

**Translational control and active transport of Vg1 mRNA in *Xenopus*  
*laevis* oocytes.**

By

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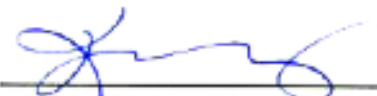
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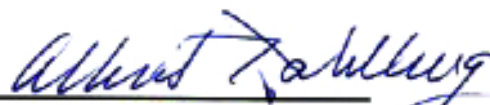
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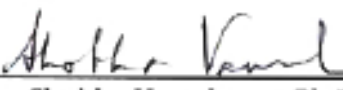
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Timothy J. Messitt, James A. Gagnon\*, Jill A. Kreiling\*, **Catherine A. Pratt\***, Young J. Yoon\*, & Kimberly L. Mowry. Multiple kinesin motors coordinate cytoplasmic RNA transport on a subpopulation of microtubules in *Xenopus* oocytes. *Developmental Cell* (2008) **15**(3): 426-436 \*equal contribution

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My committee members, Al, Jeff, and Rob, have also been there every step of the way. I would always drag my feet a little when it came time to schedule my annual committee meeting (it would usually take several emails from Tricia), but I would inevitably leave the meeting wishing I hadn't waited so long. Their insightful comments regarding the work I was doing, interpreting the weird data that made no sense to me, and the inevitable suggestions for additional experiments, have helped make this thesis what it is. I would also like to thank Shobha Vasudevan for agreeing to be my outside reader, and I look forward to hearing her feedback at the defense.

Here I would like to add a special thank you to Al. Not only has he been a wonderful committee chair, but, fresh out of college, he gave me a job as a research assistant in his lab. I can only speculate, but I think that it's likely that without that experience my career path would have been dramatically different.

Another wonderful scientist who, though she may or may not know it, really inspired me to pursue my PhD, is my aunt, Jill. When I was 16 she let me come work with her for a couple of weeks. Until then I had no idea what a real lab looked like, smelled like, sounded like. I had so much fun that I kept coming back, year after year. Her guidance, and the fantastic atmosphere in the Dahlberg lab (back on the 4<sup>th</sup> floor of J. Walter Wilson), was what made me fall in love with science. A more careful scientist I have never seen. And as my aunt and uncle, I thank her and her husband, Geoff, for all the support they have given me.

I am incredibly fortunate to have worked with some really fantastic people during my time here. The Mowry lab seems to attract practical jokers and amateur comedians, and it was a rare day when I didn't giggle at something. When I joined, Ray and Tim (Messitt) pretty much showed me everything there was to know about the way the lab worked. Jamie was relatively new to the lab at that point, and although he defended almost two years ago, he was probably my most trusted colleague. After the move to the new building we shared a bay; he is easily the best lab D.J. out there! The lab as it stands right now has a fantastic dynamic. Tim (Wood), my fellow Brit, Erin, and Xena are pretty much in charge, and then there's "the kids", Sam, Chris, and Kate. Although I'm not leaving right this second, I know I will sincerely miss the hilarious work environment they've created.

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MCB has also given me some of the greatest friends I've ever had in my life. When I first joined the Mowry lab we were in J. Walter Wilson, a building that had the most odd distribution of bathrooms. The 4<sup>th</sup> floor had a unisex loo, the second floor a men's room, and the first floor a ladies' room. This resulted in a lot of walking around, you know, when nature called. The Mowry lab was on the second floor, so I frequently had to pop down to the first floor. And this is how I met Viet. He was a technician in Kristi Wharton's lab at the time. Now he's a grad student in MCB, so I have known him almost as long as I've been here. One of our favorite activities back then was to walk past the glass-walled lecture theater in the basement and "accidentally" trip over something (trash can, step stool, imaginary step stool) to make the people in there laugh. And now he's my partner in crime in the

blogosphere, having started writing and tweeting online over the last few months. It's been great to see what he has to say, and to have conversations that are a tiny bit more serious than they used to be.

Another friend I am indebted to the J. Walter Wilson bathrooms for is Stephen. He was in the Gerbi lab on the 3<sup>rd</sup> floor, and thus had to come down to the 2<sup>nd</sup> floor every once in a while. I really don't know where to begin saying thank you to him, and I suspect it would make him awkward to read about those "feelings" anyway. But I would very much like to thank his VSGF Andy, and his family, who on countless occasions treated me like one of their own. Over the years, being so far from home, they really made me feel like I belonged here. Thank you.

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In 2008 I went to the RNA conference in Berlin. I didn't go alone. Stephen and his "team" of undergraduates, Liz and Julia, came too. Of course the conference itself was fantastic, but the week we spent in Berlin, and the following trip to Amsterdam, was probably one the best times of my life. Julia and Liz became my good friends, we discovered the wonders of a salami and cheese panini after a couple too many beers at Gecco, and several ridiculous in jokes were born. Having all those guys just down the hall for a good year or so after that made lab an even better place than it was before. I would also like to thank Janell, another Gerbi lab alum, for countless nights hanging out in her back yard, and R.J., for putting up with us.

Around that time I also began playing music with a group of Providence natives. We formed a band, recorded a couple of albums, and had a great time playing shows up and down the east coast. TDMP, and my bandmate and friend Tim (Hiles), gave me a healthy dose of confidence and the ability to stand up in front of a

crowded room without fear, something that I have no doubt will be invaluable in the years to come.

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## **Chapter 1: Introduction**

**Catherine A. Pratt** and Kimberly L. Mowry

In order to introduce my thesis work on the regulation of translation of the localized mRNA Vg1, this chapter will review the literature covering mRNA localization, eukaryotic translation, and the control of translation in a number of systems.

### **mRNA localization in *Xenopus* oocytes**

In the African clawed frog, *Xenopus laevis*, oogenesis proceeds over a period of several months. During this time, each oocyte progresses through a variety of morphological and physiological changes that have been characterized in detail, resulting in the ordering of oogenesis into six discrete stages (I—VI; Figure 1A; Dumont 1972). One of the defining moments in the development of a mature oocyte is the establishment of the animal/vegetal axis, a polarity that is defined not only by the appearance of cellular structures (such as the mitochondrial cloud, or Balbiani body, at the vegetal side of the nucleus in stages I and II) and coloration (note the distinctly pigmented animal hemisphere of stage V and VI oocytes; Figure 1A), but also through the uneven distribution of macromolecules within the oocyte cytoplasm. In particular, a number of messenger RNA (mRNA) molecules are restricted to the vegetal hemisphere of the oocyte during early oogenesis, and are essential for embryonic development (Figure 1B; reviewed in King *et al.* 2005). It is unclear precisely how or when this axis is defined, however, upon fertilization, it is the asymmetry of the various molecular determinants distributed along the animal-vegetal axis that ultimately defines the major body-plan of the tadpole.

### ***Early and late pathways of mRNA localization in *Xenopus* oocytes***

The ultimate goal of mRNA localization is subsequent spatially restricted protein expression. There are several ways that this can be accomplished, including diffusion through the cytoplasm and entrapment at the site of protein synthesis, the degradation of mRNA and/or nascent peptides outside of this region, or active transport of mRNAs to specific cellular destinations (which, like the diffusion and entrapment mechanism, requires a static anchor; reviewed by Shahbadian and Chartrand 2012). Importantly, in order to faithfully restrict protein accumulation to one part of a polarized cell, an mRNA must remain translationally silent during active transport or diffusion. I shall return to this part of the localization process later in the chapter.

mRNA localization in the *Xenopus* oocyte can be divided into the early and late pathways, which, as their names suggest, occur at different times during oogenesis. Early pathway mRNAs, such as *Xcat2*, *Xdazl*, and *DEADSouth* (Mosquera *et al.* 1993; Houston *et al.* 1998; MacArthur *et al.* 2000), and other mRNAs involved in germline specification, are restricted to the mitochondrial cloud during stages I and II of oogenesis, and in stage II this large (~30µm) cytokeleton-enclosed organelle fuses with the vegetal membrane, thus transporting associated mRNAs to their destination at the vegetal pole (Figure 1B; Heasman *et al.* 1984). The mechanism of localization of early pathway mRNAs appears to rely on diffusion and entrapment, with the mitochondrial cloud acting as a sink for various mRNAs diffusing through the oocyte cytoplasm (Kloc *et al.* 1996; Chang *et al.* 2004).

By contrast, the late pathway relies on active transport of mRNAs along a polarized microtubule cytoskeleton (Figure 1B; Yisraeli *et al.* 1990; Messitt *et al.* 2008; Chapter 3). These mRNAs are predominantly involved in specification of the three primary germ layers of the developing embryo; in particular the correct vegetal localization of Vg1 and VegT mRNAs is required for endoderm and mesoderm formation (Zhang *et al.* 1998; Birsoy *et al.* 2006). Late pathway mRNAs are found distributed throughout the cytoplasm in early oocytes, becoming restricted in a characteristic “wedge” shape in the vegetal cytoplasm of late stage II and early stage III oocytes (a phenomenon that is perhaps best viewed by confocal microscopy after microinjection of a fluorescently labeled RNA; Figure 1C (Gagnon and Mowry 2011). Once localization is complete, by stage IV, late-localizing mRNAs are found tightly anchored at the cortical cytoskeleton in a region that extends from the vegetal pole to the marginal zone of the oocyte (Figure 1B; Melton 1987; Alarcon and Elinson 2001).

### ***Vg1 mRNA localization is orchestrated by a 340-nucleotide cis-element***

Of all the localized mRNAs in *Xenopus* oocytes perhaps the most intensely studied is Vg1. This mRNA is localized to the vegetal pole via the late pathway and its encoded protein, a member of the TGF- $\beta$  superfamily of secreted growth factors, is involved in the specification of the endoderm and mesoderm of the early embryo (Melton 1987; Birsoy *et al.* 2006). Indeed, misexpression of Vg1 in the animal hemisphere of the oocyte results in an expansion of endoderm and mesoderm and a concordant loss of ectodermal tissue, an event that is fatal to the developing tadpole (Dale *et al.*

1993; Thomsen and Melton 1993). Regulated Vg1 localization and spatially restricted translation are therefore crucial processes in the oocyte.

Based upon the finding that *in vitro* transcribed Vg1 mRNA could localize after injection into cultured *Xenopus* oocytes (Yisraeli and Melton 1988), a hunt began for a localization signal contained within the mRNA itself. Through exhaustive deletion analysis, Mowry and Melton (Mowry and Melton 1992) identified a 340-nucleotide (nt) element within the ~1200-nt Vg1 3'-untranslated region (UTR) that they dubbed the vegetal localization element (VLE; Figure 1D). This element, when injected into oocytes, could direct faithful vegetal RNA accumulation in a manner identical to endogenous Vg1.

#### *Localization elements in other organisms*

While other molecular determinants of mRNA localization have been characterized (such as the translation of N-terminal signal peptides for targeting to the rough endoplasmic reticulum; (Walter *et al.* 1979)) the importance of *cis*-acting signals within a localizing transcript has been widely documented (Macdonald and Struhl 1988; Mowry and Melton 1992; Kislauskis *et al.* 1994; Long *et al.* 1997). These localization elements (LEs) are generally found within the 3'-UTR of localized mRNAs, and function predominantly to engage *trans*-acting protein factors (reviewed in Macdonald 2011). Recruited proteins, such as the molecular motors dynein and kinesin (Schnorrer *et al.* 2000; Betley *et al.* 2004) and translational regulators such as Cup (Wilhelm *et al.* 2003; see later sections), thus function to



direct the correct spatial and temporal distribution of the mRNA and its encoded protein product. Given the conservation of this mechanism in many organisms, and of the protein factors involved, surprisingly a concordant conservation of LE sequences has not been observed (reviewed in Martin and Ephrussi 2009). Instead, it has been proposed that LEs fold into conserved secondary and tertiary structures that can then be recognized by RNA-binding proteins (Mickleburgh *et al.* 2006; dos Santos *et al.* 2008; Martin and Ephrussi 2009).

Until recently, this hypothesis was based on secondary-structure predictions, with stem-loop conformations as the likely candidate for defining an active LE (Chartrand *et al.* 1999). However, in 2010, Bullock and colleagues succeeded in solving a nuclear magnetic resonance (NMR) structure for the *Drosophila K10* mRNA LE (Bullock *et al.* 2010), which localizes to the anterior of the oocyte and the apical surface of the syncytial blastoderm in embryos in a dynein- and microtubule-dependent manner (Figure 2A and B; Serano and Cohen 1995). This 44-nt LE was shown to fold into a hairpin structure (Figure 3A), but the helices found therein did not conform to the expected A-form RNA. Instead, they form an A'-form helix, which, similar to B-form DNA, has a much wider major groove than A-form helices (Figure 3B). In functional assays the widened major groove, the result of purine—purine base-stacking interactions, proved to be the key feature of the *K10* LE (Bullock *et al.* 2010). In addition, this structure is likely conserved in other anteriorly localized transcripts, such as *gurken* and *bicoid*, which contain conserved purine stretches within their LE hairpins.

For the posteriorly-localizing *Drosophila oskar* transcript, the identity of a *cis*-LE has, until very recently, remained somewhat of a mystery (Figure 2A and B; Ghosh *et al.* 2012). While the 3'-UTR was known to play a role (Kim-Ha *et al.* 1993) it was not capable of directing posterior localization in the oocyte in the absence of endogenous *osk* (Hachet and Ephrussi 2004). In addition, it was shown that splicing of the first intron in the *osk* mRNA and the deposition of the exon-junction complex (EJC) are critical events in *osk* localization (Hachet and Ephrussi 2004). A recently published paper from Ephrussi and co-workers has reconciled these data into a model whereby splicing results in the formation of the SOLE (spliced *oskar* localization element), with the two juxtaposed exons forming a hairpin structure that directs *osk* localization to the posterior pole (Ghosh *et al.* 2012). The authors also postulate that EJC components (or other spliceosomal helicases) might be important for the formation or maintenance of this structure, thus allowing for the subsequent recruitment of other *trans*-acting localization factors. Not only do these findings underscore the importance of structural elements in mRNA localization, but they also emphasize the complexity of identifying them through computational analyses.

While these short LEs fold into discrete stem-loop structures, other much longer LEs have the potential to form complex structural conformations. In neurons, several mRNAs are actively transported away from the cell body to distinct regions such as axonal growth cones and synapses (reviewed in Besse and Ephrussi 2008). With the

discovery that Fragile X Mental Retardation Protein (FMRP) is required for dendritic targeting of a number of mRNAs (Dictenberg *et al.* 2008) came a renewed interest in the RNA-recognition potential of this protein. It had previously been demonstrated that FMRP shows a propensity for binding intramolecular G-quadruplex structures (Darnell *et al.* 2001), but a biological role for this interaction had yet to be shown. Subramanian *et al.* set about testing this interaction by looking at the putative G-quadruplexes in the 3'-UTRs of two post-synaptically-localizing mRNAs, *PSD-95* and *CamKIIa* (Figure 2C; Subramanian *et al.* 2011). They found that the structural conformation of the G-quadruplex was essential for mRNA localization, and that this activity was independent of sequence information (Figure 3C and D). In addition, the G-quadruplex motif was found in almost a third of all neurite-targeted mRNAs, suggesting its function as a general LE in this process.

The notion of structural motifs in LE function is gaining significant support, but it remains a challenge to predict novel mRNA secondary and tertiary structures, especially in the context of longer localization signals (such as the 340-nt Vg1 VLE; Mowry and Melton 1992). Much progress is being made in the field of *de novo* RNA structure prediction which will likely aid in the search for conserved localization structures. Indeed, this year saw the publication of the first real test of RNA tertiary structure prediction, *RNA-Puzzles* (Cruz *et al.* 2012). Similar to the contribution of the *CASP* competition to *in silico* protein structure prediction (Moult *et al.* 1995), the goal of *RNA-Puzzles* is to improve RNA tertiary structure prediction through applying various computer programs to RNAs of known structure. The success of

this project over the coming years will likely be of great interest to the mRNA localization field, and will hopefully improve our understanding of the structural information contained within LEs.

***The VLE contains several sub-elements that are required for vegetal localization***

Whether the Vg1 VLE contains structural information is at this time unclear, however, further analyses of the VLE led to the identification of conserved sub-elements that are found not only within Vg1, but also within other late-localizing mRNAs such as VegT (Deshler *et al.* 1997; Gautreau *et al.* 1997; Bubunenko *et al.* 2002; Kwon *et al.* 2002). These sub-elements, so-called VM1 sites and E2 sites, were shown to bind the *trans*-acting RNA-binding proteins PTB (also known as hnRNPI) and Vera (also known as Vg1RBP/xVICKZ3), respectively. Lewis *et al.* subsequently showed that the presence of clusters of these 5- or 6-nt sites are required for vegetal localization (Lewis *et al.* 2004), thus delineating a minimal LE comprising a duplication of the first 135nts of the VLE; each 135-nt piece contains a pair of E2 sites and a single VM1 site.

Despite much investigation, the precise role of PTB and Vera in Vg1 localization remains unknown. Both proteins are required for localization (Deshler *et al.* 1997; Cote *et al.* 1999) and are comprised of multiple RNA-binding domains (PTB contains four RRM (Perez *et al.* 1997) and Vera has two RRM (RNA recognition motif) and four KH (hnRNPK homology) domains (Git and Standart 2002). Previous work in the Mowry lab led to the discovery that the interaction of PTB and Vera with Vg1 mRNA

is dynamic, with a remodeling event happening in the cytoplasm that is required for vegetal localization (Lewis *et al.* 2008). This interaction, combined with the requirement for multiple copies of each binding site, has led us to the hypothesis that these proteins might function in the formation of transport ribonucleoprotein (RNP) complexes. Indeed, this has been shown to be the case for *Drosophila* PTB, which promotes higher-order complex formation during *oskar* RNA localization (Besse *et al.* 2009). A similar effect has not been reported for Vera or its homologues in other species (reviewed in Yisraeli 2005), however, this protein has been implicated in translational control in other systems (Nielsen *et al.* 1999; Appendix).

#### *mRNP formation in other systems*

One of the most prominent examples of granule formation functioning in mRNA localization is that of *osk* transport in the *Drosophila* oocyte (Chekulaeva *et al.* 2006). In this instance, the formation of granules of 50—80S in size was proposed to function in the translational repression of *osk* during transport to the posterior pole of the oocyte. In contrast to the neuronal system, the oocyte is large and mRNAs are localized in sizeable quantities. Therefore, it is interesting to speculate upon the function of higher-order particle formation specifically in developmentally localized mRNAs. Recently, it was shown that single copies of *K10* mRNA LEs are localized to the apical side of the syncytial blastoderm in *Drosophila* embryos (Amrute-Nayak and Bullock 2012). While it remains to be seen if this mode of solo mRNA transport takes place in the oocyte, it will be important for future experiments addressing

granule formation in any system to realize that the transport of one mRNA at a time is a putative hypothesis.

Although RNA—protein interactions are critical in any mRNA localization pathway, regulatory RNA—RNA-interactions are entirely possible and could mediate mRNA oligomerization into higher-order particles. In the case of *osk* mRNA, where large particle formation has been documented on multiple occasions (Glutzer *et al.* 1997; Wilhelm *et al.* 2003; Chekulaeva *et al.* 2006), it appears that RNA—RNA interactions could play an important role (Jambor *et al.* 2011). Distinct from the SOLE described above, the *osk* 3'-UTR contains a second hairpin motif that is conserved among other fly species. This hairpin, by virtue of a palindromic sequence contained within it, forms intermolecular “kissing-loop” interactions, allowing *osk* mRNAs to interact without the help of *trans*-acting proteins. In addition, these data explain the previously perplexing result that unspliced *osk* could localize in the presence of *wt osk* but not in an oocyte lacking the endogenous RNA (Hachet and Ephrussi 2004). While it remains to be tested *in vivo*, this “hitch-hiking” model of RNA localization is a potential mechanism for directing cell-type specific mRNA transport by bypassing the need for conserved RNA-binding proteins.

### ***Active transport of Vg1 mRNA in the oocyte***

As mentioned previously, *Vg1* mRNA is transported via an active mechanism that depends on the microtubule cytoskeleton (Yisraeli *et al.* 1990). Microtubules are inherently polarized, as a result of the ordered unidirectional orientation of tubulin

subunits in the polymer, and have what are known as plus and minus ends. This polarity can be recognized by a variety of proteins, including those that bind the ends of a tubule (e.g., EB-1, a plus-end binding protein; Beinhauer *et al.* 1997), and motor proteins that carry cargo such as vesicles and RNPs along microtubule tracks. Kinesins are a family of plus-end directed motors (Vale *et al.* 1985), whereas dynein motors are responsible for minus-end directed transport (Gibbons and Rowe 1965). Thus, both the polarity of microtubules and the identity of a bound molecular motor can influence directional transport within a cell.

The cytoskeletal microtubules of a *Xenopus* oocyte are generally organized in a “minus-ends out” orientation (i.e., with their plus ends abutting the oocyte nucleus and their minus ends at the cell periphery; Pfeiffer and Gard 1999), a situation that does not lend itself to directional transport to a specific area of the cell. The situation became more complex when *Xenopus* kinesin-2, a plus-end directed motor, was shown to be required for Vg1 localization (Betley *et al.* 2004). However, we subsequently discovered a sub-population of microtubules within the vegetal cytoplasm that is oriented in the opposite direction; plus-ends out (Messitt *et al.* 2008). This, along with the discovery that kinesin-1 is also required for Vg1 localization, is presented in Chapter 3, and provides a compelling model for the directional transport of Vg1 mRNA localization to the vegetal cortex.

Dynein molecular motors, which move cellular cargo toward the minus-ends of microtubules (Gibbons and Rowe 1965), have also been implicated in Vg1 mRNA

transport (Gagnon *et al.*, unpublished). It would appear that the sub-population of microtubules that are oriented with their plus ends at the vegetal cortex only extend mid-way up the wedge. Dynein likely functions in the early stages of localization, directing the polarized movement of Vg1 away from the vegetal side of the oocyte nucleus. Kinesins and dynein then direct bidirectional movement as the mRNA gets closer to the vegetal pole.

### ***Vg1 at the vegetal cortex***

For mRNA localization to persist over time, the mRNA must remain anchored at its final destination. In the absence of a static attachment point, the mRNA would simply diffuse back into the bulk cytoplasm. This particular feature of the mRNA localization pathway has confounded researchers for years, and remains one of the least understood parts of the pathway. In *Drosophila*, however, recent efforts to understand mRNA anchoring have provided new insight. In 2007, Delanoue *et al.* described an anchoring activity in the minus-end-directed molecular motor dynein (Delanoue *et al.* 2007), a behavior that is reliant on the RNA-binding protein Squid. Most recently a report appeared that implicated the endocytic pathway in anchoring of the *Drosophila* pole plasm through an interaction with posteriorly localized *osk* (Tanaka *et al.* 2011). *Osk* had previously been shown to stimulate endocytosis upon localization, an activity that when disrupted resulted in the diffusion of pole plasm components into the cytoplasm (Tanaka and Nakamura 2008), but the precise mechanism for this behavior was unknown. The molecular culprit, it appears, is Mon2, a conserved protein that is associated with the Golgi apparatus and the



endosomal compartment. Mon2 was isolated in a genetic screen for pole plasm mis-localization (Tanaka *et al.* 2011), and, while it was dispensable for *osk*-induced endosomal cycling, it was critical for the formation of the long actin projections that are necessary for anchoring pole plasm components.

The involvement of the endocytic pathway in the anchoring of a localized RNA is perhaps not that unexpected. ESCRT-II (endosomal sorting complex required for transport-II), a critical component in the removal of ubiquitinated proteins from the cell membrane, was previously shown to play an important role in *bicoid* localization at the anterior pole of the *Drosophila* embryo through a specific and direct interaction with this localized transcript (Irion and St Johnston 2007). However, in this instance it is not clear exactly how ESCRT-II promotes *bicoid* accumulation at the anterior of the embryo. In the pathogenic yeast *Ustiligo maydis*, various mRNAs are localized in a microtubule-dependent manner as the cell creates invasive filaments (Becht *et al.* 2006). This process was recently linked to the endocytic pathway, with mRNPs observed to “hitch-hike” onto endosomes. Both entities were then transported through an association with various molecular motors toward the tip of the growing filament (Baumann *et al.* 2012). While the picture is far from complete, it will be important for future investigations into anchoring to consider the involvement of the endocytic pathway.

Comparatively little is known about Vg1 anchoring, although the involvement of the cortical cytoskeleton is crucial (Alarcon and Elinson 2001). The oocyte cortex is a

tough shell, composed of actin fibers and cytokeratin intermediate filaments. In addition to this proteinaceous structure mRNAs themselves have been suggested to play a role in anchoring (e.g. *Xlsirts*; (Kloc *et al.* 2007)), although it is not clear whether this effect is direct, or acts through the associated disruption of the cortical cytoskeleton. Interestingly, the evidence that dynein molecular motors have been shown to play a role in anchoring localized mRNAs (Delanoue *et al.* 2007) and are associated with the Vg1 mRNP during transport (Gagnon *et al.*, unpublished) provides a tantalizing and testable hypothesis for Vg1 anchoring.

### **Spatio-temporal control of gene expression**

As I have hinted, in order for mRNA localization to have an effect on cellular physiology it is vital that the transported mRNA is not translated until the appropriate time and in the required place. This means that stringent translational repression is essential during the transport process. To appreciate how such a mechanism might operate in the context of the eukaryotic cytoplasm, we must first consider the complexities of general eukaryotic translation.

### ***Translation in eukaryotes***

Translation can be thought of as a continuous process, with ribosomes constantly cycling from an inactive state in the cytoplasm to participating in protein synthesis on an mRNA. However, for simplicity of explanation, I will discuss the process as three discrete steps; initiation, elongation, and termination.

## *Initiation*

Initiation of translation in eukaryotes involves the recognition of the 5' end of the mRNA by the translation machinery and the subsequent assembly of an elongation-competent ribosome (Figure 4A). The 5' end of a mature mRNA is marked by a modified guanine nucleotide ( $m^7G$ ), termed the cap, that is generally added co-transcriptionally in the nucleus (Muthukrishnan *et al.* 1975). After nuclear export into the cytoplasm, the cap structure not only protects the mRNA from 5'-exonucleases (Furuichi *et al.* 1977; Shimotohno *et al.* 1977), but is also required for the initiation of translation (the exceptions to this rule will be discussed later). The cap is recognized by eIF4F, a complex of three initiation factors; eIF4E directly binds to the 5' cap, eIF4A contains RNA helicase activity, and eIF4G is a large scaffolding protein that coordinates cap recognition and ribosome recruitment (Gingras *et al.* 1999). Next, the 43S pre-initiation complex, which includes eIF3, the small (40S) ribosomal subunit, eIF2•GTP, and the initiator tRNA-methionine, engages with eIF4F (Pestova *et al.* 2001) to form a 48S complex. The 40S subunit then “scans” the 5'-UTR until, through a cognate interaction with the initiator tRNA, an AUG start codon is recognized (Kozak 1989). The process of scanning requires the helicase activity of eIF4A to remove any minor mRNA secondary structures upstream of the start codon, and additional helicase activity provided by DEAD-box helicases to generate a large single-stranded RNA “landing pad” for the 40S subunit (reviewed in Hinnebusch 2011). Upon recognition of the start codon eIF5 stimulates GTP hydrolysis on eIF2 resulting in the release of eIF2•GDP. eIF3 is also lost from the

complex at this point, and, with the help of eIF5B, the large, 60S subunit joins its 40S partner (Pestova *et al.* 2000).

From this brief overview of the initiation process it is clear that the demarcation of the 5' end of the message is a critical step. However, this is not the only important role that the cap plays in translation. Guanine addition at the 5' end of cellular mRNAs and methylation of that nucleotide are separate enzymatic events, and, in certain systems, cytoplasmic methylation has been documented (Kuge and Richter 1995; Gillian-Daniel *et al.* 1998). Furthermore, this effect was shown to be dependent on polyadenylation at the 3' end of the mRNA. This observation was perhaps the first hint toward how the 3' and 5' ends of an mRNA might communicate, and a few years later came the publication of the “closed-loop” model of eukaryotic translation (Wells *et al.* 1998). Polyadenylation is a dynamic event, and is often regulated both with regards to the cell cycle and the half-life of a particular mRNA (reviewed in Moore and Proudfoot 2009; Villalba *et al.* 2011). In addition, poly-A binding protein (PABP) was shown to directly interact with eIF4G to stimulate translation (Craig *et al.* 1998). Taken together, these observations underscore the importance of communication between the 3' and 5' ends of an mRNA in the translation of its encoded protein, an idea that I will return to later in this chapter.

While this canonical view of translation initiation can be applied to the majority of eukaryotic mRNAs, on occasion the 5' cap and a number of initiation factors are

dispensable. In these instances, structures within the 5'-UTR called IRESs (internal ribosome entry sites) direct recruitment of the small ribosomal subunit (reviewed in Vagner *et al.* 2001). IRES elements were first described in picornaviruses (Pelletier and Sonenberg 1988) and function in the translation of viral mRNAs in infected cells with reduced levels of eIF4G (through the action of a viral protease). Since this discovery, a number of cellular IRESs have been described in a variety of eukaryotic organisms and have been shown to function in situations where cap-dependent translation is compromised (including starvation, cell cycle control, and the DNA-damage response; Stoneley and Willis 2004).

### *Elongation*

Unlike initiation, which is far more complex in eukaryotes than in other organisms, the process of elongation is highly conserved in all domains of life (reviewed in Rodnina and Wintermeyer 2009). Broadly, elongation can be divided into three steps; decoding, peptide bond formation, and translocation (Figure 4B). Decoding describes the ability of the ribosome to detect a cognate codon:anti-codon interaction between the mRNA and the amino-acyl-tRNA in the A-site.

Accommodation of this interaction in the decoding center of the 40S subunit is accompanied by GTP hydrolysis and the activity of eEF1A and eEF1B. This is immediately followed by peptide bond formation, a reaction that is catalyzed by ribosomal RNA in the peptidyl transferase center of the 60S ribosomal subunit (reviewed in Beringer and Rodnina 2007), and the transfer of the nascent polypeptide chain on the P-site amino acid to the amino acid on the A-site tRNA.

Subsequent translocation, through a ratchet-like motion of the two ribosomal subunits, shifts the peptidyl-tRNA in the A-site into the P-site, and the de-acylated P-site tRNA into the E-site, from where it can be released and re-used. This final step in the elongation cycle is coordinated by eEF2, which is thought to control the direction of the ribosomal ratcheting along the mRNA in a 5' to 3' direction (Shoji *et al.* 2006; Konevega *et al.* 2007). Several 80S ribosomes can be engaged in elongation upon a single mRNA in a formation known as a polyribosome or polysome (Marks *et al.* 1962; Warner *et al.* 1963). Thus, elongation proceeds until each ribosome reaches the end of the coding region of the mRNA.

### *Termination*

Translation termination results in the release of the nascent polypeptide from the ribosome, and usually the dissociation of the two ribosomal subunits (although re-initiation is possible, see later sections). It is coordinated in eukaryotes by eRF1 and eRF3, with eRF1 entering the A-site and recognizing any of the three stop codons (UAA, UAG, and UGA; Figure 4C). eRF3•GTP binding and hydrolysis is then required for the hydrolytic release of the peptide from the P-site tRNA (Alkalaeva *et al.* 2006). In prokaryotes, EF-G, IF3, and the ribosome recycling factor (RRF) are required for separation of the ribosomal subunits (Peske *et al.* 2005; Zavialov *et al.* 2005). However, there is no eukaryotic homologue of RRF, and how eukaryotic ribosomal subunits are recycled is not well understood, although the process does involve eIF3 (Pisarev *et al.* 2007). Regardless of mechanism, correct and efficient termination

and release are critical for the completion of protein synthesis and the replenishment of cellular supplies of ribosomal subunits.

### ***General mechanisms of translational control***

Translation is a crucial process in the cell, since most processes therein rely on the activity of proteins. It should come as no surprise, therefore, that protein synthesis is a tightly regulated process. In eukaryotes, the most complex phase in the translation cycle is initiation, and this complexity lends itself to multiple levels of regulation. Through research conducted since the early 1990s, it has become clear that initiation is indeed repressed through a number of mechanisms, although perhaps the best characterized is that of a family of eIF4E-binding proteins called the 4E-BPs. These proteins are capable of binding to eIF4E and exert a steric block to eIF4G binding, thus preventing 43S recruitment. 4E-BP1 was first described as a translational repressor in rats (Pause *et al.* 1994), where hyperphosphorylation of 4E-BP1 upon insulin treatment resulted in a release of eIF4E and an increase in protein synthesis. Since then, the role of 4E-BPs in the control of the mammalian cell cycle has received considerable attention, with their role in malignant transformation being of particular interest (reviewed in Clemens 2004).

While inhibition of initiation might seem the most logical point to stop the synthesis of a new polypeptide, abrogation during elongation also serves an important biological purpose. Slowing or pausing translation in the context of an intact polysome is in effect leaving the mRNA primed for protein production upon some

sort of cellular signal reviewed in (Browne and Proud 2002). As mentioned in the previous section, eEF2 is a crucial factor in the control of translocation.

Phosphorylation of eEF2 prevents interaction with the ribosome, and thus reduces the rate of translation (Nygard and Nilsson 1990). This modification has been observed in several cases of cellular stress, including times of high energy demand when energetically costly protein synthesis must economize for the sake of other cellular processes (Homan *et al.* 2002).

### ***mRNA-specific translational control***

Global control of general cellular translation is clearly a biologically important process, but there are times when it is only a certain mRNA at a certain time that needs to be post-transcriptionally regulated in this way. In transcriptionally inactive cells, such as the cells in a cleavage-stage embryo, translational regulation is of particular importance, and therefore it is in these cells and their major precursor, the oocyte, that we tend to observe the most varied and complex mechanisms.

Where multiple examples of a given mechanism are known, I shall focus on translational repression of localized mRNAs in oocytes, however, it is important to recognize that these mechanisms are conserved in many different cell types and are not restricted to localized mRNAs.

### ***Regulation at the 5' end***

As mentioned above, all eukaryotic mRNAs are capped at their 5' ends with an m<sup>7</sup>G cap (Muthukrishnan *et al.* 1975) that is recognized by eIF4E, an event that is critical



for the initiation of canonical translation. By interfering with the interaction between eIF4E and eIF4G and thus preventing small ribosomal subunit recruitment, translation can be stopped in its initial phase, a mechanism that is exploited by the 4E-BPs (see above). The first documented case of an mRNA-specific 4E-BP was that of Maskin, a regulatory protein found in *Xenopus* oocytes (Figure 5A; Stebbins-Boaz *et al.* 1999). Maskin is recruited to the 3'-UTR of various oocyte mRNAs through its interaction with the cytoplasmic polyadenylation element binding protein (CPEB), which in turn recognizes a *cis*-element in the 3'-UTR of the RNA called the cytoplasmic polyadenylation element (CPE). Stebbins-Boaz *et al.* showed that Maskin interacts with eIF4E, thus blocking translation at this early initiation step (Stebbins-Boaz *et al.* 1999). A similar mechanism was subsequently observed in *Drosophila* oocytes, where Cup, homologous to Maskin only in function, is recruited to specific mRNAs to repress their translation (Figure 5B). In the case of *oskar* mRNA, Cup is recruited to the 3'-UTR through an interaction with the RNA-binding protein Bruno (Wilhelm *et al.* 2003; Nakamura *et al.* 2004), whereas for *nanos* mRNA Cup binds to the RNA-binding protein Smaug (Nelson *et al.* 2004). Cup has since been shown to repress the translation of other localized mRNAs in the *Drosophila* oocyte, including *gurken* (Clouse *et al.* 2008) and *orb* (Wong and Schedl 2011), with each using different adaptor proteins (Squid and Orb, respectively) to recruit Cup. This use of different adaptor proteins to bring a common translational repressor to a specific mRNA is an efficient and energetically conservative way of generating highly mRNA-specific translational repression.

Blocking small subunit recruitment to the 5' end of an mRNA is a common mechanism for translational repression at initiation, but important regulatory events can occur downstream of the cap. In the 1980s, Kozak published several detailed papers that elucidated the mechanism of start-codon recognition in eukaryotes through 40S scanning of the 5'-UTR (Kozak 1989). From this work it became clear that the context of the AUG within the 5'-UTR is critical for efficient recognition, and that the length and structural identity of the 5'-UTR could influence initiation (Kozak 1989). Indeed, hairpin structures with minimum free energy conformations of  $-30\text{kcal}$  or less, positioned close to the 5' cap, can impede the scanning process leading to a vast reduction in protein synthesis (Kozak 1989). Furthermore, as is the case for mRNAs containing an iron response element (IRE) within their 5'-UTR, such hairpin structures can recruit binding proteins that intensify this effect (Figure 5C; Hentze and Kuhn 1996).

Whereas stable secondary structure in the 5'-UTR generates a steric block to scanning, the presence of an upstream AUG or entire open reading frame can have a similarly detrimental effect on the recognition of a true start codon (reviewed in Meijer and Thomas 2002). Most true start codons are found in an optimal context ( $\text{CCA}^{\text{A}}/\text{C}^{\text{A}}\text{CCAUGG}$ ; Kozak 1986), however, upstream open reading frames (uORFs) are much less likely to be so (Suzuki *et al.* 2000). This observation led to the “leaky-scanning” hypothesis, whereby some ribosomes would initiate at the uORF resulting in non-productive translation, but some would scan past the uORF and initiate at the true AUG (Kozak 1978). Alternatively, and as is the case after translation of small

uORFs, ribosomes can re-initiate at the downstream AUG, albeit less efficiently than at upstream start codons (Kozak 1999). Translational control through the use of uORFs is most commonly found in mRNAs involved in cell stress responses (reviewed in Spriggs *et al.* 2010) with perhaps the best characterized being *GCN4* mRNA in *Saccharomyces cerevisiae*. Gcn4p is required during amino acid starvation, and positively regulates the transcription of more than 30 amino acid biosynthetic genes (reviewed in Hinnebusch 2005). The *GCN4* mRNA contains four uORFs, although only uORF1 and uORF4 are required for translational control (Mueller and Hinnebusch 1986). This realization led to a model whereby under non-starvation conditions, with an abundance of charged initiator tRNA-iMet, ~50% of ribosomes can re-initiate after translating uORF1 and recognize a second uAUG. Under starvation conditions, however, low levels of charged tRNA result in far fewer ribosomes re-initiating at uORF4. The increased time needed to receive a charged tRNA-iMet thus results in increased translation from the true start codon (Miller and Hinnebusch 1989). Further evidence for this re-initiation model stems from the fact that the distance between these uORFs and the true *GCN4* start codon affects translational repression; the closer the uORF is to the AUG, the more inhibitory it becomes, and if it is moved further upstream it loses its regulatory activity (Grant *et al.* 1994). To my knowledge, this mechanism of translational regulation does not act upon localized mRNAs, which is perhaps due to the potential for low-level translation, an outcome that could seriously affect faithful spatio-temporal protein expression.

### *Regulation during elongation*

Translation can also be blocked after initiation, while the ribosome is traversing the coding region. In *S. cerevisiae*, *ASH1* mRNA is localized along the actin cytoskeleton to the bud tip of a mitotically dividing cell (Long *et al.* 1997; Takizawa *et al.* 1997). The encoded Ash1p prevents expression of HO-endonuclease, thus preventing mating-type switching in the daughter cell but not the mother (Bobola *et al.* 1996). *ASH1* contains four discrete LEs, three of which are located in the coding region. While these coding region LEs can be moved to the 3'-UTR and still function, such manipulation results in precocious protein expression throughout both mother and daughter cells (Chartrand *et al.* 2002). As is the case for IREs, the *ASH1* LEs form hairpin structures that are stabilized by binding of the localization proteins She2p, She3p, and Myo4p (a myosin molecular motor; Figure 5D; Chartrand *et al.* 2001), thus forming a steric block to an elongating ribosome and preventing translation of *ASH1* mRNA during transport.

In neurons, the RNA-binding protein FMRP has been implicated in the translation of a number of localized mRNAs (reviewed in Costa-Mattioli *et al.* 2009). Loss of FMRP in Fragile X syndrome patients results in impaired brain function and is the leading cause of heritable mental retardation (Turner *et al.* 1996). Genetic studies in *Drosophila* suggested that FMRP might be involved in translational repression, since flies lacking the protein showed increased expression of specific neuronal mRNAs (Bolduc *et al.* 2008). FMRP is found associated with polysomal mRNA, suggesting a post-initiation block to translation (Stefani *et al.* 2004), but the protein was also

shown to interact with a non-canonical 4E-BP, CYFIP1p, thus suggesting a block to translation initiation (Napoli *et al.* 2008). Most recently, however, various mRNAs that are implicated in autism spectrum disorders and synaptic function were found associated with FMRP on stalled ribosomes (Darnell *et al.* 2011). Importantly, FMRP was found to bind to the coding region of these transcripts, suggesting a mechanism whereby binding of the protein blocks elongating ribosomes.

### *Regulators recruited to the 3'-UTR*

A common feature of many localized and translationally regulated mRNAs is that *cis*-elements within the 3'-UTR direct the binding of *trans*-acting protein factors, which in turn have an effect on the cellular metabolism of the mRNA. I have already touched upon the activity of Bruno and Smaug, which regulate the translation of *osk* and *nanos*, respectively, through the recruitment of the 4E-BP Cup (Wilhelm *et al.* 2003; Nakamura *et al.* 2004; Nelson *et al.* 2004), and of CPEB and Maskin (Stebbins-Boaz *et al.* 1999). In these cases, the recognition of elements at the 3' end of the mRNA are crucial initiating events in the assembly and recruitment of the translational control machinery. *Drosophila bicoid* mRNA is localized to the anterior of the embryo, and its encoded protein has long been known to regulate *caudal* mRNA by repressing its translation only in the anterior through binding to an element in the *caudal* 3'-UTR (Rivera-Pomar *et al.* 1996). Initially thought to directly bind eIF4E to block translation initiation (Niessing *et al.* 2002), Bicoid was subsequently found to recruit 4EHP (4E-homologous protein), which could then

directly bind the 5' cap and prevent productive initiation (Figure 5E; Cho *et al.* 2005).

An emerging area of RNA biology is the formation and function of large non-ribosomal mRNP granules. An assortment of cytoplasmic granules has been described with a variety of functions in the cell, including mRNA transport and degradation (reviewed in Erickson and Lykke-Andersen 2011). In particular, P-bodies have been implicated in several systems as a site of mRNA silencing, storage, and destruction (Eulalio *et al.* 2007). Indeed, several mRNAs have been documented to shuttle between P-bodies and polysomes, indicating a dynamic interplay between translational and silencing machinery in the cell (Brengues *et al.* 2005). While the precise composition of P-bodies (and other cytoplasmic granules) is constantly under scrutiny, their formation highlights the importance of mRNP formation in regulation of mRNA translation. For *osk* mRNA, it has been shown that the specific nucleation of large mRNPs by the 3'-UTR binding protein Bruno results in exclusion of ribosomes from the localizing mRNA and thus brings about translational repression (Chekulaeva *et al.* 2006). Furthermore, in various cell systems mRNAs are seen to localize as large granules, suggesting that this mechanism of translational repression might be widely applicable (see above).

At the 3' end of nearly all eukaryotic mRNAs is a poly-A tail of varying length. While this tail is responsible for protecting the mRNA from 3' to 5' exonuclease activity (Marbaix *et al.* 1977) it has also been shown, by virtue of its protein binding partner,

PABP, to interact with eIF4G and stimulate translation (Craig *et al.* 1998). A number of translationally regulated mRNAs are initially synthesized with a very short poly-A tail, which is then lengthened when the mRNA needs to be translated. This phenomenon has been particularly well studied in *Xenopus* oocytes, where several cell cycle mRNAs are silenced and stored until oocyte maturation (reviewed in Radford *et al.* 2008). These mRNAs all contain within their 3'-UTR a CPE that can direct polyadenylation by recruiting, among other proteins, CPEB, cytoplasmic polyadenylation stimulatory factor, and the poly-A polymerase (Dickson *et al.* 1999). As mentioned above, CPEB can also recruit the silencing factor Maskin (Stebbins-Boaz *et al.* 1999), thus there is a complex interplay between repressive and stimulatory activity at the 3' end of these CPE-containing mRNAs that regulates their translation.

In the case of Vg1 mRNA, an element within the 3'-UTR called the translational control element (TCE) has been described (Wilhelm *et al.* 2000; Otero *et al.* 2001). This ~300-nt AU-rich element is situated just downstream of the VLE and is sufficient to confer ~50% repression of a reporter mRNA (Otero *et al.* 2001). Considerable efforts have been made to understand the repression mechanism(s) underlying Vg1 translational control, but currently the only known *trans*-factor to have any effect upon TCE-mediated translational control is the *Xenopus* HuR homologue, ElrB (Colegrove-Otero *et al.* 2005). Precisely how ElrB functions to repress translation of Vg1 during early oogenesis is unclear, but it is interesting to

note that in HeLa cells, HuR has been shown to interact with the microRNA pathway (Bhattacharyya *et al.* 2006), an idea that I will return to in Chapter 2.

### ***microRNA-mediated translational control***

microRNAs (miRNAs) are 21- to 24-nt non-coding RNAs that have emerged as critical regulators of a multitude of biological processes, including developmental timing (Reinhart *et al.* 2000) and cell growth and malignancy (Miska 2005). Initially discovered in the worm *Caenorhabditis elegans* (Lee *et al.* 1993; Wightman *et al.* 1993), these small RNAs have since been characterized in vertebrates (Berezikov *et al.* 2005), other invertebrates (Lagos-Quintana *et al.* 2001), and flowering plants (Reinhart *et al.* 2002), emphasizing their potential as master regulators of gene expression throughout the eukaryotic domain. Since their discovery, the mechanism of action of miRNAs has been hotly debated (Richter 2008), but the field is beginning to reconcile the potential roles for these molecules in mRNA silencing and decay (Djuranovic *et al.* 2011). That said, it has long been clear that miRNAs are crucial regulators of specific mRNA translation in a variety of systems.

### ***Overview of miRNA biogenesis and miRISC assembly in animals\****

miRNA species originate from multiple locations within the genome, including introns and specific miRNA-encoding genes (reviewed in Olena and Patton 2010). Despite having potentially alternate genomic origins, miRNAs are transcribed, by

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\* miRNA-mediated control of gene expression is conserved in plants, however, given the significant disparities between their modes of biogenesis and action, I shall only describe these processes in animals.



either RNA polymerase II (Lee *et al.* 2004) or III (Borchert *et al.* 2006), as an ~80-nt capped and polyadenylated hairpin precursor called a primary-miRNA (pri-miRNA). While still in the nucleus, a pri-miRNA is then subjected to cleavage by the microprocessor, a complex of the RNase III enzyme Drosha and its binding partner DGCR8/Pasha (Lee *et al.* 2003), to form an ~33-nt pre-miRNA which can be exported to the cytoplasm (Yi *et al.* 2003) via the Exportin-5 complex (Lund *et al.* 2004). Next, the pre-miRNA is subjected to cleavage by Dicer, which cuts the pre-miRNA in its loop region, yielding an RNA-duplex (Hutvagner *et al.* 2001), one strand of which is then loaded into the miRNA-induced silencing complex (miRISC; (Schwarz *et al.* 2003)). This complex is comprised of the P-body component GW182 (Liu *et al.* 2005), a core effector protein from the Argonaute family (Tabara *et al.* 1999), and other regulatory proteins (Zhang *et al.* 2007).

#### *miRISC target recognition*

Target site recognition by the miRISC is entirely controlled by the sequence of the bound miRNA, however, in animals this interaction does not rely on perfect sequence complementarity as might be expected (Slack *et al.* 2000; Vella *et al.* 2004). While in plants miRNA base-pairing with a target mRNA does occur in a fully complementary fashion (Addo-Quaye *et al.* 2008), the comparable RNA duplex formed in animals generally has several mismatched bulges (reviewed in Pasquinelli 2012). Initially, it was thought that nucleotides 2—8 of the miRNA, the so-called “seed region”, did show perfect complementarity with the target site (Moss *et al.* 1997; Reinhart *et al.* 2000). It has since been shown that this is not a requirement

for activity, with increased levels of pairing at the 3' end being able to compensate for a mismatched seed (Vella *et al.* 2004). Even an miRNA:mRNA interaction with neither 3' nor 5' pairing and extensive complementarity in the centre of the site has been observed (Shin *et al.* 2010). In addition to the target sequence, the context in which it is presented to the miRISC has proved important (Thomsen *et al.* 2010). In animals, validated target sites have only been found within 3'-UTRs, often occur in multiple copies, and require an environment of minimal secondary structure (reviewed in Bartel 2009).

This level of complexity and confusion surrounding miRNA target recognition has made finding new targets of miR-mediated translational control by computational methods exceedingly difficult. Nonetheless, several target-site prediction algorithms have been devised and are often successful in finding novel mRNAs. Programs such as TargetScan, miRanda, and PicTar each have their own advantages and pitfalls in the hunt for novel targets, although the most successful parameter to date appears to be an assessment of the thermodynamic stability of the predicted duplex (reviewed in Thomas *et al.* 2010). While these prediction programs are helpful in the identification of target sites, *in vivo* validation of these sites is critically important.

### *Mechanism of miRISC-directed translational control*

In May 2012, two papers were published that finally seemed to put to rest the decade-old argument over how the miRISC regulates protein expression (Bazzini *et*

*al.* 2012; Djuranovic *et al.* 2012). Both groups were able to demonstrate, through different biochemical techniques, that miRISC acts by first blocking translation at initiation or soon after. Subsequently, through the induction of mRNA deadenylation, the target mRNA can be degraded.

But why was this a point of contention in the first place? Initial reports from Ambros and co-workers, looking at the regulation of *lin-14* mRNA by the *lin-4* miRNA during larval development in *C. elegans*, showed *lin-14* to be associated with polysomes despite being translationally repressed (Olsen and Ambros 1999). This suggested a post-initiation mechanism of translational repression. Similar results were seen by other groups for the regulation of *Drosophila hid* mRNA by the miRNA *bantam* (Brennecke *et al.* 2003) and *lin-41* mRNA by let-7a miRNA in HeLa cells (Nottrott *et al.* 2006). At the same time, however, work from Filipowicz and co-workers was suggesting that miRISC in fact blocked initiation, since let-7 miRNA could only direct repression from capped reporters (Pillai *et al.* 2005). Furthermore, human Argonaute2 was then shown to bind to eIF4E (Kiriakidou *et al.* 2007; Djuranovic *et al.* 2010). Yet more complexity was added to the miRNA puzzle when it became apparent that miRNA targets were subject not only to translational control, but also to miRISC-mediated degradation (Bagga *et al.* 2005; Giraldez *et al.* 2006).

While the most recent publications addressing the mechanism of action of miRISC address the disagreement in the field surrounding translational repression versus mRNA degradation, a definitive model for *how* translational repression is achieved

remains elusive. Nonetheless, it is clear that miRNA-mediated translational control is widespread, and a prominent feature of vertebrate biology. Examples of miRNA-mediated repression of localized mRNAs, however, are currently scarce. In *Drosophila* neurons, regulation of *CamKII* translation at synapses has been shown to depend upon components of the miRISC (Ashraf *et al.* 2006) including Armitage (an RNA helicase) and miR-280. Armitage and the miRNA pathway were also implicated in the repression of *oskar* translation during localization to the posterior pole of the oocyte (Cook *et al.* 2004; Tomari *et al.* 2004) however, this genetic interaction was subsequently shown to be indirect, with *Armitage* deletion activating a DNA-damage checkpoint that lead to the observed phenotypic defect in embryonic polarity (Klattenhoff *et al.* 2007).

### ***Multiple mechanisms of translational control can act on a single mRNA***

A common theme in spatio-temporal control of gene expression is that multiple mechanisms of translational repression act upon a single mRNA species. We can only speculate as to why this is so; in the event that one fails, perhaps a second mechanism is in place to ensure the faithful restriction of protein synthesis at the correct time and place. Alternatively, it could be that different mechanisms are active at different times during the lifetime of the RNA or development of the cell or organism. For *Drosophila oskar* mRNA, Bruno protein mediates translational control both through the recruitment of Cup (Wilhelm *et al.* 2003; Nakamura *et al.* 2004) and through the involvement in granule formation (Chekulaeva *et al.* 2006). Similarly, for the posteriorly localized *nanos* mRNA, both Smaug and Gtorund have

been implicated in translational control, with Smaug acting predominantly in the oocyte and Glorund taking over in the embryo (Andrews *et al.* 2011).

### ***Activation of translation/relief of repression***

Once localization of an mRNA is achieved, spatially appropriate expression of the protein product requires that any mechanisms that have been in place to repress translation need to be removed or modified. As mentioned above, mRNAs are often anchored at their destination for weeks or even months, and in these cases relief of repression (or activation of translation) may not occur immediately after the mRNA is localized. A cellular cue, for example activation of a phosphorylation cascade by an extracellular signal, is often required for activation. For *Xenopus* Vg1 mRNA there appears to be a two-step mechanism for translational activation, since protein accumulation can be observed once localization is complete in stage IV of oogenesis, and a further, more robust, up-regulation is seen upon progesterone-induced oocyte maturation (Tannahill and Melton 1989; Dale *et al.* 1993). This increase in translation is thought to take place in response to the activation of a mitogen-activated protein kinase cascade (MAPK), resulting in the phosphorylation of some component of the Vg1 mRNP complex. One candidate for this regulation is Vg1RBP/Vera, an RNA-binding protein that is required for Vg1 localization (Deshler *et al.* 1997; Havin *et al.* 1998) and, upon oocyte maturation, is phosphorylated by the Erk2 MAPK resulting in the release of Vg1 from the vegetal cortex (Git *et al.* 2009). A direct role for Vg1RBP/Vera in translational regulation of Vg1 has yet to be

shown, but it remains an interesting candidate for further investigation (see Appendix).

Perhaps the best understood mechanism for translational activation is through control of cytoplasmic polyadenylation. This has been extensively studied in *Xenopus* oocytes, where the activation of various mRNAs encoding proteins involved in cell cycle regulation depends upon the presence of CPEs in their 3'UTRs (reviewed in Villalba *et al.* 2011). For mRNAs such as *cyclinB1* and *Mos*, binding of CPEB to CPEs directs translational repression during oogenesis through the activity of Maskin (see above; Stebbins-Boaz *et al.* 1999). However, upon oocyte maturation CPEB is phosphorylated by the Aurora A kinase, which results in the recruitment of several stimulatory proteins and the poly-A polymerase, and results in elongation of the 3' poly-A tail (Barnard *et al.* 2004). This in turn increases translation from these mRNAs through the synergistic interaction between PABP and eIF4G (Imataka *et al.* 1998).

For miRNA-mediated translational control, relief of repression has been shown on multiple occasions to involve the intervention of accessory proteins. For example, HuR has been shown to relieve the repression of *CAT-1* mRNA by miR-122 in human hepatoma cells (Bhattacharyya *et al.* 2006) by directing the movement of this mRNA from P-bodies into the polysomal fraction. However, the actions of HuR within a cell are clearly complex, as it was also shown to be involved in the recruitment of let-7 miRISCs to induce repression of c-Myc mRNA in HeLa cells (Kim *et al.* 2009). The

relief of miRISC-dependent translational repression can also be directed through the degradation of one or more of the miRISC components, as is the case for *CamKII* regulation in *Drosophila* neurons, where the ubiquitin-dependent degradation of Argonaute2 is concomitant with the activation of translation of this mRNA (Ashraf *et al.* 2006).

### **Translational control of Vg1: this thesis**

In this introductory chapter I have attempted to set the stage for the questions addressed in my thesis research. At the outset, my goal was to understand the mechanisms underlying the translational repression of the localized mRNA Vg1 during early oogenesis in *Xenopus laevis*. Tackling this problem led me down multiple routes of inquiry. I began by addressing the *cis*-regulatory elements in the Vg1 3'-UTR in an attempt to understand whether known localization factors are involved in translational control. This project proved more complex than anticipated and as such remains an incomplete story. I shall, however, summarize the work as it stands in the Appendix. In my early work I also sought to generate a cell-free system to address translational control in early *Xenopus* oocytes. This resulted in the production of a highly active translation extract, and the published manuscript detailing the method comprises Chapter 3 of this thesis. While my major area of interest was always in the translational control of Vg1, I took a short foray into the world of RNA transport and Chapter 4 represents published work to which I contributed several pieces of data on the role of kinesin molecular motors in Vg1 transport. Finally, and most importantly, in Chapter 2, I will present data to support

my hypothesis that the miRNA pathway is involved in the silencing of Vg1 translation during the mRNA localization pathway. Taken together, this thesis supports a model whereby Vg1 mRNA is regulated at multiple levels to control translation of its encoded protein, and that this regulation is linked to its localization in the oocyte through the action of proteins previously implicated in Vg1 mRNA localization.



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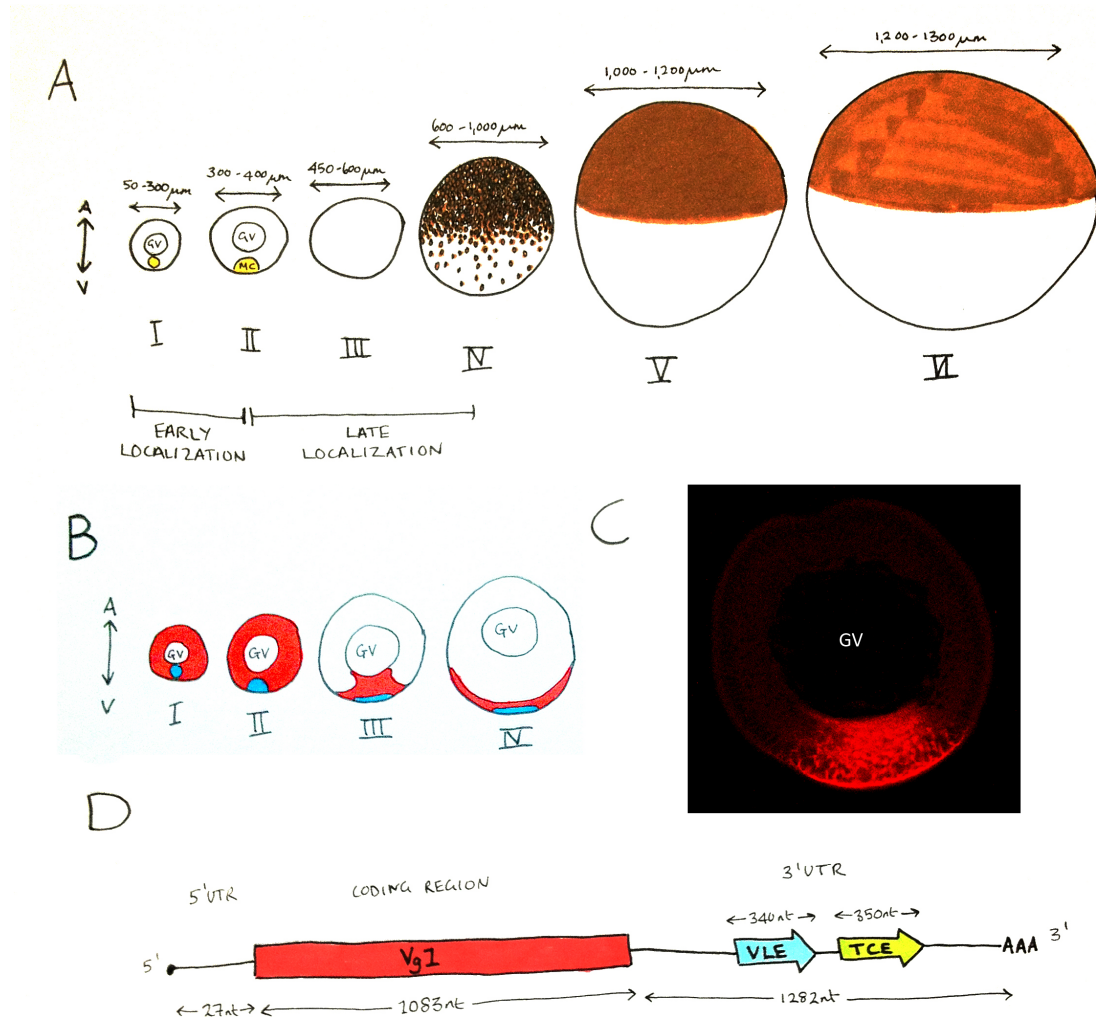
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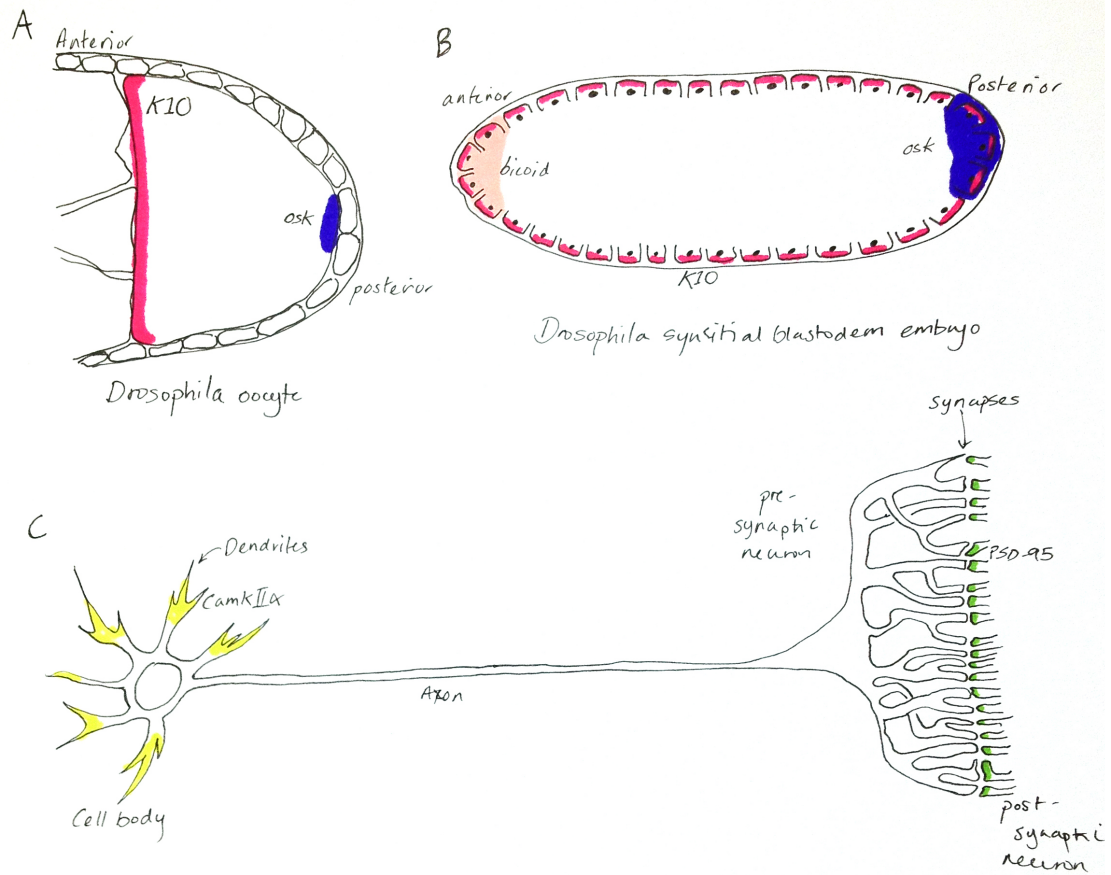
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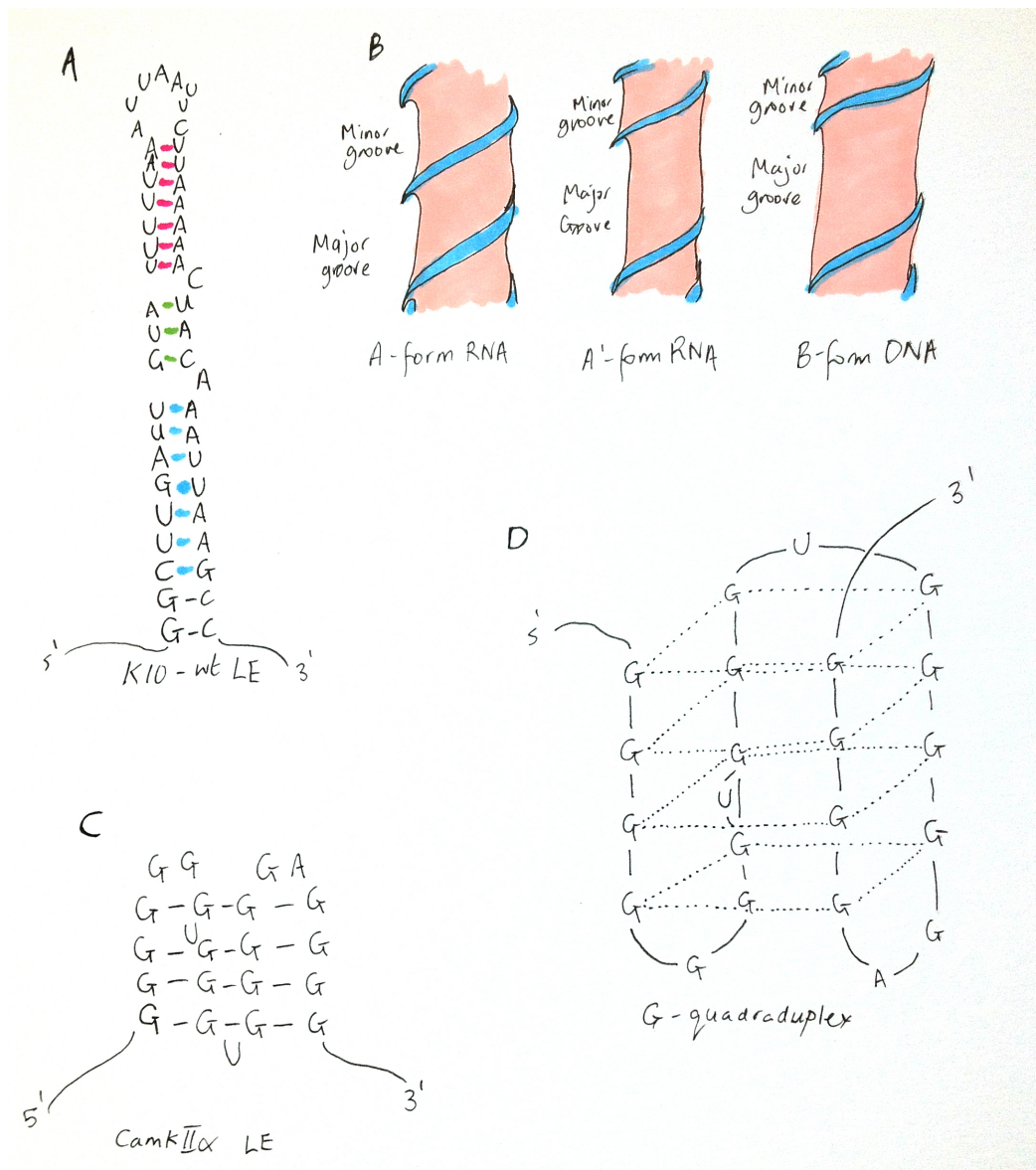


**Figure 1. Generation of asymmetry during oogenesis in *Xenopus laevis*.** **A.** Oogenesis can be split up into six stages, labeled I—VI. Polarization of the oocyte is evident as early as stage I, with the mitochondrial cloud forming on the vegetal side of the nucleus. By stage III this cloud has broken down and yolk proteins are starting to accumulate in the cytoplasm. By stage IV pigmentation can be seen, and in stages V and VI this pigmentation is clearly seen only in the animal hemisphere. **B.** mRNAs are asymmetrically distributed in the oocyte by one of two pathways. Distribution of early pathway mRNAs is shown in blue, whereas that of late localizing mRNAs, such as Vg1, is shown in red. Localization of all these RNAs is completed by stage IV of oogenesis. **C.** Fluorescently labeled VLE mRNA can be used as a proxy for endogenous mRNA localization by the late pathway. Shown is mRNA in the process of localizing in a stage III oocyte. **D.** Vg1 mRNA contains elements within its 3'-UTR that control localization (VLE) and translation (TCE).

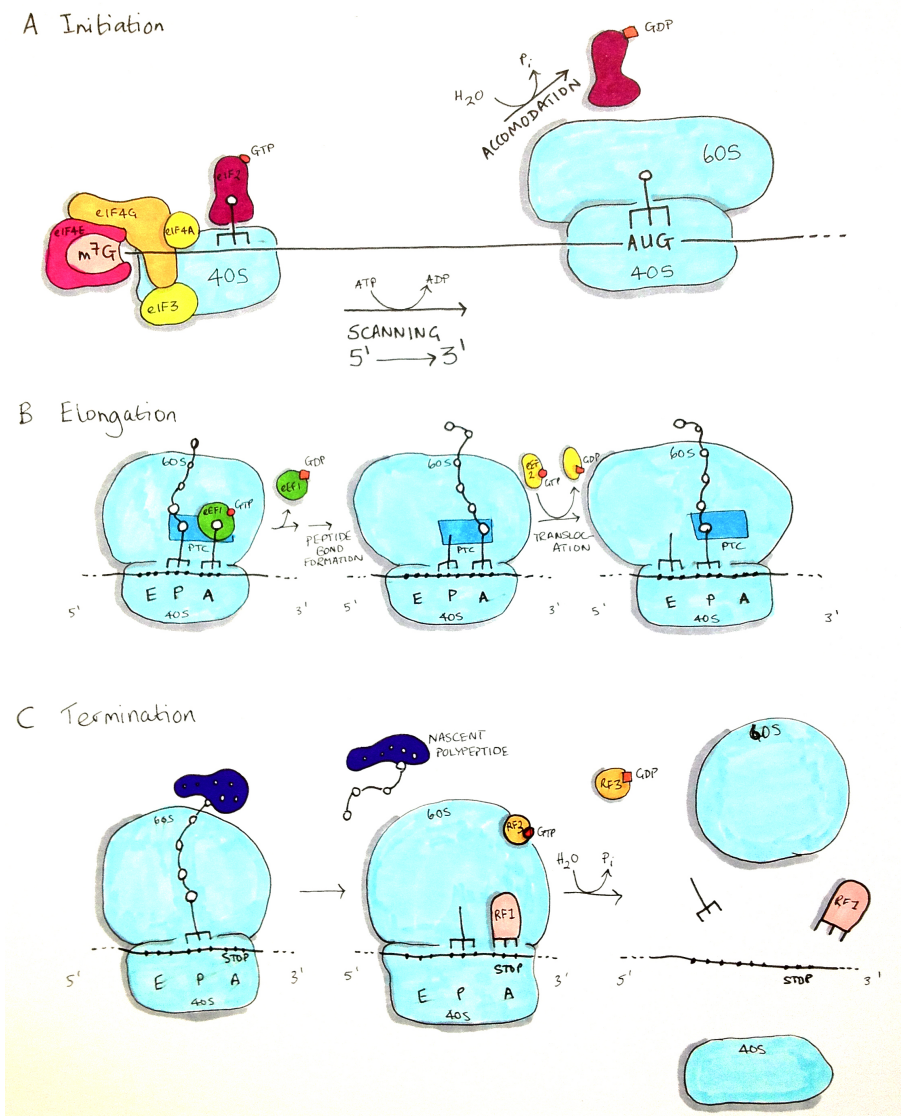


**Figure 2. mRNA localization in *Drosophila* oocytes and embryos, and animal neurons. A.** *K10* mRNA localizes to the anterior of the oocyte (magenta) and *oskar* to the posterior (purple) of a developing *Drosophila* oocyte. **B.** *Bicoid* mRNA localizes to the anterior of the early blastoderm embryo (pink), *oskar* to the posterior (purple), and *K10* is found at the apical surface of the syncytial blastomeres (magenta). **C.** *CamKIIα* mRNA localizes to the dendrites of a neuron (pale green), whereas *PSD-95* is found at post-synaptic side of a synapse (dark green).

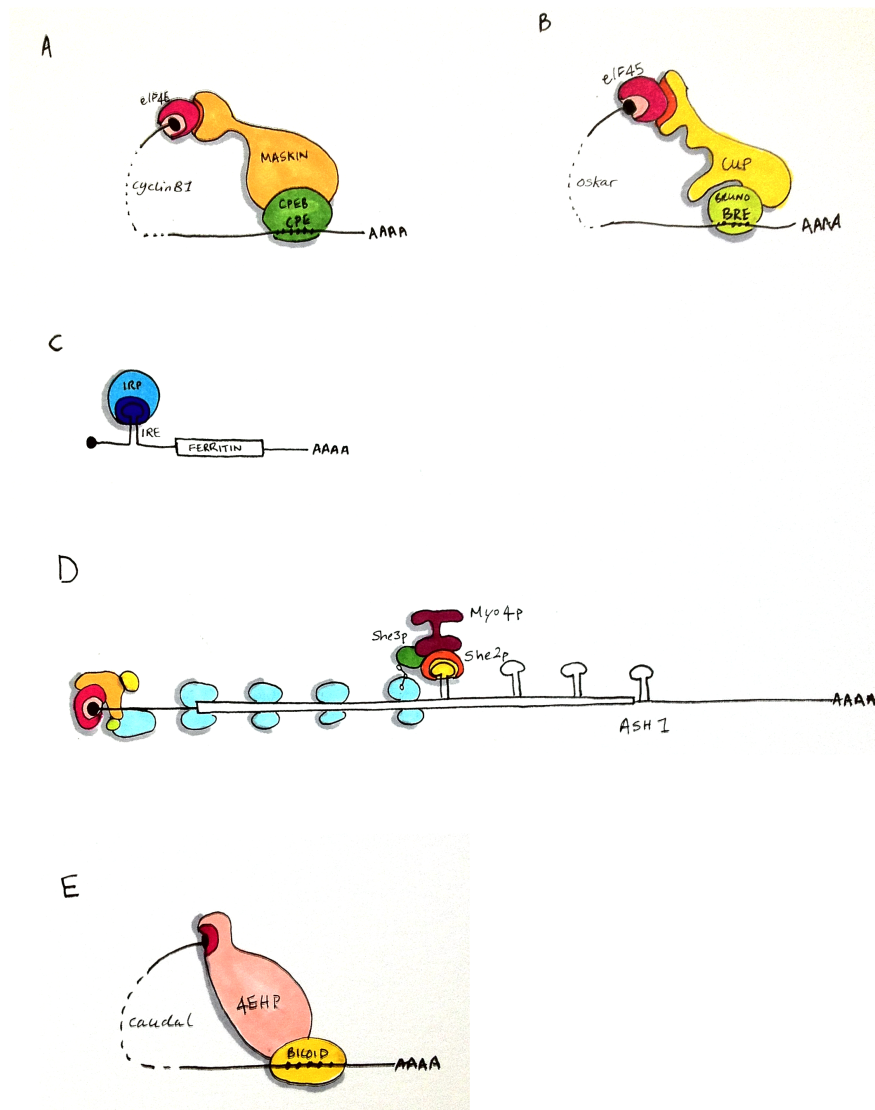




**Figure 3. Structural motifs involved in mRNA localization.** **A.** Secondary structure of the *K-10* LE from *Drosophila*. **B.** Depiction of A-form RNA, A'-form RNA, and B-form DNA helices. *K-10* folds into A'-form, which is similar to B-form DNA in the large size of the major groove. This is in contrast to the small major groove found in the more common A-form RNA helix. **C.** Secondary structure of the *CamKIIα* LE in mammals. **D.** *CamKIIα* LE folds into a G-quadruplex through hydrogen-bond interactions.



**Figure 4. Eukaryotic translation.** **A.** Initiation. The 5' cap of the mRNA is recognized by eIF4F, a complex of eIF4E, eIF4G, and eIF4A. This then recruits the 43S pre-initiation complex which contains, among other things, the initiator tRNA-iMet, eIF3, eIF2•GTP, and the small (40S) ribosomal subunit. The 40S subunit then scans the 5'-UTR for an AUG start codon, at which point hydrolysis of the GTP on eIF2 results in accommodation and the recruitment of the large (60S) ribosomal subunit. **B.** Elongation. Decoding of the mRNA takes place on the 40S subunit, and peptide bond formation takes place on the 60S subunit (the peptidyl transferase center is shown in dark blue). Through the activity of the ribosome, eEF1, and eEF2, the 80S ribosome ratchets through a cycle of peptide bond formation and translocation. **C.** Termination. Peptide synthesis comes to an end when a stop codon is recognized in the A site. Binding of RF1 in the A site results in release of the nascent peptide, and subsequent hydrolysis of GTP on RF3 results in the dissolution of the elongating complex.



**Figure 5. Mechanisms of translational control.** **A.** Maskin is recruited to *cyclinB1* mRNA through an interaction with CPEB in the 3'-UTR. Maskin recognizes eIF4E and prevents binding of eIF4G, thus preventing translation initiation. **B.** Cup is recruited to *oskar* mRNA by Bruno, and regulates translation in the same way as Maskin. **C.** The hairpin IRE in the 5'-UTR of ferritin mRNA recruits IRP and blocks ribosome access to the start codon. **D.** Four hairpin LEs of *ASH1* are bound by the localization factors She2p, She3p, and Myo4p. This complex formation blocks elongating polyribosomes therefore blocking translation of the transcript. **E.** Bicoid binds to the 3'-UTR of *caudal* mRNA and recruits 4EHP, which in turn binds the 5' cap structure to block eIF4E binding.

**Chapter 2: The miRISC and let-7 family miRNAs are required for translational repression of Vg1 mRNA in *Xenopus* oocytes.**

**Catherine A. Pratt** and Kimberly L. Mowry

## Abstract

Along the animal-vegetal axis of oocytes of the frog *Xenopus laevis*, various mRNAs are differentially deployed resulting in temporally and spatially regulated protein expression (Melton 1987). In order to prevent precocious protein expression during localization, and therefore maintain organism viability after fertilization (Birsoy *et al.* 2006), mRNAs such as Vg1 are maintained in a translationally silent state until they reach their destination or are activated by a cellular cue (reviewed in (Lipshitz and Smibert 2000)). We find that Argonaute proteins, the effector molecules of the miRNA pathway, are enriched at the vegetal cortex early in oogenesis, and immunoprecipitation experiments confirm a biochemical interaction with Vg1 mRNA. Conserved miRNA target sites are present in the translational control element (TCE) of the Vg1 3'-UTR, including 3 sites recognized by the let-7 family of miRNAs. These miRNAs are expressed throughout oogenesis, and deletion of the putative target sites and blocking let-7 function using anti-sense oligonucleotides results in the relief of repression. The observed interactions between Vg1 mRNA and Ago proteins are absent in late oogenesis, suggesting a mechanism whereby let-7 miRISCs repress translation during localization, and subsequent release of the mRNA from the vegetal cortex, after localization is complete, relieves this repression.

## Introduction

mRNA localization is a powerful mechanism used by a variety of different cell types to control gene expression in a spatially restricted manner (reviewed in (Meignin and Davis 2010)). In order for this process to be effective, the mRNA must remain translationally silent during localization in order to prevent precocious expression of the encoded protein throughout the cytoplasm of the cell. In the African clawed frog, *Xenopus laevis*, Vg1 mRNA is localized to the vegetal cortex of the developing oocyte (Melton 1987). The encoded protein is a member of the TGF- $\beta$  superfamily of growth factors and is critical for the specification of two of the primary germ layers of the embryo (endoderm and mesoderm; (Weeks and Melton 1987; Birsoy *et al.* 2006)). Vg1 mRNA localization relies on the activity of a number of proteins that are bound either directly, through the recognition of *cis* elements located within the 3'-untranslated region (UTR; (Schwartz *et al.* 1992; Deshler *et al.* 1998; Cote *et al.* 1999), or indirectly, through protein-protein interactions and ribonucleoprotein particle (RNP) formation (Elisha *et al.* 1995; Yoon and Mowry 2004). Protein recruitment to the localizing Vg1 mRNP is directed by the vegetal localization element (VLE; (Mowry and Melton 1992)), a 340nt *cis*-sequence located within the 1200nt Vg1 3'-UTR. Localization-competent RNPs are transported along a polarized microtubule cytoskeleton through an association with kinesin molecular motors (Betley *et al.* 2004) (Messitt *et al.* 2008) and subsequently anchored at the vegetal cortex by an as yet uncharacterized mechanism that relies on the cortical cytoskeleton (Yisraeli *et al.* 1990).

This process of localization and anchoring takes place during stages II-IV of oogenesis (Dumont 1972), and throughout all six stages of oogenesis the levels of Vg1 mRNA remain constant (Melton 1987). However, Vg1 protein cannot be detected by western blotting until late in stage IV (Dale *et al.* 1989); Fig. 5). Upon oocyte maturation *in vivo* or progesterone stimulation *in vitro*, there is a dramatic upregulation of Vg1 protein produced from vegetally localized mRNAs. This spatial and temporal regulation of Vg1 expression is crucial for tadpole development, as ectopic expression of Vg1 in the animal pole of the egg results in a loss of ectodermal tissue, an expansion of mesoderm and endoderm, and death of the embryo (Dale *et al.* 1993; Thomsen and Melton 1993).

A variety of different mechanisms for preventing mRNA translation, both during localization and for non-localized mRNAs, have been elucidated, but perhaps the most intuitive of these block the initiation of translation, as exemplified by the *Xenopus laevis* protein Maskin (Stebbins-Boaz *et al.* 1999). Maskin interacts directly with the cap-binding protein eIF4E and thus prevents recruitment of the 43S pre-initiation complex (Stebbins-Boaz *et al.* 1999). Cup, a *Drosophila* protein that is functionally homologous to Maskin, also binds to eIF4E and blocks translation initiation (Wilhelm *et al.* 2003). In both cases, these proteins are brought to their target mRNAs through an interaction at the 3' end of the mRNA. Maskin recognizes the cytoplasmic polyadenylation element-binding protein (CPEB) which in turn is bound to a *cis* element in the 3'-UTR of cyclin-B mRNA (Stebbins-Boaz *et al.* 1999), whereas Cup has been shown to bind different adaptor proteins depending on the

target mRNA (Wilhelm *et al.* 2003; Nakamura *et al.* 2004; Nelson *et al.* 2004). This recruitment of regulatory proteins to the 3'-UTR of specific mRNAs is used in numerous biological situations as a reliable means of controlling gene expression (reviewed in (Kuersten and Goodwin 2003).

Previous studies aimed at understanding Vg1 translational regulation have defined an approximately 300nt *cis*-element in the 3'-UTR downstream of the VLE termed the translational control element, or TCE (Wilhelm *et al.* 2000; Otero *et al.* 2001). This element, when appended to the 3' end of a luciferase reporter gene, represses translation by about 50% when compared to a control mRNA (Wilhelm *et al.* 2000; Otero *et al.* 2001). The TCE contains a target binding-site for ElrB, a member of the ELAV family of RNA-binding proteins, which is thought to play a role in the translational repression of Vg1 (Colegrove-Otero *et al.* 2005; Devaux *et al.* 2006), however the exact mechanism by which this protein might affect translation is unknown. Interestingly, ElrB is the *Xenopus* homolog of HuR, a protein that has been implicated in the translational control of over 100 mRNAs in a variety of organisms (reviewed in (Meisner and Filipowicz 2010).

Recently, a number of species of small RNAs of 20-40nts in length have been discovered and shown to play crucial roles in the control of gene expression at both the transcriptional and translational level (reviewed in (Naqvi *et al.* 2009). Of particular relevance here is the microRNA (miRNA) class of small RNAs, which are 21-23nt RNAs derived from various regions of almost all eukaryotic genomes



examined, that can regulate translation from a vast number of mRNAs (reviewed in (Djuranovic *et al.* 2011). miRNAs are known to participate in translational regulation in combination with a complex of proteins called the miRISC (miRNA-induced silencing complex; (Lee *et al.* 2004), the core effector molecule of which belongs to the Argonaute (Ago) family of proteins (Hammond *et al.* 2001). These Ago proteins have been shown to control gene expression through both the repression (Lee and Ambros 2001) and activation (Li *et al.* 2006) of translation, as well as the degradation of target mRNA (Meister *et al.* 2004), and as such are a useful molecular marker for the activity of the miRNA pathway.

While Ago proteins represent the active component of the miRISC, it is the miRNA itself that directs complex binding to a target mRNA. In animals, miRNAs have been shown to recognize and bind directly to sites within the 3'-UTRs of their target mRNA (reviewed in (Bartel 2009). The exact mode of recognition remains controversial, with miRNA/target interaction occurring in only a semi-complementary fashion (Bartel 2009). This has therefore made the identification of miRNA binding sites difficult, nonetheless various computational methods have been developed that largely rely on the overall thermodynamic stability of the putative duplex formed (reviewed in (Thomas *et al.* 2010).

A common theme in the post-transcriptional control of gene expression is the layering of complementary methods for translational control (reviewed in (Besse and Ephrussi 2008). For example, in *Drosophila melanogaster* eggs and embryos

*oskar* mRNA is silenced through the activity of Bruno (Kim-Ha *et al.* 1995). Bruno is an RNA-binding protein that is responsible not only for the recruitment of Cup, a translation initiation inhibitor (Nakamura *et al.* 2004), but also for initiating the formation of large protein granules that prevent the recognition of the *oskar* 5'-UTR by the pre-initiation complex (Chekulaeva *et al.* 2006). Given the complexity and redundancy of translational control, and the preponderance of small RNA-mediated mechanisms of such, we set out to assess whether the Vg1 TCE might possess miRNA binding sites, and if so, when they function to control translation in the *Xenopus* oocyte. Our data indicate that the let-7 family of miRNAs, in conjunction with Ago proteins, are present throughout oogenesis and are required for TCE-dependent translational control of a luciferase reporter. In addition, an antibody directed against human Ago2 indicates an enrichment of the protein at the vegetal cortex of the oocyte and a biochemical interaction with endogenous Vg1 transcripts. The timing of this localization and interaction is consistent with the known translational profile of Vg1 protein (Dale *et al.* 1989; Tannahill and Melton 1989), strongly suggesting the involvement of the let-7 miRISC in the translational control of Vg1 RNA.

## **Results**

### **Vg1 mRNA is associated with vegetal Ago protein in the oocyte**

In order to assess whether the miRISC might be affecting Vg1 mRNA translation we first asked whether Ago protein is found at the vegetal cortex of the oocyte. Using an antibody directed against human Ago2 (2A8; a gift from Z. Maurelatos) strong

staining at the vegetal cortex is observed in stage III oocytes (Fig. 1A). Weaker staining is also observed throughout the oocyte cytoplasm. This cortically enriched staining is also observed in the presence of an Alexa-fluor546-labeled injected RNA containing both VLE and TCE sequence (Fig. 1A). Levels of Ago expression remain relatively constant throughout oocyte development, whereas levels of tubulin increase in a manner consistent with the increase in cell size observed between stages I and VI (Fig. 1B). Association of the endogenous Vg1 transcript with Ago was assayed by immunoprecipitation followed by RT-PCR analysis (Fig. 1C). Indeed, Vg1 was specifically immunoprecipitated with this antibody and not an IgG control while a control mRNA, EF1 $\alpha$ , was not (Fig. 1C). Taken together these data indicate a potential role for the miRNA pathway in the regulation of Vg1 mRNA.

### **The Vg1 TCE contains several conserved putative miRNA target sites**

Ago recruitment is dependent on the presence of a bound miRNA that can recognize target sites in a target mRNA (Vella *et al.* 2004). Therefore using the software package miRanda (Enright *et al.* 2003) we analyzed the Vg1 TCE for putative miRNA target sites that could be recognized by any of the ~150 miRNAs present in a *X. tropicalis* database ((Tang and Maxwell 2008); unfortunately, such an extensive library does not currently exist for *X. laevis*). To improve the reliability of this prediction process we used Vg1 TCE sequences from three related species of frog, *X. laevis*, *X. borealis*, and *X. tropicalis*, and focused on only those sites that were conserved. Figure 2A shows a map of these conserved sites and their distribution in the *X. laevis* TCE. We next asked if these miRNAs were expressed in oocytes by

northern blotting, and by this method were only able to detect the let-7c species (Fig. 2B). Members of the let-7 family of miRNAs share a very similar sequence (Fig. 2C), and it is possible that a combination of the promiscuity of short northern blot probes and the difficulty of annealing such short probes might obscure our view of the true expression profile of each let-7 miRNA in the oocyte. Indeed, according to our miRanda predictions, four such species have the potential to bind to five somewhat overlapping target sites (Figs. 2A and 2D). We therefore used a highly specific and sensitive PCR-based assay to address the expression pattern of these four let-7 family members (let-7a, c, e, and g). Briefly, the TaqMan (Applied Biosystems) small RNA detection kit uses a hairpin primer for gene-specific cDNA synthesis, followed by real-time PCR amplification of this cDNA using one gene-specific and one hairpin-specific primer. As a control, we also assayed U6 snRNA expression by this method, and normalized let-7 species expression to that of U6. This allowed us to measure the relative expression of each miRNA across oogenesis. The results of this experiment (Fig. 2E) show that all four of the let-7 miRNAs assayed are expressed at relatively similar levels throughout oogenesis, and therefore represent viable candidates to control the translation of Vg1 mRNA.

### **Let-7 target sites are required for TCE-mediated translational repression of Vg1**

Having validated the expression of the let-7 family miRNAs throughout oogenesis, we next sought to address whether the target sites predicted by miRanda were functional in the oocyte. To do this, we created a series of luciferase reporter

constructs with varying 3'-UTR sequences (Fig. 3A). As a control for normal levels of translation in the oocyte we used a *Xenopus*  $\beta$ -globin 3'-UTR, which is not regulated during oogenesis. The remaining constructs contained iterations of the *X. laevis* TCE sequence, with the WT construct representing normal translational repression, and deletion constructs derived from this. Each 3'-UTR was cloned downstream of a firefly luciferase gene (Ow *et al.* 1986), which itself was preceded by a  $\beta$ -globin 5'-UTR that allows for efficient translation initiation in the oocyte, and an SP6 viral transcriptional promoter, which enabled us to generate mRNAs *in vitro* (Melton *et al.* 1984). After checking that the levels of translation in our mRNA mixes were equivalent in an *in vitro* rabbit reticulocyte lysate (Promega), we injected stage II oocytes as indicated in Figure 3B. Dual luciferase assays (using a Promega DLR kit) were performed 18 hours post-injection and are represented as a ratio of firefly:Renilla, with translation from the  $\beta$ -globin control reporter set to a value of 1 and the activity of the other reporters shown relative to that (Fig. 3C). As an additional control, we constructed a luciferase reporter with the miR-449 site deleted. This miRNA was not detected in our northern analysis (Fig. 2B), and as expected this deletion had no effect on translation of the reporter mRNA (Fig. 3C). Deletion of any or all of the predicted let-7 target sites resulted in an increase in firefly luciferase translation (Fig. 3C), implicating these sites in TCE-mediated translational repression of Vg1.

## **Interfering with let-7 function abrogates TCE-dependent translational repression**

If deletion of one or more let-7 target site is sufficient to disrupt TCE-mediated translational repression in our luciferase reporter system, one would expect a similar effect from interfering with the miRNA predicted to bind that site. To test this hypothesis we turned to locked nucleic acid oligonucleotide technology (LNA, Exiqon). LNA oligonucleotides have a modified backbone, and as such have an enhanced specificity for their anti-sense target, as well as an increased stability in biological scenarios. We therefore co-injected LNAs complimentary to *Xenopus* let-7c and let-7g with a WT TCE construct (Fig. 4A). As a positive control for translation we once again used a  $\beta$ -globin construct, which should also not respond to let-7 interference, and as an LNA toxicity control we used a scrambled oligonucleotide. As a guideline for gauging final LNA concentration required for interference with function in the oocyte we assessed the conditions used by (Mortensen *et al.* 2011). Based on the estimation of 1 fmol of miRNA per oocyte, that a molar excess of 300-2,500 should have a measurable effect on translation, and that there are at least four similar species of let-7 miRNAs expressed in oocytes, we injected LNAs at a concentration of 20  $\mu$ M. This, given the volume of stage II oocytes injected ( $\sim$ 250  $\mu$ m in diameter) should result in an  $\sim$ 1,250-fold molar excess of LNA over endogenous let-7 miRNA. As before, oocytes were assayed for luciferase activity 18 hours post injection. As expected, there was a significant increase in translational activity from the WT TCE reporter in the presence of either an anti-let-7c or an anti-let-7g LNA oligonucleotide, corroborating our deletion mutant data (Fig. 4B). In

addition, co-injection of both antisense oligonucleotides resulted in an additive increase in translation levels from the WT TCE reporter (Fig. 4B). These data indicate that let-7 family miRNAs are required for TCE-mediated translational control.

### **miRISC binding of Vg1 decreases at the end of oogenesis**

If let-7 family miRNAs and the miRISC are responsible for the regulation of endogenous Vg1 RNA we might expect their association with this transcript to decline towards the end of oogenesis when Vg1 protein is detected (Dale *et al.* 1989). To address this question we again turned to our IP-RT-PCR technique using the 2A8 antibody, which was raised against human Ago2 but behaves as a pan-Ago antibody. Figure 5A shows that while the antibody immunoprecipitates Vg1 during the localization process, this interaction is lost once localization is complete. This effect is coincident with the observed changes in expression levels of Vg1 protein (Fig. 5B). We also assayed the localization of the Ago by immunofluorescence using the 2A8 antibody throughout oogenesis and found that it is vegetally localized during mid-oogenesis, but this asymmetric enrichment is lost in later-stage oocytes (Fig. 5C). Taken together, these data suggest a model whereby the miRISC binds to and represses the translation of Vg1 mRNA during localization, but that loss of this interaction after transport is complete relieves that repression (Fig. 5D).

## Discussion

In this study we have uncovered a role for the let-7 family of miRNAs and the miRISC in the control of Vg1 expression. Our data suggest that during transport of the Vg1 mRNA to the vegetal pole of the *Xenopus* oocyte let-7 family miRNAs seed a silencing complex that prevents Vg1 translation. In the absence of functional miRNA or cognate let-7 binding sites within the Vg1 TCE, silencing of a reporter RNA is abolished. Furthermore, Ago protein is localized to the vegetal cortex and biochemically associates with endogenous Vg1 RNA, supporting the hypothesis that the miRISC plays an important role in the control of Vg1 translation.

The let-7 gene was first described as a crucial regulator of larval development in the worm *Caenorhabditis elegans* (Sulston and Horvitz 1977), but its identity as a small non-coding RNA was not discovered for another 23 years (Reinhart *et al.* 2000). Computational approaches have since shown that there are multiple members of the let-7 family of miRNAs that are highly conserved among metazoans (reviewed in (Roush and Slack 2008). In general, let-7 miRNAs play important roles in development and the control of cellular differentiation (Roush and Slack 2008), but their precise function in vertebrate development remains a significant area of investigation. Interestingly, the expression of let-7 family members has recently been shown in bovine oocytes (Miles *et al.* 2012), where they function in the regulation of maternal mRNAs.



A common feature of miRNA-mediated translational control is that multiple recognition sites are often required for, or at least improve, silencing (Hon and Zhang 2007). The Vg1 TCE contains three putative let-7 binding sites, although two of these overlap making only two accessible at any one time. Our data suggest that both sites are required for translational repression, although it is not entirely clear, based on the deletion series (Fig. 3), which sites are the most important. However, it is clear that interfering with either let-7g or let-7c using antisense LNA oligonucleotides diminishes TCE-dependent translational repression (Fig. 4), suggesting that these two miRNAs are functioning. Given the sequence conservation of this miRNA family the possibility remains that the sequence specificity of the LNAs is limited. Therefore, while we cannot speak to the precise binding-site requirements at this time, it is clear that the let-7 family of miRNAs is required for TCE-mediated translational repression in the *Xenopus* oocyte.

Previously, studies of the *Drosophila* nervous system have assigned a role for the miRISC in localized mRNA translation (Ashraf *et al.* 2006). CaMKII mRNA localization is critical for learning and memory through the role of its encoded protein, a calcium-dependent kinase, in synapse formation (reviewed in (Kelleher *et al.* 2004)). In this particular case, miR-280 has been implicated in recruiting the miRISC, and proteasome-mediated degradation of the protein components of the regulatory complex relieves repression upon an increase in neuronal activity (Ashraf *et al.* 2006). In *Xenopus* oocytes, however, we do not see a decrease in the

expression of Ago2 as oogenesis proceeds (Fig. 1B), disfavoring degradation as a mechanism of translation induction.

Our data suggest that Ago is released from the Vg1 mRNP in stage V oocytes (Fig. 5A), but how this release is orchestrated remains unclear. It is possible that the miRISC undergoes post-translational modification, or that an intermediary protein that is developmentally regulated dislodges the complex from the Vg1 3'-UTR. While post-translational modifications of Agos have been reported, as yet they have only been found to stabilize the protein (Qi *et al.* 2008; Kirino *et al.* 2009). On the other hand, a putative candidate for an intermediary protein regulator is ElrB. Its mammalian homolog, HuR, has been shown to be involved in recruiting the let-7 RISC to the 3'-UTR of cMyc mRNA (Kim *et al.* 2009). However the details surrounding the mode of action of HuR remain unclear, as in the case of CAT-1 mRNA regulation it appears to promote miRISC dissociation (Kundu *et al.* 2012). In that particular case, it would appear that HuR binding and subsequent polymerization along the RNA results in the eviction of the miRISC (Kundu *et al.* 2012). Nonetheless, given its documented role in TCE-dependent repression (Colegrove-Otero *et al.* 2005), ElrB represents a potential point of regulation in the interaction of the miRISC with Vg1 RNA.

An alternative hypothesis for the observed actions of the miRISC and ElrB in *Xenopus* oocyte development is that they act independently. Indeed, multiple levels of translational control have been observed for other localizing mRNAs, such as

*oskar* mRNA in *Drosophila* oocytes (Chekulaeva *et al.* 2006). In this case the *trans*-acting factor Bruno acts both in the recruitment of the eIF4E-interacting protein, Cup, and in promoting the formation of oligomeric complexes that block the access of the translation machinery. In the case of Vg1, there are two observable bursts of translation (Fig. 5B; (Dale *et al.* 1989; Tannahill and Melton 1989). The first occurs at the end of stage IV when localization of the mRNA is completed, and the second upon oocyte maturation. It is therefore conceivable that the miRISC acts predominantly during localization, whereas ElrB-mediated repression is sensitive to progesterone and thus accounts for the second, much larger, increase in Vg1 translation. In future studies, it will be important to tease apart how these two mechanisms behave over the course of oogenesis, and whether they cooperate or compete in the translational repression of Vg1.

Vg1 mRNA localization in *Xenopus* oocytes has been, and remains, a valuable model system for the study of mRNA localization. We have now contributed not only further understanding of the translational regulation of this transcript, but also added weight to the idea that the miRISC functions in the post-transcriptional regulation of localized mRNAs. In the future it will be interesting to see if this mechanism is conserved in other developmentally localized mRNAs, both in *Xenopus* and other organisms.

## **Materials and Methods**

### ***In vitro transcription***

Reporter RNAs for injection were generated using the mMessage mMachine kit (Ambion) according to manufacturers instructions. After DNase treatment to remove the linear template RNA was phenol extracted and ethanol precipitate overnight at -20°C. The concentration of the resuspended RNA was then measured using a NanoDrop microvolume spectrophotometer (ThermoFisher) and diluted to a stock concentration of 200nM. For fluorescent VLETCE RNA transcription reactions were performed as in (Mowry 1996) in the presence of 1 mM Alexa Fluor 546-14-UTP (Molecular Probes).

### ***Microinjection and oocyte manipulation***

Oocyte isolation and microinjection was performed as in (Kress *et al.* 2004). For the experiments in Figure 3, firefly luciferase reporters were injected at a concentration of 75 nM and the internal control Renilla luciferase at 5 nM. In Figure 4, firefly luciferase reporters were injected at a concentration of 150 nM with the internal control Renilla luciferase at 5 nM. Where relevant, 20 µM LNA oligo (Exiquon) was co-injected with the reporter RNAs. In all cases oocytes were incubated overnight (16-18 hours) after.

### ***Cloning***

All deletion constructs were made using the QuikChange II mutagenesis kit (Agilent Technologies) according to manufacturers guidelines. Template plasmid DNA consisted of pSP64TSN-Luc-TCE. Primer sequences were as follows: let-7a/c/e forward 5'-GATTTGTCTGTGGAAAGGCATTTGCTTCCCTATAAGATGAG-3', let-7a/c/e reverse 5'-CTCATCTTATAGGGAAGCAAATGCCTTTCCACAGACAAATC-3', let-7g1 forward 5'-CTGCCGGTCTTGATTTGTGCCTATTGCTTCTATTTGC-3', let-7g1 reverse 5'-GCAAATAGAAGCAATAGGCACAAATCAAGACCGGCAG-3', let-7g2 forward 5'-GATGAGTTTCCTTTAAAGAAAATGGAGTGCAGACAATTTTTCATTG-3', let-7g2 reverse 5'-CAATGAAAAATTGTCTGCACTCCATTTTCTTTAAAGGAACTCATC-3', let-7all forward 5'-CGCTGCCGGTCTTGATTTGGACAATTTTTCATTG-3', let-7all reverse 5'-CAATGAAAAATTGTCCAAATCAAGACCGGCAGCG-3'. For the let-7g1/g2 double deletion, the  $\Delta$ let-7g2 construct was used as a template with the let-7g1 primer pair.

### ***Immunoprecipitation***

Immunoprecipitation, RNA isolation, and RT-PCR were all carried out as in (Messitt *et al.* 2008).

### ***Immunofluorescence and Imaging***

Oocytes were fixed, permeabilized, and washed as in (Messitt *et al.* 2008). In all cases, the primary anti-human Ago2 antibody 2A8 (a kind gift from Z. Mourelatos) was used at a concentration of 1:50, and the secondary Alexa Fluor 633-conjugated

anti-mouse IgG was used at a concentration of 1:500. Oocytes were imaged by confocal microscopy using a Zeiss LSM 510 Meta.

### ***Immunoblotting***

For immunoblotting, oocytes were homogenized in WB (10 mM HEPES (pH 7.4), 10 mM KOAc, 3 mM Mg(OAc)<sub>2</sub>, 5 mM EGTA, 0.05% NP-40, 1 mM DTT, 0.1 µg/ml leupeptin (MP Biomedicals), 0.1 µg/ml antipain (MP Biomedicals), 0.1 µg/ml trypsin inhibitor (MP Biomedicals), 0.4 mM Pefabloc (Roche), 1 U/ml rRNasin (Promega), 34 g/l sucrose) at 0.5 oocytes/µl. The number of oocytes loaded is indicated in the associated Figure legends. Anti-human Ago2 (eIF2C2; Abnova) was used at a concentration of 1:100 since the 2A8 antibody does not react with *Xenopus* Ago by western blotting (unpublished data), anti-α-tubulin (Sigma) at a concentration of 1:500, and anti-Vg1 (a kind gift from D. Kessler) at 1:100.

### ***MiRanda analysis***

MiRanda analysis was performed using the *X. tropicalis* library of miRNA sequences ([http://www.mirbase.org/cgi-bin/mirna\\_summary.pl?org=xtr](http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=xtr)). Putative target sites were predicted using the following pre-set parameters: Gap Open Penalty: -9.0, Gap Extend: -4.0, Score Threshold: 140.0, Energy Threshold: -15.0 kcal/mol, Scaling Parameter: 4.0. TCE sequences from *X. laevis*, *X. Borealis*, and *X. Tropicalis* were used, and conserved target sites were identified manually.

### ***Northern blotting***

RNA was prepared from *X. tropicalis* by hot acidic phenol extraction. Briefly, oocytes were lysed in 400µl AE buffer (50mM NaOAc, 10mM EDTA) using a dounce homogenizer. 500µl of acidic phenol (pH5.3) heated to 65°C was added and the mixture was incubated at 65°C for 5 mins with vortexing every 30 seconds. After the incubation, the mixture was flash cooled in an ethanol/dry ice bath and centrifuged for 10 mins, 6,000 rpm in a room temperature microcentrifuge. To enrich for low molecular weight RNA species, high molecular weight RNA was precipitated from the aqueous phase using 10% PEG-8000 and 0.5M NaCl. The resulting supernatant was then subjected to ethanol precipitation and the pellet was resuspended in RNase free dH<sub>2</sub>O. RNA concentration was then assessed using a Nanodrop spectrophotometer. 20µg of the low molecular weight RNA prep was electrophoresed on a 15% urea gel (Invitrogen) and transferred to a supercharged nylon membrane (Millipore). The membrane was then dried and the RNA cross-linked to it using UV irradiation. For hybridization, the membrane was first incubated in 3ml pre-hybridization buffer (Sigma) for 30mins at 45°C. 100pmol of each <sup>32</sup>P-end-labelled DNA oligonucleotide probe (let-7c: 5'-AACCATACAACCTACTACCTCA-3' miR-449: 5'-AGGCAGTGTAATGTTAGCTGGT-3') was then added and allowed to anneal overnight at 45°C. The blots were then washed 3 times in 5XSSC = 0.1% SDS, wrapped in saran, and imaged by autoradiography.

### ***Luciferase assays***

Before injection reporter mRNA mixtures (75 nM or 150 nM firefly, 5 nM Renilla) were assayed in triplicate in a rabbit reticulocyte lysate (Promega) according to the manufacturer's guidelines. This procedure was implemented to ensure a "level playing field" for all experimental constructs, as TCE-dependant translational control is not active in the RRL system (Tim Messitt, unpublished data). After injection into stage II oocytes and overnight incubation, oocytes were sorted into five groups of 10 oocytes, thus allowing for luciferase analysis in pentuplicate. The oocytes were then homogenized in 20  $\mu$ l passive lysis buffer (Promega), centrifuged at 10,000 x g for 10 minutes at 4°C, and placed on ice. Luciferase assays were then performed on 5  $\mu$ l of the supernatant as in (Pratt and Mowry 2010). Firefly luciferase activity was divided by Renilla luciferase activity for each sample, and for each group of 5 the upper and lower values were removed. An average of the middle three values was then used for further analysis. Translation of the  $\beta$ -globin control construct was set to 1 and all other average values were normalized to that. Error bars represent the standard deviation from the mean in all cases, and statistical significance was calculated using Student's T test. All statistical analysis and chart generation was performed in Microsoft Excel.

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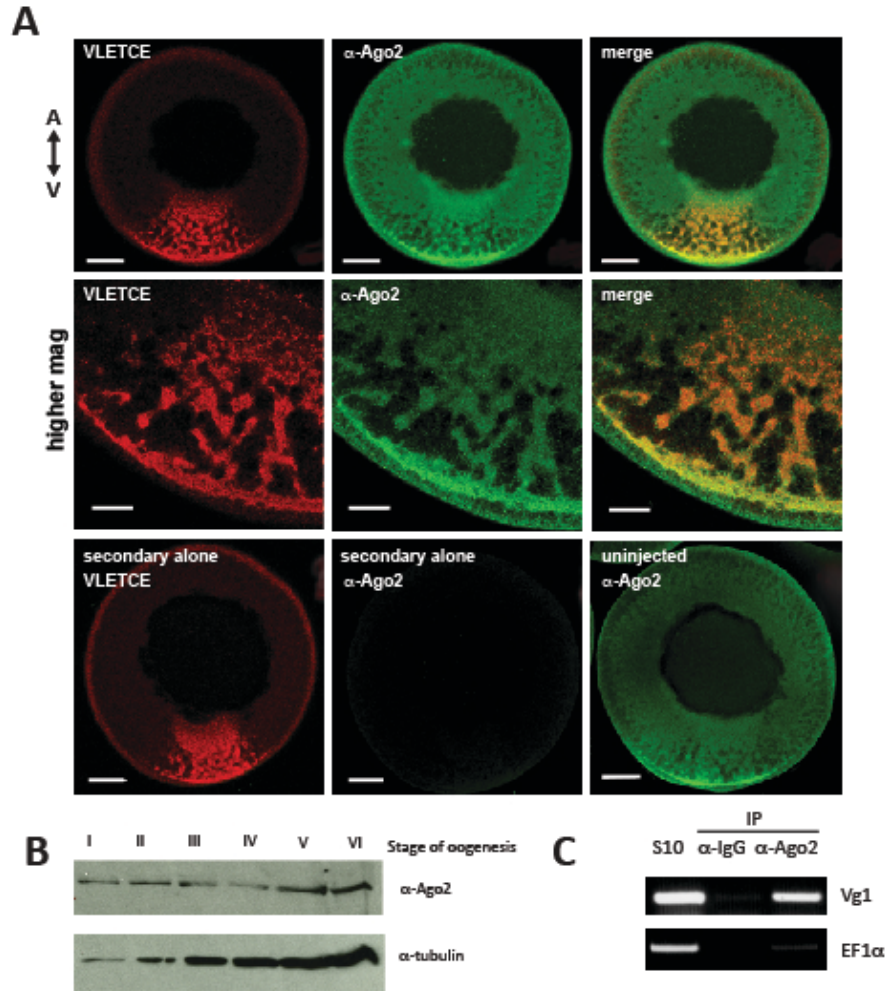
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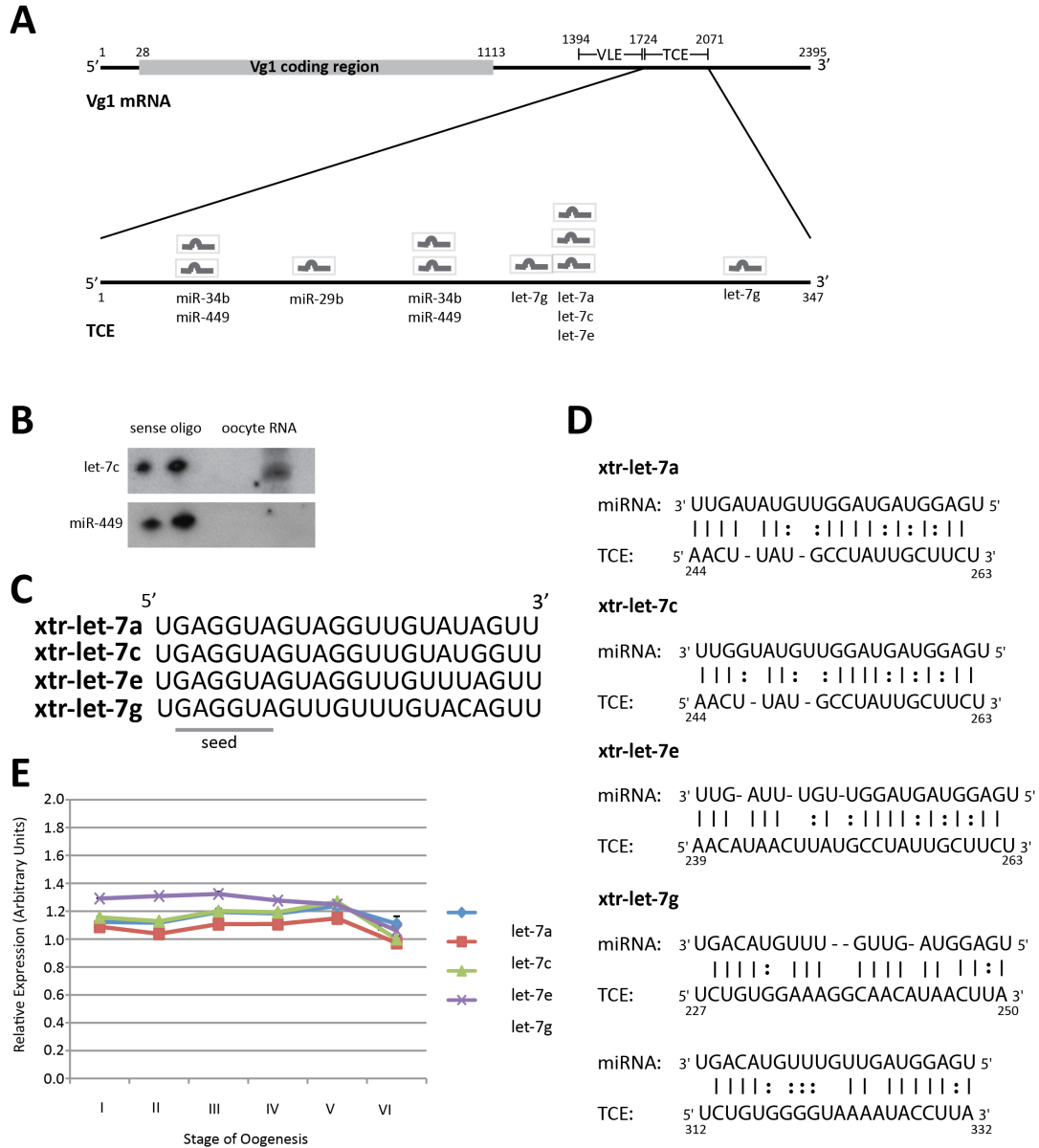
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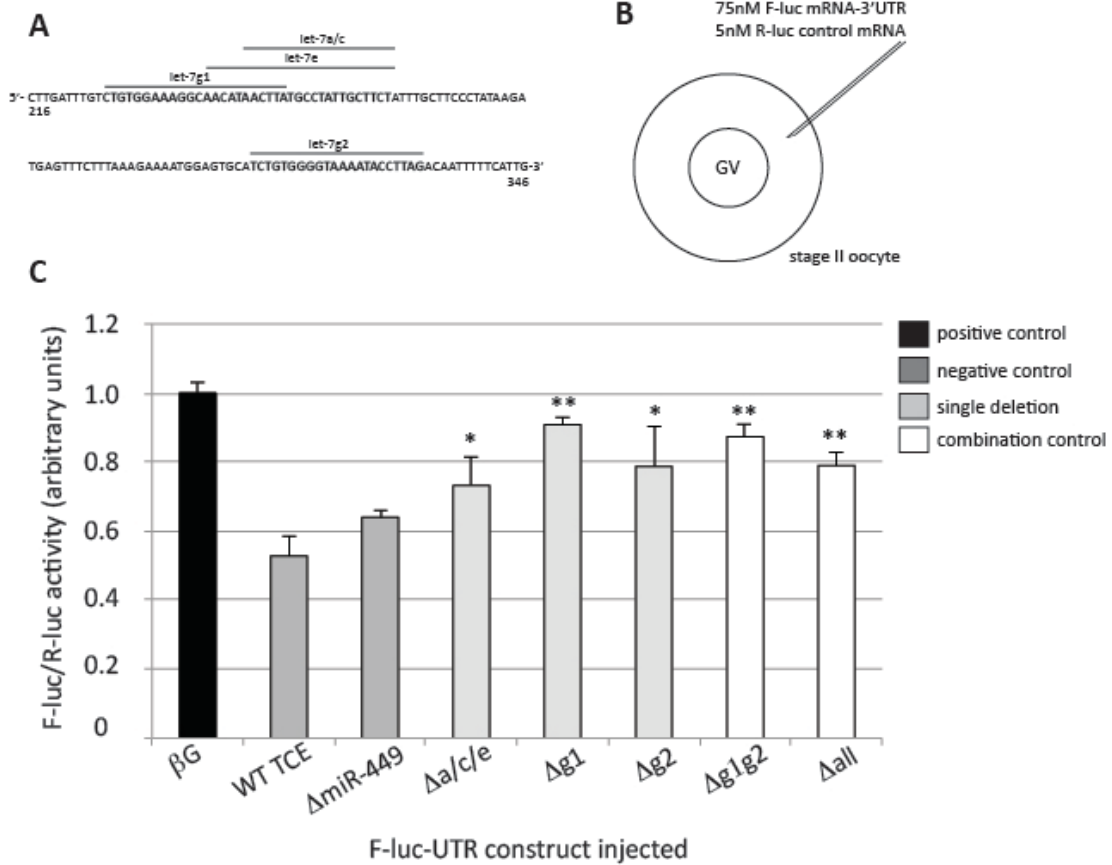




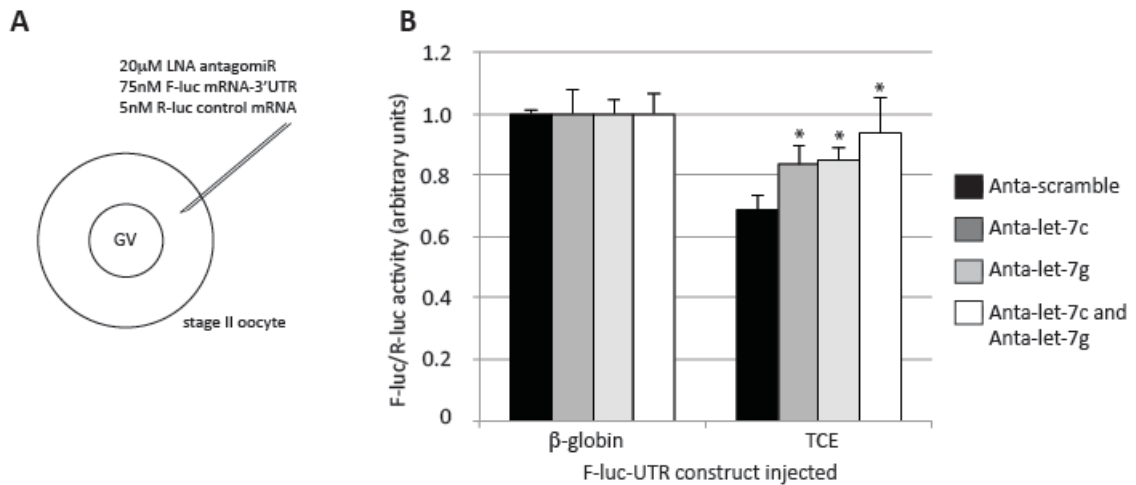
**Figure 1 The Vg1 RNP is associated with Ago in *Xenopus* oocytes.** **A.** Ago co-localizes with VLETCE RNA at the vegetal pole. Alexa-labelled VLETCE RNA was synthesized in vitro and injected into stage III oocytes. After allowing localization to proceed for 16 hours cells were fixed, permeabilized, and incubated with anti-Ago2 primary antibody overnight. Scale bars represent 50μm, or 20μm in zoomed images. **B.** Ago is expressed throughout oogenesis. Ago expression was assayed by immunoblot using an antibody against tubulin as a loading control. 30 oocyte equivalents were loaded per lane. **C.** Ago is associated with Vg1 mRNA. By IP-RT-PCR. Vg1 is specifically associated with Ago in stage III oocytes, but not with the control mRNA EF1α. S10 lane corresponds to 10% of the input RNA generated from 100 oocytes.



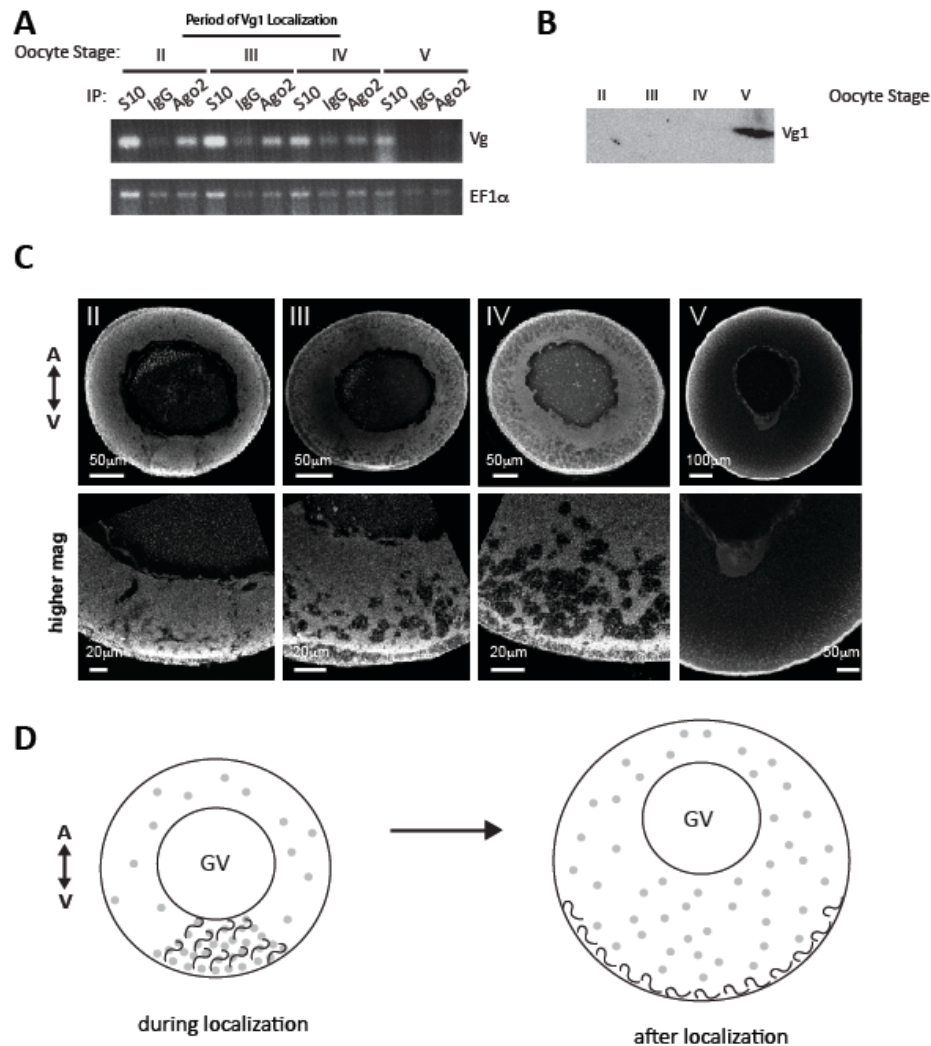
**Figure 2 The Vg1 translational control element (TCE) contains multiple conserved miRNA binding sites.** **A.** Schematic showing the distribution of conserved miRNA target sites predicted using the computational algorithm miRanda (Enright *et al.* 2003). Two 3'-UTR elements are shown; the vegetal localization element (VLE) and the translational control element (TCE). Nucleotide number relative to the 5' end of the mRNA is shown. On the enlarged TCE, numbering begins at the 5' end of the element and predicted target sites conserved in three species of frog are shown. **B.** Northern blot analysis of oocyte RNA. Oocyte RNA was prepared by hot phenol extraction followed by ethanol precipitation from a mixed population of oocytes. The RNA was then run on an 11% urea gel, transferred to a nylon membrane, and probed with a  $^{32}\text{P}$  end-labelled DNA oligo. **C.** An alignment of the four let-7 family members with putative target sites in the Vg1 TCE. **D.** miRanda interaction predictions between the *X. laevis* TCE and *X. tropicalis* miRNA sequences. Canonical base-pairing is depicted with solid lines, and non-canonical G:U base-pairing is represented as colons. **E.** TaqMan (Applied Biosystems) assay for let-7 family expression. To control for oocyte size across oogenesis (x-axis) the expression levels of each miRNA are shown relative to U6 snRNA expression on an arbitrary unit y-axis.



**Figure 3 Putative let-7 binding sites are required for translational repression of a reporter mRNA.** **A.** A segment of the Vg1 TCE is shown and the predicted let-7 target sequences are labeled and shown in bold text. Nucleotide numbering is relative to the 5' end of the TCE. Each site was deleted individually ( $\Delta$ miR-449,  $\Delta$ a/c/e,  $\Delta$ g1,  $\Delta$ g2) or in combination ( $\Delta$ g1 $\Delta$ g2,  $\Delta$ all). **B.** Stage II oocytes were co-injected with the mRNAs indicated. Firefly luciferase (F-Luc) constructs contain a downstream 3'-UTR containing a  $\beta$ -globin ( $\beta$ G) sequence, WT TCE sequence, or TCE sequence with the indicated miRNA-binding site deleted. Renilla luciferase (R-luc) constructs are not regulated in this system, and as such behave as an internal control for translation efficiency. After injection, oocytes were cultured over night in oocyte culture medium (OCM). Approximately 18 hours after injection oocytes were lysed in groups of ten and luciferase activity was assayed using a dual luciferase assay system (Promega). **C.** A representative experiment is shown. \* Corresponds to a P-value <0.05, \*\* corresponds to a P-value <0.005 as calculated using Student's T-test relative to the wt (TCE) repressed state, where  $n=30$  oocytes for each bar.  $\beta$ G expression is set to 1 and all other values are shown relative to that.



**Figure 4 Let-7c is required for TCE-dependent translational repression.** **A.** F-luc (150nM) and R-luc (5nM) mRNAs were prepared and co-injected with the indicated antagomiR antisense LNA (Exiquon) into stage III oocytes. Oocytes were incubated overnight and luciferase activity was assayed as before. **B.**  $\beta$ -globin activity was set to 1 and TCE activity is shown relative to that. A representative experiment is shown. \* Corresponds to a P-value <0.05 as calculated using Student's T-test relative to the TCE plus anta-scramble LNA, and  $n=30$  oocytes for each bar.



**Figure 5 Vg1 is associated with Ago in a developmentally regulated fashion.** **A.** Vg1 is biochemically associated with Ago during localization, but is released once the mRNA is anchored at the vegetal cortex. RNA-IP experiments were performed as in Figure 1 on lysates prepared from stages II-V of oogenesis. S10 refers to 10% of the input RNA. **B.** Vg1 protein expression across oogenesis. 20 oocyte-equivalents were loaded of each indicated stage. **C.** Ago localization correlates with a role in endogenous Vg1 translational control. Oocytes from stages II-V were fixed and stained with anti-human Ago2 (2A8) antibody as in Figure 1A. The bottom row of images depicts a higher magnification view of the vegetal cytoplasm of the same oocyte shown above. **D.** A putative model of the mechanism of miRISC-mediated translational repression of Vg1, in which the miRISC (grey circles) is recruited to Vg1 mRNA (black lines) during localization but is released once the transcript reaches the vegetal cortex.

## **Chapter 3: Preparation of a highly active cell-free translation system from immature *Xenopus laevis* oocytes**

**Catherine A. Pratt** and Kimberly L. Mowry

[Preparation of a highly active cell-free translation system from immature \*Xenopus laevis\* oocytes](#)

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## **Abstract**

Understanding mechanisms of post-transcriptional control of gene expression has come under much scrutiny in recent years. A key question in this field is how the translation of specific mRNAs is activated or repressed both spatially and temporally in a given cell. In oocytes of the frog *Xenopus laevis* a number of mRNAs are localized early in oogenesis and subsequently translated at later stages. We have developed a highly active cell-free translation system from oocytes in the early stages of oogenesis that is applicable to the study of translation and translational control of both endogenous and exogenous mRNAs.

## Introduction

The study of eukaryotic translation has been greatly aided by the generation of cell-free systems that are based upon a variety of organisms and tissues (Beckler *et al.* 1995; Van Herwynen and Beckler 1995; Castagnetti *et al.* 2000). In addition, *in vitro* translation systems have been reconstituted from purified components of both *Saccharomyces cerevisiae* and rabbit reticulocytes (Acker *et al.* 2007; Pisarev *et al.* 2007). These systems offer unique advantages to the study of translation: while *in vitro* systems reconstituted from purified factors have been extensively used to detail the mechanisms underlying general translation (Pisarev *et al.* 2007; Fan-Minogue *et al.* 2008), cell-free systems have been extremely useful in analyzing cell-specific gene expression at the level of translation (Castagnetti *et al.* 2000; Thermann and Hentze 2007). For example, regulatory components present in germ cells are likely absent or modified in somatic tissues, and *vice versa*.

Cell-free systems from eggs and fully grown oocytes (stage VI; Dumont 1972) of the frog *Xenopus laevis* have been widely used to study a variety of cellular processes including translation, translocation of secretory proteins, nuclear envelope assembly, DNA replication and repair, and cell cycle (Lohka and Masui 1983; Patrick *et al.* 1989; Matthews and Colman 1991; Murray 1991). The wide use of *Xenopus* oocytes and eggs for the preparation of these systems stems in part from the ease of acquisition of large amounts of material from these animals. A single female frog can yield thousands of oocytes that, due to their large size (50-1300  $\mu\text{m}$  in diameter), can be easily sorted by stage (I-VI; (Dumont 1972)).



During oogenesis, numerous maternal mRNAs are stored within the oocyte in preparation for fertilization and early embryogenesis when the zygotic genome is transcriptionally inactive (Woodland *et al.* 1979; Dworkin *et al.* 1985). The production of protein from these mRNAs is stringently regulated, since all cellular processes in the early stages of embryogenesis are reliant on maternally loaded transcripts and proteins. In view of the fact that these mRNAs are stored in the oocyte but not translated until after maturation of the fully-grown oocyte or upon fertilization of the egg, they must be maintained in a translationally silent, or “masked” state. A number of studies have elucidated the mechanisms underlying the translational regulation of key mRNAs in the late stages of oogenesis (Stebbins-Boaz *et al.* 1999; Otero *et al.* 2001), and this work has been much assisted by the *in vitro* translation systems developed for eggs and fully-developed *Xenopus* oocytes (Patrick *et al.* 1989).

A number of mRNAs in *Xenopus* oocytes are localized early in oogenesis (stages I-III; Melton 1987; Zhou and King 1996). mRNA localization results in a local enrichment of a particular mRNA, and ultimately of the encoded protein, and this spatial restriction of translation is crucial for the development of the embryo (Birsoy *et al.* 2006). It is therefore critical that no protein be produced from these mRNAs until they have reached their cellular destination. The mechanisms controlling translation of localized mRNAs in early oogenesis remain largely unknown, although a number of *cis*- and *trans*-acting factors have been identified through *in vivo* injection

approaches (Otero *et al.* 2001; Colegrove-Otero *et al.* 2005). In order to conduct mechanistic analyses of translational control mechanisms acting during early oogenesis, we have developed a cell-free translation system from stage I-III *Xenopus* oocytes. The lysate produced using this protocol is highly active, and can translate an exogenously supplied reporter mRNA with an efficiency equal to or greater than the commercially available rabbit reticulocyte lysate system. Given the high activity of this oocyte lysate and the ready availability of large amounts of *Xenopus* oocytes, this cell-free translation system represents a useful tool both for general studies of translational regulation and for understanding mechanisms of translational control in oocytes.

## **Description of Method**

### ***Isolation of oocytes***

For the preparation of an active translation lysate from stage I-III oocytes, it is necessary to isolate a large number of oocytes from the early stages of oogenesis. First, the entire ovary of a young *Xenopus laevis* female (6.25-7.5 cm juveniles; Nasco LM00714MX) is surgically removed (<http://www.xlaevis.com/oocyte.html>) and placed in MBSH buffer [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 0.82 mM MgSO<sub>4</sub>•7H<sub>2</sub>O, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>•4H<sub>2</sub>O, 0.41 mM CaCl<sub>2</sub>•6H<sub>2</sub>O, 10 mM HEPES (pH 7.6)]. The ovaries of frogs of this age contain predominately oocytes in the early stages of oogenesis, and therefore the majority of the harvested ovary can be used to generate translation lysate (see section 2.1.2). While older frogs can be used, it should be noted that more than one may be required to yield sufficient oocytes from

stages I-III of oogenesis, and the task of removing older oocytes will be more laborious.

Next, the ovary is cut into small pieces and defolliculated by incubation in a solution of collagenase [3 mg/ml collagenase (Sigma-Aldrich # C0130), 0.1 K<sub>2</sub>HPO<sub>4</sub> (pH 7.4)] for 15 minutes at 18°C with gentle shaking (~200 rpm). At this point, the oocytes should be liberated from the ovary. If necessary, however, the incubation time can be increased by an additional five minutes. The defolliculated oocytes are then washed three times with MBSH buffer. To remove oocytes from the later stages of oogenesis (late stage IV, stages V and VI), the oocytes are passed through a 500 µm sieve. The sieve is constructed from nylon mesh (Small Parts Inc. #CMN-0500-B) inserted into a circular hole cut into the lid of a plastic petri dish (Fig. 1). Some early stage IV oocytes may pass through the sieve, and these can be manually removed to yield a population of stage I-III oocytes (Fig. 1). The isolated stage I-III oocytes should occupy a volume of 100 µl or more when settled in a 1.5 ml microcentrifuge tube to ensure effective lysate production (see section 2.1.2).

### ***Preparation of oocyte lysate***

The ultimate outcome of the method described in this section is the production of a concentrated, translationally active oocyte lysate (Fig. 1). It is critical that the entire protocol be performed at 0-4°C, either by maintaining all components on ice or conducting the protocol in a cold room. In addition, all glassware, tips and tubes should be sterilized by autoclaving, and care should be taken to avoid any RNase

contamination. Approximately 30 minutes before beginning the oocyte lysis procedure (below) the Hypotonic Lysis Buffer [HLB; 10 mM HEPES-KOH (pH 7.5), 15 mM KCl, 1.5 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>, 2 mM DTT, 0.5 mM Pefabloc (Roche Diagnostics # 1429876)] should be prepared and chilled on ice. Aliquots of this buffer can be made in advance without DTT and Pefabloc, and stored at -20°C for up to six months. However, the DTT and Pefabloc should always be added fresh.

Place the stage I-III oocytes isolated above (section 2.1.1) in a 1.5 ml microcentrifuge tube and estimate the settled volume (at least 100 µl). Wash the oocytes twice with five volumes of cold HLB, allowing them to settle fully after each wash by placing the tube on ice for 2-5 minutes. Before the last wash, the oocytes in HLB should be transferred to a tightly fitting 1-2 ml dounce (Wheaton # 358029 or similar). After washing the oocytes, remove all the HLB with a drawn off Pasteur pipette and replace with one volume of HLB.

To lyse the oocytes use no more than three slow passes with the dounce. Excessive douncing is detrimental and will reduce the translational activity of the resulting lysate. The oocyte homogenate is then transferred to a chilled 1.5 ml microcentrifuge tube and centrifuged at  $10,000 \times g$  for 10 minutes at 4°C. After centrifugation the supernatant is carefully removed into a fresh chilled 1.5 ml microcentrifuge tube using a clean drawn off Pasteur pipette. Care should be taken to avoid the thin lipid and yolk layer that forms on top of the supernatant. This supernatant comprises the Xenopus Translation Lysate (XTL).

The translational activity of XTL as prepared in section 2.1.2 can vary from batch to batch, with variables including oocyte health and technical proficiency. For this reason it is advisable to test the activity of the lysates before they are used for further experiments, either by [<sup>35</sup>S]-methionine incorporation (Fig. 2A) or by luciferase assay (Fig. 2B; section 2.2.1). For assaying [<sup>35</sup>S]-methionine incorporation, the following reaction is set up in triplicate for each time point:

---

|        |                                                                      |
|--------|----------------------------------------------------------------------|
| 6 µl   | XTL                                                                  |
| 0.6 µl | CTX [1M KCl, 16 mM Mg(CH <sub>3</sub> COO) <sub>2</sub> , 20 mM DTT] |
| 1 µl   | CPK [200 mM creatine phosphate, 4 mg/ml creatine kinase (Sigma)]     |
| 0.2 µl | amino acid mix minus Met (Promega)                                   |
| 10 µCi | [ <sup>35</sup> S]-methionine (EasyTag, Perkin Elmer)                |
| 0.5 µl | recombinant RNasin (Promega)                                         |
| 1 µl   | DEPC-dH <sub>2</sub> O                                               |

---

To stop the reaction, 1 ml of ice cold 5% TCA is added. The reactions are then held on ice until the time course is complete. [<sup>35</sup>S]-methionine incorporation is assayed by binding to 24 mm glass microfibre filters (Whatman GF/A). After binding of the precipitated reaction, each filter is washed 10 times with 1 ml ice cold 5% TCA and dried. The filters are placed into individual scintillation vials with 3 ml scintillation fluid and counted.

If necessary, XTL can be flash frozen in liquid nitrogen and stored in aliquots at -80°C, but a reduction in translation efficiency (20-50 %) is usually observed after

only a single freeze-thaw cycle. Protein concentration should be determined using a Bradford Assay (Bio-Rad Protein Assay substrate # 500-0006). Optimal XTL translation activity requires a total protein concentration of at least 20 mg/ml. Activity of the lysate decreases dramatically below this concentration.

### ***Constructs for production of luciferase reporter mRNAs***

XTL can be programmed with any mRNA to generate the encoded protein. Of note, it is particularly tractable for quantitatively assaying translation from a firefly luciferase reporter gene. For this, we have generated the series of constructs that are diagrammed in Figure 3. pSP64-X $\beta$ LucX $\beta$  contains the firefly luciferase gene flanked by *Xenopus*  $\beta$ -globin 5'- and 3'-untranslated region (UTR) sequences (Fig. 3A). The  $\beta$ -globin 5'- and 3'-UTRs can be replaced with other UTR sequences of interest to test for a potential function in translation regulation using the 5' or 3' tester constructs pSP64-LucX $\beta$  and pSP64-X $\beta$ Luc (Figs. 3B, 3C). Each of these luciferase reporter plasmids contains an SP6 transcriptional promoter to allow for *in vitro* mRNA production (see section 2.1.4).

The dual luciferase assay (section 2.2.2) also makes use of an internal control in the form of the Renilla luciferase mRNA. The Renilla luciferase gene is available in the pRL-null plasmid from Promega (# E2271). This plasmid contains a T7 bacteriophage transcriptional promoter, and thus control reporter mRNA can be generated in a similar manner as from pSP64-X $\beta$ LucX $\beta$  (see section 2.1.4).

### ***Preparation of mRNA***

In order to generate capped mRNA for *in vitro* translation, template DNA is first linearized using an appropriate restriction enzyme to allow for runoff transcription (Melton *et al.* 1984; Yisraeli and Melton 1989). In the case of the pSP64-Luc plasmids (Fig. 3) EcoRI is used, and BamHI is used for pRL-null (Promega). The linear DNA is purified and concentrated by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation. The template DNA is resuspended in nuclease-free deionized water (dH<sub>2</sub>O). Nuclease-free dH<sub>2</sub>O (DEPC-dH<sub>2</sub>O) can be prepared as follows: 100ml dH<sub>2</sub>O is incubated for 30 minutes with 1-2 drops DEPC (Sigma-Aldrich), swirled, and autoclaved. *In vitro* transcription is carried out using a suitable (SP6 or T7, depending on the promoter sequence present in the template DNA) mMessage mMachine *in vitro* transcription kit (Ambion). RNA yield from all templates is quantified by incorporation of [ $\alpha$ -<sup>32</sup>P]UTP (1 $\mu$ Ci/ $\mu$ l, Perkin Elmer) according to the manufacturers instructions. The resulting mRNA is diluted to 500 nM and can be stored in small (~5  $\mu$ l) aliquots at -80°C for several months.

### **Using XTL to analyze translation of exogenous RNAs**

#### ***The in vitro translation assay***

XTL can be effectively and quantitatively used to assay translation from reporter mRNAs in the following way. RNAs used to program translation in XTL must first be incubated at 70°C for three minutes to disrupt any secondary structure, and placed

on ice until added to the reactions. The translation reactions are set up at room temperature as follows:

---

|        |                                                                      |
|--------|----------------------------------------------------------------------|
| 6 µl   | XTL                                                                  |
| 0.6 µl | CTX [1M KCl, 16 mM Mg(CH <sub>3</sub> COO) <sub>2</sub> , 20 mM DTT] |
| 1 µl   | CPK [200 mM creatine phosphate, 4 mg/ml creatine kinase (Sigma)]     |
| 0.1 µl | amino acid mix minus Leu (Promega)                                   |
| 0.1 µl | amino acid mix minus Met (Promega)                                   |
| 0.7 µl | DEPC-dH <sub>2</sub> O                                               |
| 0.5 µl | recombinant RNasin (Promega)                                         |
| 1 µl   | luciferase reporter RNA [75nm (see section 2.1.4)]                   |

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For comparison of activity (see Fig. 2B), translation of reporter RNAs can also be performed using commercially available rabbit reticulocyte lysate (RRL; Promega # L4960), according to the manufacturers guidelines. Translation is allowed to proceed for 90 minutes at room temperature (XTL) or at 30°C (RRL). Luciferase activity is assayed as in section 2.2.2 to quantify translation efficiency.

### ***The luciferase assay***

Firefly luciferase activity can be accurately measured upon addition of enzyme substrate (reviewed in Kain and Ganguly 2001). We routinely use commercially available reagents (Promega Luciferase Assay System # E1500) to measure luciferase activity. For the Dual-Luciferase™ assay, where Renilla mRNA is used as an internal control (see below), we use a similar system (Promega # E1910). Of



note, due to the high efficiency of translation in XTL, the substrates provided in these kits (LARII and Stop and Glo®) should be diluted 1:2.5 into dH<sub>2</sub>O prior to use. 5 µl of the translation reaction (see 2.2.1) is added to 25 µl diluted LARII (firefly luciferase substrate) in a 12x75 mm glass tube (Corning #99445-12) and light emission is measured on a Lumat 9501 luminometer for 5 seconds. As an internal control for translation in each assay, mRNA encoding the Renilla luciferase gene (see sections 2.1.3 and 2.1.4) can be used. For the Dual-Luciferase™ assay, each translation reaction therefore contains 5 nM Renilla luciferase mRNA in addition to 75 nM firefly luciferase reporter mRNA. Since each luciferase enzyme utilizes a different substrate, the activity of both can be measured in a single sample. After measuring the firefly luciferase activity, the reaction is removed from the luminometer, 25 µl diluted Stop and Glo® (Renilla luciferase substrate) is added, and the tube is replaced in the luminometer to measure Renilla luciferase activity. A ratio of firefly:Renilla luciferase activity is then generated to assess the levels of translation from different reporter constructs. Due to the sensitivity of the assay, small errors in pipetting accuracy can drastically affect the final readout. We therefore perform five individual replicates of each reaction to generate accurate, reproducible data.

### ***Testing putative translational control RNA elements***

In a variety of different cell types in eukaryotic organisms, mRNAs are known to be translationally regulated (Besse and Ephrussi 2008). Frequently this regulation is controlled by elements contained in the 5'- and/or 3'-UTRs, and can result in either

the activation or repression of translation (de Moor *et al.* 2005; Pickering and Willis 2005). Understanding the mechanisms of UTR-mediated translational control is a complex problem, and there have been many studies in recent years that focus on this question (reviewed in Besse and Ephrussi 2008). The XTL system provides a useful tool for teasing apart mechanisms of translational control, in particular with regards to the myriad mRNAs that are regulated in early oogenesis (Melton 1987; Zhou and King 1996). In order to test a UTR in this system, the putative regulatory sequence(s) can be inserted into either the pSP64-LucX $\beta$  (Fig. 3B) or pSP64-X $\beta$ Luc (Fig. 3C) constructs using the indicated restriction sites. mRNA generated through *in vitro* transcription, as described in section 2.1.4, is assayed as in section 2.2.2. Translation from pSP64-Luc constructs containing UTR sequences of interest, potentially including engineered mutations, can then be compared to pSP64-X $\beta$ LucX $\beta$  as a positive control since the  $\beta$ -globin 5' and 3' UTR sequences present in pSP64-X $\beta$ LucX $\beta$  (Fig. 3A) are unregulated in this system. Similarly, overexpression or add-back of putative *trans*-acting factors into this system can be used to assay function in the same manner, however care must be taken to avoid dilution of the XTL.

### **Concluding Remarks**

We have described the preparation of XTL, a cell-free translation system using oocytes from the early stages of oogenesis in the frog, *Xenopus laevis* (Fig. 1). The XTL system can be used to assay translation from an exogenously added reporter mRNA (Figs. 2, 3), and to analyze regulatory elements in the 3'UTR of similar

reporters. The translation efficiency of XTL is extremely high, and is comparable with commercially available *in vitro* translation systems (Fig. 2). Previously developed systems from *Xenopus* eggs and oocytes, while supporting the translation of endogenous mRNAs, are reportedly deficient in the translation of exogenous transcripts (Patrick *et al.* 1989). This may reflect a difference in initiation efficiency between oocytes early in oogenesis versus their more mature counterparts, or may be a result of the differing methods of lysate production. It is unclear which of these possibilities may be responsible, since our protocol is unsuitable to produce active translation lysates from late oocytes (data not shown). Similarly, the established protocols (Patrick *et al.* 1989) for generation of translation lysates from late stage oocytes cannot be used to produce active lysates from stage I-III oocytes (data not shown). The XTL translation lysate has also been used in our lab to study the translational status of endogenous transcripts by sucrose density centrifugation (not shown), further emphasizing the versatility of this extract. Of particular interest is the ability to dissect mechanisms of translational control that are acting on localized mRNAs early in oogenesis. This *in vitro* system can complement the array of *in vivo* experiments already in place in the *Xenopus* oocyte model system.

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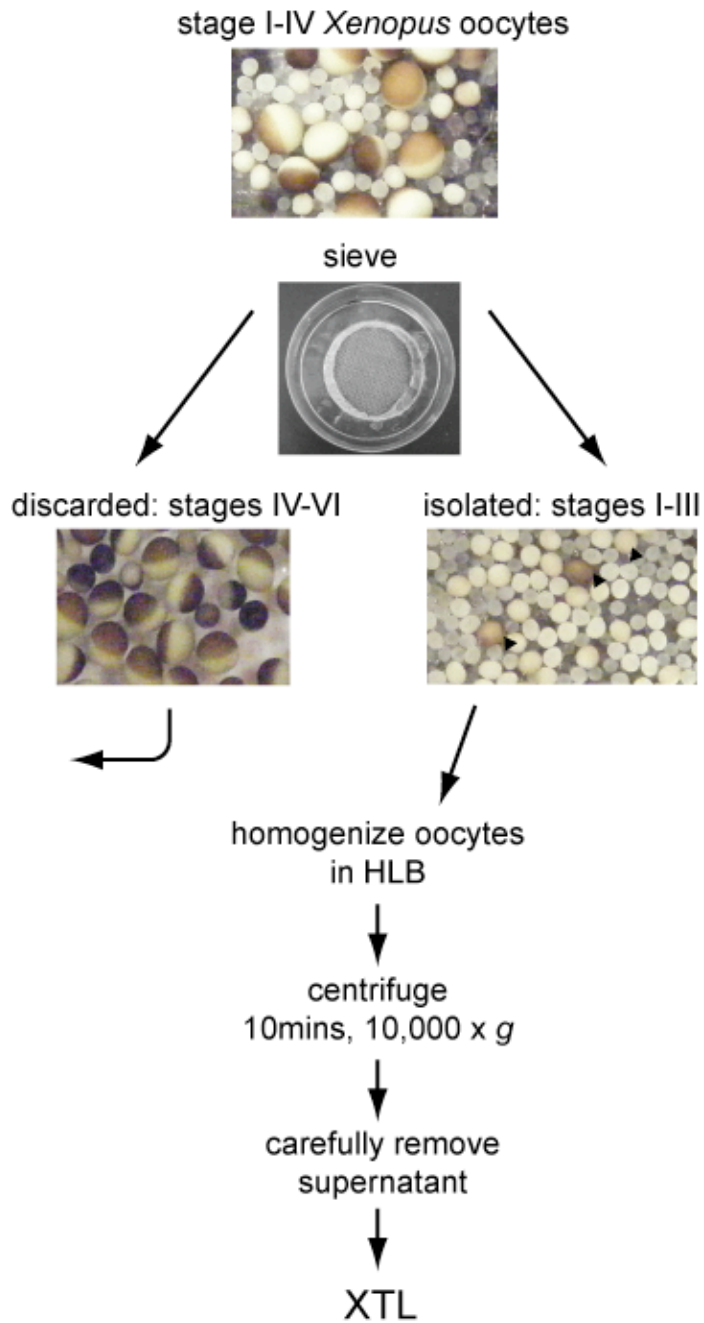
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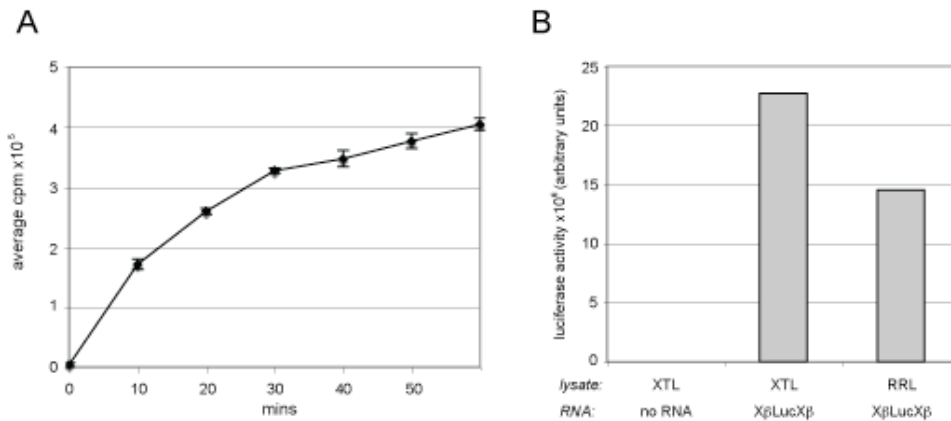
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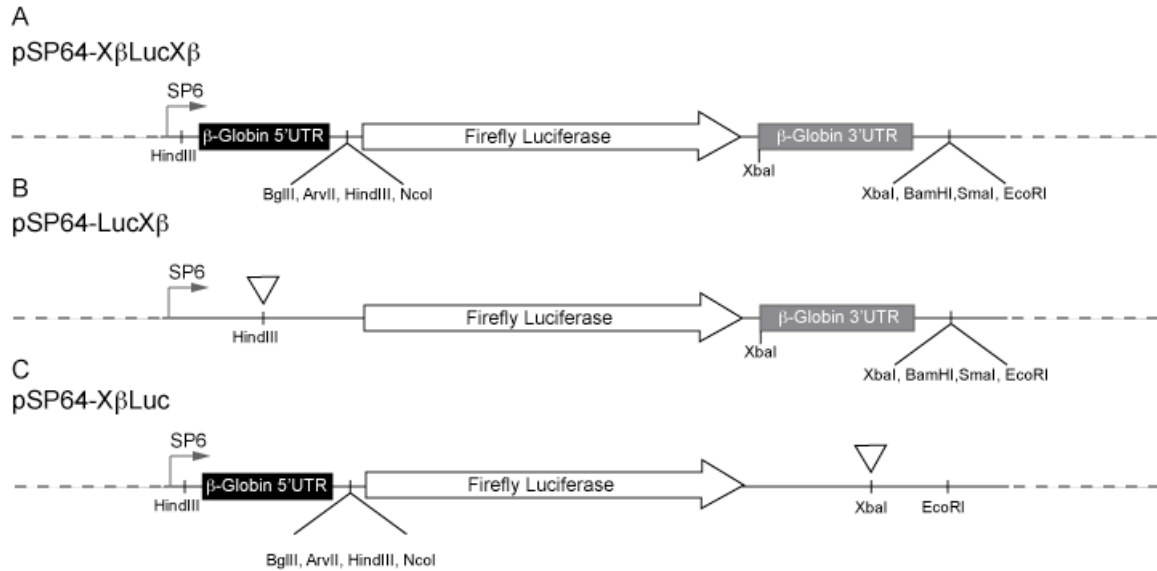


**Figure 1. Procedure for preparation of a translationally active oocyte lysate (XTL).** Stage I-III oocytes are separated from stage IV-VI oocytes by passage through a 500  $\mu\text{m}$  sieve, followed by manual removal of any pigmented oocytes that pass through the sieve (black arrow heads). Stage I-III oocytes are washed into a hypotonic lysis buffer (HLB), disrupted by dounce homogenization, and centrifuged to remove cellular debris. The supernatant (XTL) is removed and used for in vitro translation.



**Figure 2. Activity of the XTL translation lysate.** **A.** Time course of incorporation of [<sup>35</sup>S]-methionine into protein using XTL prepared as described in section 2.1.2. Translation assays were performed as described in section 2.1.2. At each time point, incorporation of the radiolabel was measured by TCA precipitation of a 10  $\mu$ l reaction and subsequent binding to a 24 mm GF/A Whatman glass microfiber filter. [<sup>35</sup>S]-methionine incorporation (cpm, y-axis) is plotted against time (minutes, x-axis). **B.** Translation efficiency of the *Xenopus* translation lysate (XTL) was compared to that of Rabbit reticulocyte lysate (RRL). XTL and RRL were programmed with RNA transcribed from pSP64-XLucX $\beta$  (Fig. 3A), and incubation of XTL without RNA (no RNA) served as a negative control. Analysis of luciferase activity demonstrates that XTL translation activity is greater than that of the commercially available (Promega) RRL.





**Figure 3. Schematic of pSP64-Luc reporters for testing UTR-mediated translation regulation.**  
**A.** pSP64-XβLucXβ contains the firefly luciferase gene (white arrow) flanked by *Xenopus* β-globin 5' (black) and 3' (gray) untranslated region (UTR) sequences. The positions of key restriction sites are marked. **B.** 5' UTR sequences of interest can be inserted into the HindIII site (marked by a white arrowhead) of pSP64-LucXβ. **C.** 3' UTR sequences of interest can be inserted into the XbaI site (marked by a white arrowhead) of pSP64-XβLuc. For the production of run-off transcripts all plasmids (A-C) are linearized with EcoRI and transcribed using SP6 RNA polymerase; the SP6 promoter regions are marked at the left.

## **Chapter 4: Multiple Kinesin Motors Coordinate Cytoplasmic RNA Transport on a Sub-population of Microtubules in *Xenopus* Oocytes**

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*I contributed Figure 1D and Figures 5E and 5F*

[Multiple kinesin motors coordinate cytoplasmic RNA transport on a subpopulation of microtubules in \*Xenopus\* oocytes.](#)

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## Abstract

RNA localization is a widely conserved mechanism for generating cellular asymmetry. In *Xenopus* oocytes, microtubule-dependent transport of RNAs to the vegetal cortex underlies germ layer patterning. Although kinesin motors have been implicated in this process, the apparent polarity of the microtubule cytoskeleton has pointed instead to roles for minus end-directed motors. To resolve this issue, we have analyzed participation of kinesin motors in vegetal RNA transport and identified a direct role for *Xenopus* kinesin-1. Moreover, *in vivo* interference and biochemical experiments reveal a key function for multiple motors, specifically kinesin-1 and kinesin-2, and suggest that these motors may interact during transport. Critically, we have discovered a sub-population of microtubules with plus ends at the vegetal cortex, supporting roles for these kinesin motors in vegetal RNA transport. These results provide a new mechanistic basis for understanding directed RNA transport within the cytoplasm.

## Introduction

Localization of mRNAs within cells is a powerful mechanism for generating cell and developmental polarity (reviewed in Du *et al.*, 2007). In somatic cells, localized mRNAs can influence both cell motility and morphology, as exemplified by localization of  $\beta$ -actin RNA in fibroblasts (reviewed in Condeelis and Singer, 2005). In many organisms, localization of maternal mRNAs in eggs and oocytes provides the basis for embryonic patterning. For example, in *Drosophila melanogaster*, localized mRNAs are necessary for anterior-posterior and dorsal-ventral patterning of the oocyte and developing embryo (reviewed in Minakhina and Steward, 2005). Among vertebrates, Vg1 mRNA is a prominent example of a localized maternal mRNA that plays a role in embryonic patterning (reviewed in King *et al.*, 2005). Vg1 mRNA, which encodes a member of the TGF- $\beta$  family of growth factors (Weeks and Melton, 1987), is localized to the vegetal hemisphere in oocytes of the frog, *Xenopus laevis*. Spatially appropriate expression of Vg1 is necessary for proper mesoderm and endoderm specification during embryonic development (Dale *et al.*, 1993; Thomsen and Melton, 1993; Birsoy *et al.*, 2006), and transport of Vg1 mRNA to the vegetal hemisphere during oogenesis initiates this process. However, mechanistic insight into the transport process itself is lacking.

Localization of Vg1 mRNA occurs during mid-oogenesis, and relies on a 340-nucleotide element in the 3'-UTR of Vg1 mRNA termed the Vg1 Localization Element (VLE; Mowry and Melton, 1992). The VLE is sufficient to direct vegetal RNA localization and associates with specific proteins to form a vegetal

ribonucleoprotein (RNP) complex ( Mowry, 1996; Deshler *et al.*, 1998; Havin *et al.*, 1998; Cote *et al.*, 1999; Kress *et al.*, 2004; Yoon and Mowry, 2004; Czaplinski and Mattaj, 2006). Notably, one component of the Vg1 RNP, *Xenopus* Staufen, has been shown to interact with the molecular motor kinesin-1 (Yoon and Mowry, 2004). An active transport mechanism for Vg1 mRNA localization was first suggested by pharmacological experiments in which depolymerization of the microtubule cytoskeleton abolished Vg1 localization (Yisraeli *et al.*, 1990). More recently, vegetal localization of Vg1 mRNA was shown to be disrupted by blocking the function of another molecular motor, kinesin-2 (Betley *et al.*, 2004). It is not yet clear whether either of these kinesin motors function directly in vegetal RNA transport.

The kinesins are a large superfamily of motor proteins containing 14 classes (reviewed in Miki *et al.*, 2005). The founding member of the kinesin superfamily, conventional kinesin, or kinesin-1, consists of two identical heavy chains, containing the motor domain responsible for ATP hydrolysis and microtubule binding, and two identical light chains, necessary for cargo binding ( Yang *et al.*, 1989; Diefenbach *et al.*, 1998). By contrast, kinesin-2 is a heterotrimeric motor protein, containing two non-identical motor subunits and a single non-motor accessory protein (Cole *et al.*, 1993; Wedaman *et al.*, 1996).

Kinesin function has been implicated in RNA transport in diverse systems, from oocytes to neurons (reviewed in Hirokawa, 2006; Bullock, 2007). For kinesin-1, specific examples include transport of CaMKII $\alpha$ , Arc, and Tau RNAs in mammalian

neurons (Aronov *et al.*, 2002; Ohashi *et al.*, 2002; Kanai *et al.*, 2004), localization of myelin basic protein mRNA in oligodendrocytes (Carson *et al.*, 1997), and transport of oskar mRNA in the *Drosophila* oocyte (Brendza *et al.*, 2000). However, direct roles for kinesin motors are, in many cases, still unclear. In the *Drosophila* oocyte for example, kinesin has been suggested to play an indirect role through regulating cytoplasmic flows or exclusion of RNA from the oocyte cortex (Glotzer *et al.*, 1997; Cha *et al.*, 2002; Palacios and St Johnston, 2002). In addition, kinesin could act in RNA localization through interactions with other motor proteins (Duncan and Warrior, 2002; Januschke *et al.*, 2002; Mische *et al.*, 2007).

Microtubules in the *Xenopus* oocyte are largely oriented with their minus ends towards the cell periphery (Pfeiffer and Gard, 1999). Thus, the oocyte cortex is rich in minus ends, and roles for minus end-directed motors in vegetal RNA transport have been postulated (Kloc and Etkin, 2005; St Johnston, 2005; Palacios, 2007). Both kinesin-1 and kinesin-2 are plus end-directed motors (reviewed in Vale, 2003), complicating models for vegetal RNA transport that rely on direct participation by such motors.

We have investigated the function of kinesin motors in vegetal RNA transport in the *Xenopus* oocyte. We show that kinesin-1 interacts with VLE RNA *in vivo* and *in vitro*, and that interfering with kinesin-1 function *in vivo* blocks VLE RNA localization, suggesting a direct role for kinesin-1 in vegetal RNA localization. Our results also demonstrate that kinesin-1 interacts with kinesin-2 and that these motors carry out

overlapping functions in RNA transport. Moreover, using expression of specific kinesin-1 and kinesin-2 mutants we have uncovered a previously unidentified step in the vegetal RNA localization pathway. Finally, using markers for microtubule polarity we have discovered a population of microtubules with plus ends directed toward the vegetal cortex, providing new mechanistic insight into this motor-driven RNA transport process.

## **Results**

### ***Kinesin-1 Interacts with Localized RNA***

Kinesin-1 is implicated in vegetal RNA transport in *Xenopus* oocytes by virtue of association with at least one critical component of a vegetally localized ribonucleoprotein (RNP) complex (Yoon and Mowry, 2004). As a test of a direct role for kinesin-1 in vegetal RNA transport, we asked if kinesin-1 associates with RNAs during transport (Fig. 1). We injected stage (st.) III oocytes with fluorescently labeled VLE RNA and cultured to allow localization. We then immunostained with SUK4 antibodies, which specifically recognize kinesin-1 heavy chain (KHC) in a variety of organisms, including *Xenopus* (Wright *et al.*, 1993; Tuma *et al.*, 1998; Lane and Allan, 1999). Confocal microscopy revealed that while kinesin-1 is located throughout the perinuclear region of the oocyte, the protein is largely absent from the outer ring of the cytoplasm and cortex (Fig. 1A). Only the vegetal cortical region of the oocyte shows enrichment of kinesin-1, precisely coincident with the

localization of VLE RNA (Fig. 1A-B). Thus, endogenous kinesin-1 is colocalized with RNA undergoing localization *in vivo*.

We further tested the interaction of kinesin-1 with both injected VLE and endogenous Vg1 RNAs biochemically. VLE RNA was injected into st. III oocytes for localization *in vivo*. Control oocytes were injected with *Xenopus*  $\beta$ -globin RNA (X $\beta$ M), which does not localize (Mowry and Melton, 1992). Immunoprecipitation with SUK4 antibodies from oocyte lysates captured complexes containing kinesin-1, and bound RNAs were detected by RT-PCR. Both Vg1 and VLE RNAs are specifically immunoprecipitated with kinesin-1 (Figs. 1C, 1D; lanes 3); the interaction cannot be detected with non-relevant antibodies (lanes 2), or with unlocalized control RNAs, X $\beta$ M (1C, lane 6) and EF1 $\alpha$  (1D, lane 6). Taken together, these results indicate that kinesin-1 is associated with vegetally localized RNP complexes.

### ***Xenopus Kinesin-1 is Necessary for Vegetal RNA Localization***

To probe the functional relevance of the interaction between kinesin-1 and VLE RNA, we used *in vivo* interference. We again used SUK4 antibodies, which recognize the motor domain of KHC and effectively block kinesin-1 function (Ingold *et al.*, 1988). We injected these antibodies into st. III oocytes, followed by injection of fluorescently labeled VLE RNA to determine the phenotypic effects of kinesin-1 inhibition on vegetal RNA localization (Fig. 2). Control oocytes were injected with either mouse IgG or SUK2 antibodies, which recognize kinesin-1 but do not block function (Wright *et al.*, 1991). In control oocytes, RNA in the process of vegetal



localization is evident by its enrichment in the vegetal cytoplasm, extending from the nucleus to the vegetal cortex (Fig. 2A,B). In the presence of kinesin-1 function-blocking antibodies (SUK4), however, vegetal localization is strongly impaired (Fig. 2C). Localization is observed in only 52% of SUK4-injected oocytes (Fig. 2D), as compared to control oocytes. These results suggest a necessary role for kinesin-1 in vegetal RNA localization.

As a further step towards testing the function of kinesin-1 in vegetal localization, we cloned the *Xenopus* kinesin-1 heavy chain (XKHC) from oocyte RNA. Sequence analysis of the XKHC cDNA (Fig. S1) confirms XKHC as a member of the kinesin-1 family (Lawrence *et al.*, 2004). *In vitro* translation of recombinant XKHC carrying a C-terminal FLAG epitope tag generates a protein that is detected by both SUK4 and FLAG antibodies (Figs. S2, S3D). When FLAG-tagged XKHC is expressed in st. III oocytes, immunofluorescence using anti-FLAG antibodies reveals an expression pattern that colocalizes with fluorescently labeled VLE RNA (Fig. S3A). Thus, recombinant XKHC is appropriately expressed in the oocyte. We next used the cloned XKHC to interfere with kinesin-1 function *in vivo*.

Kinesin-1 function can be blocked by a rigor-type mutation, which causes the motor domain to bind tightly, but rarely detach from microtubules (Nakata and Hirokawa, 1995). A point mutation (threonine to isoleucine) in the motor domain of mouse KHC inhibits kinesin-1 function in mouse fibroblasts (Nakata and Hirokawa, 1995). We engineered a similar rigor XKHC point mutant designated XKHC-T92I for *in vivo*

expression studies. To quantitate kinesin-1 rigor mutant effects on vegetal localization, we injected fluorescently labeled VLE RNA into st. III oocytes expressing either wild-type XKHC, XKHC-T92I, or control oocytes expressing no exogenous protein (Fig. 3). VLE RNA localization was assessed by confocal microscopy; oocytes scored as positive for localization exhibited accumulation of VLE RNA in the vegetal cytoplasm (Fig. 3A,B,D,E,F). Oocytes expressing wild-type XKHC displayed normal localization as compared to control oocytes (Figs. 3A,B,F). By contrast, oocytes expressing the XKHC-T92I rigor mutant displayed a dramatic inhibition of VLE localization (Figs. 3C,F). To test the specificity of the inhibitory effect on VLE localization observed for the kinesin-1 rigor mutation, we engineered and expressed a rigor mutation in another plus end-directed kinesin motor, Eg5. *Xenopus* Eg5 was chosen as a control because it has an established rigor mutation equivalent to that of KHC (Blangy *et al.*, 1998) and is expressed during mid-oogenesis (Houliston *et al.*, 1994). As shown in Figure 3, oocytes expressing either wild-type XEg5 (Fig. 3D) or the Eg5 rigor mutant, XEg5-T105N (Fig. 3E), at levels equal to XKHC and XKHC-T92I (Fig. S3D), displayed no inhibition of vegetal localization (Fig. 3F). The results of this experiment indicate that the effect of the kinesin-1 rigor mutation on vegetal localization is specific and is not a general property of plus end-directed motors, providing further evidence that kinesin-1 is necessary for vegetal RNA transport.

***An Intermediate Kinesin-1 Rigor Mutant Phenotype May Provide Mechanistic Insight into Kinesin-1 Function During Vegetal Localization***

Although the majority of oocytes expressing the kinesin-1 rigor mutant displayed a complete inhibition of VLE localization, a significant fraction was scored as positive for localization (Fig. 3F). Many of these (30-40%) display an intermediate phenotype that is not observed with wild-type kinesin-1 or the Eg5 rigor mutant, and with a frequency that is directly correlated with higher concentrations of injected XKHC-T92I-encoding RNA (Fig. S4A). Typically, VLE RNA in the process of localization adopts an hourglass-like distribution within the vegetal cytoplasm, with RNA distributed from the vegetal side of the nucleus to the vegetal cortex (Fig. 4A). Rather than resembling an hourglass, however, oocytes with this intermediate phenotype resemble a half-hourglass with RNA accumulating only from the middle of the hourglass region to the cortex (Fig. 4B). The microtubule cytoskeleton shows no apparent disruption after expression of the XKHC-T92I rigor mutant (Fig. S5), and one possible explanation for the observed RNA distribution is that the rigor mutants could disrupt transport in the upper vegetal cytoplasm. Alternatively, the phenotype could result from inhibition of transport within the lower vegetal cytoplasm due to rigor mutant motors locked onto microtubules while simultaneously bound to RNA transport cargoes. Indeed, the kinesin-1 rigor mutant is colocalized with the aberrantly localized RNA (Fig. S3B).

To further investigate the nature of the kinesin-1 rigor intermediate phenotype, we carried out a temporal analysis of VLE RNA localization, comparing oocytes

expressing either wild-type XKHC (Fig. 4C) or rigor-XKHC (Fig. 4D; XKHC-T92I).

After injection of fluorescently-labeled VLE RNA, oocytes were harvested at 2, 4, 6 or 8 hrs., and vegetal RNA localization was analyzed by confocal microscopy. By 4 hrs., a distinct difference is apparent between oocytes expressing wild-type or kinesin-1-rigor. In oocytes expressing wild-type XKHC (Fig. 4C) VLE RNA accumulates near the germinal vesicle at 4 hrs., with little accumulation in the lower vegetal cytoplasm. By contrast, in oocytes expressing kinesin-1 rigor mutant (Fig. 4D), RNA is present throughout the vegetal cytoplasm. By 6 hrs. RNA is present throughout the vegetal cytoplasm in control oocytes (Fig. 4C), but is restricted to the lower vegetal cytoplasm in oocytes expressing the kinesin-1 rigor mutant (Fig. 4D). These results indicate that movement from the upper vegetal cytoplasm is unaffected by the kinesin-1 rigor mutant, suggesting that this step does not rely on kinesin-1.

Our results revealing a role for kinesin-1 in vegetal RNA transport raise the question of whether kinesin-2, also a plus end-directed motor implicated in this process (Betley *et al.*, 2004), might act to promote a step in the vegetal localization pathway that is distinct from that mediated by kinesin-1. Alternatively, these motors could act together to promote vegetal localization, or they could function redundantly. To address whether kinesin-1 and kinesin-2 act at distinct steps in vegetal RNA localization, we exploited the intermediate phenotype that we observed upon expression of the kinesin-1 rigor mutant (Fig. 4B). The bottom-half distribution may indicate a function for kinesin-1 in the vegetal-most region of the oocyte, specifically in the vegetal cytoplasm closest to the cortex, where VLE RNA accumulates in

oocytes expressing XKHC-T92I (Fig. 4B). If kinesin-2 does not function within this region of the oocyte, we would not expect to obtain a bottom-half phenotype in the presence of a kinesin-2 rigor mutant. To test this, we constructed a kinesin-2 rigor mutation. *Xenopus* kinesin-2 is a heterotrimeric motor that contains two different motor subunits termed XKlp3a and XKlp3b (De Marco *et al.*, 2001). We introduced a threonine-to-isoleucine mutation into the ATP-binding site within the motor domain of XKlp3b (Le Bot *et al.*, 1998) to generate a kinesin-2 rigor mutant, termed XKlp3b-T103I. Using our localization assay we directly compared the phenotypes of oocytes expressing equivalent rigor mutations. As shown in Figure 4F, expression of XKlp3b-T103I, results in the same intermediate phenotype observed with XKHC-T92I (Fig. 4B) at similar levels (Fig. S4B), and additive effects are observed when XKHC-T92I and XKlp3b-T103I are co-expressed (Fig. 4G). The intermediate phenotype is specific to kinesin-1 and kinesin-2 rigor mutants and is not observed in oocytes expressing either wild-type proteins (Fig. S4B) or the Eg5 rigor mutant (data not shown). Moreover, both kinesin-1 and kinesin-2 rigor-mutant motors colocalize with VLE RNA in the lower vegetal cytoplasm (Fig. S3B,C). These data suggest that kinesin-1 and kinesin-2 mediate RNA localization in the same region of the oocyte.

### ***Kinesin-1 and Kinesin-2 May Coordinate Transport in the Oocyte***

Functions for both kinesin-1 and kinesin-2 in the same region of the vegetal cytoplasm may indicate that these motor proteins either act redundantly or have overlapping functions. To test this we constructed a kinesin-1 mutant that lacks the

motor domain, termed XKHCΔm. The XKHCΔm mutant is expected to interact with cargo but not microtubules (Berliner *et al.*, 1995; Gelfand *et al.*, 2001) and would thus exert a dominant negative effect by forming heterodimers with endogenous XKHC, thereby reducing levels of active kinesin-1 rather than by locking onto microtubules as with a rigor mutant. As shown in Figure 5A, expression of XKHCΔm inhibits localization to a similar extent as XKHC-rigor (XKHC-T92I). However, in contrast to XKHC-rigor, inhibition of VLE localization by KHCΔm results in lack of detectable vegetal RNA accumulation; no intermediate phenotype is observed. Inhibition of vegetal localization upon reduction of kinesin-1 function with the XKHCΔm mutation indicates that endogenous levels of kinesin-2 are insufficient alone to support vegetal RNA localization, suggesting that function of these motors is not simply redundant. Likewise, Betley *et al.* (2004) demonstrated inhibition of vegetal localization upon over-expression of kinesin-2 heavy chain (XKlp3b) lacking a motor domain. Thus, endogenous levels of kinesin-1 and kinesin-2 cannot rescue the effects of reduction in activity of either kinesin motor, suggesting that kinesin-1 and kinesin-2 do not function redundantly in vegetal RNA localization.

It is possible, however, that kinesin-1 and kinesin-2 may have overlapping functions, as these motors appear to function in the same region of the vegetal cytoplasm.

While reduction in kinesin-1 activity cannot be compensated for by endogenous kinesin-2 (Fig. 5A,B) and *vice versa* (Fig. 5C; Betley *et al.* 2004), over-expression of one motor could potentially rescue loss of the other motor. To test this possibility, we expressed kinesin-1 heavy chain lacking a motor domain (XKHCΔm) and asked

whether over-expression of kinesin-2 heavy chain (XKlp3b) could rescue vegetal RNA localization. As shown in Fig. 5B, inhibition of vegetal RNA localization by expression of XKHCΔm, can be rescued by the co-expression of kinesin-2 heavy chain (XKHCΔm + XKlp3b). As expected, rescue is also obtained by co-expression of wild-type kinesin-1 heavy chain (XKHCΔm + XKHC). Likewise (Fig. 5C), disruption of vegetal RNA localization caused by expression of kinesin-2 heavy chains lacking a motor domain (Xklp3bΔm) can be rescued by expression of either wild-type kinesin-2 heavy chain (XKlp3bΔm + XKlp3b) or wild-type kinesin-1 (XKlp3bΔm + XKHC). Although reduction of either kinesin-1 or kinesin-2 function can be complemented by expression of the other motor, endogenous levels of those motors are not sufficient for complementation, suggesting overlapping functions for these kinesins in vegetal RNA transport.

Overlapping functions for kinesin-1 and kinesin-2 in vegetal RNA transport in the oocyte predicts that they would be involved in the same steps of the localization pathway. If kinesin-1 and kinesin-2 are operating within the same step of localization it is possible they are interacting with the same cargo. To test this we asked whether these motors could be co-immunoprecipitated. KHC-specific SUK4 antibodies were used to immunoprecipitate kinesin-1 from oocyte lysates. As shown in Figure 5D, kinesin-2 is co-immunoprecipitated with kinesin-1, as detected with K4 antibodies, which recognize the motor domain of the XKlp3a subunit of kinesin-2 (Cole *et al.*, 1993). To test whether the association between kinesin-1 and kinesin-2 might be due to interaction with Vg1 RNA during transport, we first asked if the co-

immunoprecipitation of kinesin-1 and kinesin-2 was sensitive to RNase treatment. As shown in Figure 5E, XKHC is co-immunoprecipitated from oocyte lysates using XKlp3a-specific antibodies (lane 1), but the interaction is abolished upon treatment with RNase (lane 2). Next, we tested whether kinesin-2 is associated with endogenous Vg1 RNA. As shown in Figure 5F, Vg1 RNA is co-immunoprecipitated by XKlp3a-specific antibodies (lane 3). As demonstrated for kinesin-1 (Fig. 1D), this interaction is specific; non-relevant antibodies fail to immunoprecipitate Vg1 mRNA (lane 2) and EF1 $\alpha$  mRNA is not co-immunoprecipitated with kinesin-2 (lane 6). The results of these experiments suggest that kinesin-1 and kinesin-2 may operate by simultaneously binding the same cargo and coordination of these motor activities may be necessary to transport RNA to the vegetal cortex.

### ***A Sub-Population of Microtubules are Directed with Their Plus Ends Toward the Vegetal Cortex***

Delivery of RNA to the vegetal cortex of the oocyte by kinesins 1 and 2 requires microtubules with plus ends oriented toward the oocyte cortex. However, it has been shown that the *Xenopus* oocyte microtubule cytoskeleton is organized with minus ends primarily directed toward the cortex (Pfeiffer and Gard, 1999). This raises the question of how these plus end-directed kinesin motors could be directly involved in vegetal RNA transport if microtubule polarity is opposite to the direction of kinesin movement. To address this issue, we performed immunostaining on st. III oocytes with markers for microtubule polarity. We used antibodies specific for EB1, which has been shown to bind the plus ends of growing microtubules (Mimori-



Kiyosue *et al.*, 2000), and  $\gamma$ -tubulin, which is a component of nucleation complexes at the minus ends of microtubules (Stearns *et al.*, 1991). As shown in Figure 6A, EB1 staining is concentrated in the vegetal cortex of the oocyte, indicating an enrichment of plus ends in this region of the oocyte. Analysis of the immunostaining results by 3-D reconstruction reveals that EB1 is concentrated in a disc within the vegetal cortical cytoplasm (Movie S1). As expected, EB1 staining is abolished upon depolymerization of the microtubule cytoskeleton (Fig. S6). Analysis of EB1 and microtubule distribution at high magnification (Fig. 6D-F) reveals that EB1 is co-localized with microtubule ends (Fig. 6E-F). In contrast to the vegetal cortical enrichment of EB1 staining, visualization of  $\gamma$ -tubulin reveals a slight reduction in concentration of this minus end marker in the vegetal cortex (Fig. 6G). These data indicate that while microtubule minus ends appear to be abundant in the vegetal cortex, there is a significant concentration of microtubule plus ends, specifically in the vegetal cortex. Vegetal enrichment of EB1 is not evident at early stages in oogenesis, and is correlated temporally with the onset of Vg1 RNA localization (Fig. S7). Moreover, EB1 that is enriched in the vegetal cortex is co-localized with VLE RNA (Fig. 6H,I), and no such colocalization is evident in the animal hemisphere (Fig. 6J). These data reveal a sub-population of microtubules in the oocyte that can provide directionality for kinesin-mediated transport of vegetally localized RNAs.

## Discussion

We have studied the localization of Vg1 mRNA in the developing *Xenopus* oocyte to gain insight into the mechanisms responsible for transport of mRNA molecules to

defined regions of the cell cytoplasm. Our results support roles for two plus end-directed molecular motors, kinesin-1 and kinesin-2, in transport of Vg1 mRNA to the vegetal cortical cytoplasm. We have obtained functional evidence that *Xenopus* kinesin-1 is a necessary component of the vegetal RNA localization machinery (Figs. 1-3), and that kinesin-1 and kinesin-2 may act together to carry out vegetal RNA transport (Figs. 4, 5). Previous results (Betley *et al.*, 2004; Yoon and Mowry, 2004) implicating plus end-directed motors in transport of Vg1 mRNA to the vegetal cortical cytoplasm have been viewed with uncertainty (Kloc and Etkin, 2005; St Johnston, 2005; Palacios, 2007), as the polarity of the oocyte cytoskeleton, with microtubule minus ends throughout the oocyte cortex (Pfeiffer and Gard, 1999), was thought to be incompatible with a role for plus end-directed motors in vegetal transport. In this work, we have uncovered a population of microtubule plus ends that are enriched at the vegetal cortex (Fig. 6), consistent with a role for plus end motors in vegetal RNA transport. Based on these results, we propose a new model for vegetal RNA transport, as depicted in Figure 7. In this model, RNP complexes (red) are bound by both kinesin-1 and kinesin-2 (inset). Although the majority of microtubules in the oocyte (brown) are oriented with minus ends towards the cortex (Pfeiffer and Gard, 1999), directional transport of the RNP cargoes to the vegetal cortex is facilitated by a sub-population of microtubules (gold) in the vegetal cytoplasm with plus ends directed towards the cortex.

Active transport of mRNAs by molecular motors provides an efficient mechanism to deliver mRNAs to distinct sub-domains within the cell cytoplasm. For large cells,

such as the *Xenopus* oocyte, ~300µm in diameter at st. III, motor-driven transport is a particularly attractive model. In this work, we have obtained evidence of a necessary and direct function for *Xenopus* kinesin-1 in vegetal RNA transport. We find that kinesin-1 interacts specifically with VLE and Vg1 RNA, as evidenced by both co-localization and co-immunoprecipitation (Fig. 1). Importantly, vegetal RNA localization is inhibited by kinesin-1 function blocking antibodies (Fig. 2), and by expression of two distinct kinesin-1 heavy chain (XKHC) mutant constructs (Figs. 3-5). These results support a direct role for kinesin-1 in vegetal RNA transport. Another kinesin motor, kinesin-2, has been implicated in vegetal RNA localization (Betley *et al.*, 2004), raising the question of how these plus end-directed motors individually contribute to vegetal RNA localization. By comparing the *in vivo* effects of kinesin rigor mutations, we have discovered a previously unidentified step in the RNA transport pathway, mediated by kinesins -1 and -2. Oocytes expressing either kinesin-1 or kinesin-2 rigor mutants display identical phenotypes (Fig. 4), in which VLE RNA is restricted to a sub-region of the vegetal cytoplasm lying above the vegetal cortex. Kinesin rigor mutants have been shown to lock onto microtubules (Nakata and Hirokawa, 1995), suggesting that RNA cargoes should collect in the region where the kinesin motor is acting. Indeed, we find that VLE RNA accumulates in the bottom half of the vegetal cytoplasm in the presence of the kinesin-1 or -2 rigor mutants (Fig. 4). Kinesin function in the lower vegetal cytoplasm is further supported by the observation that endogenous kinesin-1 colocalizes with VLE RNA in this same region (Fig. 1), as do both kinesin-1 and kinesin-2 rigor-mutant motors (Fig. S3). Importantly, kinesin-1 and kinesin-2 co-immunoprecipitate in an RNA-

dependent manner (Fig. 5D,E), implying association through a shared RNA cargo.

Taken together, these data suggest that these motors function during the same step in RNA localization. This is a particularly intriguing result as it is, to our knowledge, the first indication that kinesin-1 and kinesin-2 interact during cellular transport.

The transport phase of Vg1 RNA localization has long been described as a two-step process, in which vegetally-directed transport is followed by anchoring of the RNA to the vegetal cortical cytoskeleton (Yisraeli *et al.*, 1990). Our data now suggest at least three distinct steps, as transport in the upper and lower vegetal cytoplasm appear mechanistically distinct. The “intermediate” kinesin-1 and kinesin-2 rigor mutant phenotypes (Fig. 4) indicate that transport in the upper vegetal cytoplasm can occur in the presence of kinesin-1 and kinesin-2 rigor-mutant motors, suggesting that transport from the germinal vesicle until approximately midway to the vegetal cortex is independent of kinesin-1 and kinesin-2 and may rely on an as yet unidentified motor. Thus, our results suggest that transport to the vegetal cortex consists of a step in the upper vegetal cytoplasm that is independent of kinesins -1 and -2 and a step in the lower vegetal cytoplasm that relies on these kinesin motors.

These results raise the question of why two different types of plus end-directed kinesin motors are required to coordinate transport to the vegetal cortex. If these motors are simply redundant, loss of either motor should be compensated for by the other motor. Contrary to this expectation, reduction of either kinesin-1 (Figs. 3-5) or kinesin-2 (Betley *et al.*, 2004) activity disrupts vegetal RNA localization. Thus,

kinesin-1 and kinesin-2 do not simply act redundantly in vegetal RNA localization. Further clues can be provided by cross-rescue experiments. We used kinesin mutants carrying motor domain deletions to reduce the levels of active kinesin-1 and kinesin-2 (Fig. 5), and showed that kinesin-1 was able to rescue kinesin-2 mutants and *vice versa*. These results suggest that the levels of kinesin motors in the oocyte may be a limiting factor for RNA transport, and that multiple motors may be bound to RNA cargoes to facilitate transport. Much evidence disputes a 1:1 stoichiometry of motor to cargo, with estimates of motors bound to a single cargo ranging between 1 and 11 (Bullock *et al.*, 2006; Levi *et al.*, 2006; Vershinin *et al.*, 2007). One rationale for multiple motors bound to a particle is that increased numbers of bound motors should decrease the likelihood of a cargo detaching from a microtubule. Importantly, mixed polarity of the microtubules in the vegetal oocyte cytoplasm may necessitate multiple motors bound to RNA cargoes. Our results (Fig. 6) have revealed a population of microtubules with plus ends at the vegetal cortex, which is compatible with kinesin-driven transport to the vegetal cortex. However, a significant population of microtubules is also oriented with minus ends directed towards the vegetal cortex (Figs. 6G, 7; Pfieffer and Gard, 1999). Within this mixed population of microtubules, multiple motors bound to RNA cargoes would ensure that an RNA cargo remains attached to a single microtubule regardless of whether it is oriented toward the vegetal cortex or away. In this light, continued accumulation of RNA in the upper vegetal cytoplasm in wild-type XKHC-expressing oocytes at timepoints after RNA has cleared from the upper vegetal cytoplasm in XKHC-rigor expressing oocytes (Fig. 4C,D) is intriguing, and could suggest bi-directional

transport in the lower vegetal cytoplasm. In such a model, RNAs could be transported both towards and away from the vegetal cortex, but anchoring of the RNA at the vegetal cortex could bias the flux of RNA transport vegetally. Indeed, Vg1 has been shown to be anchored to the vegetal cortex after transport (Alarcon and Elinson, 2001; Yisraeli *et al.*, 1990). Our model for vegetal RNA transport (Fig. 7) incorporates the existence of a sub-population of microtubules with plus ends at the vegetal cortex, along with multiple motors bound to RNA cargoes and anchoring at the vegetal cortex to provide a new mechanistic basis for directional RNA transport in the cytoplasm.

## **Materials and Methods**

### ***In Vitro Transcription***

Transcription of VLE and X $\beta$ M RNA for immunoprecipitation and RT-PCR were performed as in Mowry (1996). Fluorescently labeled VLE RNA was transcribed as above, with inclusion of 1 mM Alexa Fluor 546-14-UTP (Molecular Probes).

Transcription of FLAG-tagged RNA was performed using the mMessage mMachine kit (Ambion).

### ***Microinjection and Oocyte Manipulation***

Microinjection and culture of *X. laevis* oocytes was performed as in Kress *et al.* (Kress *et al.*, 2004). For RNA localization assays, 0.05 fmol of fluorescently labeled VLE RNA (2 nl at 25 nM) was injected into st. III oocytes and allowed to localize for 8 hr. FLAG-tagged rigor-mutant and control RNAs (4 nl) were injected at

concentrations ranging from 62.5-500 nM (as noted in the figure legends) and allowed to express protein for 16 hr. prior to VLE RNA injection. For antibody interference, oocytes were injected with 4 ng of Protein-G purified antibodies and incubated 2 hr. prior to VLE RNA injection. For immunoprecipitation and RT-PCR experiments, oocytes were injected with 2 nl of VLE or XβM RNA at 50 nM.

### ***Cloning***

XKHC was amplified from *Xenopus* oocyte RNA using primers designed to the predicted N- and C-termini. The full-length XKHC cDNA (deposited into GenBank; DQ680042) was cloned into pSP64TSN-FLAG (Kress *et al.*, 2004) to generate pXKHC-FLAG. XEg5 (GenBank# X54002) and XKlp3b (GenBank# AJ009839) were amplified by PCR and cloned into pSP64TSN-FLAG. XKHC-T92I-FLAG, XEg5-T105N-FLAG and XKlp3b-T103I-FLAG were cloned by PCR amplification of fragments corresponding to the N- and C- terminal halves of each protein. All primer sequences are listed in Fig. 2.S8.

### ***Immunoprecipitation***

Immunoprecipitations were carried out as in Kress *et al.* (2004), except that S10 lysates were prepared and immunoprecipitated using WB [10mM HEPES pH 7.4, 10mM KOAc, 3mM Mg(OAc)<sub>2</sub>, 5mM EGTA, 0.05% NP-40, 1mM DTT, 0.1μg/ml leupeptin (MP Biomedicals), 0.1μg/ml antipain (MP Biomedicals), 0.1μg/ml trypsin inhibitor (MP Biomedicals), 0.4mM pepabloc (Roche), 1U/ml rRNasin (Promega), 34g/l sucrose] at 0.5 μl/oocyte. Either 25 μl (for RT-PCR) or 125 μl (for co-IP) of

lysate, was mixed with Protein G-purified antibodies [SUK4 (DSHB), K2.4 (Covance)]; purified mouse IgG (Sigma) was used as a control. XKHC and XKlp3a were detected by immunoblotting using SUK4 or K2.4 antibodies at dilutions of 1:500 or 1:50, respectively. RNA isolation and RT-PCR were performed as in Kress *et al.* (2004), using primers specific for Vg1, EF1 $\alpha$ , VLE or X $\beta$ M RNAs (Fig. 2.S8).

### ***Immunolocalization and Imaging***

For immunolocalization, performed as in Yoon and Mowry (2004),  $\alpha$ SUK4 (DSHB),  $\alpha$ FLAG (Sigma), and anti- $\alpha$ -Tubulin (Sigma) were used at 1:250, and  $\gamma$ -Tubulin antibodies (Sigma) were used at 1:150. Monoclonal EB1 antibodies (BD Biosciences) were used at 1:50, and polyclonal EB1 antibodies (a kind gift of E. Karsenti; (Niethammer *et al.*, 2007) were used at 1:250. Alexa-546, Alexa-633, Alexa-647 and Alexa-546 antibodies (Molecular Probes) were used at 1:500. Oocytes were imaged by confocal microscopy using either a Leica TCS SP2 or a Zeiss LSM 510 META.

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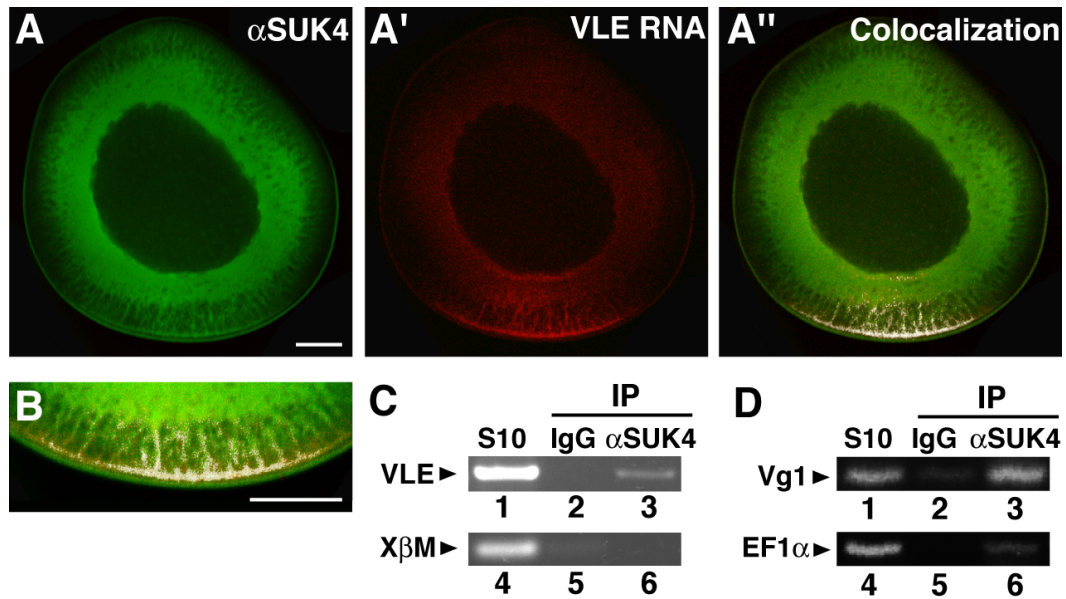
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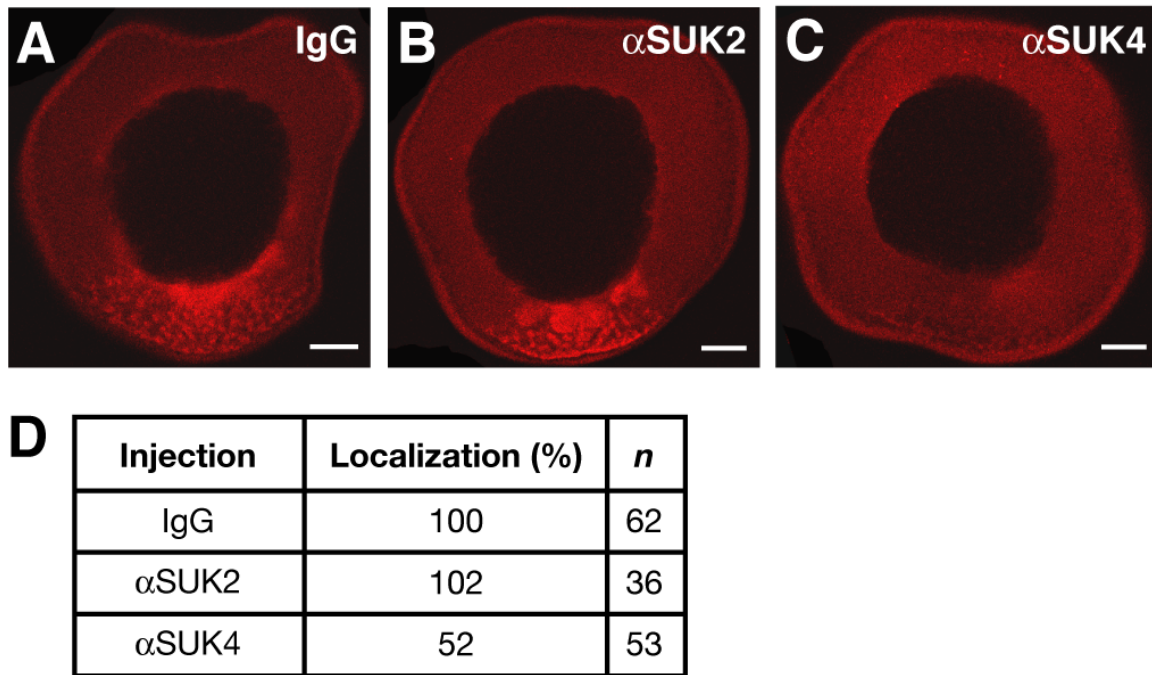
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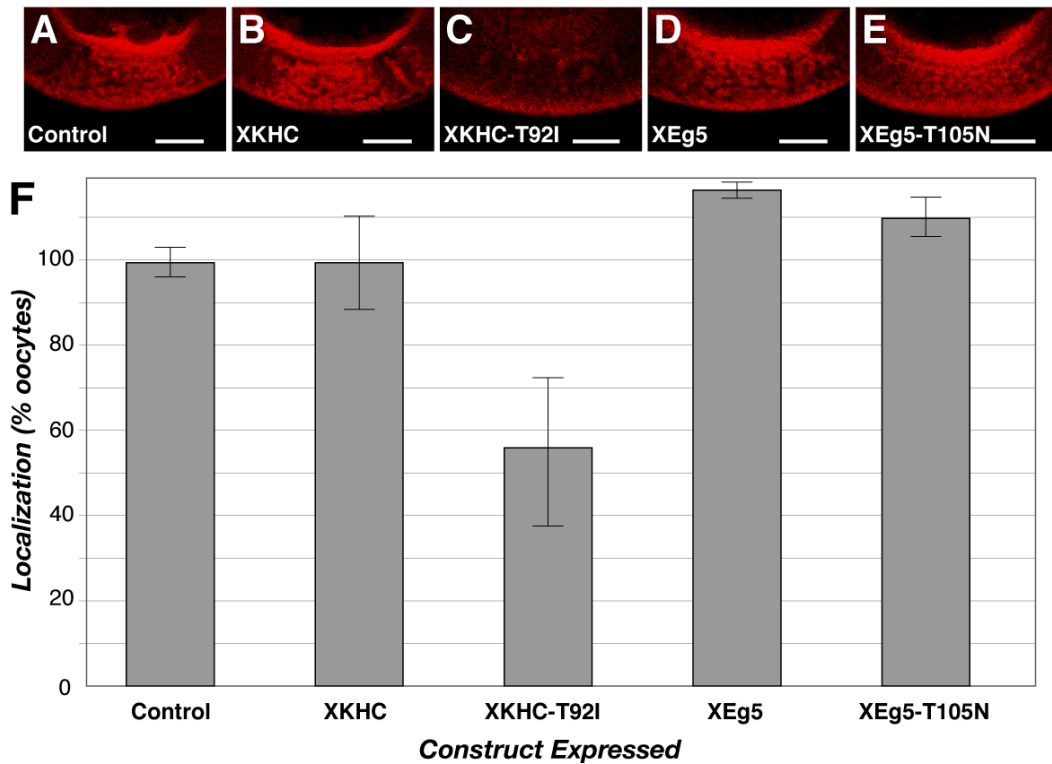


**Figure1. Kinesin-1 Interacts with VLE RNA.** A-B Immunofluorescence was carried out using SUK4 antibodies on st. III oocytes injected with fluorescently labeled VLE RNA. Shown is a confocal section with kinesin-1 shown in green (A;  $\alpha$ SUK4) and VLE RNA in red (A'); the overlap of VLE RNA and SUK4 staining (A'') is shown in white. B. Higher magnification view of VLE RNA and KHC colocalization (white) in the vegetal oocyte cytoplasm. For A-B, scale=50  $\mu$ m. C. S10 lysates from oocytes injected with VLE or X $\beta$ M RNA (lanes 1 and 4 show 10% of total) were immunoprecipitated with mouse IgG (lanes 2, 5) or  $\alpha$ SUK4 (lanes 3, 6). Bound RNAs were detected by RT-PCR using primers for VLE (lanes 1-3) or X $\beta$ M (lanes 4-6). D. S10 lysates from uninjected oocytes were immunoprecipitated with mouse IgG (lanes 2, 5) or  $\alpha$ SUK4 (lanes 3, 6). Bound RNAs were detected by RT-PCR using primers for Vg1 (lanes 1-3) or EF1 $\alpha$  (lanes 4-6). Lanes 1 and 4 show 20% of total input RNA.

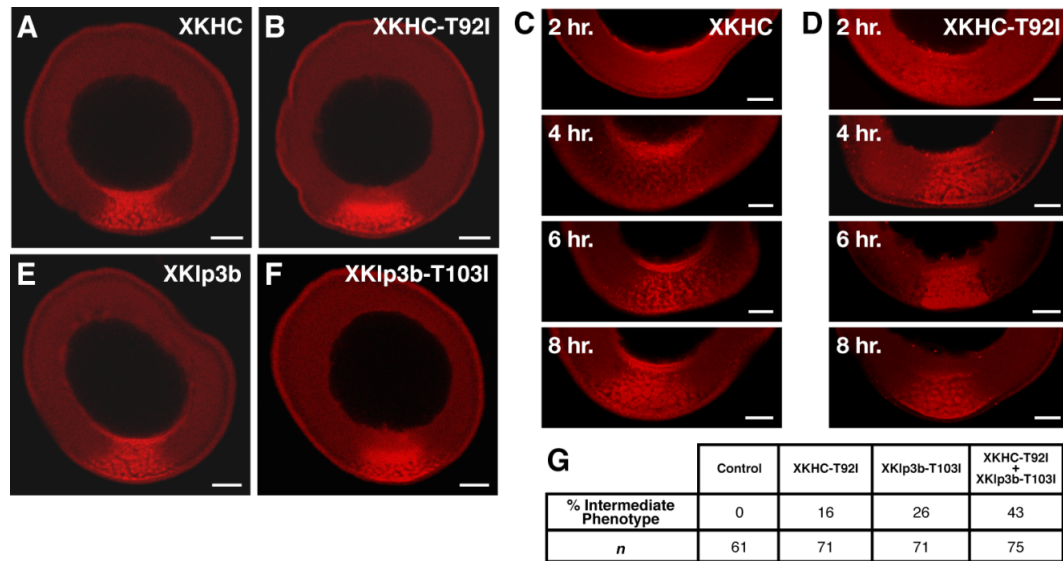




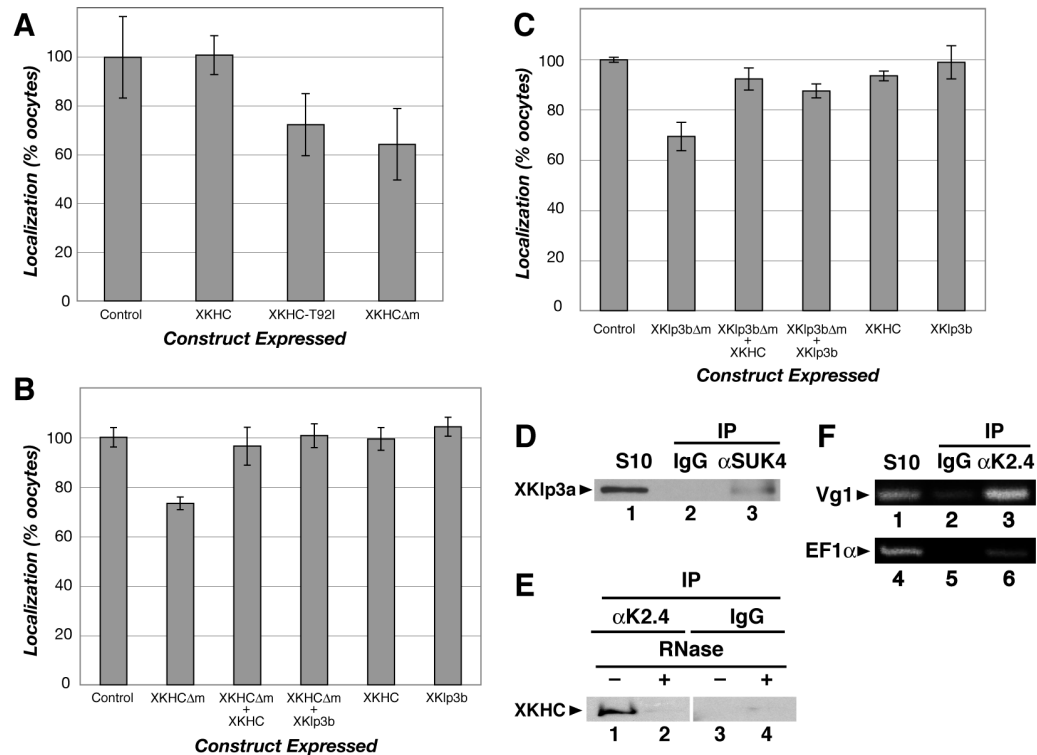
**Figure 2. Kinesin-1 Has a Role in Vegetal RNA Transport.** A-C. Confocal images of representative oocytes injected with fluorescently labeled VLE RNA following injection with (A) IgG (control), (B)  $\alpha$ SUK2 (non-function-blocking  $\alpha$ KHC) or (C)  $\alpha$ SUK4 (function blocking  $\alpha$ KHC) antibodies. Scale=50  $\mu$ m. D. Quantification of antibody interference results. Oocytes were analyzed for VLE RNA localization by confocal microscopy, with percent of oocytes exhibiting localization scored relative to the IgG control, which was set to 100%.



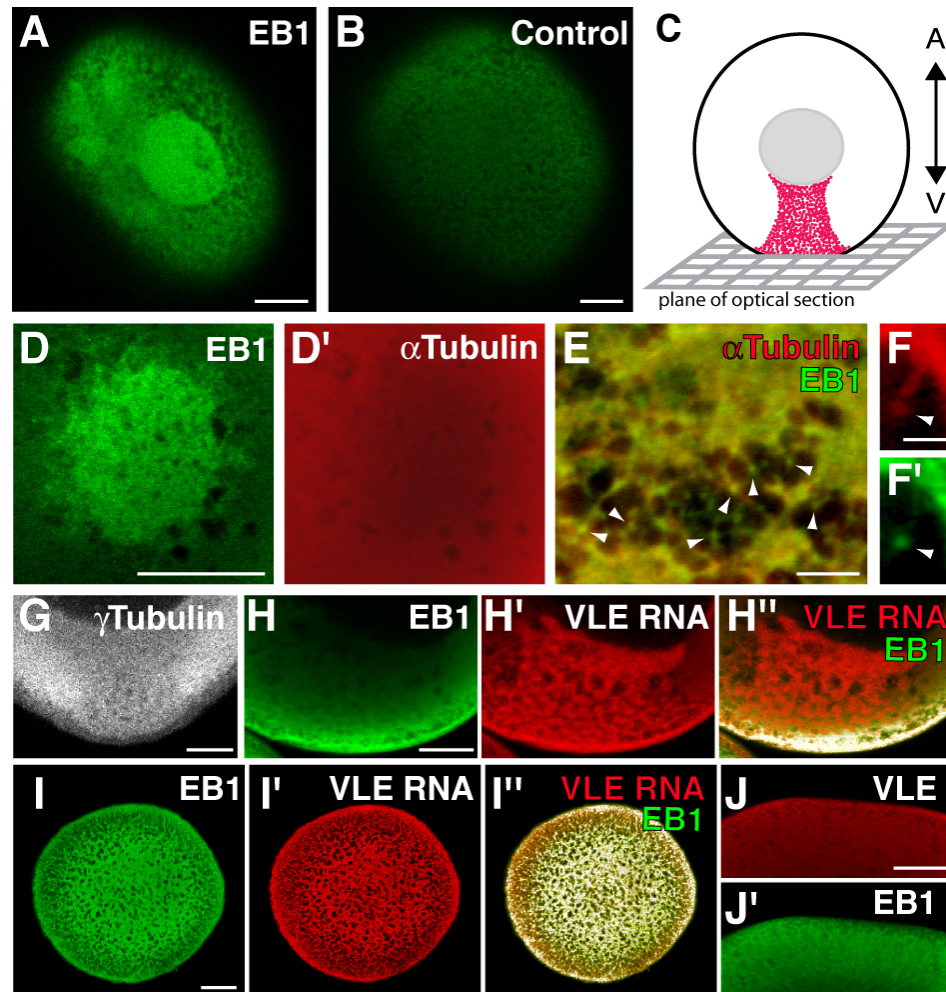
**Figure 3. Kinesin-1 Rigor Mutation Disrupts Vegetal RNA Transport.** A-E. Alexa-546 labeled VLE RNA was injected into oocytes expressing (A) no exogenous protein (control,  $n=311$ ), (B) kinesin-1 heavy chain (XKHC,  $n=253$ ), (C) kinesin-1 rigor mutant (XKHC-T92I,  $n=260$ ), (D) Eg5 (XEg5,  $n=116$ ), or (E) Eg5 rigor mutant (XEg5-T105N,  $n=123$ ), all injected at 500 nM. Representative confocal images of the vegetal hemisphere cytoplasm are shown; scale=50  $\mu$ m. F. Comparison of the percentage of oocytes (gray bars,  $\pm$  st. dev.) exhibiting vegetal localization scored relative to the average for control oocytes (A), which was set to 100%.



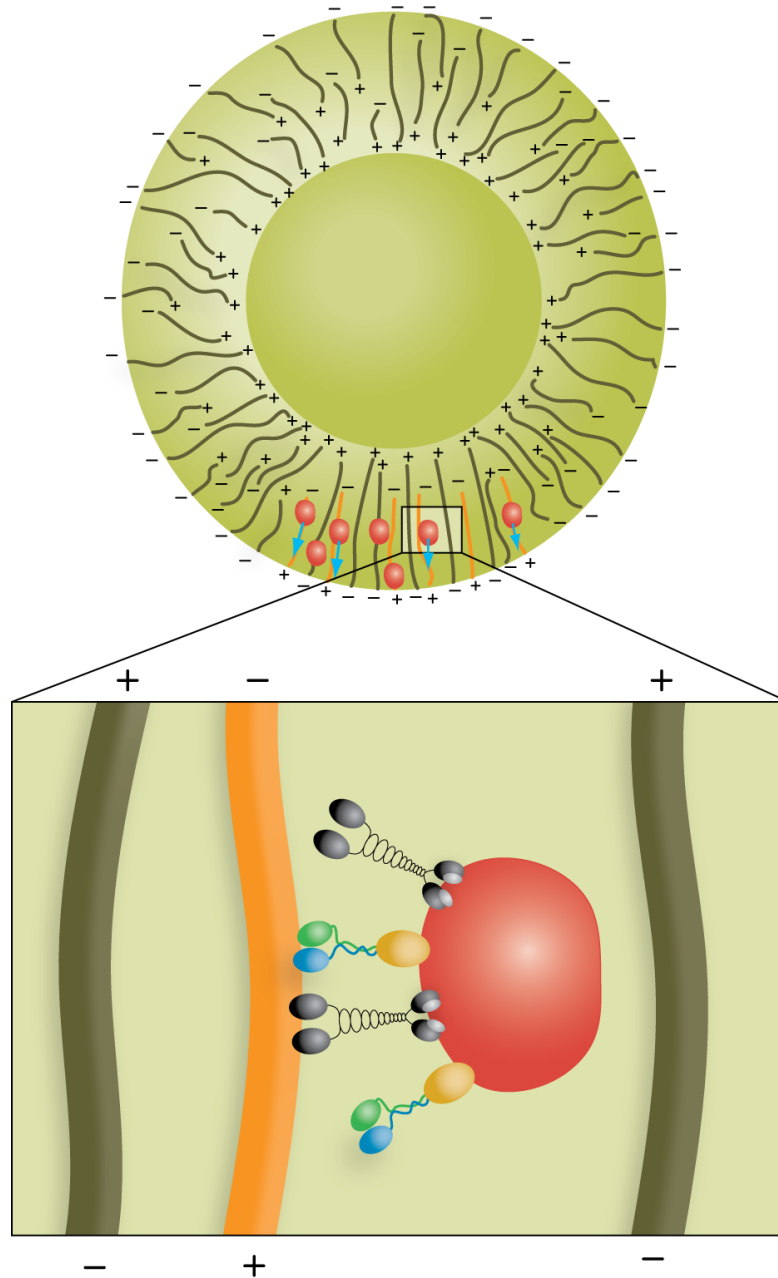
**Figure 4. The Kinesin-1 Rigor Mutation Has an Intermediate Phenotype that may Provide Insight into Kinesin-1 Function.** **A-B.** Oocytes were injected with either (A) XKHC-FLAG or (B) XKHC-T92I-FLAG at 125 nM. After 16 hr. expression, fluorescently labeled VLE RNA was injected, and localization was assayed after 8 hrs. by confocal microscopy. Representative images are shown for the “normal” phenotype (A) and “intermediate” phenotype (B), with the vegetal hemisphere towards the bottom. **C-D.** Oocytes were injected with either (C) XKHC- or (D) XKHC-T92I-encoding RNAs at 500 nM, followed by injection of fluorescently labeled VLE RNA. Oocytes were harvested at 2, 4, 6, and 8 hrs., and localization was assayed as above. Representative images of the vegetal cytoplasm are shown. **E-F.** Oocytes were injected with (E) XKlp3b- or (F) XKlp3b-T103I-encoding RNAs at 125 nM, and assayed for VLE localization as in A-B. For A-F, scale=50  $\mu$ m. **G.** Oocytes (from A,B,E,F) were scored for localization by confocal microscopy, and the percentage that exhibited the “intermediate” rigor phenotype is indicated, along with the number of oocytes (*n*) assayed.



**Figure 5. Kinesin-1 and Kinesin-2 Have Overlapping Functions.** **A.** Oocytes were injected with RNAs encoding kinesin-1 heavy chain (XKHC,  $n=168$ ), kinesin-1 rigor mutant (XKHC-T92I,  $n=155$ ) or kinesin-1 mutant lacking the motor domain (XKHCΔm,  $n=164$ ), all at 125 nM; control oocytes expressed no exogenous protein (Control,  $n=158$ ). Vegetal localization was assayed after injection of Alexa-546 labeled VLE RNA. The percentage of oocytes ( $\pm$  st. dev.) exhibiting accumulation of the injected RNA in the vegetal hemisphere is indicated by gray bars, with the average of control oocytes set to 100%. **B.** Oocytes injected with RNA encoding kinesin-1 mutant lacking the motor domain (XKHCΔm  $n=160$ ) at 50 nM were rescued by co-expression of either XKHC (XKHCΔm+XKHC  $n=168$ ) or XKlp3b (XKHCΔm+XKlp3b  $n=165$ ) at 125 nM. Control oocytes expressed no exogenous protein (Control  $n=161$ ); additional controls expressed XKHC (XKHC  $n=150$ ) or XKlp3b (XKlp3b  $n=157$ ) alone. Vegetal RNA localization was assayed as in A. The percentage of oocytes ( $\pm$  st. dev.) exhibiting vegetal localization is shown by gray bars, with the control average set to 100%. **C.** Oocytes injected with RNA encoding kinesin-2 mutant lacking the motor domain (XKlp3bΔm  $n=160$ ) were rescued by co-injection of XKHC (XKlp3bΔm+XKHC,  $n=155$ ) or XKlp3b (XKlp3bΔm+XKlp3b,  $n=155$ ) all at 125 nM. Control oocytes expressed no exogenous protein (Control,  $n=154$ ); additional controls expressed XKHC (XKHC,  $n=153$ ) or Xklp3b (XKlp3b,  $n=160$ ) alone. Vegetal RNA localization was assessed and presented as in B. **D.** Immunoprecipitation was carried out from oocyte S10 lysates (lane 1) using SUK4 (lane 3), or control IgG antibodies (lane 2). After SDS-PAGE, immunoblotting was carried out with αK2.4. **E.** S10 lysate, prepared from st. III oocytes, was treated with either RNase A (+) or RNasin (-), and immunoprecipitation was carried out using K2.4 (lanes 1-2) or control IgG antibodies (lanes 3-4). After SDS-PAGE, immunoblotting was carried out using SUK4 antibodies. **F.** Immunoprecipitation from oocyte S10 lysates was carried out using K2.4 (lanes 3 and 6) or control IgG antibodies (lanes 2 and 5). Bound RNAs were detected by RT-PCR using primers for Vg1 (lanes 1-3) or EF1α (lanes 4-6) mRNAs. Lanes 1 and 4 show 20% of total input RNA.

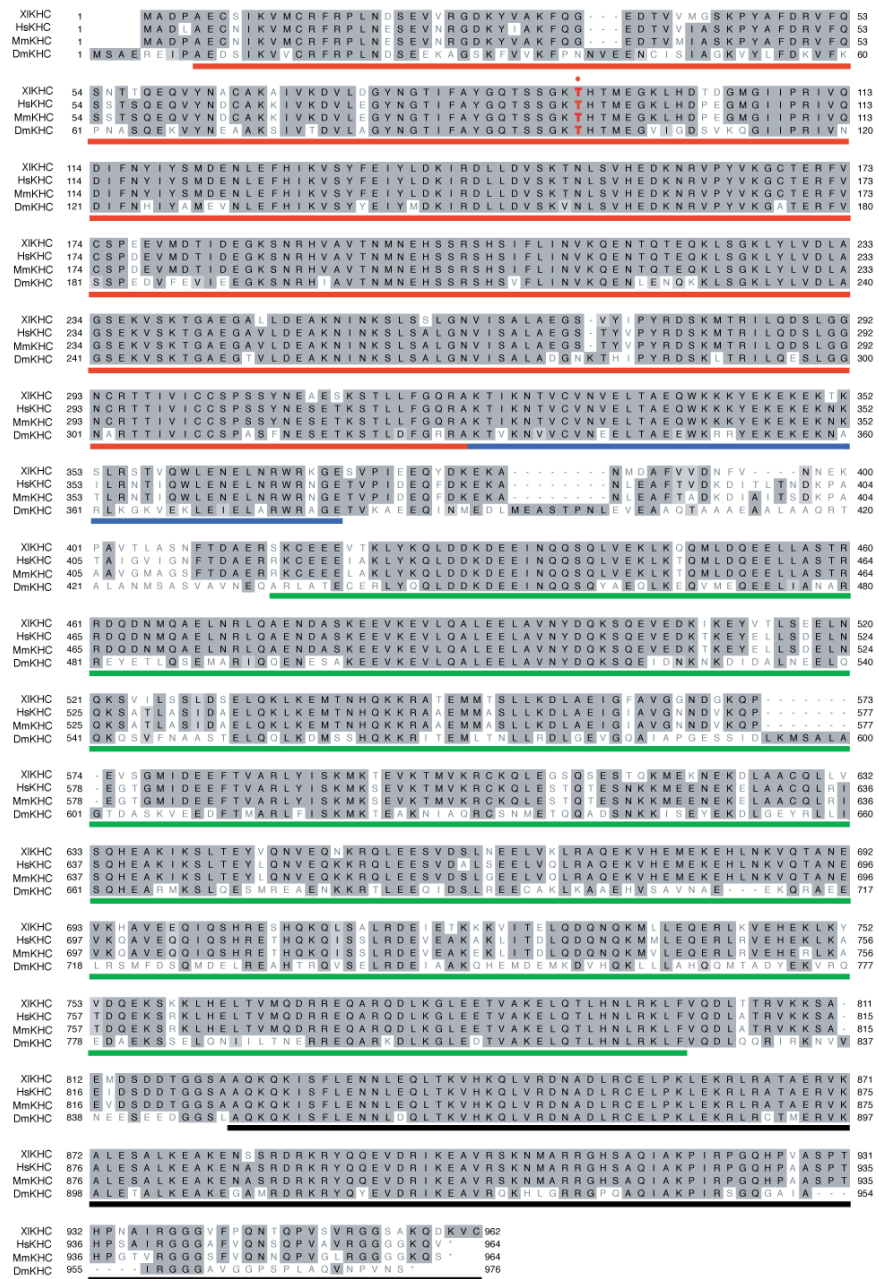


**Figure 6. Markers of Microtubule Polarity Reveal a Vegetal Population of Plus Ends.** A-B. Immunofluorescence was performed on st. III oocytes using: (A) EB1 antibodies or (B) no primary antibody. C. Depiction of vegetal views shown in A,B,D,E,I. The optical section, ~10-25  $\mu\text{m}$  from the vegetal pole, is depicted as a gray grid relative to oocyte A/V axis. D-F. Immunofluorescence was performed on st. III oocytes using both EB1 and  $\alpha$ -Tubulin antibodies. D. An optical section shows EB1 in green (D) and  $\alpha$ -Tubulin in red (D'). E. Colocalization of EB1 (green) and microtubules (red), viewed at high magnification (scale=5 $\mu\text{m}$ ). Arrowheads point to examples of microtubule ends colocalized with EB1. F. An optical section, with the vegetal pole oriented towards the bottom, shows  $\alpha$ -Tubulin in red (F) and EB1 in green (F'). Arrowhead points to a microtubule end colocalized with EB1; scale=2 $\mu\text{m}$ . G. Immunofluorescence was performed on st. III oocytes using  $\gamma$ -Tubulin antibodies. A cross section through the vegetal cytoplasm is shown, with the vegetal pole at the bottom. H. Immunofluorescence was performed on Alexa-546 VLE-injected oocytes using EB1 antibodies. An optical cross section of the vegetal cytoplasm is shown, with the vegetal pole at the bottom. EB1 is shown in green (H), VLE RNA is shown in red (H') and the overlap of VLE RNA and EB1 is visualized as white (H''). I. Vegetal view (as in C) of EB1 and VLE colocalization in a st. III oocyte. EB1 is shown in green (I), VLE RNA is shown in red (I'); VLE RNA and EB1 overlap is shown in white (I''). J. An optical cross section of the animal hemisphere from (H) is shown, with VLE RNA as red (J) and EB1 as green (J'). Scale bars represent 50 $\mu\text{m}$  (A-B,D,G-J), 5 $\mu\text{m}$  (E) or 2 $\mu\text{m}$  (F).

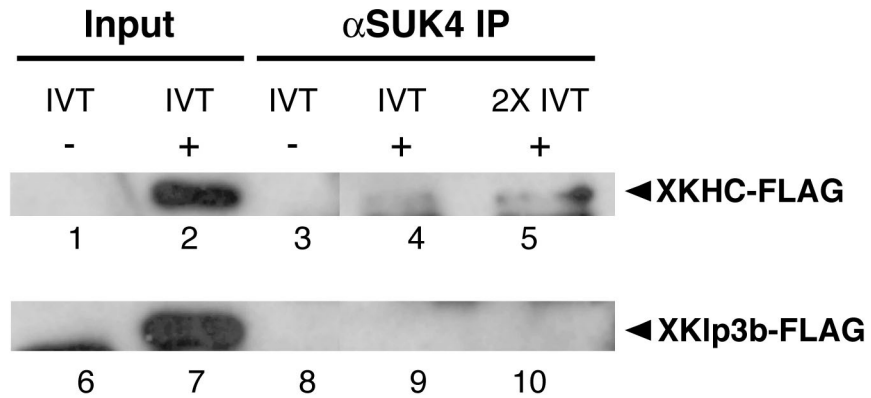


**Figure 7. Model for Vegetal RNA Localization.** A depiction of a st. III oocyte is shown, with the majority of microtubules (shown in brown) oriented with minus ends directed towards the vegetal cortex (bottom). A sub-population of microtubules (shown as gold) is present in the vegetal cytoplasm, with plus ends oriented towards the vegetal cortex. RNA-protein complexes (RNPs) transported to the vegetal cortex are shown in red. Vegetally transported RNPs are bound (see inset) by both kinesin-1 (black) and kinesin-2 (blue/green) motors. Anchoring of the RNA at the vegetal cortex is not depicted.



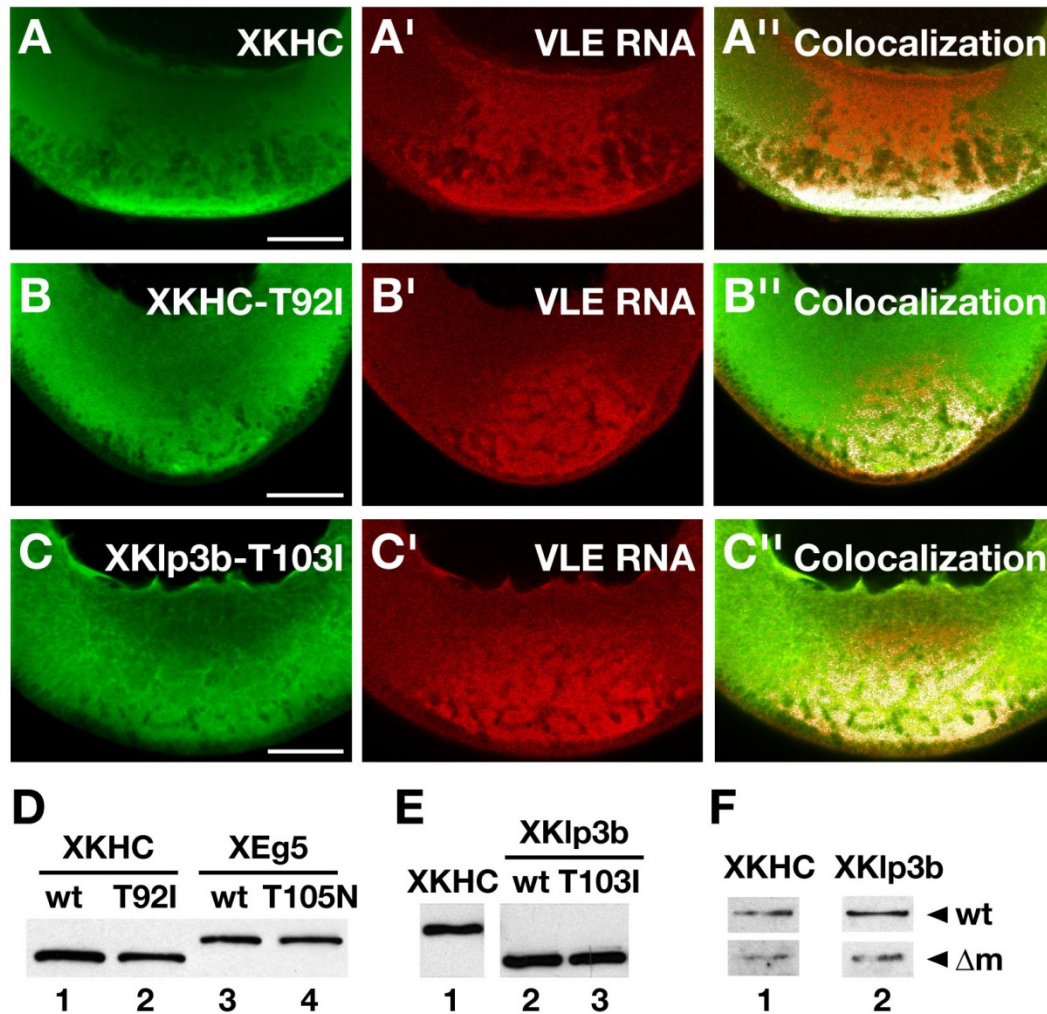


**Figure S1. Cloning of *Xenopus* kinesin-1 heavy chain.** The complete open reading frame for the XKHC cDNA encodes a protein of 962 amino acids with a predicted molecular weight of 110 kDa. Alignment of *Xenopus* kinesin heavy chain (XKHC) (GenBank Accession Number DQ680042) with human (HsKHC; Navone *et al.*, 1992), mouse (MmKHC; Gudkov *et al.*, 1994), and fly sequences (DmKHC; Yang *et al.*, 1989). Below the aligned sequences are lines representing head (red), neck (blue), stalk (green), and tail (black) domains of KHC. Comparison with mammalian sequences shows that XKHC shares 94%, 90%, 87% and 91% amino acid identity, within the N-terminal motor, neck, stalk and tail domains, respectively. For the *Drosophila* sequence, XKHC shares 75%, 66%, 50%, and 89% sequence identity with these domains, respectively. Within the motor domain the threonine residue that is mutated to isoleucine to generate a kinesin-1 rigor mutation is shown in red.

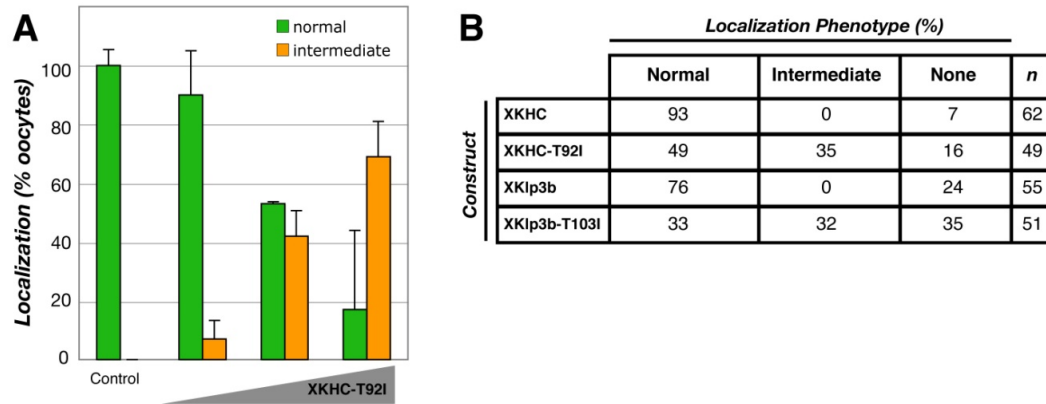


**Figure S2. SUK4 antibodies specifically recognize recombinant XKHC.** SUK4 antibodies specifically immunoprecipitate recombinant XKHC (top), but not kinesin-2 subunit XKlp3b (bottom). XKHC-FLAG (top) or XKlp3b-FLAG (bottom) RNAs were added (+; lanes 2,4-6,9,10) to rabbit reticulocyte lysate (Promega) for *in vitro* translation (IVT). Mock reactions contained no added RNA (-; lanes 1,3,6,8). 20% (lanes 3,4,8,9) or 40% (2X; lanes 5,10) of the IVT reactions were immunoprecipitated with SUK4 (DSHB) antibodies. Input represents 5% of IVT. After SDS-PAGE, translated proteins were detected by immunoblotting for the C-terminal FLAG epitope using FLAG antibodies (Sigma) at 1:1000.

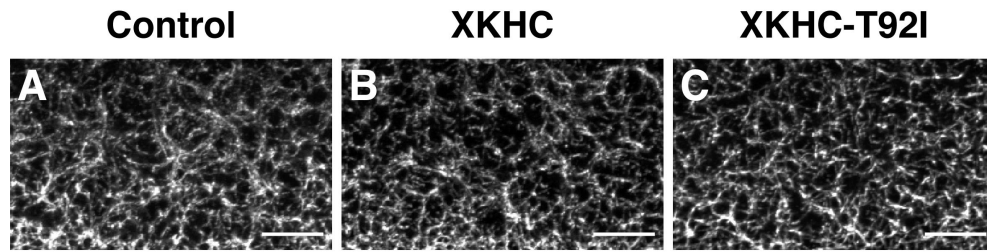




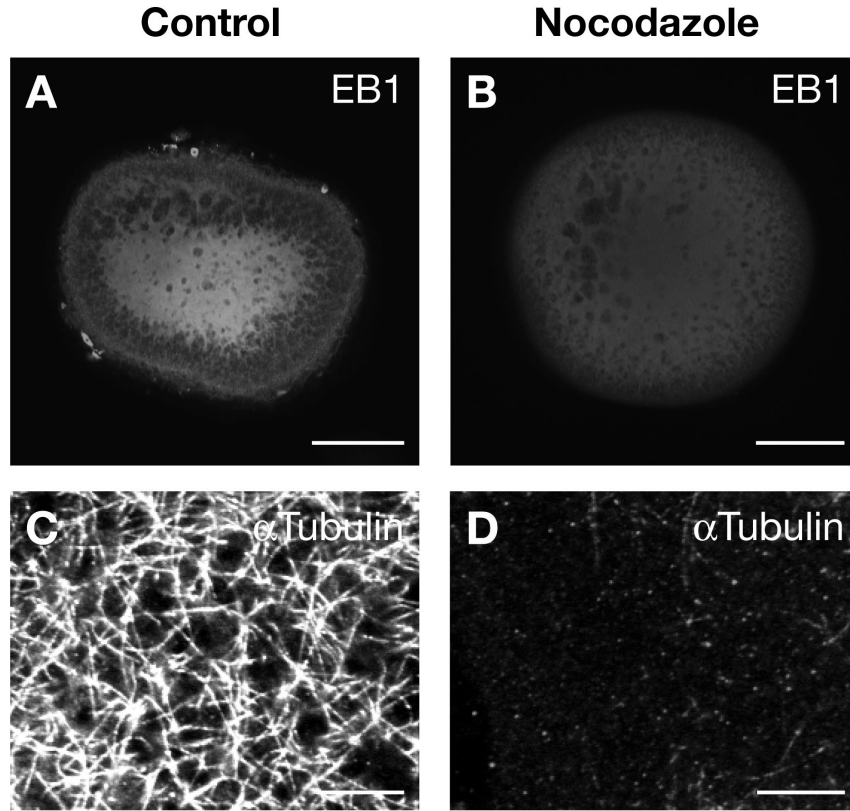
**Figure S3. Expressed FLAG-tagged wild-type and mutant kinesin motor proteins colocalize with VLE RNA.** A-C. RNAs encoding (A) XKHC-FLAG, (B) XKHC-T92I-FLAG or (C) XKlp3b-T103I-FLAG were injected into st. III oocytes and incubated overnight to allow protein expression. Oocytes were then injected with fluorescently labeled VLE RNA and cultured for 8 hr. Immunofluorescence was performed with FLAG (Sigma) and Alexa-633 (Molecular Probes) antibodies, and confocal images were obtained using a Zeiss LSM 510 META. Expressed FLAG-tagged protein is shown in green (A-C), VLE RNA is shown in red (A'-C'), and overlap of FLAG-tagged kinesin motor proteins and VLE RNA is in white (A''-C''). Cross-sections through the vegetal hemisphere are shown, with the vegetal cortex oriented towards the bottom. Scale=50  $\mu$ m. D-F. Immunoblotting was performed on lysates prepared from oocytes expressing (D) XKHC-FLAG (lane 1), XKHC-T92I-FLAG (lane 2), XEg5-FLAG (lane 3), or XEg5-T105N-FLAG (lane 4); (E) XKHC-FLAG (lane 1), XKlp3b-FLAG (lane 2) or XKlp3b-T103I (lane 3); (F) XKHC $\Delta$ m-FLAG (lane 1) or HA-XKlp3b $\Delta$ m (lane 2). Blots were probed with (D-E)  $\alpha$ FLAG (Sigma), (F)  $\alpha$ SUK4 (lane 1; DHSB) or  $\alpha$ K2.4 (lane 2; Covance) at 1:500 dilution.



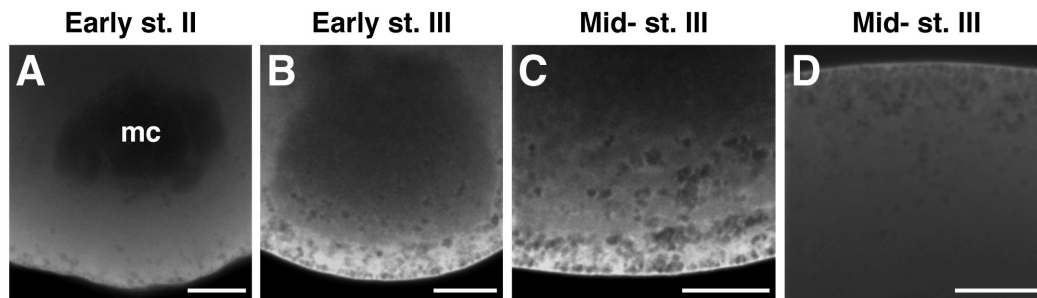
**Figure S4. The intermediate phenotype results from expression of rigor-mutant kinesin motor proteins.** **A.** Alexa-546 labeled VLE RNA was injected into oocytes expressing either no exogenous protein (Control,  $n=102$ ), 62.5 nM XKHC-T92I-FLAG RNA ( $n=75$ ), 125 nM XKHC-T92I-FLAG RNA ( $n=102$ ) or 500 nM XKHC-T92I-FLAG RNA ( $n=87$ ). After incubation for 8 hr., localization was scored by confocal microscopy. The graph shows the percentage of oocytes that exhibited normal localization (green) and the percentage showing the rigor “intermediate” phenotype (orange). The average normal localization observed for the control oocytes was set to 100%. **B.** Oocytes expressing XHHC-FLAG, XKHC-T92I-FLAG, XKlp3b-FLAG or XKlp3B-T103I were assayed and scored for localization as above (**A**), and the percentage that exhibited normal localization (normal), the intermediate rigor phenotype (intermediate), or no detectable localization (none) is shown in the table. The number of oocytes ( $n$ ) is indicated on the right.



**Figure S5. Microtubules are not disrupted in the presence of the kinesin-1 rigor mutant XKHC-T92I.** Microtubules in st. III oocytes expressing (A) no exogenous protein (Control), (B) XKHC-FLAG or (C) the kinesin-1 rigor mutant (XKHC-T92I) were labeled with antibodies directed toward  $\alpha$ -Tubulin (Sigma; 1:150). Alexa-546 anti-rabbit secondary antibodies were used at 1:500 and oocytes were imaged by confocal microscopy using a Zeiss LSM 510 META. Optical cross-sections of the vegetal cytoplasm are shown, with the vegetal hemisphere towards the bottom. Scale=10  $\mu$ m.



**Figure S6. Depolymerization of microtubules abolishes EB1 staining.** Stage III oocytes were incubated in the presence of 10  $\mu\text{g/ml}$  nocodazole to depolymerize microtubules. Immunofluorescence was performed using (A,B) EB1 antibodies (BD Biosciences) at 1:50 and (C, D)  $\alpha$ -Tubulin antibodies (Sigma) at 1:250. Enrichment of EB1 in the vegetal cortex observed in control oocytes (A), is abolished by treatment with nocodazole (B). Vegetal views (as in Fig. 6C) are shown for A-B, and Alexa-633 (Molecular Probes) anti-mouse secondary antibodies were used at 1:500. Scale=50  $\mu\text{m}$ . The filamentous network of microtubules evident in control oocytes (C) is depolymerized upon nocodazole treatment (D). Optical cross-sections are shown for C-D, and Alexa-546 anti-rabbit secondary antibodies were used at 1:500. Imaging was performed on a Zeiss LSM 510 META. Scale=10  $\mu\text{m}$ .



**Figure S7. EB1 accumulates at the vegetal cortex during early stage III of oogenesis.**

Immunofluorescence was performed with anti-EB1 antibodies (Niethammer *et al.*, 2007) on (A) early st. II, (B) early st. III, or (C-D) mid-st. III oocytes. Anti-rabbit Alexa-546 secondary antibodies were used at 1:500, and oocytes were imaged by confocal microscopy using a Zeiss LSM 510 META. The mitochondrial cloud (mc) is apparent as a dark area in the early st. II oocyte (A), but no evidence of cortical EB1 enrichment is evident. In the early st. III oocyte (B), the mc is in the process of breaking down towards the vegetal hemisphere, and accumulation of EB1 is evident at the vegetal cortex. By mid-st. III (C,D), the disc of EB1 enrichment in the vegetal cortex is larger (C; compare also, vegetal views in Fig. 6A [early st. III] and 6I [mid-st. III]), and no EB1 accumulation is evident in the animal hemisphere cortex (D). Oocyte stages are as in Dumont (1972). Oocytes are oriented with the vegetal hemisphere towards the bottom; scale = 20  $\mu$ m.

| Amplicon        | Forward Primer                           | Reverse Primer                                        |
|-----------------|------------------------------------------|-------------------------------------------------------|
| XKHC            | GAGAACCAGATCTATGGCGGACCCGGC<br>GGAG      | GAACCCTCGAGAGGCAGAC<br>TTTGTCTGTTTAGC                 |
| N- XKHC-T92I    | GAGAACCAGATCTATGGCGGACCCGGC<br>GGAG      | TTCCCTCCATGGTATGGAT<br>TTTTCCTGATGATGT                |
| C- XKHC-T92I    | GGCATCCCATGGAGGGAAAACCTCCATG             | GAACCCTCGAGAGGCAGAC<br>TTTGTCTGTTTAGC                 |
| XEg5            | GCCATCTCGAGCATGGCCAGCAAGAAG<br>GAGG      | GAACCCTCGAGGAGTTCTG<br>CATTCGCAGGGGAAG                |
| N- XEg5-T105N   | GCCATCTCGAGCATGGCCAGCAAGAAG<br>GAGG      | CATAGTAAAGTTCTTGCCG<br>GTACCAGTCTGCC                  |
| C- XEg5-T105N   | TGCGTATGGGCAGACTGGTACCGGCAA<br>GAACTTTAC | GAACCCTCGAGGAGTTCTG<br>CATTCGCAGGGGAAG                |
| XKlp3B          | GCGAGCGGAGATCTATGTCCAAGTCTA<br>AGAGCTCGG | GCGAGCTCGAGGATTTGGG<br>TACCAGGCCTCGTG                 |
| N- XKlp3B-T103I | GCGAGCGGAGATCTATGTCCAAGTCTA<br>AGAGCTCGG | CTTTTCTGGATCCCCACGC<br>ACTCCCTCCATTGTGTAAA<br>TTTTTCC |
| C- XKlp3B-T103I | GCGTGGGGATCCAGAAAAGAGAGG                 | GCGAGCTCGAGGATTTGGG<br>TACCAGGCCTCGTG                 |
| VLE RNA         | CGATGACATCCACCCAACAC                     | GAGGGTCACAGTCAGCAAG<br>G                              |
| XβM RNA         | GCAGAAGCTCAGAATAAACGC                    | GCACATAGTTTGCCCCAGG                                   |
| Vg1 mRNA        | CGATGACATCCACCCAACAC                     | ATAGGTGTTCTGGCTGAAC<br>C                              |
| EF1α mRNA       | CAGATTGGTGCTGGATATGC                     | ACTGCCTTGATGACTCCTA<br>G                              |

**Figure S8. PCR Primer Sequences.** All primer sequences are listed 5'-3'. PCR was performed using Platinum Taq (Invitrogen), according to the manufacturer's instructions.

## **Chapter 5: Conclusions and future directions**

**Catherine A. Pratt** and Kimberly L. Mowry

The work presented in this thesis represents an increased appreciation for the complexity of post-transcriptional control of gene expression. By focusing on the model system that is Vg1 mRNA localization in *Xenopus laevis* oocytes, my colleagues in the Mowry lab and I have made significant inroads into understanding how polarized protein expression is generated within this cell. The process of mRNA localization and the concordant control of translation are critical to the generation of cellular asymmetry in numerous cell types and organisms. Understanding the mechanisms underlying these processes is crucial, not only in appreciating their roles in cell growth, development, and signaling, but also for understanding how these processes can go awry in disease states such as cancer and neurological disorders. I shall therefore use this section to talk about the broader implications of my work, and how it will impact future studies in the field.

### ***miRNAs as global regulators in development and disease***

In 1998 a ground-breaking paper in Nature reported the serendipitous discovery of RNA interference (RNAi) in the worm *Caenorhabditis elegans* (Fire *et al.* 1998). Although injected RNA had been used to interfere with gene function in several systems before this publication, the mechanism behind the effect had been unclear. As it turned out, the injection of double-stranded RNAs was far more effective at knocking down gene expression than single-stranded RNA, and, the following year, Fire and Mello not only published the identity of the regulatory proteins necessary for RNAi, but also demonstrated a role for this pathway in the silencing of transposons in the *C. elegans* germline (Tabara *et al.* 1999). Since this Nobel-prize-



winning discovery, the field of RNA-mediated control of gene expression has exploded. Several different species of small regulatory RNAs have since been described, including miRNAs (Lagos-Quintana *et al.* 2001; Lee and Ambros 2001), siRNAs (Zamore *et al.* 2000), and piRNAs (Aravin *et al.* 2006), emphasizing the potential for such molecules in the control of gene expression.

Let-7 was one of the first miRNAs shown to function in translational control (Reinhart *et al.* 2000). It was identified in a genetic screen and subsequently shown to directly regulate the translation of *lin-41* through the recognition of partially complementary sites in the 3'-UTR (Vella *et al.* 2004). This interaction, and the activity of distinct miRNA species, is critical in the development of *C. elegans* larvae (Reinhart *et al.* 2000). Interestingly, the importance of let-7 in developmental timing in general was further corroborated when it was shown that the miRNA is regulated by the steroid hormone ecdysone during the larval-to-pupal transition in *Drosophila* (Sempere *et al.* 2002). Indeed, the let-7 family of miRNAs is highly conserved, with little variation found between human and worm sequences, suggesting an important and conserved role in the biology of the majority of metazoan species (Pasquinelli *et al.* 2000). That this miRNA plays a role in developmental timing in a number of species has been demonstrated, where it has been implicated in the control of cellular proliferation and differentiation (reviewed in Ambros 2011). Until now, however, a direct role for this miRNA family in oogenesis has not been demonstrated (Chapter 2).

Given the importance of let-7 family miRNAs in controlling these cellular processes, it should come as no surprise that they have also been implicated in a number of types of cancer. Most recently, let-7i was shown to be involved in the induction of squamous cell carcinomas of the head and neck (Yang *et al.* 2012), with low levels of the miRNA being linked to the epithelial to mesenchymal transition and increased invasiveness into normal tissue. This study not only highlights the role that these small RNAs play in malignant transformations, but also their potential utility as biomarkers in the clinic (reviewed in Mayer *et al.* 2011).

For miRNA-mediated control of Vg1 mRNA to be useful, the block to translation has to be lifted later in oogenesis. Our data indicate that Ago2 only interacts with Vg1 until the localization process is complete, thus implicating release from the miRISC as a mechanism of relief of repression (Chapter 2). Such a mechanism has been described recently, in which binding of HuR to the 3'-UTR of various mRNAs silenced during cell stress causes the release of bound miRISC (Kundu *et al.* 2012). The authors propose that the binding of HuR to the RNA results in HuR oligomerization and subsequent dislodging of the miRISC. This is of particular relevance, not only because we see a dissociation of the mRNA from the miRISC, but also because ElrB, the *Xenopus* orthologue of HuR, has previously been implicated in the translational control of Vg1 (Colegrove-Otero *et al.* 2005). It will be interesting to see if there is indeed a physical interaction between miRISC and ElrB in oocytes, and, if so, if it is required for Vg1 translational control.

The mechanism by which miRNAs repress translation remains a controversial topic (Chapter 1). It is clear that assembly of the miRISC and direct binding to the target mRNA are required for an effect to be observed, but precisely how the reduction in protein synthesis is achieved is unknown (reviewed in Filipowicz *et al.* 2005). One of the many proposed mechanisms is that miRISC binding directs the targeted mRNA to large cytoplasmic foci termed P-bodies (processing bodies) where they are shielded from the translational machinery (Liu *et al.* 2005; Pillai 2005). In the oocyte we routinely observe localizing RNA in large cytoplasmic granules (Cote *et al.* 1999), and more recently proteomic analysis has indicated that several components of P-bodies are found within these granules (T. Wood, manuscript in preparation). Taken together, these data hint that perhaps Vg1 coalesces into P-body-like complexes through the activity of the miRISC, thus preventing translation. Perhaps the most attractive part of this model is that mRNAs contained within P-bodies have been shown to move back into the actively translating polysomal compartment of the cell in a regulatable manner (Brenques *et al.* 2005; Bhattacharyya *et al.* 2006).

### ***Translational control of localized mRNAs***

While miRNA-mediated control of translation has been widely documented, few examples of this mechanism acting upon localized mRNAs exist (Ashraf *et al.* 2006; Klattenhoff *et al.* 2007). In Chapter 2 I uncovered a role for the miRISC and let-7 family miRNAs in the control of Vg1 translation, thus adding this transcript to the small pool of localized mRNAs that are regulated in this manner. My data show an interaction between the miRISC and the translational control element (TCE), but in

the Appendix section I show that the vegetal localization element (VLE) can also direct translational repression of a reporter RNA. This finding led us to investigate how this element might play a role in translational control. Our favored model at this time is that the RNA-binding proteins PTB and Vera are involved in this process, perhaps through the formation of mRNP granules that prevent the translation machinery from gaining access to the mRNA.

Precedent for granule formation as a mechanism for translational control of localized mRNAs exists in the literature. *Oskar* mRNA, which is localized to the posterior pole of the *Drosophila* oocyte, is regulated at the translational level by the RNA-binding protein Bruno (Kim-Ha *et al.* 1995). Bruno has been shown to not only recruit the eIF4E-binding protein Cup to repress translation (Wilhelm *et al.* 2003; Nakamura *et al.* 2004), but is also responsible for the formation of large mRNP silencing particles (Chekulaeva *et al.* 2006). More relevant to us, perhaps, is the observation that PTB can also induce particle formation and translational silencing of localizing *oskar* (Besse *et al.* 2009). Indeed, when we remove PTB binding sites from the minimal 2x135 VLE we see a reduction in particle forming ability and translational silencing is diminished (Appendix).

We have also uncovered a putative role for Vera in the translational repression of Vg1 (Appendix). Like PTB, Vera contains multiple RNA-binding domains (Git and Standart 2002) and could therefore play an important role in the formation of higher-order mRNP structures. The chicken orthologue of Vera, ZBP-1, has been

implicated in the translational control of  $\beta$ -actin mRNA (Huttelmaier *et al.* 2005), which is localized to the leading edge of motile fibroblasts (Lawrence and Singer 1986). This repression activity, which acts at the level of translation initiation, is relieved upon the phosphorylation of ZBP-1 by Src kinase (Huttelmaier *et al.* 2005). Thus, Vera remains an attractive candidate for the translational control of Vg1, since it too is phosphorylated upon oocyte maturation concurrent with an increase in Vg1 translation (Git *et al.* 2009). That PTB and/or Vera act in the control of Vg1 translation could also provide a link between localization and the control of Vg1 translation, since both proteins are required for faithful vegetal accumulation of the mRNA (Deshler *et al.* 1997; Cote *et al.* 1999).

Preliminary studies in the lab have also shown that *Xenopus* FMRP localizes to the vegetal pole of the oocyte (T. Wood, C. Carbone, unpublished). This protein has previously been shown to be involved in both the localization and translational control of a number of neuronal mRNAs (reviewed in Bassell and Warren 2008), and its malfunction through expansion of a triplet repeat cause Fragile X syndrome in humans (Verkerk *et al.* 1991). Interestingly, FMRP has a propensity for binding to G-quadruplex motifs within RNAs (Chapter 1, Fig. 1; Darnell *et al.* 2001), although no such structures have been predicted to form within the Vg1 3'-UTR. It remains to be seen how FMRP might function in Vg1 metabolism, but it is currently an intriguing candidate for the regulation of both vegetal localization and translational control.

Further studies on the components of the translational repression machinery in *Xenopus* oocytes will hopefully benefit from the *in vitro* translation system described in Chapter 3 (Pratt and Mowry 2010). Initial attempts to use immunodepletion techniques to remove candidate proteins such as Vera and PTB from the oocyte lysate have been less than successful (C. Pratt, unpublished), presumably due to the sensitive nature the *in vitro* translation system to temperature, dilution factors, and long incubation times. Optimization of the system would be a valuable endeavor, since *in vitro* translation systems have provided valuable insight into translational control processes in a number of instances (Matthews and Colman 1995; Lie and Macdonald 1999; Thermann and Hentze 2007). Indeed, with the advances being made in the field of *Xenopus* transgenesis (Rankin *et al.* 2011; Zuber *et al.* 2012), it is not outside the realm of possibility to potentially assay translational control in extracts made from Vera or PTB null or mutant oocytes.

### ***Directional transport of Vg1***

Translational repression is imperative for the control of post-transcriptional gene expression, but if the ultimate goal is to generate polarized protein expression, as is the case for Vg1 and many other localized mRNAs, enrichment of that mRNA in a specific region of the cell is critical. As described in the introduction, Vg1 relies on active mRNA transport along the microtubule cytoskeleton into the vegetal hemisphere of a stage II/III oocyte and on to the vegetal cortex where it is anchored (Yisraeli *et al.* 1989). When I joined the lab it was understood that the plus-end directed microtubule motor kinesin-2 was required for this localization process

(Betley *et al.* 2004), however it was not clear how this motor could function in a cell that contained a microtubule cytoskeleton oriented with minus-ends at the cortex and plus-ends at the nucleus (Pfeiffer and Gard 1999). We subsequently demonstrated a role for kinesin-1 in Vg1 localization, and showed that there is a subpopulation of microtubules in the vegetal cortex of stage II/III oocytes that is oriented with plus-ends at the cortex (Messitt *et al.* 2008; Chapter 4).

The presence of the plus-end out subpopulation of microtubules is transient, with its appearance in stage II oocytes coinciding with the breakdown of the mitochondrial cloud (Heasman *et al.* 1984; Messitt *et al.* 2008). This observation lends weight to the hypothesis that the early pathway of mRNA localization, or at least the activity of the mitochondrial cloud, is required to set up the late localization pathway. Indeed, the mitochondrial cloud has previously been shown to contain a gamma-tubulin-positive structure that could function as a microtubule organizing center for the plus-ends out subpopulation of microtubules in the vegetal cytoplasm (Kloc and Etkin 1998).

In conclusion, I have presented evidence implicating the miRNA pathway (Chapter 2), and PTB and Vera (Appendix), in the control of Vg1 translation in the *Xenopus* oocyte. In addition, I have generated a cell free system for the future study of translational control mechanisms in this organism (Chapter 3) and contributed to our understanding of the vegetal transport process (Chapter 4). This thesis

therefore addresses questions central to the process of polarized protein expression, a cellular event that is vital to the success of life on this planet.



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**Appendix: A potential role for VLE-dependent translational repression through RNP granule formation.**

**Catherine A. Pratt**, James A. Gagnon, and Kimberly L. Mowry

*Figure 3B by JAG. All other experiments performed by CAP.*

## Summary

Vg1 mRNA is directed to the vegetal pole of developing *Xenopus laevis* oocytes by a *cis*-element in its 3'-UTR (vegetal localization element; VLE; Fig. 1A; Mowry and Melton 1992). Contained within this element are sequences that are known to bind specific *trans*-acting proteins that are required for faithful Vg1 localization: VM1 sites recruit the polypyrimidine tract binding protein (PTB; also known as hnRNPI; Cote *et al.* 1999) and E2 sites recruit Vera (also known as Vg1RBP/xVICKZ3; Deshler *et al.* 1997). The first 135 nucleotides of the VLE contains a pair of E2 sites and a VM1 site, and a duplication of this element (2x135) is sufficient to direct vegetal localization of an injected RNA (Gautreau *et al.* 1997). Indeed, specific requirements for the clustering and spacing of the VM1 and E2 sites have been demonstrated (Lewis *et al.* 2004) which, combined with the fact that PTB and Vera both contain multiple RNA-binding domains (Fig. 2A), suggests that these proteins might direct the formation of large mRNP (messenger ribonucleoprotein particle) granules. In other systems the importance of mRNP granule formation in localization has been demonstrated, with large particles being a prerequisite for efficient mRNA transport (Glotzer *et al.* 1997; Krichevsky and Kosik 2001; Wilhelm *et al.* 2003). Vg1 translation is tightly coupled to mRNA localization, and protein accumulation cannot be detected until localization is complete in stage IV of oogenesis (Dale *et al.* 1989; Chapter 2 Fig. 5). This suggests that translational control is linked to localization. Previously, an element downstream of the VLE was shown to contain translational repression activity, and has been named the translational control element (TCE; Fig. 1A; Wilhelm *et al.* 2000; Otero *et al.* 2001), however, this element cannot completely



abrogate translation, conferring only ~50% repression over a luciferase reporter (Fig. 1). Granule formation has been linked to translational control in *Drosophila* oocytes, where Bruno binds the 3'-UTR of *oskar* mRNA, nucleates a large mRNP, and blocks translation by preventing access of the initiation machinery to the mRNA (Chekulaeva *et al.* 2006). *Oskar* is also regulated by PTB, again through granule formation, suggesting that this protein might act similarly in *Xenopus* oocytes (Besse *et al.* 2009). Given the precedent for granule formation in translational repression, the tight coupling of localization and translational control, and the requirement for putative mRNP nucleators PTB and Vera in localization, we set about testing the role of the VLE in translational repression of Vg1.

## Results and Discussion

### *The VLE can direct repression of a reporter RNA*

The dual luciferase assay system has proven an invaluable tool in the assessment of translational control mechanisms in a number of model systems. We therefore generated a series of firefly luciferase reporter constructs with downstream regulatory elements from the Vg1 3'-UTR (VLE, TCE, VLETCE) in a vector (pSP64-TSN) that allowed us to transcribe capped mRNA *in vitro* for microinjection from a viral SP6 promoter (Fig. 1B). As a positive control, we used the *Xenopus*  $\beta$ -globin 3'-UTR, which is unregulated in the oocyte. To control for injection volume we also *in vitro* transcribed Renilla mRNA from the pRL-null plasmid (Promega) using the upstream T7 promoter. For each reporter, firefly and Renilla mRNAs were assayed for concentration on a Nanodrop spectrometer and mixed together in an

appropriate manner. These mixes were then assayed for activity using an *in vitro* rabbit reticulocyte lysate translation system (Promega) to ensure that they all translated equally, and then injected into stage II oocytes (Fig. 1C). After overnight incubation, translation was measured using commercially available reagents (Promega). From the data presented in Figure 1D we concluded that the VLE is able to repress translation of the luciferase reporter in a manner comparable to the levels of repression conferred by the TCE. Interestingly, these effects are neither synergistic nor additive (Fig. 1D, VLETCE construct), perhaps suggesting that these elements repress translation in distinct fashions. Indeed, in Chapter 2, I presented evidence suggesting that the TCE exerts its effects through the miRNA pathway, and the Standart lab has also proposed a role for ElrB acting on this element (Colegrove-Otero *et al.* 2005). That multiple mechanisms of translational control could act on a single localized mRNA is not a novel concept. A prominent example of this is that of *oskar* mRNA which is regulated by the activity of the eIF4E-binding protein Cup ((Wilhelm *et al.* 2003; Nakamura *et al.* 2004), through Bruno-dependent granule formation (Chekulaeva *et al.* 2006), and through PTB-dependent granule formation (Besse *et al.* 2009).

### ***The minimal VLE, 2x135, recapitulates VLE-mediated translational repression***

Previous work from our lab has shown that a minimal 2x135 element is sufficient to direct localization of an injected RNA to the vegetal pole of the oocyte (Gautreau *et al.* 1997). We were therefore curious if the 2x135 element could also direct translational repression of a reporter mRNA. To test this hypothesis, we generated a

series of reporter constructs that contained in their 3'-UTRs either the 2x135, or a version of the element that has mutations in either the VM1 sites or the E2 sites (Fig. 2B). A version of the 2x135 with all six of these sites mutated was also included, as was the control  $\beta$ -globin control reporter used in the previous section (Fig. 2B). As before, reporter mRNAs were *in vitro* transcribed using an appropriate viral RNA polymerase and injected into oocytes (Fig. 2C). After overnight incubation, luciferase activity was assayed and the data are presented in Figure 2D. We found that the 2x135 element is indeed sufficient to repress luciferase translation in a manner similar to that of the VLE (compare Figs. 1D and 2D). While removing either the E2 sites or the VM1 sites had a measurable effect on translational repression, removal of all these sites resulted in almost complete relief of repression (Fig. 2D). These data therefore suggest that both the E2 sites (Vera-binding) and the VM1 sites (PTB-binding) are required for VLE-dependent translational repression. However, deleting only one type of site had only an intermediate effect. This suggests that each site is able to compensate somewhat for the loss of the other. If these sites are acting upon translation through the formation of granules, coordinated by PTB and Vera, one might expect that this would be the case, since one protein could compensate for the other. Alternatively, both proteins work together to nucleate granule formation, and in either the mutE2 or mutVM1 situation, protein-protein interactions are compensating for a reduction in RNA-protein interactions. A final possibility is that the injected reporter-RNAs are "piggy-backing" onto endogenous Vg1 mRNPs and thus gaining access to the translational repression machinery contained therein.

### ***E2 sites and VM1 sites are required for granule formation in vitro***

Both E2 sites and VM1 sites are required for Vg1 localization (Lewis *et al.* 2004), and we have now demonstrated a role for these sites in translational repression (Fig. 2D). These data could be reconciled in a model whereby Vera and PTB act to nucleate the formation of a large localizing granule that excludes the translational machinery. Indeed, PTB co-localizes in large structures at the vegetal pole of the oocyte with endogenous Vg1 mRNA (Cote *et al.* 1999) and Vg1 mRNA sediments in large particles after sucrose density fractionation (Fig. 3A). To test the hypothesis that PTB and Vera are required for granule formation, we developed an *in vitro* assay (Fig. 3B). Briefly, 2x135, 2x135mutE2, and 2x135mutVM1 RNAs were transcribed in the presence of <sup>32</sup>P-UTP to generate labeled transcripts. These labeled RNAs were then incubated with an oocyte lysate at room temperature to allow for protein binding. The mixtures were centrifuged at 25,000 x *g* and the pellet and supernatant were separated into scintillation vials. The radioactive label in the pellet was then compared to that in the supernatant, and a ratio of pellet:supernatant was used as a readout for granule formation, with high values corresponding to efficient granule formation. Using this assay, we determined that both VM1 sites and E2 sites are required for granule formation *in vitro* (Fig. 3C). While corroborating the importance of these sites in granule formation, it is not clear why these mutant RNAs should have such a strong effect in this assay, whereas in the translation assay it took mutation of all the sites to see a robust relief of repression (Fig. 2D). It could be that our *in vitro* granule assay lacks the resolution of the luciferase assay, in that it cannot differentiate between particles of different

size. One could imagine a scenario in which mutation of either the E2 sites or the VM1 sites reduces the size of the granules formed thus preventing them from pelleting. However, these small granules could still be competent in translational repression. This issue remains unresolved at this time, although modifications to the pelleting assay to address this question (through the use of size exclusion columns) are being made (C. Neil, T. Wood, and J. Gagnon, unpublished).

### ***A tethering assay for assessing protein contribution to translational repression***

Though experiments involving the mutation of the E2 and VM1 sites are indicative of a role for their known binding-partners, Vera and PTB, respectively, they do not conclusively show that these proteins function in translational repression and/or granule formation. The possibility remains that mutation of the sites interferes with the binding of an alternate RNA-binding protein, or that the sites themselves are the targets of other regulatory molecules. To address this possibility, we turned to a system of tethering our candidate proteins to an engineered RNA. First, we replaced all four E2 sites in the 2x135 with MS2 hairpins, a sequence of RNA that is found in the MS2 bacteriophage and specifically bound by the MS2 coat protein (MCP). We also replaced the VM1 sites with BoxB sequences, which are derived from bacteriophage  $\lambda$  and are recognized by the protein  $\lambda$ N. We then generated fusion constructs that allow us to control which protein, PTB or Vera, is recruited to the 3'-UTR of the firefly luciferase reporter (Fig. 4A). Fusion constructs were injected into stage II oocytes, either together or separately, and allowed to express overnight (Fig. 4B). The next morning, the reporter mRNAs were injected and allowed to express

for 6 hours before preparation of lysates and luciferase assay. Samples were also retained for western blotting to assess expression of the fusion proteins (Fig. 4C). The data from a representative experiment are shown in Figure 4D, and indicate that tethered PTB is able to repress translation of the modified 2x135 reporter. However Vera exhibits the opposite activity, showing an increase in translation when tethered. Furthermore, when both fusion proteins are expressed, the Vera phenotype dominates. While these results are unexpected, there are a number of possible explanations. Firstly, as is evidenced by the western blotting data (Fig. 4C), Vera-MCP is not expressed to the same level as the endogenous protein, nor to the same level as PTB- $\lambda$ N. Given that Vera has been suggested to self-associate to form a dimer *in vivo* (Git and Standart 2002), low expression of the fusion protein might prevent it from functioning in this assay. This hypothesis, however, does not account for the observed levels of translation when both fusion proteins are expressed. Indeed, if this were the case one might expect the PTB phenotype to dominate. Alternatively, it is possible that PTB binding is required to recruit Vera and thus nucleate granule formation. Such an interaction between these proteins has been documented, with both proteins binding VLE RNA in the nucleus, but upon export to the cytoplasm the complex is remodeled such that Vera no longer directly contacts the RNA but remains associated with PTB (Lewis *et al.* 2008). If this were the case, co-expression of the Vera-MCP fusion protein might act in a dominant negative manner by binding to the PTB- $\lambda$ N:2x135mod complex through a protein—protein interaction, thus occluding recruitment of endogenous Vera and preventing granule formation. Unfortunately, distinguishing between these possibilities (and a

number of others that could potentially be at play, such as the involvement of factors other than Vera and PTB) has proven extremely complicated, and as such this project remains incomplete. It will, however, be interesting to see if improvements to the *in vitro* granule assay and *in vivo* expression of Vera-MCP will, in the future, shed some light on the involvement of PTB and Vera in granule formation and translational control of Vg1.

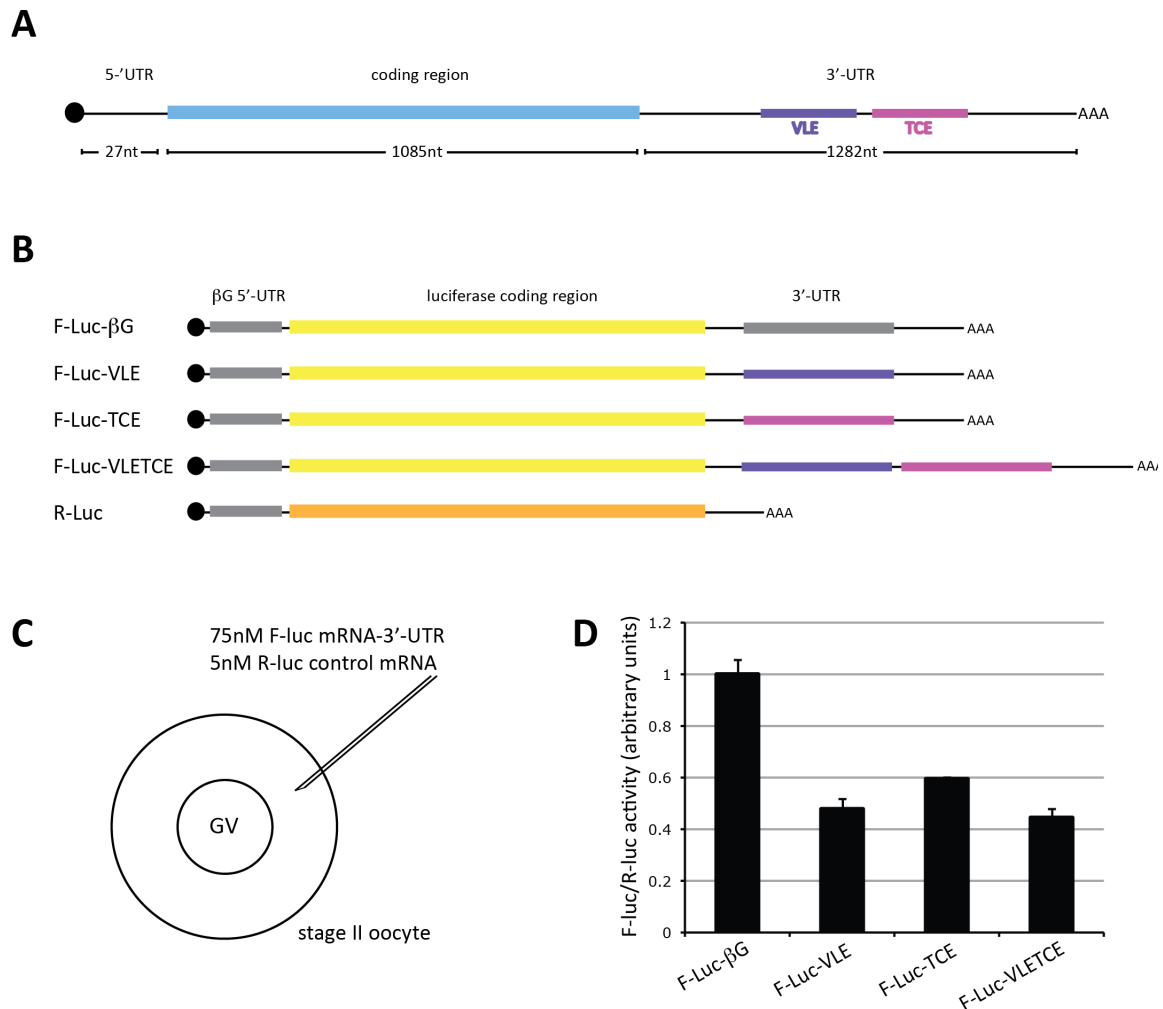
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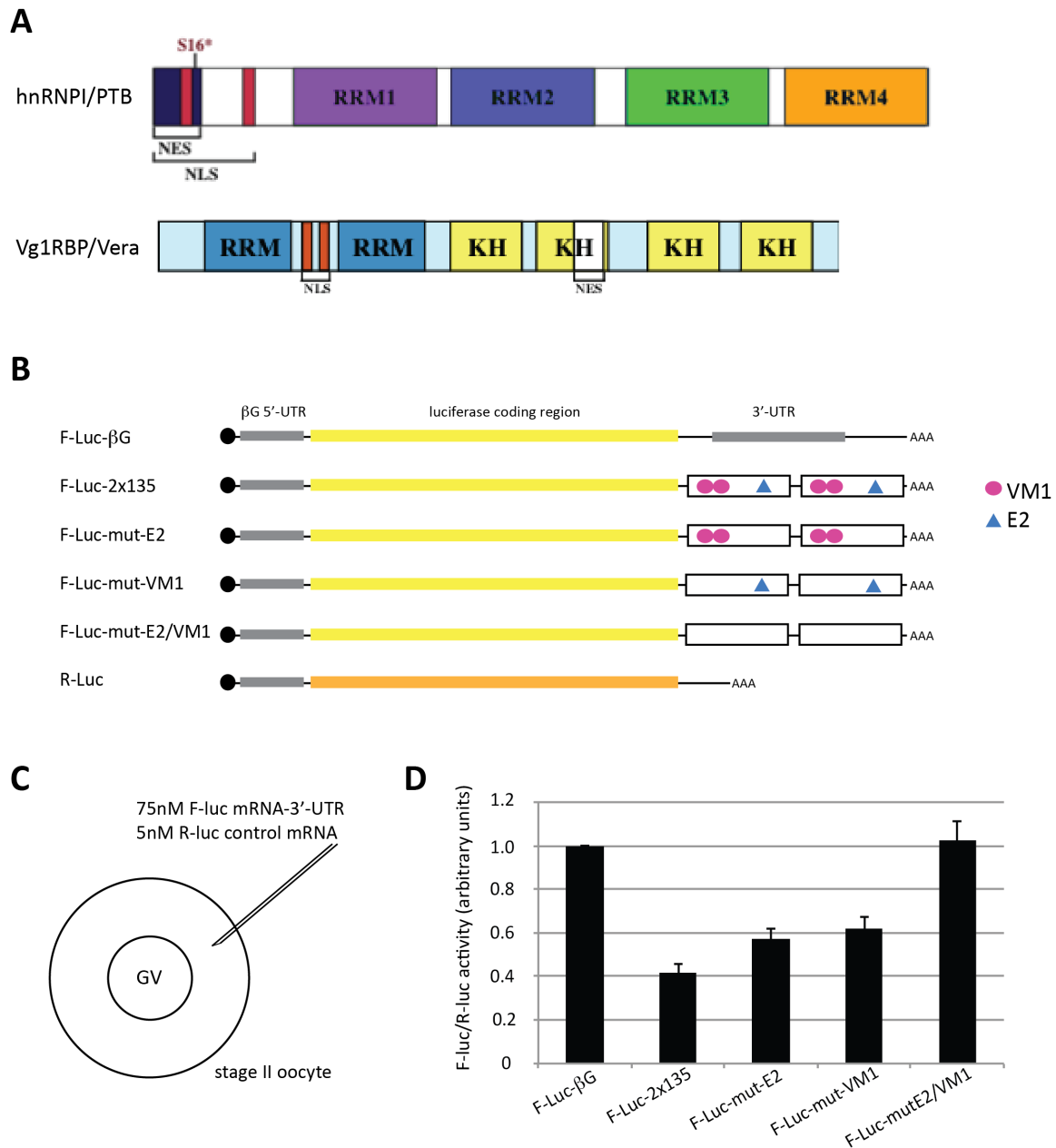


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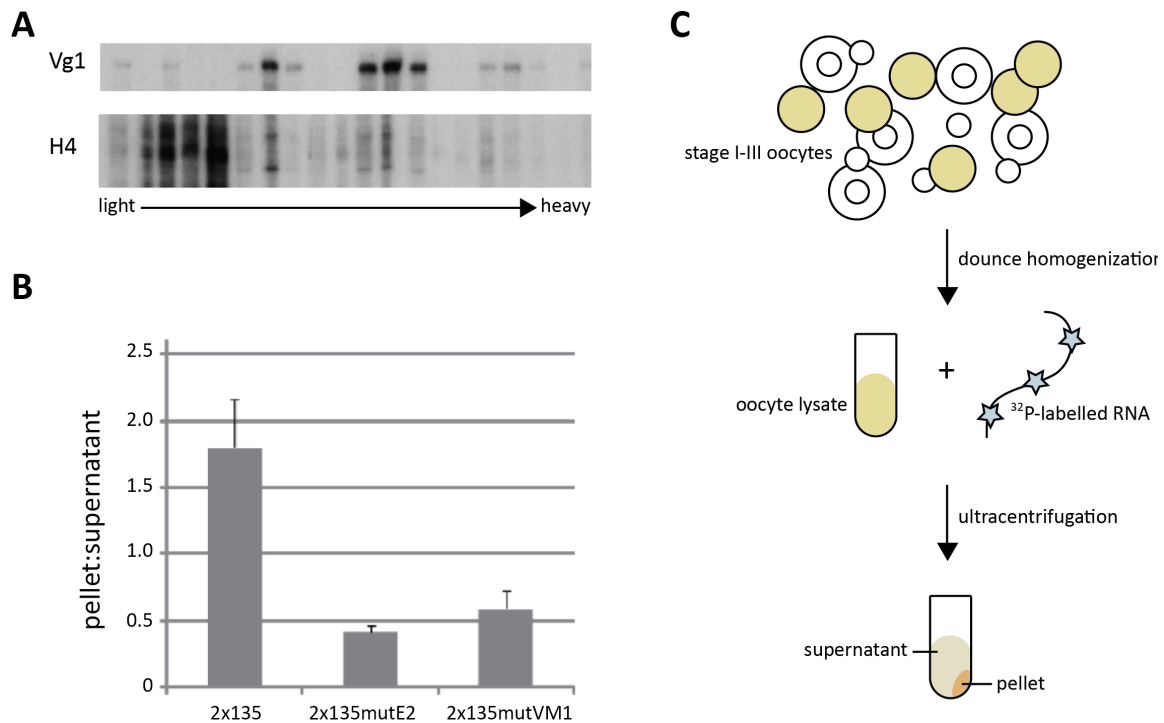
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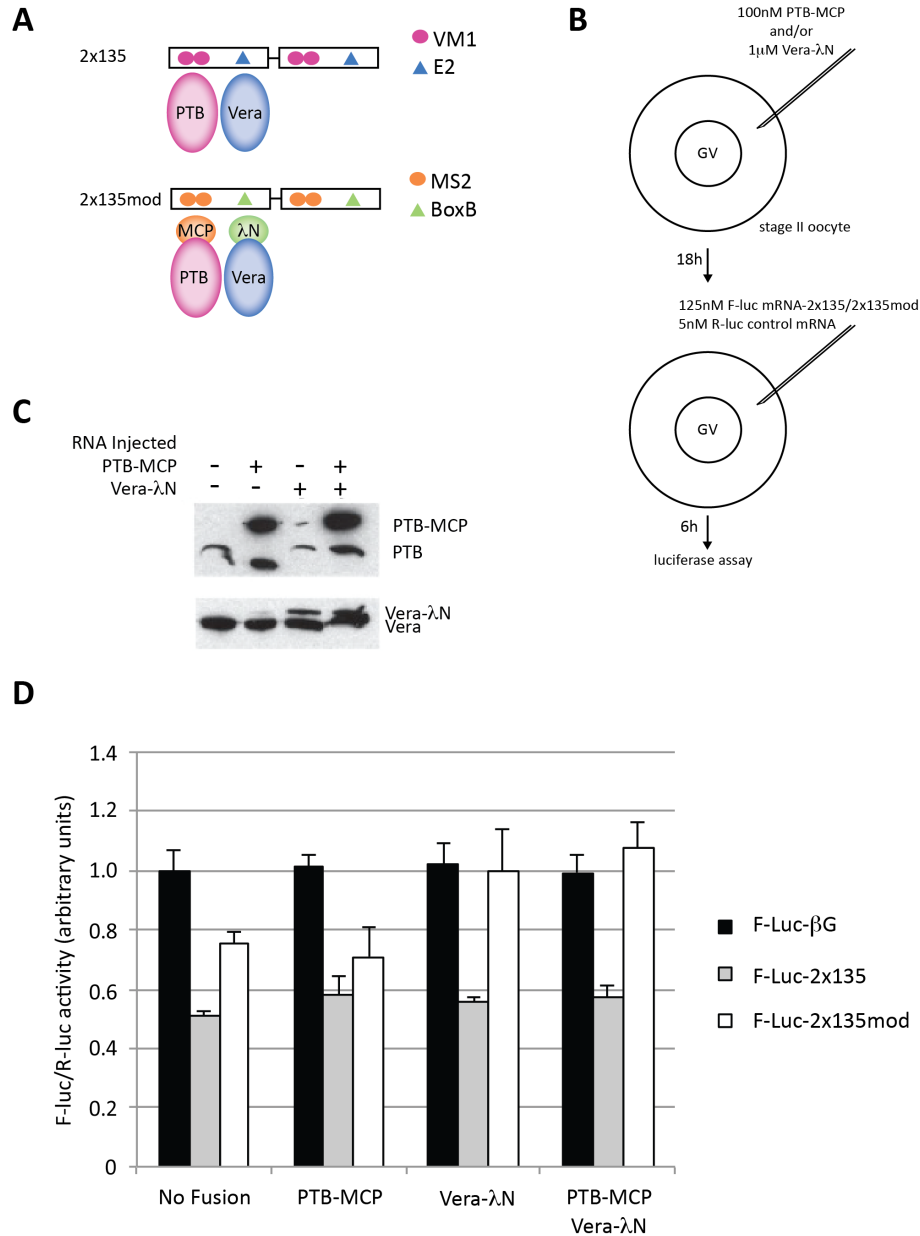
**Figure 1. The VLE contains translational repression activity.** **A.** A schematic of the Vg1 mRNA. The coding region is shown in blue, with the positions of the VLE and the TCE shown in purple and pink, respectively. **B.** A series of firefly luciferase reporters (yellow) to test translational control activity of the Vg1 3'-UTR elements. As a control, a reporter construct was made containing the unregulated  $\beta$ -globin 3'-UTR. As an internal control for injection volume, Renilla luciferase mRNA (orange) was co-injected with each firefly luciferase reporter. **C.** mRNAs were injected into stage II oocytes and allowed to express overnight before being lysed and subjected to analysis by dual luciferase assay (Promega). **D.** Results of the luciferase assay. Oocytes were assayed in three pools of 10, therefore for each bar,  $n=30$ , and error bars represent the standard deviation from the mean. Levels of translation from the  $\beta$ -globin control mRNA, normalized to Renilla luciferase expression, were set to 1 and all other data points are shown relative to that.



**Figure 2. The RNA-binding proteins PTB and Vera are required for VLE-dependent translational repression of a reporter.** **A.** A representation of the domain structure of PTB and Vera with other important elements, including nuclear import and export sequences, marked. **B.** A second series of firefly luciferase reporter constructs. The position of PTB (VM1) and Vera (E2) binding sites within the 2x135 minimal VLE are shown as pink circles and blue triangles, respectively. **C.** mRNAs were injected into stage II oocytes and allowed to express overnight before being lysed and subjected to analysis by dual luciferase assay (Promega). **D.** Results of the luciferase assay. Oocytes were assayed in three pools of 10, therefore for each bar, n=30, and error bars represent the standard deviation from the mean. Levels of translation from the  $\beta$ -globin positive control mRNA, normalized to Renilla luciferase expression, were set to 1 and all other data points are shown relative to that.



**Figure 3. PTB and Vera direct granule formation in the oocyte.** **A.** Vg1 sediments in large particles on a sucrose gradient. Oocyte lysates were layered onto 0-55% sucrose gradients and subjected to ultracentrifugation for 2 hours. The gradient was then fractionated, and the RNA in each fraction was isolated by phenol extraction and ethanol precipitation. Shown is an autoradiograph of the fractions after RNase protection and resolution on an 11% urea gel. **B.** A schematic representation of the *in vitro* granule assay that is described in the text. **C.** Wild type labeled 2x135 partitions efficiently into the pelleted fraction, whereas 2x135mutE2 and 2x135mutVM1 RNAs do not. Data are represented (in arbitrary units) as a ratio of (counts per minute in the pellet)/(counts per minute in the supernatant), as measured in a scintillation counter.



**Figure 4. A tethering assay to assess the relative contribution of PTB and Vera to VLE-dependent translational repression.** **A.** A representation of the modified 2x135. E2 sites (pink circles) were replaced with MS2 sites (orange circles) and VM1 sites (blue triangles) with BoxB sites (green triangles) to create 2x135mod. This modified UTR was then cloned downstream of the firefly luciferase gene to generate the reporter construct F-Luc-2x135mod. Fusion protein constructs coding for PTB-MCP and Vera-λN were also generated. These proteins would be expected to bind 2x135mod but not 2x135. Similarly, 2x135mod should not recruit endogenous PTB or Vera. **B.** mRNAs encoding the fusion proteins were injected into stage II oocytes and allowed to express overnight. The next day, F-Luc-2x135 or F-Luc-2x135mod RNAs were co-injected with Renilla luciferase mRNA and allowed to express for 6 hours before being lysed and subjected to analysis by dual luciferase assay (Promega). **C.** Expression levels of the fusion proteins. Oocyte lysate was separated by PAGE and subjected to western blotting for either PTB (top panel) or Vera (bottom panel). **D.** Results of the luciferase assay. Oocytes were assayed in three pools of 10, therefore for each bar, n=30, and error bars represent the standard deviation from the mean. Levels of translation from the β-globin control mRNA, normalized to Renilla luciferase expression, were set to 1 and all other data points are shown relative to that.