PERSPECTIVE

A splicer that represses (translation)

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Regulated translation and subcellular localization of maternal mRNAs underlies establishment of the antero-posterior axis in the *Drosophila* oocyte. In this issue of *Genes & Development*, Besse et al. (pp. 195–207) show that a molecule better known as a regulator of alternative splicing in the nucleus, polypyrimidine tract-binding protein (PTB), is required for repression of *oskar* mRNA in the cytoplasm. Their work suggests that PTB need not engage *oskar* mRNA in the nucleus for efficient repression, providing an important counterexample to the increasingly popular idea that cytoplasmic regulation initiates in the nucleus.

mRNAs localized to the anterior and posterior poles of the oocyte encode the proteins that initiate a now well-understood cascade of gene regulation that patterns the *Drosophila* embryo (St Johnston 2005). Localization serves to restrict accumulation of the encoded proteins to a portion of the cytoplasm in the single large cell (~500 μ m long) that is the early, syncytial embryo. This strategy is widely employed in other cells that are either unusually large (the *Xenopus* oocyte) or elongated (neurons). A recent survey shows that many mRNAs in the *Drosophila* embryo exhibit subcellular localization, suggesting that the strategy may be more widely employed than suspected previously (Lecuyer et al. 2007).

If a mRNA is localized for the purpose of driving local protein accumulation, it must be repressed during transit. Studies of *oskar* (*osk*) mRNA, which is localized to the posterior pole of the oocyte, have illuminated the coupled regulation of translation and localization for many years. *osk* mRNA is synthesized in the accessory nurse cells of the egg chamber; a subset of the mRNA is transported in mRNPs to the posterior pole of the oocyte at stage 9 of oogenesis (Zimyanin et al. 2008). Only upon arrival at the posterior pole is the mRNA translated (Kim-Ha et al. 1991; Ephrussi and Lehmann 1992); the short encoded Osk isoform (Markussen et al. 1995) then nucleates assembly of germ line determinants that are incorporated into the primordial germ cells at the posterior pole.

Osk has a second essential role at the posterior pole—directing the localization and translational activation of *nanos* (*nos*) mRNA (Ephrussi and Lehmann 1992; Smith

et al. 1992). The resulting gradient of Nos protein patterns the posterior of the embryo, directing the formation of abdominal segments (Wharton and Struhl 1991). Although the mechanism by which Osk activates *nos* mRNA is unclear (and likely to be indirect), it is clear that Osk-dependent activation must be restricted to the posterior. Otherwise, the resulting generation of Nos in anterior regions of the embryo has lethal developmental consequences. Thus, *osk* mRNA must be repressed until its arrival at the posterior.

The PTB splicing regulator as a repressor of *osk* translation

In this issue of *Genes & Development*, Ephrussi and colleagues (Besse et al. 2009) report the identification of a new factor required for repression of *osk* mRNA in the ovary—the polypyrimidine tract-binding protein (PTB). This factor has a rich, well-documented history as a regulator of alternative splicing in the nucleus and an activator of IRES-dependent translation in the cytoplasm. Reports that PTB can repress IRES-driven translation have relied primarily on overexpression experiments (at levels up to 10-fold above endogenous) in cultured cells (Kim et al. 2000; Cornelis et al. 2005). The new study from Besse et al. (2009) is the strongest evidence to date that PTB represses translation *in vivo*.

Mammalian PTB binds to CU-rich elements in intronic or exonic silencer elements near alternative exons that are only sometimes included in the mature mRNA (Singh et al. 1995; Perez et al. 1997). PTB blocks exon definition and spliceosome assembly, although the precise mechanisms by which it does so are not yet clear (Wagner and Garcia-Blanco 2002; Rideau et al. 2006; Sharma et al. 2008). PTB has also been implicated as a core *trans*-acting factor for IRES-dependent translation of many viral and cellular messages (Sawicka et al. 2008), bypassing the requirement for a m⁷GpppG cap during infection and other cellular stress. In addition to these arenas, PTB has been implicated in a number of other mRNA transactions (Sawicka et al. 2008); its role in localization of *Vg1* mRNA in the *Xenopus* oocyte (Cote et al. 1999) has been particularly influential in development of the idea that cytoplasmic mRNA regulation is initiated by events in the nucleus (a topic outlined later in this perspective). *Drosophila* PTB is closely related to the better studied human and frog proteins; but no direct demonstration that it plays a role in splicing regulation in flies has been

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reported. One way to address this issue would be to use microarrays that detect alternative splice isoforms to compare the transcriptome in wild-type and PTB-knocked-down S2 cells, as described by Rio and colleagues (Blanchette et al. 2005).

PTB emerged from a screen for factors that colocalize with *osk* mRNA, as do the previously identified *osk* regulators Staufen, Bruno, and Hrp48 (Webster et al. 1997; Martin et al. 2003; Huynh et al. 2004; Yano et al. 2004). Besse et al. (2009) found an exon-trap insertion in the *PTB* locus (*hephaestus* in FlyBase but *PTB* in this paper) that encodes a GFP fusion that is localized to the oocyte posterior in stage 9 egg chambers, like wild-type *osk*. PTB-GFP (and PTB) were shown to track *osk* mRNA in experiments where either its production or localization were altered. Thus, PTB was a good candidate to regulate *osk*.

PTB is essential for *Drosophila* viability; to test for a role in *osk* regulation, Ephrussi and colleagues (Besse et al. 2009) therefore selectively ablated its function in the ovary by preparing germ line clones. Only hypomorphic alleles were found to support the development of egg chambers to the point where *osk* mRNA localization and repression can be usefully monitored. *osk* mRNA was mislocalized in about 20% of such *PTB* hypomorphic stage 9 egg chambers, although localization was essentially normal by stage 10. This "recovery" was interpreted to result from a transient delay in reorganization of the microtubule cytoskeleton, which plays a key role in mediating transportation of *osk* mRNA (Zimyanin et al. 2008). These observations paint a picture in which PTB plays a relatively minor and indirect role in *osk* localization in contrast to its central role in the localization of *Vg1* mRNA in *Xenopus* oocytes. Whether this represents a fundamental difference between the two systems, or residual PTB activity in the ovaries studied by Besse et al. (2009) remains to be seen.

A compelling role for PTB in the repression of *osk* mRNA emerged from examination of Osk protein in the same germ line clones. Unlike wild-type egg chambers, in which Osk is found no earlier than late stage 8 and then only at the posterior pole of the oocyte, Osk was shown to accumulate both prematurely and in inappropriate (e.g., more anterior) regions of the oocyte in over half of the *PTB* mutant egg chambers examined. Similar ectopic accumulation of Osk had been observed upon mutating the Bru-binding sites in the *osk* 3' untranslated region (UTR) (Kim-Ha et al. 1995).

Besse et al. (2009) next addressed the question of whether PTB acts directly on *osk* mRNA to repress its translation. A priori, indirect regulation was a reasonable possibility, particularly if PTB, in fact, proves to regulate alternative splicing of as many pre-mRNAs as does Hrp48 (for example, Blanchette et al. 2005). However, Besse et al. (2009) found that the endogenous PTB in ovarian extracts binds preferentially to the *osk* 3' UTR shown previously to be required for translational repression and localization. Unlike repressors of other maternal mRNAs that act via one or two discrete sites in target mRNAs (e.g., Smaug or Pumilio) (Wharton and Struhl 1991; Smibert et al. 1996), PTB seemed likely to act via a different mechanism, based on two observations. First, the 1055 nt *osk* 3'

UTR was found to be littered with evolutionarily conserved CU-rich sequences similar to PTB-binding sites in well-characterized mammalian splicing silencers (Singh et al. 1995; Perez et al. 1997). Second, subdivision of the 3' UTR revealed that each of the five distinct fragments tested could bind PTB. As binding specificity controls, neither a protein-coding region of *osk* mRNA nor the *Y14* mRNA bound appreciably to PTB; and only the *osk* 3' UTR fragments with previously characterized Bruno-binding sites bound Bruno. Thus, PTB likely binds in vivo to multiple sites in the *osk* 3' UTR.

To investigate PTB binding in vitro, Besse et al. (2009) purified from bacteria a PTB fusion to the monomeric MBP carrier protein and used it in gel mobility shift experiments. These revealed selective binding to the *osk* 5' and 3' UTRs, both of which have been implicated in translational control (Gunkel et al. 1998). However, the mode of binding to each UTR appears to be different: PTB forms two discrete high-mobility complexes on the 5' UTR, and a high-order aggregate (containing an unknown number of proteins) that barely enters the gel on the 3' UTR. How might this aggregate arise? Mammalian PTB has four RNA recognition motif (RRM)-binding motifs, each capable of binding a CU-rich element (Oberstrass et al. 2005). In addition, RRM3 and RRM4 have been shown to interact with each other such that, if each is bound to a CU-rich element within a single RNA molecule, a protein cross-linked RNA loop would be formed. If the fly protein has similar properties, then either inter- or intramolecular PTB-bridged cross-links might account for the low mobility complexes observed in the experiments of Ephrussi and colleagues (Besse et al. 2009).

Besse et al. (2009) then wondered whether PTB might drive aggregation of *osk* mRNAs in vivo much as it does when incubated with the 3' UTR in vitro. Previous work had shown that reporter mRNAs bearing the *osk* 3' UTR are efficiently localized to the oocyte posterior, but only if allowed to hitchhike on endogenous *osk* mRNPs (Hachet and Ephrussi 2004). Strikingly, Ephrussi and colleagues (Besse et al. 2009) found that hitchhiking of an EGFP reporter mRNA bearing the *osk* 3' UTR is dependent on normal PTB activity, consistent with the idea that PTB-mediated cross-linking drives the formation of mRNP aggregates in vivo. Further support for this idea came from the observation that *osk* mRNP transport particles in the center of stage 9 oocytes are about one-third normal size, as measured by high-resolution in situ hybridization and immunoelectron microscopy. It would be interesting to know whether, at later stages when *osk* localization has recovered, the *osk* mRNPs are still diminutive. But, taken with the ability of PTB to promote hitchhiking and the derepression of *osk* in PTB mutants, Besse et al. (2009) concluded that PTB may repress *osk* by sequestering it from ribosomes in large mRNP aggregates.

The results outlined above raise further questions about the role of PTB in *osk* regulation. Here, I focus on two topics. First, if repression and/or localization is mediated by PTB-dependent cross-linking into high-order aggregates, might concatemered PTB-binding sites suffice to drive these events (or to allow hitchhiking of a reporter

mRNA on the native *osk* mRNP)? Oligomerized PTB-binding sites are sufficient to confer IRES activity in mammalian cells (Mitchell et al. 2005), establishing a precedent for this sort of approach. Second, what are the relative contributions of Bruno, Hrp48, and PTB toward repressing *osk*? Are the weak developmental phenotypes revealed by genetic analysis (Webster et al. 1997; Yano et al. 2004) due to redundancy? Or to the residual activity in partial loss-of-function backgrounds? Or perhaps due to sequential repressor action (much as the responsibility for repression of *nos* mRNA passes from Glorund to Smg; Kalifa et al. 2006), such that early failures in repression are masked by later successes? With the currently available alleles, this set of questions will be difficult to answer, as it is not possible to eliminate function of any one of the three factors and study the regulation of *osk* that occurs during late stages of oogenesis. Ablation of the Bruno-binding sites in *cis* appears to cause much stronger derepression than does a reduction in activity of any of the three *trans*-acting factors (Kim-Ha et al. 1995). However, some of the Bruno- and Hrp48-binding sites are close to each other and some overlap (Yano et al. 2004), raising the possibility that the mutations studied affect binding of both factors to the *osk* 3' UTR. Perhaps these issues can be addressed in vitro by selectively depleting each factor from the ovarian extracts that support regulation of *osk* mRNA.

Other dual function regulators of splicing and translation in *Drosophila*

Many RNA-binding proteins shuttle between the nucleus and cytoplasm, even if they prefer to reside in one of the compartments. Is it surprising, then, that PTB has dual roles in regulating splicing and translation? The simple answer is no—of the other well-characterized repressors of posteriorly localized mRNAs (*osk* and *nos*), one certainly plays a direct role in splicing and two others probably do. The known or suspected dual function regulators all have multiple RRM, versatile motifs for RNA recognition and protein-protein interaction.

Of the other *osk* repressors, Hrp48 is better known for its role in splicing regulation than translation. Rio and colleagues (Blanchette et al. 2005) have studied its function in regulation of the splicing of P-element transposase pre-mRNA in some detail. They concluded recently that it regulates at least 90 pre-mRNAs, some in collaboration with the PSI cofactor critical for transposase splicing and some not (Blanchette et al. 2005). *Drosophila* Bruno has not yet been shown to play a role in splicing regulation. But the protein appears to shuttle (Snee et al. 2008) and mammalian orthologs have been implicated in splicing (Suzuki et al. 2002).

Of the two well-characterized *nos* repressors, Glo (the *Drosophila* hnRNP F/H homolog) has been shown to interact directly with Hrp48 and the splicing factor Halfpint in vitro, consistent with observations that mammalian hnRNP H is involved in regulation of alternative splicing (Kalifa et al. 2008). Only Smg seems to steer clear of the nucleus, sticking to the cytoplasm where its main

role is to direct the degradation of a large subset of maternal mRNAs during the transition from maternal to zygotic control of development (Tadros et al. 2007).

In summary, dual function may become the rule rather than the exception, particularly as sophisticated genetic tools reveal functions for essential RNA-binding proteins in repression (and localization).

Is the nuclear experience overrated?

The idea that cytoplasmic mRNA transactions are obligatorily set up in the nucleus has become generally accepted (Giorgi and Moore 2007), despite documented exceptions (perhaps most notably, the apical localization of various mRNAs microinjected into the cytoplasm of the *Drosophila* embryo) (Bullock and Ish-Horowicz 2001; Wilkie and Davis 2001). One broad reason for the wave of enthusiasm has been the demonstration that nonsense mediated decay (NMD) in the cytoplasm (or at the nuclear/cytoplasmic boundary during a pioneer round of translation) is initiated by deposition of the exon junction complex (EJC) during splicing. Following is a brief summary of two additional influential contributions: the discovery that the EJC is required for *osk* localization in *Drosophila*, and isomerization of the *Vg1* mRNP during its localization in the *Xenopus* oocyte.

Genetic experiments revealed that *osk* mRNA localization requires factors that proved to be core components of the EJC (Hachet and Ephrussi 2001; Palacios et al. 2004). These observations were initially somewhat puzzling, since the EJC is thought to be deposited on every spliced mRNA, even though very few of these are localized. Subsequent experiments showed that splicing of *osk* pre-mRNA is essential for its localization, and that the position of the first intron is critical, presumably for the deposition of an EJC at a privileged position where it facilitates the assembly of a localization-competent RNP (Hachet and Ephrussi 2004).

Work on the localization of *Vg1* mRNA to the vegetal pole of the *Xenopus* oocyte has led to a convincing argument by Mowry and colleagues (Kress et al. 2004) that its cytoplasmic fate is determined by earlier events in the nucleus. Localization of *Vg1* is dependent on binding of two factors to a *cis*-acting element in the 3' UTR-PTB and *Vg1*RBP/Vera (Deshler et al. 1998; Cote et al. 1999). Upon immunoprecipitation of separated nuclear and cytoplasmic fractions, both proteins are found in association with each other and with the mRNA (Kress et al. 2004). However, although *Vg1*RBP/Vera is part of the nuclear mRNP, it is not directly bound to the RNA until the complex is exported to the cytoplasm. Direct binding of *Vg1*RBP/Vera (assayed by UV cross-linking) is dependent on PTB binding, presumably a prior "good" act committed in the nucleus (Lewis et al. 2008).

In light of the nuclear role of *Xenopus* PTB in subsequent cytoplasmic trafficking, Besse et al. (2009) asked whether PTB must engage *osk* mRNA in the nucleus to establish a cytoplasmic repression complex in *Drosophila*. The experimental approach was to express a nuclear localization signal (NLS)-deleted PTB derivative in *PTB*

mutant egg chambers, assaying *osk* mRNA localization and translational repression as described above. Somewhat surprisingly, expression of a fusion of either wild-type PTB or the Δ NLS derivative to GFP restored translational repression. The Δ NLS derivative appeared to be effectively excluded from the nucleus, since it fails to repress accumulation of the endogenous protein, a well-documented autoregulatory activity of mammalian PTB (Wollerton et al. 2004). (This observation further increases the likelihood that the fly and mammalian proteins have very similar nuclear functions, since autoregulation is achieved by controlling the splicing of *PTB* pre-mRNA.) The Δ NLS protein also rescued the minor *osk* localization defects in mutant egg chambers, albeit somewhat less efficiently than did wild-type PTB, suggesting that nuclear engagement may be important for localization.

Nevertheless, a major conclusion from the work of Besse et al. (2009) is that nuclear recruitment of PTB is not required for cytoplasmic repression in *Drosophila*, with the caveat that residual wild-type PTB in the hypomorphic background employed (of necessity) might initiate the process in the nucleus. Perhaps an attractive parallel with the action of PTB on *Vg1* mRNA first in the nucleus and then in the cytoplasm of the *Xenopus* oocyte will yet carry the day.

Is formation of large mRNPs particles/aggregates an essential aspect of repression or an epiphenomenon?

Many RNA transactions occur in large assemblies: mRNA is translated on ribosomes, degraded in P bodies, and localized in particles large enough to be visible by light microscopy. Might an essential aspect of translational repression occur by sequestration in large aggregates/particles that exclude ribosomes?

The properties of PTB reported by Besse et al. (2009) make an attractive correlative case for the importance of particle formation. In vitro, PTB forms high-order aggregates specifically on the *osk* 3' UTR; in vivo, it is required for translational repression and promotes hitchhiking of mRNAs bearing the *osk* 3' UTR to the posterior pole. Strengthening the argument will likely require the isolation of mutants that selectively interfere with oligomeric binding to the *osk* 3' UTR (and not, for example, formation of discrete complexes on the *osk* 5' UTR). Such an approach is undoubtedly easier to propose than carry out, particularly on a molecule with four RNA-binding domains.

Two other examples of large, repressed *Drosophila* mRNP particles have been reported. First, binding sites for Bru (Bru response elements or BREs) were shown (1) to promote the formation of fast-sedimenting mRNP aggregates or particles, and (2) to mediate cap-independent repression (Chekulaeva et al. 2006). The BREs also were shown to mediate formation of cross-linked complexes containing multiple RNA species in ovarian extracts (e.g., RNA hitchhiking). Taken together, the experiments suggested a model in which Bru cross-links mRNAs into large silencing aggregates/particles as part of its repressive activity. However, Besse et al. (2009) report that the BREs are neither necessary nor sufficient for hitchhiking

of mRNAs bearing the *osk* 3' UTR in vivo. Perhaps in vitro Bru can promote aggregation by binding to BREs, whereas in vivo Bru mediates protein-protein interactions that stabilize aggregates primarily cross-linked by PTB.

A second report of large, silenced mRNP particles involves miR2-dependent repression in embryonic extracts (Thermann and Hentze 2007). The mechanism by which micro-RNAs inhibit translation has been somewhat controversial; the experiments of Thermann and Hentze (2007) demonstrated that miR2 acts by blocking formation of the 48S initiation complex. In the course of experiments to resolve both 48S pre- and 80S post-initiation complexes, they made the surprising observation that miR2 promotes the formation of heavy, puromycin-insensitive but EDTA-sensitive aggregates that they named "pseudopolysomes." The relevance of these aggregates to repression is not clear, since miR2 promoted their formation even on a substrate refractory to repression by virtue of possession of an AppG 5' cap.

In summary, it seems likely that PTB will be important for at least one step in the assembly of silenced *osk* mRNP particles, given its role in mediating mRNA hitchhiking in vivo. Future work is likely to focus on the identification of other components of these particles and efforts to pin down the mechanism by which they block translation.

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