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Review

Mechanisms of translational regulation in *Drosophila*

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Translational regulation plays an essential role in many phases of the *Drosophila* life cycle. During embryogenesis, specification of the developing body pattern requires co-ordination of the translation of oskar, gurken and nanos mRNAs with their subcellular localization. In addition, dosage compensation is controlled by Sex-lethal-mediated translational regulation while dFMR1 (the *Drosophila* homologue of the fragile X mental retardation protein) controls translation of various mRNAs which function in the nervous system. Here we describe some of the mechanisms that are utilized to regulate these various processes. Our review highlights the complexity that can be involved with multiple factors employing different mechanisms to control the translation of a single mRNA.

Introduction

Regulated translation controls a wide range of processes in eukaryotes. Global regulation can be exercised through modification of the basic translation machinery via events such as phosphorylation. In other cases, control is more selective with sequence-specific RNA-binding proteins recognizing target transcripts, thereby regulating translation. While the number of such factors is growing rapidly, the molecular details of how most regulate translation are not well understood. The binding sites for many of these regulatory proteins are located in the target transcript's 3′ untranslated region (UTR). Thus any proposed mechanism must explain how a protein bound to the 3′ end of a mRNA is able to interact with the translation machinery.

Some of the best characterized examples of 3′ UTR-binding proteins functioning as translational regulators comes from studies of the *Drosophila* mRNAs that specify the anteroposterior and dorsal-ventral axes of the embryo. The expression of these mRNAs is controlled by a combination of translational regulation and mRNA localization to ensure the encoded proteins only accumulate at the correct place and at the correct time. In general, translational repression mediated by sequence-specific 3′ UTR-binding proteins ensures these mRNAs are not expressed until they reach their final destination. Translational activation of these transcripts upon localization requires mechanisms to relieve their translational repression. Both genetic and biochemical approaches have been employed to identify the relevant *cis*- and *trans*-acting factors, and recent work has made inroads into the molecular mechanisms that are involved. In the first part of this review we will focus on the translational control of three localized mRNAs, oskar (osk), gurken (grk) and nanos (nos).

In addition to the translational control of localized transcripts, studies in *Drosophila* have identified a role for translational control in a variety of processes ranging from spermatogenesis to fat metabolism. In the second part of this review, we will turn our attention to the role of translational regulation in dosage compensation and neuronal function, as exemplified by two RNA-binding proteins, Sex-lethal (SXL) and

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Key words: Cup, *Drosophila* fragile X mental retardation 1 (dFMR1), RNA interference (RNAi), Smaug, Sex-lethal.

Abbreviations used: *armi, armitage*; *aub*, *aubergine*; Bcd, Bicoid; BicC, Bicaudal C; Bru, Bruno; Btz, Barentsz; CPEB, cytoplasmic-polyadenylation-element-binding protein; 4E-BP, eIF4E-binding protein; eIF, eukaryotic initiation factor; FMRP, fragile X mental retardation protein; *grk*, *gurken*; KH, K homology; ME31B, Maternal expression at 31B; miRNA, microRNA; MSL, male specific lethal; NAC, nascent polypeptide-associated complex; nos, nanos; orb, oo18 RNA-binding protein; osk, oskar; PABP, poly(A)-binding protein; poly(A)$^+$, polyadenylated; RISC, RNA-induced silencing complex; RNAi, RNA interference; RNP, ribonucleoprotein; Smg, Smaug; siRNA, short interfering RNA; *spn-E*, *spindle-E*; SXL, Sex-lethal; UTR, untranslated region; Vas, Vasa; Yps, Ypsilon Schachtel.

dFMR1, the *Drosophila* homologue of the human fragile X mental retardation protein (FMRP).

Regulation of osk translation

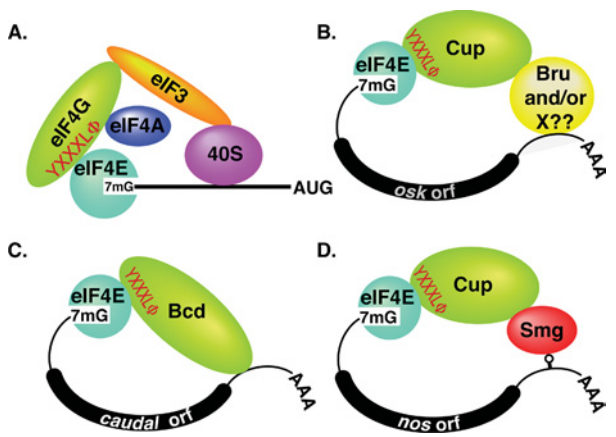
One of the major steps in establishing the anteroposterior axis of the *Drosophila* embryo is the localization of Osk protein to the posterior of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991). The localized Osk protein, in turn, nucleates the assembly of a ribonucleoprotein (RNP) complex that directs development of the posterior (Ephrussi et al., 1991; Kim-Ha et al., 1991; Smith et al., 1992; Webster et al., 1994; Kobayashi et al., 1995; Breitwieser et al., 1996). Inappropriately localized Osk protein directs ectopic posterior development leading to lethal body patterning defects. Thus it is not surprising that multiple mechanisms ensure that Osk protein only accumulates at the posterior (Benton and St Johnston, 2002). For example, osk mRNA is transported to the posterior via a microtubule-based transport mechanism and its localization is co-ordinated with its translation to ensure that Osk protein is only synthesized at the posterior pole (Tekotte and Davis, 2002). Here we will focus on the factors and mechanisms that ensure that only correctly localized osk transcripts are translated.

The role of eIF4E-BP (eukaryotic initiation factor 4E-binding protein) in translational control of localized messages: Cup

While a large number of factors that control the localization or translation of osk mRNA have been identified, only recently have biochemical functions been assigned to components of the osk RNP complex. The best understood of these factors is Cup, an eIF4E-BP that is required for both the translational repression and localization of osk mRNA (Wilhelm et al., 2003; Nakamura et al., 2004; Nelson et al., 2004). eIF4E is the component of the translation initiation machinery that recognizes the m^7GpppN cap at the 5′ end of the transcript and nucleates the assembly of the translation initiation complex on the message (Gingras et al., 1999). The critical step in this assembly process is the binding of the scaffolding protein eIF4G to eIF4E (Figure 1). The recruitment of eIF4G is crucial because it links the initiation complex (eIF4A, eIF4E) to the ribosome via its interaction with eIF3

Figure 1 | The mechanism of Cup-dependent translation repression

Cup-dependent translation (**A**) involves the recognition of the 5′ cap by eIF4E which in turn recruits eIF4G to the transcript through an eIF4E-binding motif (YXXXLΦ) within eIF4G. eIF4G functions as a scaffolding protein to recruit eIF4A and eIF3, and eIF3 recruits the 40S ribosomal subunit. Cup also interacts with eIF4E through an eIF4E-binding motif and Cup recruitment to osk (**B**) and nos (**D**) mRNAs resulting in translational repression by blocking the eIF4E/eIF4G interaction. Recruitment of Cup to nos mRNA is mediated by Cup's interaction with Smg bound to the nos 3′ UTR. The mechanism of Cup recruitment to osk may involve Bru and/or some other as yet uncharacterized mechanism. Repression of caudal translation (**C**) differs from osk and nos in that a single protein, Bcd, interacts with both eIF4E and the caudal 3′UTR. Again Bcd's ability to interact with eIF4E is mediated via an eIF4E-binding motif, and as such Bcd also blocks recruitment of eIF4G to the caudal mRNA.



(Haghighat et al., 1995; Lamphear et al., 1995). Cup is a member of a diverse group of 4E-BPs that have been found to bind to eIF4E *in vitro* through a conserved eIF4E-binding motif, YxxxxLϕ (x is any amino acid and ϕ is a hydrophobic amino acid) (Mader et al., 1995; Wilhelm et al., 2003; Nakamura et al., 2004; Nelson et al., 2004). Because eIF4G also binds to eIF4E via the YxxxxLϕ motif, these 4E-BPs are thought to repress translation by acting as competitive inhibitors for the eIF4G-eIF4E interaction (Figure 1). In the case of Cup, deletion of this motif causes premature translation of osk, indicating that the interaction between Cup and eIF4E is critical for repressing translation of osk mRNA (Nakamura et al., 2004).

Surprisingly, Cup also plays a role in the last step of osk mRNA localization. During early oogenesis (stages 1–6), osk mRNA accumulates at the posterior pole of the oocyte, where the minus ends of the microtubule array are concentrated (Chekulaeva and Ephrussi, 2004). At stages 7 and 8, the microtubules reorganize so that microtubule nucleation occurs over most of the oocyte cortex with the majority of the minus ends being concentrated at the anterior of the oocyte. Tracking the minus ends of the microtubules, osk mRNA transiently localizes to the anterior of the oocyte during these stages. During stages 9 and 10, however, osk mRNA transits back to the posterior pole in a plus-end directed transport step that requires *kinesin heavy chain* (*khc*). The defect in plus-end directed transport, seen with particular alleles of *cup,* is most probably due to a failure to correctly assemble the osk localization complex, since *cup* mutants block the transport of the localization factor Barentsz (Btz) into the oocyte (Wilhelm et al., 2003). Because the defect in Btz transport is apparent during the early stages of oogenesis when osk mRNA localization is normal, it is likely that the late stage osk mRNA localization defects observed in *cup* mutants are due to a requirement for Btz only in late stage oocytes. Consistent with this interpretation, *btz* and *cup* mutants display osk mRNA localization defects at the same stages of oogenesis (van Eeden et al., 2001; Wilhelm et al., 2003). Since *cup* function is not required for the recruitment of other components of the osk RNP, such as Ypsilon Schachtel (Yps), or for the early minus-end directed microtubule transport of osk mRNA, it has been proposed that Cup is specifically required to recruit plus-end directed transport factors, such as Btz, to the complex (Wilhelm et al., 2003). Thus Cup is a translational repressor that is required to assemble the osk mRNA localization machinery. In this regard it is worth noting that the weakest alleles of *cup* are those that completely delete the eIF4E-binding domain of Cup, and that these alleles appear to localize osk mRNA normally (Nakamura et al., 2004). This favours the argument that the localization and repression functions of Cup are separable and that a structure–function analysis of Cup might help to identify new domains required to assemble the osk localization complex.

The requirement for *cup* in assembling the osk RNP raises the question of how Cup itself is recruited to the osk mRNA. Biochemical studies of Cup indicate that it binds directly to Bruno (Bru), an RNA-binding protein that was identified by its ability to bind directly to the osk 3′ UTR (Kim-Ha et al., 1995; Webster et al., 1997; Nakamura et al., 2004). This observation has led to an attractive model where the binding of Bru to the osk 3′ UTR recruits Cup and leads to translational repression via sequestration of eIF4E (Nakamura et al., 2004). Consistent with this model, mutating all of the Bru response elements in the osk 3′ UTR leads to translational derepression of osk transcripts (Kim-Ha et al., 1995). However, it has recently been noted that this derepession is not as severe as that observed in *cup* mutants (Macdonald, 2004). Furthermore, while Bru is a translational regulator of the localized message, grk, Cup is not (Filardo and Ephrussi, 2003; Wilhelm et al., 2003; Nakamura et al., 2004). Thus Cup and Bru do not always act in concert. Together, these observations suggest that Bru may not be sufficient for Cup recruitment and that the Cup–Bru interaction is not absolutely required for the function of either protein: a proposal that is borne out by the recent discovery (described below) that Cup is recruited to nos transcripts by an unrelated RNA-binding protein, Smaug (Smg) (Nelson et al., 2004).

Multifunctional 4E-BPs

While Cup may be targeted to messages by a suite of RNA-binding proteins, not all 4E-BPs function this way. A multifunctional 4E-BP has been found in the case of Bicoid (Bcd). Bcd is a homeodomain transcription factor that was identified on the basis of its role in anterior pattern formation (Ephrussi and St Johnston, 2004). However, subsequent work has shown that Bcd is also a RNA-binding protein that binds the 3′ UTR of caudal as well as an eIF4E-binding protein that functions in translational repression of caudal *in vivo* (see Figure 1 and Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996; Niessing et al., 2002). Furthermore, unlike Cup, the eIF4E-binding activity of Bcd requires it be bound to RNA, indicating a new way that repression by 4E-BPs can be targeted to a select group of transcripts (Niessing et al., 2002). Thus, Bcd is a transcription factor, RNA-binding protein, and translational repressor: all rolled into one! While the case of Bcd may be the exception, it could also be the rule. It is currently unclear what context is required for a canonical 4E-binding motif to be functional within any given

protein. However, if the sequence requirements for allowing the 4E-binding motif to function are as degenerate as the 4E-binding motif itself, then a great number of proteins may have acquired eIF4E-binding activity in addition to their other roles. For instance, out of 5886 separate entries in the *Saccharomyces* genome database, 2389 have a canonical 4E-binding motif! Obviously, not all of these motifs are in a functional context, but what additional sequence features are required to make a 4E-binding motif functional?

Recent structural studies of eIF4G–eIF4E complexes have shown that the binding of eIF4E to eIF4G induces a conformational change in eIF4G that allows it to form a 'molecular bracelet' around the N-terminus of eIF4E (Gross et al., 2003). Using these structures as a base, homology modelling of human 4E-BP1–eIF4E complex suggests that a similar extended pattern of contacts may also exist for 4E-binding proteins and that the residues flanking the canonical 4E-binding motif might be critical for high affinity binding. Identifying these sequence features should make the computerized identification of 4E-binding proteins possible.

The role of 4E-BPs in mRNA localization

Evidence that other 4E-BPs in addition to Cup may play a role in both mRNA localization and translational control has come from work on the translational regulator, cytoplasmic-polyadenylation-element-binding protein (CPEB). CPEB is an RNA-binding protein that recruits the eIF4E-binding protein maskin, to transcripts and translationally represses them (Stebbins-Boaz et al., 1999). Surprisingly in neurons, inserting the CPEB-binding sequence into an unlocalized message is sufficient to cause the message to be transported into dendrites (Huang et al., 2003). This result implies that CPEB binding is sufficient to assemble an active transport complex. Consistent with this interpretation, overexpression of a truncated CPEB that only contains the RNA-binding domains dominantly interferes with the transport of endogenous messages into dendrites (Huang et al., 2003). How do the additional domains of CPEB act to assemble the transport machinery? One possible explanation is that maskin, like Cup, is a critical factor for assembling the transport complex. Thus the main role of CPEB in transport could be to recruit maskin to localized messages. Experiments with mutant forms of CPEB that are incapable of binding to maskin should help address whether or not there is a broadly conserved role for 4E-BPs in both translational repression and mRNA localization.

RNA interference (RNAi)-mediated repression of osk translation

Recently another mechanism of translational control, RNA silencing, has been implicated in the repression of osk translation. RNA silencing, also referred to as RNAi, is an evolutionarily conserved process that represses gene expression and can function at the level of transcription, translation, or mRNA stability (Lai, 2003; Bartel, 2004). Repression is mediated by small non-coding RNAs that come in two forms, short interfering RNAs (siRNAs) and microRNAs (miRNAs), that are generated by Dicer, an RNase III endonuclease. siRNAs are processed by Dicer cleavage of long double-stranded RNA molecules while miRNAs are processed by Dicer cleavage of hairpins. Once cleaved, one strand of the duplex is incorporated into the RNA-induced silencing complex (RISC) and RISC then uses the incorporated RNA strand as a guide to identify targets for repression based on complementarity to the small RNA. When regulation occurs post-transcriptionally, a target mRNA can be degraded or translationally repressed. Usually perfect complementarity, most commonly seen with siRNAs, is associated with target degradation. Translational repression, normally seen with miRNAs, results when several sites of partial complementarity are found within the target's 3′ UTR.

One of the characteristics of translational repression by miRNAs is that the repression acts at a step after translation initiation (Olsen and Ambros, 1999). As a result, the target mRNA is associated with polysomes in a puromycin-sensitive manner, but no protein accumulates. This behaviour suggests that the block in translation may be at the elongation step. Recent work has shown that osk mRNA is associated with polysomes even when it is translationally repressed, suggesting that miRNA-mediated translational control might also play a role in the regulation of osk expression (Braat et al., 2004). At first glance, such a translational control mechanism, would be at odds with Cup-mediated translational repression which acts to prevent translation initiation. However, recent work on the role of the RNAi machinery in oogenesis has pointed the way to integrating these two mechanisms.

Clues that RNAi might play a role in osk translational regulation first came with the identification of mutations in the gene *armitage* (*armi*) which disrupt oocyte polarity and cause Osk protein to accumulate prematurely (Cook et al., 2004). Since *armi* mutants appear to express similar levels of osk mRNA, this suggested that it is required to repress osk translation early in oogenesis. The Armi protein contains a putative RNA helicase domain that is most closely related to the *Arabidopsis* silencing defective 3 protein (Dalmay et al., 2001; Willmann, 2001). Because silencing defective 3 protein is required for posttranscriptional gene silencing, an RNAi-like mechanism that functions in plants, the similarity between Armi and silencing defective 3 protein suggested that osk translation might be regulated by the RNAi machinery. To test this hypothesis, the authors examined mutations in the RNAi pathway for defects in osk translation. They found that mutations in *aubergine* (*aub*), *spindle-E* (*spn-E*), and *maelstrom* all result in precocious Osk protein accumulation. Aub is an member of the Argonaute family of proteins which have been implicated in RNAi-like processes in a variety of organisms (Harris and Macdonald, 2001; Carmell et al., 2002), while Spn-E is a member of the DE-H-box RNA helicase family (Gillespie and Berg, 1995). Both Aub and Spn-E are required for RNAi in the early embryo as well as the silencing of the Stellate genes through a mechanism thought to involve naturally occurring siRNAs (Aravin et al., 2001; Kennerdell et al., 2002). Maelstrom is a novel protein that is required for the normal localization of the RNAi factors Dicer and Argonaute 2 during oogenesis (Clegg et al., 1997; Findley et al., 2003). Intriguingly, all of these mutants in the RNAi pathway display premature translation of osk before such defects are usually seen in *cup* mutants. This suggests that RNAi-mediated repression may predominate early in oogenesis while Cup-mediated repression acts late in oogenesis, providing a possible resolution as to how two different mechanisms of translational repression can both act on osk (Wilhelm et al., 2003; Cook et al., 2004; Nakamura et al., 2004).

Taken together these data suggest that osk translational repression involves an RNAi-dependent mechanism, and that *armi* may function in RNAi. Confirmation of the role of *armi* in RNAi came from experiments showing that *armi* is required for Stellate silencing *in vivo* and for RNAi *in vitro* (Tomari et al., 2004). The latter experiment showed that ovary extracts from *armi* mutants were defective for siRNA-dependent cleavage of an exogenous mRNA. This defect results from a failure to form fully functional RISC and argues that RISC is required to repress osk translation.

Armi, *aub*, *maelstrom*, and *spn-E* mutants all show defects in osk mRNA localization which probably result from the cytoskeletal defects associated with these mutations (Cook et al., 2004). Thus the RNAi machinery may also regulate expression of cytoskeletal components, perhaps at the level of translation. The cytoskeletal defects in these mutants underscore the complexity of their phenotypes and raises the possibility that the defects in osk translation are not direct. However, the fact that translationally repressed osk messages are polysome associated, a characteristic of miRNA translational repression, supports the argument that the RNAi machinery may act directly on osk (Braat et al., 2004). However, polysome association of translationally repressed osk mRNA could reflect an RNAi independent mechanism of translational repression. Proof that osk translation is directly regulated by an RNAi-dependent mechanism would come with the identification of the *cis*-acting elements within the osk mRNA that are required for RNAi-dependent repression and the miRNA(s) that recognize these elements. A recent bioinformatic effort to assign targets to *Drosophila* miRNAs suggests that miRNA280 could regulate osk (Stark et al., 2003); however, experimental evidence supporting this predication is lacking.

A dual role for Aub in osk translation

In addition to functioning in osk translational repression, Aub is also required for efficient translation of osk mRNA upon its ultimate localization to the posterior. Translation of osk requires localization of the mRNA to the posterior. Thus the role of Aub in efficient osk translation could be an indirect consequence of the defect in osk mRNA localization seen in *aub* mutants. Alternatively, the requirement for Aub in osk translation might be direct. A direct role is suggested when one considers that osk mRNA localization can be divided into two distinct phases: (1) the transport phase which delivers the RNA to the posterior, and (2) the maintenance phase which ensures that osk transcripts stay localized to the posterior. Maintenance requires Osk protein and *aub*

mutants also display defects in osk mRNA maintenance (Kim-Ha et al., 1991; Webster et al., 1994; Rongo et al., 1995). Thus the inefficient translation of Osk protein in *aub* mutants could be the primary defect which leads to a secondary defect in osk mRNA maintenance. If the direct model is true, then Aub serves a dual role in osk translational control, functioning as a repressor early in oogenesis, and an activator later on. Since translational activation may simply result from inactivation of the repressive machinery, Aub may be a component of the RNAi machinery that is targeted for inactivation upon localization of osk mRNA to the posterior.

Translational activation via the poly(A) (polyadenylated) tail

Once osk mRNA is localized to the posterior, translational repression must be overcome to allow Osk protein to be synthesized. One mechanism that has been implicated in derepressing osk translation is cytoplasmic polyadenylation. The phenomenon of cytoplasmic polyadenylation was first discovered in *Xenopus* oocytes, when it was found that a number of translationally silent messages have short poly(A) tails, and that elongation of these tails is required for their translation (Richter, 2000). Biochemical studies in *Xenopus* have identified a collection of factors that are required for this polyadenylation to occur, including CPEB (Hake and Richter, 1994), poly(A) polymerase (Bilger et al., 1994; Gebauer and Richter, 1995), and the cleavage and polyadenylation specificity factor (Dickson et al., 1999). As mentioned previously, *Xenopus* CPEB recruits a 4E-BP called maskin to repress translation, in addition to its role in promoting translation by assembling the cytoplasmic polyadenylation complex (Stebbins-Boaz et al., 1999). CPEB's role in mediating the switch between translational repression and translational activation appears to be regulated by phosphorylation by the aurora kinase Eg2. In its unphosphorylated state, CPEB recruits maskin which in turn represses translation by sequestering the eIF4E–cap complex. However, when CPEB is phosphorylated it recruits cleavage and polyadenylation specificity factor and poly(A) polymerase, which lengthens the poly(A) tail (Mendez et al., 2000). Because poly(A)-binding protein (PABP) binds directly to eIF4G, a longer poly(A) tail should increase the effective concentration of both PABP and eIF4G. Thus, lengthening of the poly(A) tail is thought to stabilize eIF4E–eIF4G complexes in preference to eIF4E–maskin complexes and lead to translational activation (Cao and Richter, 2002).

While work in *Xenopus* has given us a clear understanding of how cytoplasmic polyadenylation can be combined with 4E-BPs to generate a translational switch, the details of how an equivalent switch may be generated for osk mRNA remain unclear. *Drosophila* has a CPEB homologue, oo18 RNA-binding protein (Orb), and *orb* mutants display both impaired osk translation as well as osk transcripts with shortened poly(A) tails (Chang et al., 1999; Castagnetti and Ephrussi, 2003). However, Orb has not been shown to interact with *Drosophila* cleavage and polyadenylation specificity factor or poly(A) polymerase, nor has a *Drosophila* homologue of maskin been identified. Furthermore, Eg2 phosphorylation sites are not conserved from *Xenopus* to *Drosophila*, which suggests that the trigger for switching between translationally repressed and activated states might be quite different. One possible solution to the differences between CPEBs in *Xenopus* and *Drosophila* is that the translational activation and repression functions of CPEB have been segregated into different complexes. In this model, translational repression is maintained by the eIF4E–Cup–Bru complex and Orb acts as a translational activator that breaks the eIF4E–Cup interaction via elongation of the poly(A) tail. Thus, the translational derepression functions of CPEB would be conserved between species, while the repression functions would be segregated into a different complex.

Pieces in search of a puzzle: BicC (Bicaudal C), Hrp48, Yps, and ME31B

Not all known regulators of osk translation (Table 1) fit neatly into the three well characterized translational control mechanisms. A number of factors, such as Staufen, Vasa (Vas), and Apontic, have been shown to be required for efficient translational control of osk *in vivo*, but their mode of action remains mysterious (Breitwieser et al., 1996; Markussen et al., 1997; Lie and Macdonald, 1999; Micklem et al., 2000). However, there are four factors, BicC, Hrp48, Yps, and Maternal expression at 31B (ME31B), that on the basis of their phenotype or proposed biochemical function are likely to yield new insights into osk regulation in the near future.

BicC is a K homology (KH) domain RNA-binding protein that is required for normal

Table 1 | Roles of various factors in osk translation
Refer to text for further explanation and primary references.

Protein	Biochemical function	Role in osk translation
Armi	RNAi pathway	Early translational repression
Aub	RNAi pathway	Early translational repression/translational activation
Spn-E	RNAi pathway	Early translational repression
Maelstrom	RNAi pathway	Early translational repression
BicC	RNA-binding protein	Late translational repression
Cup	eIF4E-binding protein	Late translational repression
Bru	RNA-binding protein; Cup-binding protein	Late translational repression
ME31B	RNA helicase	Late translational repression
Hrp48	RNA-binding protein	Late translational repression
Apontic	RNA-binding protein; Bruno-binding protein	Translational repression
Orb	RNA-binding protein	Translational activation
Yps	RNA-binding protein	Antagonizes Orb translational activation
Staufen	RNA-binding protein	Translational activation
Vas	RNA helicase	Required for efficient osk translation

anterior-posterior patterning. While ovaries from homozygous females display premature osk translation similar to that observed in *cup* mutants, *BicC* is unusual in that females heterozygous for a deletion of the *BicC* locus produce embryos with a range of anterior-posterior patterning defects, including bicaudal embryos (Mahone et al., 1995; Saffman et al., 1998). The dominant effects associated with removing a single copy of *BicC*, together with its effects on translational control of osk suggest that it may be a limiting factor for translational repression of osk.

Hrp48, also known as hrb27C, is the *Drosophila* homologue of the heterogeneous nuclear RNP A/B family of RNA-binding proteins. Previous work on Hrp48 had implicated it in regulating alternative splicing (Siebel et al., 1994; Hammond et al., 1997; Burnette et al., 1999). However, it has been shown recently that *hrp48* is required for proper localization and translational control of osk mRNA, as well as the proper localization of grk mRNA (Goodrich et al., 2004; Huynh et al., 2004; Yano et al., 2004). Hrp48 was identified biochemically as a protein that specifically interacts with the translational control sequences of osk transcripts (as assayed via UV cross-linking; see the definition box for an explanation of this method), as well as genetically in a screen for genes required for anterior-posterior axis formation. The *hrp48* alleles isolated in this screen have specific defects in osk mRNA localization and do not display defects in translational control, splicing, or in the organization of the cytoskeleton (Huynh et al., 2004). In contrast, characterization of a different set of alleles showed defects in osk mRNA localization, polarization of the microtubule cytoskeleton, and weaker effects on translational repression of osk (Yano et al., 2004). Two explanations for this range of phenotypes have been proposed. First, because a number of components of the exon junction complex are required for osk mRNA localization, and *hrp48* is a known splicing regulator, it is possible that *hrp48*'s effects on osk mRNA localization are due to its effects on assembling or stabilizing the exon junction complex (Huynh et al., 2004). Secondly, since Hrp48 can homodimerize, and has binding sites in both the 5′ and 3′ UTRs of osk, it could promote circularization of the transcript and translational repression via Cup (Yano et al., 2004). Further experiments directed at the requirement of Hrp48 for Cup or exon junction complex function should help clarify *hrp48*'s role in osk regulation.

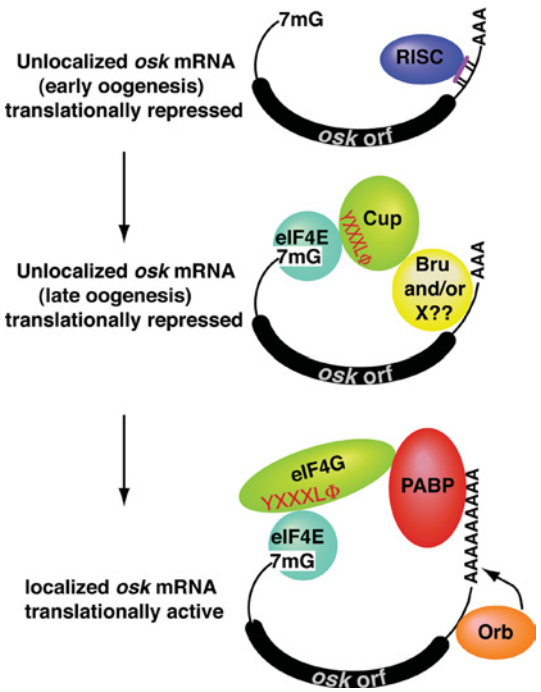
UV cross-linking: A method commonly employed to search for proteins in an extract that interact with a fragment of RNA. It exploits the fact that irradiation of many protein/RNA complexes with UV light covalently cross-links the protein to the RNA. After irradiation the complexes are treated with RNase which digests the bulk of the RNA except for a small fragment in the proximity of the cross-link that is protected by the protein. As this method normally employs a radiolabelled RNA it results in the labelling of RNA-binding proteins which can be detected after resolution by SDS/PAGE.

Yps is a member of the cold shock family of RNA-binding proteins, which was purified as part of a large protein complex that contains osk mRNA (Wilhelm et al., 2000). While mutations in Yps do not cause any obvious defect in osk translation, they do suppress the localization and translational control defects observed in *orb* mutants. Intriguingly, this suppression is specific for osk, since the grk mRNA localization defects observed in *orb* mutants are not rescued in the double mutant (Mansfield et al., 2002). Recently, it has also been shown that *orb* mutants disrupt cytoskeleton function and that *yps* mutants can suppress this defect. This has led to the proposal that *yps* and *orb* act indirectly on osk via their effects on the cytoskeleton (Martin et al., 2003). However, the fact that both Orb and Yps have been demonstrated to be in biochemical complexes with osk mRNA supports the arguement that osk is a direct target of both proteins (Chang et al., 1999; Mansfield et al., 2002). The most likely interpretation of this set of results is that *orb* and *yps* regulate a cluster of transcripts, in addition to osk, some of which are required for proper cytoskeletal function. The identification of the targets that are common to both *yps* and *orb* should yield more insights into how both of these proteins regulate translation and localization of mRNA.

ME31B is a putative RNA helicase that is required to establish translational repression of osk mRNA and Bicaudal D mRNA within the *Drosophila* ovary (Nakamura et al., 2001). ME31B has also been purified as part of a large RNP complex that contains Exuperantia, Yps, Cup, eIF4E, Bru, and osk mRNA, indicating that it acts directly in translationally repressing osk. How does an RNA helicase establish translational repression? One possibility is that it is required to unwind target messages so that the repression machinery can be loaded on the transcript. While this may be one of the roles of ME31B, it is interesting to note that ME31B appears to be stably incorporated into the complex (Nakamura et al., 2001). This suggests that ME31B may play additional regulatory roles within the osk RNP complex. Consistent with this, the *Xenopus* homologue of ME31B, Xp54, is part of a large, RNAse resistant complex that contains CPEB and eIF4E (Minshall and Standart, 2004). Furthermore, tethering Xp54 to a reporter transcript via the RNA-binding protein, MS2, is sufficient to repress the reporter and, surprisingly, mutating the helicase domains actually activates translation as compared with the untethered reporter (Minshall et al., 2001). These results suggest that Xp54 and, by extension, ME31B may act as a switch to change the activity of the complex between repression and active translation.

Figure 2 | A speculative model for translational regulation of osk mRNA

Early in oogenesis unlocalized osk mRNA is recognized by an as yet unidentified miRNA which recruits RISC, thereby blocking translation through a mechanism that functions post-initiation. Later in oogenesis, repression of unlocalized osk mRNA shifts to a Cup-mediated mechanism. Upon localization of osk mRNA to the posterior, Orb protein stimulates polyadenylation of osk mRNA. This results in the recruitment of PABP, and PABP's ability to interact with eIF4G displaces Cup through the formation of a stable eIF4E–eIF4G–PABP complex leading to translation of localized osk mRNA.



Overview of osk translation

Taken together, recent data would suggest that both a RNAi-mediated mechanism and a Cup-dependent mechanism repress osk translation, and cytoplasmic polyadenylation relieves this repression (see Table 1 and Figure 2). Comparison of the data from different laboratories would suggest that while the RNAi mechanism is dominant during early oogenesis, Cup

is dominant at later stages (Wilhelm et al., 2003; Cook et al., 2004; Nakamura et al., 2004). This would imply that repression of osk mRNA also involves a mechanism that shifts control from the RNAi machinery to Cup.

grk translational control and the double-stranded break repair pathway

Grk is a transforming growth factor α family member whose restricted translation is required during oogenesis for both anterior-posterior and dorsal-ventral polarity (Neuman-Silberberg and Schüpbach, 1993; Gonzáles-Reyes et al., 1995). A number of genes, such as *encore*, *bru*, and *squid*, have been implicated in translational repression or activation of *grk*, but their mode of action has remained unclear (Norvell et al., 1999; Van Buskirk et al., 2000; Filardo and Ephrussi, 2003). In contrast, recent studies have demonstrated that a meiotic checkpoint that is activated by the double-stranded break repair pathway causes a block in Grk protein accumulation (Ghabrial et al., 1998; Ghabrial and Schupbach, 1999; Abdu et al., 2002; Doronkin et al., 2002). This block in translation correlates with the posttranslational modification of the RNA helicase, Vas, a protein known to be required for grk translation (Ghabrial and Schupbach, 1999).

While this work provides great insight into how regulation of oocyte polarity is coupled to progression through meiosis, the more mechanistic questions of how *grk* is translationally repressed have remained elusive. Recent work on Vas, however, has begun to address this deficit. eIF5B, a translation factor required for the 60 S ribosomal subunit joining step of translation initiation, has been found to be a Vas-binding protein (Carrera et al., 2000). Furthermore, specific *vas* alleles that disrupt the Vas–eIF5B interaction also disrupt the accumulation of Grk protein (Johnstone and Lasko, 2004). These results argue strongly that a Vas–eIF5B complex is required to activate translation of grk transcripts, but leave open the question of what is preventing the normal translation of grk.

One possible solution to this problem comes from the finding that the 5′ UTR of grk contains RNA localization elements required for its transport into the oocyte (Saunders and Cohen, 1999; Thio et al., 2000). This is unusual, since the majority of RNA localization elements are found in the 3′ UTR. This distribution of localization elements also presents a special problem for translation, since the localization complex probably needs to be removed in order to allow efficient translation. Thus grk might represent a special case where the localization complex is also the repression complex by virtue of its binding site. If this model is true, mutations in the 5′ UTR that block oocyte transport should also cause premature translation. Because of the novel role of Vas–eIF5B in activating grk translation, understanding how grk is repressed should yield new insights into the translational control of localized messages.

Regulation of nos translation

Once Osk protein accumulates in the oocyte, it is thought to direct the formation of a RNP complex which recruits nos mRNA to the posterior (Wang and Lehmann, 1991; Gavis and Lehmann, 1992; Wang et al., 1994). While osk mRNA is probably localized via active transport along microtubules, nos mRNA is localized by trapping and anchoring nos mRNA that is diffusing throughout the oocyte (Forrest and Gavis, 2003). This mechanism is apparently very inefficient, with only ~4% of the transcripts localized to the posterior while the remaining are found distributed throughout the bulk of the embryo (Bergsten and Gavis, 1999). However, Nos protein is only detected at the posterior suggesting that unlocalized nos mRNA is not expressed (Gavis and Lehmann, 1994). Preventing the expression of these transcripts requires spatial regulation of both nos translation and mRNA stability: while posteriorly localized nos mRNA is stable and translated, unlocalized nos mRNA is translationally repressed and degraded (Dahanukar and Wharton, 1996; Gavis et al., 1996; Smibert et al., 1996; Bashirullah et al., 1999). Thus regulation of translation and mRNA stability are co-ordinated with transcript localization.

Initial efforts to identify the *cis*- and *trans*-acting factors that regulate nos and osk translation seemed to suggest that different mechanisms may be involved; however, more recent work, as detailed below, suggests otherwise.

Mechanisms of nos translational repression

Repression of nos translation is mediated by *cis*-acting stem/loop structures in the nos transcript's 3′ UTR. One stem/loop appears to represent the binding site for an as yet unidentified translational repressor

(Crucs et al., 2000). The other two stem/loops are binding sites for a sequence-specific RNA-binding protein known as Smg which functions as a translational repressor (Dahanukar et al., 1999; Dahanukar and Wharton, 1996; Smibert et al., 1996, 1999). Smg is conserved from yeast to humans, and recent work has shown that Smg homologues represent a family of post-transcriptional regulators that employ a common RNA-binding domain for transcript recognition (Aviv et al., 2003; Green et al., 2003).

What is the mechanism that underlies Smg's ability to repress translation? As work was proceeding which demonstrated a role for Cup protein in osk regulation (described above), parallel efforts demonstrated that Cup also plays a role in the regulation of nos translation. Using a biochemical approach, Cup was identified as a Smg-binding protein and Cup was shown to mediate an indirect interaction between Smg and eIF4E (Nelson et al., 2004). The ability of Cup to block the interaction between eIF4E and eIF4G, combined with *in vivo* evidence demonstrating that Cup plays a role in Smg-mediated translational repression is consistent with the Smg/Cup/eIF4E complex playing a role in nos translational repression (Figure 1).

The interaction of Cup with both Bru and Smg suggests that Cup may serve as a general adaptor between 3′ UTR-binding proteins and eIF4E. Indeed, Cup interacts with Nos protein, which itself functions as a translational repressor (Verrotti and Wharton, 2000). The *cup* mutant phenotype is complex, showing defects in oocyte growth, maintenance of chromosome morphology and egg chamber polarity (Keyes and Spradling, 1997). These defects may result from disruption of translational control of other mRNAs that are regulated by different RNA-binding proteins.

nos translation is repressed at multiple levels

The role of Cup in Smg function would suggest that nos translational repression would occur at the level of initiation. However, like osk mRNA, translationally repressed nos mRNA is also associated with polysomes (Clark et al., 2000). One possible explanation for this contradiction is that nos translation is repressed by at least two *trans*-acting factors, Smg and an as yet unidentified factor that interacts with the nos 3′ UTR (Crucs et al., 2000). Alternatively, Smg may use both Cup-dependent and Cup-independent mechanisms to repress nos translation. While an explanation for this apparent contradiction vis-à-vis osk relates to a switch from RNAi-mediated repression to repression via Cup, data supporting such a switch for nos does not exist at present.

Implication of polysome association of repressed mRNAs

The polysomal association of repressed osk and nos mRNAs complicates the possible mechanisms that are involved in regulating their expression (Clark et al., 2000; Braat et al., 2000). If one assumes that translation elongation and termination occur at normal rates on these repressed mRNAs, this would imply that translation is not regulated. Instead, repression would have to result from degradation of the protein produced by these mRNAs. Given that repression is mediated by *cis*-acting 3′ UTR elements, this would imply that the 3′ UTR recruits a protease that degrades the protein, perhaps as it emerges from the ribosome. Alternatively, elongation or termination might be slowed by the 3′ UTR elements. In this case, degradation of the nascent peptide could result from either lengthening the time the unfolded portion of the nascent peptide remains accessible to general proteases, or through 3′ UTR-mediated protease recruitment as described above.

Interestingly, repression of nos translation appears to require the nascent polypeptide-associated complex (NAC) (Markesich et al., 2000). NAC is a heterodimeric protein complex that associates with the ribosome and is thought to interact with the nascent peptide as it emerges from the ribosome (Rospert et al., 2002). NAC is also required for both osk and nos mRNA localization (Markesich et al., 2000; Braat et al., 2004). It is unclear if NAC's role in the regulation of either mRNA is direct; however, the association of repressed nos and osk transcripts with polysomes is consistent with NAC being able to interact with the nascent peptides produced by these transcripts, which in turn may regulate their localization and/or expression.

Activation of nos translation

While unlocalized nos mRNA is translationally repressed, localized nos mRNA is translated (Gavis and Lehmann, 1994). As described above, activation of osk translation is associated with an increase in the length of the osk poly(A) tail. In contrast, measurement of the length of the nos poly(A) tail suggests that regulation of nos does not involve changes in

poly(A) tail length (Sallés et al., 1994; Gavis et al., 1996). However, the strength of this conclusion is undermined by the fact that if nos translation were regulated via the poly(A) tail, one would expect the nos mRNA at the posterior to have a longer tail than the nos mRNA in the bulk of the embryo. Given that only 4% of nos mRNA is actually localized (Bergsten and Gavis, 1999), this could mean that relevant differences in length of the tail between localized and unlocalized nos mRNA might have been missed.

Both Cup and Smg are distributed throughout the embryo and are not excluded from the posterior (Keyes and Spradling, 1997; Dahanukar et al., 1999; Smibert et al., 1999). This suggests that translation of nos mRNA at the posterior, in part, involves blocking Smg and/or Cup function at the posterior. Translation of nos at the posterior requires Osk protein and Osk is also required to localize nos mRNA to the posterior (Wang and Lehmann, 1991; Gavis and Lehmann, 1992, 1994; Wang et al., 1994). This might suggest that Osk's role in translational activation is indirect, simply reflecting the requirement for Osk to localize nos mRNA. However, the interaction of Osk protein with Smg is also consistent with a direct role for Osk in translational activation (Dahanukar et al., 1999). Interestingly, Smg's ability to bind to RNA, and interact with both Cup and Osk, all maps to the same region of Smg (Dahanukar et al., 1999; Nelson et al., 2004). If Osk binding were to block either Smg's ability to bind to mRNA and/or Cup, this could block Smg repression leading to activation of nos translation specifically at the posterior. This might be consistent with one model for the activation of nos translation, which suggests that the *cis*-acting elements required for nos mRNA localization partially overlap with those required for translational repression (Bergsten and Gavis, 1999; Crucs et al., 2000). Thus binding of the localization machinery would prevent binding of the factors required for translational repression thereby coupling localization and translation of nos mRNA.

Translational regulation, dosage compensation and SXL

Dosage compensation is the process which ensures that equal amounts of X-linked gene products are expressed in males that carry one X chromosome and females that carry two. In *Drosophila,* this equalization results from hypertranscription of the single male X chromosome, which is mediated by the male specific lethal (MSL) proteins, MSL-1, MSL-2, MSL-3 and Maleless (Lucchesi, 1998). These proteins are part of a RNP complex that binds to numerous sites on the male X chromosome resulting in its hypertranscription (Franke and Baker, 2000). Dosage compensation is controlled by SXL, an RNA-binding protein containing two RNA recognition motifs, that also regulates sex determination (Penalva and Sanchez, 2003). In females, functional SXL protein acts as both a splicing regulator to control sex determination and a translational repressor to control dosage compensation.

SXL protein expressed in females represses msl-2 translation by interacting with U-rich SXL-binding sites in the transcripts 5′ and 3′ UTR (Bashaw and Baker, 1995, 1997; Kelley et al., 1995, 1997). Efficient repression requires the cooperation of sites in both UTRs (Bashaw and Baker, 1997; Kelley et al., 1997) and this distinguishes SXL from most translational repressors, which function through sites located in either the 5′ or the 3′ UTR, but not in both. The lack of MSL-2 protein prevents formation of a functional MSL complex, which in turn prevents hypertranscription of the female X chromosomes. In males, where functional SXL is not produced, msl-2 is translated allowing the MSL complex to form and trigger hypertranscription of the male X chromosome.

In vitro extracts that recapitulate SXL-dependent repression have been used to investigate the mechanism involved (Gebauer et al., 1998). These experiments have shown that SXL inhibits stable binding of the 40 S ribosomal subunit to the mRNA (Gebauer et al., 2003). On its own this would suggest that SXL prevents one of the following steps in translation initiation: initial recruitment of the 40 S subunit to the mRNA, 40 S subunit scanning, or stable association of the 40 S subunit with the initiator codon. Additional experiments have shown that SXL-mediated repression does not require either the 5′ cap or the poly(A) tail (Gebauer et al., 1999, 2003). As both of these structures function in translation initiation via their ability to stimulate the initial recruitment of the 40 S subunit, these results would suggest that SXL must function after this initial recruitment by either disrupting scanning or the stable association of the 40 S subunit with the initiator codon. This distinguishes SXL-mediated repression from mechanisms

that employ Maskin, Cup or Bcd where repression is achieved by blocking the initial recruitment of the 40 S subunit to the target mRNA.

As mentioned above, a key question related to the function of any *trans*-acting translational repressor is: what mechanism ensures that a repressor only blocks the translation of target transcripts? An explanation in the case of SXL comes from experiments that generated SXL protein tagged with a heterologous RNA-binding domain (Grskovic et al., 2003). This protein still repressed the translation of a reporter mRNA carrying wild-type 5′and 3′ msl-2 UTR sequences, demonstrating that the heterologous RNA-binding domain had no effect on SXL function. In contrast, tagged SXL protein was unable to recapitulate repression via binding sites for the heterologous RNA-binding domain. Thus SXL's ability to repress translation requires recognition of the target transcript via the genuine SXL RNA-binding domain. These results suggest the binding of the SXL RNA recognition motifs to RNA results in a conformational change that creates a surface necessary for translational repression.

Experiments *in vitro* suggest also that other factors interact with the msl-2 3′ UTR and function in translational repression. For example, sites within the msl-2 3′ UTR that are not involved in SXL binding are required for translational repression (Gebauer et al., 2003). This implies that the 3′ UTR interacts with other essential factors. Consistent with this model, UV-cross-linking experiments have identified proteins that interact simultaneously with both SXL and the msl-2 3′ UTR (Grskovic et al., 2003). The binding of these proteins strongly correlates with the ability of the 3′ UTR to support translational repression, suggesting that they function as translational co-repressors with SXL. While SXL can interact with RNA on its own, the putative co-repressors interact with the msl-2 3′ UTR only in the presence of SXL. Thus, SXL appears to stabilize the interaction of these proteins with the msl-2 3′ UTR. Taken together, these data suggest a model whereby binding of SXL to the msl-2 mRNA creates a surface on SXL that recruits co-repressor RNA-binding proteins that interact with sites in the msl-2 3′ UTR. Once all the relevant factors are bound, this complex is able to interact either directly or indirectly with components of the translation machinery, thereby preventing the 40 S ribosomal subunit from either scanning the 5′UTR or associating with the initiator codon. Identification of the co-repressor proteins should provide a starting point for understanding how the SXL–co-repressor complex interacts with the translation machinery and may shed light on the requirement for SXL binding to both the 5′ and 3′ end of msl-2 mRNA.

Translational regulation, *Drosophila* and fragile X syndrome

Fragile X syndrome is the most common form of inherited mental retardation and is caused by inactivation of FMRP (O'Donnell and Warren, 2002). This loss of function is usually caused by expansion of CGG triplet repeats in the 5′ UTR of the gene encoding FMRP. Patients typically have greater than 200 repeats, while normal individuals carry 5–50. Abnormal methylation of these repeats spreads into the surrounding genomic sequences leading to transcriptional silencing.

FMRP is a RNA-binding protein with several RNA-binding motifs, including two KH domains and an arginine- and glycine-rich region (RGG box) (Ashley et al., 1993; Siomi et al., 1993). Consistent with the presence of these domains, the protein is associated with mRNAs in large RNP complexes and these RNPs are associated with translating polysomes (Corbin et al., 1997; Feng et al., 1997). A missense mutation within the second KH domain (I304N) results in the incorporation of FMRP into abnormal RNPs and abolishes its association with polysomes (Feng et al., 1997). This missense mutation results in a particularly severe fragile X syndrome phenotype, suggesting that association of the protein with both RNPs and elongating polysomes is critical for its function (De Boulle et al., 1993). In addition, FMRP is also thought to interact directly with the 60 S ribosomal subunit (Siomi et al., 1996). Taken together these results suggest that FMRP functions as a translational regulator.

Both the molecular mechanisms that underlie the function of FMRP and the molecular and cellular bases of fragile X syndrome remain poorly understood, but investigations into the *Drosophila* homologue, dFMR1 (also known as dFXR), have begun to provide some insights. *Drosophila* provides a number of advantages for the dissection of FMRP function. Firstly, the *Drosophila* genome encodes only one

FMRP family member, while the human genome encodes 3 FMRP-like proteins. Secondly, dFMR1 is very similar to FMRP, particularly in regions that are thought to be important in FMRP function including both KH domains and the RGG box (Wan et al., 2000). Thirdly, dFMR1 and FMRP share similar binding profiles to homopolymers of RNA. Finally, comparisons of the defects associated with loss of the *Drosophila* and mammalian proteins suggest that they play a conserved role in the nervous system and the testis (Dockendorff et al., 2002; Morales et al., 2002; Zhang et al., 2001, 2004). Taken all together these results suggest that understanding dFMR1 function will have a profound effect on our understanding of fragile X syndrome.

dFMR1 and RNAi

Investigation of dFMR1 has provided several lines of evidence suggesting that it mediates its function through the RNAi machinery. Two studies, employing independent biochemical approaches, found evidence for the association of dFMR1 with components of RISC. As described above, miRNAs and siRNAs are incorporated into RISC where they serve to guide the complex to target mRNAs, resulting in either translational repression or transcript degradation. The purification of RISC from *Drosophila* tissue culture cells demonstrated that dFMR1 is associated with RISC (Caudy et al., 2002), while another group purified dFMR1 complexes from the same cells and found Argonaute 2, a RISC component, associated with dFMR1 (Ishizuka et al., 2002). Both groups showed that Argonaute 2′s interaction with dFMR1 is resistant to RNase, suggesting that it is mediated by protein–protein interactions and not due to binding of these proteins to the same mRNA. Other RNAi components were also found to interact with dFMR1, including Dicer protein and both siRNAs and miRNAs. In addition, purified dFMR1 complexes displayed RISC activity *in vitro*. Both groups tested whether dFMR1 is required for RNAi, and while their results were not in complete agreement (probably owing to the different assays they employed) they would suggest that dFMR1 is not essential for RNAi.

Testing components of the RNAi machinery for their ability to enhance or suppress phenotypes associated with dFMR1 confirmed that dFMR1 function is linked to the RNAi pathway (Jin et al., 2004). In particular, Argonaute 1 was required for dFMR1 overexpression phenotypes, while the dFMR1 loss-of-function phenotype was enhanced by the loss of Argonaute 1. These results demonstrate that dFMR1 function in flies requires Argonaute 1, consistent with the model that dFMR1′s function *in vivo* involves the RNAi machinery.

Other experiments have shown that the RISC/dFMR1 interaction is conserved in mammals (Jin et al., 2004). Biochemical approaches demonstrated that all three mammalian FMRP proteins, including FXR1P and FXR2P, interact with the mammalian Argonaute 1 homologue, eIF2C. In addition, they showed that FMRP and FXR2P immunoprecipitated small RNAs that are the correct size to be miRNAs, and both proteins also immunoprecipitated a Dicer-like activity that cleaved double-stranded RNAs into short siRNA-sized duplexes. Interestingly, FXR1P, which is 60% similar to its mammalian homologues, failed to pulldown short RNAs or a Dicer activity in spite of its interaction with eIF2C, suggesting that its function may be distinct.

Taken together, these results suggest that dFMR1 and its homologues may regulate translation using an RNAi-related mechanism. Thus these proteins might interact with RISC and use an associated miRNA to identify targets that would result in translational repression. Such a model would raise questions about the role of the various RNA-binding domains within these proteins. For example, the RGG box of FMRP has been shown to specifically interact with RNAs containing G quartets (Darnell et al., 2001). Therefore, does target recognition involve binding to an mRNA that contains both G quartets and a miRNA recognition site? Alternatively, these proteins might employ multiple mechanisms to regulate translation: one could involve RISC-mediated recognition and regulation while the other could involve recognition of mRNAs via RGG-box-binding to G quartets and could employ an independent mechanism of translational control. Differentiating between these models will require investigations to determine: (1) target mRNAs whose regulation is directly controlled by dFMR1 and FMRP; (2) the mechanisms of target recognition; (3) the effects that dFMR1 and FMRP have on the metabolism of their targets (i.e. translational repression or activation, mRNA destabilization); and (4) the molecular mechanisms that underlie dFMR1 and FMRP-mediated regulation.

dFMR1 targets

The identification of dFMR1 targets will provide a detailed understanding of the molecular and cellular functions of dFMR1 which will in turn provide insights into the underlying causes of fragile X syndrome. The dFMR1 loss-of-function phenotype is complex, including defects in circadian rhythm, synaptic function, dendrite development and development of the testis (Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002; Lee et al., 2003; Xu et al., 2004; Zhang et al., 2001, 2004). Thus dFMR1 probably regulates the expression of multiple mRNAs.

Three mRNAs have been identified in dFMR1 immunoprecipitates which may be direct targets. The first encodes the *Drosophila* microtubule-associated protein MAP1B homologue Futsch (Zhang et al., 2001). Futsch protein levels show an inverse correlation to the levels of dFMR1 (i.e. when dFMR1 is overexpressed Futsch levels go down, while loss of dFMR1 results in overexpression of Futsch). Therefore, these results suggest that dFMR1 downregulates Futsch expression. Genetic studies are consistent with this model and suggest that dFMR1 regulation of Futsch controls synaptic structure. A second mRNA present in dFMR1 immunoprecipitates encodes the small GTPase Rac1, and genetic evidence suggests that dFMR1 might downregulate Rac1 expression (Lee et al., 2003). In this case, dFMR1 regulation of Rac1 controls dendritic development; however, direct evidence showing that dFMR1 effects levels of Rac1 protein is not available. Finally, the third mRNA associated with dFMR1 encodes Pickpocket1, a multiple dendritic neuron subtype-specific degenerin/epithelial sodium channel (Xu et al., 2004). Here, genetic experiments suggest that dFMR1 downregulates Pickpocket1 expression to control the crawling behaviour of *Drosophila* larvae. In this case dFMR1 appears to decrease the levels of pickpocket1 mRNA, suggesting that it may be functioning at the level of mRNA stability. Alternatively, dFMR1′s primary effect could be at the level of translation, and the decrease in mRNA levels could be secondary to translational repression. Consistent with the role of the RNAi machinery in dFMR1 function Argonaut 2 is required for dFMR1 to reduce levels of pickpocket1 mRNA.

The identification of dFMR1 targets is the first step in developing a more complete understanding of how dFMR1 regulates mRNA expression. The next step for the three mRNAs discussed here will be to confirm that they are indeed direct dFMR1 targets. This will involve mapping the *cis*-acting elements required for dFMR1-mediated regulation and determining whether they represent sites for direct binding by dFMR1, or if they are recognized by miRNAs. In addition, where it has not been done, assaying the amounts of target mRNA and protein will allow for a more definitive statement as to the level at which dFMR1 regulates their expression. Also testing the role of various factors known to function in RNAi, including various miRNAs, will provide mechanistic insights into how repression is achieved.

Concluding remarks

While some progress has been made in understanding the molecular mechanisms that control the translation of specific transcripts, many issues remain unresolved. Work to date has highlighted the fact that translational regulation of even a single message can be complex, involving many factors which function at multiple steps in translation. Thus the identification of the full repertoire of factors that regulate an mRNA will be required to permit a detailed understanding of how these different mechanisms function together on the same target. Perhaps the most eagerly anticipated results will be those that shed light on the mechanisms that underlie repression of polysome-associated mRNAs.

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