DROSOPHILA UNR:

A Factor Involved in the Translational Regulation of Dosage Compensation

Irina Abaza

PhD Thesis

Barcelona, November 2006

Front cover: *Visualization of UNR localization (by immunostaining) in Drosophila embryos at blastoderm stage (by S. Patalano, Abaza et al., 2006).*

International PhD Program in Health and Life Sciences, BasicBiomedical Research
Department of Health and Experimental Sciences
UNIVERSITAT POMPEU FABRA

DROSOPHILA UNR:

A Factor Involved in the Translational Regulation of Dosage Compensation

Irina Abaza

Memòria presentada per optar al grau de Doctor
per la Universitat Pompeu Fabra.
Aquesta tesi Doctoral ha estat realitzada sota la direcció de la
Dra. Fátima Gebauer al Centre de Regulació Genòmica (CRG)

Dr. Fátima Gebauer **Irina Abaza**

Barcelona, November 2006

The research in this thesis has been carried out in the group of Dr. Fátima Gebauer at the CRG, Gene and Regulation Program.

The research carried out in this thesis has been supported by a FI-IQUC predoctoral fellowship from the Generalitat de Catalunya and by grants BMC203-04108 from the Spanish Ministry of Science and Technology and 2005SGR00669 from the Departament of Universities, Information and Sciences of the Generalitat de Catalunya (DURSI).

Generalitat de Catalunya

Mojim roditeljima i braći
(To my parents and brothers)

CONTENTS

FOREWORD

“Alice looked round her in great surprise. `Why, I do believe we've been under this tree the whole time! Everything's just as it was!'

`Of course it is,' said the Queen, `what would you have it?'

`Well, in our country,' said Alice, still panting a little, `you'd generally get to somewhere else -- if you ran very fast for a long time, as we've been doing.'

`A slow sort of country!' said the Queen. `Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!' “

‘Through the Looking Glass, and What Alice Found There’

by Lewis Carroll (1871)

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

Most thesis authors put in a page of thanks to those who have helped them in matters scientific, and also indirectly by providing such essentials as food, education, genes, money, help, advice, friendship etc. It is difficult to adequately convey the depth of my gratitude to the many people who participated in this project by virtue of their generous support and encouragement. At the top of the list is Josep. Thank you for your enduring patience and for surrounding me with love and encouragement. A special portion of my gratitude goes to Dr. Fátima Gebauer for taking me under her wings and keeping me ever focused on the principles of good theory and research. I whish also to thank the members of my thesis committee for their wisdom and council throughout my research. There have been countless others who have contributed in part to the completion of this work and collectively to the development of my mind and spirit. Among them is my lab P66: Solenn, Rafa, Aida and with special thanks to Olga. I cannot claim credit for many of the ideas contained within these lines. Many of them have come from the other guides that I've looked at -- I've listed them below. I have tried to give credit where credit is due, but I may have left some people out. My sincere apologies to those individuals and my thanks to the authors of all those guides. Finally, I give thanks and honor to my mother and father for sustaining me through the various trials, setbacks, and rewards that I have encountered along the way without which this project of ours would not be brought to completion.

Irina Abaza, Barcelona 2006

ABSTRACT

Dosage compensation is a mechanism that equalizes the expression of X-linked genes in those organisms in which males and females differ in the number of X chromosomes. In *Drosophila melanogaster*, dosage compensation is achieved by up-regulating the transcription of the single male X chromosome. This effect is mediated by a chromatin remodeling complex known as the Male Specific Lethal (MSL) complex or Dosage Compensation Complex (DCC). In female flies, dosage compensation is inhibited primarily because of the translational repression of the mRNA encoding one of the DCC subunits, MSL-2, by the female-specific RNA binding protein Sex-lethal (SXL). To inhibit translation, SXL binds to poly(U) stretches present in both the 5' and 3' UTRs of *msl-2* mRNA. Sequences adjacent to those SXL-binding sites in the 3´UTR are also required for translation inhibition and are bound by co-repression.

In this thesis work, we have designed an affinity chromatography assay to isolate the putative co-repressor(s), and have identified the protein Upstream of N-ras (UNR). *Drosophila* UNR (dUNR) is an ubiquitous, conserved protein that contains 5 cold shock domains (CSD) and a glutamine- (Q) rich amino- terminal extension. We show that dUNR is a necessary co-factor for SXL-mediated *msl-2* repression. SXL recruits dUNR to the 3' UTR of *msl-2* mRNA, imparting a sex-specific function to this ubiquitous protein. Domain mapping experiments indicate

that dUNR interacts with SXL and *msl-2* mRNA through CSD1, and that the domains for translation inhibition and SXL interaction can be distinguished. Our data indicate that the Q-rich domain, together with CSDs 1 and 2, plays an important role in translational repression, and suggest that factors in addition to dUNR and SXL are required for repression of *msl-2* mRNA. Using a combination of □UNR immunoprecipitation and microarray analysis, we have identified the mRNAs that are bound to dUNR in male and female flies. Our results suggest that dUNR is not only a novel regulator of dosage compensation, but also a general post-transcriptional regulator of gene expression.

1. INTRODUCTION

1.1 The Mechanism of Translation

mRNAs lead a precarious existence. During their synthesis in the nucleus, mRNAs have their heads altered (capping), their bodies reduced (splicing) and their tails successively clipped and extended (cleavage and polyadenylation). Sometimes the sequence of the mRNA is also altered (mRNA editing) and the result is mature mRNA different from the corresponding sequence in the genome. Although much of the regulation of gene expression has been described to occur at the time of transcription, the regulation of mRNA stability, localization and translation are crucial steps. In particular, translational control has been shown to be relevant in a wide variety of biological situations, such as embryonic development, metabolism, synaptic plasticity, cell differentiation, proliferation and cancer (reviewed in Kuersten and Goodwin, 2003; Wilhelm and Smibert, 2005).

This section gives an overview of the molecular mechanics of mRNA translation in eukaryotes. Emphasis is given to the initiation step because it is the target of most translation regulatory mechanisms described to date. A description of the elements in the mRNA that may influence the efficiency of translation is also provided.

1.1.1 The mRNA

An mRNA basically consists of an open reading frame (ORF) flanked by untranslated regions (5' and 3' UTRs) (Figure 1.1). The ORF is delimited by an AUG initiation codon and one of three termination codons (UAA, UAG or UGA). Although the 5' UTR may contain several AUGs (also called upstream AUGs) the ribosome preferentially recognizes that embedded in a good Kozak initiation context, composed of CANC**AUG**G. Although rare, alternative initiation codons such as CUG, GUG and UUG may be used (Blattner et al., 1997; Kunst et al., 1997 and Lobocka et al., 2004). Recent evidence also suggests a moderate context for the termination codon, consisting of a bias against citidine immediately downstream (Brown et al., 1990, 1993; reviewed in Welch et al., 2000). Typically, the 5' end of the mRNA contains a 7-methylguanosine (m^7G) cap (Furuichi and Shatkin, 2000). The presence and availability of the cap structure is important, although not absolutely essential for translation (Gunnery and Mathews, 1995; Palmer et al., 1993). The cap provides the initial contact of the ribosome with the mRNA (see below), and uncapped mRNAs are generally translated with lower efficiency than their capped counterparts *in vitro* (Both et al. 1975; Muthukrishnan et al., 1975; Gallie, 1991). The cap is also important for other processes, including pre-mRNA splicing, nucleo-cytoplasmic transport, and mRNA stability (Varani, 1997). Although most mRNAs initiate translation in a cap-dependent manner, some contain structures in their 5' UTRs called *internal ribosome entry sites* (IRES) that can bind ribosomal subunits directly. IRESs were first described in picornaviruses and were subsequently shown to function in several cellular mRNAs encoding oncoproteins, growth factors and products usually translated in situations where cap-dependent translation is compromised (e.g. cellular stress) (Pickering and Willis, 2005). The IRESs are distinguished by their particular requirements on initiation factors for translation (Jackson, 2005; reviewed in Pickeing and Willis, 2005).

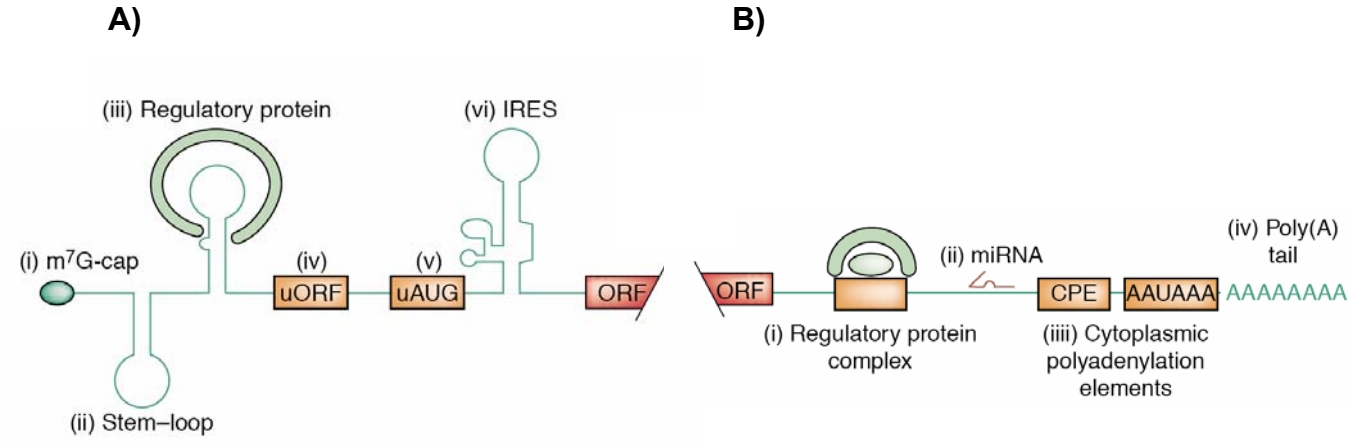


Figure 1.1. mRNA cis-acting sequences that can regulate translation. The open reading frame (ORF; red) denotes the main ORF. **A)** 5' UTR sequences. (i) The m^7GpppG cap is a crucial determinant for translational efficiency because it is recognized by the cap-binding complex eIF4F. (ii) Secondary structure, or stem-loops, negatively affect translation by impeding the binding or migration of 40S ribosomal subunits. (iii) Regulatory proteins interact with specific elements within the 5' UTR. (iv) Upstream ORFs (uORF) and (v) upstream AUGs (uAUG) normally down-regulate translation at the main ORF by providing alternative start sites. (vi) Internal ribosome entry sites (IRES) promote cap-independent initiation. **B)** 3' UTR sequences. (i) Elements can act as recognition sites for regulatory proteins (green). These elements can be structured or unstructured. Often, 3' UTR regulation requires a complex of regulatory proteins rather than a single protein. (ii) Short 21-nucleotide anti-sense microRNA (miRNAs) repress translation by targeting complementary sequences within the 3'UTR. (iii) Cytoplasmic polyadenylation elements (CPE) and the hexanucelotide AAUAAA are required to activate poly(A)-tail lengthening of an mRNA. In addition, CPEs have been suggested to play a role in translational repression. (iv) The poly(A) tail stimulates translation. Adapted from Wilkie et al., 2003.

The 3' UTR is the major repository site for regulatory elements for translation, localization and stability. All eukaryotic mRNAs -except those for histones- contain a 3' poly(A) tail, a structure that stimulates nucleo-cytoplasmic transport, translation and stability (reviewed in Wickens et al., 2000; Kuersten and Goodwin, 2003). The regulation of the poly(A) tail length is widely used to

modulate translation during early development (reviewed in Wilkie et al., 2003, de Moor et al., 2005).

Translation can be subdivided in four steps: initiation, elongation, termination and recycling. Some of these, especially initiation, occur differently in the various kingdoms of life. The translation mechanism in eukaryotes is summarized below.

Table 1.1. Translation initiation factors

Eukaryotic initiation factor	*Function*
eIF1	AUG recognition, processivity of scanning
eIF1A	Increases Met-tRNA$_i$ binding to 40S subunit, processivity of scanning, AUG recognition, 60S subunit joining
eIF2 (α, β and γ)	Binds Met-tRNA$_i$ to 40S subunit; GTPase activity
eIF2B (α, β, γ, δ and ε)	Guanidine- nucleotide exchange factor for eIF2
eIF3 (10 polypeptides)	Promotes Met-tRNA$_i$ and mRNA binding to 40S subunit, promotes dissociation of 80S ribosome into 40S and 60S
eIF4A (I and II)	DEAD-box helicase; binding of pre-initiation complex to the mRNA and scanning
eIF4B	Promotes eIF4A activity
eIF4E	m^{7}GpppG cap binding protein; binding of pre-initiation complex to the mRNA
eIF4F	Cap-binding complex consisting of eIFs 4A, 4E and 4G
eIF4G (I and II)	Scaffold protein, interacts with eIFs 4E, 4A, 3 and PABP; binding of pre-initiation complex to the mRNA
eIF4H	Promotes eIF4A activity
eIF5	AUG recognition, promotes eIF2 GTPase activity; assembly of pre-initiation complex
eIF5B	60S subunit joining; GTPase activity stimulated by the 80S ribosome
eIF6	Promotes dissociation of 80S ribosomes into 40S and 60S

1.1.2 Initiation

Translation initiation consists of the positioning of the ribosome at the AUG initiator codon. Initiation can be divided into four sub-steps: i) formation of a 43S pre-initiation complex, ii) recruitment of the 43S complex to the 5' end of the mRNA iii) scanning of the 5' untranslated region (UTR) and start codon recognition, and iv) assembly of the 80S ribosome (for a more detailed description see Preiss and Hentze, 2003; Sonenberg and Dever, 2003) (Figure 2). Initiation depends on the activity of about 25 polypeptides known as eukaryotic initiation factors (eIFs; see Table 1.1), and is the step of translation most frequently targeted for regulation.

Translation initiation starts with the selection of the initiator tRNA (called Met-$tRNA_i$) by eIF2 coupled to GTP, yielding the ternary complex. This complex joins the 40S small ribosomal subunit together with other eIFs (eIFs 3, 5, 1 and 1A) to generate the 43S pre-initiation complex. Binding of the 43S complex to the mRNA occurs by a mechanism that involves the initial recognition of the m^7G cap at the 5' end. This recognition, at least in higher eukaryotes, takes place by a bridging interaction between eIF3 (a component of the 43S complex) with eIF4G, the "scaffold" component of the cap-binding factor (eIF4F). Other components of eIF4F are eIF4E, which directly binds to the cap, and eIF4A, a DEAD box RNA helicase that unwinds secondary structure in the 5' UTR to allow for ribosome binding and scanning (Figure 1.2). eIF4G also binds to the poly(A) binding protein (PABP), an interaction that is believed to shape the mRNA in a pseudo-circular or "closed-loop" conformation (see section 1.2.2; Tarun and Sachs, 1996, reviewed in Hershey and Merrick, 2000).

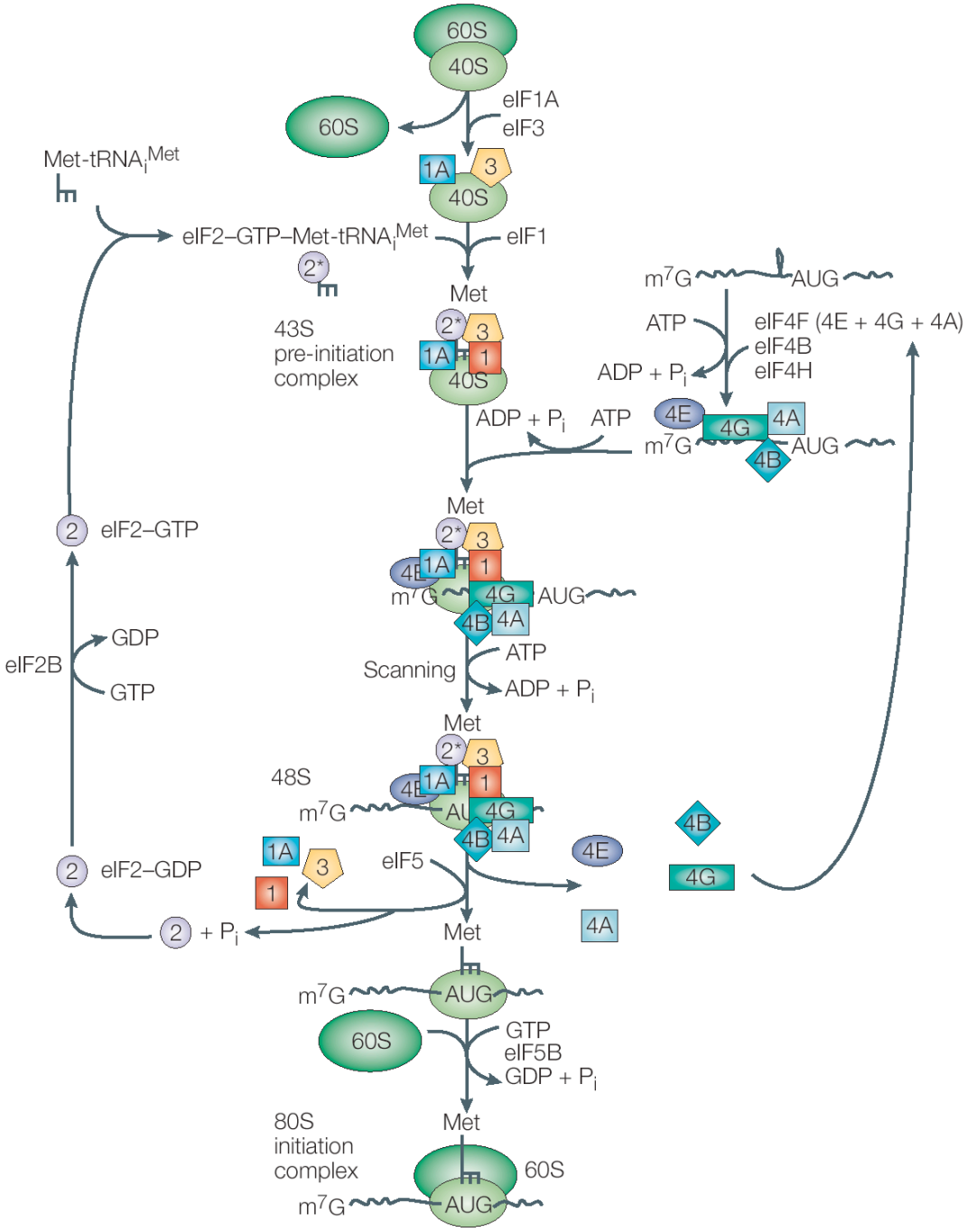


Figure 1.2. The initiation of translation. Current model for translation initiation in eukaryotes. The translation of requires the assembly of the 80S ribosome on the mRNA. This process is mediated by proteins that are known as eukaryotic initiation factors (eIFs) (see Table 1.1 and text for details). Taken from Holcik and Sonenberg, 2005.

Once the 43S complex binds to the 5' end of the mRNA, it is thought to scan the 5' UTR until it finds the first initiation codon in "good" context (Kozak, 2002). It is unclear whether the entire package assembled at the cap moves towards the AUG or whether the 43S complex moves alone. The factors eIF1 and eIF1A contribute to the processivity of scanning and play an important role in the correct recognition of the AUG (i.e. fidelity). The 43S complex recognizes the initiation codon through the formation of base pairs between that codon and the initiator tRNA. Once the 43S complex recognizes the AUG, it forms a stable complex known as the 48S initiation complex. Recognition of the AUG results in a conformational change in the small ribosomal subunit that induces eIF5 to stimulate the hydrolysis of the GTP bound to eIF2 (by eIF2 itself) (Maag et al., 2005, 2006; Algire et al., 2005), promoting the release of most initiation factors from the complex. The hydrolysis of a second GTP molecule on eIF5B is stimulated by the ribosome upon large (60S) ribosomal subunit binding, and is required to release eIF5B and leave an 80S ribosome (supposedly free of initiation factors) assembled at the AUG competent for polypeptide elongation (Pestova et al., 2000).

1.1.3 Elongation

The ribosome contains three sites: the A site, where the aminoacyl-tRNA (aa-tRNA) is placed, the P site, which holds the tRNA appended to the growing polypeptide chain, and the E or exit site, where the empty tRNA awaits to be discarded (Figure 1.3). Translation initiation leaves the Met-$tRNA_i$ placed at the P site (an unusual position for an aa-tRNA) and a free A site. Elongation consists of the recruitment of an aa-tRNA to the A site of the ribosome, the formation of a peptide bond between the incoming amino acid and the methionine carried by the initiator tRNA (or the growing polypeptide chain in the case of subsequent peptide bonds), and the translocation of the ribosome to the next codon in the

mRNA (Spahn and Nierhaus, 1998). This process is highly conserved across the three kingdoms of life (Ramakrishnan, 2002) and is assisted by three elongation factors (eEFs) that act as catalysts, mediating the speed and accuracy of elongation (reviewed in Merrick and Nyborg, 2000). eEF1A bound to GTP selects the aa-tRNA and takes it to the ribosomal A site. Once the aa-tRNA is correctly placed, hydrolysis of GTP results in the release of eEF1A-GDP, allowing for peptide bond formation by the peptidyl transferase center of the ribosome. eEF2-GTP then binds to the ribosome and stimulates translocation (again through GTP hydrolysis), leaving the empty tRNA at the E site, the peptidyl-tRNA at the P site, and a free A site available for another round of elongation. eEF1A-GDP cannot bind aa-tRNA and is recycled to the active form by eEF1B.

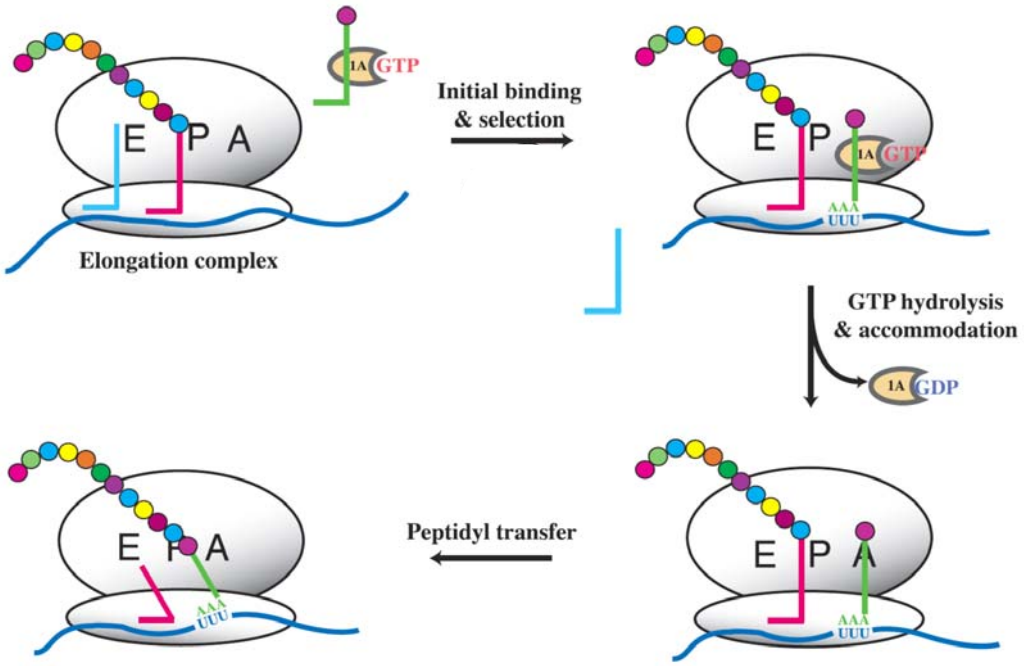


Figure 1.3. Translation elongation. Current model for translation elongation in eukaryotes. Elongation consists of the positioning of the peptidyl-tRNAs on the ribosomal A site, the formation of peptidyl bond by the large ribosomal subunit, and the translocation of the ribosome. See text for details. Adapted from Kapp and Lorsch, 2004.

1.1.4 Termination and Recycling

How does the synthesis of a polypeptide chain comes to an end? Termination and recycling are the least-investigated steps of translation. Aminoacyl-tRNA does not normally bind to the A site of a ribosome if the codon is UAA, UGA or UAG. Rather, termination occurs when the stop signal on the mRNA is read by a release factor (RF), which hydrolyzes the ester bond between the polypeptide and the tRNA in the P site. In eukaryotes there are two release factors, eRF1 and eRF3, which bind as a complex to the ribosomal A site. After polypeptide release, GTP hydrolysis on eRF3 leads to dissociation of the RFs from the ribosome, followed by tRNA and mRNA release and the dissociation of the ribosomal subunits (reviewed in Kapp and Lorch, 2004). eIF3 then binds the small ribosomal subunit, preventing its re-association with the large subunit in the absence of a properly assembled initiation complex. An interaction between PABP and eRF3 has been described that may influence ribosome recycling (Uchida et al., 2002).

1.2 Translational Control by RNA - Binding Proteins

In principle, translational regulation could lead to the activation or repression of translation by affecting any of the steps described above. However, most regulatory mechanisms known so far are of the inhibitory type and target translation initiation. Despite the plethora of initiation factors that could be targeted for regulation, the majority of mechanisms affect early steps of translation initiation, notably by modifying the activity or availability of eIF2 and eIF4E (Gingras et al., 1999; Dever et al., 2002). This suggests that only those mechanisms that are most frequently used have been grasped in the scientific literature and much remains to be discovered.

Two general modes of translational control can be distinguished: global control, where the translation of most mRNAs in the cell is regulated, and mRNA-specific control, where the translation of a group of mRNAs is regulated without changing the translational status of the transcriptome as a whole. Global control is generally achieved by the modification (phosphorylation/ dephosphorylation), physical blockage or cleavage of general translation factors (Dever, 2002). For example, phosphorylation of the alpha subunit of eIF2 is a mechanism used to reduce protein synthesis during stress (reviewed in de Haro et al., 1996, Hinnebusch, 1997; Hinnebusch and Natarajan, 2002). Other mechanisms include the binding of inhibitory proteins to eIF4E (4E- binding proteins or 4EBPs) on the site of interaction with eIF4G, or the cleavage of eIF4G and PABP during viral infection and apoptosis (Holcik and Sonenberg, 2005; reviewed in Sachs, 2000). Global mechanisms of translational control will not be further discussed here. For

more information see (reviewed in Gray and Wickens, 1998; Gebauer and Hentze, 2004; Hentze et al., 2007).

mRNA-specific translational control allows the expression of specific mRNAs at particular times and/ or locations within the cell, and is achieved by trans-acting factors (proteins or miRNAs) binding to sequence elements usually located in the UTRs, often the 3' UTR. Although many examples have been described, the molecular mechanisms of translational regulation are known for only a few. This section intends to review the examples for which molecular data is available.

1.2.1 Regulation by 5' UTR Binding Proteins

Iron regulatory protein

Our understanding of how a 5' UTR-bound protein can affect the translation of its target comes mostly from the analysis of a single protein-RNA complex: the iron regulatory protein (IRP) binding to a stem-loop in the 5' UTR of *ferritin* mRNAs termed the iron responsive element (IRE) (reviewed in Hentze and Kuhn, 1996; Hentze et al., 2004). In conditions of low iron, IRP binds to the IRE (Figure 1.4). Because the IRE is located close to the cap structure, IRP sterically blocks the landing to the 43S complex on the mRNA. Indeed, eIF4F but not eIF3 or 40S ribosomal subunits are detected on repressed ferritin mRNPs *in vitro* (Muckenthaler et al., 1998). The position of the IRE is critical for regulation, because when displaced to a cap-distal position translation inhibition is partially relieved (Goossen et al., 1990; Goossen and Hentze, 1992). Steric inhibition of translation also occurs when proteins with no apparent role in translation (e.g. U1A) are artificially placed close to the cap structure (Stripecke et al., 1994).

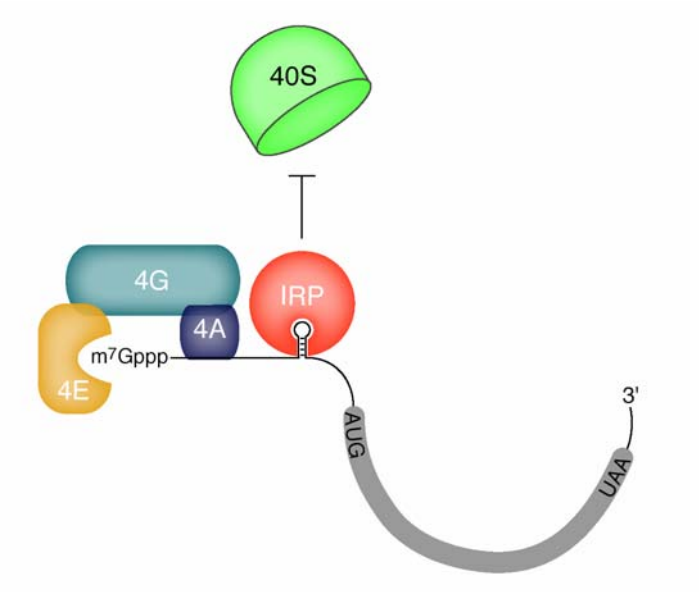


Figure 1.4. Regulation by IRP. IRP binds to a 5' stem-loop and sterically blocks the binding of the 43S pre-initiation complex to eIF4F. Taken from Hentze et al., 2007.

PABP

Although PABP binds to the 3' poly(A) tail of most mRNAs and stimulates translation, it also binds to the 5' UTR of its own message repressing its translation (Patel et al., 2005). A mechanism distinct from that for IRP must operate in this case, because the binding site for PABP is located in a cap-distal position, close to the AUG. PABP binds in a complex with at least two other proteins, Upstream of N-ras (UNR) and insulin-like growth factor 2 mRNA binding protein 1 (IMP-1) (Patel et al., 2005). The role of these factors in translational repression of PABP mRNA is unknown.

IRES trans-acting factors (ITAFs)

An interesting case of translational stimulation by 5' UTR binding proteins occurs during IRES-dependent translation. As mentioned above, this type of translation is independent of the cap structure and the cap-binding factor eIF4E, but requires specific translation factors (which may differ depending on the

particular IRES) and IRES-binding proteins called ITAFs (for IRES trans-acting factors) (reviewed in Spriggs et al, 2005a; Martínez-Salas and Fernández-Miragall, 2004). ITAFs generally act as RNA chaperones, modifying the structure of the IRES to make it accessible to initiation factors and, ultimately, the ribosome. An apparently general ITAF used in several IRESs is the pyrimidine-tract-binding protein-1 (PTB-1). PTB-1 binds to a (CCU)n motif present in all PTB-dependent IRESs which permits internal ribosome entry on its own provided that it is present within a stem-loop (Mitchell et al., 2005; Spriggs et al., 2005b). This motif is usually not accessible, but it is made available by the binding of other ITAFs to the IRES. For example, UNR and poly(C) binding protein 1 (PCBP-1) bind to the IRES of *apaf-1* and *bag-1* mRNAs, respectively, and change the structure of the IRES so that PTB can bind (Mitchell et al., 2003; Pickering et al., 2004). It has been proposed that PTB-1 itself is involved in ribosome recruitment and it is part of a pre-initiation complex that assembles on IRES-containing mRNAs (Pickering et al., 2004).

1.2.2 Regulation by 3' UTR Binding Proteins

In most cases, proteins or complexes binding to the 3' UTR regulate translation. Understanding how complexes placed at such a far distance from the 5' end of the mRNA influence translation is still a challenge. In this respect, the closed-loop model for translation is appealing because it provides a physical framework for the action of 3' UTR binding factors: perhaps, the 5' and 3' UTRs of a message are not as distant as previously thought. Although, again, most known examples are of the inhibitory type, a positive role for 3' UTR elements has been observed in certain plant viruses, where they have been suggested to be functional equivalents of the cap or poly(A) tail, recruiting intitation factors (reviewed in Gray and Wickens, 1998).

3' UTR binding proteins may act directly on the translational machinery, or indirectly by changing the poly(A) status or the stability of the mRNA. For example, cytoplasmic polyadenylation is a major mechanism to control translation of maternal mRNAs during early development and is exerted by a protein complex binding to the cytoplasmic polyadenylation element (CPE) in the 3' UTR (Fox et al., 1989; Decker and Parker, 1995; Mendez et al., 2000a; Mendez et al., 2000b; reviewed in Mendez and Richter, 2001; Groisman et al., 2001; de Moor et al, 2005;). Below, the role of PABP and the poly(A) tail is briefly reviewed, as well as that of 3' UTR binding proteins whose mechanisms of regulation are relatively well known.

PABP

The poly(A) tail can recruit 43S complexes and 60S ribosomal subunits, and synergizes with the cap structure for translation (Munroe and Jacobson, 1990; Tarun and Sachs, 1995). The known trans-acting factors for poly(A) tail function are the poly(A)-binding proteins (PABPs) (Mangus et al. 2003; Gorgoni and Gray 2004). They exist in cytoplasmic or nuclear forms, which bear little resemblance to each other. Here we will focus on the ubiquitous cytoplasmic PABPC1, which we will simply call PABP. PABP contains 4 conserved RNA recognition motifs (RRMs) joined to a C-terminal region by a proline-rich linker (Figure 1.5). RRMs 1+2 bind to poly(A) with high affinity and specificity, while RRMs 3+4 exhibit more generic RNA-binding activity. Early studies indicated that PABP interacts with eIF4G through RRMs 1+2 (Tarun and Sachs, 1996; Tarun et al., 1997), and lead to the suggestion of the closed-loop model for translation. Indeed, a closed-loop mRNA could provide an explanation for the cooperative interactions within the cap-eIF4E-eIF4G-PABP-poly(A) assembly, would facilitate ribosome recycling to the 5' end of the message in virtue of the interaction of PABP with eRF3, and would ensure that only full length mRNAs are translated efficiently.

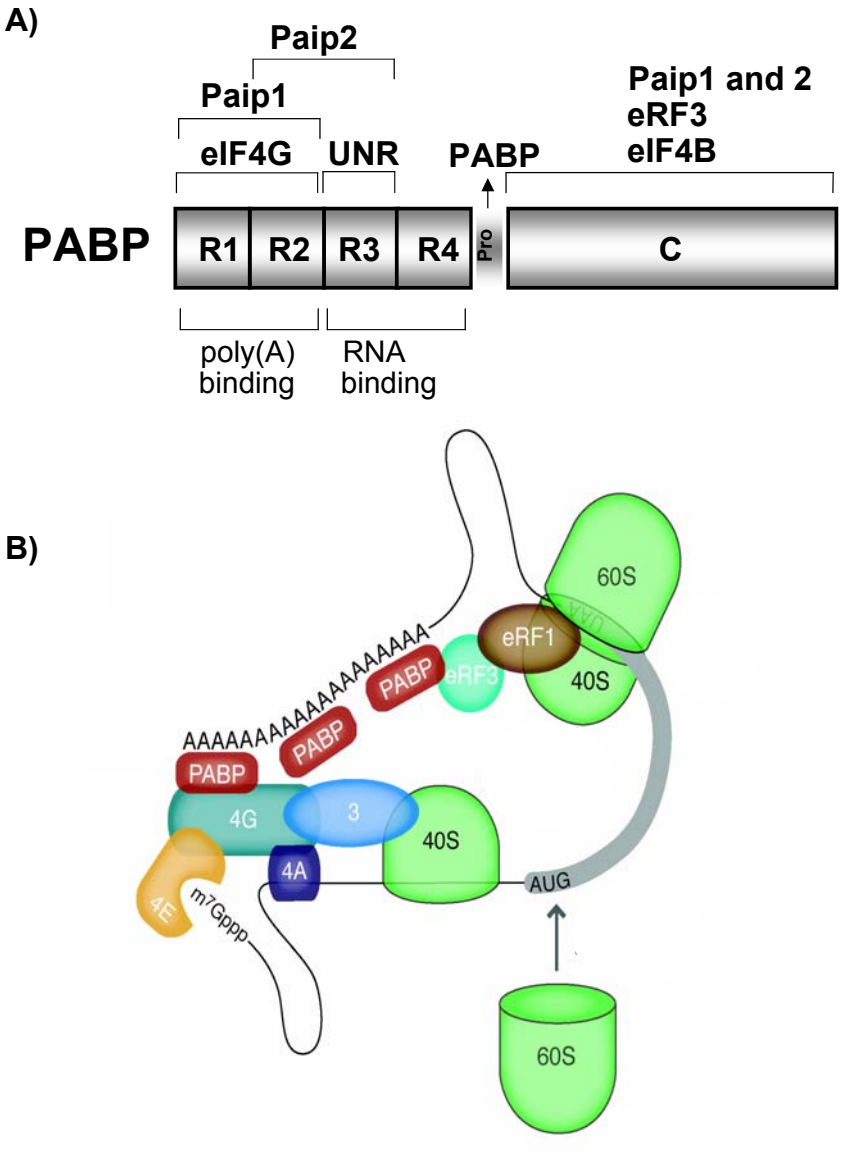


Figure 1.5. PABP and the closed-loop model. A) PABP domains and interactors. Schematic representation of the PABP showing four RNA binding domains (R1-4), the C-terminal (C) (depicted by gray boxes) and the proline-rich linker (Pro). **B)** The closed-loop model. The concurrent interactions between the m^7G cap- eIF4E- eIF4G- PABP- poly(A) tail are thought to pseudo-circularize the mRNA, bringing the 3' UTR in proximity to the 5' end of the mRNA and providing a physical framework for the action of 3' UTR binding regulatory factors on translation initiation. Taken from Hentze et al., 2007.

The proline-rich linker of PABP is implicated in homodimerisation and the C-terminal domain provides important regulatory contacts to other proteins, such as eIF4B, a factor that stimulates the helicase activity of eIF4A, or eRF3

(reviewed in Sachs, 2000; Hentze et al., 2007). PABP- interacting proteins 1 and 2 (PAIP-1 and PAIP-2) also interact with PABP through its C-terminal domain, although other domains may stabilize these interactions. PAIP-1 is believed to stimulate translation via the recruitment of eIF4A, while PAIP-2 titrates PABP from the poly(A) tail and, thus, represses translation. The recent finding that depletion of PABP decreases the translational efficiency of nonadenylated mRNA suggests that PABP may have roles in translation independently of the poly(A) tail (Kavehjian et al., 2005). In summary, PABP is an initiation factor critical for the efficient translation of polyadenylated -and perhaps nonadenylated- mRNAs.

CPEB

In *Xenopus laevis* oocytes, a large fraction of the maternal mRNA pool is stored in an inactive form. Many of these mRNAs contain usually short poly(A) tails and have CPEs in their 3' UTRs. The CPE is bound by CPEB, a bifunctional regulator that also stimulates polyadenylation and translation at a later time, during oocyte maturation (see above), that contains two RNA recognition motifs (RRMs). For repression CPEB is thought to recruit Maskin, a protein that binds to eIF4E on the same site used by eIF4G blocking eIF4G recruitment (Stebbins-Boaz et al., 1999, Cao and Richter, 2002). Hence, Maskin can be considered as a message-specific 4EBP (Figure 1.6).

Bruno

Oskar (Osk) is a morphogen essential for the formation of germ cells and abdominal structures in *Drosophila*, which is translated during late oogenesis from an mRNA localized at the posterior pole. *Osk* mRNA is synthesized by the nurse cells and is silenced during its transport to the oocyte posterior by the RNA-binding protein Bruno (Kim-Ha et al., 1995). Bruno binds to defined, repeated sequence elements in the 3' UTR of *osk* mRNA, referred to as the Bruno Response Elements (BREs). Additional factors to Bruno are necessary to repress translation of the *oscar* mRNA binding to the Bruno and/or directly to the RNA 3’UTR (Apontic, Bicaudal-C and p50; Lie and Macdonald, 1999).

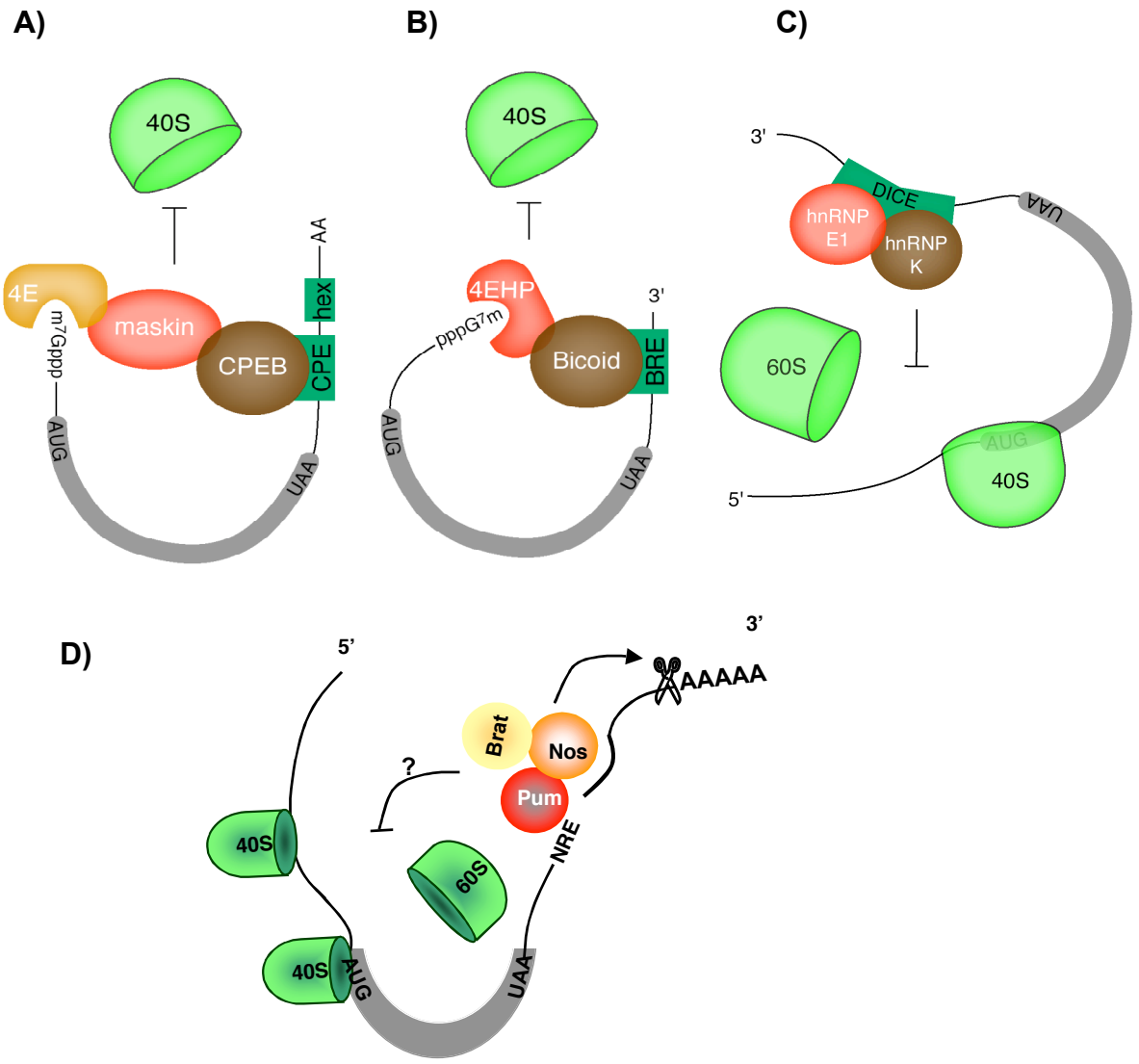


Figure 1.6. Regulation by 3' UTR binding proteins. A) The CPEB/ Maskin complex binds to the CPE in the 3' UTR of the mRNA and competitively inhibits the association of eIF4G with eIF4E. Taken from Hentze et al., 2007. **B)** Bicoid binds to the BRE in the 3' UTR and recruits the translation incompetent cap-binding factor 4EHP, thereby inhibiting the association of eIF4F to the 5' cap structure. Taken from Hentze et al., 2007. **C)** hnRNPs K and E1 bind to the DICE in the 3' UTR of *lox* mRNA and block the joining of the 60S ribosomal subunit to the 40S subunit placed at the initiation codon. Taken from Hentze et al., 2007. **D)** Pumilio binds to the NREs in the 3' UTR of *hunchback* mRNA and recruits Nanos and Brat. This complex promotes the deadenylation of the message and inhibits an early, still unknown, step of translation initiation.

There is conflicting evidence on the mechanism of translational repression of *osk* mRNA. Sucrose density gradient analysis showed that the repression mechanism targets the recruitment of the 43S pre-initiation complex (Chekulaeva et al., 2006). Consistent with this, Bruno binds Cup, which precludes the interaction of eIF4E with eIF4G (see above) (Wilhelm et al., 2003; Nakamura et al., 2004; Zappavigna et al., 2004). However, another report suggested that translational repression by Bruno is independent of the cap structure (Lie and Macdonald, 1999). A second, cap- and Cup- independent mechanism to control *osk* mRNA translation was uncovered recently. This second mechanism consists of Bruno-driven mRNA oligomerisation and the formation of repressed higher order "silencing particles" (up to 80S) that are inaccessible for ribosomes (Chekulaeva et al., 2006). Therefore, Bruno seems to be a translational regulator that uses multiple mechanisms to repress translation. Another example of a multi-functional regulator is that of Sex-lethal (SXL), which is discussed extensively in section 1.4.

Smaug

Smaug (Smg) is an embryonic *Drosophila* factor involved in the translational repression of *nanos (nos)* mRNA. Nos itself is a translational regulator involved in posterior patterning. *Nos* mRNA is produced in the nurse cells and deployed in the oocyte, where a small fraction of it (~4%) is localized to the posterior. The rest of *nos* is distributed throughout the oocyte and must be translationally repressed for proper anterior-posterior axis formation (Bergsten and Gavis, 1999). Smg binds to two Smaug responsive elements (SREs) in *nos* 3'UTR through its SAM (sterile alpha motif) domain (Smibert et al., 1996). In addition to the SREs, the translational control element (TCE) of *nos* mRNA contains a distal loop that is not bound by Smg but is required for translational repression during late oogenesis (reviewed in Hentze et al., 2007). Smg recruits Cup, an eIF4E-binding protein that –similar to Maskin- blocks the interaction of eIF4E with eIF4G (Nelson et al., 2004) (Figure 1.6). One intriguing observation is that, although translationally repressed at an early step, ~50% of *nos* mRNA still associates with polysomes. Perhaps this association is "residual", reflecting

polysomes that were associated to *nos* mRNA when it was translationally active in the nurse cells and remained bound after translational repression by Cup. Smg has also been described to have a role in degradation of unlocalized maternal mRNAs in the early embryo by recruiting the CCR4-POP2-NOT deanylase complex (Semotok et al., 2005).

Pumilio

Hunchback mRNA (*hb*) encodes a transcription factor required for anterior development in the fly. Zygotic *hb* is only transcribed at the anterior, but maternal *hb* (hb^{mat}) mRNA is distributed throughout the embryo and is translationally repressed at the posterior by Nos (Murata and Wharton, 1995). hb^{mat} contains two copies of a 32 nucleotide element just downstream of the termination codon that are necessary for translational repression and have been referred to as the Nanos response elements or NREs. Nos does not bind directly to the NREs, but its interaction requires the previous binding of the RNA-binding protein Pumilio (Pum) (Murata and Wharton, 1995; Sonoda and Wharton, 1999) (Figure 1.f6). The NRE-Pum-Nos complex subsequently recruits Brat, another factor required for repression of hb^{mat} mRNA (Sonoda and Wharton, 2001). Whether Brat interacts directly with the translation machinery is an open question: although early evidence suggested that translational repression of hb^{mat} involves deadenylation (Wreden et al., 1997), these findings have been challenged (Chagnovich and Lehmann, 2001).

Pum and Brat are uniformly distributed throughout the embryo so spatial restriction of translation cannot be attributed to any of them. Rather it is a consequence of localized posterior expression of Nos protein. Therefore it is thought that one of possible explanation is that translational repression requires recruitment of Nos to a pre-existing complex containing, at least, Pum and Brat prebound to an NRE. Pum is the founding member of a large family of proteins containing the PUF domain (named after Pum and the *C. elegans* protein FBF), a rainbow-shaped RNA-binding domain (Edwards et al., 2001; Wang et al., 2001). The Pum/Nos complex is highly conserved in evolution, and controls mRNA

translation and/or stability in other organisms (Parisi et al., 2000; Jaruzelska et al., 2003).

Bicoid

Bicoid (Bcd) is a homeodomain transcription factor that represses the translation of *caudal* mRNA for proper anterior development of *Drosophila* embryos (Rivera-Pomar et al., 1996). Bcd binds to the Bicoid-binding region (BBR) in the 3' UTR of *caudal* mRNA and recruits an eIF4E-related protein called 4EHP (eIF4E- homologous protein) (Figure 1.6). 4EHP exhibits cap-binding activity, but is unable to bind eIF4G (Lasko et al., 2005; Cho et al., 2005). Thus, Bcd represses translation by blocking the cap structure with a translationally impaired cap-binding protein.

HnRNPs K and E1

Lox (15-lipoxygenase) mRNA remains silenced during erythroid differentiation until a terminal stage, when LOX is translated and used to destroy mitochondrial membranes (Ostareck-Lederer et al., 2004). Translational repression requires an element in the 3' UTR of *lox* mRNA called the differentiation control element (DICE). The DICE is bound by heterogenous nuclear (hn) ribonucleoprotein (RNP) K and hnRNP E1, which appear to repress LOX translation independently of the poly(A) tail. Sucrose gradient and toe-print assays indicate that hnRNPs K and E1 impair the joining of the 60S ribosomal subunit to the 48S complex (Ostareck- Lederer et al., 1994; Ostareck et al., 1997, 2001) (Figure 1.6). The exact molecular mechanism that underlies this translational regulation event is still unknown, but the observation that translational repression functions when translation is driven by the EMCV and CSFV IRESs, while repression does not occur when driven by the CrPV IRES (which does not require known initiation factors) suggests that an initiation factor -rather than the ribosome- is targeted for inhibition.

1.3.3 RNA as Regulator

Translation can also be regulated by a class of conserved, small (~20- 24 nt) RNAs called microRNAs (miRNAs). In addition to translation, miRNA-mediated regulation has been shown for other steps of gene expression, including chromatin methylation and mRNA stability (Carthew, 2006; Vaucheret et al., 2006). The founding member of the miRNA family, *lin-4*, was shown to repress the translation of *lin-14* mRNA, an event crucial for appropriate developmental timing of C. elegans (Lee et al., 1993; Wightman et al., 1993; Olsen and Ambros, 1999; reviewed in Wickens et al., 2000). To date, several hundred miRNAs have been described in plants and animals that are important for a broad spectrum of biological processes.

miRNAs hybridize usually to multiple sites in the 3' UTR of target mRNAs by imperfect base-pairing (Figure 1.7). miRNAs are biochemically indistinguishable from another class of small RNAs called small interfering RNAs (siRNAs). siRNAs normally hybridize perfectly with their targets and function by degrading the mRNA. miRNAs and siRNAs arise from different precursors, but share some processing steps and are found in complexes with common proteins (e.g. the Argonaute proteins) (Neslon et al., 2003; Chen and Meister, 2005; Vazquez, 2006). These have led some authors to speculate that similar complexes might direct both mRNA degradation and translational repression. Indeed a miRNA can induce mRNA degradation when modified to match perfectly its target (reviewed in Gebauer and Hentze, 2004; Hutvagner, 2005; Valencia-Sanchez et al., 2006). Thus, the general belief is that when the si/miRNA hybridizes perfectly with its target it induces degradation, and when it hybridizes imperfectly it leads to translational repression. miRNA encoding genes may account for as much as 1% of the total plant or animal genome, therefore arguing that miRNAs could represent a major class of regulators of gene expression (Nakahara et al., 2004) but the mechanism of translational repression by miRNAs is unknown.

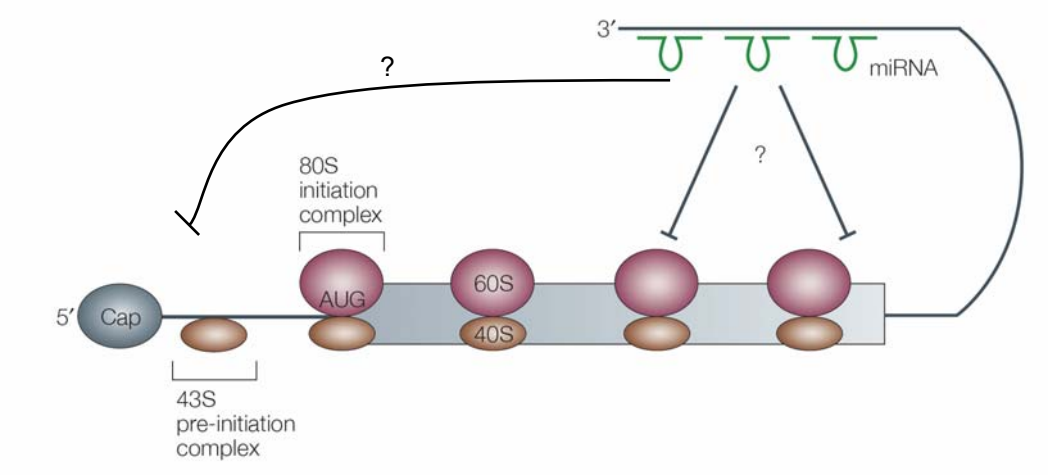


Figure 1.7. Translational control by microRNAs. MicroRNAs (miRNAs; shown in green) engage in imperfect base-pairing interactions with the 3' UTR and cause translational arrest. it is unclear at present which step of translation is affected by miRNAs. Adapted from Gebauer and Hentze, 2004.

Conflicting evidence have identified miRNAs both as initiation or elongation/termination regulators. Some studies argue that repression by miRNAs does not occur when translation is driven by IRESs, suggesting that miRNAs target the cap structure or eIF4E for repression and inhibit translation initiation (Humphreys et al, 2005; Pillai et al. 2005). However, repressed *lin-14* mRNA is associated to polysomes, suggesting that miRNAs regulate elongation or termination of translation (Seggerson et al., 2002; Boehm and Slack, 2005). The possibility that miRNAs reduce the stability of the nascent polypeptide chain has not been excluded.

1.3 Dosage Compensation

More than 70 years ago, Herman Muller discovered the phenomenon of dosage compensation working with fruit flies (Muller, 1932). Since then, dosage compensation has been studied at the molecular level in three model organisms: *Caenorhabditis elegans*, *Drosophila melanogaster* and the mouse. In diploid organisms, the genetic information responsible for sexual differentiation is stored on the sex chromosomes. Often, one of the sex chromosomes has become structurally modified during evolution while the other has remained practically unchanged. This modification is restricted to one sex, so in some organisms is transmitted from father to son (Y chromosome), in others from mother to daughter (W chromosome). The other sex chromosome, called X when the male is heterogametic (XY), or Z when the female is heterogametic (ZW) is present in two doses in the homogametic sex. The chromosome was originally named X for “unknown”, remaining an oddity that has puzzled geneticists for decades. This system of sex determination results in an inequality in the dosage of genes linked to the X or Z chromosomes. Different mechanisms have evolved to prevent such inequalities and to compensate for the dosage of X-linked or Z-linked genes between sexes. Mammalian females randomly select and shut down one of the X chromosomes to form a Barr body.

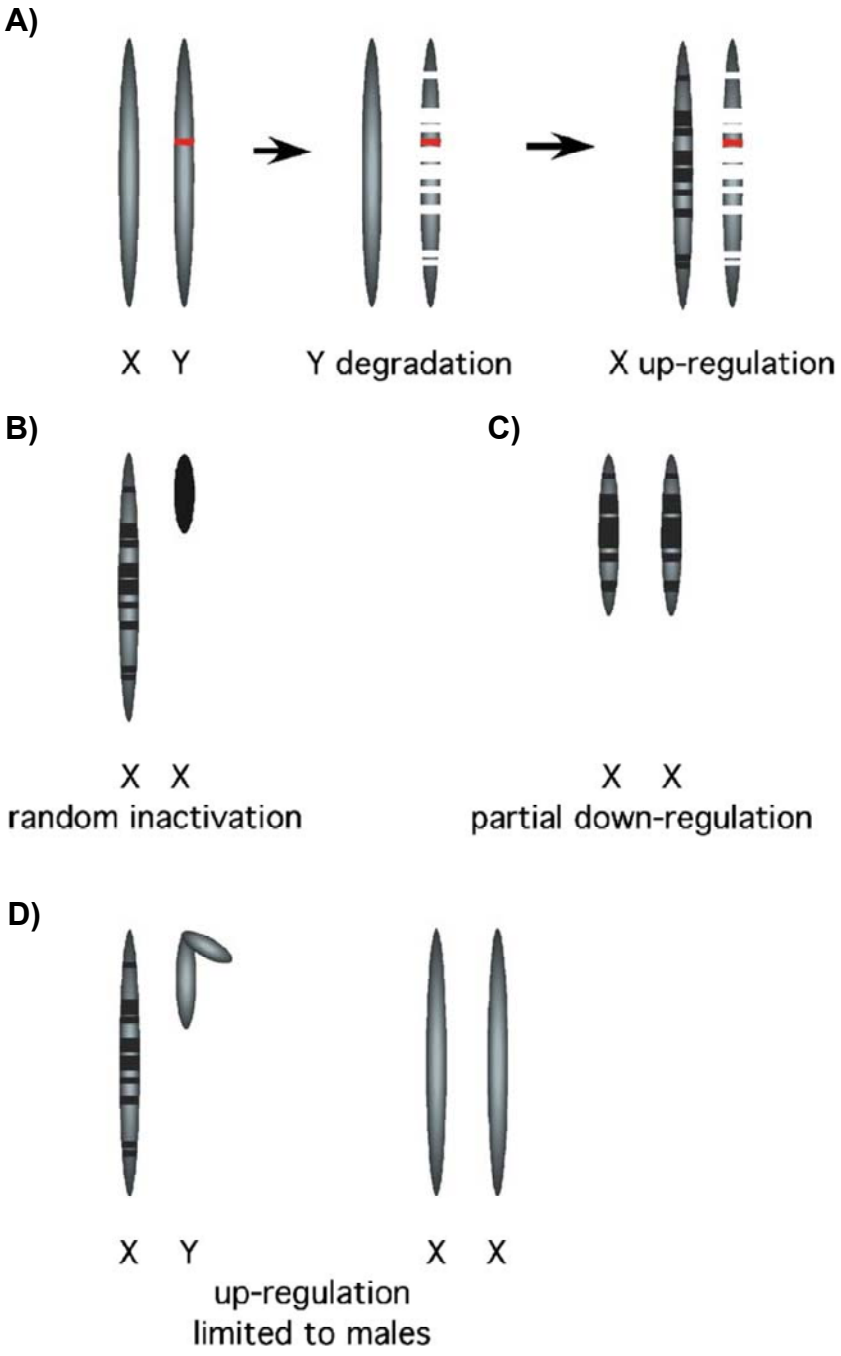


Figure 1.8. Dosage compensation in model organisms. A) Model for the origin of dosage compensation. The establishment of sex-determining locus (red) is followed by degradation of the chromosome bearing it (the Y chromosome). X-linked genes then increase expression to compensate for the reduction of gene dosage in males (dark bars on X). **B)** Mammalian females randomly inactivate a single X (black oval). The active X is expressed at the same level as the male X chromosome. **C)** *C. elegans* hermaphrodites partially repress both X- chromosomes. **D)** *Drosophila* males activate transcription from the single X chromosome. Taken from Larsson and Meller, 2006.

C.elegans hermaphrodites (XX) down-regulate transcription from both X chromosomes to about half, and *Drosophila* males increase transcription from the single X chromosome 2-fold. A brief description of the dosage compensation mechanisms is included below (Figure 1.8).

1.3.1 Mammals

The mammalian X and Y chromosomes arose from one pair of autosomes 300 million years ago. The X and Y still share a few genes, mainly in the pseudoautosomal region on the top of the chromosomes. Over time, the Y chromosome has been devoted to preserve only genes for maleness, while the X contains many genes important for cell homeostasis. To preserve the gene dose, females randomly inactivate one of the Xs by using a mechanism that includes counting of X chromosomes, inactivation, and maintenance of the inactive state (Valley and Willard, 2006; Clerc and Avner, 2006; Graves, 2006; reviewed in Lucchesi et al., 2005). This choice is permanent and consequently human females are mosaic of the X chromosomes from each parent. Inactivation requires a region of the chromosome known as Xic (X inactivation center). This locus includes the *Xist* gene, whose product is a large non-coding RNA that spreads in *cis* along the X chromosome resulting in hetherochromatization. Although necessary and sufficient for X-inactivation, *Xist* is not required to maintain the inactive state, which is characterized by histone H2A, H3 and H4 hypoacetylation and hypermethylation. Recent studies indicate that about 15% of genes escape permanent inactivation (Ross et al., 2005; Carrel and Willard, 2005).

1.3.2 C. elegans

In *C.elegans* (males X0, hermaphrodites XX) dosage compensation uses a complex that is an adapted form of the condensin complex functioning during cell division (Bean et al., 2004; Gupta et al., 2006; reviewed in Lucchesi et al., 2005). The dosage compensation complex (DCC) is encoded by genes apparently involved only in dosage compensation, the *dpy* or *dumpy* genes (so called because of the short and fat appearance of dpy mutants), and genes that also have a function in sex determination, and hence are called the *sex determination and dosage compensation* (*sdc*) genes. Interestingly, the DCC binds to a single autosomal site in addition to the X, the gene *her-1*. Expression of *her-1* is one of the initial steps leading to male differentiation and, thus, binding of the DCC to both the X chromosome and *her-1* provides a link between dosage compensation and sex determination. The DCC is targeted to the X by the proteins SDC-2 and SDC-3. Expression of SDC-2 is inhibited by the master sex-determining gene *xol-1* (male-specific *XO lethal-1)*. Males express high levels of XOL-1 and, thus, inhibit dosage compensation. Another component of the DCC is the protein MIX-1. MIX-1 and DPY-27 are members of the SMC (structural maintenance of chromosome) family of proteins required for condensation and segregation of mitotic chromosomes.

1.3.3 D. melanogaster

In *Drosophila* (males XY, females XX), the ~ 2-fold transcriptional increase of the single male X chromosome results from the action of a DC complex composed of proteins whose absence affect the viability only of male flies. These proteins have been collectively called the MSL (for male-specific lethal) proteins since mutations in any of them cause male-specific lethality (Table 1.2) (Kuroda

et al., 1991; Gorman et al., 1995; Hilfiker et al., 1997). The MSL complex contains five proteins: *msl-1*, *msl-2*, *msl-3, mle (maleless)* and *mof* (*males absent on first*), and two non-coding RNAs: *roX1* and *roX2* (reviewed in Amrein, 2000; Akhtar, 2003). Together, they bind to hundreds of specific sites on the male X as early as the blastoderm stage of embryonic development, when zygotic transcription commences (Rastelli et al., 1995; Franke et al., 1996).

MSL-2 is a ring-finger (a special type of Zinc-finger probably involved in protein-protein interactions) protein that binds to MSL-1 and stabilizes it (Palmer et al., 1994; Chang and Kuroda, 1998). The MSL-1::MSL-2 complex then nucleates the assembly of the other components on the X chromosome. MSL-3 contains a chromodomain, a conserved domain present in chromatin remodeling factors that serves protein-protein and protein-RNA interactions. MLE is an RNA helicase with ATPase activity, and MOF is the acetyl- transferase responsible for acetylating histone H4 on lysine 16, a modification only found associated to the male X chromatin and thought to represent the molecular “signature” of the DCC (Hilfiker et al., 1997; Smith et al., 2000; Akhtar and Baker, 2000; reviewed in Akhtar et al., 2003). JIL-1 kinase also associates to the MSL complex and promotes phosphorylation of Ser10 on histone H3 (Jin et al., 2000; Wang et al., 2001), although the role of this modification in dosage compensation is unclear. This chromatin remodeling consequently results in diffuse chromatin structure typical for chromosome undergoing transcription with reduced chromatin condensation. *Rox1* and *rox2* anchor MOF to the DCC complex. Initial studies suggested that the *Rox1* and *Rox2* loci (located on the X) were “entry” sites from which the DCC would spread to the rest of the chromosome, because they could promote spreading of the DCC when transferred to an autosomal site, and because the DCC was preferentially associated to these loci when the MSL components were limiting (Demakova et al., 2003). Recent findings have challenged this view, and it is now thought that the X has high affinity (e.g. *rox1* and *rox2*) and low affinity sites for the DCC (Dahlsveen et al., 2006). How the DCC is targeted to these sites remains a mystery. Surprisingly, chromatin-IP followed by microarray analysis showed that the DCC is associated to the 3’ end

of genes, preferentially the ORFs and 3' UTRs, which defies the previous idea that the DCC modulates the initiation of transcription at the promoters (Gilfillan et al., 2006; Alekseyenko et al. 2006; Legube et al., 2006).

Dosage compensation does not occur in female flies because the expression of MSL-2 is repressed. All the other components are present in females, albeit at lower amounts (Bashaw and Baker, 1995; Kelley et al., 1995). Indeed, forced expression of MSL-2 in females leads to the assembly of the DCC on both X chromosomes and female-specific lethality (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995). Thus, inhibiting the expression of *msl-2* is essential for female viability. The factor responsible for this repression is the master regulator of sex determination and dosage compensation in the fly: Sex-lethal (SXL). In the following section, the properties and mechanisms of action of this regulatory protein are discussed.

Table 1.2. Dosage compensation complex components and their properties.

DCC components	*Properties*
MSL-1	X chromosome localization, interacts with MSL-2, MOF and MSL-3
MSL-2	Ring finger protein, X chromosome localization, interacts with MSL-1
MSL-3	Chromodomain, interacts with MSL-1 and *rox* RNAs
MOF	Chromodomain, HAT activity, interacts with *rox 2*, C2HC zinc finger that interacts with H4 tail
MLE	ATP-dependent helicase, GGY domain important for targeting to X chromosome via rox RNA
rox 1 and rox 2	non-coding RNA components
JIL-1	H3 kinase, interacts with MSL-1 and MSL-3

1.4 SXL

Fly development is like a one ring circus that occasionally complicates an already exciting scene by adding a second and a third ring. Varieties of different acts are taking place at the same time and are coordinated by the circus master SXL. At least three acts are under its direction: female somatic development, the sex-specific development of germ cells, and dosage compensation.

1.4.1 Who is SXL?

SXL is a female specific RNA-binding protein that preferentially binds to U-rich stretches (Sakashita and Sakamoto, 1994; Singh et al., 1995). In vitro SELEX (systematic evolution of ligands by exponential enrichment) experiments indicated that the U-rich stretches could be interrupted by a few guanidines, but not by citidines (Singh et al., 1995). SXL contains 354 amino acids that are organized into four distinct domains (Figure 1.9). Two RNP-type RNA binding domains (RBDs) are located in the center of the protein and are necessary and sufficient for site-specific RNA binding (Samuels et al., 1998). Individual RBDs interact with RNA significantly more weakly and somewhat non-specifically

(Kannar et al., 1995). NMR and X-ray structures of SXL have been obtained in the presence and absence of RNA, showing that the two RBDs are flexibly tethered in solution (Crowder et al., 1999; Lee et al., 1997), while their structure changes upon RNA binding adopting a V-shaped conformation around the RNA (Handa et al., 1999; Kim et al, 2000; Antson, 2000). The RBDs of SXL also participate in protein-protein interactions with the snRNP component SNF (see below), and the protein of unknown function SIN (Dong and Bell, 1999). SXL has also been shown to dimerize via its RBDs (Sakashita and Sakamoto, 1996). The amino terminal domain of SXL (aa 1-121) is referred to as the GN domain because of its high glycine and asparagine content. This domain mediates cooperative binding of SXL monomers to adjacent sequences on target mRNAs (Wang and Bell, 1994), and interacts with the splicing factor SPF45 (Lallena et al., 2002). No function has been assigned to the carboxy terminal domain of SXL (aa 295-354).

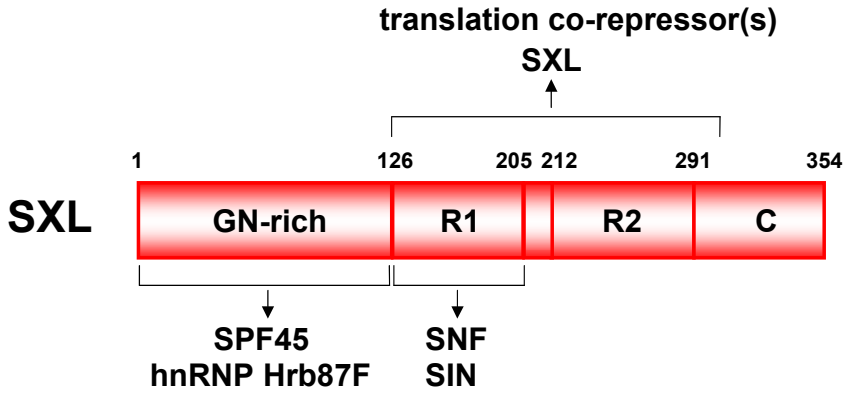


Figure 1.9. Domain organization of SXL and interacting proteins. Schematic representation of the SXL protein showing the GN-rich N-terminal domain (GN), two RNP-type RBDs (R1 and R2), and the C-terminal tail (C). The amino acid numbers at boundaries among these domains are shown. The GN domain is implicated in SXL cooperative binding to RNA substrates and interactions with splicing factor SPF45 and heterogenous nuclear ribonucleoprotein (hnRNP) Hrb87F. R1 mediates interactions with the snRNP component SNF and with SIN, a protein of unknown function. SXL dimerizes through the RBDs and interacts with co-repressors in *msl-2* mRNA translational silencing.

1.4.2 Regulation of SXL Expression

Shortly after fertilization, there is an assessment of the X-chromosome-to-autosomes (X:A) ratio that establishes whether a fly will become male (1X:2A= 0.5) or female (2X:2A=1). The X:A ratio is determined by the balance between positive transcriptional regulators of *sxl* located on the X, also called *numerator* genes, and negative regulators located on the autosomes called *denominator* genes (reviewed in Schütt and Nöthiger, 2000) that have been shown to bind to specific sites in the promoter of *sxl*, thereby activating or repressing the gene, respectively. The double dose of *numerators* in females results in the activation of the early or *establishment* promoter of *sxl* (PE) and shunts the female mode of sexual determination, as well as the repression of dosage compensation (Figure 1.10). In a fly with an X:A ratio of 0.5, no functional SXL protein is produced and, consequently, the fly develops as a male.

So far, four activating numerator genes have been identified: *sisterless* (*sis)* A, B and C, and *runt* (*run*). The only known zygotic negative regulator of *sxl* is the transcription factor *deadpan* (*dpn*). In addition, at least four maternal genes help to interpret the X:A signal: the activators *daughterless* (*da*) and *hermaphrodite* (*her*), and the repressors *extramacrochaetae* (*emc*) and *groucho* (*gro*). These maternal products are not discriminatory since they are present in equal dose in both sexes. When transcription from the establishment promoter ceases, a late or *maintenance* promoter (PL) becomes active in both sexes. From this point on, sex-specific expression of *sxl* is shifted from transcriptional regulation to modulation at the level of RNA alternative splicing. Pre-existing active SXL positively regulates the female- specific alternative splicing of its own late transcript, generating an auto-regulatory loop that reinforces SXL expression throughout development (Figure 1.10).

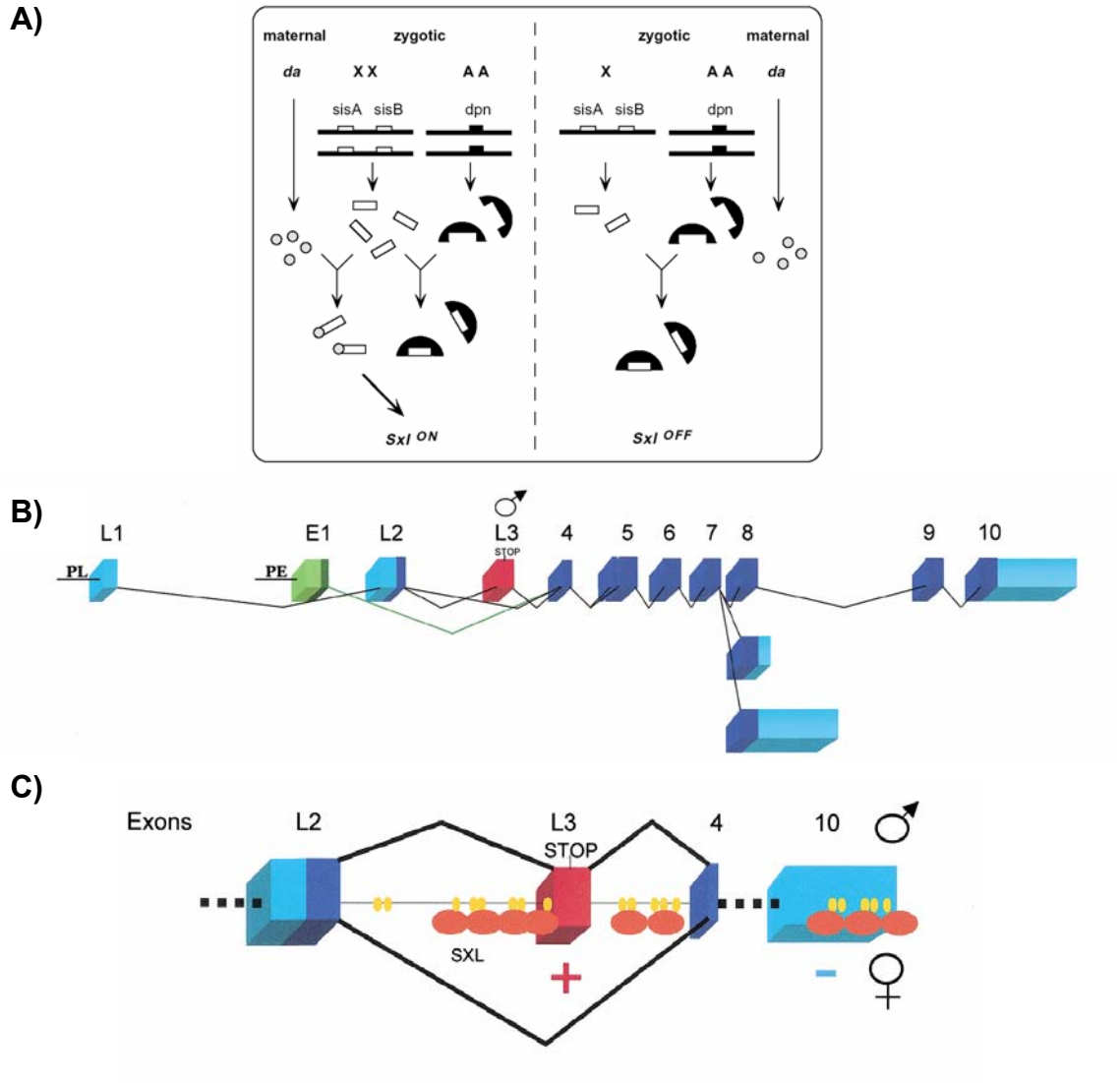


Figure 1.10. Regulation of SXL expression. A) Simplified titration model to illustrate how the X:A ratio regulates transcription of *Sxl* in *Drosophila*. If the X:A ratio is 0.5, all numerator molecules (only SISA and SISB are shown here) are bound by the negatively acting denominator (DPN). If the ratio is 1.0, some numerators remain free and can form a heterodimeric complex with maternal daughterless (da), which activates the early *Sxl* promoter. Taken from Schütt and Nöthiger, 2000. **B)** Schematic representation of *sxl* transcripts. The *Sxl* gene produces two separate sets of transcripts, deriving from the two different promoters (early or PE, and maintenance or PL). The early and the late transcripts differ in the first 25 amino acids. Alternative splicing and polyadenylation give rise to several mRNAs. The embryo-specific exon (E1) is presented in green (light green for the noncoding region, and dark green for the coding region). The male specific exon is presented in red. Other exons are presented in blue (light blue for the untranslated region, and dark blue for the coding region). Taken from Penalva and Sánchez, 2003. The SXL protein used in this study includes exon 1 to exon 8. **C)** SXL controls splicing of its own pre-mRNA. SXL binds to poly(U) sequences in introns 2 and 3 and excludes exon L3, which contains translational stop codons, from the mature transcript. This process establishes a positive autoregulatory loop. Boxes represent exons, and horizontal lines represent introns. Taken from Penalva and Sánchez, 2003.

Autoregulation of *sxl* splicing involves its interaction with the second-step splicing factor SPF45, resulting in the skipping of an exon 3 (male-specific exon) containing several stop codons (Lallena et al., 2002). Males do not express SXL from the early promoter, thus synthesizing only nonproductive mRNAs encoding truncated products from the late promoter.

Genetic studies have implicated three other genes in *sxl* splicing regulation: *sans-fille* (*snf*) a splicing factor, *female-lethal-2d* (*fl2d)* and *virilizer* (*vir*) proteins of unknown function (Granadino et al., 1992; Flickinger and Salz, 1994; Hilfiker et al., 1995). At least, SNF interacts with the RBDs of SXL (see above). Consistent with the fact that SPF45 interacts with the GN domain of SXL, SXL missing this domain is unable to autoregulate its own splicing (Wang and Bell, 1994).

1.4.3 Regulation of Somatic Sexual Differentiation

SXL modulates alternative splicing of downstream genes in the cascade of sex differentiation (Forch and Valcarcel, 2003). The most proximal gene in the casdade is *transformer* (*tra*), which, in turn, is a splicing regulator of *doublesex* (*dsx*), of which both are required for female differention pathway. Similar to *sxl*, *tra* pre-mRNA is present in both sexes, but it is only spliced appropriately in females. SXL directs the skipping of an exon (exon 2) containing in-frame stop codons, resulting in the synthesis of functional TRA. SXL inhibits the recognition of exon 2 by the splicing machinery using a mechanism that includes SXL binding to the pirimidine tract at the 5' of exon 2, blocking the binding of the necessary splicing factor U2AF (Valcárcel et al., 1993) (Figure 1.11). TRA, together with TRA2 (a protein present in both sexes), activates the female specific splicing of *dsx* pre-mRNA. The female version of DSX (DSX^F) activates the transcription of genes required for female development and represses those

required for male development (reviewed in Schütt and Nöthiger, 2000 and MacDougall et al., 1995).

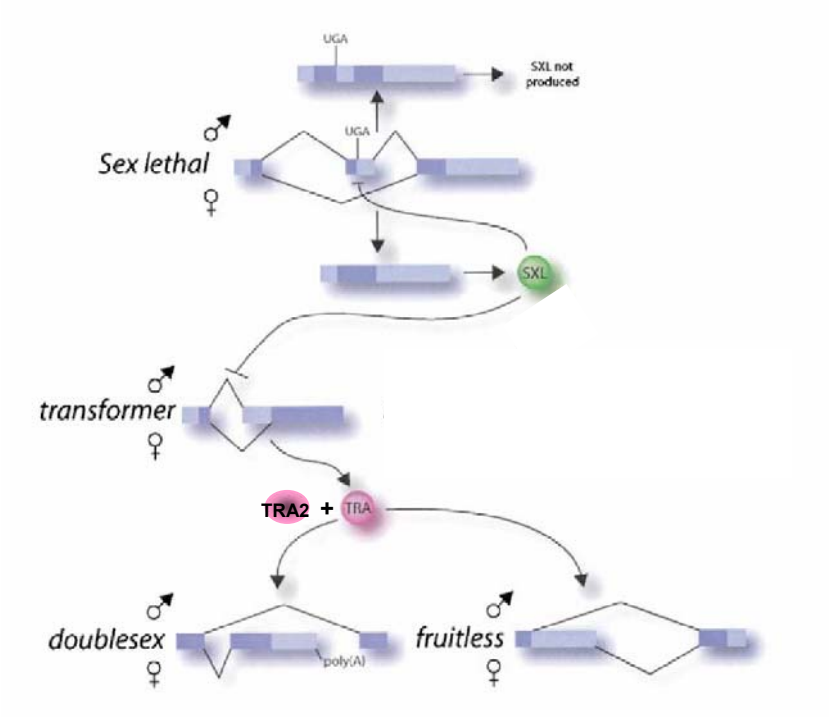


Figure 1.11. SXL regulation of somatic sex determination. The Drosophila sex determination alternative splicing cascade. SXL autoregulates its own splicing and represses the male-specific splice sites in the *transformer* (*tra*) pre-mRNA. Female-specific splicing of tra results in the synthesis of TRA protein that together with TRA2 positively regulates alternative splicing of the *doublesex* (*dsx*) and *fruitless* (*fru*) pre-mRNAs. Adapted from Graveley, 2002.

1.4.4 Regulation of Germline Development

Ovaries represent a completely different tissue from somatic cells and, consequently, *sxl* splicing demands a different set of factors (Granadino, 1993). Germline SXL function requires the X:A signal (1.0), a female soma surrounding, and the action of the genes *ovo*, *otu (ovarian tumor)* and *snf* (Penalva and Sanchez, 2003). The only one that can act in soma and germ line is SNF, but *tra,*

tra2 and *dsx* are responsible for the inductive signal given by the somatic cells (Nothiger et al., 1989). The downstream targets of SXL in the germline are less clear. A recent study indicates that SXL directs sex-specific germ development by alternative polyadenylation of *enhancer of rudimentary* (*e(r)*) in the ovary, but the biological significance of this is unclear (Gawande et al., 2006). Recently, it was reported that gene *gutfeeling* (*guf*) – the Drosophila homologue of the ornithine decarboxylase antizyme- is a target of SXL in the germ line (Vied et al., 2003). Moreover, *guf* itself apparently controls SXL expression in the germline by localization and translocation from the cytoplasm to the nucleus. However, molecular mechanism remains to be resolved.

1.4.5 Regulation of Dosage Compensation

In the center of the spotlight after setting the proper sex determinants comes the regulation by SXL of dosage compensation in female flies. SXL inhibits dosage compensation in females by repressing the expression of *msl-2* (Gorman et al., 1993; Bashaw and Baker, 1995; Kelley et al., 1995). To this end, SXL inhibits the splicing of a facultative 5' UTR intron in *msl-2* pre-mRNA, and subsequently inhibits *msl-2* mRNA translation. Thus SXL is an RNA binding protein with multiple functions in mRNA metabolism (splicing, polyadenylation and translation) that uses a dual mechanism to repress *msl-2* expression.

msl-2 pre-mRNA contains multiple consensus SXL binding sites: two in the 5' UTR, close to the splices sites (ss) of a small intron (Figure 1.12), sites A and B), and a cluster of sites in the 3' UTR (sites C-D-E-F). In HeLa extracts, SXL binding to site A prevents 5'ss recognition by the splicing regulator TIA-1, necessary for U1snRNP recruitment to *msl-2* pre-mRNA (Forch et al., 2001). The *Drosophila* homologue of TIA-1 is called ROX8 and its function is still unclear. SXL binding to site B (which is in fact the pirimidine tract) blocks U2AF binding and inhibits the recognition of the 3' ss (Merendino et al., 1999). This double

block of splice site recognition leads to intron retention in females (Bashaw and Baker, 1995; Gebauer et al., 1998; Forch et al., 2001). Translational repression of *msl-2* requires SXL-binding to both the 5' and 3' UTRs (Bashaw and Baker, 1997; Kelley et al., 1997; Gebauer et al, 1998). Because *msl-2* from *D. virilis* lacks an intron in the 5' UTR while retaining SXL-binding sites, it has been speculated that the primary function of splicing inhibition is to preserve functional SXL-binding sites in the 5' UTR for subsequent translational repression (Bashaw and Baker, 1997; Gebauer et al., 1998).

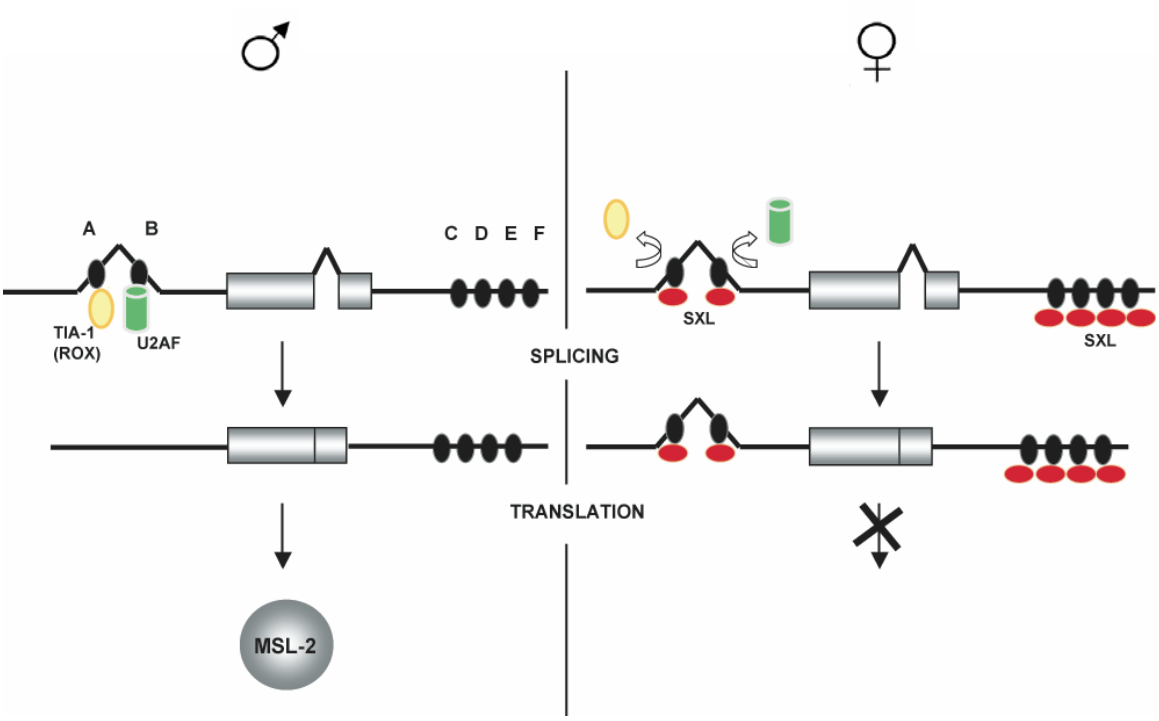


Figure 1.12. *msl-2* pre-mRNA and its regulation in males and females. Inhibition of *msl-2* expression in females occurs in two steps. First, SXL inhibits splicing of *msl-2* pre-mRNA. SXL prevents splicing of the 5' UTR intron by competing with U2AF and ROX8 for binding to two poly(U) sequences (sites A and B) located close to 5' and 3' splice sites. Second, binding of SXL to sites in the 5' and 3' UTRs represses translation. Boxes represent the ORF (gray), and horizontal lines represent UTRs or introns. Poly(U) sequences are represented as black ovals and denoted A to F.

In vitro translation experiments performed in rabbit reticulocyte lysates and *Drosophila* extracts showed that SXL inhibits translation of *msl-2* mRNA directly without affecting its stability (Gebauer et al., 1998, 1999). Unlike most previously described examples of translational regulation by RNA binding proteins (see Chapter 1.2), translational repression of *msl-2* by SXL seems to be independent of the cap structure and the poly(A) tail (Gebauer et al., 1999,2003). In addition, efficient repression requires the binding of SXL to both UTRs of *msl-2* (Gebauer et al., 1999). Indeed, mutation of the *msl-2* 5' or 3' UTR SXL-binding sites leads to a substantial assembly of dosage compensation complexes on the female X chromosomes (Bashaw and Baker, 1997; Kelley et al., 1997). Not all SXL binding sites contribute equally to translational repression. In the 5' UTR, mutation of site A had minor effects on translational repression while site B is critical for regulation *in vitro* (Gebauer et al., 1999). In the 3’ UTR, deletion of sites C and D did not affect translation inhibition while sites E and F proved to be essential (Gebauer et al., 2003). Sites E and F are flanked by sequences that do not contribute to SXL binding but are required for translational repression, suggesting that they are binding sites for co-repressors. Two high molecular weight polypeptides were found to cross-link to these sequences and to co-immunoprecipitate with SXL (Grskovic et al., 2003).

Sucrose gradient analysis indicated that SXL inhibits the stable recruitment of the 43S ribosomal complex to the mRNA (Gebauer et al., 2003). Using toe-print assays, Beckmann et al. (2005) showed that SXL follows a "redundant" or "fail-safe" mechanism of translational control: SXL bound to the 3’ UTR reduces the initial recruitment of 43S ribosomal complexes to the mRNA, while 5’ UTR- bound SXL blocks the scanning of 43S complexes that may have escaped the first control (Beckmann et al., 2005) (Figure 1.13). SXL is the first regulator reported to inhibit scanning. In addition, how 43S recruitment is affected without targeting the cap is an unsolved mystery. The mechanism of repression probably does not involve steric hindrance, because the highly conserved SXL homologue from *Musca domestica* (mSXL) does not repress translation despite

binding to *msl-2* with a similar affinity (Grskovic et al., 2003; Beckmann et al., 2005). This points to a specific initiation factor(s) as a putative target for control.

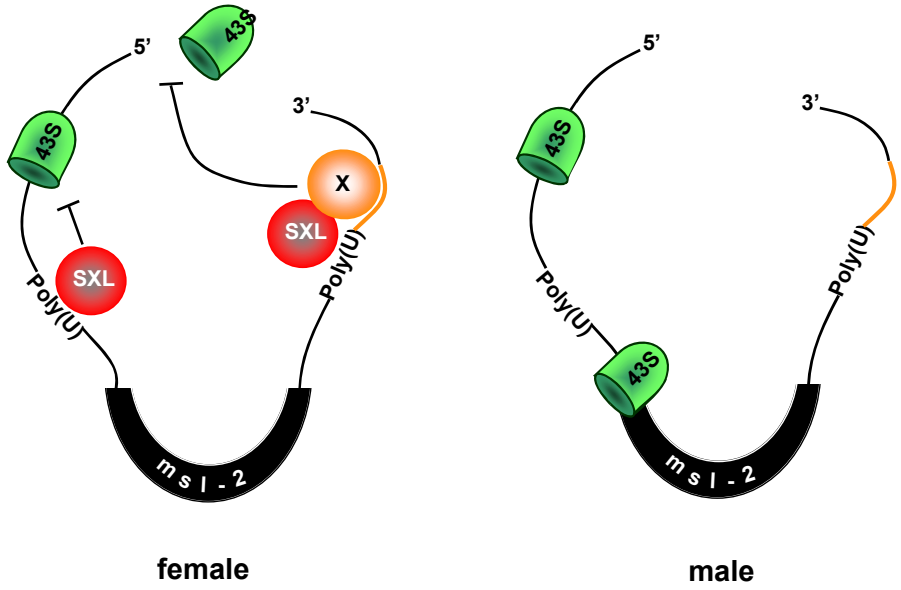


Figure 1.13. Model for *msl-2* mRNA translational repression. The female-specific protein SXL binds to poly(U) stretches in the 5' and the 3' UTRs of *msl-2* mRNA. SXL bound to the 3' UTR recruits co-repressor(s) to adjacent sequences and inhibits the association of the 43S ribosomal complex with the 5' end of the message, thereby repressing translation. SXL bound to the 5' UTR inhibits the scanning of ribosomal complexes that might have escaped the first block (Beckmann et al., 2005). In male flies, which lack SXL, no inhibitory complexes as assembled at the UTRs of *msl-2* mRNA and translation proceeds.

2. OBJECTIVES

The aim of this thesis is:

1. To identify and characterize the components of the co-repressor complex nucleated by SXL in the 3' UTR of msl-2 mRNA in order to gain insight into the mechanism of translational repression by SXL.

2. To determine putative broader functions of the co-repressors.

3. RESULTS

In this section, the scientific results on *Drosophila* UNR are presented in the form of research papers.

3.1 Publication 1.

Abaza, I., Coll, O., Patalano, S. and Gebauer, F. *Genes Dev. 2006 Feb 20:380-389.* "*Drosophila* UNR is required for translational repression of *male specific-lethal 2* mRNA during regulation of X chromosome dosage compensation*"*

3.2 Publication 2.

Duncan, K., Grskovic, M., Strein, C., Beckmann, K., Niggeweg, R., **Abaza, I.**, Gebauer, F., Wilm, M. and Hentze, M.W. *Genes Dev. 2006 Feb 20:368-379*."Sex-lethal Imparts a Sex-specific Function to UNR by Recruiting it to the *msl-2* mRNA 3' UTR: Translational Repression for Dosage Compensation"

3.3 Publication 3.

Abaza, I. and Gebauer, F. "Functional Domains of *Drosophila* UNR" (in preparation).

Abaza, I., Coll, O., Patalano, S. and Gebauer, F. "*Drosophila* UNR is required for translational repression of *male specificlethal 2* mRNA during regulation of X chromosome dosage compensation". *Genes Dev. 2006 Feb; 20(3):380-389.*

Duncan, K., Grskovic, M., Strein, C., Beckmann, K., Niggeweg, R., Abaza, I., Gebauer, F., Wilm, M. and Hentze, M.W. *"Sex-lethal Imparts a Sex-specific Function to UNR by Recruiting it to the msl-2 mRNA 3' UTR: Translational Repression for Dosage Compensation"*. *Genes Dev. 2006 Feb; 20(3):368-379*.

Functional Domains of Drosophila UNR

Irina Abaza and Fátima Gebauer*

Running title: Domains of dUNR

Keywords: msl-2; SXL; UNR; dosage compensation; translational control.

Centre de Regulació Genòmica
Passeig Marítim 37-49
08003 Barcelona
Spain

(*) Corresponding author:
phone: 0034-93-2240920
FAX: 0034-93-2240899
email: fatima.gebauer@crg.es

Abstract

Translational repression of *male-specific-lethal 2* (*msl-2*) mRNA by Sex-lethal (SXL) is an essential regulatory step of dosage compensation in *Drosophila*. Translation inhibition requires that SXL recruits the protein Upstream of N-ras (UNR) to the 3' UTR of *msl-2* mRNA. UNR is a conserved, ubiquitous protein that contains five cold shock domains. Here we dissect the domains of UNR required for translational repression and complex formation with SXL and *msl-2* mRNA. Using gel-mobility shift assays we have mapped the region involved in SXL and *msl-2* interactions specifically to the first cold shock domain (CSD1). Indeed, excess of a peptide containing this domain de-represses *msl-2* translation *in vitro*. This effect is reverted by addition of excess SXL, suggesting that the stoichiometry between SXL and UNR is critical for translational repression. The CSD1 of human UNR can also form a complex with SXL and *msl-2*. Analyses of the CSDs of the *Drosophila* and human proteins indicates that a specific set of exposed residues is responsible for complex formation. UNR can repress translation when tethered to *msl-2* mRNA, although at a lesser efficiency than when it is recruited by SXL. Tethering assays indicate that the translational effector domain of UNR is contained within the first 397 amino acids of the protein, and that complex formation with SXL and *msl-2* can be distinguished from translational repression.

Introduction

Translational control is widely used in development to regulate processes such as cell differentiation, synaptic plasticity, embryonic patterning, sex determination or dosage compensation (reviewed in Kuersten and Goodwin, 2003; Wilhelm and Smibert, 2005; Hentze et al., 2007). Dosage compensation is the process that equalizes the expression of X-linked genes in those organisms in which sex determination relies on highly dimorphic sex chromosomes (for review, see Larsson and Meller, 2006). In *Drosophila,* dosage compensation is achieved by increasing the transcriptional output of the single male X chromosome by ~ 2 fold, as a result of the activity of a ribonucleoprotein assembly known as the dosage compensation complex (DCC) or male-specific lethal (MSL) complex (Hamada et al., 2005; Straub et al., 2005). The DCC fails to assemble in females because the expression of one of its subunits, the protein MSL-2, is blocked (Kelley and Kuroda, 1995). The female-specific RNA-binding protein Sex-lethal (SXL) prevents *msl-2* expression via a dual mechanism that includes the inhibition of the splicing of a facultative intron in the 5' UTR of *msl-2* pre-mRNA, and the subsequent translational repression of the unspliced message (Bashaw and Baker, 1997; Kelley et al., 1997; Gebauer et al., 1998). Translational repression requires SXL binding to specific U-rich sequences in both the 5' and 3' UTRs of *msl-2* mRNA. SXL binding to the 3' UTR inhibits the recruitment of the small ribosomal subunit to the mRNA, while SXL binding to the 5' UTR blocks the scanning towards the AUG initiation codon of those subunits that presumably have escaped the 3' UTR control (Beckmann et al., 2005). How SXL inhibits these steps of translation initiation is unknown. Recently, a factor necessary for SXL-mediated translational repression has been identified as the protein Upstream of N-ras (UNR) (Abaza et al., 2006; Duncan et al., 2006). UNR is a conserved, ubiquitous protein that is recruited to the 3' UTR of *msl-2* by SXL, but its mechanism of action remains obscure.

Most of the current knowledge about UNR derives from the mammalian

system. Human UNR (hUNR) is involved in the destabilization of *c-fos* mRNA and the translational repression of *pabp* mRNA (Chang et al., 2004; Patel et al., 2005). In both cases, UNR interacts with PABP within complexes that bind to distinct regions in the target transcripts. Mammalian UNR also stimulates translation driven by the internal ribosome entry sites (IRESs) of a number of viral and cellular transcripts, including rhinovirus, poliovirus, c-myc, PITSLRE protein kinase, and the pro-apoptotic factor Apaf-1 (Hunt et al., 1999; Evans et al., 2003; Boussadia et al., 2003; Mitchell et al., 2003; Brown and Jackson, 2004; Tinton et al., 2005). At least in the case of Apaf-1, hUNR acts as an RNA chaperone, changing the conformation of the IRES to make it accessible to the activator PTB and, ultimately, the ribosome (Mitchell et al., 2003). RNA binding by hUNR is mediated by its five cold shock domains (CSDs), an ancient b-barrel fold containing RNP1 and RNP2 motifs (Brown and Jackson, 2004). Drosophila UNR (dUNR) contains an additional Q-rich amino-terminus that is absent in its mammalian counterpart.

In order to gain insight into the mechanism of *msl-2* translational repression, we have dissected the functional domains of dUNR. We show that dUNR interacts with SXL via a dedicated CSD, CSD1. A comparison between the SXL-binding activities of the CSDs of Drosophila and human UNR indicates that residues exposed outside the β-barrel drive the interaction. Tethering experiments show that the translational effector domain of dUNR is contained within the first 397 amino acids of the protein and can be distinguished from the SXL interaction domain. Indeed, the Q-rich N-terminus confers translational repression activity to CSDs 1 and 2, suggesting that the lack of this domain in mammalian UNR is responsible for the poor translational repression of *msl-2* observed in mammalian extracts.

Results

dUNR interacts with SXL via the CSD1

Although dUNR can interact with SXL in the absence of RNA, the interaction is stabilized by the binding of both factors to specific sites in *msl-2* 3' UTR that are contained within a region referred to as the EF RNA fragment (Abaza et al., 2006). In addition, binding of dUNR to *msl-2* mRNA requires the presence of SXL (Abaza et al., 2006; Duncan et al, 2006). Thus, interactions between dUNR and SXL can be monitored by the formation of an appropriate complex on EF RNA, which can be visualized by a super-shift of the SXL::EF complex in gel-mobility shift assays (GEMSA). Indeed, such super-shift is observed when a complex between a SXL fragment fully functional in translational repression (dRBD4) and EF RNA is challenged with full-length dUNR (Abaza et al., 2006). To determine the minimal region of dUNR that interacts with SXL -and *msl-2* mRNA- a collection of dUNR fragments were expressed as MBP-fusion proteins (Figure 1A) and tested by GEMSA (Figure 1B). As previously reported, dRBD4 binds to EF RNA with an apparent Kd in the nanomolar range, and addition of full-length dUNR super-shifts the complex (Figure 1B, lanes 2-6). While addition of MBP or dUNR fragments corresponding to the C-terminal two thirds of the protein has no effect (lanes 15-18 and 23-33), addition of fragments containing CSD1 or CSD1 alone super-shift of the dRBD4::EF complex (lanes 7-14 and 19-22, respectively). These results indicate that CSD1 mediates the interaction of dUNR with SXL and *msl-2* mRNA.

Exposed residues within the CSD1 interact with SXL

To map more finely the residues that interact with SXL within dUNR CSD1, we took advantage of the fact that mammalian UNR shares an overall identity of 45% with its *Drosophila* homologue. The conservation is higher in the CSDs, rising to 70.6% in the case of CSD1, while hUNR is devoid of Q-rich

regions (Figure 2A). GEMSA assays indicate that, contrary to dUNR, hUNR binds to *msl-2* in the absence of dRBD4 (Figure 2B, lanes 1-4). However, similar to *Drosophila*, hCSD1 in isolation does not bind to *msl-2* but requires dRBD4 (compare lanes 5-8 with 9-12). In addition, as for the negative controls, hCSD2 does not produce a super-shift of the dRBD4::RNA complex (lanes 13-24). These data indicates that, similar to *Drosophila*, hCSD1 -but not hCSD2- interacts with SXL. Thus, residues present in the first CSDs but absent in the second CSDs of UNR from both species should be responsible for the interaction with SXL. This group of residues is shown in Figure 3A (highlighted in yellow). Modeling the position of these residues on the structure of the bacterial cold shock protein A (CspA), which is basically composed of one CSD, shows that they are highly exposed in the outer surface of the b-barrel, concentrated in loops 2 and 4 at one edge of the cylinder (Figure 3B) and outside of RNPs.

The relative stechiometry between SXL and dUNR is important for translational repression *in vitro*

Although CSD1 is responsible for the interaction of dUNR with SXL, other domains could contribute to translational repression. To evaluate the functional significance of the various dUNR fragments in translation inhibition, we first tested the effect of adding them in excess to an *in vitro* translation reaction programmed with BLEF mRNA. The BLEF transcript contains the ORF of *Firefly* luciferase fused to the minimal sequences of *msl-2* mRNA required for translational repression (see Figure 5A). Translation of BLEF was efficiently repressed by dRBD4 (Figure 4A, compare lanes 1 and 2). Addition of increasing amounts of full-length dUNR de-repressed translation in a dose-dependent manner (lanes 3-7). The same effect was observed when CSD1 or dUNR fragments containing CSD1 were added to the reaction (lanes 18-22 and data not shown). However, addition of dUNR deletion derivatives lacking CSD1 did not alter translational repression (lanes 8-17 and 23-30), as observed with the unrelated protein MBP (lanes 31-35). Although these data do not rule out a function for other dUNR fragments in translational repression, they highlight the

functional relevance of CSD1 and suggest that CSD1 alone can titrate the interaction of dUNR with SXL within the translation repressor complex.

Adding increasing amounts of dRBD4 to a reaction de-repressed by excess dUNR recovered translation inhibition, indicating that a given stechiometry between dUNR and SXL must be preserved for efficient translational repression and suggesting that SXL and dUNR are the limiting components of the repressor complex assembled on *msl-2* 3' UTR (Figure 4B).

The Q-rich N-terminal domain of dUNR confers the translational repressor activity

To identify the translational repressor domain of dUNR, we performed functional tethering analysis. dUNR was fused to the bacteriophage MS2 coat protein, which binds with high affinity to a specific stem-loop structure -the MS2 site- that was placed in several copies at the 3' UTR of *msl-2* reporters (Figure 5A). Tethering dUNR to the 3' UTR of *msl-2* in this manner allows the analysis of the dUNR domains on translation, independently on RNA- binding or SXL interaction. The ability of the MS2-dUNR fusion protein to repress the translation of reporters containing or lacking MS2 and SXL binding sites was tested *in vitro* (Figure 5). Tethered dUNR repressed the translation of the MS2 reporter mRNAs by 40% (Figure 5B, middle and right panel, blue lines). This repression was specific because: i) MS2-dUNR did not repress the translation of BLEF (left panel, blue line), and ii) the translation of reporter mRNAs containing MS2 sites was not repressed by an unrelated MS2 fusion protein (MS2-MBP, observe the black lines in the three panels). As expected, dRBD4 did not contribute to translational repression by the 3' UTR of MS2 reporters (right panel, red line). However, when SXL-binding sites were provided in the 5' UTR, dRBD4 repressed translation to a similar level as with the reference BL(EF)mut mRNA (compare the red line and the discontinuous black line in the middle panel), indicating that the MS2 sites placed at the 3' UTR do not interfere with repression by the 5' UTR. Furthermore, when dRBD4 and dUNR were added together to a reporter containing binding sites for both, an additive effect was observed (Figure

5C). Repression by tethered dUNR was less efficient than repression by SXL, both when the 5' and 3' UTRs where provided, or only the 3' UTR were considered. Middle and right panels are referenced to the BL(EF)mut and BmutLEF inhibition by dRBD4, respectively representing 5’ and 3’ UTR contribution to inhibition strength. Only when SXL binding sites were provided in the 5’ UTR (B site) the MS2 reporter mRNA is repressed to 20% by dRBD4 (Figure 3B, middle panel, red line). The level of inhibition is increased by 20% after addition of dRBD4 to the already inhibited RNA by MS2dUNR (Figure 3C). These results indicate that: MS2dUNR can repress translation in the absence of SXL and that the tethered complex at the 3’ UTR does not interfere with translational repression mediated by the 5’ UTR.

Translational repressor domain of dUNR is distinct from dSXL interacting domain

To determine which domains of UNR are required for translational repression of msl-2 mRNA we fused fragments of dUNR to the MS2 coat protein (Figure 1B), and analyzed their ability to inhibit translation of the same test RNA described above (Figure 3A). The domain of dUNR that attained translational repression activity proved not to encompass only the dSXL minimal interacting domain (CSD1). Derivative containing N terminal Q rich region fused to the CSD1 and CSD2 showed to be sufficient for translational repression of *msl-2* mRNA (Figure 4, NCSD12), while by itself N termini could not deliver any effect on translation. Even so, the repression level obtained is not strong as having SXL and the dUNR bound to the wild type 3’UTR sequences of the *msl-2* mRNA (Figure 3C, grey bar), suggesting necessity of a proper complex formation.

Discussion

Inhibition of *msl-2* expression is essential for development of female *Drosophila* flies, since expression of MSL-2 causes the assembly of the DCC on both X chromosomes and lethality (Kelley and Kuroda, 1995). A complex of SXL and dUNR binds to the 3' UTR of *msl-2* mRNA and plays an essential role in the repression of its translation (Abaza et al., 2006; Duncan et al., 2006). We have analyzed the functional domains of dUNR and show that interaction with SXL and translational repression are carried by distinguishable domains.

Gel mobility-shift assays indicate that dUNR interacts with SXL and *msl-2* mRNA via CSD1 (Figures 1-2). The CSD is a domain highly conserved in evolution used to bind single stranded nucleic acids (Ermolenko and Makhatadze, 2002). Indeed all five CSDs of hUNR are required to bind to the rhinovirus IRES (Brown and Jackson, 2004). In addition, the CSD can support protein-protein interactions. For example, hUNR has been shown to interact with the trithorax homolog ALL-1 via a region encompassing CSD2. In the case of dUNR, CSD1 sustains both functions (Figure 1). The ability to do so probably resides in that the particular residues involved in the interaction with SXL are exposed at one edge of the b-barrel (Figure 3). Our data, however, do not rule out the possibility that other domains of dUNR contribute separately to either SXL interaction or RNA binding, because the detection of a super-shifted complex in GEMSA depends on the faculty to attain both activities. Despite the conservation of the CSDs, human and *Drosophila* UNRs have different RNA binding properties because-unlike its *Drosophila* homolog- hUNR can bind to *msl-2* mRNA in the absence of SXL (Figure 2).

Although CSD1 is sufficient for stable recruitment of dUNR to the 3' UTR of *msl-2*, it does not suffice for translational repression (Figure 6). Rather, the Q-rich N-terminal domain confers translational repression to the CSDs. Q-rich domains are present in proteins with diverse roles in gene expression, and serve as protein-protein interaction and multimerisation modules (Emili et al., 1994; Pascal and Tjian, 1991; Stott et al., 1995; Strom et al., 1996; Wilkins and Lis, 1999; McBride and Silver, 2001). TIA-1, a splicing and translation regulator, contains a Q-rich C-terminal domain that interacts with the protein U1C facilitating the recruitment of the U1 snRNP to the 5' splice site (Forch et al., 2002). By analogy, the Q-rich domain of dUNR could facilitate the recruitment of co-repressors, or components of the translation machinery that are so sequestered, to the 3' UTR of *msl-2*. One such component could be PABP. This translation factor has been shown to interact with hUNR in complexes binding to the coding region of *c-fos* mRNA and the 5' UTR of *pabp* mRNA, which are involved in destabilization and translational repression, respectively (Chang et al., 2004; Patel et al., 2005). However, it is not immediately obvious how PABP recruitment to the 3' UTR of *msl-2* would result in its inactivation, because PABP stimulates translation when tethered to the 3' as it does when it binds to the poly(A) tail (Gray et al., 2000).

Translational repression by tethered dUNR is less efficient than that by SXL (Figure 5), suggesting that SXL function in 3' UTR-mediated repression is not limited to the recruitment of dUNR. Alternatively, the lesser efficiency of dUNR could be due to improper conformation of the recombinant protein or to geometry constraints imposed on the tethered complex. Indeed, even though SXL is critical for *msl-2* translational repression, it does not function when tethered to the 3' UTR (Grskovic et al., 2003).

The balance between SXL and dUNR is critical for translation inhibition. Excess dUNR de-represses translation in a dose dependent manner, and translational repression is recovered by restituting the relative levels of SXL

(Figure 4). The observation that excess CSD1, which does not function in translational repression, has a similar squelching effect argues that it is the SXL::dUNR complex, and not a translation factor, that is limiting for repression in vitro.

In summary, our data delimit the functional domains of dUNR in *msl-2* translational repression. Finding out which factors interact with the translational effector domain will help us gain insight into the molecular mechanism of translation inhibition by this essential factor.

Materials and Methods

Plasmids

BLEF and BL(EF)mut have been previously described (Gebauer et al., 2003). BmutLEF was obtained by exchanging the 5' UTR of BLEF with a similar segment generated by hybridization of complementary oligonucleotides containing site B (T_{16}) substituted by $(CT)_8$. BLMS2 and BmutLMS2 were generated by replacing the 3' UTRs of BLEF and BmutLEF, respectively, with 9 copies of the MS2 binding site obtained from plasmid pLucMS2(9) (Collier et al., 2005).

dUNR deletion derivatives were obtained by PCR amplification of relevant fragments from plasmid pET15b-dUNR(Abaza et al., 2006) and were cloned into pET-15b (Novagen) , pET-30a (Novagen) and pMALc (New England Biolabs).
To generate MS2-dUNR, MS2 was amplified by PCR from pMALc-MS2 (a kind gift from Josep Vilardell) and cloned into the NdeI site of the FL plasmid, containing full-length dUNR (Abaza et al., 2006). The resulting protein contains MS2 fused to His-FLAG-tagged dUNR. MS2-dUNR deletion derivatives were obtained by PCR amplification of dUNR fragments that were cloned into the AgeI and XhoI sites of pET30a-MS2 GFP (Bertrand et al., 1998) that in addition to His

contains S• tag.

The pET-hUNR plasmid has been previously described (Mitchell et al., 2001). hUNR deletion derivatives were obtained by PCR amplification and cloned into pET30a-MS2 to obtain MS2-hUNR fusions.

Protein expression and purification

dRBD4 was expressed in *E. coli* as an N-terminal GST-tagged fusion and purified as described previously (Grskovic et al., 2003). Full-length dUNR, hUNR, MS2 fusion proteins, and deletion derivatives were expressed as N-terminal His-tagged fusions and purified following the pET system user´s manual (Novagen). In the case of full-length dUNR, its derivatives (N, csd2, csd12, Ncsd12 and csd345), hUNR and its derivative hcsd2, the proteins were purified under denaturing conditions. In addition to this Drosophila UNR derivatives (csd2, csd12,Ncsd12 and csd345) were submitted to an additional purification using S-protein agarose following user´s manual for elution under low pH conditions (Novagen). MBP- dUNR derivatives were purified following the pMALc system user's manual (New England Biolabs). All proteins were dialyzed against buffer D (20 mM HEPES pH 8.0, 20% glycerol, 1 mM DTT, 0.2 mM EDTA, 0.01% NP-40).

In vitro translation

In vitro translation reactions in *Drosophila* embryo extracts were performed as described (Abaza et al, 2006). *Renilla* mRNA was co-translated as an internal control. The translation efficiency was determined by measuring the luciferase activity using the Dual Luciferase Assay System (Promega), and *Firefly* luciferase values were corrected for *Renilla* expression.

For translation competition assays, *Drosophila* embryo extracts were supplemented with increasing amounts of recombinant full-length dUNR, or its derivatives, prior to assemble of translational reaction adding dRBD4 and test RNAs.

Gel mobility-shift assays

^{32}P-labelled msl-2 3´UTR RNA (positions 909- 954, containing SXL-binding sites E and F) was incubated with increasing amounts of recombinant dRBD4 and/or dUNR, hUNR or their derivatives, and processed as described previously (Valcarcel et al., 1993).

Acknowledgments

We are grateful to Drs Nicola Gray, Anne Willis, Raúl Mendez, and Josep Vilardell for generously providing pBSK-Luc-MS2, pET21d-hUNR, pET31a-MS2-GFP and pMAL-MBP-MS2 plasmids, respectively. I. A. was supported by a FI-IQUC fellowship from the Generalitat of Catalunya. This work was supported by grants BMC203-04108 from the Spanish Ministry of Science and Technology and 2005SGR00669 from the Department of Universities, Information and Sciences of the Generalitat of Catalunya (DURSI).

References

Abaza, I., Coll, O., Patalano, S., and Gebauer, F. (2006). Drosophila UNR is required for translational repression of male-specific lethal 2 mRNA during regulation of X-chromosome dosage compensation. Genes Dev *20*, 380-389.

Bashaw, G. J., and Baker, B. S. (1997). The Regulation of the Drosophila msl-2 Gene Reveals a Function for Sex-lethal in Translational Control. Cell *89,*, 789–798.

Beckmann, K., Grskovic, M., Gebauer, F., and Hentze, M. W. (2005). A dual inhibitory mechanism restricts msl-2 mRNA translation for dosage compensation in Drosophila. Cell *122*, 529-540.

Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S. M., Singer, R. H., and Long, R. M. (1998). Localization of ASH1 mRNA particles in living yeast. Mol Cell *2*, 437-445.

Boussadia, O., Niepmann, M., Creancier, L., Prats, A. C., Dautry, F., and Jacquemin-Sablon, H. (2003). Unr is required in vivo for efficient initiation of translation from the internal ribosome entry sites of both rhinovirus and poliovirus. J Virol *77*, 3353-3359.

Brown, E. C., and Jackson, R. J. (2004). All five cold-shock domains of unr (upstream of N-ras) are required for stimulation of human rhinovirus RNA translation. Journal of General Virology *85*, 2279–2287.

Chang, T. C., Yamashita, A., Chen, C. Y., Yamashita, Y., Zhu, W., Durdan, S., Kahvejian, A., Sonenberg, N., and Shyu, A. B. (2004). UNR, a new partner of poly(A)-binding protein, plays a key role in translationally coupled mRNA turnover mediated by the c-fos major coding-region determinant. Genes Dev *18*, 2010-2023.

Collier, B., Gorgoni, B., Loveridge, C., Cooke, H. J., and Gray, N. K. (2005). The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells. Embo J *24*, 2656-2666.

Duncan, K., Grskovic, M., Strein, C., Beckmann, K., Niggeweg, R., Abaza, I., Gebauer, F., Wilm, M., and Hentze, M. W. (2006). Sex-lethal imparts a sex-specific function to UNR by recruiting it to the msl-2 mRNA 3' UTR: translational repression for dosage compensation. Genes Dev *20*, 368-379.

Emili, A., Greenblatt, J., and Ingles, C. J. (1994). Species-specific interaction of the glutamine-rich activation domains of Sp1 with the TATA box-binding protein. Mol Cell Biol *14*, 1582-1593.

Ermolenko, D. N., and Makhatadze, G. I. (2002). Bacterial cold-shock proteins. Cell Mol Life Sci *59*, 1902-1913.

Evans, J. R., Mitchell, S. A., Spriggs, K. A., Ostrowski, J., Bomsztyk, K., Ostarek, D., and Willis, A. E. (2003). Members of the poly (rC) binding protein family stimulate the activity of the c-myc internal ribosome entry segment in vitro and in vivo. Oncogene *22*, 8012-8020.

Forch, P., Puig, O., Martinez, C., Seraphin, B., and Valcarcel, J. (2002). The splicing regulator TIA-1 interacts with U1-C to promote U1 snRNP recruitment to 5' splice sites. Embo J *21*, 6882-6892.

Gebauer, F., Gskovic, M., and Hentze, M. (2003). *Drosophila* Sex-Lethal inhibits the stable association if the 40S ribosomal subunit with msl-2 mRNA. Molecular Cell *11*, 1397-1404.

Gebauer, F., Merendino, L., Hentze, M., and Valcarcel, J. (1998). The *Drosophila* splicing regulator sex-lethal directly inhibits translation of *male-specific-lethal 2* mRNA. RNA *4*, 142-150.

Gray, N. K., Coller, J. M., Dickson, K. S., and Wickens, M. (2000). Multiple portions of poly(A)-binding protein stimulate translation in vivo. Embo J *19*, 4723-4733.

Grskovic, M., Hentze, M. W., and Gebauer, F. (2003). A co-repressor assembly nucleated by Sex-lethal in the 3'UTR mediates translational control of Drosophila msl-2 mRNA. Embo J *22*, 5571-5581.

Hamada, F. N., Park, P. J., Gordadze, P. R., and Kuroda, M. I. (2005). Global regulation of X chromosomal genes by the MSL complex in Drosophila melanogaster. Genes Dev *19*, 2289-2294.

Hunt, S. L., Hsuan, J. J., Totty, N., and Jackson, R. J. (1999). unr, a cellular cytoplasmic RNA-binding protein with five cold-shock domains, is required for internal initiation of translation of human rhinovirus RNA. Genes & Development *13*, 437-448.

Hentze, M. W., Gebauer, F., and Preiss, T. (2007). Cis-regulatory sequences and trans-acting factors in translational control. In Translational control in Biology and Medicine (Cold Spring Habor Laboratory Press).

Kelley, R., Wang, J., Bell, L., and Kuroda, M. (1997). *Sex-lethal* controls dosage copensation in *Drosophila* by a non-splicing mechanism. Nature *387*, 195-199.

Kelley, R. L., and Kuroda, M. (1995). Equality for X chromosomes. Science *270*, 1607-1610.

Kuersten, and Goodwin (2003). The power of the 3' UTR: translational control and development. Nat Rev Genet *4*, 626-637.

Larsson, J., and Meller, V. H. (2006). Dosage compensation, the origin and the afterlife of sex chromosomes. Chromosome Res *14*, 417-431.

McBride, A. E., and Silver, P. A. (2001). State of the arg: protein methylation at arginine comes of age. Cell *106*, 5-8.

Mitchell, S. A., Brown, E. C., Coldwell, M. J., Jackson, R. J., and Willis, A. E. (2001). Protein factor requirements of the Apaf-1 internal ribosome entry

segment: roles of polypyrimidine tract binding protein and upstream of N-ras. Mol Cell Biol *21*, 3364-3374.

Mitchell, S. A., Spriggs, K. A., Coldwell, M. J., Jackson, R. J., and Willis, A. E. (2003). The Apaf-1 internal ribosome entry segment attains the correct structural conformation for function via interactions with PTB and unr. Mol Cell *11*, 757-771.

Pascal, E., and Tjian, R. (1991). Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev *5*, 1646-1656.

Patel, G. P., Ma, S., and Bag, J. (2005). The autoregulatory translational control element of poly(A)-binding protein mRNA forms a heteromeric ribonucleoprotein complex. Nucleic Acids Res *33*, 7074-7089.

Stott, K., Blackburn, J. M., Butler, P. J., and Perutz, M. (1995). Incorporation of glutamine repeats makes protein oligomerize: implications for neurodegenerative diseases. Proc Natl Acad Sci U S A *92*, 6509-6513.

Straub, T., Gilfillan, G. D., Maier, V. K., and Becker, P. B. (2005). The Drosophila MSL complex activates the transcription of target genes. Genes Dev *19*, 2284-2288.

Strom, A. C., Forsberg, M., Lillhager, P., and Westin, G. (1996). The transcription factors Sp1 and Oct-1 interact physically to regulate human U2 snRNA gene expression. Nucleic Acids Res *24*, 1981-1986.

Tinton, S. A., Schepens, B., Bruynooghe, Y., Beyaert, R., and Cornelis, S. (2005). Regulation of the cell-cycle-dependent internal ribosome entry site of the PITSLRE protein kinase: roles of Unr (upstream of N-ras) protein and phosphorylated translation initiation factor eIF-2alpha. Biochem J *385*, 155-163.

Valcarcel, J., Singh, R., Zamore, P. D., and Green, M. R. (1993). The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of transformer pre-mRNA. Nature *362*, 171-175.

Wilhelm, J. E., and Smibert, C. A. (2005). Mechanisms of translational regulation in Drosophila. Biol Cell *97*, 235-252.

Wilkins, R. C., and Lis, J. T. (1998). GAGA factor binding to DNA via a single trinucleotide sequence element. Nucleic Acids Res *26*, 2672-2678.

Figure legends

Figure 1. dUNR interacts with SXL and *msl-2* mRNA via CSD1. **(A)** Schematic diagram of dUNR and the derivatives used in this study. dUNR contains five cold shock domains (1-5, blue boxes) and two Glutamine-rich regions (Q, yellow boxes). The positions of the first and last amino acid residues of each construct are indicated. **(B)** Binding of dUNR and its derivatives to radiolabelled EF RNA in GEMSA. Recombinant dUNR derivatives were expressed as MBP fusions and were added in increasing amounts (indicated at the bottom) in the absence or presence of 10 nM dRBD4. MBP alone was included as a control. Full length UNR (FL) was expressed as a His-tagged fusion. The positions of the different complexes are indicated.

Figure 2 . The CSD1 of hUNR interacts with SXL. **(A)** Schematic representation of hUNR and its fragments. The CSDs are denoted in blue, and the positions of the first and last amino acids of each construct as well as the conservation of each CSD are indicated. The hUNR construct contains a small deletion of 31 amino acids between CSDs 1 and 2, and hence is called hUNRΔ. **(B)** Binding of hUNRΔ and its derivatives to radiolabelled EF RNA in GEMSA. Recombinant proteins were expressed as MS2 fusions and were added in increasing amounts in the absence or presence of 10 nM dRBD4. MS2MBP alone was included as a negative control. The positions of the different complexes are indicated.

Figure 3. Residues of dUNR interacting with SXL. **A)** Alignment of the CSDs 1-5 of dUNR with hCSD1 using ClustalW (EBI). The RNP motifs (RNP1 and RNP2) are underlined in green. Dark yellow color indicates the putative SXL interacting amino acids. **B)** Stereo-view of CSD1 taking as a model the structure of bacterial CspA. Five anti-parallel β-strands (pink arrows) give an overall β-barrel fold. The putative SXL interacting residues are depicted in yellow matching the alignment

shown in A). Amino acid residues of interest localize outside of RNA binding motifs.

Figure 4. The relative stoichiometry of SXL and dUNR is important for translational repression of *msl-2* mRNA *in vitro*. **(A)** Excess recombinant dUNR, or CSD1, de-represses translation. BLEF mRNA was incubated in typical translation reactions in the absence or presence of 15-fold molar excess of dRBD4, and increasing amounts (0.5, 1, 3, 10 and 30 molar excess over dRBD4) of recombinant full-length dUNR (FL) or its deletion derivatives (N, CSD345, CSD1 and CSD2). MBP was carried as a negative control. *Firefly* luciferase values were corrected for co-translated *Renilla* expression. The activity obtained in the absence of recombinant protein was taken as 100%. **(B)** Translation inhibition by SXL is restored upon addition of recombinant dRBD4 to a de-repressed reaction. BLEF mRNA was incubated in typical translation reactions containing 10-fold molar excess of dUNR over the initial amount of the dRBD4. To this reaction, increasing amounts of dRBD4 (0.5, 1, 3 10 and 30 molar excess over dUNR) where added, and the translational rate was measured and plotted as described in A).

Figure 5. Tethered dUNR represses translation. **A)** Schematic diagram of the RNA constructs used in this study. BLEF mRNA contains the minimal *msl-2* sequences required for translational repression, consisting of 69 nt in the 5' UTR containing site B and 46 nt in the 3' UTR containing sites E and F, fused to the *Firefly* luciferase open reading frame (Gebauer et al., 2003). The SXL binding sites are indicated by black ovals. Mutated SXL binding sites are indicated by red ovals. Hairpins denote the substitution of the 3' UTR by 9 MS2 binding sites. **B)** Tethered dUNR specifically represses translation in the absence of dRBD4. BLEF, BLMS2 and BmutLMS2 mRNAs were translated in *Drosophila* embryo extracts in the presence of increasing amounts of either MS2dUNR (blue lines) or dRBD4 (red lines). MS2MBP was used as a negative control (black lines). The translation efficiencies of BL(EF)mut and BmutLEF RNAs in the presence of

increasing amounts of SXL were taken as a reference (dashed black lines). The translation rate was measured as indicated in the legend of Figure 4, and was plotted against the molar ratio of recombinant protein/RNA. **C)** Tethered dUNR can cooperate with dRBD4 to inhibit *msl-2* mRNA translation. BLMS2 mRNA was incubated in the absence or presence of 15 nM MS2-dUNR and 910 nM dRBD4. The translation efficiency of BLEF in the presence of 30 nM dRBD4 is shown as a reference (grey bar).

Figure 6. The translational repressor domain of dUNR is embedded within its amino-terminal 397 amino acids. The translation inhibition of BmLMS2 mRNA by increasing amounts of tethered MS2-dUNR derivatives was measured. The result obtained at a 30-fold molar excess of protein to RNA is shown. Consistent results where obtained when BLMS2 mRNA was used (not shown). Translation assays were performed as described in the legend of Figure 4.

Figure 1.

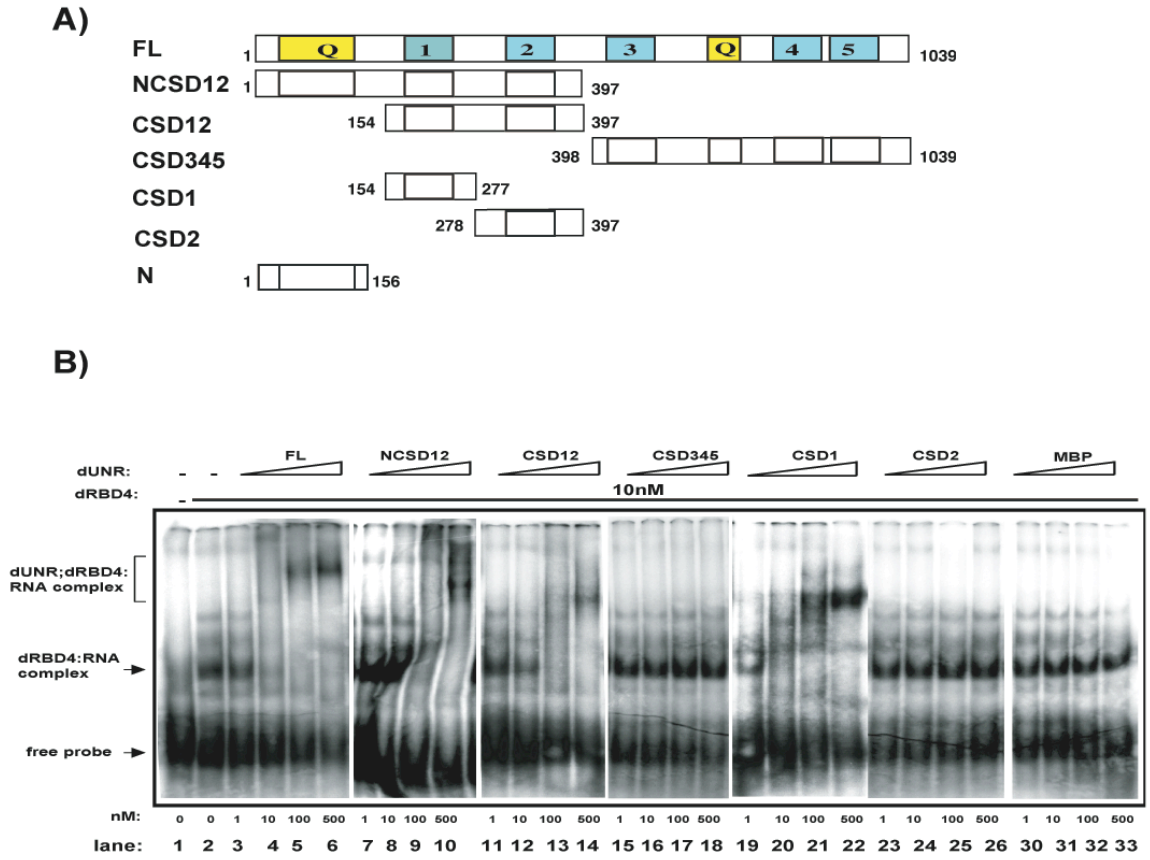

A)
FL
NCSD12
CSD12
CSD345
CSD1
CSD2
N
Q
1
2
3
Q
4
5
1039
397
154
398
277
278
156
B)
dUNR:
dRBD4:
FL
NCSD12
CSD12
CSD345
CSD1
CSD2
MBP
10nM
dUNR;dRBD4: RNA complex
dRBD4:RNA complex
free probe
nM:
lane:

Figure 2.

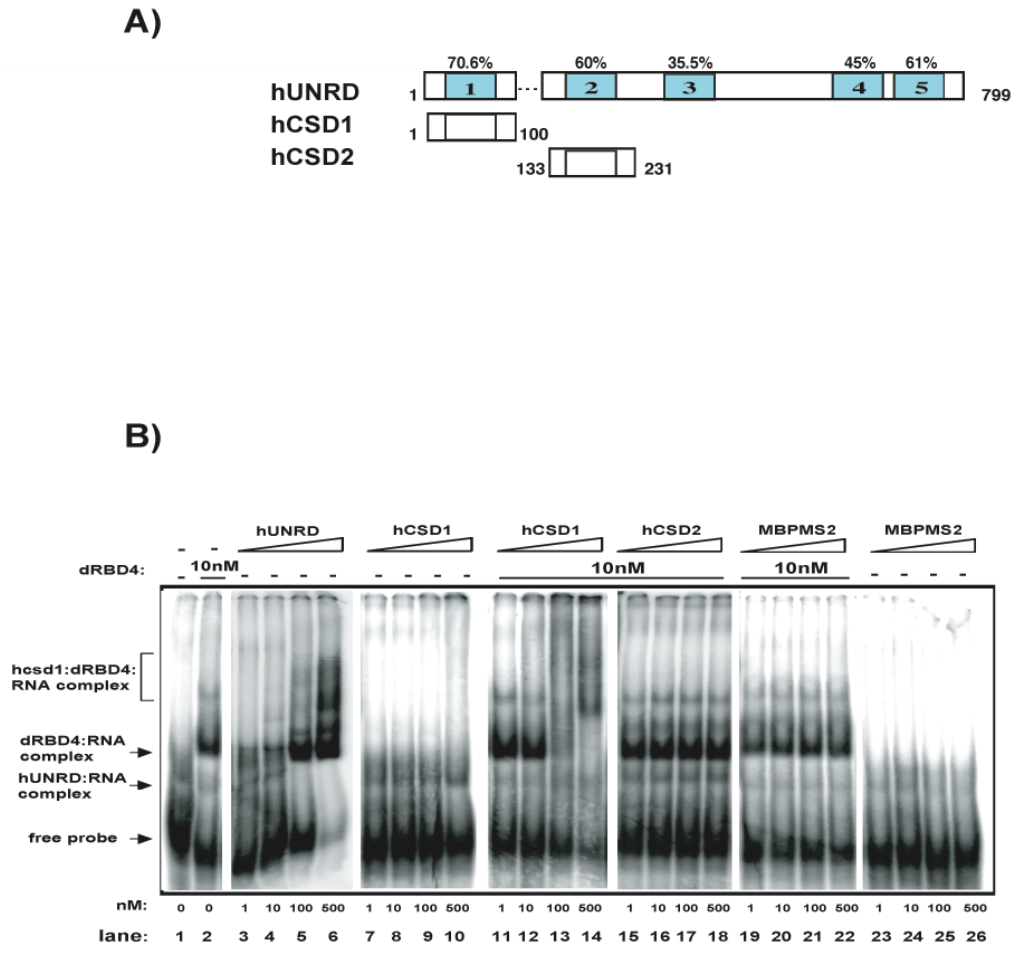

A)
70.6%
60%
35.5%
45%
61%
hUNRD
1
1
2
3
4
5
799
hCSD1
1
100
hCSD2
133
231
B)
hUNRD
hCSD1
hCSD1
hCSD2
MBPMS2
MBPMS2
dRBD4:
10nM
10nM
10nM
hcsd1:dRBD4:
RNA complex
dRBD4:RNA
complex
hUNRD:RNA
complex
free probe
nM:
0 0 1 10 100 500 1 10 100 500 1 10 100 500 1 10 100 500 1 10 100 500 1 10 100 500
lane:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Figure 3.

A)

```
dCSD1   198 ----------YGFIQC-CERQARLFFHFSQFSGNIDHLKIGDPVEFEMTYDRRTGKPIASQV- 248
hCSD1    37 ----------YGFIQC-SERQARLFFHCSQYNGNLQDLKVGDDVEFEVSSDRRTGKPIAVKL- 87
dCSD3   520 EQGTIASLKEGFGFLRC-VERQARLFFHFTEVLDTSREIDINDEVEFTVIQEP---------- 569
dCSD5   923 ----------QFGFLNFEVEDGKKLFFHMSEVQGNTVALHPGDTVEFSVVTNQRNGKSSACNVL 984
dCSD2   354 --------KESFGFIER-ADVVKEIFFHFSEAEGNVE-LRPGDDVEFTIQTRSSASVPPQ---- 405
hCSD2   197 ----------FGFIER-GDVVKEIFFHYSEFKGDLETLQPGDDVEFTIKDRNGKEVATDV--- 245
dCSD4   775 ----------FGFIET-LSHDEEVFFHFSNYMGNPNWLELGQEVEYTLARNGNTSVSGNCL-- 824
                     RNP1          RNP2
```

B)

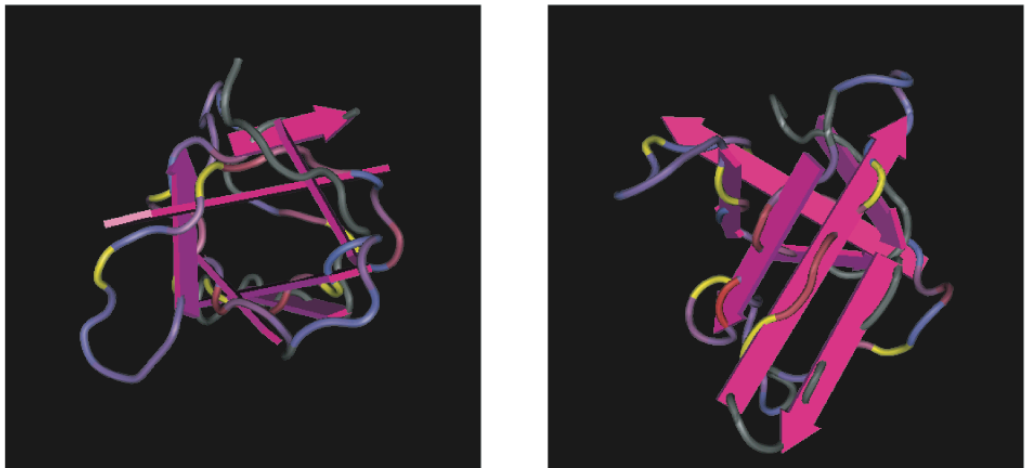


Figure 4.

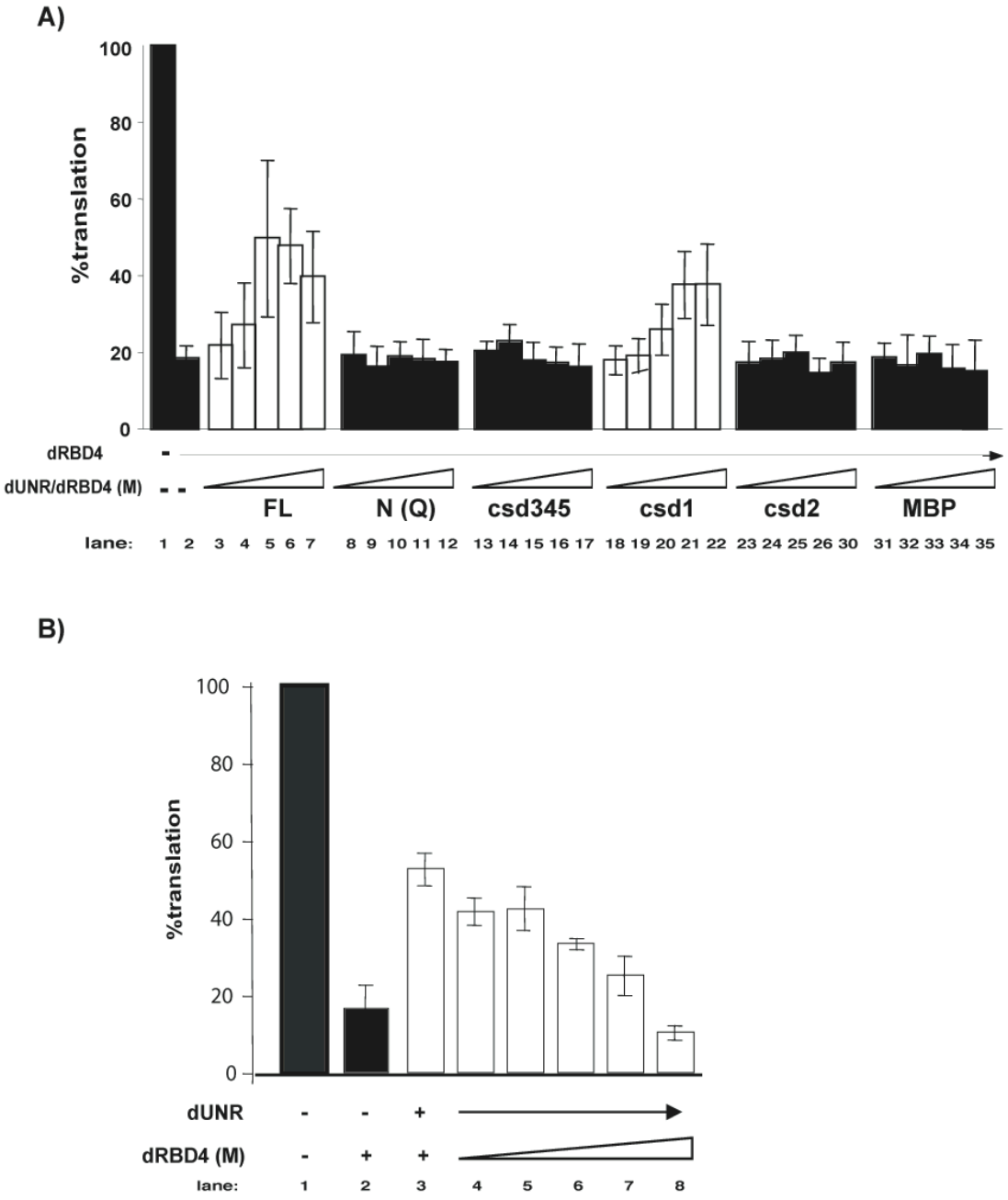

A)
100
80
60
40
20
0
%translation
dRBD4
-
dUNR/dRBD4 (M)
- -
FL
N (Q)
csd345
csd1
csd2
MBP
lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 30 31 32 33 34 35
B)
100
80
60
40
20
0
%translation
dUNR - - +
dRBD4 (M) - + +
lane: 1 2 3 4 5 6 7 8

Figure 5.

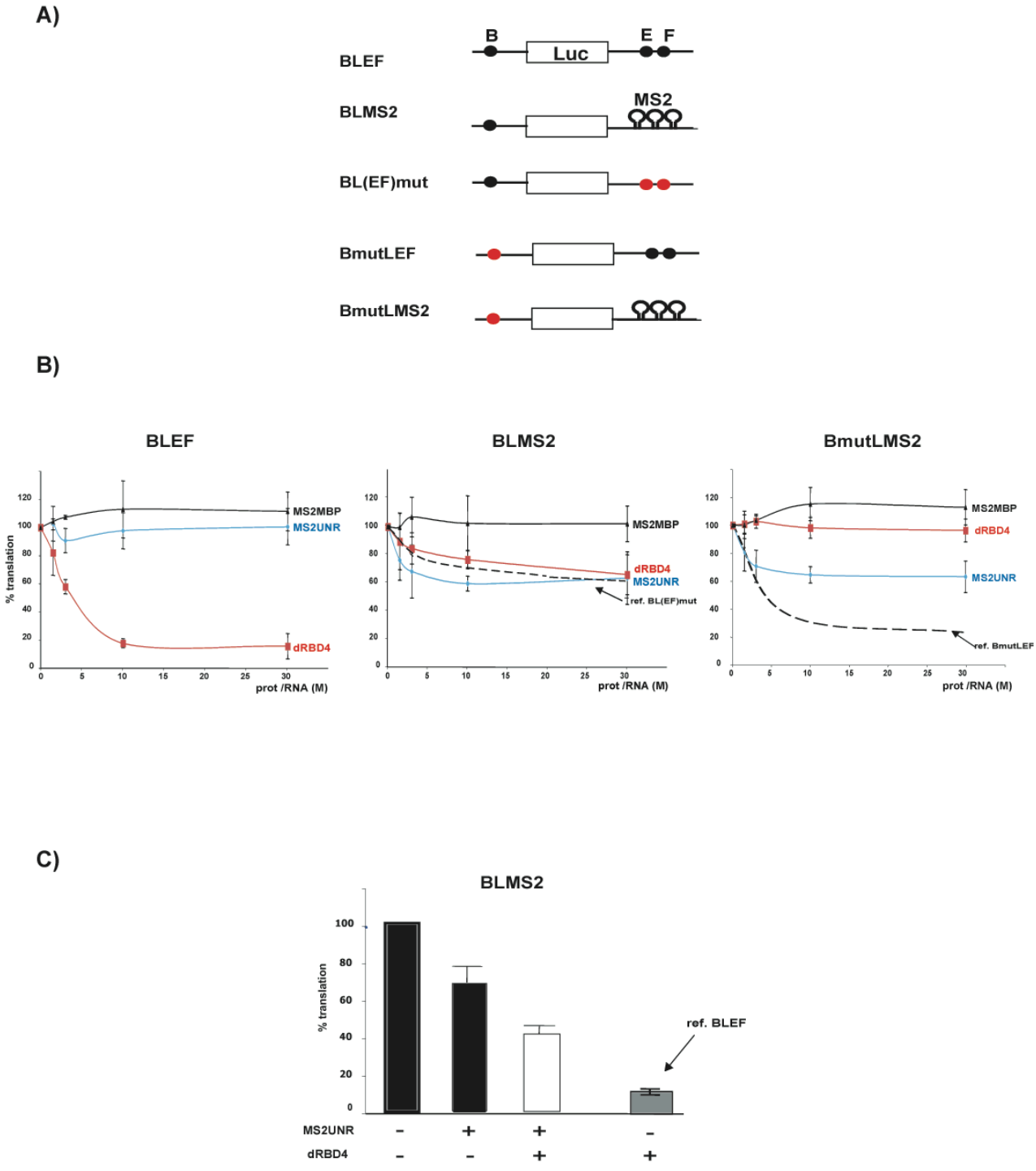
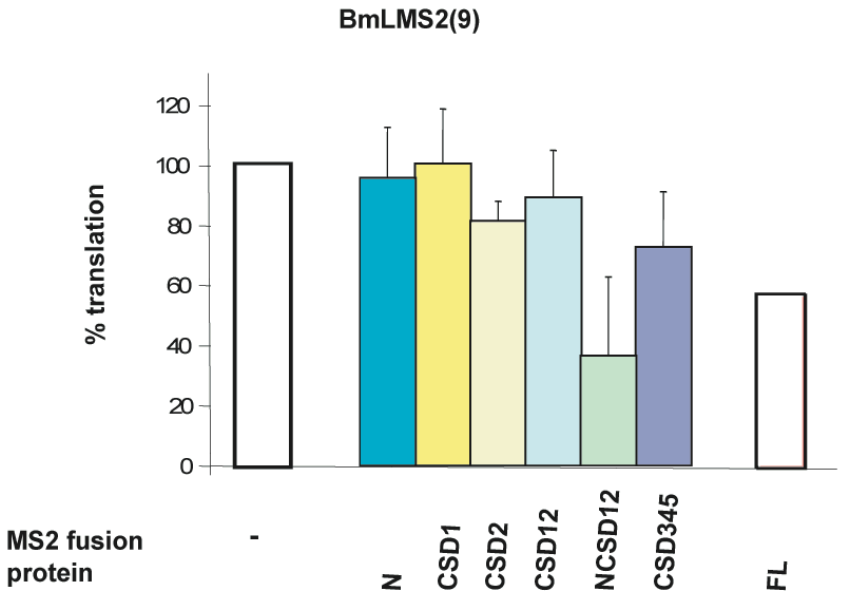

A)
B
Luc
E F
BLEF
BLMS2
MS2
BL(EF)mut
BmutLEF
BmutLMS2
B)
BLEF
BLMS2
BmutLMS2
% translation
prot /RNA (M)
MS2MBP
MS2UNR
dRBD4
ref. BL(EF)mut
ref. BmutLEF
C)
BLMS2
% translation
ref. BLEF
MS2UNR
dRBD4

Figure 6.


BmLMS2(9)
% translation
120
100
80
60
40
20
0
MS2 fusion protein
-
N
CSD1
CSD2
CSD12
NCSD12
CSD345
FL

3.4 APPENDICES

Appendices are used here to present additional data not yet published. They are relevant to the research performed and offer new possibilities for future studies.

APPENDIX I

Factors in Addition to SXL and dUNR are Required for Translational Repression of *msl-2* mRNA.

APPENDIX II

Effect of The Poly(A) Tail on SXL- mediated Repression.

APPENDIX III

dUNR Regulatory Networks.

APPENDIX I

Evidence for Additional Factor(s) Required for Translational Repression of msl-2 mRNA

The minimal fragment of SXL that exerts full translational repression activity consists of the RNA-binding domains followed by 7 amino acids (dRBD4, Grskovic et al., 2003). The equivalent fragment of the SXL homologue from *Musca domestica* (mRBD), despite showing 90% identity, is unable to repress translation (Grskovic et al., 2003). The co-repressor dUNR was originally detected by its ability to interact with dRBD4, but not with mRBD in crosslink- IP analysis (Grskovic et al., 2003). dUNR was enriched in a 25% ammonium sulfate cut of *Drosophila* embryo extracts and was efficiently retained in dRBD4 columns, as evidenced by their absence in the flow-through (Abaza et al., 2006; Figure IA, lane 2). However, we noticed that a 50% ammonium sulfate cut contained significant levels of dUNR but showed no dUNR retention in the dRBD4 column (Figure IA, compare lanes 2 and 5). These results suggest that the retention of dUNR in the dRBD4 column and, by extension, the formation of a

repressor complex in the 3' UTR of *msl-2,* depends on additional factors present in the 25% ammonium sulfate cut. In addition, functional tethering of dUNR did not work efficiently in dUNR-depleted extracts: compared to 60% repression obtained in untreated extracts, tethered dUNR only repressed translation 20% in depleted extracts (Figure IB). These results suggest that factors required for efficient repression are co-depleted with dUNR. The results also correlate with the fact that translational repression of BLEF was not fully restored by addition of recombinant dUNR to depleted extracts (Abaza et al., 2006, Figure 7).

Two other in vitro models were tested for translational repression of *msl-2* mRNA by SXL: rabbit reticulocyte lysates (RRL) and HeLa cytoplasmic extracts. Translational repression does not function in Hela extracts, while it works only partially in RRL (Gebauer et al., 2001). Specifically, RRL supports 5'- but not 3'-mediated *msl-2* repression when supplemented with SXL (Gebauer et al., 2001; data not shown). We reasoned that the inability of RRL to sustain 3' UTR-dependent *msl-2* repression was due to the lack of detectable UNR in this system (Mitchell et al., 2001). To check for this possibility, we tested the ability of SXL to repress translation in RRL supplemented with recombinant dUNR or its derivative NCSD12, which supports both translational repression and SXL interaction (see Publication 2). Indeed, neither of the two dUNR proteins conferred significant 3'-dependent repression to RRL (Figure IC). Although these results could be interpreted in many ways, together with those of Figures IA and IB, they suggest that *Drosophila*- specific factors in addition to UNR are necessary for *msl-2* translational repression by 3' UTR-bound SXL.

Figure I

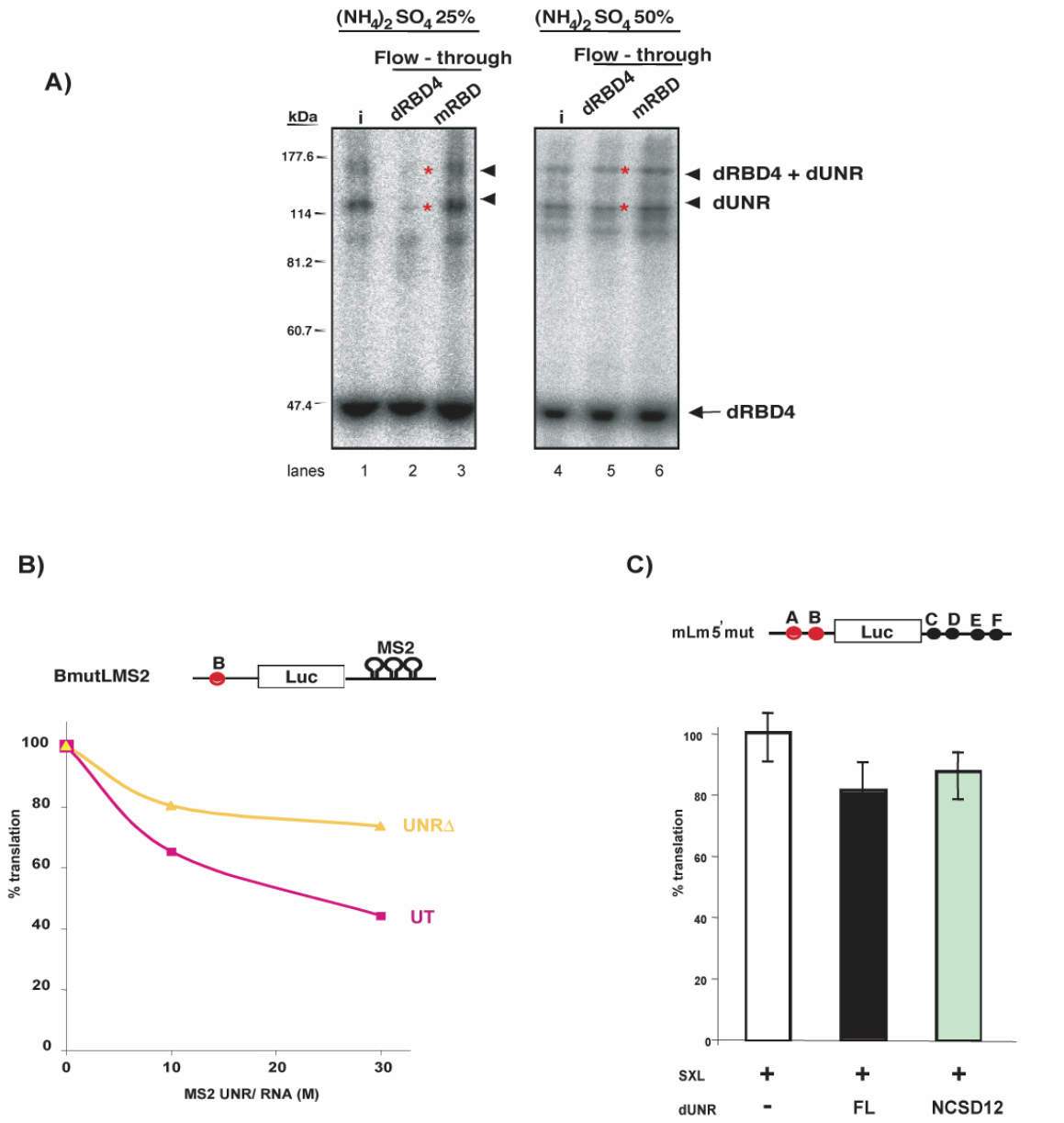


Figure I. Factors in addition to SXL and dUNR are required for translational repression of *msl-2* mRNA. A) Retention of dUNR in SXL column. dUNR was enriched by saturation of *Drosophila* extracts with 25% and 50% ammonium sulfate. Enriched dUNR precipitates were then resuspended and loaded onto dRBD4 and mRBD columns as described in Abaza et al. (2006). Retention of dUNR was tested by analyzing its presence in the column flow-through by UV crosslink-IP assay. (i) Input. The dUNR and dUNR-SXL complex are highlighted (red asterisks). **B)** Tethered dUNR represses translation less efficiently in dUNR-depleted extracts. Bmut LMS2 was incubated in typical translation reactions containing increasing amounts of MS2-dUNR in either dUNR-depleted (yellow line) or untreated (pink line) extract. *Renilla* mRNA was co-translated as an internal control. **C)** Supplementing RRL with dUNR does not improve translation inhibition of *msl-2* mRNA. mLm 5' mut RNA (containing functional sites only in the 3'UTR) was incubated in typical translation reactions containing 10-fold molar excess of SXL over RNA. To this reaction 40-fold molar excess of dUNR over the amount of SXL were added and translational rate was measured. The activity obtained in the absence of proteins was taken as 100%. UV crosslink- IP assays, immunodepletions and translation reactions have been performed as described (Abaza et al., 2006; Gebauer et al., 2001).

APPENDIX II

Role of The Poly(A) Tail in Translational Repression of msl-2 mRNA

Mammalian UNR has been shown to interact with PABP in several settings (Chang et al., 2004; Patel et al., 2005). We, therefore, tested whether an analogous interaction could be important for 3' UTR- mediated translational inhibition of *msl-2* mRNA. We first examined the poly(A) tail requirements of 5' and the 3' UTR- mediated inhibition of *msl-2* using full-length (mLm, Gebauer et al., 1999) and minimal (BLEF, Gebauer et al., 2003) chimeric constructs. The mRNAs contained either a full set of SXL binding sites (WT) or lacked SXL binding sites at the 5' (5' mut) or 3' (3' mut) UTRs to measure 3' and 5' SXL-dependent repression, respectively, and were used in deadenylated or polyadenylated (A= 73) versions. The results show that mLm WT mRNA is repressed at a somewhat lesser efficiency than its adenylated counterpart, indicating that there is a minor effect of the poly(A) tail on the efficiency of translational repression (Figure II, WT panel). This effect of the poly(A) tail is completely 3'- dependent: SXL bound to the 5' UTR inhibits translation of *msl-2* to the same extent whether a poly(A) tail is present or not, while SXL bound to

the 3' UTR represses translation more efficiently if a poly(A) tail is present (compare 5' mut and 3' mut panels). Similar results were obtained for BLEF mRNA constructs (not shown). No effect of the poly(A) tail was observed in previous experiments performed with mLm WT mRNA (Gebauer et al., 1999). We conclude that the poly(A) tail is not critical for repression. Our attempts to detect a PABP::UNR interaction in *Drosophila* have failed so far (data not shown).

Figure II

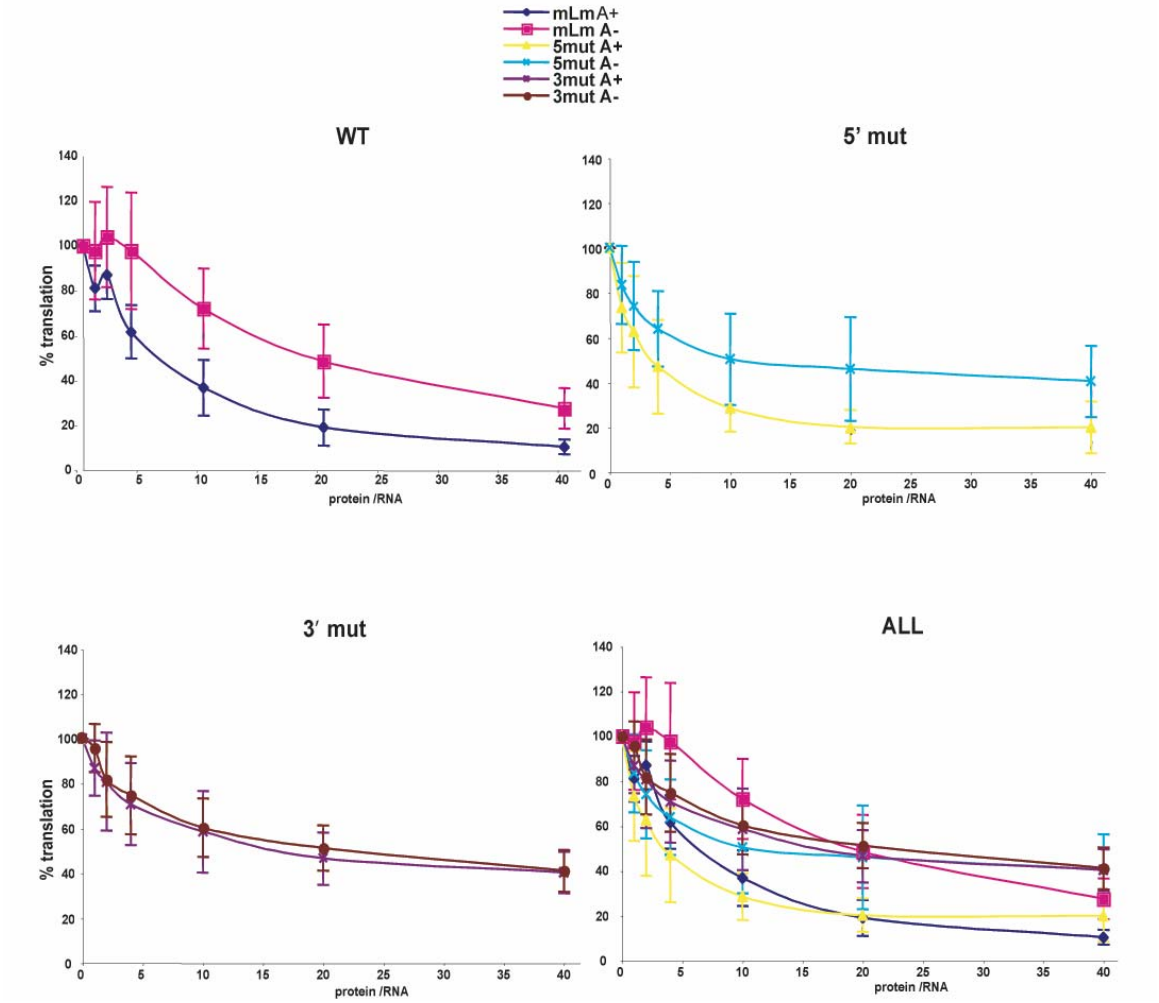


Figure II. Effect of the poly(A) tail on SXL-mediated repression. Translation of mLM, 5' mut and 3' mut RNAs were tested in the presence of increasing amounts of SXL. The RNAs either contained or lacked a poly(A) tail of 73 residues. *Firefly* luciferase values were corrected for co-translated *Renilla* and plotted against the molar ratio SXL/RNA. The activity obtained in the absence of recombinant protein was taken as 100%. Similar results were obtained for BLEF mRNA constructs.

APPENDIX III

dUNR Regulatory Networks

Over-expression of dUNR causes lethality of both male and female flies, suggesting that dUNR has roles in addition to the regulation of dosage compensation (S. Patalano and F. Gebauer, unpublished). To identify other regulatory targets of dUNR besides *msl-2*, we performed high scale immunoprecipitation of dUNR from either male or female adult extract (Abaza et al. 2006), selected the mRNAs associated to dUNR and identified them using microarrays. Male and female -selected mRNAs were hybridized against each other, so that an idea of mRNAs bound by dUNR in a sex-specific fashion could be obtained. As a control, total male versus female mRNA was hybridized to account for the relative abundance of specific mRNAs in both sexes. A total of 2.179 mRNAs (of the 16.548 cDNAs present in the chip) were selected by dUNR. Of those, 47 were clearly specifically selected in males and 185 in females (Figure IIIA, see blue and pink for male and female- specific mRNAs, respectively). Selection was specific because many abundant mRNAs were not selected by dUNR, and some mRNAs that were more abundant in males were selected by dUNR in females (Figure IIIA, blue dots in the pink section). A big portion of the mRNAs is included in what we call the “grey” area, that contains all

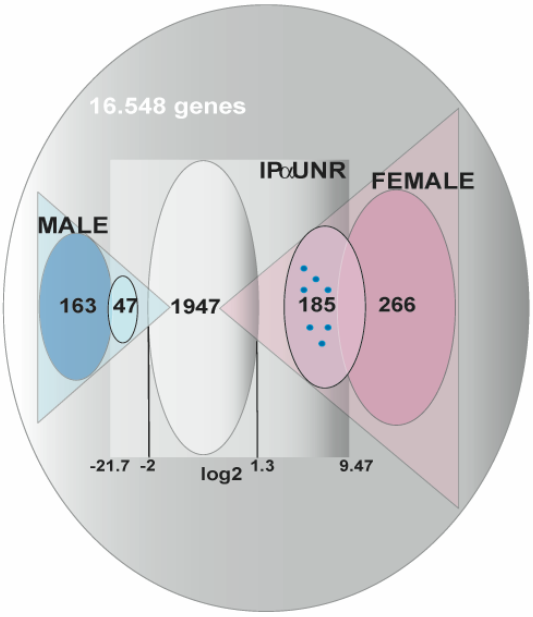
mRNAs for which the enrichment thresholds (>1.3 fold change for female-selected mRNAs and >2.0 fold change for male-selected mRNAs) were not met. The data is preliminary, because no independent check by qRT-PCR has been performed yet. In addition, an error was likely introduced by adding the same amount of male and female probe during hybridization. Indeed, *msl-2* mRNA - which we have previously detected associated to dUNR specifically in females by RT-PCR- falls within the grey area. These data suggest that the proportion of sex-specifically selected mRNAs may increase once we curate the data.

GO annotation suggests that most dUNR-selected genes are implicated in cell metabolism and localization (Figure IIIB). In general, the distribution of biological processes and molecular functions is maintained in sex-specific immunoprecipitates, although genes encoding products with oxidoreductase activity or sexual reproduction are enriched in females (not shown).

Interestingly, 22 genes involved in translational regulation or dosage compensation were selected by dUNR (Table III). Pattern discovery analysis was carried out in order to check the occurrence of SXL and dUNR binding sites on this small set of mRNAs using the UTResource web page (http://bighost.ba.itb.cnr.it/UTR/). This algorithm found UNR binding sites in the 3' UTRs and/or ORFs of 9 of the 22 transcripts (Table III). Co-occurrence of SXL and UNR binding sites was found only for *msl-2*. The fact that dUNR was associated to the mRNAs encoding other DCC components suggests that dUNR is a general post-transcriptional regulator of dosage compensation.

Figure III

A)



B)

UNR immunoprecipitated RNAs	BIOLOGICAL PROCESSES	41% metabolism 28% localization 11% signal transduction 7% gametogenesis 5% development 3% cell cycle 5% the rest
	MOLECULAR FUNCTIONS	58% protein binding 15% hydrolase activity 15% nucleic acid binding 12% the rest

Figure III. Microarray analysis of dUNR- bound mRNAs. A) Schematic representation of dUNR-selected mRNAs and specific gene distribution in male and female flies. Dark blue and dark pink spheres represent mRNAs that are more abundant in males and females, respectively. The light gray quadrant represents 2.179 mRNAs selected by dUNR. Light blue and light pink spheres represent dUNR-associated mRNAs that were specifically selected in male or females, respectively. Eight mRNAs that were more abundant in males were selected by dUNR in females (blue dots in the light pink sphere). 1.947 RNAs (grey oval) do not fall in the enrichment thresholds (>1.3 fold change for female- selected mRNAs and >2.0 fold change for male-selected mRNAs). Immunoprecipitation and RNA selection from adult males and females was performed as described (Abaza et al., 2006). Undiluted immunoprecipitated RNA from three independent experiments was amplified using the pico version of the ExpressArt ® mRNA Amplification Kit (Artus) to generate antisense- oriented Cy3/ Cy5 labeled RNA, which was used to hybridize 70-mer oligo arrays (OPERON Drosophila collection). For each experiment, the same amount of male and female probe was hybridized and a dye swap control was included. The slides were scanned with an Agilent scanner, and images were processed using the Genepix Image Analysis Software v6.0. For analysis of ratios, the Cy3:Cy5 signal for each spot was averaged over biological replicate experiments with the corresponding dye-swaps, and was calculated as a median. Log2 value data from eight sample sets representing three biological replicates for male and female immunoprecipitates was calculated. **B)** Distribution of GO annotations of biological processes and molecular functions for dUNR-selected genes. Analysis of gene function was done using the vocabulary of the Gene Ontology Consortium (www.geneontology.org). We queried our element species list with the GO term analysis tool 'FatiGO' (Bioinformatics Department at the Centro de Investigacion Principe Felipe, Spain). The statistical probability for representation of genes within a given category relative to the total number of genes with GO function terms (EASE score) was calculated for each category using the Drosophila- specific database as a background filter. Drosophila features with GO term categories with EASE scores of P< 0.001 are shown.

Table III. dUNR- bound mRNAs involved in translational regulation or dosage compensation.

Gene ID	name	GO function§	ch.	zone✣	UNR bs*	SXL bs*
CG3506	vasa	regulation of oskar mRNA translation	2L	G	ND	ND
CG4659	Srp54k(Signal recognition particle protein 54k)	SRP-dependent cotranslational protein targeting to membrane	3L	G	ND	ND
CG3481	Adh (Alcohol dehydrogenase)		2L	G	ND	ND
CG11886	Slbp (Stem-loop binding protein)	regulation of translation	3R	G	ORF	ND
CG31762	aret (arrest, Bruno)	regulation of alternative nuclear mRNA splicing and oskar mRNA translation	2L	G	ORF; 3'	ND
CG8846	Thor	negative regulation of translational initiation; regulation of cell growth	2L	G	ND	ND
CG9054	Ddx1 (Dead-box-1)	regulation of translational initiation; spliceosome assembly	3L	G	ND	ND
CG9325	hts (hu li tai shao)	oocyte fate determination; protein complex assembly	2R	G	3'	ND
CG5393	apt (apontic)	central nervous system development; regulation of oskar mRNA translation	2R	G	ND	ND
CG4262	elav (embryonic lethal, abnormal vision)	RNA metabolism; histone mRNA 3'-end processing; nuclear mRNA splicing	X	G	3'	ND
CG2922	eIF-5C (eukaryotic initiation factor 5C)	long-term memory; regulation of translational initiation	3R	G	ND	ND
Cg17166	mRpL39 (mitochondrial ribosomal protein L39)	protein biosynthesis	3L	G	ND	ND
CG12186	Aats-pro		3L	G	ORF	ND
CG4647	mRpL49 (mitochondrial ribosomal protein L49)	protein biosynthesis; translational initiation	X	G	ND	ND
CG31739	CG31739	aspartyl-tRNA aminoacylation	2L	G	ORF	ND
CG5414	CG5414	tRNA aminoacylation for protein translation	3L	G	ND	ND
CG4954	eIF3-S8	regulation of translation	2R	G	ND	ND
CG4918	RpLP2(Ribosomal protein LP2)	translational elongation	2R	G	ND	ND
CG8257	CG8257	cysteinyl-tRNA aminoacylation	2R	G	ND	ND
CG5735	orb2	RNA localization; mRNA polyadenylylation	3L	M	ND	3'
CG9412	rin (rasputin)	regulation of translation	3R	G	3'	ND
CG5099	msi (musashi)	cell fate determination; mRNA processing; regulation of translation	3R	F	5'; 3'	ND
CG10719	brat (brain tumor)	cell proliferation; regulation of translation	2L	F	ND	ND
CG7490	RpP0 (Ribosomal protein LP0)	DNA repair; translational elongation	3L	F	ND	ND
CG8280	Ef1alpha48D (Elongation factor 1alpha48D)	regulation of translation	2R	F	ND	ND
CG10306	CG10306	translational initiation	2R	F	ND	ND
CG4087	RpLP1 (Ribosomal protein LP1)	translational elongation	2L	F	ND	ND
CG31426	ligatin	translational initiation	3R	F	ND	ND
CG3241	msl-2 (male-specific lethal 2)	dosage compensation complex assembly	2L	G	3'	5'; 3'
CG11680	mle (maleless)	dosage compensation complex assembly	2R	G	ND	ND
CG8631	msl-3 (male-specific lethal 3)	dosage compensation complex assembly	3R	F	ND	3'

§ Predicted function for selected genes given by 'FatiGO' GO term analysis.
✣ Sex-specific distribution according to Figure IIIA, where 'F', 'M' and 'G' denotes female, male and gray zone, respectively
* Presence of binding sites (bs) for SXL and dUNR carried out by UTResource algorithm (http://bighost.ba.itb.cnr.it/UTR/). 5', 3' and ORF denotes localization of binding sites in the mRNA. ND stands for 'not detected'.

5. DISCUSSION

Inhibition of *msl-2* expression is essential for development of female *Drosophila* flies, since expression of MSL-2 causes the assembly of the DCC on both X chromosomes and lethality (Kelley and Kuroda, 1995). In this study, we identify the protein dUNR as a co-repressor that acts in conjunction with SXL to inhibit *msl-2* mRNA translation, and characterize its functional domains. Further, we identify the set of mRNA targets of dUNR in male and female flies. Our data suggest that, in addition to controlling dosage compensation, dUNR regulates other processes essential for cell viability. Thus, dUNR is a fundamental regulator of mRNA metabolism.

dUNR is a Cold Shock Protein

dUNR is a large protein containing five CSDs. The CSD is a five-stranded b-barrel fold that mediates binding to single stranded nucleic acids (Figure 3, Publication 3, reviewed in Ermolenko and Makhatadze, 2002). It is the most evolutionally conserved nucleic acid binding domain and, as the RRM, contains RNP1 and RNP2 motifs. Proteins containing CSDs have an apparently low RNA binding affinity, as is the case for dUNR (Figure 6B, Publication 1). The CSD was found originally in bacterial cold shock protein A (CspA), a 70 aa protein

consisting of basically one CSD that facilitates transcription and translation at low temperatures. Members of the CSD family have been subsequently found throughout the three kingdoms of life and appear to have diverse functions. They have been implicated in various cellular processes, including adaptation to low temperatures, cellular growth and differentiation, and nutrient stress (Graumann and Marahiel, 1998). In addition, CSD and CSD-related proteins have been shown to play a role in cap-dependent (Matsumoto et al., 1996; Braun, 2000; Davies et al., 2000; Giorgini et al., 2001; Nekrasov et al., 2003; Evdokimova et al., 2006) and IRES-dependent (Hunt et al., 1999a, 1999b; Mitchell et al., 2001, 2003) translation, pre-mRNA splicing (Stickeler et al., 2001; Chansky et al., 2001), transcription (La Teana et al., 1991; Jones et al., 1992; Brandi et al., 1994; Graumann and Marahiel, 1997), and mRNA stability (Evdokimova et al., 2001; Leeanne et al., 2004; Chang et al., 2004).

Prominent members of the CSD family are the vertebrate Y-box proteins YB-1 and FRGY2, which constitute major components of mRNP particles and have been proposed to play a role in mRNA packaging and translational silencing (Davydova et al., 1997; Skabkina et al., 2005; Evdokimova et al., 2006). The ability to package mRNA while regulating its activity has been considered analogous to the role of core histones in packaging and regulating DNA (reviewed in Sommerville, 1999). Similar to CspA, Y-box proteins have RNA chaperone activity that is thought to be important for their function (Evdokimova et al., 1995; Skabkin et al., 2001). Y-box proteins are also transcriptional regulators that are loaded onto the mRNA in the nucleus, providing a connection between the nuclear life of an mRNA and its cytoplasmic fate (Wolffe and Meric, 1996). As the Y-box proteins, dUNR represses translation and appears to be an abundant, ubiquitous protein (Publication 1). However, dUNR is primarily cytoplasmic, concentrated around the nucleus (Figure 4, Publication 1).

UNR was initially identified in mammals as a gene unusually close to, and upstream of, *N-ras* (thereby the name upstream of N-ras) (Jeffers et al., 1990). Its disruption in mice results in embryonic lethality (Boussadia et al., 1997), while

its over-expression in mammalian cell lines induces apoptosis (Cornelis et al., 2005). Consistent with a role in viability, enforced expression of UNR in *Drosophila* results in lethality of both male and female flies (S. Patalano and F. Gebauer, unpublished data). UNR is a conserved protein, with homologues in vertebrates (human, rat, mouse, chicken) and insects (mosquito, fruit fly). dUNR is 45% identical to its human counterpart, and contains an amino terminal extension rich in glutamines that is absent in hUNR (Figure 1E, Publication 1). Similar to vertebrates, several transcripts are detected for dUNR which may arise by alternative splicing and/or polyadenylation (Publication 1) (Boussadia et al., 1993; Ferrer et al., 1999). Interestingly, while dUNR protein levels are comparable in male and female flies, transcript levels are considerably higher in females suggesting that dUNR could be sex-specifically regulated at the level of translation (i.e. repressed in females or activated in males) (Figure 3, Publication 1). Indeed, YB-1 specifically inhibits its own synthesis at the step of translation initiation (Skabkina et al., 2005), providing a cellular homeostasis mechanism to finely tune the levels of a protein that is broadly involved in the regulation of gene expression.

dUNR Represses msl-2 mRNA Translation

Drosophila extracts depleted of dUNR cannot support SXL- mediated *msl-2* translational repression. Importantly, addition of recombinant dUNR to depleted extracts restores *msl-2* translation inhibition, indicating that dUNR is a necessary factor for *msl-2* repression (Publication 1). Consistent data were obtained in *Drosophila* cell lines with male (SL2) and female (Kc) properties. Kc cells are considered of female origin because: i) they express endogenous SXL, ii) they lack MSL-2 and iii) they splice *doublesex* pre-mRNA in the female mode (Burtis and Baker, 1989; Publication 2). Conversely, SL2 cells are considered male because they do not express SXL and contain high levels of MSL-2 (Gebauer et

al., 1998; Buscaino et al., 2003). Both cell lines express dUNR (Publication 2). Addition of SXL to SL2 cells results in *msl-2* inhibition, while ablation of dUNR by RNAi in Kc cells leads to MSL-2 expression (Publication 2). These data indicate that dUNR is required for *msl-2* translation inhibition both *in vitro* and *in vivo*, and suggest that dUNR is a novel regulator of dosage compensation. Whether interfering with dUNR function results in the assembly of the DCC in female flies has not been directly assessed. However, it is interesting to note that mRNAs encoding other DCC components are associated to dUNR in females (Table III), suggesting that dUNR is a master regulator of dosage compensation in *Drosophila*.

dUNR does not bind to *msl-2* by itself, but requires the assistance of SXL (Publication 1). Indeed, SXL recruits dUNR to the 3' UTR of *msl-2*, conveying a sex-specific function to this ubiquitous protein (Publications 1 and 2). In this respect, the RNA-binding properties of dUNR differ from those of hUNR, because hUNR can bind to *msl-2* mRNA in the absence of SXL (Publication 3). The requirement for complex formation with SXL to bind RNA has allowed us to use GEMSA in order to map the domain of dUNR that sustains both *msl-2* and SXL binding. This domain was identified as CSD1 (Publication 3). That CSD1 of dUNR suffices for RNA binding seems to contrast with the observation that all five CSDs of hUNR are necessary for binding to rhinovirus IRES (Brown and Jackson, 2004). However, the CSD1 of human and *Drosophila* UNR share similar properties in terms of *msl-2* binding, because they both require SXL to bind to *msl-2* when tested in isolation (Publication 3). We have taken advantage of this observation to map putative residues within CSD1 involved in complex formation with SXL. Our current analysis indicates that these residues are mostly exposed and concentrated towards one side of the b-barrel. Further mutational analysis should allow us to identify a (few) specific residue(s) involved in SXL contact.

Tethering experiments indicate that, although CSD1 is sufficient for complex formation with SXL, it is not enough to support translational repression (Publication 3). The Q-rich N-terminal domain together with CSDs 1 and 2 (i.e.

the NCSD12 fragment) is required for translation inhibition. These data indicate that translational repression can be distinguished from SXL interaction, and that interaction with SXL does not suffice for translational repression. The translational effector domain of dUNR could contact either another co-repressor or a component of the translation machinery that is targeted for repression. Indeed, our unpublished evidence suggests that additional co-repressors are required (Figure I, see below). Alternatively, the Q-rich domain could mediate multimerisation of dUNR that could be required for repression. Several lines of evidence exist for multimerization and interactions with heterologous proteins mediated by Q-rich regions of transcription and splicing factors (Emili et al., 1994; Pascal and Tjian, 1991; Stott et al., 1995; Strom et al., 1996; Wilkins and Lis, 1998; McBride and Silver, 2001; Förch et al., 2002). However, it is important to note that the Q-rich domain of dUNR alone does not show translational repression activity when tethered to *msl-2*. Thus, the recruitment of additional factors –or the putative dUNR multimerization- needs residues from the first cold shock domains. Interestingly, hUNR lacks the Q-rich domain. Tethering hUNR or hybrid hUNR/dUNR molecules to *msl-2* should let us finely map the translational effector domain of dUNR and understand the role of the *Drosophila* Q-rich extension.

Tethered dUNR represses translation less efficiently than SXL (Publication 3). Two alternative explanations could be envisaged for this observation: i) SXL provides repressor functions in addition to the recruitment of dUNR, and ii) tethered dUNR does not attain the proper conformation or geometry to fully repress translation. Certainly, not all proteins involved in translation can function when tethered to the mRNA. A salient example of this is precisely SXL, which is completely inactive when tethered to the 3' UTR of *msl-2* (Grskovic et al., 2003).

Additional Factor(s) are Required for msl-2 Translational Repression

Several lines of evidence suggest that factors in addition to SXL and dUNR are required for repression of *msl-2* (Appendix I). First, although dUNR present in a 25% ammonium sulfate cut from an embryo extract can be retained in dRBD4 columns (i.e. can form repressive complexes), dUNR from a 50% ammonium sulfate cut cannot (Figure IA). Second, tethered dUNR represses translation less efficiently in dUNR-depleted extracts, suggesting that additional factors have been co-depleted with dUNR (Figure IB). Third, attempts to reconstitute 3' UTR-dependent *msl-2* repression in RRL, either by adding SXL and dUNR or the NCSD12 fragment (Figure IC), or by tethering dUNR (not shown), have failed.

These factors could be contacting dUNR thought its NCSD12 region. A literature search for factors binding to hUNR revealed three candidates. UNR-interacting protein (UNRIP) was shown to form a complex with hUNR that binds to the rhinovirus IRES and stimulates translation (Hunt et al., 1999b). UNRIP shows 25% identity to the S2 subunit of eIF3 (Hunt et al., 1999b) that bears 60% of homology with *Drosophila* counterpart. ALL-1, the human homologue of *Drosophila* trithorax, is involved in acute leukemia and interacts with CSD3 of hUNR in yeast two-hybrid assays (Leshkowitz et al., 1996). hUNR also interacts with PABP in complexes involved in the destabilization of *c-fos* mRNA and the translational repression of *pabp* mRNA (Chang et al., 2004; Patel et al., 2005). Because of the role of PABP in translation initiation, which is the step targeted by the SXL::dUNR complex, we paid special attention to a possible interaction between PABP and SXL or dUNR. So far, our co-immunoprecipitation and pull-down assays have failed to detect a specific interaction (data not shown). Other proteins apparently associated with the silencing complex assembled in the 3' UTR of *msl-2* are Rigor mortis, Rm62, dFMR1, Rasputin, Me31B, La and YB

(Publication 2). However, RNAi experiments in Kc cells showed no effect of down-regulation of any of those proteins in *msl-2* translation (Publication 2).

Another protein frequently functionally associated with hUNR is PTB. Although no direct interaction between these two proteins has been reported, PTB and hUNR synergistically stimulate translation mediated by the rhinovirus IRES (Hunt et al., 1999b). In addition, PTB and hUNR co-operate for translation dependent on the *apaf-1* IRES (Mitchell et al., 2001). hUNR binds to the *apaf-1* IRES and modifies its conformation so that PTB, a general stimulator of IRES-dependent translation, can bind (Mitchell et al., 2003). An analogy could be drawn between the functional relationship of PTB and hUNR during mammalian *apaf-1* translation, and that of SXL and dUNR during *Drosophila msl-2* repression. As PTB, SXL is a pyrimidine-tract binding protein: while in mammals hUNR binding allows for the recruitment of PTB, in *Drosophila* SXL binding allows for the recruitment of dUNR.

Role of The Poly(A) Tail

As mentioned above, one of the usual partners of hUNR is PABP. As another way to test a possible involvement of PABP in *msl-2* translational repression, we measured whether inhibition of *msl-2* translation depends on the poly(A) tail. The results showed that nonadenylated *msl-2* mRNA was repressed at a somewhat lesser efficiency (~ 20%) than its adenylated counterpart (Figure II). This effect was entirely 3' UTR-dependent, because a similar difference was observed when repression occurred only via the 3' UTR while no difference was detected when repression occurred only via the 5' UTR. The difference observed in these experiments, although specific, was not detected in a previous report (Gebauer et al., 1999). Indeed, substantial repression takes place even in the absence of a poly(A) tail (Figure II). These data do not rule out a role for PABP

per se, because PABP is thought to have roles in translation independent of the poly(A) tail. Indeed, depletion of PABP reduces translation of nonadenylated mRNAs, while addition of recombinant PABP restores their translation efficiency (Kavehjian et al., 2005). However, as already mentioned, our attempts to detect an interaction (direct or indirect) between PABP and dUNR or SXL have failed. We, thus, speculate that other translation initiation factors are targeted by the SXL::dUNR complex. It will be intriguing to decipher the identity of those targets. Toe-print and sucrose gradient analysis showed that the SXL::dUNR complex assembled at the 3' UTR of *msl-2* mRNA inhibits the stable recruitment of 43S ribosomal complexes to the 5' end of the mRNA (Beckmann et al., 2005). Curiously, this recruitment is cap-dependent, but translational repression is cap-independent (Gebauer et al., 2003). Presumably, identifying the translational targets of SXL will reveal factors involved in cap-dependent translation distinct from those that directly bind to the cap structure (eIF4E). It will be also important to understand the functional coordination between repression mediated by the 5' and 3' UTR- bound complexes. SXL bound to the 5' UTR has been shown to inhibit ribosomal scanning, a process that is not well understood (Beckmann et al., 2005). Our model proposes that 3' UTR- bound SXL recruits dUNR and possibly other factors, promoting the formation of an assembly that inhibits the initial recruitment of 43S complexes to the 5' end of the mRNA, while 5' UTR-bound SXL inhibits the scanning of those subunits that escape the first control (Figure 4.1). Figuring out the molecular mechanism of repression by SXL and dUNR will improve our understanding of the translation process itself.

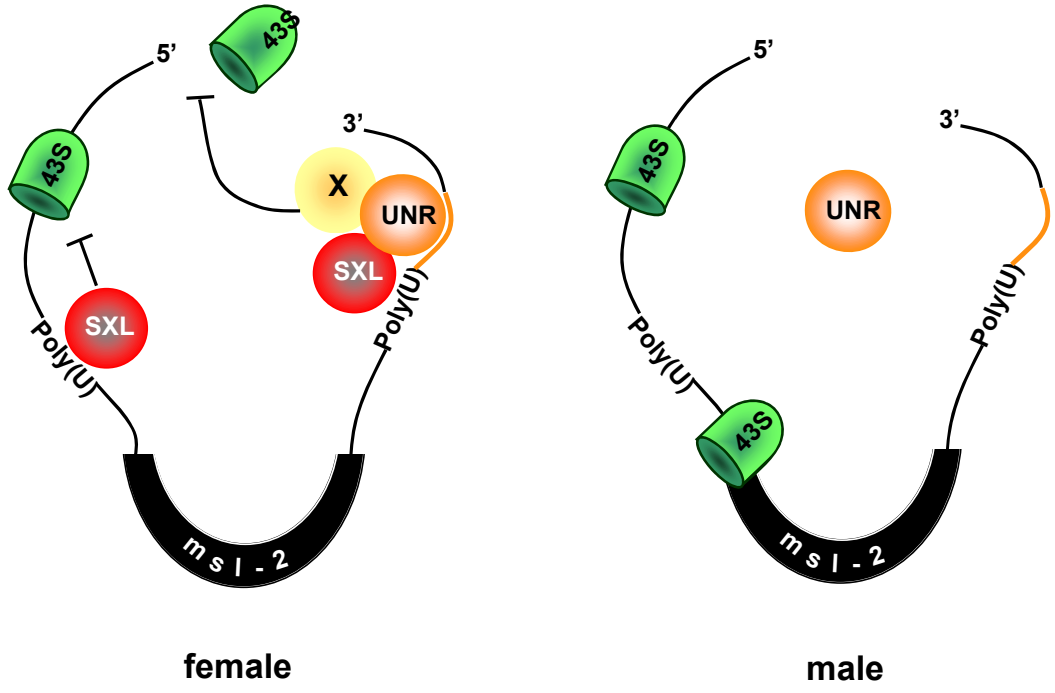


Figure 4.1. Current model for *msl-2* mRNA translational repression. In female flies, SXL binds to poly(U) stretches in the 5' and 3' UTR of *msl-2* mRNA. 3' UTR-bound SXL further recruits dUNR and other co-repressor(s) to the adjacent sequences. The 3' UTR complex then blocks the association of the 43S ribosomal complex with the 5' end of the *msl-2* mRNA, thereby repressing translation. The 5' UTR-bound SXL blocks scanning that escaped the blockage mediated by the 3' UTR (Beckmann et al., 2005). Male flies, which lack SXL, do not assemble inhibitory complex in the 3' UTR of the *msl-2* mRNA and translation proceeds.

dUNR Regulatory Networks

dUNR is an ubiquitous protein present in males and females in equivalent amounts (Publication 1). The fact that over-expression of dUNR causes lethality of both sexes (S. Patalano and F. Gebauer, unpublished) suggests that, in addition to its role in the repression of dosage compensation in females, dUNR plays other roles common for both sexes. In order to gain insight on additional

functions for dUNR, we identified its regulatory targets. Using anti-dUNR polyclonal antibodies, dUNR::RNA complexes were pulled down from adult male and female extracts and the co-purified mRNAs were identified by microarray analysis. In addition, the general abundance of mRNAs in males versus females was determined as a control.

Selection of mRNAs by dUNR was specific because many abundant mRNAs were not immunoprecipitated with the anti-dUNR antibodies, and some mRNAs that were more abundant in males were selected by dUNR in females (Figure III). Female and male threshold changes of 1.3 and 2-fold, respectively, were chosen because the overall fold change in female immunoprecipitates appeared smaller than that in males (9.47 versus 21.7, respectively). Selected mRNAs falling within these thresholds were considered to be in a “grey” area in which the male or female specificity is unclear. The difference in overall fold change is likely due to the Cy5:Cy3 signal normalization procedure, which we believe introduces errors. For example, *msl-2* mRNA should be selected by dUNR only in females (Publication 1) but falls in the grey area in our microarray analysis (fold change 1.13). Thus, confirmation of the microarray data by qRT-PCR is needed to establish the limit between male- and female- selected mRNAs.

Although the majority of dUNR-selected genes have unknown functions, GO annotation shows that the list of dUNR-interacting transcripts includes genes involved in cell metabolism, localization and signal transduction, with molecular functions converging on protein and nucleic-acid binding, as well as hydrolase activity (Figure IIIB). The distribution of biological processes and molecular functions is maintained in sex-specifically selected mRNAs, although female immunoprecipitates also contain transcripts encoding products with oxidoreductase activity, structural constituents of the ribosome, or involved in sexual reproduction (data not shown). Putative dUNR targets involved in translational regulation and dosage compensation are shown in Table III. Important translational regulators of early development are included in the list, suggesting that dUNR might coordinate translational regulation at this time of

development. Interestingly, the mRNAs for two other DCC components (*msl-3 and mle*) were selected with anti-dUNR antibodies, which seeds a new light on the role of dUNR in dosage compensation. Both products are down-regulated in females (Bashaw and Baker, 1995; Kelley et al., 1995). *msl-3* mRNA also contains SXL-binding sites in the 3' UTR and, although not detected by the UTResource algorithm, visual inspection suggests that a dUNR-binding site lies close to the SXL-binding site, raising the possibility that *msl-3* is regulated in a manner analogous to *msl-2*. These data suggest that dUNR is a global regulator of dosage compensation in females. The mRNAs coding for other transcriptional and post-transcriptional regulators are associated to dUNR (not shown). Altogether, these data suggest that dUNR is a general post-transcriptional regulator of gene expression.

6. CONCLUSIONS

1. UNR is a novel regulator of dosage compensation required for SXL-mediated translational repression of msl-2 mRNA in females of Drosophila melanogaster.

2. UNR is an ubiquitous, cytoplasmic protein present in similar amounts in male and female flies.

3. In order to repress translation, SXL recruits UNR to the 3' UTR of msl-2 mRNA, thereby imparting a sex-specific function to ubiquitous UNR.

4. UNR interacts with SXL and msl-2 mRNA via the cold shock domain 1.

5. The SXL- interacting and translational repressor domains of UNR are distinct. The Q-rich amino terminal region of UNR is important for translational repression.

6. Factors in addition to SXL and UNR are likely to be required for translational repression of msl-2 mRNA. These factors could be interacting with the translational repression domain of UNR.

7. A large set of mRNAs are associated with UNR, including those encoding other components of the dosage compensation complex, and important translational regulators. This suggests that UNR is a general post-transcriptional regulator of gene expression in Drosophila.

6. REFERENCES

A

Abaza, I., Coll, O., Patalano, S., and Gebauer, F. (2006). Drosophila UNR is required for translational repression of male-specific lethal 2 mRNA during regulation of X-chromosome dosage compensation. Genes Dev 20, 380-389.

Akhtar, A. (2003). Dosage compensation: an intertwined world of RNA and chromatin remodelling. Curr Opin Genet Dev 13, 161-169.

Akhtar, A., and Becker, P. B. (2000). Activation of transcription through histone H4 acetylation by MOF, an acetyltransferase essential for dosage compensation in Drosophila. Mol Cell 5, 367-375.

Alekseyenko, A. A., Larschan, E., Lai, W. R., Park, P. J., and Kuroda, M. I. (2006). High-resolution ChIP-chip analysis reveals that the Drosophila MSL complex selectively identifies active genes on the male X chromosome. Genes
Dev 20, 848-857.

Algire, M. A., Maag, D., and Lorsch, J. R. (2005). Pi release from eIF2, not GTP hydrolysis, is the step controlled by start-site selection during eukaryotic translation initiation. Mol Cell 20, 251-262.

Amrein, H. (2000). Multiple RNA-protein interactions in Drosophila dosage compensation. Genome Biol 1, REVIEWS1030.

Antson, A. A. (2000). Single-stranded-RNA binding proteins. Curr Opin Struct Biol 10, 87-94.

B

Baker, B., Gorman, M., and Marin, I. (1994). Dosage compensation in Drosophila. Annu Rev Genet 28, 491-521.

Baker, B. S., Burtis, K., Goralski, T., Mattox, W., and Nagoshi, R. (1989). Molecular genetic aspects of sex determination in Drosophila melanogaster. Genome 31, 638-645.

Bashaw, G. J., and Baker, B. S. (1995). The msl-2dosage compensation gene of Drosophilaencodes a putative DNAbinding
protein whose expression is sex specifically regulated by Sex-lethal. Development 121, 3245-3258.

Bashaw, G. J., and Baker, B. S. (1997). The Regulation of the Drosophila msl-2
Gene Reveals a Function for Sex-lethal
in Translational Control. Cell 89,, 789–798.

Bean, C. J., Schaner, C. E., and Kelly, W. G. (2004). Meiotic pairing and imprinted X chromatin assembly in Caenorhabditis elegans. Nat Genet 36, 100-105.

Beckmann, K., Grskovic, M., Gebauer, F., and Hentze, M. W. (2005). A dual inhibitory mechanism restricts msl-2 mRNA translation for dosage compensation in Drosophila. Cell 122, 529-540.

Bergsten, S. E., and Gavis, E. R. (1999). Role for mRNA localization in translational activation but not spatial restriction of nanos RNA. Development 126, 659-669.

Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S. M., Singer, R. H., and Long, R. M. (1998). Localization of ASH1 mRNA particles in living yeast. Mol Cell 2, 437-445.

Blattner, F. R., Plunkett, G., 3rd, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., et al. (1997). The complete genome sequence of Escherichia coli K-12. Science 277, 1453-1474.

Boehm, M., and Slack, F. (2005). A developmental timing microRNA and its target regulate life span in C. elegans. Science 310, 1954-1957.

Both, G. W., Banerjee, A. K., and Shatkin, A. J. (1975). Methylation-dependent translation of viral messenger RNAs in vitro. Proc Natl Acad Sci U S A 72, 1189-1193.

Boussadia, O., Amiot, F., Cases, S., Triqueneaux, G., Jacquemin-Sablon, H., and Dautry, F. (1997). Transcription of unr (upstream of N-ras) down-modulates N-ras expression in vivo. FEBS Lett 420, 20-24.

Boussadia, O., Jacquemin-Sablon, H., and Dautry, F. (1993). Exon skipping in the expression of the gene immediately upstream of N-ras (unr/NRU). Biochim Biophys Acta 1172, 64-72.

Brandi, A., Pon, C. L., and Gualerzi, C. O. (1994). Interaction of the main cold shock protein CS7.4 (CspA) of Escherichia coli with the promoter region of hns. Biochimie 76, 1090-1098.

Braun, R. E. (2000). Temporal control of protein synthesis during spermatogenesis. Int J Androl 23 Suppl 2, 92-94.

Brown, C. M., Dalphin, M. E., Stockwell, P. A., and Tate, W. P. (1993). The translational termination signal database. Nucleic Acids Res 21, 3119-3123.

Brown, C. M., Stockwell, P. A., Trotman, C. N., and Tate, W. P. (1990). The signal for the termination of protein synthesis in procaryotes. Nucleic Acids Res 18, 2079-2086.
Brown, E. C., and Jackson, R. J. (2004). All five cold-shock domains of unr (upstream of N-ras) are required for stimulation of human rhinovirus RNA translation. Journal of General Virology 85, 2279–2287.

Buscaino, A., Kocher, T., Kind, J. H., Holz, H., Taipale, M., Wagner, K., Wilm, M., and Akhtar, A. (2003). MOF-regulated acetylation of MSL-3 in the Drosophila dosage compensation complex. Mol Cell 11, 1265-1277.

C

Cao, Q., and Richter, J. D. (2002). Dissolution of the maskin-eIF4E complex by cytoplasmic polyadenylation and poly(A)-binding protein controls cyclin B1 mRNA translation and oocyte maturation. Embo J 21, 3852-3862.

Carrel, L., and Willard, H. F. (2005). X-inactivation profile reveals extensive variability in X-linked gene expression in females. Nature 434, 400-404.

Carthew, R. W. (2006). Gene regulation by microRNAs. Curr Opin Genet Dev 16, 203-208.

Chagnovich, D., and Lehmann, R. (2001). Poly(A)-independent regulation of maternal hunchback translation in the Drosophila embryo. Proc Natl Acad Sci U S A 98, 11359-11364.

Chang, K. A., and Kuroda, M. I. (1998). Modulation of MSL1 abundance in female Drosophila contributes to the sex specificity of dosage compensation. Genetics 150, 699-709.

Chang, T. C., Yamashita, A., Chen, C. Y., Yamashita, Y., Zhu, W., Durdan, S., Kahvejian, A., Sonenberg, N., and Shyu, A. B. (2004). UNR, a new partner of poly(A)-binding protein, plays a key role in translationally coupled mRNA turnover mediated by the c-fos major coding-region determinant. Genes Dev 18, 2010-2023.

Chansky, H. A., Hu, M., Hickstein, D. D., and Yang, L. (2001). Oncogenic TLS/ERG and EWS/Fli-1 fusion proteins inhibit RNA splicing mediated by YB-1 protein. Cancer Res 61, 3586-3590.

Chekulaeva, M., Hentze, M. W., and Ephrussi, A. (2006). Bruno acts as a dual repressor of oskar translation, promoting mRNA oligomerization and formation of silencing particles. Cell 124, 521-533.

Chen, P. Y., and Meister, G. (2005). microRNA-guided posttranscriptional gene regulation. Biol Chem 386, 1205-1218.

Cho, P. F., Poulin, F., Cho-Park, Y. A., Cho-Park, I. B., Chicoine, J. D., Lasko, P., and Sonenberg, N. (2005). A new paradigm for translational control: inhibition via 5'-3' mRNA tethering by Bicoid and the eIF4E cognate 4EHP. Cell 121, 411-423.

Clerc, P., and Avner, P. (2006). Random X-chromosome inactivation: skewing lessons for mice and men. Curr Opin Genet Dev 16, 246-253.

Coles, L. S., Bartley, M. A., Bert, A., Hunter, J., Polyak, S., Diamond, P., Vadas, M. A., and Goodall, G. J. (2004). A multi-protein complex containing cold shock domain (Y-box) and polypyrimidine tract binding proteins forms on the vascular endothelial growth factor mRNA. Potential role in mRNA stabilization. Eur J Biochem 271, 648-660.

Collier, B., Gorgoni, B., Loveridge, C., Cooke, H. J., and Gray, N. K. (2005). The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells. Embo J 24, 2656-2666.

Cornelis, S., Tinton, S. A., Schepens, B., Bruynooghe, Y., and Beyaert, R. (2005). UNR translation can be driven by an IRES element that is negatively regulated by polypyrimidine tract binding protein. Nucleic Acids Res 33, 3095-3108.

Crowder, S. M., Kanaar, R., Rio, D. C., and Alber, T. (1999). Absence of interdomain contacts in the crystal structure of the RNA recognition motifs of Sex-lethal. Proc Natl Acad Sci U S A 96, 4892-4897.

D

Dahlsveen, I. K., Gilfillan, G. D., Shelest, V. I., Lamm, R., and Becker, P. B. (2006). Targeting determinants of dosage compensation in Drosophila. PLoS Genet 2, e5.

Davies, H. G., Giorgini, F., Fajardo, M. A., and Braun, R. E. (2000). A sequence-specific RNA binding complex expressed in murine germ cells contains MSY2 and MSY4. Dev Biol 221, 87-100.

Davydova, E. K., Evdokimova, V. M., Ovchinnikov, L. P., and Hershey, J. W. (1997). Overexpression in COS cells of p50, the major core protein associated with mRNA, results in translation inhibition. Nucleic Acids Res 25, 2911-2916.

de Haro, C., Mendez, R., and Santoyo, J. (1996). The eIF-2alpha kinases and the control of protein synthesis. Faseb J 10, 1378-1387.

de Moor, C. H., Meijer, H., and Lissenden, S. (2005). Mechanisms of translational control by the 3' UTR in development and differentiation. Semin Cell Dev Biol 16, 49-58.

Decker, C. J., and Parker, R. (1995). Diversity of cytoplasmic functions for the 3' untranslated region of eukaryotic transcripts. Curr Opin Cell Biol 7, 386-392.

Demakova, O. V., Kotlikova, I. V., Gordadze, P. R., Alekseyenko, A. A., Kuroda, M. I., and Zhimulev, I. F. (2003). The MSL complex levels are critical for its correct targeting to the chromosomes in Drosophila melanogaster. Chromosoma 112, 103-115.

Dever, T. E. (2002). Gene-specific regulation by general translation factors. Cell 108, 545-556.

Dong, Z., and Bell, L. R. (1999). SIN, a novel Drosophila protein that associates with the RNA binding protein sex-lethal. Gene 237, 421-428.

Duncan, K., Grskovic, M., Strein, C., Beckmann, K., Niggeweg, R., Abaza, I., Gebauer, F., Wilm, M., and Hentze, M. W. (2006). Sex-lethal imparts a sex-specific function to UNR by recruiting it to the msl-2 mRNA 3' UTR: translational repression for dosage compensation. Genes Dev 20, 368-379.

E

Edwards, T. A., Pyle, S. E., Wharton, R. P., and Aggarwal, A. K. (2001). Structure of Pumilio reveals similarity between RNA and peptide binding motifs. Cell 105, 281-289.

Emili, A., Greenblatt, J., and Ingles, C. J. (1994). Species-specific interaction of the glutamine-rich activation domains of Sp1 with the TATA box-binding protein. Mol Cell Biol 14, 1582-1593.

Ermolenko, D. N., and Makhatadze, G. I. (2002). Bacterial cold-shock proteins. Cell Mol Life Sci 59, 1902-1913.

Evdokimova, V., Ruzanov, P., Anglesio, M. S., Sorokin, A. V., Ovchinnikov, L. P., Buckley, J., Triche, T. J., Sonenberg, N., and Sorensen, P. H. (2006). Akt-mediated YB-1 phosphorylation activates translation of silent mRNA species. Mol Cell Biol 26, 277-292.

Evdokimova, V., Ruzanov, P., Imataka, H., Raught, B., Svitkin, Y., Ovchinnikov, L. P., and Sonenberg, N. (2001). The major mRNA-associated protein YB-1 is a potent 5' cap-dependent mRNA stabilizer. Embo J 20, 5491-5502.

Evdokimova, V. M., Wei, C. L., Sitikov, A. S., Simonenko, P. N., Lazarev, O. A., Vasilenko, K. S., Ustinov, V. A., Hershey, J. W., and Ovchinnikov, L. P. (1995). The major protein of messenger ribonucleoprotein particles in somatic cells is a member of the Y-box binding transcription factor family. J Biol Chem 270, 3186-3192.

F

Ferrer, N., Garcia-Espana, A., Jeffers, M., and Pellicer, A. (1999). The unr gene: evolutionary considerations and nucleic acid-binding properties of its long isoform product. DNA Cell Biol 18, 209-218.

Flickinger, T. W., and Salz, H. K. (1994). The Drosophila sex determination gene snf encodes a nuclear protein with sequence and functional similarity to the mammalian U1A snRNP protein. Genes Dev 8, 914-925.

Forch, P., Merendino, L., Martinez, C., and Valcarcel, J. (2001). Modulation of msl-2 5' splice site recognition by Sex-lethal. Rna 7, 1185-1191.

Forch, P., Puig, O., Martinez, C., Seraphin, B., and Valcarcel, J. (2002). The splicing regulator TIA-1 interacts with U1-C to promote U1 snRNP recruitment to 5' splice sites. Embo J 21, 6882-6892.

Forch, P., and Valcarcel, J. (2003). Splicing regulation in Drosophila sex determination. Prog Mol Subcell Biol 31, 127-151.

Fox, C. A., Sheets, M. D., and Wickens, M. P. (1989). Poly(A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by the sequence UUUUUAU. Genes Dev 3, 2151-2162.

Franke, A., Dernburg, A., Bashaw, G. J., and Baker, B. S. (1996). Evidence that MSL-mediated dosage compensation in Drosophila begins at blastoderm. Development 122, 2751-2760.

Furuichi, Y., and Shatkin, A. J. (2000). Viral and cellular mRNA capping: past and prospects. Adv Virus Res 55, 135-184.

G

Gallie, D. R. (1991). The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. Genes Dev 5, 2108-2116.

Gawande, B., Robida, M. D., Rahn, A., and Singh, R. (2006). Drosophila Sex-lethal protein mediates polyadenylation switching in the female germline. Embo J 25, 1263-1272.

Gebauer, F., Corona, D., Preiss, T., BEcker, P., and Hentze, M. (1999). Translational control of dosage compensation in Drosophila by Sex-ltehal: cooperative silencing via the 5' and 3'UTRs of msl-2 mRNA is independent of the poly(A). The EMBO Journal 18, 6146-6154.

Gebauer, F., Gskovic, M., and Hentze, M. (2003). Drosophila Sex-Lethal inhibits the stable association if the 40S ribosomal subunit with msl-2 mRNA. Molecular Cell 11, 1397-1404.

Gebauer, F., and Hentze, M. (2004). Molecular Mechanism of Translational control. review: Nature : Molecular Cell Biology 5, 827-836.

Gebauer, F., Merendino, L., Hentze, M., and Valcarcel, J. (1998). The Drosophila splicing regulator sex-lethal directly inhibits translation of male-specific-lethal 2 mRNA. RNA 4, 142-150.

Gilfillan, G. D., Straub, T., de Wit, E., Greil, F., Lamm, R., van Steensel, B., and Becker, P. B. (2006). Chromosome-wide gene-specific targeting of the Drosophila dosage compensation complex. Genes Dev 20, 858-870.

Gingras, A. C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 68, 913-963.

Giudice, G. (2001). Conserved cellular and molecular mechanisms in development. Cell Biol Int 25, 1081-1090.

Goossen, B., Caughman, S. W., Harford, J. B., Klausner, R. D., and Hentze, M. W. (1990). Translational repression by a complex between the iron-responsive element of ferritin mRNA and its specific cytoplasmic binding protein is position-dependent in vivo. Embo J 9, 4127-4133.

Goossen, B., and Hentze, M. W. (1992). Position is the critical determinant for function of iron-responsive elements as translational regulators. Mol Cell Biol 12, 1959-1966.

Gorgoni, B., and Gray, N. K. (2004). The roles of cytoplasmic poly(A)-binding proteins in regulating gene expression: a developmental perspective. Brief Funct Genomic Proteomic 3, 125-141.

Gorman, M., Franke, A., and Baker, B. S. (1995). Molecular characterization of the male-specific lethal-3 gene and investigations of the regulation of dosage compensation in Drosophila. Development 121, 463-475.

Gorman, M., Kuroda, M. I., and Baker, B. S. (1993). Regulation of the sex-specific binding of the maleless dosage compensation protein to the male X chromosome in Drosophila. Cell 72, 39-49.

Granadino, B., San Juan, A., Santamaria, P., and Sanchez, L. (1992). Evidence of a dual function in fl(2)d, a gene needed for Sex-lethal expression in Drosophila melanogaster. Genetics 130, 597-612.

Granadino, B., Santamaria, P., and Sanchez, L. (1993). Sex determination in the germ line of Drosophila melanogaster: activation of the gene Sex-lethal. Development 118, 813-816.

Graumann, P., and Marahiel, M. A. (1997). Effects of heterologous expression of CspB, the major cold shock protein of Bacillus subtillis, on protein synthesis in Escherichia coli. Mol Gen Genet 253, 745-752.

Graumann, P. L., and Marahiel, M. A. (1998). A superfamily of proteins that contain the cold-shock domain. Trends Biochem Sci 23, 286-290.

Graveley, B. R. (2002). Sex, AGility, and the regulation of alternative splicing. Cell 109, 409-412.

Graves, J. A. (2006). Sex chromosome specialization and degeneration in mammals. Cell 124, 901-914.

Gray, N. K., and Wickens, M. (1998). Control of translation initiation in animals. Annu Rev Cell Dev Biol 14, 399-458.

Groisman, I., Huang, Y. S., Mendez, R., Cao, Q., and Richter, J. D. (2001). Translational control of embryonic cell division by CPEB and maskin. Cold Spring Harb Symp Quant Biol 66, 345-351.

Grskovic, M., Hentze, M. W., and Gebauer, F. (2003). A co-repressor assembly nucleated by Sex-lethal in the 3'UTR mediates translational control of Drosophila msl-2 mRNA. Embo J 22, 5571-5581.

Gunnery, S., and Mathews, M. B. (1995). Functional mRNA can be generated by RNA polymerase III. Mol Cell Biol 15, 3597-3607.

Gupta, V., Parisi, M., Sturgill, D., Nuttall, R., Doctolero, M., Dudko, O. K., Malley, J. D., Eastman, P. S., and Oliver, B. (2006). Global analysis of X-chromosome dosage compensation. J Biol 5, 3.

H

Handa, N., Nureki, O., Kurimoto, K., Kim, I., Sakamoto, H., Shimura, Y., Muto, Y., and Yokoyama, S. (1999). Structural basis for recognition of the tra mRNA precursor by the Sex-lethal protein. Nature 398, 579-585.

Hentze, M. W., Gebauer, F., and Preiss, T. (2007). Cis-regulatory sequences and trans-acting factors in translational control. In Translational control in Biology and Medicine (Cold Spring Habor Laboratory Press).

Hentze, M. W., and Kuhn, L. C. (1996). Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc Natl Acad Sci U S A 93, 8175-8182.

Hentze, M. W., Muckenthaler, M. U., and Andrews, N. C. (2004). Balancing acts: molecular control of mammalian iron metabolism. Cell 117, 285-297.
Hershey, J. W. B., and Merrick, W. C. (2000). The Pathway and Mechanism of Initiation of Protein Synthesis. In Translational Control of Gene Expression (Cold Spring Harbor Laboratory Press).

Hilfiker, A., Amrein, H., Dubendorfer, A., Schneiter, R., and Nothiger, R. (1995). The gene virilizer is required for female-specific splicing controlled by Sxl, the master gene for sexual development in Drosophila. Development 121, 4017-
4026.

Hilfiker, A., Hilfiker-Kleiner, D., Pannuti, A., and Lucchesi, J. C. (1997). mof, a putative acetyl transferase gene related to the Tip60 and MOZ human genes and to the SAS genes of yeast, is required for dosage compensation in Drosophila. Embo J 16, 2054-2060.

Hinnebusch, A. G. (1997). Translational regulation of yeast GCN4. A window on factors that control initiator-trna binding to the ribosome. J Biol Chem 272, 21661-21664.

Hinnebusch, A. G., and Natarajan, K. (2002). Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. Eukaryot Cell 1, 22-32.

Holcik, M., and Sonenberg, N. (2005). Translational control in stress and apoptosis. Nat Rev Mol Cell Biol 6, 318-327.

Humphreys, D. T., Westman, B. J., Martin, D. I., and Preiss, T. (2005). MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc Natl Acad Sci U S A 102, 16961-16966.

Hunt, S. L., and Jackson, R. J. (1999a). Polypyrimidine-tract binding protein (PTB) is necessary, but not sufficient, for efficient internal initiation of translation of human rhinovirus-2 RNA. Rna 5, 344-359.

Hunt, S. L., Hsuan, J. J., Totty, N., and Jackson, R. J. (1999b). unr, a cellular cytoplasmic RNA-binding protein with five cold-shock domains, is required for internal initiation of translation of human rhinovirus RNA. Genes & Development 13, 437-448.

Hutvagner, G. (2005). Small RNA asymmetry in RNAi: function in RISC assembly and gene regulation. FEBS Lett 579, 5850-5857.

Jackson, R. J. (2005). Alternative mechanisms of initiating translation of mammalian mRNAs. Biochem Soc Trans 33, 1231-1241.

Jaruzelska, J., Kotecki, M., Kusz, K., Spik, A., Firpo, M., and Reijo Pera, R. A. (2003). Conservation of a Pumilio-Nanos complex from Drosophila germ plasm to human germ cells. Dev Genes Evol 213, 120-126.

Jeffers, M., Paciucci, R., and Pellicer, A. (1990). Characterization of unr; a gene closely linked to N-ras. Nucleic Acids Res 18, 4891-4899.

Jin, Y., Wang, Y., Johansen, J., and Johansen, K. M. (2000). JIL-1, a chromosomal kinase implicated in regulation of chromatin structure, associates with the male specific lethal (MSL) dosage compensation complex. J Cell Biol 149, 1005-1010.

Jones, P. G., Krah, R., Tafuri, S. R., and Wolffe, A. P. (1992). DNA gyrase, CS7.4, and the cold shock response in Escherichia coli. J Bacteriol 174, 5798-5802.

K

Kahvejian, A., Svitkin, Y. V., Sukarieh, R., M'Boutchou, M. N., and Sonenberg, N. (2005). Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. Genes Dev 19, 104-113.

Kanaar, R., Lee, A. L., Rudner, D. Z., Wemmer, D. E., and Rio, D. C. (1995). Interaction of the sex-lethal RNA binding domains with RNA. Embo J 14, 4530-4539.

Kapp, L. D., and Lorsch, J. R. (2004). The molecular mechanics of eukaryotic translation. Annu Rev Biochem 73, 657-704.

Kelley, R., Wang, J., Bell, L., and Kuroda, M. (1997). Sex-lethal controls dosage copensation in Drosophila by a non-splicing mechanism. Nature 387, 195-199.

Kelley, R. L., and Kuroda, M. (1995). Equality for X chromosomes. Science 270, 1607-1610.

Kim, I., Muto, Y., Watanabe, S., Kitamura, A., Futamura, Y., Yokoyama, S., Hosono, K., Kawai, G., Takaku, H., Dohmae, N., et al. (2000). Interactions of a didomain fragment of the Drosophila sex-lethal protein with single-stranded uridine-rich oligoribonucleotides derived from the transformer and Sex-lethal messenger RNA precursors: NMR with residue-selective [5-2H]uridine substitutions. J Biomol NMR 17, 153-165.

Kim-Ha, J., Kerr, K., and Macdonald, P. M. (1995). Translational regulation of oskar mRNA by bruno, an ovarian RNA-binding protein, is essential. Cell 81, 403-412.

Kozak, M. (2002). Pushing the limits of the scanning mechanism for initiation of translation. Gene 299, 1-34.

Kuersten, and Goodwin (2003). The power of the 3' UTR: translational control and development. Nat Rev Genet 4, 626-637.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., et al. (1997). The complete genome sequence of the gram-positive bacterium Bacillus subtilis. Nature 390, 249-256.

Kuroda, M. I., Kernan, M. J., Kreber, R., Ganetzky, B., and Baker, B. S. (1991). The maleless protein associates with the X chromosome to regulate dosage compensation in Drosophila. Cell 66, 935-947.

L

La Teana, A., Brandi, A., Falconi, M., Spurio, R., Pon, C. L., and Gualerzi, C. O. (1991). Identification of a cold shock transcriptional enhancer of the Escherichia coli gene encoding nucleoid protein H-NS. Proc Natl Acad Sci U S A 88, 10907-10911.

Lallena, M. J., Chalmers, K. J., Llamazares, S., Lamond, A. I., and Valcarcel, J. (2002). Splicing regulation at the second catalytic step by Sex-lethal involves 3' splice site recognition by SPF45. Cell 109, 285-296.

Larsson, J., and Meller, V. H. (2006). Dosage compensation, the origin and the afterlife of sex chromosomes. Chromosome Res 14, 417-431.

Lasko, P., Cho, P., Poulin, F., and Sonenberg, N. (2005). Contrasting mechanisms of regulating translation of specific Drosophila germline mRNAs at the level of 5'-cap structure binding. Biochem Soc Trans 33, 1544-1546.

Lee, A. L., Volkman, B. F., Robertson, S. A., Rudner, D. Z., Barbash, D. A., Cline, T. W., Kanaar, R., Rio, D. C., and Wemmer, D. E. (1997). Chemical shift mapping of the RNA-binding interface of the multiple-RBD protein sex-lethal. Biochemistry 36, 14306-14317.

Lee, R. C., Feinbaum, R. L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75, 843-854.

Legube, G., McWeeney, S. K., Lercher, M. J., and Akhtar, A. (2006). X-chromosome-wide profiling of MSL-1 distribution and dosage compensation in Drosophila. Genes Dev 20, 871-883.

Leshkowitz, D., Rozenblatt, O., Nakamura, T., Yano, T., Dautry, F., Croce, C. M., and Canaani, E. (1996). ALL-1 interacts with unr, a protein containing multiple cold shock domains. Oncogene 13, 2027-2031.

Lie, Y. S., and Macdonald, P. M. (1999). Translational regulation of oskar mRNA occurs independent of the cap and poly(A) tail in Drosophila ovarian extracts. Development 126, 4989-4996.

Lobocka, M. B., Rose, D. J., Plunkett, G., 3rd, Rusin, M., Samojedny, A., Lehnherr, H., Yarmolinsky, M. B., and Blattner, F. R. (2004). Genome of bacteriophage P1. J Bacteriol 186, 7032-7068.

Lucchesi, J. C., Kelly, W. G., and Panning, B. (2005). Chromatin remodeling in dosage compensation. Annu Rev Genet 39, 615-651.

M

Maag, D., Algire, M. A., and Lorsch, J. R. (2006). Communication between eukaryotic translation initiation factors 5 and 1A within the ribosomal pre-initiation complex plays a role in start site selection. J Mol Biol 356, 724-737.

Maag, D., Fekete, C. A., Gryczynski, Z., and Lorsch, J. R. (2005). A conformational change in the eukaryotic translation preinitiation complex and release of eIF1 signal recognition of the start codon. Mol Cell 17, 265-275.

MacDougall, C., Harbison, D., and Bownes, M. (1995). The developmental consequences of alternate splicing in sex determination and differentiation in Drosophila. Dev Biol 172, 353-376.

Mangus, D. A., Evans, M. C., and Jacobson, A. (2003). Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. Genome Biol 4, 223.

Martinez-Salas, E., and Fernandez-Miragall, O. (2004). Picornavirus IRES: structure function relationship. Curr Pharm Des 10, 3757-3767.

Matsumoto, K., Meric, F., and Wolffe, A. P. (1996). Translational repression dependent on the interaction of the Xenopus Y-box protein FRGY2 with mRNA. Role of the cold shock domain, tail domain, and selective RNA sequence recognition. J Biol Chem 271, 22706-22712.

McBride, A. E., and Silver, P. A. (2001). State of the arg: protein methylation at arginine comes of age. Cell 106, 5-8.

Mendez, R., Hake, L. E., Andresson, T., Littlepage, L. E., Ruderman, J. V., and Richter, J. D. (2000a). Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA. Nature 404, 302-307.

Mendez, R., Murthy, K. G., Ryan, K., Manley, J. L., and Richter, J. D. (2000b). Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. Mol Cell 6, 1253-1259.

Mendez, R., and Richter, J. D. (2001). Translational control by CPEB: a means to the end. Nat Rev Mol Cell Biol 2, 521-529.

Merendino, L., Guth, S., Bilbao, D., Martinez, C., and Valcarcel, J. (1999). Inhibition of msl-2 splicing by Sex-lethal reveals interaction between U2AF35 and the 3' splice site AG. Nature 402, 838-841.

Merrick, W. C., and Nyborg, J. (2000). The Protein Biosynthesis Elongation Cycle. In Translational Control of Gene Expression (Cold Spring Harbot Laboratory Press).

Mitchell, S. A., Brown, E. C., Coldwell, M. J., Jackson, R. J., and Willis, A. E. (2001). Protein factor requirements of the Apaf-1 internal ribosome entry segment: roles of polypyrimidine tract binding protein and upstream of N-ras. Mol Cell Biol 21, 3364-3374.

Mitchell, S. A., Spriggs, K. A., Bushell, M., Evans, J. R., Stoneley, M., Le Quesne, J. P., Spriggs, R. V., and Willis, A. E. (2005). Identification of a motif that mediates polypyrimidine tract-binding protein-dependent internal ribosome entry. Genes Dev 19, 1556-1571.

Mitchell, S. A., Spriggs, K. A., Coldwell, M. J., Jackson, R. J., and Willis, A. E. (2003). The Apaf-1 internal ribosome entry segment attains the correct structural conformation for function via interactions with PTB and unr. Mol Cell 11, 757-771.

Muckenthaler, M., Gray, N. K., and Hentze, M. W. (1998). IRP-1 binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the cap-binding complex eIF4F. Mol Cell 2, 383-388.

Muller, H. J. (1932). Furhter studies on the nature and causes of gene mutations. Proc 6th Int Congr Genetics 1, 213-255.

Munroe, D., and Jacobson, A. (1990). mRNA poly(A) tail, a 3' enhancer of translational initiation. Mol Cell Biol 10, 3441-3455.

Murata, Y., and Wharton, R. P. (1995). Binding of pumilio to maternal hunchback mRNA is required for posterior patterning in Drosophila embryos. Cell 80, 747-756.

Muthukrishnan, S., Both, G. W., Furuichi, Y., and Shatkin, A. J. (1975). 5'-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation. Nature 255, 33-37.

N

Nakahara, K., and Carthew, R. W. (2004). Expanding roles for miRNAs and siRNAs in cell regulation. Curr Opin Cell Biol 16, 127-133.

Nakamura, A., Sato, K., and Hanyu-Nakamura, K. (2004). Drosophila cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis. Dev Cell 6, 69-78.

Nekrasov, M. P., Ivshina, M. P., Chernov, K. G., Kovrigina, E. A., Evdokimova, V. M., Thomas, A. A., Hershey, J. W., and Ovchinnikov, L. P. (2003). The mRNA-binding protein YB-1 (p50) prevents association of the eukaryotic initiation factor eIF4G with mRNA and inhibits protein synthesis at the initiation stage. J Biol Chem 278, 13936-13943.

Nelson, M. R., Leidal, A. M., and Smibert, C. A. (2004). Drosophila Cup is an eIF4E-binding protein that functions in Smaug-mediated translational repression. Embo J 23, 150-159.

Nelson, P., Kiriakidou, M., Sharma, A., Maniataki, E., and Mourelatos, Z. (2003). The microRNA world: small is mighty. Trends Biochem Sci 28, 534-540.

Nothiger, R., Jonglez, M., Leuthold, M., Meier-Gerschwiler, P., and Weber, T. (1989). Sex determination in the germ line of Drosophila depends on genetic signals and inductive somatic factors. Development 107, 505-518.

O

Olsen, P. H., and Ambros, V. (1999). The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol 216, 671-680.

Ostareck, D. H., Ostareck-Lederer, A., Shatsky, I. N., and Hentze, M. W. (2001). Lipoxygenase mRNA silencing in erythroid differentiation: The 3'UTR regulatory complex controls 60S ribosomal subunit joining. Cell 104, 281-290.

Ostareck, D. H., Ostareck-Lederer, A., Wilm, M., Thiele, B. J., Mann, M., and Hentze, M. W. (1997). mRNA silencing in erythroid differentiation: hnRNP K and hnRNP E1 regulate 15-lipoxygenase translation from the 3' end. Cell 89, 597-606.

Ostareck-Lederer, A., and Ostareck, D. H. (2004). Control of mRNA translation and stability in haematopoietic cells: the function of hnRNPs K and E1/E2. Biol Cell 96, 407-411.

Ostareck-Lederer, A., Ostareck, D. H., Standart, N., and Thiele, B. J. (1994). Translation of 15-lipoxygenase mRNA is inhibited by a protein that binds to a repeated sequence in the 3' untranslated region. Embo J 13, 1476-1481.

P

Palmer, M. J., Richman, R., Richter, L., and Kuroda, M. I. (1994). Sex-specific regulation of the male-specific lethal-1 dosage compensation gene in Drosophila. Genes Dev 8, 698-706.

Palmer, T. D., Miller, A. D., Reeder, R. H., and McStay, B. (1993). Efficient expression of a protein coding gene under the control of an RNA polymerase I promoter. Nucleic Acids Res 21, 3451-3457.

Parisi, M., and Lin, H. (2000). Translational repression: a duet of Nanos and Pumilio. Curr Biol 10, R81-83.

Pascal, E., and Tjian, R. (1991). Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev 5, 1646-1656.

Patel, G. P., Ma, S., and Bag, J. (2005). The autoregulatory translational control element of poly(A)-binding protein mRNA forms a heteromeric ribonucleoprotein complex. Nucleic Acids Res 33, 7074-7089.

Penalva, L. O., and Sanchez, L. (2003). RNA binding protein sex-lethal (Sxl) and control of Drosophila sex determination and dosage compensation. Microbiol Mol Biol Rev 67, 343-359, table of contents.

Pestova, T. V., Lomakin, I. B., Lee, J. H., Choi, S. K., Dever, T. E., and Hellen, C. U. (2000). The joining of ribosomal subunits in eukaryotes requires eIF5B. Nature 403, 332-335.

Pickering, B. M., Mitchell, S. A., Spriggs, K. A., Stoneley, M., and Willis, A. E. (2004). Bag-1 internal ribosome entry segment activity is promoted by structural changes mediated by poly(rC) binding protein 1 and recruitment of polypyrimidine tract binding protein 1. Mol Cell Biol 24, 5595-5605.

Pickering, B. M., and Willis, A. E. (2005). The implications of structured 5' untranslated regions on translation and disease. Semin Cell Dev Biol 16, 39-47.

Pillai, R. S. (2005). MicroRNA function: multiple mechanisms for a tiny RNA? Rna 11, 1753-1761.

Preiss, T., and Hentze, M. W. (2003). Starting the protein synthesis machine: eukaryotic translation initiation. BioEssays 25, 1201-1211.

R

Ramakrishnan, V. (2002). Ribosome structure and the mechanism of translation. Cell 108, 557-572.

Rastelli, L., Richman, R., and Kuroda, M. I. (1995). The dosage compensation regulators MLE, MSL-1 and MSL-2 are interdependent since early embryogenesis in Drosophila. Mech Dev 53, 223-233.

Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W. J., and Jackle, H. (1996). RNA binding and translational suppression by bicoid. Nature 379, 746-749.

Ross, M. T., Grafham, D. V., Coffey, A. J., Scherer, S., McLay, K., Muzny, D., Platzer, M., Howell, G. R., Burrows, C., Bird, C. P., et al. (2005). The DNA sequence of the human X chromosome. Nature 434, 325-337.

S

Sachs, A. (2000). Physical and Functional Interactions between the mRNA Cap Structure and the Poly(A) tail. In Translational Control of Gene Expression (Cold Spring Harbor Laboratory Press).

Sakashita, E., and Sakamoto, H. (1994). Characterization of RNA binding specificity of the Drosophila sex-lethal protein by in vitro ligand selection. Nucleic Acids Res 22, 4082-4086.

Sakashita, E., and Sakamoto, H. (1996). Protein-RNA and protein-protein interactions of the Drosophila sex-lethal mediated by its RNA-binding domains. J Biochem (Tokyo) 120, 1028-1033.

Samuels, M., Deshpande, G., and Schedl, P. (1998). Activities of the Sex-lethal protein in RNA binding and protein:protein interactions. Nucleic Acids Res 26, 2625-2637.

Schütt, C., and Nöthiger, R. (2000). Structure, function and evolution of sex-determining systems in Dipteran insects. Development 127, 667-677.

Seggerson, K., Tang, L., and Moss, E. G. (2002). Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. Dev Biol 243, 215-225.

Semotok, J. L., Cooperstock, R. L., Pinder, B. D., Vari, H. K., Lipshitz, H. D., and Smibert, C. A. (2005). Smaug recruits the CCR4/POP2/NOT deadenylase complex to trigger maternal transcript localization in the early Drosophila embryo. Curr Biol 15, 284-294.

Singh, R., Valcarcel, J., and Green, M. R. (1995). Distinct binding specificities and functions of higher eukaryotic polypyrimidine tract-binding proteins. Science 268, 1173-1176.

Skabkin, M. A., Evdokimova, V., Thomas, A. A., and Ovchinnikov, L. P. (2001). The major messenger ribonucleoprotein particle protein p50 (YB-1) promotes nucleic acid strand annealing. J Biol Chem 276, 44841-44847.

Skabkina, O. V., Lyabin, D. N., Skabkin, M. A., and Ovchinnikov, L. P. (2005). YB-1 autoregulates translation of its own mRNA at or prior to the step of 40S ribosomal subunit joining. Mol Cell Biol 25, 3317-3323.

Skabkina, O. V., Lyabin, D. N., Skabkin, M. A., and Ovchinnikov, L. P. (2005). YB-1 autoregulates translation of its own mRNA at or prior to the step of 40S ribosomal subunit joining. Mol Cell Biol 25, 3317-3323.

Smibert, C. A., Wilson, J. E., Kerr, K., and Macdonald, P. M. (1996). smaug protein represses translation of unlocalized nanos mRNA in the Drosophila embryo. Genes Dev 10, 2600-2609.

Smith, E. R., Pannuti, A., Gu, W., Steurnagel, A., Cook, R. G., Allis, C. D., and Lucchesi, J. C. (2000). The drosophila MSL complex acetylates histone H4 at lysine 16, a chromatin modification linked to dosage compensation. Mol Cell Biol 20, 312-318.

Sommerville, J. (1999). Activities of cold-shock domain proteins in translation control. Bioessays 21, 319-325.

Sonenberg, N., and Dever, T. E. (2003). Eukaryotic translation initiation factors and regulators. Curr Opin Struct Biol 13, 56-63.

Sonoda, J., and Wharton, R. P. (1999). Recruitment of Nanos to hunchback mRNA by Pumilio. Genes Dev 13, 2704-2712.

Sonoda, J., and Wharton, R. P. (2001). Drosophila Brain Tumor is a translational repressor. Genes Dev 15, 762-773.

Spahn, C. M., and Nierhaus, K. H. (1998). Models of the elongation cycle: an evaluation. Biol Chem 379, 753-772.

Spriggs, K. A., Bushell, M., Mitchell, S. A., and Willis, A. E. (2005a). Internal ribosome entry segment-mediated translation during apoptosis: the role of IRES-trans-acting factors. Cell Death Differ 12, 585-591.

Spriggs, K. A., Mitchell, S. A., and Willis, A. E. (2005b). Investigation of interactions of polypyrimidine tract-binding protein with artificial internal ribosome entry segments. Biochem Soc Trans 33, 1483-1486.

Stebbins-Boaz, B., Cao, Q., de Moor, C. H., Mendez, R., and Richter, J. D. (1999). Maskin is a CPEB-associated factor that transiently interacts with eIF-4E. Mol Cell 4, 1017-1027.

Stickeler, E., Fraser, S. D., Honig, A., Chen, A. L., Berget, S. M., and Cooper, T. A. (2001). The RNA binding protein YB-1 binds A/C-rich exon enhancers and stimulates splicing of the CD44 alternative exon v4. Embo J 20, 3821-3830.

Stott, K., Blackburn, J. M., Butler, P. J., and Perutz, M. (1995). Incorporation of glutamine repeats makes protein oligomerize: implications for neurodegenerative diseases. Proc Natl Acad Sci U S A 92, 6509-6513.

Stripecke, R., Oliveira, C. C., McCarthy, J. E., and Hentze, M. W. (1994). Proteins binding to 5' untranslated region sites: a general mechanism for translational regulation of mRNAs in human and yeast cells. Mol Cell Biol 14, 5898-5909.

Strom, A. C., Forsberg, M., Lillhager, P., and Westin, G. (1996). The transcription factors Sp1 and Oct-1 interact physically to regulate human U2 snRNA gene expression. Nucleic Acids Res 24, 1981-1986.

T

Tarun, S. Z., Jr., and Sachs, A. B. (1995). A common function for mRNA 5' and 3' ends in translation initiation in yeast. Genes Dev 9, 2997-3007.

Tarun, S. Z., Jr., and Sachs, A. B. (1996). Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. Embo J 15, 7168-7177.

Tarun, S. Z., Jr., Wells, S. E., Deardorff, J. A., and Sachs, A. B. (1997). Translation initiation factor eIF4G mediates in vitro poly(A) tail-dependent translation. Proc Natl Acad Sci U S A 94, 9046-9051.

U

Uchida, N., Hoshino, S., Imataka, H., Sonenberg, N., and Katada, T. (2002). A novel role of the mammalian GSPT/eRF3 associating with poly(A)-binding protein in Cap/Poly(A)-dependent translation. J Biol Chem 277, 50286-50292.

V

Valcarcel, J., Singh, R., Zamore, P. D., and Green, M. R. (1993). The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of transformer pre-mRNA. Nature 362, 171-175.

Valencia-Sanchez, M. A., Liu, J., Hannon, G. J., and Parker, R. (2006). Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev 20, 515-524.

Valley, C. M., and Willard, H. F. (2006). Genomic and epigenomic approaches to the study of X chromosome inactivation. Curr Opin Genet Dev 16, 240-245.

Varani, G. (1997). A cap for all occasions. Structure 5, 855-858.

Vaucheret, H. (2006). Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes Dev 20, 759-771.

Vazquez, F. (2006). Arabidopsis endogenous small RNAs: highways and byways. Trends Plant Sci.

Vied, C., Halachmi, N., Salzberg, A., and Horabin, J. I. (2003). Antizyme is a target of sex-lethal in the Drosophila germline and appears to act downstream of hedgehog to regulate sex-lethal and cyclin B. Dev Biol 253, 214-229.

W

Wang, J., and Bell, L. R. (1994). The Sex-lethal amino terminus mediates cooperative interactions in RNA binding and is essential for splicing regulation. Genes Dev 8, 2072-2085.

Wang, X., Zamore, P. D., and Hall, T. M. (2001). Crystal structure of a Pumilio homology domain. Mol Cell 7, 855-865.

Wang, Y., Zhang, W., Jin, Y., Johansen, J., and Johansen, K. M. (2001). The JIL-1 tandem kinase mediates histone H3 phosphorylation and is required for maintenance of chromatin structure in Drosophila. Cell 105, 433-443.

Welch, E. M., Wang, W., and Peltz, S. W. (2000). Translation Termination; It's Not the End of the Story. In Translational Control of Gene Expression (Cold Spring Harbor Laboratory Press).

Wickens, M., Goodwin, E. B., Kimble, J., Strickland, S., and Hentze, M. W. (2000). Translational Control of Developmental Decisions. In Translational Control of Gene Expression (Cold Spring Harbor Laboratory Press).

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75, 855-862.

Wilhelm, J. E., Hilton, M., Amos, Q., and Henzel, W. J. (2003). Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz. J Cell Biol 163, 1197-1204.

Wilhelm, J. E., and Smibert, C. A. (2005). Mechanisms of translational regulation in Drosophila. Biol Cell 97, 235-252.

Wilkie, G. S., Dickson, K. S., and Gray, N. K. (2003). Regulation of mRNA translation by 5'- and 3'-UTR-binding factors. Trends Biochem Sci 28, 182-188.

Wilkins, R. C., and Lis, J. T. (1998). GAGA factor binding to DNA via a single trinucleotide sequence element. Nucleic Acids Res 26, 2672-2678.

Wolffe, A. P., and Meric, F. (1996). Coupling transcription to translation: a novel site for the regulation of eukaryotic gene expression. Int J Biochem Cell Biol 28, 247-257.

Wreden, C., Verrotti, A. C., Schisa, J. A., Lieberfarb, M. E., and Strickland, S. (1997). Nanos and pumilio establish embryonic polarity in Drosophila by promoting posterior deadenylation of hunchback mRNA. Development 124, 3015-3023.

Z

Zappavigna, V., Piccioni, F., Villaescusa, J. C., and Verrotti, A. C. (2004). Cup is a nucleocytoplasmic shuttling protein that interacts with the eukaryotic translation initiation factor 4E to modulate Drosophila ovary development. Proc Natl Acad Sci U S A 101, 14800-14805.

Zhou, S., Yang, Y., Scott, M. J., Pannuti, A., Fehr, K. C., Eisen, A., Koonin, E. V., Fouts, D. L., Wrightsman, R., Manning, J. E., and et al. (1995). Male-specific lethal 2, a dosage compensation gene of Drosophila, undergoes sex-specific regulation and encodes a protein with a RING finger and a metallothionein-like cysteine cluster. Embo J 14, 2884-2895.

IN CLOSING….

‘Zog the Martian steered her craft carefully into its new orbit and prepared to reenter the hole in the back of the planet, the one that had never been seen from Earth. She had done it many times before and was not so much nervous as impatient to be home. It had been a long stay on Earth, longer than most Martians made, and she looked forward to a long argon bath and a glass of cold chlorine. It would be good to see her colleagues again. And her children. And her husband- she caught herself and laughed. She had been on Earth so long she had even begun to think like an earthling. Husband indeed! Every Martian knew that no Martian had a husband. There was no such thing as sex on Mars. Zog thought with pride of the report in her knapsack: “Life on Earth: The Reproduction Enigma Solved.” It was the finest thing she had ever done; promotion could not be denied her now, whatever Big Zag said.

A week later, Big Zag opended the door of the Earthstudy Inc. committee room and asked the secretary to send Zog in. Zog entered and sat in the seat assigned to her. Big Zag avoided her eyes as she cleared her throat and began.

“Zog, this committee has read your report carefully, and we are all, I think I can say, impressed with its thoroughness. You have certainly made an exhaustive survey of reproduction on Earth. Moreover, with the possible exception of Miss Zeeg here, we are all agreed that you have made an overwhelming case for your hypothesis. I consider it now beyond any doubt that life on Earth reproduces in the way you describe, using this strange device called ‘sex’. Some of the committee are less happy with your conclusion that many of the peculiar facets of the earthling species known as human beings are a consequence of this sex thing: jealous love, a sense of beauty, male aggression, even what they laughingly call intelligence.” The committee chuckled sycophantically at this old joke. “But, “ said Big Zag suddenly and loudly, looking up from the paper in front of her, “we have one major difficulty with your report. We believe you have entirely failed to address the most interesting issue of all. It is a three-letter question of great simplicity.” Big Zag’s voice dripped sarcasm. “*Why?*”

Zog stammered: “What do you mean, why?”

“I mean why do earthlings have sex? Why don’t they just clone themselves as we do? Why do they need two creatures to have one baby? Why do males exist? Why? Why? Why?’

‘The Red Queen: Sex and The Evolution of Human Nature’
by Matt Ridley (1993)

While Watson- Crick DNA structure *per se* provides fundamental understanding of straightforwardness, gene expression on the other hand, surely cannot be so easily explained. It appears a Rube Goldberg machine, a complex and fascinating, but somewhat molecular toy. Where are we in all this? An other way to phrase this question would be to say, What question did I ask, and what was my answer?

Charles Darwin once said " A naturalist's life would be a happy one if he had only to observe and never to write." There is a great deal more to writing than arranging well-chosen words in a clear, concise way. The process actually begins long before the first words go on paper. All of us can suffer from the normal human failings of inflating the importance of a message and overestimating its audience.

Irina Abaza