma-Aldrich (St. Louis, USA) and VWR (Leuven, Belgium). Restriction enzymes as well as enzymes for nucleic acid modifications, nucleotides and molecular weight markers were supplied by Aglient Technologies (Santa Clara, USA), Epicentre (Madison, USA), Invitrogen (Carlsbad, USA), New England Biolabs (Ipswich, USA), Promega (Madison, USA), Roche (Basel, Switzerland) and Thermo Fisher Scientific (Waltham, USA). Radiochemicals were ordered from Hartmann Analytics (Braunschweig, Germany). DNA oligonucleotides were obtained from Thermo Fisher Scientific (Waltham, USA), whereas RNA oligonucleotides were purchased from Metabion (Munich, Germany) and Biomers (Ulm, Germany).

4.2 Kits, membranes and reagents

Name	Supplier
Amersham Protan Premium 0.45 NC	GE Healthcare (Buckinghamshire, UK)
Amersham Hybond P 0.2 PVDF	GE Healthcare (Buckinghamshire, UK)
Anti-FLAG® M2 Magnetic Beads	Sigma-Aldrich (St. Louis, USA)
Clarity Western ECL Substrate	Biorad (Hercules, USA)
DC Protein Assay	Biorad (Hercules, USA)
Dual-Luciferase Reporter Assay System	Promega (Madison, USA)
FuGENE HD Transfection Reagent	Promega (Madison, USA)
Illustra MicroSpin G-25 Columns	GE Healthcare (Buckinghamshire, UK)
Mono S 5/50 GL	GE Healthcare (Buckinghamshire, UK)
Phusion® High-Fidelity DNA Polymerase	New England Biolabs (Ipswich, USA)
Protein Sepharose A 4B Beads	Thermo Fisher Scientific (Waltham, USA)
Protino® GST/4B Column 5 mL	Macherey Nagel (Düren, Germany)
Protino® Ni-NTA Column 5 mL	Macherey Nagel (Düren, Germany)
QIAfilter Plasmid Midi Kit	Qiagen (Hilden, Germany)
QIAprep Spin Miniprep Kit	Qiagen (Hilden, Germany)
QIAquick Gel Extraction Kit	Qiagen (Hilden, Germany)

QIAquick PCR Purification Kit	Qiagen (Hilden, Germany)
QIAshredder	Qiagen (Hilden, Germany)
RQ 1 RNase-free DNase	Promega (Madison, USA)
Spectra/Por Dialysis Membrane (6-8 kDa)	Spectrum Labs (Los Angeles, USA)
SsoFast EvaGreen Supermix	Biorad (Hercules, USA)
SuperScript II Reverse Transcriptase	Invitrogen (Carlsbad, USA)
Taq DNA Polymerase with ThermoPol® Buffer	New England Biolabs (Ipswich, USA)
Trizol Reagent	Thermo Fisher Scientific (Waltham, USA)
30% Acrylamide/Bis Solution 19:1	Biorad (Hercules, USA)
30% Acrylamide/Bis Solution 37:5:1	Biorad (Hercules, USA)

4.3 Buffer and Solutions

Phosphate buffered saline (PBS)

130mM	NaCl
774mM	Na_2HPO_4
226mM	NaH_2PO_4

Tris/Borate/EDTA buffer (TBE)

89mM	Tris pH 8.3
89mM	Boric acid
2.5 mM	EDTA

Tris buffered saline (TBS)

10mM	Tris pH 7.5
150mM	NaCl

Western Blotting buffer anode 1

300mM	Tris pH 10.4
10%	Methanol

Western Blotting buffer anode 2

25mM	Tris pH 10.4
10%	Methanol

Western Blotting buffer cathode

25mM	Tris pH 9.4
10%	Methanol

Western Blot blocking buffer

10%	milk powder
add	TBS

6-15% SDS-PAGE separation gel

6-15%	Acrylamide/Bis Solution 37:5:1
375mM	Tris-HCl pH 8.8
0.1%	SDS
0.1%	APS
0.05%	TEMED

5% SDS-PAGE stacking gel

17%	Acrylamide/Bis Solution 37:5:1 30%
130mM	Tris-HCl pH 6.8
0.1%	SDS
0.1%	APS
0.05%	TEMED

SDS running buffer

25mM	Tris
190mM	Glycine
1%	SDS

2xSDS-PAGE loading dye

100mM	Tris pH 6.8
2%	SDS
20%	Glycerol
add	Bromphenol blue
200mM	DTT (add freshly)

2x modified RIPA

40mM	Tris pH 8.0
300mM	NaCl
2%	NP-40
10mM	EDTA
4%	SDS

Coomassie staining solution

45%	Methanol
10%	Acetic acid
1% (w/v)	Coomassie Brilliant Blue R-250, filtered

Coomassie destaining solution

20%	Methanol
10%	Acetic acid
add	water

IP Lysis buffer

50mM	Tris pH 8.0
150mM	NaCl
2mM	$MgCl_2$
1%	NP40

1x	cOmplete EDTA-free protease inhibitor cocktail
add	RNAsin

IP Wash buffer

50mM	Tris pH 8.0
150mM	NaCl
2mM	$MgCl_2$
0.1%	NP40
1x	cOmplete EDTA-free protease inhibitor cocktail
add	RNAsin

IP Wash buffer iCLIP

50mM	Tris pH7.4
800mM	NaCl
0.05%	Tween 20
1x	cOmplete EDTA-free protease inhibitor cocktail
Add	RNAsin

Proteinase K buffer

50mM	Tris pH 8.0
10	mM EDTA
1.3%	SDS

Transcription Buffer

40mM	Tris pH 7.9
6mM	$MgCl_2$
10mM	DTT
2mM	spermindine

Buffer X

20mM	Tris pH 7.5
1M	NaCl
0.2mM	EDTA
1mM	DTT

Buffer D0-D1000

20mM	HEPES pH 8.0
20%	Glycerol
0,2mM	EDTA
0.01%	NP40
1mM	DTT
0-1M	KCl

Elution buffer (for GST purifications)

50mM	Glutathione
100mM	HEPES/KOH pH 8.0
50mM	NaCl
1mM	DTT

Lysis buffer for UNR-CSD1

50mM	KH_2PO_4
300mM	NaCl
adjust to	pH 8.0

Wash buffer 1 for UNR-CSD1

50mM	KH_2PO_4
300mM	NaCl
10mM	Imidazole
adjust to	pH 8.0

Wash buffer 2 for UNR-CSD1

50mM	KH_2PO_4
300mM	NaCl
20mM	Imidazole
adjust to	pH 8.0

Elution buffer for UNR-CSD1

50mM	KH_2PO_4
300mM	NaCl
120mM	Imidazole
adjust to	pH 8.0

Buffer for UNR-CSD1(after $(NH_4)_2SO_4$ precipitation)

10mM	KH_2PO_4
50mM	NaCl
1mM	DTT
adjust to	pH 6

Dialysis Buffer for UNR-CSD1

10mM	KH_2PO_4
50mM	NaCl
1mM	DTT
adjust to	pH 6

EMSA Incubation buffer

10mM	Tris pH 7.4
50mM	KCl
1mM	EDTA
0.05%	Glycerol
1mM	DTT

EMSA running buffer

1X	TBE

In vitro translation buffer

40%	*Drosophila* embryo extract
24mM	HEPES/KOH pH 7.4
100mM	KOAc
500µM	MgOAc
60mM	Aminoacids
20mM	Creatine phosphate
800ng	Ceatine kinase
0.1875µg/µl	Renilla mRNA

LB medium

1%	Tryptone
0.5%	Yeast extract
1%	NaCl
adjust to	pH 7.5

LB plates

1%	Tryptone
0.5%	Yeast extract
1%	NaCl
1.5%	Agar-Agar
adjust to	pH 7.5

4.4 DNA and RNA Oligonucleotides

4.4.1 DNA oligonucleotides for cloning

All oligonucleotides used for cloning are listed in table 1.1.

Table 1.1: Table of primers with respective restriction sites, primer sequence indicated in 5' to 3' direction.

name	sequence 5'→3'
Homology I Ssx NdeI fwd	AGTACATATGTTGAACGCGCAGGCAATTTATCG
Homology I Ssx NdeI rev	AGTACATATGGTGTCAAGCCTTGATAGCTCCTG
Homology II Ssx XhoI fwd	AGGACTCGAGCGTAGGCAATGGCATTTGCCACA
Homology II Ssx XhoI rev	AGGATCTCGAGGTCCGGCCGGAATAGTCGCCACATC
5' guide RNA Ssx fwd	CTTCGTATCAAGGCTTGACACAGA
5' guide RNA Ssx rev	AAACTCTGTGTCAAGCCTTGATAC
3' guide RNA Ssx fwd	CTTCGCCAGCCAGCCGCATCCCGT
3' guide RNA Ssx rev	AAACACGGGATGCGGCTGGCTGGC
3xFlagHA Tag EcoRI fwd	AATTGAACATGGACTACAAGGACGACGATGACAAGTACCCTTATGACGTGC CCGATTACGCTG
3xFlagHA Tag EcoRI rev	AATTCAGCGTAATCGGGCACGTCATAAGGGTACTTGTCATCGTCGTCCTTGT AGTCCATGTTC
3xFlagHA NotI fwd	ATCAGCGGCCGCAACATGGACTACAAGGACGACGATGACAAGTACCCTTAT G
Ssx 1-443aa XhoI rev	TGACTCGAGTCAAATAAATTTCTGTGCATGG
Ssx 1-443aa+Kozak EcoRI fwd	CGATCCGAATTCAACATGTCCAACGCGGATAAGATGCAG
Ssx 1-443aa XbaI rev	CGCTCTAGAAATAAATTTCTGTGCATGGTTAT
Sxl 1-354aa+Kozak EcoRI fwd	CGATCCGAATTCAACATGTACGGCAACAATAATCCGG

Sxl 1-354aa XbaI rev	TGCTAAAAAGTTTATCTCTAGAGGATGC
GFP EcoRI fwd	ATAGAATTCCTCGAGATGGTGAGC
GFP XbaI rev	ATGGACGAGCTGTACAAGTCTAGAATA
Sxl 122-301aa BamHI fwd	CCCCTGGGATCCGCAAGCAACACC
Sxl 122-301aa XhoI rev	GCGGCCGCTCGAGTTATTACATAAAG
Ssx 92-354aa BamHI fwd	CCCCTGGGATCCACCAATCTGATC
Ssx 92-354aa XhoI rev	GCGGCCGCTCGAGTTATTACATAAACTGGGCC
Ssx C1 mut fwd	GTACGCCCTCTTTCGCGCCATCGGGCCCATCAACACCTGC
Ssx C1 mut rev	GATGGCGCGAAAGAGGGCGTACAGTTCGCGGTCGGTCATG
Ssx C2 mut fwd	GGACTTCACATCGGAGTCGGACTCGGAGGAC
Ssx C2 mut rev	CGGCTACGGCTTCGTGGACTTCACATCGGAGTCGGAC
Ssx C3 mut fwd	GATGGACTCGCAGCGCGCCATCAAGGTGCTAAATGGCTTCTATGTGCGCAA
Ssx C3 mut rev	GGCTTCGTGGACTACAAAACGGAGATGGACTCGCAGCGCGCCATCAAGGTG
Ssx C4 mut fwd	GCTAAATGGCATCACTGTGCGCAACAAGCGATTAAAGG
Ssx C4 mut rev	CGCCATCCAGAAGCTAAATGGCATCACTGTGCGC
Sxl-Ssx chimera border fwd	CACATACAGATTGGTGTCCTTGATCGATTCCCCACCCGGTCGAGCATACG
Sxl-Ssx chimera border rev	GACGTACAAATTCGTATCCTTAATGGACTGTCCGCCGGGACGTGCATAGG
Ssx-Sxl chimera border fwd	CGTATGCTCGACCGGGTGGGGAATCGATCAAGGACACCAATCTGTATGTG
Ssx-Sxl chimera border rev	CACATACAGATTGGTGTCCTTGATCGATTCCCCACCCGGTCGAGCATACG
Ssx K123R F128Y fwd	ACCTGCAGGATAATGCGCGACTACAAGACC
Ssx K123R F128Y rev	GGTCTTGTAGTCGCGCATTATCCTGCAGGT
Ssx N111A fwd	GTACGCCCTCTTTAGCGGCTGCGGGCCCATCAACACCTGC
Ssx N111A rev	GCAGCCGCTAAAGAGGGCGTACAGTTCGCGGTCGGTCATG
Ssx K123R fwd	ACCTGCAGGATAATGCGCGACTTCAAGACC
Ssx K123R rev	GGTCTTGAAGTCGCGCATTATCCTGCAGGT
Ssx F128Y fwd	ACCTGCAAGATAATGCGCGACTACAAGACC
Ssx F128Y rev	GGTCTTGTAGTCGCGCATTATCTTGCAGGT
Sxl R155K Y160F fwd	CGTGCAAAATCATGCGAGACTTTAAGACTGGCTA
Sxl R155K Y160F rev	TAGCCAGTCTTAAAGTCTCGCATGATTTTGCACG
Sxl A143N fwd	ACCGATCGCGAGCTGTACAACCTATTCAGAGCCATTGGAC
Sxl A143N rev	GTCCAATGGCTCTGAATAGGTTGTACAGCTCGCGATCGGT
T7 Sxl KD fwd	TTAATACGACTCACTATAGGGAGAGCATGTACGGCAACAATAATCC
T7 Sxl KD rev	TTAATACGACTCACTATAGGGAGAGCGACAATCCGCAGAG ATTATTC
T7 Ssx KD fwd	TAATACGACTCACTATAGGGAGACCACGGTGGTAACTTCCACCACATG
T7 Ssx KD rev	TAATACGACTCACTATAGGGAGACCACAATGCGCAATATATCTGATGG
Sxl-Ssx border Nterm fwd	TCATGAACGATCCTCGGACCAATCTGATCATC
Sxl-Ssx border Nterm rev	GATGATCAGATTGGTCCGAGGATCGTTCATGA
Ssx-Sxl border Nterm fwd	GGACCGAACCAGCGCCGCAAGCAACACCAACC
Ssx-Sxl border Nterm rev	GGTTGGTGTTGCTTGCGGCGCTGGTTCGGTCC

4.4.2 DNA oligonucleotides for quantitative real time PCR

All oligonucleotides listed in table 1.2 were specifically designed for *D. melanogaster* and were used for qRT-PCR.

Table 1.2: List of primers for the indicated templates, primer sequence indicated in 5' to 3' direction.

name	sequence 5'→3'
Alpha tubulin fwd	GCTTCCTCATCTTCCACTCG
Alpha tubulin rev	AATCAGACGGTTCAGGTTGG
Msl-2 fwd	ATGTTCGCACTGGCACACT
Msl-2 rev	CCTGGGCTAGTTACCTGCAA

4.4.3 DNA oligonucleotides for RT-PCR

All oligonucleotides used for RT-PCR are listed in table 1.3.

Table 1.3: Table of primers for the indicated templates, primer sequence indicated in 5' to 3' direction.

name	sequence 5'→3'
Msl-2 intron fwd	ACTGGGGAAGGGAACCGAAGCC
Msl-2 intron rev	CTTCTGCCCCCATAAGCCTAGTGCCG
Msl-2 fwd	ATGTTCGCACTGGCACACT
Msl-2 rev	CCTGGGCTAGTTACCTGCAA
Tra fwd	ATGAAAATGGATGCCGACAG
Tra rev	GCTGTCCCTCTCGCTTGAT
Sxl fwd	GCAACTCACCTCATCATCCTT
Sxl rev	GATGGCAGAGAATGGGAC
Ssx isoform C fwd	GTACAACCTCTTTAGCGCCTGCGGGCCCATCAACACCTGC
Ssx isoform C rev	CGGCTACGGCTTCGTGGACTTCACATCGGAGTCGGAC
Ssx full-length transcript fwd	CGATCCGAATTCAACATGTCCAACGCGGATAAGATGCAG
Ssx full-length transcript rev	AATCTGCTGTTCTTGTTGGGGCTGTG
Ssx RB/RE-RD isoform fwd	ATGGGCATGCCCATTCAC
Ssx RB/RE-RD transcript rev	AATCTGCTGTTCTTGTTGGGGCTGTG

4.4.4 RNA oligonucleotides for electro mobility shift assay

All oligonucleotides were designed for radioactive labeling and binding studies via EMSAs and are shown in table 1.4.

Table 1.4: List of RNAs used for binding studies, sequence indicated in 5' to 3' direction.

name	sequence 5'→3'
Msl-2 Bsite	CCAAUUUUUUUUUUUUUUUUGCAC
Msl-2 Bsite mut	CCAACUCUCUCUCUCUCUCUGCAC
Msl-2 Esite	UUUUUUUGAGCACGUGAA
Msl-2 Esite mut	UCUCUCUGAGCACGUGAA

4.5 Plasmids

List of plasmids used in the experiments which were already available in the lab.

Table 2.1: List of used plasmids which were purchased or were already available in the lab.

name	promotor	application	source
pAc 5.1/V5	Ac 5.1	Expression in *Drosophila* cell culture	Thermo fisher scientific
pCaSpeR-hs msl-2 Bm-RL-EFm	Hsp70	Expression in *Drosophila* cell culture	Jan Medenbach
pCaSpeR-hs msl-2 B-RL-EFm	Hsp70	Expression in *Drosophila* cell culture	Jan Medenbach
pCaSpeR-hs msl-2 Bm-RL-EF	Hsp70	Expression in *Drosophila* cell culture	Jan Medenbach
pCaSpeR-hs msl-2 Bm-FF-EFm	Hsp70	Expression in *Drosophila* cell culture	Jan Medenbach
PGEX-6P-Sxl-RBD4	tac	GST-tagged bacterial protein overexpression of GST-Sxl-RBD4	Jan Medenbach
PGEX-6P-Ssx-RBD4	tac	GST-tagged bacterial protein overexpression of GST-Ssx-RBD4	Jan Medenbach
pBS-msl-2Bm-FF-EFm	T3	Run-off transcription	Jan Medenbach
pBS-msl-2B-FF-EFm	T3	Run-off transcription	Jan Medenbach
pBS-msl-2Bm-FF-EF	T3	Run-off transcription	Jan Medenbach
pBS-Renilla pA	T7	Run-off transcription	Gebauer et al. 2003
pU6-BbsI-gRNA	U6-2	gRNA vector for Ssx Ko	Gratz et al. 2013
pHD-DsRed-attP		Donor vector for Ssx Ko	Gratz et al. 2014

4.6 Antibodies

List of primary and secondary antibodies used for Western Blot and immunoprecipitations.

Table 3.1: List of used antibodies for Western Blot analysis and Immunoprecipitation.

antibody	source	dilution
rabbit-anti-Sxl	polyclonal, Sxl-RBD4, Hentze laboratory EMBL (Iowa, USA)	1:1000
mouse-anti-Sxl	monoclonal, M18, DSHB Hybridoma Bank (Iowa, USA)	1:50
rabbit-anti-Ssx	polyclonal, SY6156, raised at Eurogentec (Lüttich, Belgium)	1:1000
rabbit-anti-Ssx	polyclonal, SY6157, raised at Eurogentec (Lüttich, Belgium)	1:1000
rabbit-anti-Ssx	polyclonal, SY6158, raised at Eurogentec (Lüttich, Belgium)	1:1000
rabbit-anti-Ssx	polyclonal, SY6159, raised at Eurogentec (Lüttich, Belgium)	1:1000
rabbit-anti-GAPDH	polyclonal, GTX100118 Genetex (Irvine, USA)	1:1000
mouse-anti-tubulin	monoclonal, E7, DSHB Hybridoma Bank (Iowa, USA)	1:2
mouse-anti-HA	monoclonal, HA-7, Sigma Aldrich (St. Louis, USA)	1:1000
mouse-anti-rabbit	monoclonal, light-chain specific, Jackson Immuno Research	1:10 000
goat-anti-mouse	monoclonal, light-chain specific, Jackson Immuno Research	1:10 000

4.7 Bacterial strains, *D. melanogaster* cell lines, *D. melanogaster* strains

4.7.1 Bacterial strains

Following bacterial strain was used for molecular cloning:

***E. coli* TOP10** F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG

Following bacterial strain was used for protein expression:

***E. coli* BL21* transformed with a Rosetta2 plasmid (Jan Medenbach)** F-ompT hsdSB (rB-, mB-) galdcmrne131 (DE3)

4.7.2 Cell lines

Following cell lines were used for cell culture experiments:

Dmel-2 Embryo, 20-24h, near hatching, male, corresponds to Schneider's line S2, Schneider 1972 (S2-R)

Kc167 Embryo, 8-12h, dissociated, female, Cherbas et al. 1988

4.7.3 Fly alleles

Following fly strains were used for fly crossing and generation of applied fly strains:

P{EPgy2}ssx^{EY14203}	Gene Disruption Project members 2001, chromosome 1, Bloomington #20792
Vasa::Cas9	Gratz et al. 2014, chromosome 1 and 3, Bloomington #51323
da::GAL4	Wodarz et al. 1995, chromosome 3
Gla/CyO	Michael Ashburner, University of Cambridge, chromosome 2
attp 25C	Bateman et al. 2006, chromosome 2

5 Methods

5.1 Molecular biological methods

5.1.1 General methods

If not otherwise described, general methods were performed according to the manuals of Sambrook et al. 1989 or according to manufactures instructions of used kits. For DNA isolation from *E.coli* Top10 the QIAfilter Plasmid Midi Kit or QIAprep Spin Miniprep Kit from Qiagen (Hilden, Germany) was used. For PCR purification the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and for DNA extraction from agarose gels the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was applied.

5.1.2 Molecular cloning

5.1.2.1 Polymerase chain reaction (PCR) and overlapping PCR

For standard polymerase chain reaction (PCR) Phusion Polymerase or Taq Polymerase (New England Biolabs, Ipswich, USA) were used according to the following pipetting scheme with indicated standard cycling conditions:

Table 4.1: Composition of a PCR reaction

PCR Mix, 50µl	Phusion	Taq
DNA template	<250ng	<1000ng
Buffer	1x	1x
dNTPs	0.2mM	0.2mM
Primer fwd	0.5µM	0.5µM
Primer rev	0.5µM	0.5µM
Polymerase	1 U	1.25 U
Water	to 50µl	to 50µl

Table 4.2: Thermocycler program

25-35 cycles	Phusion	Taq
Initial denaturation	98°C; 30s	95°C; 30s
Denaturation	98°C; 10s	95°C; 30s
Annealing	45-72°C;30s	45-68°C;1min
Elongation	72°C; 30s/1kb	68°C; 1min/1kb
Final extention	72°C 10min	68°C 5min

Single point mutations were generated using the overlapping PCR procedure. In sum, two outer and two inner primers were needed to amplify the final PCR product. The terminal cloning primers flank the 5’ or 3’ end of the PCR product whereby the internal mutation primers align to the sequence to be mutated and introduce the desired mutation. The first amplification step resulted in two PCR products. One PCR product was amplified using the 5’

terminal cloning primer and the reverse internal mutation primer, whereas the other PCR product was generated using the 3' terminal cloning primer and the forward internal mutation primer. To perform the second amplification step, both purified PCR products were applied as templates in an equimolar ratio and were amplified as one template using the outer terminal cloning primers. Final PCR products were separated and purified on a 0.5-2% agarose-TBE gel and subsequently excised from the gel. DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's guidelines.

5.1.2.2 Restriction digest

Restriction digest of PCR products or vector backbones for ligation were performed using respective restriction enzymes from the companies New England Biolabs (Ipswich, USA) and Thermo Fisher Scientific (Waltham, USA) and were incubated at 37°C for at least 2h. The digested vector DNA was agarose purified and eluted with the kit QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany); The insert was purified from the PCR reaction with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following manufacturer's recommendations.

5.1.2.3 Ligation and transformation of *E. coli*

Using T4 ligase enzyme (New England Biolabs, Ipswich, USA) , 100ng of the digested and purified vector was ligated to a 3x molar excess of the digested insert product for 2h at room temperature or at 4°C overnight. 5µl of the ligated product were subsequently transformed into chemically competent *E.coli* TOP10. Transformation of the ligated product was initiated by 30min incubation on ice, afterwards a 90s heat-shock at 42°C was followed by 2min cool-down on ice. When selecting for ampicillin resistant cells, the transformed bacteria were directly plated out on 1xLB-amplicillin agar plates, while bacteria with transformed plasmids encoding other resistance genes were recovered with 500µl 1xLB medium for 1h at 37°C with continuous shaking (700rpm) before plating. 1xLB agar plates (+respective antibiotic) were incubated overnight at 37°C. The next day, single colonies were inoculated in 3ml 1xLB medium (+respective antibiotic) for mini preparation or in 200ml 1xLB medium (+respective antibiotic) for midi preparation and were incubated overnight at 37°C with continuous shaking. Retransformation of chemically competent *E.coli* TOP10 with pure plasmid DNA was performed according to the transformation protocol described above, using small amounts of DNA.

LB medium	1% Tryptone; 0.5% Yeast extract; 1% NaCl; adjust to pH 7.5
LB plates	1% Tryptone; 0.5% Yeast extract; 1% NaCl; 1.5% Agar-Agar; adjust to pH 7.5

5.1.2.4 Extraction of plasmid DNA from *E.coli* and sequencing

Extraction of plasmid DNA was performed using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Large scale DNA purification was performed using QIAfilter Plasmid Midi Kit (Qiagen, Hilden, Germany). Purified DNA (300-500ng) was sequenced using a suitable sequence primer (2.5µM) by the companies GATC Biotech AG (Konstanz, Germany) or Macrogen (Amsterdam, Netherlands).

5.1.2.5 Generated DNA plasmids

Cloning procedures of plasmids generated in this work. Used primers are listed in table 1.1.

pAc5.1 3xFlagHA The 3xFlagHA tag was generated by annealing of the phosphorylated oligos 3xFlagHA EcoRI fwd and 3xFlagHA EcoRI rev and was subsequently cloned into pAc5.1 using the restriction enzyme EcoRI disrupting the 5' EcoRI site in the process.

pAc5.1 3xFlagHA SxlFl, pAc5.1 3xFlagHA SxlLOFfl, pAc5.1 3xFlagHA SsxFl, pAc5.1 3xFlagHA SsxGOFfl, pAc5.1 3xFlagHA GFP, paAc5.1 3xFlagHA NtermSxl-Ssx and pAc5.1 3xFlagHA NtermSsx-Sxl. SsxFl was amplified from male embryonic *D. melanogaster* cDNA. SxlFl was amplified from the plasmid pGEX6P-Sxl1-354aa (available in the lab, Jan Medenbach). Mutations and fusion constructs were generated with suitable mutation primers by overlap PCR. The codon-optimized GFP coding region was amplified from a *Drosophila* 3xGFP plasmid (gift from Prof. Dr. Scheuwly). Constructs were generated using forward primer Sxl 1-354aa+Kozak EcoRI fwd for SxlFl, SxlLOFfl and NtermSxl-Ssx, Ssx 1-443aa+Kozak EcoRI fwd for SsxFl, SsxGOFfl and NtermSsx-Sxl and GFP EcoRI fwd for GFP. The reverse primer Sxl 1-354aa XbaI rev was used for SxlFl, SxlLOFfl and NtermSsx-Sxl, Ssx 1-443aa XbaI rev for SsxFl, SsxGOFfl and NtermSxl-Ssx and GFP XbaI rev for GFP. Inserts were cloned via EcoRI and XbaI into pAc5.1 3xFlagHA.

PGEX-6P-Sxl-Ssx and PGEX-6P-Ssx-Sxl The chimeric protein Sxl-Ssx was generated by overlapping PCR using primers Sxl 122-301aa BamHI fwd plus Sxl-Ssx chimera border rev and Ssx 92-354aa XhoI rev plus Ssx-Sxl chimera border fwd. Ssx-Sxl was cloned with primer pairs Ssx 92-354aa BamHI fwd/ Ssx-Sxl chimera border rev and Sxl 122-301aa XhoI rev/ Ssx-Sxl chimera border fwd. Chimeric inserts were cloned into PGEX-6P via BamHI and XhoI.

PGEX-6P-Ssx-RBD4-C1, PGEX-6P-Ssx-RBD4-C2, PGEX-6P-Ssx-RBD4-C3 and PGEX-6P-Ssx-RBD4-C4 Cluster mutations of Ssx RBD4 were generated using the overlapping PCR procedure using the plasmid PGEX-6P-Ssx-RBD4 as template. As terminal cloning primers Ssx 92-354aa BamHI fwd and Ssx 92-354aa XhoI rev were used and applied cluster

mutation primers are named accordingly and are listed in table 1.1. Generated inserts were cloned via BamHI and XhoI into PGEX-6P.

PGEX-6P-Ssx-RBD4-C1 K123R F128Y, PGEX-6P-Ssx-RBD4-K123R F128Y, PGEX-6P-Ssx-RBD4-K123R, PGEX-6P-Ssx-RBD4-F128Y, PGEX-6P-Ssx-RBD4-N111A K123R F128Y, PGEX-6P-Ssx-RBD4-N111A K123R, PGEX-6P-Ssx-RBD4-N111A F128Y, PGEX-6P-Ssx-RBD4-N111A and PGEX-6P-Sxl-RBD4-A143N R155K Y160F Point mutations of Ssx RBD4 and Sxl RBD4 were generated using overlapping PCR procedure with PGEX-6P-Ssx-RBD4 or PGEX-6P-Sxl-RBD4 as template. As terminal cloning primers Ssx 92-354aa BamHI fwd and Ssx 92-354aa XhoI rev were used for Ssx and Sxl 122-301aa BamHI fwd and Sxl 122-301aa XhoI rev were applied for Sxl. For single or combined point mutations internal mutation primers are named accordingly and their sequences are listed in table 1.1. Generated inserts were cloned via BamHI and XhoI into PGEX-6P.

pU6-BbsI-Ssx5'gRNA and pU6-BbsI-Ssx3'gRNA Ssx 5' and 3'gDNA were generated by annealing of the phosphorylated oligos 5' guide RNA Ssx fwd/5' guide RNA Ssx rev and 3' guide RNA Ssx fwd/3' guide RNA Ssx rev, respectively. They were subsequently cloned via BbsI into the vector pU6-BbsI-gRNA (kindly provided from Prof. Dr. med. vet. Dr. rer. nat. Michael Krahn)

pHD-DsRed-HomI-II Ssx Homology regions I and II of Ssx were amplified from male embryonic *D. melanogaster* gDNA with primers Homology I Ssx NdeI fwd/Homology I Ssx NdeI rev and Homology II Ssx XhoI fwd/ Homology II Ssx XhoI rev and were cloned via NdeI and XhoI into the vector pHD-DsRed-attP (kindly provided from Prof. Dr. med. vet. Dr. rer. nat. Michael Krahn).

pDest-UASt-3xFlagHA-SSX Ssx fl 3xFlagHA was directly cloned via NotI and XhoI into the UASp destination vector (kindly provided from Prof. Dr. med. vet. Dr. rer. nat. Michael Krahn) using primers 3xFlagHA NotI fwd and Ssx 1-443aa XhoI rev. Since shuttling between Entry- and Destination-vectors alters recombination sequences, an already cloned Destination-vector was used (kindly provided from Prof. Dr. med. vet. Dr. rer. nat. Michael Krahn) and the coding region was replaced by 3xFlagHA Ssxfl.

5.1.3 RNA-based methods

5.1.3.1 RNA isolation

RNA isolation from *Drosophila* cell culture, fly embryos or adult flies for RT-PCR analyses was performed using TRIzol® reagent (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. For cell culture samples, 1ml TRIzol® was used for one well of a 6-well plate. For RNA extraction from embryos, approx. 100µl of embryos were

homogenized in 1ml Trizol. Similarly, 20-30 adult animals were meshed with a pestle in 1ml TRIzol® and were subsequently centrifuged for 5min at 14000rpm at 4°C. The supernatant was further processed according to the manufacturer's recommendations.

After RNA-immunoprecipitation analysis, RNA was isolated after protein digestion with Proteinase K. Here, 200µl Proteinase K buffer was added to the beads together with 160U of Porteinase K. The sample was incubated for 30min at 50°C and 300rpm and the remained supernatant was phenolized with 200µl Roti aqua-phenol/chloroform/isoamylalcohol 25:24:1 (Roth, Karlsruhe, Germany). Subsequently, the sample was vortexed and centrifuged for 5min at 14000rpm at room temperature. The aqueous phase was transferred into a new reaction tube and RNA was precipitated by addition of 10% NaoAc, linear acrylamide and 50% ice cold isopropanol and storage for at least 2h at -20°C. RNA was pelleted by centrifugation at 14000rpm and 4°C for 30min and the RNA pellet was washed with 75% ice cold ethanol before air-dried and resuspended in nuclease-free water.

Proteinase K buffer	50mM Tris pH 8.0; 10mM EDTA; 1,3% SDS

5.1.3.2 DNase digestion, reverse transcription and PCR for splicing pattern analysis

5µg of total RNA were DNase-digested for 1h at 37°C using 5µl of RQ1 DNase and 1xRQ1 DNase buffer (Promega, Madison, USA). Next, RNA was precipitated as described before (5.1.3.1). 2µg of RNA were reverse transcribed with random hexamer oligos using the reverse transcriptase enzyme Superscript II (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions. PCR for splicing analysis was performed according to the Taq Polymerase (New England Biolabs, Ipswich, USA) standard operations. Primers used for specific template splicing pattern analysis are listed in table 1.3. RT-PCRs were performed at 59°C annealing temperature, except *tra* RT-PCR which was performed at 54.5°C. Elongation time was routinely set to 30s, except for *Ssx* fl transcripts (90s). RT-PCRs were amplified for max. 29 cycles.

5.1.3.3 Quantitative RT-PCR

1µg total RNA or 1/3 of the total volume of immunoprecipitated RNA was applied to qRT-PCR analysis. First, RNA was digested with RQ1 DNase for 15min at 37°C. The reaction was stopped by the addition of 5mM EDTA and heat inactivation at 75°C for 10min. Next, the RNA sample was reverse transcribed with random hexamers using Superscript III reverse transcriptase (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. QPCR analysis was performed with the Sso Fast Eva Green Mix (Biorad, Hercules, USA), with 1.8µM forward and 1.8µM reverse primer (listed in Table 1.2). Q-RT-PCRs were run on a CFX96 cycler (Biorad, Hercules, USA) using a standard Eva Green program with 95°C initial denaturation for 3min, 95°C denaturation for 15sec, 60°C annealing for 30sec and 72°C elongation for 30sec for 40 cycles and a 65-95°C melting curve. Analysis

of fold changes were calculated using $\Delta\Delta C_t$ ratios of sample triplicates and $\Delta\Delta C_t$ ratios of control triplicates. Error bars were calculated from all received ratios (Livak and Schmittgen 2001).

5.1.3.4 *In vitro* transcription

RNA used for *in vitro* luciferase assays was transcribed from a HindIII-linearized pBluescript vector (Table 2.1) or from a BamHI-linearized pBS-renilla pA plasmid (Table 2.1). Here, 3µg of digested plasmid were incubated with 4.4µl of 40mM 3'-O-Me-m^7G(5')ppp(5')G RNA cap structure analog, RNasin, 1mM CTP, 1mM UTP, 1mM ATP and 1.5µl of T3/T7 polymerase in 1xtranscription buffer in a total reaction volume of 22.5µl for 5min at 37°C. To ensure efficient capping of the *in vitro* transcribed RNA 2mM GTP was added after 5min. After 1h incubation at 37°C the reaction was phenol-extracted (Roth, Karlsruhe, Germany) and precipitated.
RNA used for knockdown experiments in *Drosophila* cell culture was transcribed from a PCR product with flanking T7 promoter sequences (primers are listed in table 1.1). *In vitro* transcription reaction was performed using 500ng of PCR product as described above, but omitting the cap analog. Phenolized and precipitated RNA was denatured for 3min at 65°C and slowly cooled down to room temperature before storing at -80°C.

Transcription Buffer	40mM Tris pH 7.9; 6mM $MgCl_2$; 10mM DTT; 2mM spermindine

5.1.3.5 Probe labeling

RNA labeling for electro mobility shift assays was done using 15pmol of RNA oligonucleotide (listed in table 1.4) with 10µCi of γ^{32}P-ATP (Hartmann Analytics, Braunschweig, Germany) in a 10µl T4 PNK (Thermo Fisher Scientific, Waltham, USA) reaction following the manufacturer's instructions. The reaction was incubated for 30min at 37°C, and then T4 PNK was heat-inactivated for 10min at 75°C. Afterwards, the total sample volume was transferred onto an Illustra Microspin G-25 column (GE Healthcare, Buckinghamshire, UK) and the flow-through was stored at -20°C and diluted to 10fmol/µl for EMSA application.

5.2 Tissue culture methods

5.2.1 Cell culture

Drosophila cell lines Dmel-2 and Kc167 were cultured under standard conditions (25°C) using Express Five™ SFM medium supplemented with 10x Glutamax without antibiotics and were splitted 1:5 every other day.

5.2.1.1 Transfection using FugeneHD

Dmel-2 cells and Kc167 cells were transfected at a confluency of approximately 50% using Fugene®HD with a DNA to FugeneHD ratio of 1:3. Per well of a 6-well plate, 2µg of DNA were used in 50µl of water and 6µl of FugeneHD were added to the mixture, followed by

immediate vortexing. Per 15cm plate, 15µg of DNA and 45µl FugeneHD were used. Per well of a 48-well plate, 150µg DNA were transfected with 0.75µl FugeneHD. After 20min of incubation, the transfection mixture was added in droplets to the cells and incubated for 48-72h. For Kc167 cells, two subsequent rounds of transfection were performed.

5.2.1.2 Knockdown via dsRNA

Knockdown of Ssx and control knockdown of Sxl in Dmel-2 cells was performed using 30µg of dsRNA added to a freshly seeded well of a 6-well plate with $1.2x10^6$ cells and was incubated for 72h according to Clemens et al. 2000.

5.2.1.3 UV-Crosslinking of Dmel-2 cells

Dmel-2 cells were grown in 15cm plates to a confluency of about 90%. Next, medium was aspirated and cells were quickly washed with ice cold PBS. The remaining PBS was carefully removed and crosslinking of cells occurred at 300mJ/cm^2 at 254nm on ice using a UV stratalinker 2400 (Stratagene). Afterwards, cells were immediately harvested in 500µl PBS, pelleted for 5min at 1500rpm at 4°C, snap frozen in liquid nitrogen and stored at -80°C.

5.2.1.4 *In vivo* luciferase assay

Luciferase reporter assays were performed in Dmel-2 cells using the plasmids of pCaSpeR-hs msl2 renilla as reporters containing 5' and 3' UTR of *msl-2* and pCaSpeR-hs-msl-2-Bm-FF-EFm as control reporter for normalization. Cells were co-transfected in 48-well plates with 30ng firefly control reporter, 45ng renilla reporter and 75ng pAc5.1 expression plasmids according to the protocol described in 5.2.1.1. Luciferase activity was determined were measured 48-72h post transfection. For this, cells were detached by vigorous agitation with a pipet and pelleted for 1min at 2000rpm and 4°C. The pellet was washed with ice-cold PBS, resuspended in 50µl 1xpassive lysis buffer and incubated for 15min at room temperature while shaking (500rpm). 10µl of lysate were measured on a Mithras LB 940 luminometer (Berthold technologies, Bad Wildbad, Germany) using the Dual-Luciferase Reporter Assay System (Promega, Madison, USA). Data were analyzed by calculation of renilla to firefly ratios for each single well and normalized to the unregulated renilla reporter control.

5.3 Biochemical methods

5.3.1 SDS PAGE, Coomassie staining and Western Blot

Lysate preparation for SDS-PAGE was done using 1xmodified RIPA buffer supplemented with proteinase inhibitors. Cell culture samples were pelleted and resuspended in 100µl 1xRIPA buffer and incubated for 10min on ice. For embryonic samples (100µl), 100µl 1xmodified RIPA was added, for fly samples (30 bodies) 150µl 1xmodified RIPA was added and samples were homogenized with a pestle on ice before 10min incubation on ice.

Genomic DNA of cell culture samples and embryonic samples was removed by centrifugation in QIAshredders (Qiagen, Hilden, Germany), flow through was supplemented with 2x SDS loading dye and boiled for 5min at 95°C before centrifugation for 1min at full speed to remove cell debris. Fly samples were centrifuged 3 times for 6min at 14000rpm at 4°C to remove debris and fat particles. Afterwards, 2x SDS loading dye was added to the purified sample and proteins were denatured for 5min at 95°C. Protein concentrations were measured using DC Protein Assay (Biorad, Hercules, USA) according to manufacturer's instructions.

2x modified RIPA	40mM Tris pH 8.0; 300mM NaCl; 2% NP-40; 10mM EDTA; 4% SDS
2xSDS-PAGE loading dye	100mM Tris pH 6.8; 2% SDS; 20% Glycerol; add Bromphenol blue; 200mM DTT freshly

Depending on the molecular weight of the target proteins, 6% to 15% SDS polyacrylamide gels were used. For detection of purified proteins using Coomassie staining 3µg of total protein were loaded. For cell culture and embryonic samples 10-50µg of total protein were loaded, for fly samples 150µg of total protein were loaded. Gels were run at 90-130 V in 1x SDS running buffer.

SDS-PAGE separation gel	Acrylamide/Bis Solution (37:5:1) 6%-15%; 375mM Tris- pH 8.8; 0.1 % SDS; 0.1% APS; 0.05% TEMED
SDS-PAGE stacking gel	17% Acrylamide/Bis Solution 37:5:1 30%; 130mM Tris- pH 6.8; 0.1% SDS; 0.1% APS; 0.05% TEMED
SDS running buffer	25mM Tris; 190mM Glycine; 1% SDS

For Coomassie staining, the SDS PAGE gel was incubated overnight in Coomassie staining solution with continuous shaking and was destained the next day using Coomassie destaining solution until protein bands were detectable. The destaining reaction was stopped by water supplemented with 5% acetic acid.

Coomassie staining	45% Methanol; 10% Acetic acid; 1% (w/v) Coomassie Brilliant Blue R-250, filtered
Coomassie destaining	20% Methanol; 10% Acetic acid; add water

For Western Blot analysis, 3 Whatman papers were soaked in blotting buffer anode I, anode II or cathode. Using a nitrocellulose membrane, the membrane was shortly incubated in

anode buffer II whereas PVDF membrane was activated in methanol before blotting. The assembly order for blotting was 3 Whatman papers soaked in anode I, 3 Whatman papers soaked in anode II, membrane, SDS-gel, 3 Whatman papers soaked in cathode buffer. Blotting of proteins was performed at 25V for 30min. The blotted membrane was blocked in 5% milk in TBS for at least 30min and was incubated with the respective primary antibody (listed in Table 3.1) overnight in 5% milk in TBS under constant shaking. The next day, the membrane was washed three times for 10min with TBS and was subsequently incubated with a suitable secondary antibody (listed in Table 3.1) for 1h at room temperature in 5% milk in TBS. After three washing steps in TBS for 10min, the proteins were visualized using Clarity Western ECL Substrate (Biorad, Hercules, USA) on a ChemiDoc Touch Imaging System (Biorad, Hercules, USA).

Blotting buffer anode 1	300mM Tris pH 10.4; 10% Methanol
Blotting buffer anode 2	25mM Tris pH 10.4; 10% Methanol
Blotting buffer cathode	25mM Tris pH 9.4; 10% Methanol
Blocking buffer	10% Milk powder; add TBS
Tris buffered saline	10mM Tris pH 7.5; 150mM NaCl

5.3.2 RNA-Immunoprecipitation (RIP)

For IPs of overexpressed FlagHA-tagged proteins in Dmel-2 cells anti-FLAG® M2 Magnetic Beads (Sigma-Aldrich, St. Louis, USA) were used according to manufacturer's recommendations. In brief, medium of transfected cells was removed, cells were washed with ice cold PBS and were harvested in 500µl cold PBS. After 3min of centrifugation at 500g 4°C, supernatant was removed and 500µl IP lysis buffer was added. The sample was lysed for 20min on ice and subsequently centrifuged for 30min at 14000rpm and 4°C. 100µl anti-FLAG® M2 Magnetic beads were used for lysate from two 15cm plates and were slowly rotated for 3h at 4°C, while 10% of the lysed sample was taken as input control and stored on ice. Next, the supernatant was discarded and beads were washed once with lysis buffer and then transferred into a new reaction tube. After four additional washing steps with IP wash buffer, 1/5 of the sample was applied to Western Blot analysis and supplemented with 2xSDS loading dye. The remaining beads were treated with Proteinase K for 30min at 50°C, using 200µl Proteinase K buffer and 160U Proteinase K enzyme. Afterwards the protein-bound RNA was extracted using 200µl phenol/chloroform/isoamylalcohol (25:24:1) followed by precipitation (see also 5.1.3.1 RNA isolation). Isolated RNA was subjected to sequencing library preparation using the Pico Input SMARTer Stranded Total RNA-Seq Kit (Clontech). Libraries were prepared and sequenced by the Genomics Core Facility at the EMBL,

Heidelberg (Germany). Bioinformatic analysis for the RIP-seq data was performed by Dr. Nicholas Strieder, Institute of statistical bioinformatics, working group of Prof. Dr. Rainer Spang, University of Regensburg. In brief, using the program trimmomatic (Bolger et al. 2014), adapter sequences were removed from raw reads, and reads were subsequently aligned to the *Drosophila* dm6 genome with the help of the program Tophat2.0 (Kim et al. 2013). Reads were summarized per gene using featureCounts from the Rsubread R-library (Liao et al. 2014). Using DESeq2 (Love et al. 2014), differential enrichment of genes bound to Ssx and Sxl vs GFP and Ssx vs Sxl was analyzed.

IP lysis buffer	50mM Tris pH 8.0; 150mM NaCl; 2mM $MgCl_2$; 1% NP40; add Protease inhibitors; add RNAsin
IP wash buffer	50mM Tris pH 8.0; 150mM NaCl; 2mM $MgCl_2$; 0.1% NP40; add Protease inhibitors; add RNAsin
Proteinase K buffer	50mM Tris pH 8.0; 10mM EDTA; 1.3% SDS

5.3.3 Individual-nucleotide resolution crosslinking-immunoprecipitation (iCLIP)

For iCLIP experiments, proteins were crosslinked to their target RNAs using UV-light according to chapter 5.2.1.3 (UV-crosslinking of Dmel-2 cells), cells were lysed and treated with 36U RNaseI. Next, endogenous Ssx protein was immunoprecipitated using the antibody SY6158 coupled to Dynabeads protein G (Thermo Fisher Scientific, Waltham, USA) for 2h at 4°C under constant rotation. An experiment performed with a non-specific antibody served as negative control. After four washing steps with IP wash buffer, the co-immunoprecipitated RNA was dephosphorylated and ligated to a 3' RNA-linker. Subsequently, the ligated RNA fragments were radioactively labeled with [γ-^{32}P]-ATP and Ssx-bound RNA complexes were separated by a neutral SDS PAGE (NuPAGE, Invitrogen). After blotting to a nitrocellulose membrane, the protein-bound RNA fragments were detected by audioradiography and Ssx/RNA complexes were excised. After a Proteinase K digestion, isolated RNA was used for iCLIP library preparation according to König et al. 2010. Sequencing occurred on a MiSeq® (Illumina, 130nt single read). All steps after UV-crosslinking of Dmel-2 cells were performed by Dr. Oliver Rossbach, University of Gießen (Germany). Sequencing of libraries and bioinformatic analysis of the iCLIP data was performed by Norbert Eichner and Gerhard Lehmann at the Institute of Biochemistry I, working group of Prof. Dr. Gunter Meister, University of Regensburg. In brief, adapter of raw sequences were trimmed using Cutadapt (Martin 2011), the unique molecular identifier (UMI) was extracted, and ribosomal RNA reads were removed. Sequences were aligned to the *Drosophila* dm6 genome sequence with the help of the program bowtie (Langmead et al. 2009), while duplicate reads were removed.

Differential gene expression analysis was performed using the DeSeq2 package (Love et al. 2014). Peaks were scored using ASpeak (Kucukural et al. 2013) and sequences located 30nt upstream and downstream of the peak were conducted for motif analysis using MEME (Bailey et al. 2009).

IP Wash buffer iCLIP	50mM Tris pH7.4; 800mM NaCl; 0.05% Tween 20; add Protease inhibitors; add RNAsin
Proteinase K buffer	50mM Tris pH 8.0; 10mM EDTA; 1.3%SDS

5.3.4 Expression and purification of recombinant proteins

5.3.4.1 Purification of Sxl RBD4, Ssx RBD4 and mutations thereof

For preparation of recombinant proteins GST-Sxl-RBD4, GST-Ssx-RBD4 (and mutations thereof), protein expression was induced by IPTG for 4h and 23°C in *E.coli* BL21* (Invitrogen) (transformed with the Rosetta 2 plasmid). Cells were pelleted and resuspended in buffer X, lysed and centrifuged for 30min at 40000rpm. GST-tagged proteins were purified using a 5ml Protino® GST/4B Column (Macherey Nagel, Düren, Germany) and an ÄKTA FPLC system. Bound proteins were eluted with elution buffer, supplemented with 3C protease (Prescission) and dialyzed against Buffer D100. Next, an ion exchange chromatography was performed using a MonoS column (GE Healthcare, Buckinghamshire, UK). After elution with a salt gradient, the protein was dialyzed against buffer D50 and stored at -80°C.

Buffer X	20mM Tris pH 7.5; 1M NaCl; 0.2mM EDTA; 1mM DTT
Buffer D0-D1000	20mM HEPES pH 8.0; 20%Glycerol; 0.2mM EDTA; 0.01% NP40; 1mM DTT (0-1M KCL)
Elution buffer	50mM Glutathione; 100mM HEPES/KOH pH 8.0; 50mM NaCl; 1mM DTT

5.3.4.2 Purification of UNR-CSD1

UNR-CSD1 was expressed and purified as described previously (Hennig et al. 2013). In brief, the recombinant protein was expressed by IPTG induction in *E.coli* BL21* (Invitrogen; transformed with the Rosetta 2 plasmid) overnight at 20°C. Cells were harvested, lysed and sonicated. After clarification by centrifugation, the supernatant was loaded onto a Protino® Ni-NTA Column 5ml (Macherey Nagel, Düren, Germany) and the protein was eluted with elution buffer. The His-tag was cleaved off using a TEV protease and the protein was dialyzed against lysis buffer overnight at 4°C. The tag was removed by a second Ni-NTA column purification, collecting the flow through and precipitating the untagged and purified

protein with ammonium sulfate. The pelleted protein was dissolved in rehydration buffer and dialyzed overnight at 4°C against UNR dialysis buffer before concentrating the UNR-CSD1 protein via a gelfiltration S75 column in dialysis buffer lacking DTT.

Lysis buffer	50mM KH_2PO_4; 300mM NaCl; adjust to pH 8.0
Wash buffer 1	50mM KH_2PO_4; 300mM NaCl; 10mM Imidazole; adjust to pH 8.0
Wash buffer 2	50mM KH_2PO_4; 300mM NaCl; 20mM Imidazole; adjust to pH 8.0
Elution buffer	50mM KH_2PO_4; 300mM NaCl; 120mM Imidazole; adjust to pH 8.0
Rehydration buffer	10mM KH_2PO_4; 50mM NaCl; 1mM DTT; adjust to pH 6
Dialysis buffer	10mM KH_2PO_4; 50mM NaCl; 1mM DTT; adjust to pH 6

5.3.5 Electro-mobility-shift-assays (EMSA)

10fmol of P^{32}-labeled RNA was incubated with the indicated protein amounts diluted in buffer D100 and were subsequently analyzed for ribonucleoprotein complex formation. For this, each sample was supplemented with 1xEMSA incubation buffer and 0.2µg/µl yeast tRNA and was incubated for 30min on ice. Subsequently, samples were loaded on an 8% native polyacrylamide gel and were run in 1xTBE for 2h at 4°C and 230V. The polyacrylamide gel was vacuum-dried on a Whatman paper for 2h and 80°C and ribonucleoprotein complex formation was detected with the Personal Molecular Imager System (Biorad, Hercules, USA).

EMSA Incubation buffer	10mM Tris pH 7.4; 50mM KCl; 1mM EDTA; 0.05% glycerol; 1mM DTT
Native 8% EMSA gel	8% acrylamide (37:5:1); 1xTBE; 1%APS; 0.1% TEMED
EMSA running buffer	0.5xTBE

5.3.6 *In vitro* luciferase assay

For *in vitro* translation, 130fmol of *in vitro* transcribed reporter RNA was supplemented with 10pmol of recombinant protein in 1x *in vitro* translation buffer in a total volume of 10µl and samples were incubated for 90min at 25°C to allow translation to occur. Samples were supplemented with 1x passive lysis buffer and incubated for 10min at room temperature. Experiments were performed in triplicates and were measured on a Mithras LB 940 luminometer (Berthold technologies, Bad Wildbad, Germany) using the Dual-Luciferase

Reporter Assay System (Promega, Madison, USA). Data were analyzed calculating firefly to renilla ratios for each well, which were then normalized to an unregulated firefly reporter control.

In vitro translation buffer	40% *Drosophila* embryo extract; 24mM HEPES/KOH pH 7.4;100mM KOAc; 500µM MgOAc; 60mM aminoacids; 20mM creatine phosphate; 800ng creatine kinase; 0.1875µg/µl renilla mRNA

5.4 Fly genetics

The fly work was conducted at the Department of Anatomy, laboratory of Junior Prof. Dr. med. vet. Dr. rer. nat. Michael Krahn at the University of Regensburg.

5.4.1 Maintaining fly stocks and breeding conditions

Drosophila flies were maintained in glass vials containing standard food (0.8% agar, 2.2% sugar beet molasses, 8.0% malt extract, 1.8% brewer's yeast, 1.0% soy flour, 8.0% maize meal, 0.3% nipagin) with particles of dry yeast on top at 18°C, 21°C (room temperature) or 25°C depending on the experimental setup. The fly stocks were exchanged at the latest every 5 weeks at room temperature; a 12 hour day/night rhythm was simulated by an artificial light source.

For collection of *Drosophila* embryos, flies were kept in cages of two different sizes depending on the experimental requirements. The bottom of each cage was an apple juice agar plate (∅ 6cm or 10cm) with yeast paste made out of water and baker's yeast. After defined time points (injections: 30min; embryo lysate preparation: 12-24h), embryos were collected and used for different experimental set-ups.

5.4.2 Generation of transgenic fly lines

Injection of embryos was applied, in order to achieve a transgenic fly line using the PhiC31 integrase system and to generate a transgenic knock out fly line using CRISPR/Cas9 mediated genomic engineering.

5.4.2.1 PhiC31 integrase system

The PhiC31 integrase, a recombinase isolated from the bacteriophage PhiC31, mediates site-specific recombination between two specific attachment sites. This site-specific recombination is carried out between an *attB* site, encoded within a donor plasmid and an *attP* site, a target site integrated in the fly genome (Thorpe et al. 2000). After recombination of *attB* and *attP* sites, two different sites are produced (*attR* and *attL*), ensuring uni-directionality of the recombination event. In this study, the fly strain UASt::Ssx was generated using the PhiC31 integrase system.

5.4.2.2 CRISPR/Cas9 system

The CRISPR/Cas9 system is based on dsDNA breaks generated by the Cas9-nuclease targeted to specific genomic sites by chimeric RNAs (chiRNAs). Subsequently, these double strand breaks are subject to homology-directed repair (HDR) employing a supplemented donor vector. Using this system which is originally derived from the adaptive immune system in bacteria and archea (Ishino et al. 1987; Makarova et al. 2006; Barrangou et al. 2007; Jinek et al. 2012), the genome of *Drosophila* can easily be edited and modified. Here, the adapted system is composed of a Cas9 nuclease, already integrated into the fly genome, two guideRNAs, marking cleavage sites for the Cas9 nuclease, and a repair donor template containing a 3xdsRed marker flanked by two homologous regions of the to be edited genome region for HDR. This system was used to generate a Ssx knock out fly strain (Δssx), replacing the entire Ssx coding region by a 3xdsRed cassette.

5.4.2.3 Injection of embryos

DNA for transgenic recombination via PhiC31 integrase or CRISPR/Cas9 mediated knock out was injected into the pole cell region of early embryos (preblastoderm). Here, embryos containing an attp40 landing site (for PhiC31 driven integration, listed in 4.7.3) or embryos harboring a *vasa*-promoter driven GFP-tagged Cas9 nuclease (for Ssx knock out, listed in 4.7.3) were used. An injection mix composed of DNA and 1x injection buffer was centrifuged for 30min at 15000rpm at 16°C before usage. Embryos were collected every 30min and were dechorionized, oriented on apple juice agar in rows and were transferred on a coverslip coated with embryo glue. After a drying step of 15-25min, the embryos were covered with oil 10 S VOLTALEF®. Next, the injection mix was injected into the embryos pole cell region using Femptotips®II microinjection capillary and a micromanipulator InjectMan NI2. The injected embryos were incubated overnight at 18°C. After hatching, animals were transferred into new glass vials supplemented with loosened standard food the next day. Adult flies were crossed with w-; Gla/CyO flies for transgenic fly selection by eye color (wt vs. w- for PhiC31 integrated FlagHA tagged-Ssx and 3xdsRed for Ssx knock out) and back-crossed for stable or homozygous fly stocks.

Injection buffer	0.5 mM KCl, 0.01 mM sodium phosphate, pH 6.8

5.4.2.4 UAS::GAL4 system

The UAS-GAL4 system is used for controlled protein overexpression in *Drosophila* (Brand and Perrimon 1993). The system is based on the GAL4 protein, a transcription factor from yeast which binds to an upstream activating sequence (UAS) driving transcription of the associated downstream gene. Combination of various GAL4 driver strains with transgenic strains that harbor a UAS expression cassette, allows precise spatial and temporal control of the expression of a gene of interest. Combination of various GAL4 driver strains with

transgenic strains that harbor a UAS expression cassette, allows precise spatial and temporal control of the expression of a gene of interest. In this study, the GAL4/UAS system was applied for ubiquitous overexpression of FlagHA-tagged Ssx in flies. The UAS::Ssx fly strain was generated as described in 5.4.2.1 and 5.4.2.3 and was crossed to a da::GAL4 driver line, leading to a ubiquitous overexpression of Ssx starting in late stages of embryonic development. Since forced homozygous overexpression of Ssx was not viable, the fly strain was balanced over CyO on chromosome 2 and TM3 on chromosome 3 (UAS::Ssx/CyO; da::GAL4/TM3), thus reducing the expression level of Ssx.

5.4.3 Lethality assay

To score survival of transgenic or knockout flies, 3x150 embryos (12h) per fly strain were collected and arranged in rows on apple juice agar plates. Between each embryo row, a line of yeast paste was placed and plates were incubated in cages at 25°C. Embryos were analyzed for survival on a daily basis and developmental stages were monitored in parallel (embryo, larval stage 1, larval stage 3, pupa, adult fly). Plates were supplemented with water every day, to avoid drying out of the plates. Deceased individuals and surviving animals were counted at every developmental stage and overall percentages were calculated.

Appendix

Table A.1: List of significantly enriched Ssx target mRNAs identified by iCLIP.

Loci significantly enriched in Ssx iCLIP	chromosome	location	log change in RIP	position
Act5C	chrX	Intron		
ade5	chrX	3'-UTR	2,222214721	
alpha-Man-I	chrX	3'-UTR	3,3279458	
ap	chr2R	Intron	2,755120056	
Atg8a	chrX	3'-UTR	2,796714225	
B4	chr2L	5'-UTR	2,194862135	
ben	chrX	3'-UTR	3,115791774	311
beta-Spec	chrX	3'-UTR		
bif	chrX	Intron	3,021150483	365
brat	chr2L	3'-UTR	4,474534906	46
CG10077	chr3L	3'-UTR	4,210012031	74
CG10970	chrX	5'-UTR		
CG11151	chrX	3'-UTR		
CG11360	chr4	3'-UTR	3,581132461	176
CG12592	chr3R	3'-UTR		
CG12643	chrX	3'-UTR		
CG1572	chrX	3'-UTR		
CG1673	chrX	3'-UTR	3,494423367	195
CG17018	chr2L	Intron	2,14213332	840
CG17691	chr2R	Intron	2,015925311	941
CG17912	chr2L	3'-UTR	3,941789375	115
CG32373	chr3L	Intron		
CG32638	chrX	3'-UTR	2,439646663	652
CG4165	chrX	3'-UTR	3,159550746	308
CG42324	chr3L	Intron	4,009350465	104
CG42335	chr3R	Intron		
CG4239	chrX	3'-UTR	3,610445906	162

CG43759	chrX	3'-UTR	3,212125105	289
CG44325	chrX	Intron		
CG45086	chr2R	3'-UTR		
CG7231	chr2L	5'-UTR		
Clic	chrX	3'-UTR	3,294294628	271
CoRest	chrX	3'-UTR	3,005098134	373
CrebA	chr3L	Intron	3,292145366	270
cta	chr2L	Intron	2,518679682	595
CtBP	chr3R	Intron		
cwo	chr3R	5'-UTR	2,105242112	872
Cyp1	chrX	3'-UTR		
dm	chrX	3'-UTR	4,376195203	57
Dok	chrX	3'-UTR	3,375230856	232
Dsp1	chrX	3'-UTR	4,288227791	68
Fim	chrX	3'-UTR	3,64508059	155
fs(1)h	chrX	3'-UTR	3,048262336	356
Galphaq	chr2R	3'-UTR	2,780678349	477
GlcAT-I	chrX	3'-UTR	2,516686231	598
Gprk1	chr2R	Intron		
hbs	chr2R	Intron		
Hers	chrX	3'-UTR	2,962103953	388
Hex-A	chrX	3'-UTR	2,525331096	591
His3.3B	chrX	3'-UTR	2,883353341	429
Hr4	chrX	Intron		
Hsc70-3	chrX	3'-UTR	3,373363986	234
Jafrac1	chrX	3'-UTR		
Jhl-21	chr2L	3'-UTR		
kirre	chrX	Intron		
Klp10A	chrX	3'-UTR	2,950570562	391
l(1)G0320	chrX	3'-UTR	2,268627482	757
larp	chr3R	3'-UTR	2,505520066	605
mab-21	chrX	3'-UTR	4,099915273	91
Mec2	chrX	3'-UTR		
mgl	chrX	Intron	2,661664435	542
Mid1	chr2R	Intron		

Moe	chrX	3'-UTR	2,065376053	904
mspo	chr2R	3'-UTR		
NFAT	chrX	3'-UTR	3,822123375	129
nmo	chr3L	Intron	3,599467446	166
pigs	chrX	3'-UTR	3,230742474	284
Pka-R1	chr3L	Intron		
prtp	chrX	3'-UTR	3,302948452	262
Rala	chrX	3'-UTR	4,284063553	69
Ran	chrX	3'-UTR	2,666447521	539
Rbp2	chrX	3'-UTR	2,31769766	728
RhoGAP18B	chrX	3'-UTR	3,322859156	249
RpL17	chrX	5'-UTR/Intron		
RpL28	chr3L	Intron		
RpS19a	chrX	5'-UTR		
RpS5a	chrX	5'-UTR		
RpS6	chrX	5'-UTR		
Sk2	chr3L	3'-UTR	2,637422444	554
SkpA	chrX	3'-UTR	2,498597945	609
spoon	chrX	3'-UTR	3,812414813	134
sqh	chrX	3'-UTR		
stai	chr2L	3'-UTR		
Sxl	chrX	Intron	3,305423482	260
tara	chr3R	5'-UTR	2,48551297	622
Teh1	chr3R	Intron		
tlk	chrX	3'-UTR	2,773462303	479
TRAM	chrX	3'-UTR		
Trf2	chrX	3'-UTR	2,899256943	422
Ubqn	chrX	3'-UTR		
Uev1A	chr3L	3'-UTR	2,249464121	733
v(2)k05816	chr2L	Intron		

List of Figures

List of Abbreviations

A	adenine
A	alanine
Aa	amino acid
AML-1 like	acute myeloid leukemia 1
APS	ammonium persulfate
ARE	AU-rich element
bHLH	basic helix-loop-helix
BPS	branch point sequence
C	cytosine
C	cysteine
C. elegans	*Caenorhabditis elegans*
CGRP	calcitonin gene-related peptide
chiRNA	chimeric RNA
Cpsf160	cleavage and polyadenylation specificity factor 160
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSD1	cold shock domain 1
Cstf64	cleavage stimulation factor 64
C-terminus	carboxyl-terminus
CTP	cytidine triphosphate
CyO	curly of Oyster
D	aspartic acid
Da	daughterless
DCC	dosage compensation complex
D. melanogaster	*Drosophila melanogaster*
DNA	deoxyribonucleic acid
Dpn	deadpan
Dscam	down syndrome cell adhesion molecule
dsRNA	double-stranded DNA
Dsx	doublesex
DTT	dithiothreitol
E.coli	*Escherichia coli*

EDTA	ethylenediaminetetraacetic acid
ELAV	embryonic lethal abnormal visual system
EMSA	electro mobility shift assay
EMC	extramachrochetae
E(r)	enhancer of rudimentary
ESE	exonic splice enhancer
ESS	exonic splice silencer
Ewg	erect wing
EiF	eukaryotic initiation factor
F	phenylalanine
Fl(2)d	female-lethal(2)d
FNE	found in neurons
Fru	fruitless
G	guanine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
Gla	glace
GOF	gain of function
GST	glutathione S-transferase
GTP	guanosine triphosphate
H	histidine
HA	hemagglutinin
HDR	homology-directed repair
hnRNP	heterogeneous nuclear ribonucleoprotein
How	held out wings
Hu	human antigen
iCLIP	individual-nucleotide resolution crosslinking-immunoprecipitation
Ime4	inducer of meiosis 4
IP	immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISS	intronic splice silencer
Jak/Stat	januskinase/signal transducers and activators of transcription
K	lysine
LB	lysogeny broth
LOF	loss of function
Mettl	methyl-transferase like
Met-tRNAi	methionine-transfer-RNAi

mRNA	messenger RNA
Msl-2	male specific lethal-2
m6A	N^6-methyladenosine
m7G	7-methylguanosine
N	asparagine
NHEJ	non-homologous-end joining
N-terminus	amino-terminus
OH	hydroxyl
ORF	open reading frame
PABP	poly(A) binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIC	preinitiation complex
PPS	protein partner of snf
PPT	polypyrimidine tract
PTB	polypyrimidine tract-binding protein
R	arginine
RBP9	RNA binding protein 9
RBM15	RNA-binding motif protein 15
RFP	red fluorescent protein
RGG	arginine glycine glycine
RIP	RNA immunoprecipitation
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RNAi	RNA interference
Rpm	rounds per minute
RRM	RNA recognition motif
RS	arginine serine
RT	reverse transcription
S	serine
Sdc-2	syndecan-2
SDS	sodium dodecyl sulfate
SF	splicing factor
Sis	sisterless
Snf	sans fille
SnRNP	small nuclear ribonucleoprotein particle
SR	serine arginine

SRY	sex determining region of Y
Stat92E	signal-transducer and activator of transcription protein at 92E
Ssx	sister of sex lethal
Sxl	sex lethal
TBE	tris/borate/EDTA
TBS	tris-buffered saline
TEV	tobacco etch virus
Tra	transformer
tRNA	transfer RNA
UAS	upstream activating sequence
U	uridine
UNR	upstream of N-ras
uORF	upstream open reading frame
UTP	uridine triphosphate
UTR	untranslated region
U1-70K	U1 small nuclear ribonucleoprotein 70
U2AF	U2 auxiliary factor
Vir	virilizer
Wt	wildtype
Wtap	Wilms' tumor 1-associating protein
Xist	X-inactive specific transcript
Xol-1	XO lethal protein 1
XSE	X-linked signal elements
Y	tyrosine
YTHDC-1	YTH domain-containing protein 1

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Contributions

CLIP experiments were performed by Dr. Oliver Rossbach from the Institute of Biochemistry at the Justus-Liebig-University of Gießen (Germany).

Sequencing of the CLIP experiments was performed by Norbert Eichner using a MiSeq™ platform.

Data analysis of iCLIP experiments was performed by Gerhard Lehmann und Norbert Eichner from the working group of Prof. Dr. Gunter Meister, Institute of Biochemistry I at the University of Regensburg (Germany).

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