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Translational Control via Protein-Regulated Upstream Open Reading Frames

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SUMMARY

Analysis of the regulation of *msl-2* mRNA by Sex lethal (SXL), which is critical for dosage compensation in *Drosophila*, has uncovered a mode of translational control based on common 5′ untranslated region elements, upstream open reading frames (uORFs), and interaction sites for RNA-binding proteins. We show that SXL binding downstream of a short uORF imposes a strong negative effect on major reading frame translation. The underlying mechanism involves increasing initiation of scanning ribosomes at the uORF and augmenting its impediment to downstream translation. Our analyses reveal that SXL exerts its effect controlling initiation, not elongation or termination, at the uORF. Probing the generality of the underlying mechanism, we show that the regulatory module that we define experimentally functions in a heterologous context, and we identify natural *Drosophila* mRNAs that are regulated via this module. We propose that protein-regulated uORFs constitute a systematic principle for the regulation of protein synthesis.

INTRODUCTION

Regulation of translation represents a critical layer of gene expression control in essentially all cells. It allows rapid and localized changes in the expression of proteins in response to extra- and intracellular stimuli, thus being crucial for a large number of important cellular processes. Translational control can occur on a global basis by modifications of the basic translation machinery, or selectively target defined subsets of messenger RNAs. The latter commonly involves the sequence-specific recognition of target mRNAs by *trans*-acting factors such as miRNA complexes or RNA-binding proteins (RBPs) (reviewed in Jackson et al., 2010; Pestova et al., 2007; Sonenberg and Hinnebusch, 2009).

In *Drosophila*, translational control of *male-specific lethal (msl)-2* mRNA provides an intricate paradigm for understanding how RBPs can control translation. Silencing of *msl-2* mRNA is essential for the survival of female flies, preventing formation of the dosage compensation complex that is required in male flies for hypertranscription of the single X chromosome (reviewed in Bashaw and Baker, 1995; Kelley et al., 1995, 1997). In female flies translational silencing is achieved by association of the protein Sex lethal (SXL) with poly(U) stretches present in both the 5′ and 3′ untranslated regions (UTRs) of the *msl-2* mRNA (Duncan et al., 2006; Gebauer et al., 2003).

Two distinct and mutually reinforcing mechanisms are employed by SXL to block *msl-2* mRNA translation. Bound to the 3′UTR regulatory elements, SXL recruits the corepressor protein UNR (upstream of N-ras) to adjacent binding sites and in conjunction with it blocks recruitment of the 43S preinitiation complex to the 5′ end of the mRNA (Abaza et al., 2006; Beckmann et al., 2005; Duncan et al., 2006; Duncan et al., 2009). Preinitiation complexes that escape this first regulatory intervention are challenged by SXL molecules bound to the regulatory elements in the 5′UTR. This challenge stalls and destabilizes the small ribosomal subunit upstream of the SXL-binding site (Beckmann et al., 2005). This bifunctional mode of SXL action ensures proper control over *msl-2* translation in the form of a failsafe mechanism to prevent developmental and viability defects in female flies (Kelley et al., 1995).

Although regulation via the 3′UTR regulatory mechanism has been extensively studied both in terms of cofactor requirement and mechanism of action (Abaza et al., 2006; Beckmann et al., 2005; Duncan et al., 2006; Duncan et al., 2009), regulation via the 5′UTR remains less well understood. Making use of the cell-free translation system (derived from *Drosophila* embryos) that has enabled all previous mechanistic work, we analyzed 5′UTR-mediated translational control in the absence of 3′UTR regulatory elements. We uncover the importance of a short upstream open reading frame (uORF) in vicinity of the SXL-binding site that acts in conjunction with SXL. Based on the experimental definition of the regulatory module and the identification of similar modules in naturally occurring *Drosophila* RNAs, we show that SXL controls a family of *Drosophila* mRNAs by regulation of uORFs, and suggest that the underlying regulatory principle may apply more broadly to eukaryotic gene regulation.

RESULTS

A Conserved uORF Mediates Translational Control of *msl-2* mRNA by SXL

Previous analyses of SXL-mediated translational control of *msl-2* mRNA revealed an integrated mechanism with a block to two

consecutive initiation steps operating independently via the 5′ or 3′UTRs (Beckmann et al., 2005). This regulation involves several SXL-binding sites, short poly(U) stretches, present in both the 5′ and 3′UTR of the *msl-2* transcript. In addition to two SXL-binding sites, the 5′UTR of *msl-2* mRNA harbors uORFs. Although noting small changes in translation, earlier experiments could not establish a clear role of these uORFs in translational repression of *msl-2* mRNA using transgenic flies, cultured cells, or an in vitro translation system based on *Drosophila* embryo extract (Bashaw and Baker, 1997; Gebauer et al., 1999, 2003). However, these experiments were performed before the realization that regulation via the 5′UTR and 3′UTR occurs by two independent, separable mechanisms, and the contribution of 3′UTR regulation might have masked impediments to 5′UTR-mediated regulation. We reexamined 5′UTR-mediated regulation using firefly luciferase reporters for the *msl-2* 5′UTR (including three uORFs) in the absence of 3′UTR regulatory elements (Figure 1A, minΔ3′). To determine the regulatory contribution of individual uORFs, mutations to AUU were introduced in either the first three (AUG123m), the first two (AUG12m), or the third (AUG3m) upstream initiation codon, and tested with increasing molar excess of recombinant SXL protein in *Drosophila* embryo extracts (Figure 1B). Regulation is severely compromised when all three uAUGs are mutated (compare AUG123m to min Δ3′, Figure 1B). Although mutation of the first two uAUGs (AUG12m) does not impair translational control, a point mutation in the third uAUG alone (AUG3m) suffices for derepression.

To test whether additional sequences are required for the regulatory effect of uORF3, shortened versions of the RNA were produced including mutations of the B site that abrogate SXL-binding (denoted Bm). When regulation is assayed, we observe the strong contribution of the uAUG3 (compare B and uORF-B RNAs) that depends entirely on a functional SXL-binding site (Figure 1C). Moreover, the observed effects are independent of the open reading frame used as a reporter (see Figure S1 available online). Finally, we confirmed the importance of the uORF for SXL-mediated regulation in cultured *Drosophila* cells (Figure S2).

In the absence of SXL, uORF3 reduces translation of the downstream cistron by only ~2-fold, indicating a high rate of reinitiation after the uORF and/or weak upstream initiation due to leaky scanning. In the presence of SXL, downstream translation is 14.4-fold repressed (Figure 1D), revealing that SXL acts by increasing the repressive effect of the uORF.

To exclude effects of the uORF on mRNA stability (Ruiz-Echevarria and Peltz, 2000 and references therein), we tested whether the uORF or SXL affects mRNA levels. Neither the uORF nor SXL addition impacts on RNA stability (Figure 1E), demonstrating that regulation occurs at the level of translation.

Female-specific regulation of gene expression by SXL is conserved in other Drosophilid species (Bopp et al., 1996). We inspected the genomes of the 12 sequenced Drosophilids for evolutionary conservation of the uORF. Although the SXL-binding sites (poly(U) stretches) differ slightly in length and are interspersed by other nucleotides (nt) in some species (Table 1, column B site), the combination of the short uORF followed by a SXL-binding motif is universally conserved.

Although the presence and length of the uORF are completely conserved (Table 1, column uORF), the neighboring upstream and downstream sequences exhibit no apparent sequence conservation. The distance that separates the SXL-binding site from the uAUG ranges from 27 nt in *Drosophila melanogaster* to 35 nt in *Drosophila willistoni*. To test if this spacing is critical for regulation, we successively shortened the distance or introduced stretches of nonstructured sequence (CAA repeats, Figure S3). Shortening of the spacer by 10 nt (to a distance of 17 nt) and introduction of 40 nt (to 67 nt separating the uAUG from the B site) both still support robust (albeit not maximal) repression, demonstrating that SXL can act over a relatively wide range of distances. Only when the uAUG is less than 17 nt upstream of the B site is translational repression severely compromised (Figure S3).

The uORF Operates via Initiation, Not Elongation or Termination

To probe the underlying mechanism and to test if uORF-mediated regulation of downstream translation acts at the level of initiation, elongation, or termination, we constructed a series of RNAs with mutations in the uORF sequence or its surrounding nucleotides.

In the natural context of *msl-2* mRNA, the uORF encodes the dipeptide Met-Thr (Table 1). By swapping the ACU (Thr) codon and the subsequent translation termination codon (UGA), we created a construct that allows translation initiation, but not elongation (termed AUGstop, Figure 2A). As seen in Figure 2B, the AUGstop-B RNA, similar to uORF-B RNA, is subject to strong SXL-mediated regulation, which again depends on the presence of a functional SXL-binding site (compare panels AUGstop-B and -Bm). This result demonstrates that the uORF acts in an amino acid sequence-independent manner and that translation elongation is dispensable for its effect on downstream translational control.

Next, we examined if translation initiation at the uAUG is required for the regulatory mechanism. The frequency of ribosomal recognition of a translation initiation codon is determined by its sequence context (Kozak, 1987). Positioning of a translation initiation codon within a "poor" sequence context results in inefficient ribosomal recognition and bypassing ("leaky scanning"). To understand if translation initiation at the upstream AUG is required for regulation by the uORF, we altered its initiation codon sequence context (Figure 2A). The natural context (AACA) was replaced by either an "optimal" sequence for *Drosophila* (CAAC, +consensus) (Cavener, 1987) or the complementary sequence (GUUG, −consensus) placing the uAUG into a nonfavorable context. Because these changes directly affect initiation at a generally inhibitory uORF, changes in downstream translation are expected to occur even in the absence of the regulatory SXL protein. When a "weak" initiation context is introduced at the uAUG, more ribosomes fail to recognize ("leaky scan" past) the uAUG, resulting in an increase of downstream translation (Figure 2C, compare lane 7 with lanes 1 and 4). In contrast, when the uAUG is placed in a favorable context, downstream translation becomes more dependent on reinitiation, resulting in reduced translation of the downstream cistron (lane 4) (reviewed in Hinnebusch, 2005). When testing

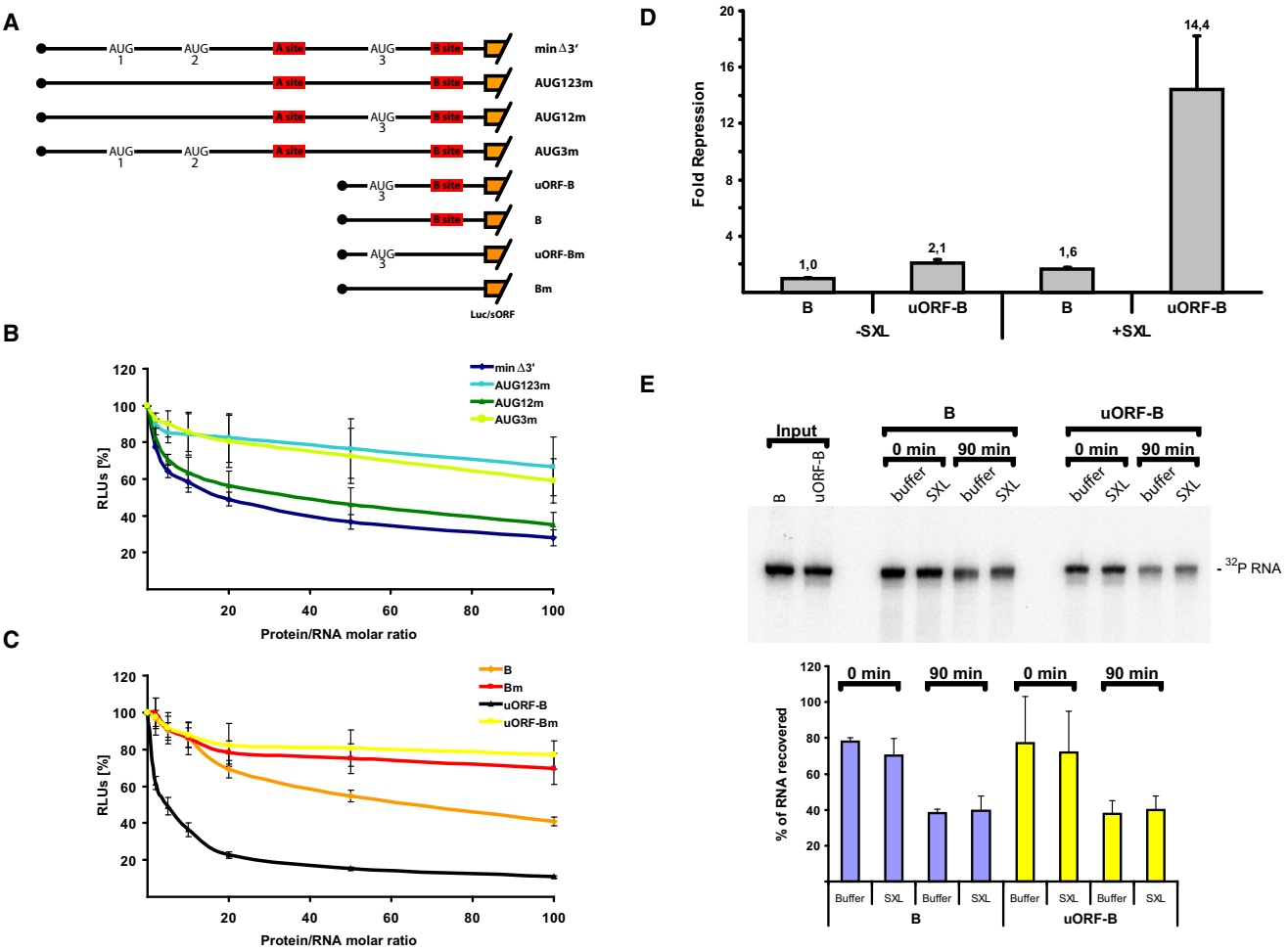

Figure 1. An Upstream Translation Initiation Codon Is Critical for SXL-Mediated Translational Control of *msl-2* mRNA

(A) Schematic representation of the 5′UTRs of the reporter mRNAs used. SXL-binding sites (A and B site) are shown as red boxes; upstream translation initiation codons (AUG) are depicted when present. The beginning of the ORF used for experimental readout, either firefly luciferase (Luc) or an artificial sORF (Gebauer et al., 2003), is shown in orange. For simplicity, downstream sequences such as the 3′UTR and a 73 nt poly(A) tail are not depicted.

(B) min Δ3′ mRNA (see A) and derivatives thereof that carry a firefly luciferase open reading frame were translated in *Drosophila* embryo extract in the presence of increasing amounts of recombinant SXL protein (up to 100× molar ratio of protein relative to RNA). *Renilla* luciferase mRNA was employed as an internal control and used for normalization of firefly luciferase activity. min Δ3′ mRNA is shown in dark blue, RNAs with mutated uAUGs in light blue (all three uAUGs mutated, AUG123m), dark green (the first two uAUGs mutated, AUG12m), or light green (the third uAUG mutated, AUG3m). Normalized luciferase counts in the absence of protein were set to 100%. The experiment was performed in three biological replicates with three replicates each.

(C) Assay similar to the one described for (B), this time using reporter mRNAs with a shortened 5′UTR that lack the A site and the first two uAUGs (depicted in A). Both remaining regulatory elements, the uAUG3 and the B site, were mutated individually (see A), yielding reporters that carry none of the regulatory elements (Bm), a uORF (uORF-Bm), a SXL-binding site only (B), or both regulatory elements (uORF-B). See also Figures S1 and S2.

(D) Translational repression of B and uORF-B RNAs (see A and C) was determined *in Drosophila* embryo extract either in the absence (−SXL) or in presence of a 40× molar excess of SXL over the RNA (+SXL). *Renilla* luciferase served as an internal control; the experiment was repeated three times with three replicates each.

(E) ^{32}P body-labeled B and uORF-B RNAs (see A)—containing a shortened firefly luciferase open reading frame (sORF) (Gebauer et al., 2003) (see also Figure S1 for regulation of the reporter RNAs)—were incubated under translation conditions in *Drosophila* embryo extract either in the presence (SXL) or absence (buffer) of a 40× molar excess of recombinant SXL protein. RNAs were recovered either immediately or after 90 min of incubation at 25°C by Phenol/Chloroform extraction and subsequently separated by denaturing polyacrylamide-urea gel electrophoresis. An autoradiography of a representative gel is shown at the top, the first two lanes showing the RNA input. After phosphorimager quantification of three independent experiments, mean values were plotted in percent relative to the input (shown below).

Error bars represent SD.

translational repression by SXL on either luciferase reporters or shortened versions thereof, regulation is unaffected when the natural sequence is replaced by a "better" initiation consensus (Figure 2C, compare AUGstop-B to +consensus, and Figure 2D for reporters bearing a luciferase ORF). Conversely, placement of the uAUG into a nonfavorable context severely compromises

Table 1. The *msl-2* uORF/SXL-Binding Site Motif Is Conserved among Drosophilids

Species	AUG Context	uORF M H/T *	Spacer	B Site
D. simulans	ACCATTAACA	ATGACTTGA	GACCTCTCAAACATAAACAACATA	$(T)_{16}$
D. sechellia	ACCATTAACA	ATGACTTGA	GACCTCTCAAACAGAAACAACATA	$(T)_{15}$
D. melanogaster	ACCATTAACA	ATGACTTGA	GACCTCTCAAACGTAAACCAA	$(T)_{16}$
D. yakuba	AACCCTAACA	ATGACTTGA	GACCACTCAAACATAAACCAAATA	$(T)_{17}$
D. erecta	ACCATTAACA	ATGACTTGA	GAGCTCTCAAACATAAACCACATA	$(T)_{18}$
D. ananassae	TCTATTAACC	ATGACTTGA	AAATACATAAACATAAACCCCTAC	$(T)_{16}$
D. pseudoobscura	ATATTTAAAA	ATGACTT*AA*	TCTTAAGAATATATAAACATAAACC	$(T)_{18}$
D. persimilis	ATATTTAAAA	ATGACTT*AA*	TCTTAAGAATATATAAACATAAACC	$(T)_{17}$
D. willistoni	AATAATAATA	ATG<u>CAT</u>TGA	TTCCCAGACACATATATAAACATAAACCC	$(T)_8$ G $(T)_{11}$
D. mojavensis	AAAATTAAAA	ATGACTTGA	ATAACTTTTAGAATACATAACCATC	$(T)_{11}$ G $(T)_{11}$ $(N)_4$ $(T)_9$
D. virilis	ATAATTAAAA	ATGACTTGA	ATAACATTGAGACTACATATCCATC	$(T)_{11}$ G $(T)_{12}$ $(N)_3$ $(T)_9$
D. grimshawi	ATAATTTACA	ATGACTTGA	ATAACATTTAACATACTACAAAC	$(T)_{19}$ $(N)_3$ $(T)_9$

The conserved uORF (column uORF, amino acid sequence depicted below) and putative SXL-binding site (B site) found in the 5′UTR of *msl-2* mRNA sequences are depicted for 12 *Drosophilid* species. The nt separating both elements are also shown (spacer). A single nucleotide difference in the translation termination codon of the uORF in the *Drosophila obscura* group is italicized; the change of the ACT (Thr) codon to a CAT (His) codon in *D. willistoni* is underlined. Additionally, the upstream sequence context of the uORF is shown (AUG Context).

SXL-mediated repression (Figures 2C and 2D), approaching the degree of regulation of a construct without the uAUG altogether (Figure 1C). These data strongly suggest that regulation requires uAUG recognition by scanning ribosomes.

Finally, we tested whether the regulation mechanism also involves translation termination at the uORF. We created a series of reporter RNAs where the translation termination codon of the uORF is removed (UGA to CGA, Arg) and the uAUG placed in frame with the downstream major ORF (uORF fusion, Figure 3A). Thus, these reporters allow to directly monitor upstream translation initiation, prevent uORF termination and, hence, reinitiation, and downstream translation initiation becomes solely dependent on leaky scanning. Two translation products are encoded by these RNAs, one from the main short open reading frame (sORF) (denoted S) and an N-terminally elongated version derived from initiation at the upstream AUG (Figures 3A and 3B, denoted L; see Figure S4 for a stability assay of the translation products). In the absence of SXL, the uAUG is used inefficiently, allowing a fraction of ribosomes to initiate further downstream by leaky scanning. We also tested these constructs in the context of the improved translation initiation consensus (+consensus) and the complementary (−consensus) sequence at the uAUG, now allowing to directly monitor their effect on uAUG usage. As predicted, the near-optimal consensus sequence shifts the L/S protein ratio in favor of upstream initiation, whereas the nonconsensus sequence drastically increases leaky scanning (Figure 3B, lanes 4, 7, and 10).

SXL strongly represses translation of the downstream cistron (i.e., the "S-protein"), and these constructs confirm the earlier conclusion that a "strong" uAUG context favors regulation, whereas this is severely blunted for a reporter with poor upstream initiation (Figure 3B). These data also clearly show that SXL-mediated regulation of downstream translation does not require translation termination at the uORF.

Role of SXL in uORF-Mediated Regulation

When directly monitoring translation initiation at the uAUG on the in-frame reporter RNAs, we noticed that SXL promotes translation initiation at the uAUG (Figure 3B, compare lanes "−" versus "20×" SXL). This effect requires a functional SXL-binding site in the 5′UTR of the reporter RNA (Figures 3B and 3C) and could be independently confirmed by ribosomal toeprinting (Figure S5).

RNA secondary structure elements per se can promote ribosomal recognition of a translation initiation codon when placed at an appropriate distance downstream (Kozak, 1990). The spacing requirements for such hairpins relative to the initiation codon to promote uAUG usage are quite narrowly defined, possibly because they act by transiently arresting the scanning 43S preinitiation complex when the AUG is in the decoding center (Kozak, 1990). By contrast, the effective spacing requirements for SXL-mediated enhancement of ribosomal uAUG recognition are far less constrained (Figure S6). RNAs with spacers ≥19 nt between the uAUG and the 5′ end of the SXL-binding site show strong (>6-fold) repression and SXL-mediated increase in uAUG recognition. When the linker length is reduced to 16 nt (uORF fusion-12-B), this effect is lost and translational regulation compromised (Figure S6), possibly because the leading edge of the ribosome encounters the regulatory protein before the uAUG reaches the decoding center.

To test whether SXL-mediated translational repression can be mimicked by an unrelated high-affinity RBP, we replaced the B site by a binding motif for the polypyrimidine tract-binding protein (PTB). PTB specifically recognizes and binds to poly(CU) stretches (Oberstrass et al., 2005). Thus, mutation of the B site (U_{16}) to $(CU)_8$ abolishes SXL binding and simultaneously creates a canonical PTB site (denoted Bm in the reporter RNAs). For experiments with PTB we used constructs where PTB only binds to the 5′UTR (Bm) site. Recombinant *Drosophila* PTB robustly and specifically represses translation of the appropriate

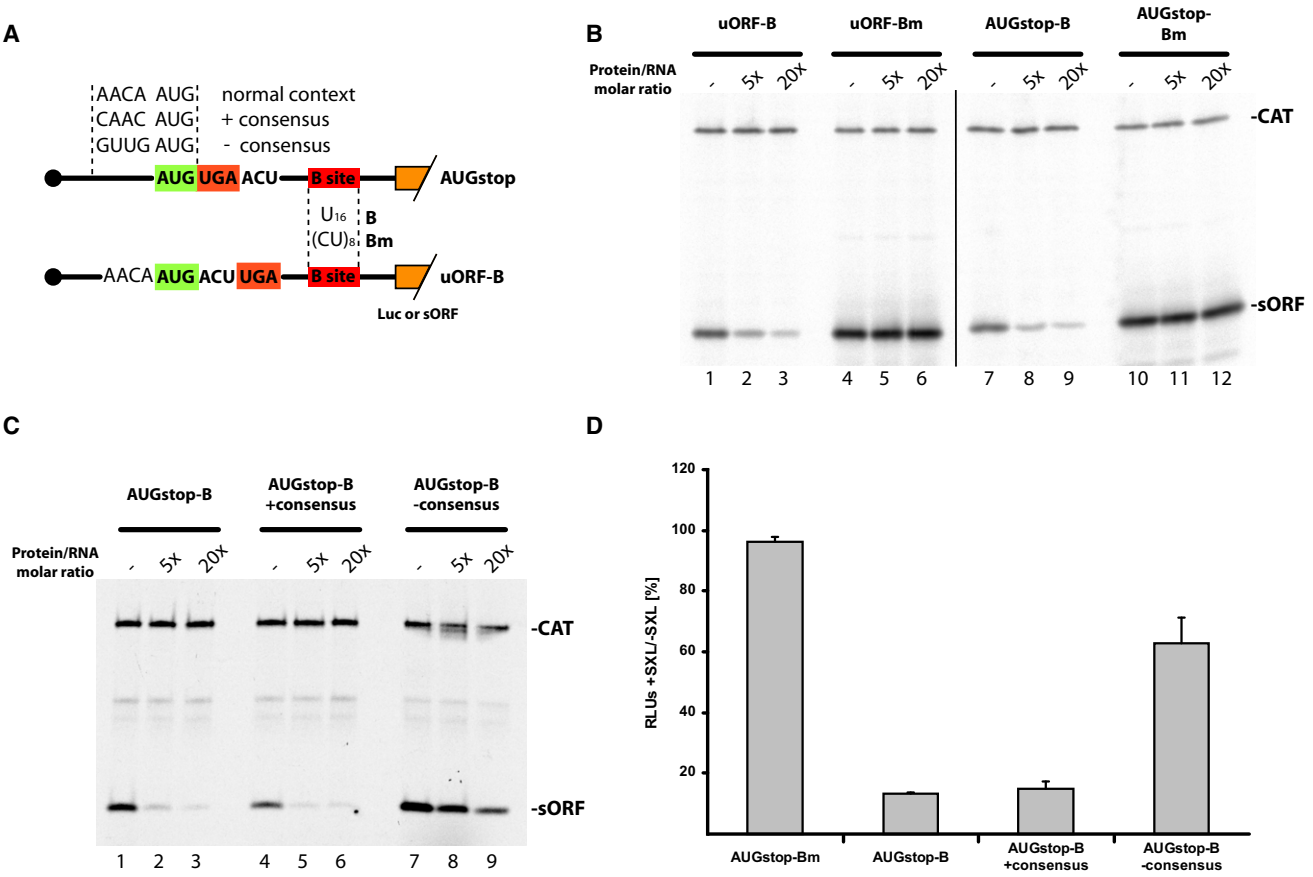

Figure 2. SXL-Mediated Translational Control Involves Initiation at the uAUG, Not uORF Elongation

(A) Schematic representation of AUGstop reporter RNAs with swapped translation termination (UGA, red box) and the Threonine codons (ACU, white); the wild-type sequence is shown below (uORF-B). The altered sequence contexts of the upstream translation initiation codon (AUG, green box) are shown above: CAAC representing the favored consensus (+consensus), GUUG the complementary sequence (−consensus). The SXL-binding site (B site) is depicted in red; the sequences for the wild-type and mutant version (Bm) are indicated.

(B) Reporter RNAs containing the uAUG either in the absence or presence of a functional SXL-binding site (uORF-B and uORF-Bm, left panel) and related reporters with the second codon of the uORF swapped for the subsequent stop codon (AUGstop-B and -Bm) were translated in vitro in the presence of ^{35}S methionine. To assay for translational repression, either buffer (lanes denoted "−") or increasing amounts of SXL (5× and 20× molar excess of protein over RNA) were added to the reactions. CAT mRNA was cotranslated as an internal control. After immunoprecipitation of the translation products by an excess of α-CAT and α-FLAG antibodies, the peptides were resolved by SDS-PAGE using a 15% Tricine gel and detected by autoradiography.

(C) Translation assay as in (B) using reporter RNAs with altered uAUG context (see A).

(D) Quantification of translational repression by a 40× molar excess of SXL on AUGstop mRNA reporters as in (B) and (C) except that a full-length firefly luciferase open reading frame was used for readout. The experiment was repeated three times with three replicates each.

Error bars represent SD. See also Figure S3.

construct with the PTB (= mutated SXL B site)-binding sequence (Figures 4A and 4B), but repression by PTB is not uORF mediated. These results further suggest that the uORF/SXL regulatory module does not act by a simple steric arrest of scanning ribosomes at the uAUG.

Scope of uORF-Mediated Regulation by SXL

To explore whether additional natural *Drosophila* RNAs may be regulated in a manner similar to *msl-2* mRNA, we screened the almost 20,000 annotated 5′UTR sequences of the *Drosophila melanogaster* transcriptome biocomputationally for regulatory motifs similar to the one defined for *msl-2* mRNA.

We find that 58.7% of the *Drosophila* 5′UTRs contain one or more upstream translation initiation codons, whereas 4.3% exhibit sequences that resemble putative SXL-binding sites, stretches rich in U residues (U_{12} or 12 out of 14 nt U). Of the annotated sequences, 1.3% contains a SXL-binding motif associated with an uAUG spaced at an appropriate distance (a total of 268 mRNAs), possible targets for SXL-controlled translational repression. We next cloned the complete 5′UTRs of a dozen of these candidate RNAs and tested them for SXL-mediated repression of a luciferase reporter RNA. Six of these (50%) mediate translational repression in response to SXL to various extents (Figure 5A and Figure S7). One of these, *lrr47* (Figure 5B), was tested further by mutational analysis. SXL represses translation via the *lrr47* 5′UTR ~4-fold (Figure 5C, uAUG, gray bar). This repression depends on the predicted SXL-binding site in *lrr47* mRNA—a sequence of 17 nt rich in uracil—because

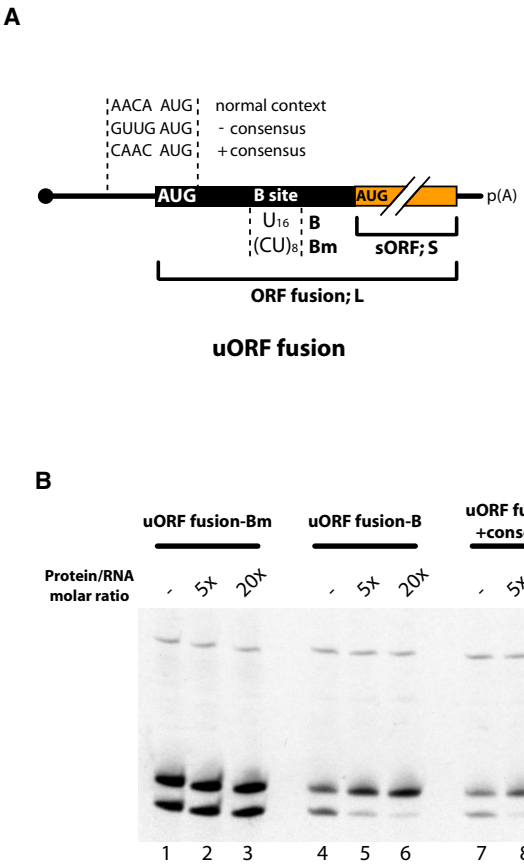

Figure 3. SXL-Mediated Repression without Termination following the uAUG

(A) The uORF fusion reporter RNAs are depicted schematically where the upstream termination codon was mutated rendering the uAUG (white on black background) in frame with the AUG initiating the major ORF (black on orange background). The fused open reading frame (uORF fusion) is depicted at the bottom versus the short ORF (sORF). Sequences for the SXL-binding site (B site) or the mutant version are indicated below.

(B) In vitro translation assay of the uORF fusion RNAs depicted in (A). After translation in the absence (lanes denoted "−") or presence of SXL (5× or 20× molar excess relative to RNA), the ^{35}S methionine-labeled translation products were immunoprecipitated and resolved by PAGE (as described in Figure 2B). CAT mRNA was cotranslated and served as an internal control. The slightly larger translation product of the fused open reading frame is marked L (see A), below the shorter version derived from downstream translation (S).

(C) Translation assay (as in B) using an RNA with a mutated SXL-binding site.

See also Figures S4, Figure S5, and Figure S6.

mutation thereof abolishes SXL-mediated translational control (black bar). Moreover, mutation of the upstream translation initiation codon to AUC severely blunts the translational response (Figure 5C, uAUG mut) as also seen with *msl-2* reporters (Figures 1B–1D). Thus, the *lrr47* and potentially other natural 5′UTRs in addition to *msl-2* mRNA are responsive to SXL-mediated translational repression via the defined uORF regulatory motif.

To further explore the scope of uORF-mediated translational control by SXL, we inserted the bare regulatory motif (*msl-2* uORF—21 unrelated nt—U_n SXL-binding site) into a completely heterologous context and assayed for regulation. For this purpose we used the 5′UTR sequence of human β-globin mRNA fused to a luciferase open reading frame (Figure 6A). A SXL-binding site in the absence of a uORF inhibits translation only negligibly in the presence of SXL (Figure 6B, no uORF); generalizing our findings with *msl-2* and *lrr47* mRNAs, the uORF at a comparable distance upstream of the SXL-binding site strongly augments regulation in response to SXL (Figure 6B, RNA uORF). Optimization of the uAUG context further improves the degree of translational repression (Figure 6B, uORF +consensus), reflecting the previously established hallmark of AUG context dependence. We also note that the degree of regulation of the β-globin reporter is less than that of the corresponding *msl-2* reporters. Thus, whereas these data help to define the uORF/SXL-binding site motif as a module sufficient to confer this regulatory mechanism, additional features appear to modulate its quantitative expression.

DISCUSSION

SXL-mediated translational repression of *Drosophila msl-2* mRNA is essential to prevent deleterious hypertranscription of the two X chromosomes in female flies (Bashaw and Baker, 1997; Kelley et al., 1995, 1997). Using *Drosophila* embryo translation extracts, we have studied the underlying mechanisms. We found that SXL employs two independent and complementary mechanisms to interfere with translation initiation (Beckmann et al., 2005): one via the 3′UTR and involving UNR and the poly(A)-binding protein (PABP) (Beckmann et al., 2005; Duncan et al., 2009); and the other via the 5′UTR by some form of regulated scanning (Beckmann et al., 2005). Here, we investigate how SXL regulates ribosomal scanning for the *msl-2* initiation codon.

Extending the Functional Role of uORFs

uORFs are common features found in many eukaryotic mRNAs. They are widely recognized as *cis*-regulatory elements that can affect mRNA translation from the main or physiological open reading frame, thus fine-tuning the levels of protein expression.

In humans and rodents approximately half of the transcripts contain uAUGs, and their presence generally correlates with reduced protein expression. Moreover, mutations that disrupt or create uORFs can result in or increase susceptibility to severe disorders such as hereditary thrombocythemia, cancer, bipolar affective disorders, or Alzheimer's disease (reviewed in Chatterjee and Pal, 2009). uORFs can regulate translation by multiple mechanisms (Hood et al., 2009; Morris and Geballe,

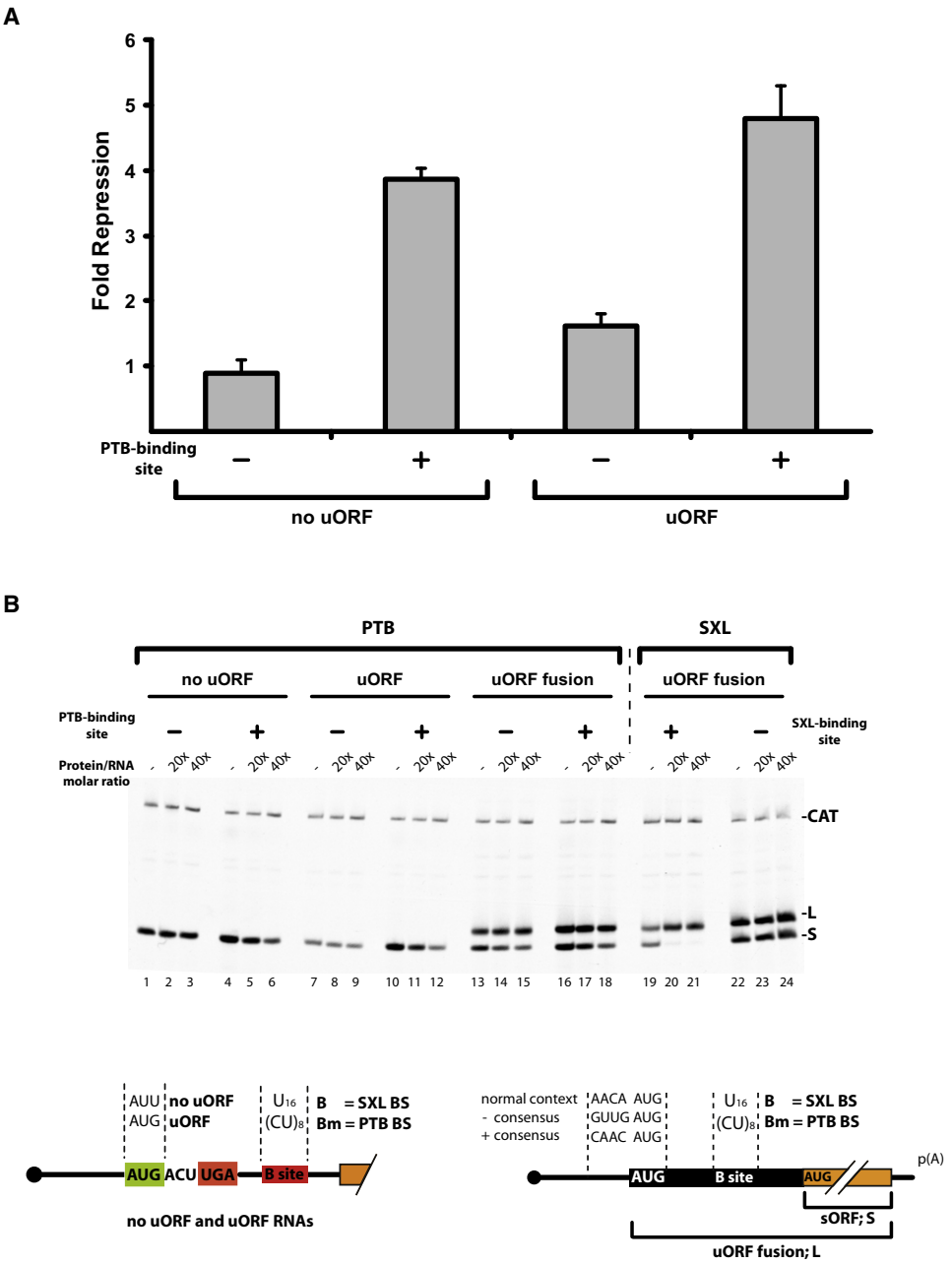

Figure 4. SXL Cannot Functionally Be Replaced by PTB

(A) Luciferase reporter RNAs that contain a single canonical PTB-binding site in the 5′UTR instead of the SXL-binding site (as indicated below) were translated in the absence or presence of recombinant PTB (40× molar excess of PTB over RNA) to assay for translational repression. RNAs lacking a high-affinity PTB-binding site (U_{16}, marked below as "−") served as a control. *Renilla* luciferase RNA was cotranslated and served as an internal normalization standard. Error bars represent SD.

(B) Translation assay as in Figure 2B using reporter RNAs with a canonical high-affinity PTB-binding site. The RNAs were translated in the presence of buffer or recombinant *Drosophila* PTB (20× or 40× molar excess relative to RNA as indicated; lanes 1–18). As a positive control, SXL was used on analogous reporter RNAs that contain a single SXL-binding site in the 5′UTR (lanes 19–24, compare also Figure 3B). The presence or absence of the respective PTB- or SXL-binding sites in the reporter RNAs is indicated above each set of experiments. Translation products derived from initiation at the uAUG are denoted L, the translation product from the smaller main open reading frame S (as described in Figure 3). CAT mRNA was cotranslated in every reaction and served as an internal control. As a reference the reporter RNAs used in this experiment are depicted below (see Figure 2A and Figure 3A for a brief description).

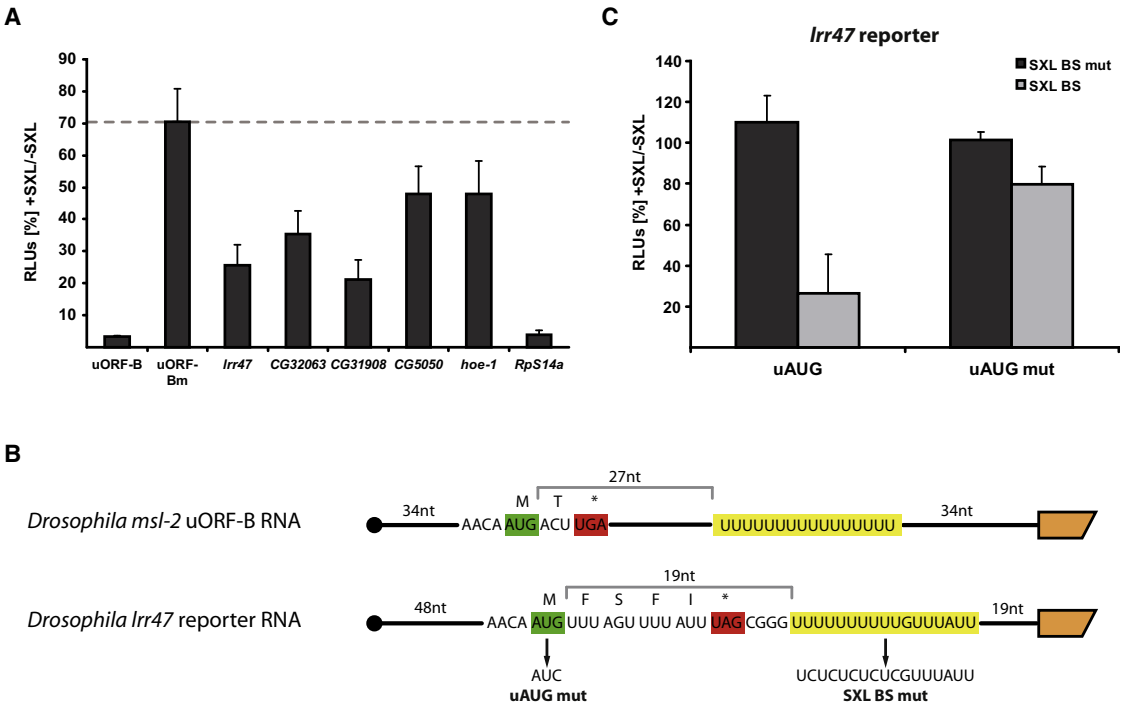

Figure 5. uORF/SXL Regulation of Additional *Drosophila* mRNAs

(A) Translation assay using the newly identified SXL target mRNA 5′UTR sequences (see also Figure S7) as indicated below each bar. Relative light units in the presence of a 40× molar excess of recombinant SXL are expressed in percent relative to control reactions that were supplemented with buffer only. uORF-B and uORF-Bm RNAs served as positive and negative control, respectively (compare Figure 1); *Renilla* mRNA was cotranslated in every reaction and used for normalization. Error bars represent SD.

(B) Comparison of the *msl-2* and *lrr47* reporter RNAs. The translation initiation (green) and termination codons (red) of the uORFs of *msl-2* and *lrr47* mRNAs, respectively, in close proximity to the SXL binding motifs (yellow) are highlighted; the encoded peptide sequence is depicted above each sequence. The distances of the regulatory elements relative to each other, to the cap structure, and to the physiological open reading frame are shown. For the control reporter RNAs, every second uracil residue of the putative SXL-binding site was mutated to C to abolish protein binding (SXL BS mut reporters). Additionally, mutations of the upstream translation initiation codon were introduced (AUG to AUC, denoted uAUG mut), yielding RNAs that carry a SXL-binding site only (or mutated version thereof) in the absence of a uORF (depicted at the bottom).

(C) RNAs bearing the *lrr47* 5′UTR or mutated versions thereof were assayed for translational repression as described in Figure 1. Reporters containing the SXL-binding motif are depicted in gray, analogous RNAs with a mutated SXL-binding motif in black (SXL BS mut). The reporters depicted on the right (uAUG mut) carry a mutation of the upstream translation initiation codon (AUG to AUC). Firefly luciferase activity of reactions with a 40× molar excess of recombinant SXL over RNA is plotted in percent relative to reactions that were supplemented with buffer only. *Renilla* luciferase RNA served as a normalization control. Error bars represent SD.

See also Figure S7.

2000 and references therein). The GCN4 mRNA in budding yeast represents a particularly well-studied example with four uORFs that regulate the expression of the main open reading frame by a sophisticated termination reinitiation mechanism without the involvement of RNA-binding *trans*-acting factors (reviewed in Hinnebusch, 2005). In contrast the *S. cerevisiae* CPA1 and the *N. crassa arg-2* uORF function via ribosomal stalling at the uORF termination codon, which is dependent on the amino acid sequence of the uORF-encoded peptide—the arginine attenuator peptide (AAP). Although the GCN4 uORFs do not influence mRNA stability, ribosomal stalling at the uORF termination codon triggers CPA1 and *arg-2* RNA degradation via the NMD pathway (reviewed in Hood et al., 2009).

In *Drosophila*, similar to mammals, uAUGs are common (Hayden and Bosco, 2008). Here, we show that a uORF is critical for the regulation of *msl-2* mRNA via its 5′UTR. In contrast to all other known examples, the uORF does not regulate translation on its own, but in conjunction with a *trans*-acting regulatory protein (SXL) that binds to a second *cis*-regulatory mRNA element, the B site. Furthermore, regulation is independent of the uORF-coding sequence, and indeed elongation altogether, and it does not require a translation termination codon (Figures 2 and 3). These characteristics clearly separate *msl-2* translational repression from previously reported uORF-regulatory mechanisms that operate via decay, reinitiation, or peptide-mediated ribosomal stalling during uORF translation. Moreover, the uORF is only part of a binary regulatory module that responds in *trans* to the RBP SXL (Figure 6C).

Functional Role of SXL in uORF-Mediated Regulation

For the uORF to function as an efficient negative regulator of downstream (*msl-2*) translation, it requires the cooperation of

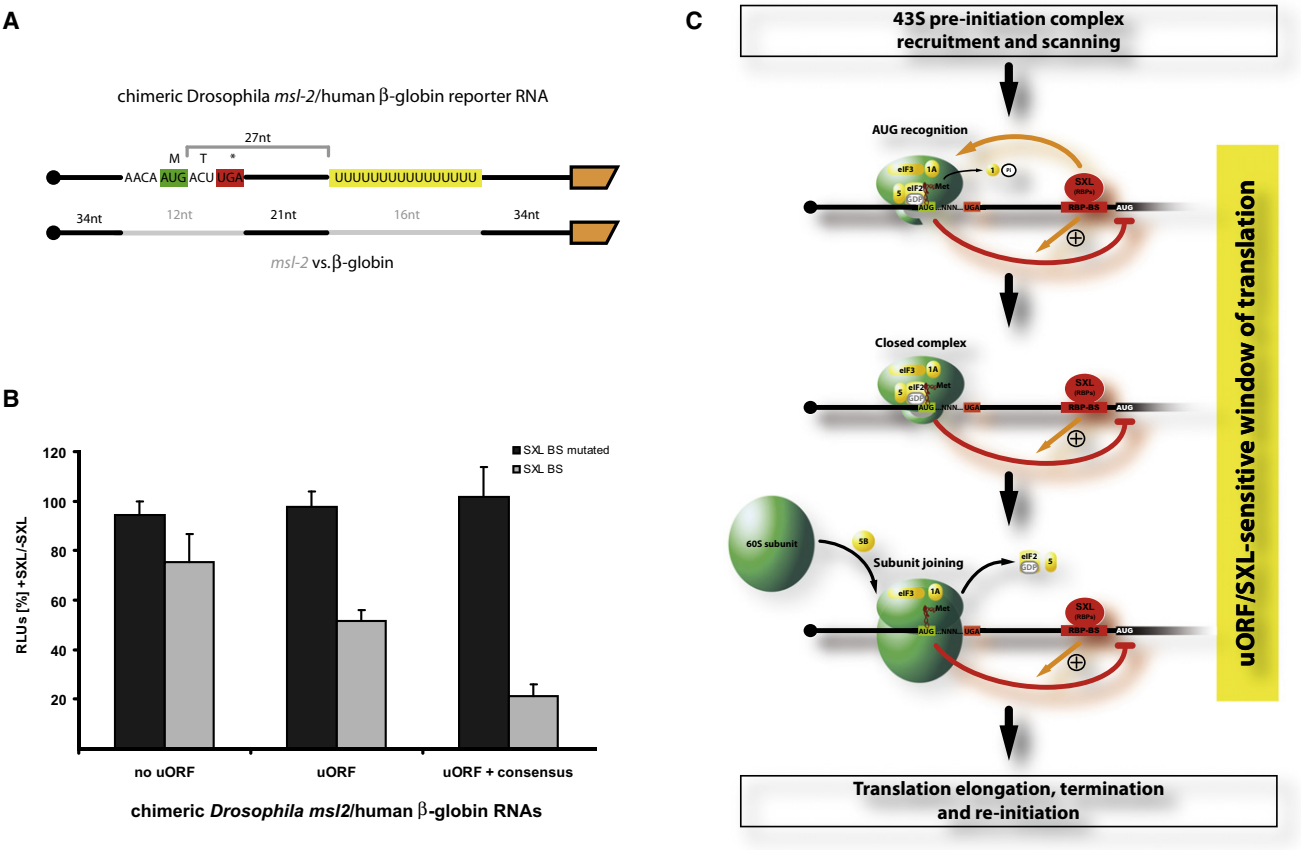

Figure 6. The uORF/SXL Regulatory Motif Functions in a Heterologous Context

(A) Schematic representation of the chimeric *msl-2*/β-globin reporter RNAs. The SXL-binding site is highlighted in yellow; important features of the uORF are shown in green (translation initiation codon) and red (termination codon). A graphical overview depicting the origin of the constituents of the chimeric 5′UTR is shown below: *msl-2*-derived sequences are in gray versus human β-globin sequences in black (not drawn to scale).

(B) Chimeric *msl-2*/β-globin reporter mRNAs were assayed for translational repression by SXL (40× molar excess of protein relative to RNA) as described before. The RNAs carry a SXL-binding site (light-gray bars) or a β-globin-derived control sequence (black bars) in the absence of the *msl-2* uORF (RNAs denoted no uORF) or in presence of the uORF with the uAUG positioned either in a "weak" (reporters denoted uORF) or a "strong" initiation context (denoted uORF +consensus). Cotranslated *Renilla* luciferase mRNA served as an internal control. Normalized firefly luciferase units in presence of SXL are plotted relative to control reactions supplemented with buffer only. The experiment was repeated three times with three biological replicates. Error bars represent SD.

(C) Translational control via protein-regulated uORFs. The cartoon depicts the experimentally defined window for uORF/SXL-mediated regulation between uAUG recognition and the onset of translation elongation. When scanning 43S preinitiation complexes (ribosomal subunits are depicted green, eukaryotic translation initiation factors in yellow) encounter the *msl-2* uORF, SXL (orange) promotes ribosomal recognition of the uAUG (upper orange arrow). This in turn diminishes translation from the major ORF (red line). In addition SXL acts by converting the uORF from a relatively weak to a potent inhibitor for translation of the physiological *msl-2*-coding sequence (lower orange arrow and "+" sign). We suggest that similar mechanisms may operate with other RBPs.

the RBP SXL. As shown in Figures 1–3, SXL cooperates with the uORF via initiation at the uAUG, not elongation or termination, based on the following results: (1) a "uORF" through which no elongation is possible (AUGstop reporters) is fully functional; and (2) when the uAUG is placed in frame with the major ORF, such that an upstream translation termination codon is absent (uORF fusion reporters), repression of initiation at the downstream AUG of the major ORF is unaffected. Note that SXL cannot inhibit expression of the N-terminally extended translation product that initiates at the uAUG. This directly shows that SXL fails to regulate ribosomes that complete the initiation phase and proceed to elongation. The critical importance of the initiation events at the uAUG for SXL-mediated regulation is further reflected by the consequences of changing the sequence context surrounding the uAUG. Already in the 1980s, Kozak (1987) discovered that the recognition of a translation initiation codon is governed by its context, and can be enhanced by downstream secondary structures that may slow down scanning (Kozak, 1990). Our data show that SXL can promote recognition of the uAUG (Figure 3). Replacing SXL with PTB does not recapitulate this effect, indicating that the SXL-mediated increase of uAUG recognition is unlikely to be solely explained by a steric block to scanning ribosomes (Figure 4B). Furthermore, such a block would be predicted to be highly sensitive to changes in the spacing between the two regulatory elements (Kozak, 1990), whereas SXL functions over a wider range of distances

(Figure S3 and Figure S6). Only on RNAs with critically short linkers ≤15 nt is SXL-mediated regulation severely impaired (Figure S3 and Figure S6), possibly because the leading edge of the ribosome encounters the regulatory protein before the uAUG reaches the decoding center.

Nonetheless, SXL appears to have some ability to stall scanning because earlier toeprinting analyses of SXL-repressed mRNPs indicated the presence of stalled ribosomal initiation complexes in the 5′UTR of *msl-2* mRNA positioned upstream of the SXL binding site, even in the absence of an uAUG (Beckmann et al., 2005).

Our experiments define a relatively narrow window of the translation initiation pathway during which SXL acts (Figure 6C). Scanning preinitiation complexes need to recognize a translation initiation codon to become susceptible to SXL-mediated repression, but elongating ribosomes are evidently already resistant to SXL. Recognition of a translation initiation codon is thought to trigger a conformational change of the ribosomal preinitiation complex from an "open" and scanning-competent to a "closed" and scanning-arrested conformation (Passmore et al., 2007). The translation initiation factors eIF1, eIF1A, eIF3, and eIF5, among others, play important roles in this dynamic process. Upon recognition of an initiation codon, dissociation of eIF1 allows phosphate release from eIF2, which is promoted by the GTPase-activating protein eIF5, leading to a conformational change of the ribosomal 43S complex and commitment to subunit joining (Nanda et al., 2009; references therein). We hypothesize that this event and one of the factors involved in it is targeted by SXL.

To investigate if SXL directly interacts with the translation machinery and interferes with its function, we conducted a yeast two-hybrid assay with SXL. Notably, SXL specifically interacts with two subunits of the *Drosophila* eIF3 complex: eIF3i and h (data not shown). The yeast counterpart of eIF3i has been demonstrated to play an important role in ribosomal scanning (Cuchalova et al., 2010), whereas *Arabidopsis thaliana* eIF3h (although being nonessential for translation) mitigates the inhibitory effects of certain uORFs by promoting ribosomal reinitiation (Kim et al., 2004; Roy et al., 2010), qualifying eIF3i and/or eIF3h as highly attractive candidate targets for SXL. However, despite extensive efforts, we have not obtained experimental evidence to support the functional importance of these interactions.

A More Widespread Mechanism of Translational Control?

We dissected the mechanism of how SXL regulates *msl-2* mRNA via its 5′UTR-binding site. Our results pose the question of whether this mechanism may apply more widely to: other *Drosophila* mRNAs that could be regulated by SXL, other *Drosophila* RBPs, and other species including mammals. Although a full exploration of this question is beyond the scope of this work, our experiments led to the identification of several additional *Drosophila* 5′UTRs that mediate SXL control (Figure 5A). For one of these (*lrr47*), we verified the preeminent features of *msl-2* control, the requirement of a uAUG followed by a SXL-binding site (Figure 5C). In fact the regulatory motif defined from *msl-2* mRNA can function in the completely heterologous context of the human β-globin 5′UTR (Figure 6), attesting further to its broader utility in principle. We also tested whether *Drosophila* PTB can substitute for SXL (Figure 4). Although the data demonstrate specificity of the SXL effect, they fail to support the notion that other RBPs can act similarly. Knowledge of the responsible SXL regulatory domain(s) and its target will inform biocomputational searches and functional experiments that may further broaden the scope of the translational regulatory mechanism described here.

EXPERIMENTAL PROCEDURES

Plasmids

The plasmids min Δ3′, BL(EF)mut, and BS(EF)mut have been described (Gebauer et al., 2003). To generate AUG123m, AUG12m, and AUG3m, the individual translation initiation codons were mutated to ATT by PCR site-directed mutagenesis, and the resulting products were inserted between the SacI and NcoI sites in min Δ3′, replacing the original sequence. In a similar fashion uORF-B and uORF-Bm plasmids were created on the basis of the BL(EF)mut and BS(EF)mut vectors, respectively, reverting the ATT codon back to the original ATG sequence. Subsequently, changes in the AUG context were introduced yielding AUGstop-B +consensus and −consensus plasmids. The uORFfusion plasmids were created on the basis of uORF-B mutating the TGA stop codon to CGA and by simultaneous insertion of an additional A upstream of the sORF changing GACCATG to GAACCATG, thus placing the two initiation codons in frame. Subsequently, the context of the uAUG was changed by PCR site-directed mutagenesis. To change the distance separating the uORF from the SXL-binding site, different multiples of the CAA trinucleotide were introduced, or the physiological spacer was shortened by deletion using PCR-directed mutagenesis.

The human β-globin 5′UTR was generated by annealing of two oligonucleotides followed by a fill-in reaction using *Taq* polymerase under standard conditions. The resulting double-stranded product was digested with SacI and NcoI and ligated into the BL(EF)m plasmid replacing the *msl-2*-derived 5′UTR sequence. The SXL-binding site and the uORF were introduced by replacing the respective β-globin-derived positions in the oligonucleotides. The *lrr47* reporter plasmids were generated in a analogous fashion.

For reporters carrying the 5′UTRs of *CG31908-RB*, *CG5050*, *hoe-1-RC*, and *RpS14a-RA*, the respective sequences were PCR amplified either from *Drosophila* genomic DNA or cDNA generated from total embryo RNA using an oligo-dT Primer and Superscript II RT (Invitrogen) under standard conditions (see also Figure S7). For *CG32063-RB* a 5′UTR fragment lacking exon 1 was generated (nt 225–551) in an analogous fashion. PCR products were subsequently inserted between the SacI and NcoI restriction sites of the BL(EF)mut vector.

Recombinant Proteins

SXL was expressed in *E. coli* as described (Grskovic et al., 2003); the N-terminal GST tag was cleaved off with TEV protease. Further purification was performed by ion-exchange chromatography using a MonoS column and salt elution. The peak fractions containing SXL were pooled and dialyzed against the original buffer.

The *Drosophila* PTB open reading frame was PCR amplified from pETG-40A-dPTB (a generous gift from Anne Ephrussi) (Besse et al., 2009) and cloned between the BamHI and XhoI restriction sites of the pGEX6P vector (GE Healthcare). Recombinant protein was expressed by IPTG induction in BL21 Star *E. coli* (Invitrogen) that was transformed with the Rosetta 2 plasmid (Merck). GST-PTB was purified as described above for GST-SXL. After cleavage of the GST tag by 3C protease, pure PTB was obtained by ion-exchange chromatography using a MonoS column as described above.

In Vitro Transcription

Plasmid templates for the *msl-2*, *lrr47*, or other reporter mRNAs were linearized with HindIII and transcribed in vitro in the presence of a 3′-O-Me-m^7(5′)Gppp(5′)G ("anti-reverse") cap analog (NEB) using T3 RNA polymerase (Stratagene) as described before (Gray et al., 1993). After purification using RNeasy columns (QIAGEN), aliquots were analyzed by gel electrophoresis to confirm integrity. All RNAs contained a poly(A) tail of 73 nt.

Renilla luciferase and CAT control mRNAs were synthesized in a similar fashion, using BamHI and T7 RNA polymerase or HindIII and SP6 RNA polymerase, respectively.

Translation Assays

Drosophila embryo translation extract was prepared as described before, without micrococcal nuclease treatment for translation of reporter RNAs to occur in a competitive mode as occurs in vivo (Gebauer et al., 1999). Briefly, overnight embryos were collected and dechorionated using 3% sodium hypochlorite solution. After extensive washing, embryos were disrupted by 20 strokes of a Potter-Elvehjem homogenizer at ~1500 rpm in a buffer containing 10 mM HEPES/KOH (pH 7.4), 5 mM DTT, and 1× Complete-protease inhibitors (Roche). After centrifugation for 20 min at 40,000 × g at 4°C, the clear aqueous phase was collected and supplemented with 10% Glycerol before storage at −80°C.

For luciferase assays, 65 fmol of firefly and 23 fmol of *Renilla* luciferase reporter mRNA were translated in a final volume of 10 μl containing 24 mM HEPES/KOH (pH 7.4), 100 mM KOAc, 0.6 mM $Mg(OAc)_2$, 60 μM amino acids, 20 mM creatine phosphate, 800 ng creatine kinase, and 40% *Drosophila* embryo extract for 90 min at 25°C. SXL was added at the indicated molar excess (0×, 2.5×, 5×, 10×, 20×, 50×, or 100×) with respect to template RNA. Luciferase activities were assayed with the Dual Luciferase Assay System (Promega) in a microplate luminometer (Berthold).

When using reporter mRNAs containing the artificial sORF, experiments were performed as described before (Beckmann et al., 2005). Translation products were resolved using 15% Tricine-SDS-PAGE (Schagger and von Jagow, 1987).

RNA Stability Assay

^{32}P trace-labeled sORF mRNAs were synthesized and used to prepare translation reactions essentially as described above. Subsequently, the reactions were treated with 5 μg of Proteinase K for 15 min at 50°C in a buffer containing 100 mM Tris/HCl (pH 8), 12.5 mM EDTA, 150 mM NaCl, and 1% SDS. After phenol/chloroform extraction and ethanol precipitation, RNAs were resolved on a 10% denaturing urea polyacrylamide gel and quantified by 2D densitometry using a phosphorimager.

Bioinformatic Search for Putative SXL Target mRNAs

Drosophila melanogaster sequence data were downloaded from the FlyBase FTP server (FB2010_03 release, at http://flybase.org in March 2010) (Tweedie et al., 2009). The annotated 5′UTRs were analyzed for the occurrence of a translation initiation codon upstream of a uridine-rich sequence (12 or more uridines within 14 nt) allowing a maximum distance of 70 nt between the two elements. For this purpose a pattern search was performed using the fuzzy pattern search tool 3of5 (Seiler et al., 2006).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at doi:10.1016/j.cell.2011.05.005.

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