

Insights into Maternal mRNA Localization:
Characterization of a New Cytoplasmic RNA Granule

By

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B.A., Colby College, 2007

Thesis

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This dissertation by Christopher Richer Neil is accepted in its present form by the Department of Molecular Biology, Cell Biology, and Biochemistry as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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Laviolette LA, Wilson J, Koller J, **Neil C**, Hulick P, Rejtar T, Karger B, The BT, Iliopoulos O. Human folliculin delays cell cycle progression through late S and G2/M-phases: effects of phosphorylation and tumor associated mutations. PLoS One. 2013 Jul 11;8(7):e66775.

Zimmer M, Lamb J, Ebert BL, Lynch M, **Neil C**, Schidt E, Golub TR, Iliopoulos O. The Connectivity Map links iron regulatory protein-1-mediated inhibition of hypoxia inducible factor-2a translation to the anti-inflammatory 15-deoxy- $\Delta^{12,14}$ -Prostaglandin J₂. Cancer Res. 2010 Apr 15;70(8):3071-9.

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To Erin, I do not think I have met a more passionate person in my life. You bring fire and dedication to all that you do, both professionally and personally. There is a reason everyone goes to you for advice and I hope that you never stop being a source of wisdom for me.

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Chapter One: Introduction

“The living Protoplasm ... is a liquid, or rather a mixture of liquids,
in the form of a fine emulsion consisting of continuous substance
in which are suspended drops ... of different chemical nature”
- Edmund B. Wilson, 1899

Organization of the intracellular space is fundamental to life. Through it, control over both spatial and temporal expression of genes allows cells to orchestrate complex biochemical processes in a dynamic manner — capable of responding to cellular cues associated with growth, development, and stress, as well as providing a framework for specifying a diversity of cellular functions and identities. Taking a eukaryote-centric view, the most obvious distinction in cellular organization is the sequestration of genomic DNA from the cytoplasm by a selectively permeable nuclear envelope. By physically separating DNA from the translational machinery, cells are able to apply layers of regulation between the transcription of mRNA in the nucleus and its subsequent translation into protein in the cytoplasm. Beyond the nucleus, cellular organization continues to be a prominent biological mechanism in the cytoplasm. Membrane delimited organelles spatially restrict specific cellular processes in order to regulate flux and protect the cell from potentially harmful byproducts. Intriguingly, while traditionally thought to be a homogenous space for biological activity, the surrounding cytoplasm is emerging as an active medium, capable of functioning in the control of gene expression. Shifting away from the view of the cytoplasm as a homogenous fluid to a more dynamic view of cytoplasmic organization has given new life to the concept of a “living protoplasm,” and reshaped our understanding of cell and molecular biology.

Highly polarized cells, such as germ cells or neurons, represent an extreme example of cytoplasmic organization, often relying on the transport and localization of mRNAs to create compartments with specific cellular functions or blueprints (Martin and Ephrussi 2009; Holt and Bullock 2009; Buxbaum et al. 2015; Chin and Lécuyer 2017). However, RNA localization is not unique to polarized cells. The need to organize functional space is fundamental to the capacity of cells to coordinate a multitude of biochemical reactions while remaining responsive to environmental cues. Within the cytoplasm, stress granules and P-bodies have emerged as hubs for sequestering mRNAs and proteins away from the general cytoplasmic milieu (Balagopal and Parker 2009; Decker and Parker 2012). In doing so these structures organize mRNAs along with specific protein factors that allow the cell to finely-tune control of gene expression. While differing with respect to the ultimate functional goal of transcript organization, these cytoplasmic structures are conceptually similar to localized complexes of mRNA and protein, in that they represent translationally repressed higher-order assemblies that the cell has designated for a specific function.

Intriguingly, while functionally distinct, macromolecular assemblies of RNA and protein, termed ribonucleoprotein (RNP) bodies, including localization competent RNPs and traditionally immobile structures such as stress granules and P-bodies, share many compositional similarities. RNP granules are thought to be extremely heterogeneous in composition and held together by weak interactions between components (Banani et al. 2017; Shin and Brangwynne 2017). Moreover, the components of these systems appear to be in dynamic exchange with the surrounding cytoplasm. How these structures are able to concentrate molecules, maintain and regulate their structure, and control their composition in the absence of a physical barrier has been a question that has perplexed

researchers investigating the challenges of spatiotemporal control of gene expression. However, within the last ten years, seminal papers examining the physical nature of P granules in *C. elegans* and nucleoli in *Xenopus laevis* have produced a new physical model of membrane-less compartments based on principals of polymer chemistry and soft matter physics (Brangwynne et al. 2009, 2011, 2015). In this model, RNP granules can be viewed not as continuations of the cytoplasm, but rather as phase distinct structures demonstrating differing degrees of fluidity based on the physical properties of their constituents (Banani et al. 2017; Shin and Brangwynne 2017). Work both *in vitro* and *in vivo* has demonstrated the capacity of RNP granule constituents to phase separate from their surroundings given an appropriate concentration of reactants (Li et al. 2012; Kato et al. 2012; Brangwynne et al. 2015; Lin et al. 2015; Banani et al. 2016; Protter et al. 2018). In this way, scientific fields that had previously been distinguished based on characterization of specific cellular functions have converged, with demixed phases of proteins and RNAs serving as a unifying principle of subcellular organization and as a powerful new mechanism for generating localized function.

In this thesis, I will define the structure in which maternally loaded mRNA transcripts localize in *Xenopus laevis* oocytes as a phase-separated transport RNP granule. Using a new imaging strategy, my work shows that these granules are large heterotypic complexes, containing multiple mRNA transcripts. Through a combination of biochemical purification and mass spectrometry, this work reveals the protein composition of the transport granule as bearing significant overlap with other classes of cytoplasmic granules, as well as possessing a set of unique granule proteins. Moreover, examination of the relationship between both the protein and RNA components of the *Xenopus* vegetal

transport granule reveals insights into phase separated bodies, and hints at a novel multiphase structure with dynamic protein constituents and a solid RNA-containing scaffold.

mRNA Localization

Subcellular localization of mRNAs in conjunction with regulated translation is a vital biological mechanism for the generation and maintenance of polarized gene expression. Employed in both somatic and germ cells, mRNA localization has traditionally been viewed in the context of generating specialized subcellular compartments, which have been functionally linked to a diverse array of cellular processes. Some of the most prominent examples include the control of mating type switching in yeast, cytoskeletal-mediated motility in fibroblasts, axonal guidance and synaptic plasticity in neurons, and the establishment of developmental patterning in both vertebrate and invertebrate animals, and (reviewed in Martin and Ephrussi 2009; Holt and Bullock 2009; Buxbaum et al. 2014).

While the aforementioned examples are limited to mRNAs encoding proteins with specialized local functions, several studies have suggested that mRNA localization may be a more general aspect of RNA biology. A high-throughput, high-resolution *in situ* hybridization screen in *Drosophila* embryos revealed that up to 70% of endogenous transcripts exhibit distinct subcellular localization (Lécuyer et al. 2007). Similarly, in mammalian neurons fully half of mRNA transcripts are enriched in axons and dendrites relative to the cell body (Eberwine et al. 2002; Cajigas et al. 2012), in stark contrast to the previously held belief that only a small handful of mRNAs localized to synapses. Similar analyses in migrating fibroblasts (Mili et al. 2008) and *Xenopus* oocytes (Blower et al. 2007)

hint at a broader class of localized RNA transcripts. These studies, in combination with expanding data sets, may reveal previously unrecognized subcellular compartments and a more dynamic understanding of cellular architecture.

Organizing the spatial distribution of mRNAs offers a host of advantages to the cell (reviewed in Martin and Ephrussi 2009; Holt and Bullock 2009; Buxbaum et al. 2014). The first, and most powerful, is that it allows the cell to have exquisite spatiotemporal control over gene expression. Pairing spatial restriction of genes with tight translational controls, allows cells to mediate protein synthesis in response to dynamic cues generated either intrinsically or in response to external stimuli. By decoupling translation from transcription, mRNA localization can decentralize the control of gene expression, and forego the need for retrograde signaling from the cytoplasm to the nucleus, allowing for local control of translation. Second, mRNA localization can prevent proteins from acting ectopically, where their expression could be toxic or deleterious. Site specific translation also has the advantage of controlling the activity of nascent proteins. This can allow the cell to bias protein interactions to specific partners by generating local enrichment of specific proteins and RNAs. This may facilitate the assembly of macromolecular protein complexes that rely on appropriate stoichiometric concentrations of components for efficient formation. Lastly, there is an economic advantage to mRNA localization. It is energetically favorable to localize a small number of mRNAs to a specific site, where their continuous local translation can generate a locally high concentration of protein, rather than to localize each distinct protein from disparate sites to achieve the same cellular polarity.

Mechanisms of mRNA Localization

Targeting of a transcript for localization is initiated by *cis*-acting elements within the mRNA sequence. These localization elements, or zipcodes, are most frequently found in the 3' untranslated region (3'UTR) of mRNAs, although some have been identified in the 5'UTR or in the coding sequence (reviewed in Jambhekar and Derisi 2007). *Cis*-sequence elements are recognized by *trans*-acting protein factors, usually RNA-binding proteins (RBPs). Generally, these RBPs have roles in both the localization and translational control of their target mRNAs. As such, combinations of *cis*-elements can drive the assembly of specific RNA-protein complexes, ribonucleoproteins (RNPs), that can either independently, or in higher-order assemblies, mediate a diversity of functions. mRNA localization is achieved through three general mechanisms: (1) by local capture of freely diffusing mRNAs; (2) by local stabilization and regulated degradation of mRNAs; and (3) by active and directed transport of mRNA to a subcellular site. Either individually or in combination, these mechanisms act to generate biases in the intracellular distribution of mRNAs, as reviewed below.

Diffusion and Capture

The local capture mechanism acts on populations of mRNA that are initially evenly distributed throughout the cytoplasm (Martin and Ephrussi 2009; Holt and Bullock 2009). Stochastic interactions with anchoring complexes spatially restrict mRNAs, with local enrichment being driven by the total number and strength of capture events. An example of this strategy can be found in the localization of *nanos* mRNA to the posterior pole of

developing *Drosophila* oocytes. Here, *nanos* mRNA becomes entrapped in RNP complexes, termed germ granules, which are required for the specification of germline fate during embryogenesis (Gavis and Lehmann 1992; Kobayashi et al. 1996). While motor-based transport of the germ plasm organizing *oskar* mRNA and its subsequent translation is a prerequisite for the posterior accumulation of *nanos* mRNA (Ephrussi et al. 1991; Kim-Ha et al. 1991; Ephrussi and Lehmann 1992; Rongo et al. 1995), *nanos* mRNA localization itself is not dependent on microtubules (Forrest and Gavis 2003). *nanos* mRNA is maternally loaded from anteriorly-positioned nurse cells with localization occurring during a period in which transcripts largely circulate throughout the cytoplasm by diffusion, facilitated by a concerted microtubule-dependent streaming of the oocyte cytoplasm (Forrest and Gavis 2003). By this process, *nanos* mRNA becomes captured at the posterior of the oocyte (Forrest and Gavis 2003). Single-molecule fluorescence *in situ* hybridization (smFISH) studies of *nanos* mRNA, in addition to other germ granule mRNAs, demonstrate that they enter the oocyte as single-transcript RNPs that interact at the posterior of the oocyte to produce complex germ granules (Little et al. 2015; Trcek et al. 2015). Interestingly, while germ granule proteins appear to be homogeneously distributed within these structures, constituent mRNAs are present in distinct homotypic clusters (Little et al. 2015; Trcek et al. 2015). Germ granule mRNA capture is observed to occur in two distinct steps. Initially, mediated by a piRNA-Piwi adhesion trap present within posterior *oskar* complexes, pioneer mRNAs seed germ granules (Vourekas et al. 2016; Niepielko et al. 2018). Following this initial stochastic event, homotypic mRNA cluster growth proceeds through self-recruitment and is dependent on acquisition of additional *oskar* protein (Niepielko et al. 2018). While this diffusion capture method is

only able to sequester approximately 4% of bulk *nanos* mRNA to the posterior of developing oocytes, it is sufficient to generate a locally distinct population of germ granule specific mRNAs (Bergsten and Gavis 1999).

A *nanos* homolog, *xcat2* mRNA, also uses a capture-based method for localization in early *Xenopus* oogenesis (Kloc and Etkin 1995; Chang et al. 2004). *Xcat2* mRNA is concentrated in a mitochondria-rich, perinuclear region of the vegetal cytoplasm (Forristall et al. 1995). This structure, termed the Balbiani body, detaches from the nucleus and, in a microtubule-independent manner, moves to the vegetal cortex (Houston and King 2000). Accumulation of *xcat2* mRNA in the mitochondrial cloud is not accompanied by a decline in its overall transcript number in the oocyte, suggesting that its localization is accomplished through a diffusion-capture mechanism, rather than local protection from degradation (Chang et al. 2004). In zebrafish, *Danio rerio*, the protein Bucky Ball is required for the organization of the evolutionarily conserved Balbiani body (Marlow and Mullins 2008; Bontems et al. 2009). In the absence of Balbiani formation, vegetally localized mRNAs remain dispersed in the oocyte, leading to a lack of cell polarity (Marlow and Mullins 2008; Bontems et al. 2009). It is interesting to note that the Balbiani body, like *Drosophila* germ granules, relies on the assembly of a macromolecular RNP to facilitate mRNA capture (Chang et al. 2004; Vourekas et al. 2016; Niepielko et al. 2018).

Local Stabilization and Regulated Degradation

By selectively stabilizing an mRNA in a specific location and actively degrading transcripts outside of this space, a cell can effectively localize specific mRNAs. Localization of the mRNA encoding the heat shock protein 83 (*hsp83*) to the posterior pole plasm of

Drosophila embryos presents a clear example of this mechanism. Outside of the posterior, the RBP Smaug binds *cis*-elements in both the open reading frame and 3'UTR of *hsp83* mRNA and recruits the CCR4/Not deadenylase, effectively targeting transcripts for degradation (Holdeman et al. 1998; Tadros et al. 2007; Semotok et al. 2008). It has been suggested that the local expression of *oskar* mRNA inhibits Smaug binding and prevents *hsp83* degradation at the posterior pole (Chen et al. 2014).

Similarly, regulated degradation of *nanos* mRNA plays a role in its restriction to the posterior of *Drosophila* embryos. While previously noted that entrapment can sequester approximately 4% of bulk *nanos* mRNA at the posterior of developing oocytes, a Smaug-mediated degradation elsewhere in the embryo is able to amplify the concentration imbalance to greater than one hundred-fold enrichment relative to the surrounding cytoplasm (Bashirullah et al. 2001; Bergsten and Gavis 1999). In addition to driving local enrichment, regulated degradation also prevents the aberrant expression of mRNA, using control of mRNA stability as a means of post-transcriptional gene regulation.

Active and Directed Transport

Motor-based transport along cytoskeletal elements is the predominant mechanism of mRNA localization (reviewed in Gagnon and Mowry 2011). Directed motility allows mRNAs to move significantly faster than by simple diffusion alone (Fusco et al. 2003), and relies on complexes of motor proteins, RBPs, and adaptors. Coordination of RBP binding and function is essential during active transport, enabling fine-tuned control over distinct steps in the transport of RNPs (Amrute-Nayak and Bullock 2012; Soundararajan and

Bullock 2014). Localization elements can specify both the composition and stoichiometry of an RNP, with specific combinations leading to corresponding changes in the processivity and run length of motor associated-RNPs along microtubules (Amrute-Nayak and Bullock 2012; Sladewski et al. 2013). Directional specificity is achieved by exploiting the polarity of microtubule or actin filaments, with asymmetries in cytoskeletal structure providing a roadmap for motor-bound RNP complexes to shuttle mRNAs to targeted destinations (Gagnon and Mowry 2011).

In budding yeast, active transport of *ASH1* mRNA along actin filaments establishes cellular asymmetries that allow for mating type switching (Long et al. 1997; Bertrand et al. 1998). In this example, a localization element in *ASH1* mRNA is recognized directly by the RBP She2, which recruits *ASH1* mRNA to a myosin motor protein (Myo4) via the adaptor protein She3. This RNP complex is transported along the actin cytoskeleton to the bud tip during cell division, where it is subsequently anchored (Bobola et al. 1996; Takizawa et al. 1997; Bertrand et al. 1998; Beach et al. 1999; Takizawa and Vale 2000). Importantly, *ASH1* mRNA remains translationally repressed during transport (Bertrand et al. 1998; Chartrand et al. 2002). Binding of the RBPs Khd1 and Puf6 prevent ribosomal subunits from associating with the mRNA, thus inhibiting translation. At the bud tip, the membrane-associated casein kinase Yck1 phosphorylates Puf6 and Khd1 exclusively, leading to the translational derepression of *ASH1* mRNA (Gu et al. 2004; Paquin et al. 2007; Deng et al. 2008). By coupling translational control with localization, Ash1 protein expression is restricted to the daughter cells of dividing yeast, establishing a clear distinction between the mother and daughter cells (Long et al. 1997). This control is predicated on the ability

of *cis*-acting sequences in *ASH1* mRNA to recruit a specific set of *trans*-acting proteins, generating a functional higher-order transport RNP.

In vertebrate cells, motor protein-based localization of β -actin mRNA generates highly responsive cellular subdomains capable of mediating active actin polymerization in response to external stimuli (Kislauskis et al. 1997; Leung et al. 2006; Yao et al. 2006). In both chicken and mammalian fibroblasts, localization of β -actin mRNA and additional actin regulator mRNAs to the ARP2/3 actin-nucleating complex establishes a distinct leading edge lamellipodia (Kislauskis et al. 1997). Here, dramatic cytoskeletal rearrangements drive polarized cell movement (Kislauskis et al. 1997; Mingle et al. 2005; Mili et al. 2008). A localization element within the β -actin 3'UTR is recognized by the RBP ZBP1 and transported along actin filaments by the motor protein myosin (Ross et al. 1997; Mingle et al. 2005). In response to phosphorylation by the protein kinase SRC at the end of transport, translational repression of β -actin mRNA is relieved and a high local concentration of monomeric actin promotes polymerization (Hüttelmaier et al. 2005). Intriguingly, β -actin mRNA localization is also essential for steering axons in response to attractive and repulsive cues in the developing brain (Leung et al. 2006; Yao et al. 2006). β -actin in *Xenopus* growth cones is rapidly recruited to the near-stimulus side in response to attractive gradients. Disruption of either β -actin mRNA translation or transport abolishes attractive turning (Leung et al. 2006; Yao et al. 2006). The mechanistic and functional similarities of β -actin mRNA localization among cell types and biological processes highlights the conserved utility of motor-based transport and demonstrates the dynamic control that RNP localization provides in gene expression.

Active transport is also used in adult neurons, where mRNA localization is essential for general function. The importance of mRNA localization as a mechanism for posttranscriptional regulation can be observed in the high degree of autonomy exhibited by neuronal processes, which often extend significant distances from the cell body. Local translation at synapses is thought to underlie persistent changes in neuronal transmission, which are crucial for learning and memory (Martin et al. 2000). Neuronal mRNAs are transported in RNP granules along polarized microtubules by both dynein and kinesin motors (Kanai et al. 2004; Xu et al. 2013). Neuronal granules can be further sorted into distinct post-synaptic spines by myosin motors moving along the actin cytoskeleton (Mitsumori et al. 2017). This complex level of positional sorting is dictated by the specific components of transport RNP complexes. Isolation of these complexes has identified over 40 protein constituents, with few individual proteins being required for the actual localization of mRNA (Kanai et al. 2004). Instead, it has been proposed that the majority of these proteins function in translational control of the cargo mRNA (Kanai et al. 2004; Fritzsche et al. 2013). Translation is blocked within neuronal transport granules during their localization and is not relieved until synaptic activation (Wu et al. 2016). Interestingly, while the protein composition of neuronal granules is heterogeneous and complex, the mRNA content is not, consisting of single mRNAs (Mikl et al. 2011; Batish et al. 2012; Buxbaum et al. 2014). Each target mRNA contains a localization element sufficient to specify a unique RNP complex capable of directing both its localization and translation (Tübing et al. 2010; Mitsumori et al. 2017).

Active transport is also an essential mechanism for mRNA localization in germ cells. In *Drosophila* oocytes, localization of mRNAs is essential for developmental

patterning (reviewed in Weil 2014). *oskar*, *gurken*, and *bicoid* mRNAs are produced in nurse cells, and subsequently accumulate in the oocyte, where their specific localization specifies both the anterior-posterior and dorsal-ventral axis (reviewed in Weil 2014). Initial transport of *oskar* mRNA to the posterior of the oocyte is kinesin-dependent (Brendza et al. 2000), and establishes an initial population of Oskar protein capable of directing both the future capture of germ granule mRNAs, and preventing their Smaug-mediated degradation, as discussed earlier. *Drosophila* Oskar-containing germ granules are maintained at the posterior of the oocyte by persistent trafficking using both dynein and kinesin motors (Sinsimer et al. 2013). This motility relies on cortical microtubules in coordination with the actin cytoskeleton (Sinsimer et al. 2013). The anterodorsal- and anterior-localized mRNAs, *gurken* and *bicoid*, respectively, use the minus-end directed motor dynein in coordination with dramatic reorganizations of the microtubule network to direct their ultimate accumulation (Theurkauf et al. 1992; Kugler and Lasko 2009). Pairing end-directed motors with a highly dynamic polarized cytoskeletal network allows for fine-tuning of transport destinations using a largely conserved set of *trans*-acting factors (Weil 2014).

Xenopus oocytes are a powerful model for the study of motor-based mRNA transport in vertebrates. Localization of mRNAs to the vegetal cortex of *Xenopus* oocytes occurs through two distinct pathways (Kloc and Etkin 1995). While an initial, or “early”, localization pathway relies on capture of mRNA in the Balbiani body, as described for *xcat2* mRNA, a number of additional maternal mRNAs are actively transported to the vegetal cortex along microtubules as part of a “late-localizing” pathway (Messitt et al. 2008;

Gagnon et al. 2013). Work with one such mRNA, encoding the TGF β -growth family member, Vg1, will be the focus of this thesis. Specifically, my work builds on the previous body of knowledge characterizing *vg1* mRNA localization, reviewed below, and explores the composition of and relationship between components of a large transport-competent mRNP granule.

***vg1* mRNA Localization in *Xenopus laevis* oocytes**

Xenopus laevis oogenesis is classified into six distinct morphological stages, designated I-VI (Dumont 1972). While maternally loaded *vg1* mRNA is initially ubiquitously distributed throughout the cytoplasm of stage I oocytes, its localization to the vegetal cortex is required for the appropriate specification of germ layers (Thomsen and Melton 1993; Joseph and Melton 1998; Birsoy et al. 2006). During stages II and III of oogenesis, *vg1* mRNA becomes confined to the vegetal hemisphere, where it is subsequently anchored to the vegetal cortical cytoskeleton by stages IV-VI of oogenesis (Figure 1.1; Weeks and Melton 1987; Yisraeli et al. 1990). A 340-nucleotide vegetal localization element (VLE) found within the 3'UTR of the transcript is sufficient to specify vegetal RNA transport (Figure 1.2A; Mowry and Melton 1992), and contains clusters of short sequence motifs that are required for vegetal localization (Figure 1.2A; Gautreau et al. 1997; Deshler et al. 1998; Bubunencko et al. 2002; Lewis et al. 2004). The VLE interacts with a number of *trans*-acting RBPs in a sequence specific manner (Mowry 1996) including: Vera/Vg1RBP/Igf2bp3 (Deshler et al. 1998; Havin et al. 1998), PTB/hnRNPI (Cote et al. 1999), and hnRNPAB/40LoVe (Czaplinski et al. 2005), which will hereafter be

referred to as Vera, PTB, and hnRNPAB (Figure 1.2). Binding of PTB and Vera are essential for *vg1* mRNA localization, as mutations in their cognate binding sites within the VLE result in a complete loss of localization (Deshler et al. 1998; Havin et al. 1998; Cote et al. 1999; Lewis et al. 2004). Additionally, the RBPs Elavl-1 and Elavl-2 interact with sites within the *vg1* 3'UTR outside of the VLE, and are thought to play a role in the translational control of *vg1* mRNA (Figure 1.2B; Colegrove-Otero et al. 2005). While this principal set of proteins interacts with RNAs localizing to both the animal and vegetal cytoplasm of oocytes, interaction of the double stranded RBP Staufen with the VLE has been proposed to confer directional specificity to *vg1* and other “late-localizing” pathway mRNAs (Figure 1.2; Snedden et al. 2013).

vg1 mRNA localization is initiated in the nucleus with the formation of core RNP complexes consisting of *vg1* mRNA, Vera, and PTB (Kress et al. 2004). In the cytoplasm, these complexes are remodeled (Lewis and Mowry 2007), initiating recruitment of additional cytoplasmic RBPs, including Staufen (Yoon and Mowry 2004). Motor-based transport of *vg1* mRNA occurs in two stages. An initial unidirectional, dynein-mediated, transport step carries *vg1* mRNA to the lower vegetal cytoplasm (Gagnon et al. 2013). Here a kinesin-mediated second step takes advantage of a subpopulation of microtubules with their plus-ends directed towards the vegetal pole, to deliver *vg1* mRNA to the vegetal cortex (Messitt et al. 2008). Photoactivation and *in vivo* inhibition experiments indicate that *vg1* mRNA transport is bidirectional in the lower cytoplasm, which has been proposed to offer fine-tuned control over target site selection and prevent RNA escape from the vegetal cytoplasm (Gagnon et al. 2013).

Following transport, *vg1* mRNA is retained at the vegetal cortex in an, as yet, poorly understood anchoring step (Yisraeli et al. 1990). Anchoring is known to be dependent on an enriched actin and cytokeratin network at the cortex (Pondel and King 1988; Klymkowsky et al. 1991; Elinson et al. 1993; Forristall et al. 1995; Alarcón and Elinson 2001). Prior deposition of early RNAs, such as *xlsirts*, by the Balbiani body is also required for proper *vg1* mRNA anchoring (Kloc and Etkin 1994).

Organization of a large localization-competent RNP appears to be an early or initiating event in the *vg1* mRNA localization pathway. While RNP complexes have been functionally linked to the control of mRNA localization, mRNA translation, and mRNA stability in a number of systems (reviewed in Buchan 2014), a role for the *vg1* RNP, and possible higher-order assemblages thereof, has not been addressed. The dynamic nature of RNP granules offers an attractive regulatory mechanism, with changes in composition and remodeling events potentially mediating different stages in the localization pathway. However, while several *trans*-acting proteins have been identified, the full composition and molecular nature remains to be determined. Such characterization could potentially offer insights into *vg1* mRNA localization.

Interestingly, Many of the known *vg1* mRNA-interacting proteins are conserved amongst other localized RNPs: with PTB and Vera promoting oligomerization of higher-order structures in *Drosophila* germ cells and motile fibroblasts, respectively (Besse et al. 2009; Farina et al. 2003), and Staufen required for mRNA localization in neuronal granules and *Drosophila* oocytes (St Johnston et al. 1991; Tang et al. 2001; Mitumori et al. 2017). This compositional conservation extends beyond traditionally localized RNP structures to include other classes of cytoplasmic granules. Both stress granules and P-bodies, important

post-transcriptional regulators of gene expression (reviewed in Balagopal and Parker 2009; Decker and Parker 2012), contain nearly all of the known *vg1* RBPs (Jain et al. 2016; Hubstenberger et al. 2017). This raises the possibility that a greater understanding of the *vg1* RNP complex may provide insights into more general aspects of cellular regulation of gene expression.

Ribonucleoprotein Granules as Phase Separated Bodies

RNP granules have traditionally been classified in accordance with their cellular context and their presumed cellular function. In this way, germ granules (such as germ plasm in *Drosophila*, P-granules in *C. elegans*, Balbiani body, and nuage) have been considered distinct from other classes of cytoplasmic RNP granules (such as stress granules, P-bodies, and neuronal transport granules) (reviewed in Buchan 2014). Studying these structures independently has revealed much about their biological activity and identified many of the protein and RNA components involved in RNP granule assembly, maintenance, and function. Yet, while differences have emerged, fundamental commonalities between different RNP granules are also apparent. Granules are distinct cellular compartments that are not bound by membranes but are still capable of concentrating protein and RNA components, maintaining and regulating their structures, and controlling their composition to modulate biochemical activities (reviewed in Banani et al. 2017). In all cases, they contain repressed mRNAs that are capable of (re)-entering translation in response to appropriate signals (Hüttelmaier et al. 2005; Brengues et al. 2005; Bhattacharyya et al. 2006; Nagamori et al. 2011). And perhaps most intriguing, mRNP granules display broad overlap in protein composition (reviewed in Buchan 2014).

By shifting away from a pluralistic view of mRNP granules, a clearer picture of post-transcriptional gene regulation has emerged which unites many of the observed behaviors of membraneless compartments. Seminal work presented by Clifford Brangwynne and Anthony Hyman in 2009 proposed a new physical model for membraneless compartments, demonstrating that germline specifying P granules in *C. elegans* exhibit liquid-like behaviors which can be best understood using principles of polymer chemistry and soft matter physics (Brangwynne et al. 2009, 2015). P granules fuse with one another and subsequently relax back to a spherical shape, condense to solid surfaces in a process called wetting, deform under shear force, and are composed of proteins that are highly mobile within the granule and exchange rapidly with the surrounding cytoplasm as assessed by photobleaching experiments (Brangwynne et al. 2009). Such experimentation allowed for estimates of viscosity and surface tension, and demonstrated that P granules are materially distinct from the extra-granular space (Brangwynne et al. 2009). Brangwynne *et al.* went on to propose that P granules form through liquid-liquid demixing (phase separation) (Figure 1.2A) from the surrounding cytoplasm in response to polarity protein gradients which vary the condensation point across the cell (Brangwynne et al. 2009). P granules are effectively demixed at the posterior of embryos by a local concentration of reactants that favor phase separation, while prevented from doing so in the rest of the cell. Under this model, even weak gradients of regulator proteins can drive position-dependent phase separation and create a sharp threshold point between mixed and demixed phases (Figure 1.3; Brangwynne et al. 2009; Lee et al. 2013). Through their efforts, Brangwynne and Hyman present phase structuring as a potential general mechanism for organizing the

cytoplasm into dynamic RNP assemblies held together by weakly “sticky” molecules (Brangwynne et al. 2009).

Extending the phase-separation model, other RNP granules have been shown to exhibit liquid-like properties. Both Nucleoli (Brangwynne et al. 2011) and cytoplasmic stress granules (Patel et al. 2015) demonstrate many of the same dynamic characteristics as P granules. Further analysis of each of these RNP granules has revealed that they are non-homogenous structures, composed of subcompartments with unique physical traits (Wang et al. 2014; Jain et al. 2016; Feric et al. 2016). Encapsulation of one condensed phase by another is enabled by differences in viscosity and surface tension arising from distinct underlying compositions (Wang et al. 2014). Phase separated structures thus have the capacity to assume a diverse array of physical properties, ranging from liquid-like to solid (Figure 1.3). Recent classification of the Balbiani body in *Xenopus* oocytes as a solid amyloid-like assembly, suggests that having less fluid properties may be a mechanism by which oocytes are able to function as long-lived germ cells (Boke et al. 2016).

Beyond RNP granules, additional phase-separated compartments have been identified. Phase separations function in nuclear-cytoplasmic shuttling (Frey et al. 2006; Hülsmann et al. 2012), membrane receptor clustering and signal transduction (Banjade and Rosen 2014; Su et al. 2016), DNA damage repair (Patel et al. 2015; Altmeyer et al. 2015), mitotic and meiotic chromosomal segregations (Jiang et al. 2015; Berchowitz et al. 2015), both centrosomal and noncentrosomal microtubule nucleation (Woodruff et al. 2017; Hernández-Vega et al. 2017), epigenetic inheritance (Wan et al. 2018), transcriptional elongation and mRNA processing (Lu et al. 2018), and post-transcriptional gene-regulation through the microRNA-induced silencing complex (Sheu-Gruttadauria and

MacRae 2018). As this list continues to expand, it is clear that phase separation is a fundamental mechanism by which membraneless compartments both form and function. While these structures lack the complexity of much larger RNP granules, they do offer insights into the mechanisms and potential functions of phase separation.

Phase separation of nucleoporins into a semi-solid hydrogel generates a permeability barrier between the nucleus and cytoplasm (Frey et al. 2006). Under this “Selective Phase Model,” a sieve structure is created through reversible cross-linking between phenylalanine and glycine (FG)-rich nucleoporin repeats, which excludes diffusive passage over a size limit (Frey et al. 2006; Hülsmann et al. 2012). Selective passage of cargo above this threshold is facilitated by local disruption of FG interactions by nuclear transport receptors (Hülsmann et al. 2012). While not an RNP granule, the nuclear pore provides insights into two fundamental aspects of phase separated bodies. First, phase separation generates structures with unique properties of molecular diffusion and permeability (Frey et al. 2006; Updike et al. 2011; Wei et al. 2017). The molecular interactions that underlie phase separation create an effective mesh, capable of controlling the dynamics of materials that attempt to move through them (Wei et al. 2017). Control over both composition and intra-body dynamics offers tremendous regulatory potential for RNP granules. Second, phase separation is reversible. Phase-distinct structures are capable of both condensing and dissolving based on cellular context. Progression of an RNP granule to a physical state that is no longer able to disperse is believed to be a hallmark of a number of degenerative diseases (reviewed in Ramaswami et al. 2013; Shin and Brangwynne 2017). This phase flexibility is made possible by reliance on a conserved set of underlying types of molecular interactions that drive phase separation.

Molecular Mechanisms of Phase Separation

Phase transitions occur at a threshold concentration where attractive interactions between constituent molecules outweighs the thermodynamic benefits of entropy-driven mixing (Goldenfeld and Halley 1986). In living cells, interactions between protein and RNA macromolecules mediate such phase changes (Figure 1.2B; reviewed in Banani et al. 2017). Extensive research both *in vivo* and *in vitro* has identified several fundamental mechanisms that govern phase separation, as reviewed below.

Intrinsically Disordered Protein Regions

RNP granules are enriched in proteins that contain extended stretches of low sequence complexity, termed intrinsically disorder regions (IDRs) (Castello et al. 2012; Kato et al. 2012; Uversky 2017). These areas lack canonical domains and exhibit conformational heterogeneity, which traditionally has been thought incapable of function (Uversky 2017). However, serendipitous work presented from the laboratory of Steven McKnight in 2012 demonstrated that IDRs play an important role in intracellular RNP granule phase transitions (Kato et al. 2012; Han et al. 2012). While conducting a high-throughput drug screen to identify chemicals capable of specifying particular stem cell fates, the McKnight laboratory identified 5-aryl-isoxazole-3-carboxamide. Addition of a biotinylated form of the isoxazole (b-isox) to lysate resulted in the accumulation of white precipitate, which upon further analysis proved to be highly enriched in low sequence complexity containing RBPs that populate RNP granules (Kato et al. 2012). Using a series of recombinant GFP fusion proteins, Kato *et al.* demonstrated that the IDRs of seven RNP

granule RBPs were both necessary and sufficient for b-isox-mediated aggregation (Kato et al. 2012). Furthermore, these IDRs were capable of undergoing concentration-dependent phase transitions to hydrogels in the absence of b-isox (Kato et al. 2012). These hydrogels were comprised of amyloid-like fibers which were able to selectively retain both the IDRs of other RBPs as well as mRNAs commonly found in neuronal granules (Kato et al. 2012; Han et al. 2012). Kato *et al.* proposed that polymerization of IDRs could serve as an organizing principle in RNP granule assembly (Kato et al. 2012).

IDRs are generally enriched in a limited number of amino acids; including glycine, glutamine, serine, asparagine, phenylalanine and tyrosine. Repeated sequence elements within these stretches provides the basis for weakly adhesive homo- and heterotypic intermolecular interactions (Brangwynne et al. 2015). Many IDR-containing proteins enriched in RNP granules are able to phase separate on their own *in vitro* under various solution conditions (Nott et al. 2015; Elbaum-Garfinkle et al. 2015; Molliex et al. 2015). As the diversity of IDR-based interactions and underlying sequence motifs continues to emerge, the role of IDRs as a driver of phase separation is an increasingly prevalent model.

Multivalent Proteins

The ability of a molecule to engage in multiple intra- and inter-molecular interactions is critical in the assembly of large oligomers. Using combinations of modular interaction domains and IDRs with several weakly adhesive sequence elements, proteins can generate high levels of multivalency. Work in the laboratory of Michael Rosen explored the relationship between valency and phase separation in the context of

intracellular molecules (Li et al. 2012). Using synthetic repeats of the SRC homology 3 (SH3) domain and its proline-rich motif (PRM) ligand, Li *et al.* were able to produce sharp liquid-liquid phase separations whose concentration thresholds were directly related to the valency of the interacting species (Figure 1.3; Li et al. 2012). Phase separation was observed both *in vitro* and within living cells expressing complementary pairs of interacting constructs (Li et al. 2012). Additional work on the actin-regulatory protein neural Wiskott-Aldrich syndrome protein (N-WASP) and its interacting partners NCK and phosphorylated nephrin provided further evidence that intracellular phase separation can be modulated by the tunable valencies and affinities of interacting proteins (Li et al. 2012; Banjade and Rosen 2014). Higher valency and affinity both promote assembly into larger structures, enabling phase separation at lower concentrations and decreasing the dynamic rearrangements of molecules within phase-separated droplets (Figure 1.3; Li et al. 2012; Banjade and Rosen 2014).

The Role of RNA

While much focus has been placed on the role of proteins in phase separation, RNA is by no means passive in the process (Genot 2017). Often RNA appears to promote phase separation. Using the polypyrimidine tract binding protein (PTB), an RNA-binding protein with four RNA recognition motif (RRM) domains, phase separation can be induced by RNA in the absence of any IDRs (Lin et al. 2015). Similarly, the two RRM domains of hnRNPA1 can phase separate in the presence of RNA (Molliex et al. 2015). More commonly, RNP granule proteins contain both RNA-binding motifs and IDRs (Castello et al. 2012; Protter et al. 2018), and interactions with RNA promotes phase separation by

lowering the saturation concentration needed for demixing to occur (Molliex et al. 2015; Zhang et al. 2015; Lin et al. 2015; Saha et al. 2016). RNA is able to act as a multivalent platform, mediating intermolecular interactions between *trans*-acting proteins (Figure 1.2B; Banani et al. 2016). In addition to controlling threshold concentration for phase separation, RNA has also been shown to modulate droplet viscosity and dynamics (Elbaum-Garfinkle et al. 2015; Zhang et al. 2015)

Recent studies have suggested that beyond facilitating protein-protein interactions, RNA may play a more direct role in phase separation. Nucleotide repeat expansions, common in inherited diseases (Gatchel and Zoghbi 2005; La Spada and Taylor 2010), can act as a source of multivalent base-pairing, causing purified RNA to phase separate *in vitro* (Jain and Vale 2017). These repetitive RNA transcripts can phase separate in cells, and can be dissolved by agents that disrupt RNA gelation, suggesting that direct RNA-RNA interactions may play a critical role in droplet formation *in vivo* (Jain and Vale 2017). Further support for this idea comes from the finding that purified yeast RNA, under physiologically relevant conditions, forms *in vitro* assemblies comprised of RNAs enriched in stress granules *in vivo* (Van Treeck et al. 2018). This suggests that RNA-RNA interactions contribute to both stress granule assembly and to the selectivity of RNA incorporation (Van Treeck et al. 2018).

Composition and Dynamics of Phase Separated Bodies

While phase-separation is understood to be mediated by multivalent interactions amongst protein and RNA molecules, the overall compositional structure of RNP granules remains poorly understood. While a growing number of RNP granule constituents have

been identified, very few have been shown to be essential for structural integrity (Kedersha et al. 2000; Clemson et al. 2009; Hanazawa et al. 2011). A model has been proposed, wherein these less dynamic structural elements, termed scaffolds, mediate passage of more dynamic components that are dispensable for body assembly, termed clients, through heterotypic interactions (Banani et al. 2016). Using synthetic multivalent protein and RNA scaffolds, Banani *et al.* were able to generate phase separated structures both *in vitro* and *in vivo*, which were able to concentrate low valency client proteins (Banani et al. 2016). Clients were differentially recruited to bodies based on both scaffold concentration and valency, as well as their own interaction facilities (Banani et al. 2016). Minor alterations of these variables caused sharp changes in client recruitment and dramatic compositional changes on a cellular timescale (Banani et al. 2016). While this model provides for a dynamic view of multiphase RNP granule bodies, identification of scaffold proteins and the mechanisms that mediate changes in scaffold valency are lacking.

The Dynamic RNP Granule

Thinking about RNP granules as phase separated bodies invites a reimagining of the underlying molecular biology that governs post transcriptional gene regulation. In a shift from the previous perception of RNP granules as static structures, capable of mediating a diversity of tasks through specific rearrangements, phase separated granules emerge as uniquely dynamic bodies. The utility of this model can be appreciated through recent studies of P granules and stress granules, where their recognition as liquid-like structures has not only informed their own basic biology, but also provided a broader context for its understanding.

In *C. elegans*, the germline is specified by P granules, structures that are functionally analogous to mammalian nuage or *Drosophila* germ granules (Voronina et al. 2011). P granules are initially distributed uniformly throughout the unpolarized one-cell embryo. However, upon symmetry breakdown, the embryo becomes polarized along the anterior-posterior axis and P granules are observed to dissolve (Brangwynne et al. 2009; Voronina et al. 2011). Cortical and cytoplasmic flows develop as a consequence of cytoskeletal reorganization (Schneider and Bowerman 2003), and establish two cortical domains consisting of PAR-6/PAR-3/PKC at the anterior and PAR-2/LGL/PAR-1 at the posterior of the embryo (Hoege and Hyman 2013). Signals from the posteriorly localized PAR-1 protein dictate the establishment of an anterior-posterior cytoplasmic concentration gradient of the RBPs MEX-5 and MEX-6 (Schubert et al. 2000; Pagano et al. 2007; Tenlen et al. 2008; Daniels et al. 2010). MEX-5 competes with the P granule scaffold proteins, PGL-1 and PGL-3, for binding of mRNA in the cytoplasm, with the reduced MEX-5 levels at the posterior of the embryo providing a sufficient concentration of unbound mRNA to drive PGL-mediated P granule phase separation (Brangwynne et al. 2009; Hanazawa et al. 2011; Saha et al. 2016). Translationally repressed mRNA, along with the accompanying RBP CAR-1, preferentially accumulate within P granules (Noble et al. 2008; Saha et al. 2016). The MEG (maternal-effect germline defective) proteins 1 and 3 are also required for P granule formation, and localize to a dynamic domain that both surrounds and penetrates the PGL domain of the granule (Wang et al. 2014). Having consolidated a core set of germ specifying mRNAs, the posteriorly positioned P granules are selectively inherited by the presumptive germline, where they help to contribute to its integrity and function (Voronina et al. 2011).

The viscosity of the P granule is developmentally regulated, and believed to control an effective mesh which mediates passage of client proteins through the granule (Wei et al. 2017). Viscosity is sensitive to RNA content, and relies on the DEAD-box helicases LAF-1/DDX3 and CGH-1/RCK/DDX6, both widely conserved amongst RNP granules, to fine-tune the dynamic properties of the body, and prevent it from polymerizing into a nondynamic solid (Hubstenberger et al. 2013; Elbaum-Garfinkle et al. 2015; Hubstenberger et al. 2015). Phosphorylation of MEG proteins by the kinase MBK-2 /DYRK disrupts IDR interactions and leads to the disassembly of the granule during late embryogenesis, offering a mechanism whereby germline mRNA are released from translational repression (Wang et al. 2014).

Like P granules, stress granules are a liquid-like RNP granule that functions in the control of gene expression (Patel et al. 2015). However, unlike P granules, stress granules are not regulated by developmental cues designed to restrict gene expression to a specific spatial pattern, but rather are distributed cytoplasmic bodies that transiently form in response to cellular stresses such as heat shock, amino acid starvation, hypoxia, and chemical perturbation (Decker and Parker 2012). Stress granules are assemblies of untranslating mRNPs that form from mRNAs stalled in translational initiation (Protter and Parker 2016). Stress granules are thought to assemble via nontranslating mRNAs serving as multivalent scaffolds for RBPs, such as G3BP1 and ATXN2, which interact with one another through protein-protein interactions to form higher order assemblies (Nonhoff et al. 2007; Kedersha et al. 2016; Panas et al. 2016). Additionally, IDRs function to recruit and concentrate the scaffold protein TIA-1 which engages in homotypic IDR interactions to drive phase separation (Kedersha et al. 2000; Gilks et al. 2004). Stress

granules contain a liquid-like shell that surrounds more stable cores (Jain et al. 2016). The dynamics and assembly of these structures are mediated through the activity of ATP consuming complexes, with chaperone proteins as well as members of the common RNP granule DEAD-box helicase family, including DDX3, featuring prominently in stress granule maintenance (Jain et al. 2016). The self-assembly capabilities of RNA are believed to modulate partitioning of mRNAs into stress granules, and may play structural roles beyond complexing protein-protein interactions (Van Treeck et al. 2018).

Stress granules are thought to function in two key ways. First, by concentrating reactants, stress granules can shift the equilibrium of interacting proteins towards associated states. This is believed to aid in the translational repression of mRNAs within the granule, while simultaneously priming transcripts to quickly reinitiate translation upon relief from cellular stress by promoting the formation of translation initiation complexes (Buchan et al. 2008). The second function of stress granules is to limit the interactions of sequestered components with the bulk cytoplasm. In this way, stress granules modulate signaling pathways by sequestering components of TOR, RACK1, or TRAF2 signaling pathways (Kim et al. 2005; Arimoto et al. 2008; Takahara and Maeda 2012; Wippich et al. 2013). Through the control of stress granule assembly and disassembly, global patterns of translation can be post transcriptionally directed to respond to specific cellular stresses.

Using a core set of underlying principles, phase separated RNP granules are capable of regulating gene expression in diverse biological settings. Building from the tremendous conservation of protein constituents observed between cytoplasmic mRNP granules, it is likely that additional structures will be revealed to have phase separated properties. The large number of neuronal proteins that have been shown to phase separate *in vitro* and

form solid pathological inclusions *in vivo*, has focused much attention on the role of phase separation in neuronal function (Ramaswami et al. 2013). Indeed dynamic properties have previously been noted in neuronal granules, where tagged-Staufen was observed to rapidly exchange with the surrounding cytoplasm in dendritically targeted bodies (Barbee et al. 2006). Yet, the mechanisms by which phase separation effect granule function remain poorly understood. Recent work in germ cells has demonstrated that phase separation may be a conserved mechanism for germline specification, as the formation of solid amyloid-like aggregates is essential for both Balbiani body formation in *Xenopus* and gametogenesis in yeast (Berchowitz et al. 2015; Boke et al. 2016). While their functions may be distinct, it is becoming increasingly clear that knowledge gained through the study of individual mRNP granule types has the potential to extend to the broader class of phase separated bodies.

The Emerging Vegetal Transport Granule

Vegetal transport complexes in *Xenopus laevis* oocytes represent a unique class of mRNP, displaying features of both germ and somatic granule types. Capable of acting as long-term storage sites for specific translationally repressed mRNA, vegetal transport RNPs are governed by developmental cues. Additionally, they require motor-mediated transport over an extended distance and interact extensively with the cellular cytoskeleton. Through the work presented in this thesis, I have characterized the *Xenopus* vegetal transport RNP as a multiphase granule, composed of a non-dynamic mRNA-containing substructure enmeshed in a dynamic liquid-like layer. Using biochemical purification and imaging techniques in combination with traditional approaches of cell and molecular

biology, this work provides a framework for understanding *Xenopus laevis* mRNA localization within the context of a phase separated mRNP granules. In this way, regulation of distinct transport and developmental steps operate within the context of a system in nonequilibrium, where liquid-like properties can be fine-tuned to control granular composition and function. *Xenopus* vegetal transport granules represent a unique model for the study of such dynamic systems, as granule function can be separated both spatially within individual oocytes, as well as temporally moving through oogenesis. By applying previous knowledge of vegetal mRNA transport, *Xenopus* oocytes can be used to study aspects of granule function that have remained largely inaccessible to the research community and provide a new understanding of regulated gene expression through cellular organization.

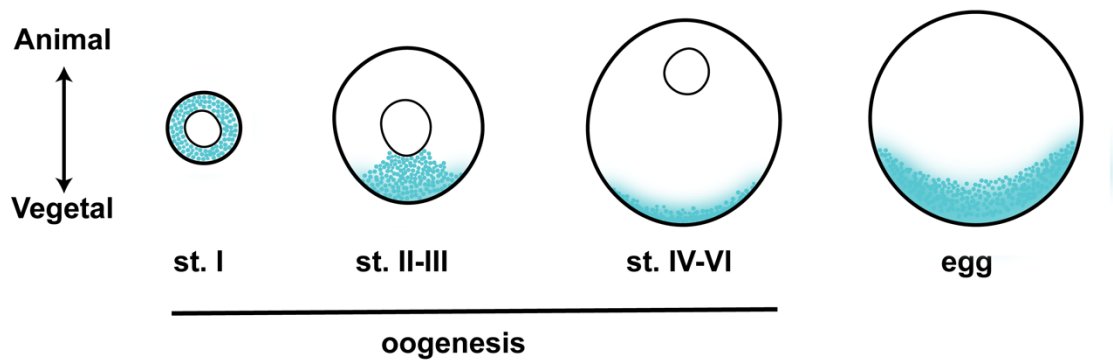
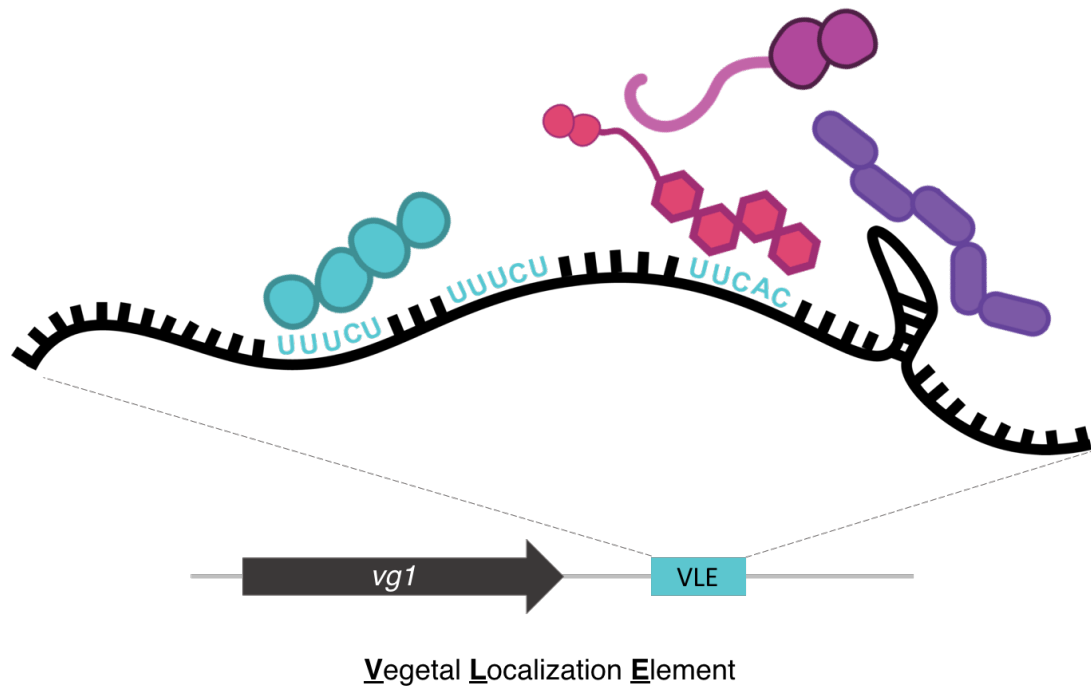


Figure 1.1 Vegetal mRNA Transport in *Xenopus* oocytes. Oogenesis can be divided into six morphologically distinct stages, labeled I-VI (Dumont 1972). The maternally loaded *vg1* mRNA (cyan stipples) is initially ubiquitously distributed throughout the cytoplasm in early oogenesis (st. I). However, during mid-oogenesis (st. II-III) *vg1* mRNA becomes confined to the vegetal hemisphere, where it is subsequently anchored to the vegetal cortical cytoskeleton (st. IV-VI). In the egg (pictured at right) and early embryo (not shown), Vg1 protein is restricted to the vegetal hemisphere and vegetal blastomeres, respectively.

A



B

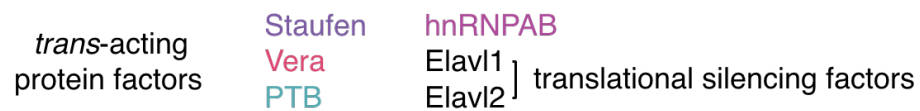


Figure 1.2 A *cis*-acting localization element mediates the organization of a multivalent *vg1* ribonucleoprotein complex. (A) A 340-nucleotide vegetal localization element (VLE) found within the 3'UTR of *vg1* mRNA is sufficient to specify vegetal localization. Composed of multiple clusters of short sequence motifs, the *cis*-acting VLE mediates interactions with a number of *trans*-acting RNA-binding proteins to form a large

ribonucleoprotein (RNP) complex. **(B)** Six core *trans*-acting proteins have been previously identified as *vg1* RNP components: including Staufen, Vera, PTB, hnRNPAB, Elavl1, and Elavl2. Each of these proteins harbors multiple RNA and/or protein interaction domains. Staufen (purple) contains five double-stranded RNA-binding domains (schematically represented as ovals). Vera (pink) contains four K Homolgy (KH) domains (schematically represented as hexagons) and two RNA Recognition Motif (RRM) domains (schematically represented as circles). PTB (cyan) contains four RRM domains. And hnRNPAB (magenta) contains two RRM domains and an extended region of low sequence complexity (schematically represented as a thick line).

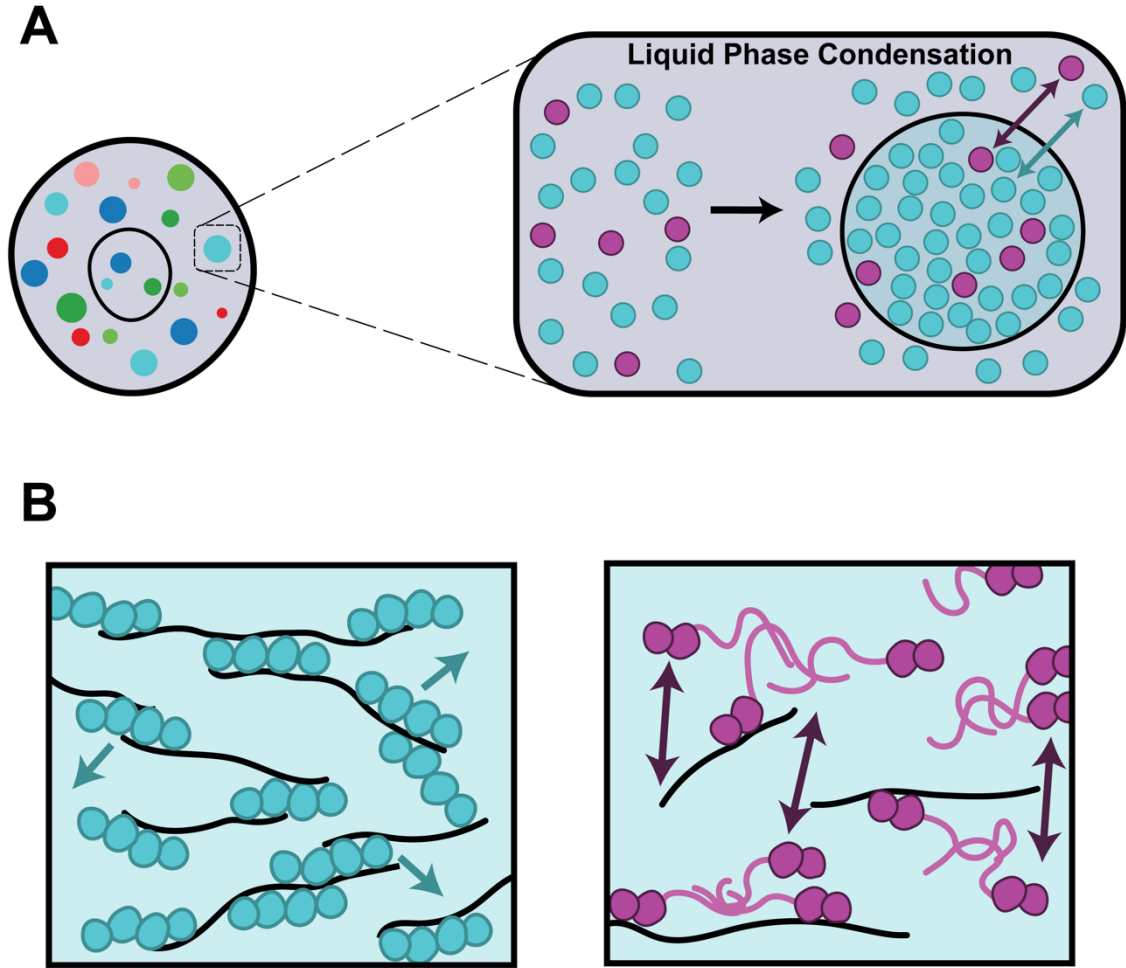


Figure 1.3 Liquid phase condensation: An emerging mechanism of cellular organization. (A) Living cells contain a diversity of condensed liquid-like structures that are compositionally and materially distinct from the surrounding cellular milieu (represented by colored dots). Liquid-like bodies are capable of concentrating components (cyan and magenta dots) into discrete, nonmembrane-bound, cellular compartments for extended periods, while remaining in dynamic exchange with the surrounding medium. (B) Interactions between multivalent protein (cyan and magenta structures) and RNA (black lines) macromolecules mediate such phase changes based on cellular context. Using

combinations of modular interaction domains (colored circles) and intrinsically disordered regions with several weakly adhesive sequence elements (bold line segments on proteins), proteins can generate high levels of multivalency which dictate their intra- and inter-granule behaviors. Increased networks of interactions can restrict motility (represented by arrows) of resident proteins relative to less valent structures.

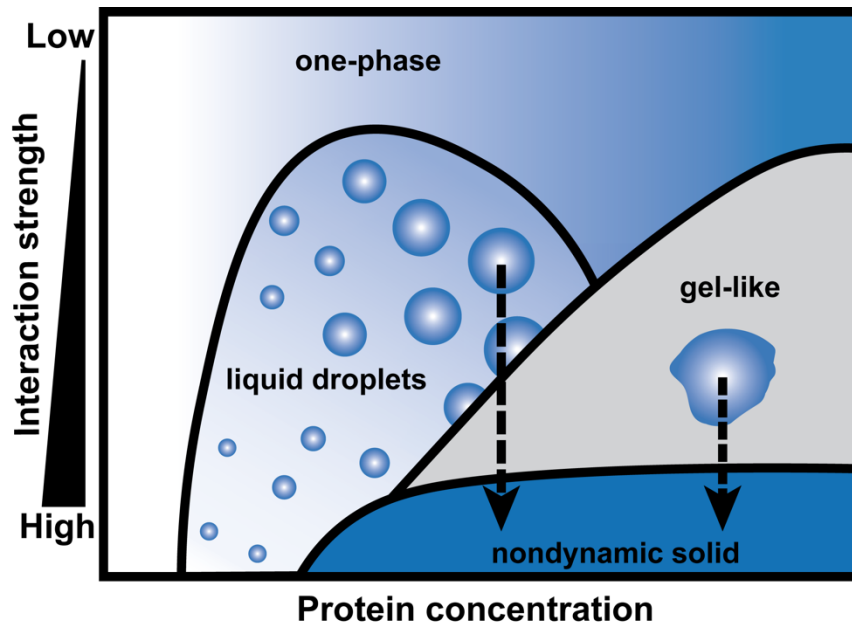


Figure 1.4 Phase Diagram: Material properties of phase-separated bodies. The material properties of mRNP granule components can assume diffuse (one-phase), liquid-like (liquid droplets), or more solid states (gel-like or nondynamic solid) in response to surrounding context. Mediated by the capacity to form networks of inter-molecular interactions, the phase behavior of a protein is directly related to its concentration and interaction strength. Within the two-phase region, dynamically arrested solids appear to form which may represent matured versions of liquid and/or gel-like bodies. [Figure adapted from (Shin and Brangwynne 2017)]

References

- Alarcón VB, Elinson RP. 2001. RNA anchoring in the vegetal cortex of the *Xenopus* oocyte. *J Cell Sci* **114**: 1731–41.
- Altmeyer M, Neelsen KJ, Teloni F, Pozdnyakova I, Pellegrino S, Grøfte M, Rask M-BD, Streicher W, Jungmichel S, Nielsen ML, et al. 2015. Liquid demixing of intrinsically disordered proteins is seeded by poly(ADP-ribose). *Nat Commun* **6**: 8088.
- Amrute-Nayak M, Bullock SL. 2012. Single-molecule assays reveal that RNA localization signals regulate dynein-dynactin copy number on individual transcript cargoes. *Nat Cell Biol* **14**: 416–23.
- Arimoto K, Fukuda H, Imajoh-Ohmi S, Saito H, Takekawa M. 2008. Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways. *Nat Cell Biol* **10**: 1324–32.
- Balogopal V, Parker R. 2009. Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs. *Curr Opin Cell Biol* **21**: 403–8.
- Banani SF, Lee HO, Hyman AA, Rosen MK. 2017. Biomolecular condensates: organizers of cellular biochemistry. *Nat Rev Mol Cell Biol* **18**: 285–98.
- Banani SF, Rice AM, Peeples WB, Lin Y, Jain S, Parker R, Rosen MK. 2016. Compositional control of phase-separated cellular bodies. *Cell* **166**: 651–63.
- Banjade S, Rosen MK. 2014. Phase transitions of multivalent proteins can promote clustering of membrane receptors. *Elife* **3**: e04123.
- Barbee SA, Estes PS, Cziko A-M, Hillebrand J, Luedeman RA, Collier JM, Johnson N, Howlett IC, Geng C, Ueda R, et al. 2006. Staufen- and FMRP-containing neuronal RNPs are structurally and functionally related to somatic P bodies. *Neuron* **52**: 997–1009.
- Bashirullah A, Cooperstock RL, Lipshitz HD. 2001. Spatial and temporal control of RNA stability. *Proc Natl Acad Sci U S A* **98**: 7025–28.
- Batish M, van den Bogaard P, Kramer FR, Tyagi S. 2012. Neuronal mRNAs travel singly into dendrites. *Proc Natl Acad Sci U S A* **109**: 4645–50.
- Beach DL, Salmon ED, Bloom K. 1999. Localization and anchoring of mRNA in budding yeast. *Curr Biol* **9**: 569–78.

- Berchowitz LE, Kabachinski G, Walker MR, Carlile TM, Gilbert W V., Schwartz TU, Amon A. 2015. Regulated formation of an amyloid-like translational repressor governs gametogenesis. *Cell* **163**: 406–18.
- Bergsten SE, Gavis ER. 1999. Role for mRNA localization in translational activation but not spatial restriction of *nanos* RNA. *Development* **126**: 659–69.
- Bertrand E, Chartrand P, Schaefer M, Shenoy SM, Singer RH, Long RM. 1998. Localization of *ASH1* mRNA particles in living yeast. *Mol Cell* **2**: 437–45.
- Besse F, López de Quinto S, Marchand V, Trucco A, Ephrussi A. 2009. *Drosophila* PTB promotes formation of high-order RNP particles and represses *oskar* translation. *Genes Dev* **23**: 195–207.
- Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. 2006. Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells. *Cold Spring Harb Symp Quant Biol* **71**: 513–21.
- Birsoy B, Kofron M, Schaible K, Wylie C, Heasman J. 2006. Vg1 is an essential signaling molecule in *Xenopus* development. *Development* **133**: 15–20.
- Blower MD, Feric E, Weis K, Heald R. 2007. Genome-wide analysis demonstrates conserved localization of messenger RNAs to mitotic microtubules. *J Cell Biol* **179**: 1365–73.
- Bobola N, Jansen RP, Shin TH, Nasmyth K. 1996. Asymmetric accumulation of Ash1p in postanaphase nuclei depends on a myosin and restricts yeast mating-type switching to mother cells. *Cell* **84**: 699–709.
- Boke E, Ruer M, Wühr M, Coughlin M, Lemaitre R, Gygi SP, Alberti S, Drechsel D, Hyman AA, Mitchison TJ. 2016. Amyloid-like self-assembly of a cellular compartment. *Cell* **166**: 637–50.
- Bontems F, Stein A, Marlow F, Lyautey J, Gupta T, Mullins MC, Dosch R. 2009. Bucky ball organizes germ plasm assembly in zebrafish. *Curr Biol* **19**: 414–22.
- Brangwynne CP, Eckmann CR, Courson DS, Rybarska A, Hoege C, Gharakhani J, Julicher F, Hyman AA. 2009. Germline P granules are liquid droplets that localize by controlled dissolution/condensation. *Science* **324**: 1729–32.
- Brangwynne CP, Mitchison TJ, Hyman AA. 2011. Active liquid-like behavior of nucleoli determines their size and shape in *Xenopus laevis* oocytes. *Proc Natl Acad Sci U S A* **108**: 4334–39.

- Brangwynne CP, Tompa P, Pappu R V. 2015. Polymer physics of intracellular phase transitions. *Nat Phys* **11**: 899–904.
- Brendza RP, Serbus LR, Duffy JB, Saxton WM. 2000. A function for kinesin I in the posterior transport of *oskar* mRNA and Staufen protein. *Science* **289**: 2120–22.
- Bregues M, Teixeira D, Parker R. 2005. Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. *Science* **310**: 486–89.
- Bubunencko M, Kress TL, Vempati UD, Mowry KL, King ML. 2002. A consensus RNA signal that directs germ layer determinants to the vegetal cortex of *Xenopus* oocytes. *Dev Biol* **248**: 82–92.
- Buchan JR. 2014. mRNP granules. Assembly, function, and connections with disease. *RNA Biol* **11**: 1019–30.
- Buchan JR, Muhlrads D, Parker R. 2008. P bodies promote stress granule assembly in *Saccharomyces cerevisiae*. *J Cell Biol* **183**: 441–55.
- Buxbaum AR, Haimovich G, Singer RH. 2015. In the right place at the right time: visualizing and understanding mRNA localization. *Nat Rev Mol Cell Biol* **16**: 95–109.
- Buxbaum AR, Wu B, Singer RH. 2014. Single β -actin mRNA detection in neurons reveals a mechanism for regulating its translatability. *Science* **343**: 419–22.
- Cajigas IJ, Tushev G, Will TJ, tom Dieck S, Fuerst N, Schuman EM. 2012. The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging. *Neuron* **74**: 453–66.
- Castello A, Fischer B, Eichelbaum K, Horos R, Beckmann BM, Strein C, Davey NE, Humphreys DT, Preiss T, Steinmetz LM, et al. 2012. Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell* **149**: 1393–406.
- Chang P, Torres J, Lewis RA, Mowry KL, Houliston E, King ML. 2004. Localization of RNAs to the mitochondrial cloud in *Xenopus* oocytes through entrapment and association with endoplasmic reticulum. *Mol Biol Cell* **15**: 4669–81.
- Chartrand P, Meng XH, Huttelmaier S, Donato D, Singer RH. 2002. Asymmetric sorting of Ash1p in yeast results from inhibition of translation by localization elements in the mRNA. *Mol Cell* **10**: 1319–30.
- Chen L, Dumelie JG, Li X, Cheng MH, Yang Z, Laver JD, Siddiqui NU, Westwood JT, Morris Q, Lipshitz HD, et al. 2014. Global regulation of mRNA translation and stability in the early *Drosophila* embryo by the Smaug RNA-binding protein. *Genome Biol* **15**: R4.

- Chin A, Lécuyer E. 2017. RNA localization: Making its way to the center stage. *Biochim Biophys Acta* **1861**: 2956–70.
- Clemson CM, Hutchinson JN, Sara SA, Ensminger AW, Fox AH, Chess A, Lawrence JB. 2009. An architectural role for a nuclear noncoding RNA: *NEAT1* RNA is essential for the structure of paraspeckles. *Mol Cell* **33**: 717–26.
- Colegrove-Otero LJ, Devaux A, Standart N. 2005. The *Xenopus* ELAV protein ElrB represses *vg1* mRNA translation during oogenesis. *Mol Cell Biol* **25**: 9028–39.
- Cote C a., Gautreau D, Denegre JM, Kress TL, Terry N a., Mowry KL. 1999. A *Xenopus* protein related to hnRNP I has a role in cytoplasmic RNA localization. *Mol Cell* **4**: 431–37.
- Czaplinski K, Köcher T, Schelder M, Segref A, Wilm M, Mattaj IW. 2005. Identification of 40LoVe, a *Xenopus* hnRNP D family protein involved in localizing a TGF-beta-related mRNA during oogenesis. *Dev Cell* **8**: 505–15.
- Daniels BR, Dobrowsky TM, Perkins EM, Sun SX, Wirtz D. 2010. MEX-5 enrichment in the *C. elegans* early embryo mediated by differential diffusion. *Development* **137**: 2579–85.
- Decker CJ, Parker R. 2012. P-bodies and stress granules: possible roles in the control of translation and mRNA degradation. *Cold Spring Harb Perspect Biol* **4**: a012286.
- Deng Y, Singer RH, Gu W. 2008. Translation of *ASH1* mRNA is repressed by Puf6p-Fun12p/eIF5B interaction and released by CK2 phosphorylation. *Genes Dev* **22**: 1037–50.
- Deshler JO, Highett MI, Abramson T, Schnapp BJ. 1998. A highly conserved RNA-binding protein for cytoplasmic mRNA localization in vertebrates. *Curr Biol* **8**: 489–96.
- Dumont JN. 1972. Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J Morphol* **136**: 153–79.
- Eberwine J, Belt B, Kacharmina JE, Miyashiro K. 2002. Analysis of subcellularly localized mRNAs using *in situ* hybridization, mRNA amplification, and expression profiling. *Neurochem Res* **27**: 1065–77.
- Elbaum-Garfinkle S, Kim Y, Szczepaniak K, Chen CC-H, Eckmann CR, Myong S, Brangwynne CP. 2015. The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics. *Proc Natl Acad Sci U S A* **112**: 7189–94.

- Elinson RP, King ML, Forristall C. 1993. Isolated vegetal cortex from *Xenopus* oocytes selectively retains localized mRNAs. *Dev Biol* **160**: 554–62.
- Ephrussi A, Dickinson LK, Lehmann R. 1991. Oskar organizes the germ plasm and directs localization of the posterior determinant *nanos*. *Cell* **66**: 37–50.
- Ephrussi A, Lehmann R. 1992. Induction of germ cell formation by *oskar*. *Nature* **358**: 387–92.
- Farina KL, Huttelmaier S, Musunuru K, Darnell R, Singer RH. 2003. Two ZBP1 KH domains facilitate *beta-actin* mRNA localization, granule formation, and cytoskeletal attachment. *J Cell Biol* **160**: 77–87.
- Feric M, Vaidya N, Harmon TS, Mitrea DM, Zhu L, Richardson TM, Kriwacki RW, Pappu R V., Brangwynne CP. 2016. Coexisting liquid phases underlie nucleolar subcompartments. *Cell* **165**: 1686–97.
- Forrest KM, Gavis ER. 2003. Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for *nanos* mRNA localization in *Drosophila*. *Curr Biol* **13**: 1159–68.
- Forristall C, Pondel M, Chen L, King ML. 1995. Patterns of localization and cytoskeletal association of two vegetally localized RNAs, *vg1* and *xcat-2*. *Development* **121**: 201–08.
- Frey S, Richter RP, Görlich D. 2006. FG-rich repeats of nuclear pore proteins form a three-dimensional meshwork with hydrogel-like properties. *Science* **314**: 815–17.
- Fritzsche R, Karra D, Bennett KL, Ang FY, Heraud-Farlow JE, Tolino M, Doyle M, Bauer KE, Thomas S, Planyavsky M, et al. 2013. Interactome of two diverse RNA granules links mRNA localization to translational repression in neurons. *Cell Rep* **5**: 1749–62.
- Fusco D, Accornero N, Lavoie B, Shenoy SM, Blanchard J-M, Singer RH, Bertrand E. 2003. Single mRNA molecules demonstrate probabilistic movement in living mammalian cells. *Curr Biol* **13**: 161–67.
- Gagnon J a, Mowry KL. 2011. Molecular motors: directing traffic during RNA localization. *Crit Rev Biochem Mol Biol* **46**: 229–39.
- Gagnon JA, Kreiling J a, Powrie EA, Wood TR, Mowry KL. 2013. Directional transport is mediated by a Dynein-dependent step in an RNA localization pathway. *PLoS Biol* **11**: e1001551.
- Gatchel JR, Zoghbi HY. 2005. Diseases of unstable repeat expansion: mechanisms and common principles. *Nat Rev Genet* **6**: 743–55.

- Gautreau D, Cote C a, Mowry KL. 1997. Two copies of a subelement from the *vg1* RNA localization sequence are sufficient to direct vegetal localization in *Xenopus* oocytes. *Development* **124**: 5013–20.
- Gavis ER, Lehmann R. 1992. Localization of *nanos* RNA controls embryonic polarity. *Cell* **71**: 301–13.
- Genot E. 2017. ARF1 at the crossroads of podosome construction and function. *J Cell Biol* **216**: 13–15.
- Gilks N, Kedersha N, Ayodele M, Shen L, Stoecklin G, Dember LM, Anderson P. 2004. Stress granule assembly is mediated by prion-like aggregation of TIA-1. *Mol Biol Cell* **15**: 5383–98.
- Goldenfeld, Halley. 1986. Goldenfeld and Halley respond. *Phys Rev Lett* **56**: 1315.
- Gu W, Deng Y, Zenklusen D, Singer RH. 2004. A new yeast PUF family protein, Puf6p, represses *ASH1* mRNA translation and is required for its localization. *Genes Dev* **18**: 1452–65.
- Han TW, Kato M, Xie S, Wu LC, Mirzaei H, Pei J, Chen M, Xie Y, Allen J, Xiao G, et al. 2012. Cell-free formation of RNA granules: bound RNAs identify features and components of cellular assemblies. *Cell* **149**: 768–79.
- Hanazawa M, Yonetani M, Sugimoto A. 2011. PGL proteins self associate and bind RNPs to mediate germ granule assembly in *C. elegans*. *J Cell Biol* **192**: 929–37.
- Havin L, Git A, Elisha Z, Oberman F, Yaniv K, Schwartz SP, Standart N, Yisraeli JK. 1998. RNA-binding protein conserved in both microtubule- and microfilament-based RNA localization. *Genes Dev* **12**: 1593–98.
- Hernández-Vega A, Braun M, Scharrel L, Jahnel M, Wegmann S, Hyman BT, Alberti S, Diez S, Hyman AA. 2017. Local nucleation of microtubule bundles through Tubulin concentration into a condensed Tau phase. *Cell Rep* **20**: 2304–12.
- Hoege C, Hyman AA. 2013. Principles of PAR polarity in *Caenorhabditis elegans* embryos. *Nat Rev Mol Cell Biol* **14**: 315–22.
- Holdeman R, Nehrt S, Strome S. 1998. MES-2, a maternal protein essential for viability of the germline in *Caenorhabditis elegans*, is homologous to a *Drosophila* Polycomb group protein. *Development* **125**: 2457–67.
- Holt CE, Bullock SL. 2009. Subcellular mRNA localization in animal cells and why it matters. *Science* **326**: 1212–16.

- Houston DW, King ML. 2000. A critical role for *xdazl*, a germ plasm-localized RNA, in the differentiation of primordial germ cells in *Xenopus*. *Development* **127**: 447–56.
- Hubstenberger A, Cameron C, Noble SL, Keenan S, Evans TC. 2015. Modifiers of solid RNP granules control normal RNP dynamics and mRNA activity in early development. *J Cell Biol* **211**: 703–16.
- Hubstenberger A, Courel M, Bénard M, Souquere S, Ernoult-Lange M, Chouaib R, Yi Z, Morlot J-B, Munier A, Fradet M, et al. 2017. P-Body purification reveals the condensation of repressed mRNA regulons. *Mol Cell* **68**: 144–57.
- Hubstenberger A, Noble SL, Cameron C, Evans TC. 2013. Translation repressors, an RNA helicase, and developmental cues control RNP phase transitions during early development. *Dev Cell* **27**: 161–73.
- Hülsmann BB, Labokha AA, Görlich D. 2012. The permeability of reconstituted nuclear pores provides direct evidence for the selective phase model. *Cell* **150**: 738–51.
- Hüttelmaier S, Zenklusen D, Lederer M, Dichtenberg J, Lorenz M, Meng X, Bassell GJ, Condeelis J, Singer RH. 2005. Spatial regulation of *beta-actin* translation by Src-dependent phosphorylation of ZBP1. *Nature* **438**: 512–15.
- Jain A, Vale RD. 2017. RNA phase transitions in repeat expansion disorders. *Nature* **546**: 243–47.
- Jain S, Wheeler JR, Walters RW, Agrawal A, Barsic A, Parker R. 2016. ATPase-modulated stress granules contain a diverse proteome and substructure. *Cell* **164**: 487–98.
- Jambhekar A, Derisi JL. 2007. *Cis*-acting determinants of asymmetric, cytoplasmic RNA transport. *RNA* **13**: 625–42.
- Jiang H, Wang S, Huang Y, He X, Cui H, Zhu X, Zheng Y. 2015. Phase transition of spindle-associated protein regulate spindle apparatus assembly. *Cell* **163**: 108–22.
- Joseph EM, Melton D a. 1998. Mutant Vg1 ligands disrupt endoderm and mesoderm formation in *Xenopus* embryos. *Development* **125**: 2677–85.
- Kanai Y, Dohmae N, Hirokawa N. 2004. Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. *Neuron* **43**: 513–25.
- Kato M, Han TW, Xie S, Shi K, Du X, Wu LC, Mirzaei H, Goldsmith EJ, Longgood J, Pei J, et al. 2012. Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. *Cell* **149**: 753–67.

- Kedersha N, Cho MR, Li W, Yacono PW, Chen S, Gilks N, Golan DE, Anderson P. 2000. Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules. *J Cell Biol* **151**: 1257–68.
- Kedersha N, Panas MD, Achorn CA, Lyons S, Tisdale S, Hickman T, Thomas M, Lieberman J, McInerney GM, Ivanov P, et al. 2016. G3BP-Caprin1-USP10 complexes mediate stress granule condensation and associate with 40S subunits. *J Cell Biol* **212**: 845–60.
- Kim-Ha J, Smith JL, Macdonald PM. 1991. *oskar* mRNA is localized to the posterior pole of the *Drosophila* oocyte. *Cell* **66**: 23–35.
- Kim WJ, Back SH, Kim V, Ryu I, Jang SK. 2005. Sequestration of TRAF2 into stress granules interrupts tumor necrosis factor signaling under stress conditions. *Mol Cell Biol* **25**: 2450–62.
- Kislauskis EH, Zhu X, Singer RH. 1997. *beta-actin* messenger RNA localization and protein synthesis augment cell motility. *J Cell Biol* **136**: 1263–70.
- Kloc M, Etkin LD. 1994. Delocalization of *vg1* mRNA from the vegetal cortex in *Xenopus* oocytes after destruction of *xlsirt* RNA. *Science* **265**: 1101–03.
- Kloc M, Etkin LD. 1995. Two distinct pathways for the localization of RNAs at the vegetal cortex in *Xenopus* oocytes. *Development* **121**: 287–97.
- Klymkowsky MW, Maynell LA, Nislow C. 1991. Cytokeratin phosphorylation, cytokeratin filament severing and the solubilization of the maternal mRNA *vg1*. *J Cell Biol* **114**: 787–97.
- Kobayashi S, Yamada M, Asaoka M, Kitamura T. 1996. Essential role of the posterior morphogen *nanos* for germline development in *Drosophila*. *Nature* **380**: 708–11.
- Kress TL, Yoon YJ, Mowry KL. 2004. Nuclear RNP complex assembly initiates cytoplasmic RNA localization. *J Cell Biol* **165**: 203–11.
- Kugler J-M, Lasko P. 2009. Localization, anchoring and translational control of *oskar*, *gurken*, *bicoid* and *nanos* mRNA during *Drosophila* oogenesis. *Fly (Austin)* **3**: 15–28.
- La Spada AR, Taylor JP. 2010. Repeat expansion disease: progress and puzzles in disease pathogenesis. *Nat Rev Genet* **11**: 247–58.
- Lécuyer E, Yoshida H, Parthasarathy N, Alm C, Babak T, Cerovina T, Hughes TR, Tomancak P, Krause HM. 2007. Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. *Cell* **131**: 174–87.

- Lee CF, Brangwynne CP, Gharakhani J, Hyman AA, Jülicher F. 2013. Spatial organization of the cell cytoplasm by position-dependent phase separation. *Phys Rev Lett* **111**: 088101.
- Leung K-M, van Horck FPG, Lin AC, Allison R, Standart N, Holt CE. 2006. Asymmetrical *beta-actin* mRNA translation in growth cones mediates attractive turning to netrin-1. *Nat Neurosci* **9**: 1247–56.
- Lewis RA, Kress TL, Cote CA, Gautreau D, Rokop ME, Mowry KL. 2004. Conserved and clustered RNA recognition sequences are a critical feature of signals directing RNA localization in *Xenopus* oocytes. *Mech Dev* **121**: 101–09.
- Lewis RA, Mowry KL. 2007. Ribonucleoprotein remodeling during RNA localization. *Differentiation* **75**: 507–18.
- Li P, Banjade S, Cheng H-C, Kim S, Chen B, Guo L, Llaguno M, Hollingsworth J V., King DS, Banani SF, et al. 2012. Phase transitions in the assembly of multivalent signalling proteins. *Nature* **483**: 336–40.
- Lin Y, Protter DSW, Rosen MK, Parker R. 2015. Formation and maturation of phase-separated liquid droplets by RNA-binding proteins. *Mol Cell* **60**: 208–19.
- Little SC, Sinsimer KS, Lee JJ, Wieschaus EF, Gavis ER. 2015. Independent and coordinate trafficking of single *Drosophila* germ plasm mRNAs. *Nat Cell Biol* **17**: 558–68.
- Long RM, Singer RH, Meng X, Gonzalez I, Nasmyth K, Jansen RP. 1997. Mating type switching in yeast controlled by asymmetric localization of *ASH1* mRNA. *Science* **277**: 383–87.
- Lu H, Yu D, Hansen AS, Ganguly S, Liu R, Heckert A, Darzacq X, Zhou Q. 2018. Phase-separation mechanism for C-terminal hyperphosphorylation of RNA polymerase II. *Nature* **558**: 318–23.
- Marlow FL, Mullins MC. 2008. Bucky ball functions in Balbiani body assembly and animal-vegetal polarity in the oocyte and follicle cell layer in zebrafish. *Dev Biol* **321**: 40–50.
- Martin KC, Barad M, Kandel ER. 2000. Local protein synthesis and its role in synapse-specific plasticity. *Curr Opin Neurobiol* **10**: 587–92.
- Martin KC, Ephrussi A. 2009. mRNA localization: gene expression in the spatial dimension. *Cell* **136**: 719–30.
- Messitt TJ, Gagnon JA, Kreiling JA, Pratt CA, Yoon YJ, Mowry KL. 2008. Multiple kinesin motors coordinate cytoplasmic RNA transport on a subpopulation of microtubules in *Xenopus* oocytes. *Dev Cell* **15**: 426–36.

- Mikl M, Vendra G, Kiebler MA. 2011. Independent localization of *MAP2*, *CaMKII α* and *β -actin* RNAs in low copy numbers. *EMBO Rep* **12**: 1077–84.
- Mili S, Moissoglu K, Macara IG. 2008. Genome-wide screen reveals APC-associated RNAs enriched in cell protrusions. *Nature* **453**: 115–19.
- Mingle LA, Okuhama NN, Shi J, Singer RH, Condeelis J, Liu G. 2005. Localization of all seven messenger RNAs for the actin-polymerization nucleator Arp2/3 complex in the protrusions of fibroblasts. *J Cell Sci* **118**: 2425–33.
- Mitsumori K, Takei Y, Hirokawa N. 2017. Components of RNA granules affect their localization and dynamics in neuronal dendrites. *Mol Biol Cell* **28**: 1412–17.
- Molliex A, Temirov J, Lee J, Coughlin M, Kanagaraj AP, Kim HJ, Mittag T, Taylor JP. 2015. Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. *Cell* **163**: 123–33.
- Mowry KL. 1996. Complex formation between stage-specific oocyte factors and a *Xenopus* mRNA localization element. *Proc Natl Acad Sci U S A* **93**: 14608–13.
- Mowry KL, Melton DA. 1992. Vegetal messenger RNA localization directed by a 340-nt RNA sequence element in *Xenopus* oocytes. *Science* **255**: 991–94.
- Nagamori I, Cruickshank VA, Sassone-Corsi P. 2011. Regulation of an RNA granule during spermatogenesis: acetylation of MVH in the chromatoid body of germ cells. *J Cell Sci* **124**: 4346–55.
- Niepielko MG, Eagle WVI, Gavis ER. 2018. Stochastic seeding coupled with mRNA self-recruitment generates heterogeneous *Drosophila* germ granules. *Curr Biol* **28**: 1872–81.
- Noble SL, Allen BL, Goh LK, Nordick K, Evans TC. 2008. Maternal mRNAs are regulated by diverse P body-related mRNP granules during early *Caenorhabditis elegans* development. *J Cell Biol* **182**: 559–72.
- Nonhoff U, Ralser M, Welzel F, Piccini I, Balzereit D, Yaspo M-L, Lehrach H, Krobitsch S. 2007. Ataxin-2 interacts with the DEAD/H-box RNA helicase DDX6 and interferes with P-bodies and stress granules. *Mol Biol Cell* **18**: 1385–96.
- Nott TJ, Petsalaki E, Farber P, Jervis D, Fussner E, Plochowietz A, Craggs TD, Bazett-Jones DP, Pawson T, Forman-Kay JD, et al. 2015. Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles. *Mol Cell* **57**: 936–47.

- Pagano JM, Farley BM, McCoig LM, Ryder SP. 2007. Molecular basis of RNA recognition by the embryonic polarity determinant MEX-5. *J Biol Chem* **282**: 8883–94.
- Panas MD, Ivanov P, Anderson P. 2016. Mechanistic insights into mammalian stress granule dynamics. *J Cell Biol* **215**: 313–23.
- Paquin N, Ménade M, Poirier G, Donato D, Drouet E, Chartrand P. 2007. Local activation of yeast *ASH1* mRNA translation through phosphorylation of Khd1p by the casein kinase Yck1p. *Mol Cell* **26**: 795–809.
- Patel A, Lee HO, Jawerth L, Maharana S, Jahnelt M, Hein MY, Stoyanov S, Mahamid J, Saha S, Franzmann TM, et al. 2015. A liquid-to-solid phase transition of the ALS protein FUS accelerated by disease mutation. *Cell* **162**: 1066–77.
- Pondel MD, King ML. 1988. Localized maternal mRNA related to transforming growth factor beta mRNA is concentrated in a cytokeratin-enriched fraction from *Xenopus* oocytes. *Proc Natl Acad Sci U S A* **85**: 7612–16.
- Protter DSW, Parker R. 2016. Principles and properties of stress granules. *Trends Cell Biol* **26**: 668–79.
- Protter DSW, Rao BS, Van Treeck B, Lin Y, Mizoue L, Rosen MK, Parker R. 2018. Intrinsically disordered regions can contribute promiscuous interactions to RNP granule assembly. *Cell Rep* **22**: 1401–12.
- Ramaswami M, Taylor JP, Parker R. 2013. Altered ribostasis: RNA-protein granules in degenerative disorders. *Cell* **154**: 727–36.
- Rongo C, Gavis ER, Lehmann R. 1995. Localization of *oskar* RNA regulates *oskar* translation and requires Oskar protein. *Development* **121**: 2737–46.
- Ross AF, Oleynikov Y, Kislauskis EH, Taneja KL, Singer RH. 1997. Characterization of a *beta-actin* mRNA zipcode-binding protein. *Mol Cell Biol* **17**: 2158–65.
- Saha S, Weber CA, Nusch M, Adame-Arana O, Hoege C, Hein MY, Osborne-Nishimura E, Mahamid J, Jahnelt M, Jawerth L, et al. 2016. Polar positioning of phase-separated liquid compartments in cells regulated by an mRNA competition mechanism. *Cell* **166**: 1572–84.
- Schneider SQ, Bowerman B. 2003. Cell polarity and the cytoskeleton in the *Caenorhabditis elegans* zygote. *Annu Rev Genet* **37**: 221–49.
- Schubert CM, Lin R, de Vries CJ, Plasterk RHA, Priess JR. 2000. MEX-5 and MEX-6 function to establish soma/germline asymmetry in early *C. elegans* embryos. *Mol Cell* **5**: 671–82.

- Semotok JL, Luo H, Cooperstock RL, Karauskakis A, Vari HK, Smibert CA, Lipshitz HD. 2008. *Drosophila* maternal *hsp83* mRNA destabilization is directed by multiple SMAUG recognition elements in the open reading frame. *Mol Cell Biol* **28**: 6757–72.
- Sheu-Gruttadauria J, MacRae IJ. 2018. Phase transitions in the assembly and function of human miRISC. *Cell* **173**: 946–57.
- Shin Y, Brangwynne CP. 2017. Liquid phase condensation in cell physiology and disease. *Science* **357**.
- Sinsimer KS, Lee JJ, Thiberge SY, Gavis ER. 2013. Germ plasm anchoring is a dynamic state that requires persistent trafficking. *Cell Rep* **5**: 1169–77.
- Sladewski TE, Bookwalter CS, Hong M-S, Trybus KM. 2013. Single-molecule reconstitution of mRNA transport by a class V myosin. *Nat Struct Mol Biol* **20**: 952–57.
- Snedden DD, Bertke MM, Vernon D, Huber PW. 2013. RNA localization in *Xenopus* oocytes uses a core group of *trans*-acting factors irrespective of destination. *RNA* **19**: 889–95.
- Soundararajan HC, Bullock SL. 2014. The influence of dynein processivity control, MAPs, and microtubule ends on directional movement of a localising mRNA. *Elife* **3**: e01596.
- St Johnston D, Beuchle D, Nüsslein-Volhard C. 1991. *stau*, a gene required to localize maternal RNAs in the *Drosophila* egg. *Cell*.
- Su X, Ditlev JA, Hui E, Xing W, Banjade S, Okrut J, King DS, Taunton J, Rosen MK, Vale RD. 2016. Phase separation of signaling molecules promotes T cell receptor signal transduction. *Science* **352**: 595–99.
- Tadros W, Goldman AL, Babak T, Menzies F, Vardy L, Orr-Weaver T, Hughes TR, Westwood JT, Smibert CA, Lipshitz HD. 2007. SMAUG is a major regulator of maternal mRNA destabilization in *Drosophila* and its translation is activated by the PAN GU kinase. *Dev Cell* **12**: 143–55.
- Takahara T, Maeda T. 2012. Transient sequestration of TORC1 into stress granules during heat stress. *Mol Cell* **47**: 242–52.
- Takizawa PA, Sil A, Swedlow JR, Herskowitz I, Vale RD. 1997. Actin-dependent localization of an RNA encoding a cell-fate determinant in yeast. *Nature* **389**: 90–93.
- Takizawa PA, Vale RD. 2000. The myosin motor, Myo4p, binds *Ash1* mRNA via the adapter protein, She3p. *Proc Natl Acad Sci U S A* **97**: 5273–78.

- Tang SJ, Meulemans D, Vazquez L, Colaco N, Schuman E. 2001. A role for a rat homolog of Staufen in the transport of RNA to neuronal dendrites. *Neuron* **32**: 463–75.
- Tenlen JR, Molk JN, London N, Page BD, Priess JR. 2008. MEX-5 asymmetry in one-cell *C. elegans* embryos requires PAR-4- and PAR-1-dependent phosphorylation. *Development* **135**: 3665–75.
- Theurkauf WE, Smiley S, Wong ML, Alberts BM. 1992. Reorganization of the cytoskeleton during *Drosophila* oogenesis: implications for axis specification and intercellular transport. *Development* **115**: 923–36.
- Thomsen GH, Melton DA. 1993. Processed Vg1 protein is an axial mesoderm inducer in *Xenopus*. *Cell* **74**: 433–41.
- Trcek T, Grosch M, York A, Shroff H, Lionnet T, Lehmann R. 2015. *Drosophila* germ granules are structured and contain homotypic mRNA clusters. *Nat Commun* **6**: 7962.
- Tübing F, Vendra G, Mikl M, Macchi P, Thomas S, Kiebler MA. 2010. Dendritically localized transcripts are sorted into distinct ribonucleoprotein particles that display fast directional motility along dendrites of hippocampal neurons. *J Neurosci* **30**: 4160–70.
- Urdike DL, Hachey SJ, Kreher J, Strome S. 2011. P granules extend the nuclear pore complex environment in the *C. elegans* germ line. *J Cell Biol* **192**: 939–48.
- Uversky VN. 2017. Intrinsically disordered proteins in overcrowded milieu: Membraneless organelles, phase separation, and intrinsic disorder. *Curr Opin Struct Biol* **44**: 18–30.
- Van Treeck B, Protter DSW, Matheny T, Khong A, Link CD, Parker R. 2018. RNA self-assembly contributes to stress granule formation and defining the stress granule transcriptome. *Proc Natl Acad Sci U S A* **115**: 2734–39.
- Voronina E, Seydoux G, Sassone-Corsi P, Nagamori I. 2011. RNA granules in germ cells. *Cold Spring Harb Perspect Biol* **3**.
- Vourekas A, Alexiou P, Vrettos N, Maragkakis M, Mourelatos Z. 2016. Sequence-dependent but not sequence-specific piRNA adhesion traps mRNAs to the germ plasm. *Nature* **531**: 390–94.
- Wan G, Fields BD, Spracklin G, Shukla A, Phillips CM, Kennedy S. 2018. Spatiotemporal regulation of liquid-like condensates in epigenetic inheritance. *Nature* **557**: 679–83.

- Wang JT, Smith J, Chen B-C, Schmidt H, Rasoloson D, Paix A, Lambrus BG, Calidas D, Betzig E, Seydoux G. 2014. Regulation of RNA granule dynamics by phosphorylation of serine-rich, intrinsically disordered proteins in *C. elegans*. *Elife* **3**: e04591.
- Weeks DL, Melton DA. 1987. A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF-beta. *Cell* **51**: 861–67.
- Wei M-T, Elbaum-Garfinkle S, Holehouse AS, Chen CC-H, Feric M, Arnold CB, Priestley RD, Pappu R V., Brangwynne CP. 2017. Phase behaviour of disordered proteins underlying low density and high permeability of liquid organelles. *Nat Chem* **9**: 1118–25.
- Weil TT. 2014. mRNA localization in the *Drosophila* germline. *RNA Biol* **11**: 1010–18.
- Wippich F, Bodenmiller B, Trajkovska MG, Wanka S, Aebersold R, Pelkmans L. 2013. Dual specificity kinase DYRK3 couples stress granule condensation/dissolution to mTORC1 signaling. *Cell* **152**: 791–805.
- Woodruff JB, Ferreira Gomes B, Widlund PO, Mahamid J, Honigsmann A, Hyman AA. 2017. The Centrosome is a selective condensate that nucleates microtubules by concentrating Tubulin. *Cell* **169**: 1066–77.
- Wu B, Eliscovich C, Yoon YJ, Singer RH. 2016. Translation dynamics of single mRNAs in live cells and neurons. *Science* **352**: 1430–35.
- Xu X, Brechbiel JL, Gavis ER. 2013. Dynein-dependent transport of *nanos* RNA in *Drosophila* sensory neurons requires Rumpelstiltskin and the germ plasm organizer Oskar. *J Neurosci* **33**: 791–800.
- Yao J, Sasaki Y, Wen Z, Bassell GJ, Zheng JQ. 2006. An essential role for *beta-actin* mRNA localization and translation in Ca²⁺-dependent growth cone guidance. *Nat Neurosci* **9**: 1265–73.
- Yisraeli JK, Sokol S, Melton D a. 1990. A two-step model for the localization of maternal mRNA in *Xenopus* oocytes: involvement of microtubules and microfilaments in the translocation and anchoring of *vg1* mRNA. *Development* **108**: 289–98.
- Yoon YJ, Mowry KL. 2004. *Xenopus* Staufén is a component of a ribonucleoprotein complex containing *vg1* RNA and kinesin. *Development* **131**: 3035–45.
- Zhang H, Elbaum-Garfinkle S, Langdon EM, Taylor N, Occhipinti P, Bridges AA, Brangwynne CP, Gladfelter AS. 2015. RNA controls polyQ protein phase transitions. *Mol Cell* **60**: 220–30.

Chapter Two: Fluorescence *in situ* Hybridization of Cryosectioned *Xenopus* Oocytes

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Abstract

Xenopus laevis oocytes are widely used to study mechanisms of RNA function and biogenesis. While the large size of *Xenopus* oocytes is amenable to both biochemical and imaging approaches, the relative opacity of the yolk-rich cytoplasm has limited high-resolution imaging of endogenous RNAs. Here, we present a protocol that combines multi-probe fluorescence in situ hybridization with cryosectioning to provide a highly sensitive means of imaging endogenous oocyte RNAs.

Materials

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

- Formamide (Fisher Scientific, BP228-100)
- MBSH Buffer <R>*
- MEMFA <R>
- Nail polish
- O.C.T. Compound (Fisher Scientific, 4585)
- PBS <R>
- PBT <R>
- PBT-30S <R>
- Prolong Gold™ Antifade Mountant (Thermo Fisher Scientific, P36934)
- Richard-Allan Scientific™ Cytocool™ II Aerosol (Thermo Fisher Scientific, 8323)
- Silicone Adhesive Sealant (Loctite, 595)
- RNA FISH probe set for gene of interest (*These are commercially available e.g., LGC Biosearch Technologies, or can be synthesized as in Raj et al., 2008*).
- Stellaris® RNA FISH Hybridization Buffer (LGC Biosearch Technologies, SMF-HB1-10)
- Stellaris® RNA FISH Wash Buffer A (LGC Biosearch Technologies, SMF-WA1-60)

* Recipes for reagents marked <R> are included.

- Stellaris® RNA FISH Wash Buffer B (LGC Biosearch Technologies, SMF-WB1-20)
- *Xenopus* oocyte Culture Medium (XOCM) <R>

Equipment

- Applicators (cotton-tipped, sterile)
- Cryostat (such as Leica CM3050 S Research Cryostat)
- Embedding molds (disposable, Peel-A-Way [Truncated-T12]; Polysciences, 18986)
- Eppendorf centrifuge tubes (5 mL , RNase-free; Fisher Scientific, 14-282-300)
- Forceps
- Glass Insert Anti-Roll Plate (70 mm)
- Microscope Coverslips (24 × 60 mm)
- Microscope, fluorescence (widefield or confocal)
- Microscope Slide Box
- Microscope slides (Superfrost Plus Gold, 25 × 75 × 1.0 mm; Thermo Fisher Scientific, 15-188-48)
- Microtome Blades (high-profile, disposable 818; Leica Biosystems, 14035838926)
- Rotator
- Slide Chamber (humidified)
- Specimen Disc (30 mm)

Method

Fixation and Freezing

1. Defolliculate and sort oocytes of the desired stage (see Protocol: “Isolation of *Xenopus* Oocytes” [Newman et al. 2018]).
2. Incubate the oocytes for at least 1 hour in XOCM to allow for recovery. (*Note: oocytes can be cultured overnight, if desired.*)
3. Transfer a settled volume of 300-500 μ L of cultured oocytes in XOCM to a 5 mL Eppendorf tube. Remove the OCM and wash oocytes with 5 mL of MBSH.
4. Remove the MBSH and add 5 mL of MEMFA. Rotate the oocytes for 1 hour at room temperature.
5. Remove the MEMFA and replace with 5 mL of PBT. Wash oocytes for 20 minutes, rotating, at room temperature. Repeat PBT washes 2 times, for a total of 3 washes.
6. Following the final wash, allow oocytes to settle to the bottom of the Eppendorf tube. (*Note: some oocytes may adhere to the side of the Eppendorf and require gentle prodding with a pipette tip to be dislodged.*)
7. Remove 2.5 mL of PBT and replace with 2.5 mL of PBT-30S. Allow oocytes to equilibrate for 30 minutes at room temperature. (*Note: the oocytes will float to the top of the liquid volume upon addition of the PBT-30S and slowly sink to the bottom of the tube as they equilibrate.*)
8. Remove 5 mL of PBT/PBT-30S solution and replace with 5 mL of PBT-30S. Allow oocytes to equilibrate for 30 minutes at room temperature. Replace PBT-30S 3 times more for a total of 4 equilibrations.

9. Transfer the oocytes to a Peel-A-Way® Disposable Embedding Mold. Allow oocytes to settle to the base of embedding mold and carefully remove the PBT-30S from the embedding mold using a Pasteur pipette.
10. Gently add O.C.T. over the oocytes, filling the embedding mold to approximately the half waypoint. (*Note: avoid generating bubbles in the O.C.T, particularly near the sample.*) Allow oocytes to equilibrate in O.C.T. for 30 minutes at room temperature.
11. Snap freeze samples in a dry ice-ethanol bath by submerging the embedding mold to its midway point until the O.C.T. becomes uniformly solid. Store at -80°C.

Cryosectioning

12. Upon removal from -80°C all samples should be stored on dry ice prior to sectioning.
13. Place the oocytes (in embedding molds) into a precooled (-20°C) cryostat and equilibrate for approximately 30 minutes.
14. Remove sample from embedding mold and freeze it to a specimen disc using liquid O.C.T. and Cytocool II aerosol spray.
15. Cut 30-50 µM sections under a glass anti-roll plate.
16. Transfer each section to a room temperature microscope slide by touching the slide to the sample. (*Note: Each slide can hold 2-4 sections*)
17. Cure the sections to the slides by storing slides at 18-20°C overnight, shielded from light.

RNA Fluorescence in situ Hybridization (FISH)

18. Using a sterile cotton tipped applicator coated in silicone adhesive sealant, create a hydrophobic barrier around sectioned sample on the slide (see Fig. 2.1A).
19. Coat the sections by applying 1 mL of PBS to coat the sample within the hydrophobic well for 20 minutes at room temperature. (*Note: avoid application of liquid directly to the sectioned samples.*)
20. Remove the PBS and apply 1 mL of PBT to the sample. Incubate for 20 minutes at room temperature.
21. Remove the PBT and apply 1 mL of Wash Buffer A (prepared with 10% formamide, final concentration) to the sample. Incubate for 5 minutes at room temperature. Repeat once.
22. Remove the second wash of buffer A (with 10% formamide). Add 50-100 μ L of hybridization buffer containing (prepared with 10% formamide, final concentration) containing the probe(s) of interest at a concentration of 125-500 nM.
23. Carefully place a clean coverglass over the hybridization buffer. Make sure the buffer is distributed evenly and covers the sample completely.
24. Place the slide in a humidified slide chamber and incubate in the dark for 16-20 hours at 37°C.
25. Immerse the slide in wash buffer A (with 10% formamide). Use forceps to gently remove the coverslip.
26. Remove the slide from the wash buffer. Remove excess buffer surrounding the sections.

27. Apply 1 mL of wash buffer A (with 10% formamide) to the slide. Incubate in the dark for 30 minutes at 37°C.
28. Remove wash buffer A (with 10% formamide) and apply 1 mL of wash buffer B. Incubate in the dark for 5 minutes at room temperature. Repeat the wash with buffer B once, shielded from light.
29. Remove wash buffer B. Add approximately 50-100 µL of Prolong Gold antifade mountant onto the sample sections. Cover with a clean coverslip.
30. Seal corners using nail polish. Cure the samples at room temperature for 24 hours shielded from light.
31. Seal the coverglass perimeter with nail polish and allow to dry shielded from light.
32. Samples are now suitable for imaging using a fluorescence microscope (examples are shown in Fig. 2.1B,C). For short-term storage (up to a week), slides are stable at 4°C.

Troubleshooting

Problem (Steps 19-29): There is poor retention of oocytes on slides during RNA FISH treatment.

Solution: Increasing the density of oocytes on the slide will significantly improve sample retention during processing. Ensuring that oocytes are uniformly settled along the base of the embedding mold prior to snap freezing generates an optimal cutting surface to produce more durable sections.

Discussion

Xenopus laevis oocytes provide an excellent resource for the study of RNA biology in vertebrate cells. Their large size provides advantages for both biochemical and imaging-based approaches. This strength has been applied to the study of RNA-protein interactions (Hake and Richter 1994; Gall et al. 1999; Yoon and Mowry 2004), polyadenylation (McGrew et al. 1989), translational control (Colegrove-Otero et al. 2005) and subcellular localization (Melton 1987; Mowry and Melton 1992) and transport of mRNA (Messitt et al. 2008; Gagnon et al. 2013). The ease with which distinct stages can be separated based on the morphology of the oocyte (Dumont 1972) allows for investigation of specific events in oogenesis in order to probe key developmental questions. However, the yolk-rich oocyte cytoplasm can present a barrier to high-resolution imaging of endogenous RNAs. Approaches to overcome these limitations include both electron microscopy and optical clearing of whole-mount preparations of oocytes and embryos (Kloc et al. 2005; Bilinski et al. 2010; Agricola and Cha 2016). Advances in fluorescence in situ hybridization (FISH), using probe sets of multiple fluorescently labeled oligonucleotides, has allowed for increased sensitivity and higher specificity (Raj et al. 2008). In addition, direct comparison of multiple RNA species in a single cell are possible through multiplexing of probe sets. This protocol combines the strengths of the multi-probe FISH approach with cryosectioning as a means of circumventing the need for clearing agents and increasing target accessibility in *Xenopus* oocytes.

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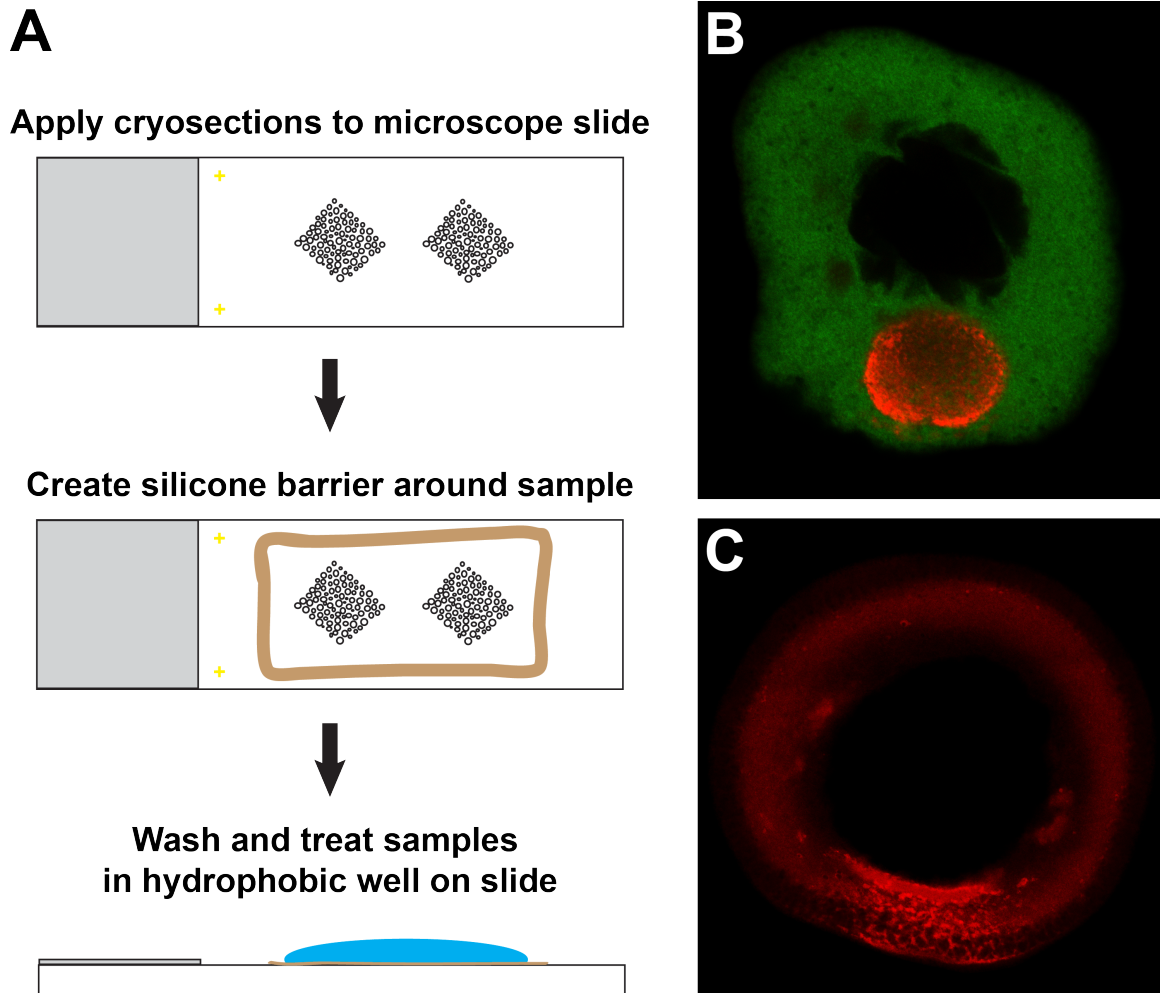


Figure 2.1. Endogenous mRNA FISH in *Xenopus* oocytes. (A) A workflow diagram depicts how oocyte samples cryosectioned onto a microscope slide outlined with silicone adhesive sealant (Step 18), which creates a hydrophobic well over the sample in which FISH treatments (Steps 19-28) are conducted. (B-C) Detection and localization of endogenous *vg1* (Weeks and Melton 1987), *Xpat* (Hudson and Woodland 1998) and GAPDH mRNA in a Stage I-II oocytes were performed using Stellaris gene specific probe sets designed and provided by LGC BioSearch Technologies. The *vg1* (Quasar® 570), *xpat* (Quasar® 570) and *gapdh* (Quasar® 670) probe sets were applied at a concentration of 250 μ M and imaged using a Zeiss LSM 800 Confocal Laser Scanning Microscope with a 20 $\times$ objective. (B) A

stage I oocyte is shown with ubiquitously distributed *gapdh* mRNA (green) and *xpat* mRNA (red), which is localized to the Balbiani body. (C) A stage II oocyte is shown with *vg1* mRNA (red), which is enriched in the vegetal hemisphere. Oocytes are oriented with the vegetal pole at the bottom, and scale bars = 50 μ M.

Recipes

MBSH (10x)

Store as a sterilized 10x stock solution and prepare fresh 1x MBSH prior to use.

880 mM NaCl

10 mM KCl

24 mM NaHCO₃

8.2 mM MgSO₄·7H₂O

3.3 mM Ca(NO₃)₂·4H₂O

4.1 mM CaCl₂·6H₂O

100 mM HEPES (pH 7.6)

MEMFA

Prepare fresh, and protect from light.

1 ml 10× MEM (1 M MOPS, pH 7.4, 20 mM EGTA, 10 mM MgSO₄)

1 ml microfiltered 37% formaldehyde (Electron Microscopy Science, 15686)

8 ml deionized H₂O

Oocyte Culture Media

50% Leibovitz's L-15 Medium (Thermo Fisher Scientific, 11415064)

15 mM HEPES, pH 7.6

1 mg/ml Insulin

Sterilize through a 0.22 µm filter—OCM without antibiotics can be stored at 4°C for up to two months.

Add antibiotics to OCM just prior to culture. Do not store. Do not reuse.

50 U/ml Nystatin (Sigma-Aldrich, N1638)

100 U/ml Penicillin/Streptomycin (Life Technologies, 15070063)

0.1 mg/ml Gentamicin (Thermo Fisher Scientific, 15750060)

PBS

137 mM NaCl

2.7 mM KCl

10 mM Na₂HPO₄

1.8 mM KH₂PO₄

PBT

1× PBS supplemented with

0.2mg/mL BSA (Sigma-Aldrich, A2153)

0.1% (v/v) Triton X-100 in 1×PBS.

Mix thoroughly and sterilize through 0.22 µm filter. Store at 4°C.

PBT-30S

1× PBT supplemented with

30% (w/v) Sucrose

Mix thoroughly. Store at 4°C

References

- Agricola Z, Cha S-W. 2016. Two-color fluorescence *in situ* hybridization using chromogenic substrates in *Xenopus*. *Biotechniques* **61**: 263–68.
- Bilinski SM, Jaglarz MK, Dougherty MT, Kloc M. 2010. Electron microscopy, immunostaining, cytoskeleton visualization, *in situ* hybridization, and three-dimensional reconstruction of *Xenopus* oocytes. *Methods* **51**: 11–19.
- Colegrove-Otero LJ, Devaux A, Standart N. 2005. The *Xenopus* ELAV protein ElrB represses *vgl* mRNA translation during oogenesis. *Mol Cell Biol* **25**: 9028–39.
- Dumont JN. 1972. Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J Morphol* **136**: 153–79.
- Gagnon JA, Kreiling JA, Powrie EA, Wood TR, Mowry KL. 2013. Directional transport is mediated by a Dynein-dependent step in an RNA localization pathway. *PLoS Biol* **11**: e1001551.
- Gall JG, Bellini M, Wu Z, Murphy C. 1999. Assembly of the nuclear transcription and processing machinery: Cajal bodies (coiled bodies) and transcriptosomes. *Mol Biol Cell* **10**: 4385–402.
- Hake LE, Richter JD. 1994. CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. *Cell* **79**: 617–27.
- Hudson C, Woodland HR. 1998. Xpat, a gene expressed specifically in germ plasm and primordial germ cells of *Xenopus laevis*. *Mech Dev* **73**: 159–68.
- Kloc M, Wilk K, Vargas D, Shirato Y, Bilinski S, Etkin LD. 2005. Potential structural role of non-coding and coding RNAs in the organization of the cytoskeleton at the vegetal cortex of *Xenopus* oocytes. *Development* **132**: 3445–57.
- McGrew LL, Dworkin-Rastl E, Dworkin MB, Richter JD. 1989. Poly(A) elongation during *Xenopus* oocyte maturation is required for translational recruitment and is mediated by a short sequence element. *Genes Dev* **3**: 803–15.
- Melton DA. 1987. Translocation of a localized maternal mRNA to the vegetal pole of *Xenopus* oocytes. *Nature* **328**: 80–82.
- Messitt TJ, Gagnon JA, Kreiling JA, Pratt CA, Yoon YJ, Mowry KL. 2008. Multiple kinesin motors coordinate cytoplasmic RNA transport on a subpopulation of microtubules in *Xenopus* oocytes. *Dev Cell* **15**: 426–36.
- Mowry KL, Melton DA. 1992. Vegetal messenger RNA localization directed by a 340-nt RNA sequence element in *Xenopus* oocytes. *Science* **255**: 991–94.
- Newman K, Agüero T, King ML. 2017. Isolation of *Xenopus* oocytes. *Cold Spring Protoc* doi :10.1101/pdb.prot095851.

- Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. 2008. Imaging individual mRNA molecules using multiple singly labeled probes. *Nat Methods* **5**: 877–79.
- Weeks DL, Melton DA. 1987. A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF- β *Cell* **51**: 861–67.
- Yoon YJ, Mowry KL. 2004. *Xenopus* Staufén is a component of a ribonucleoprotein complex containing *vg1* RNA and kinesin. *Development* **131**: 3035–45.

Chapter Three: Dynamic Multiphase RNP Granules Drive RNA Localization in *Xenopus laevis* Oocytes

The work within this chapter is currently being prepared as a manuscript for submission:

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*The first two authors contributed equally to this work.

My work contributed to Figures 3.1(A-F, I-J), 3.2, 3.3(B-F), 3.4, 3.5 (D-J), 3.6, and 3.S1

Abstract

Cytoplasmic mRNA localization is understood as an important biological strategy for establishing cell and developmental polarity in a variety of organisms and cell types. In diverse examples including oocytes, fibroblasts and neurons, mRNA localization results in spatially restricted gene expression through local protein synthesis. In *Xenopus laevis* oocytes, maternal mRNAs are transported to the vegetal hemisphere of developing oocytes where their local translation is critical for proper embryonic patterning. Motor-based vegetal transport of such mRNAs relies on the assembly of RNP transport cargos, a conserved feature of RNA localization pathways. However, the molecular and physical nature of these structures, or other transport RNP cargos, remains as yet unknown. Using both biochemical purification and *in vivo* and fixed cell imaging techniques, our results characterize vegetal transport RNPs as multiphase structures that represent a new class of cytoplasmic RNP granule, bearing both compositional and functional overlap with an expanding set of phase separated RNP granules that function in post-transcriptional gene regulation.

Introduction

Subcellular localization of mRNAs in conjunction with regulated translation is a conserved mechanism among eukaryotes for the generation and maintenance of polarized gene expression (Chin and Lécuyer 2017). Employed in both somatic and germ cells, mRNA localization has traditionally been viewed in the context of generating specialized subcellular compartments, which have been functionally linked to a diverse array of cellular processes. Some of the most prominent examples include the control of mating type switching in yeast (Buxbaum et al. 2015), cytoskeletal-mediated motility in fibroblasts (Eliscovich et al. 2013), axonal guidance and synaptic plasticity in neurons (Holt and Schuman 2013), and the establishment of developmental patterning in both vertebrate and invertebrate animals (Medioni et al. 2012; Schisa 2012).

While the aforementioned examples are limited to mRNAs encoding proteins with specialized local functions, several studies have suggested that mRNA localization may be a more general aspect of RNA biology. A high-throughput, high-resolution *in situ* hybridization screen in *Drosophila* embryos revealed that up to 70% of endogenous transcripts exhibit distinct subcellular localization (Lécuyer et al. 2007). Similarly, in mammalian neurons fully half of mRNA transcripts are enriched in axons and dendrites relative to the cell body (Eberwine et al. 2002; Cajigas et al. 2012), in stark contrast to the previously held belief that only a small handful of mRNAs localized to synapses. Similar analyses in migrating fibroblasts (Mili et al. 2008) and *Xenopus* oocytes (Blower et al. 2007) hint at a broader class of localized RNA transcripts. By pairing spatial restriction of genes with tight translational controls, cells are able to exert exquisite spatiotemporal control over

gene expression. This, in turn, allows cells to mediate protein synthesis in response to dynamic cues generated either intrinsically or in response to external stimuli.

Targeting of a transcript for localization is initiated by *cis*-acting elements within the mRNA sequence. These localization elements, or zipcodes, are most frequently found in the 3' untranslated region (3'UTR) of mRNAs, although some have been identified in the 5'UTR or in the coding sequence (reviewed in Jambhekar and Derisi 2007). *Cis*-sequence elements are recognized by *trans*-acting protein factors, usually RNA-binding proteins (RBPs). Generally, these RBPs have roles in both the localization and translational control of their target mRNAs. As such, combinations of *cis*-elements can drive the assembly of specific RNA-protein complexes, ribonucleoproteins (RNPs), that can either independently, or in higher-order assemblies, often termed granules, mediate a diversity of functions.

Xenopus laevis oocytes are an important model system for the study of mRNA localization (Medioni et al. 2012). One of the first identified maternally localizing vertebrate mRNAs, *vg1* (also known as *gdf1* and *dvr1*) becomes restricted to the vegetal hemisphere of developing *Xenopus laevis* oocytes (Rebagliati et al. 1985; Weeks and Melton 1987), where it specifies endoderm and mesoderm formation (Birsoy et al. 2006). Initially ubiquitously expressed throughout the oocyte cytoplasm in early oogenesis, *vg1* mRNA becomes spatially restricted to the vegetal hemisphere during stages II-III of oogenesis and is tightly localized to the vegetal cortex by the end of oogenesis (stage VI) where it will ultimately be inherited by vegetal blastomeres during embryogenesis (Melton 1987). Vegetal localization is mediated by a set of *vg1* RNA-binding proteins (RBPs), including

hnRNPAB, PTB, Staufen, and Vera, which interact with *vg1* 3'UTR sequences (Deshler et al. 1997; Gautreau et al. 1997; Havin et al. 1998; Cote et al. 1999; Yoon and Mowry 2004; Czaplinski et al. 2005; Lewis et al. 2004). Transport of these RNPs to the vegetal cortex is achieved through active transport along microtubules, mediated by the motor proteins Kinesin and Dynein (Betley et al. 2004; Messitt et al. 2008; Gagnon et al. 2013). However, the molecular and physical nature of these and other RNP transport cargos remains unknown. The *vg1* RBPs hnRNPAB, PTB, Staufen, and Vera have all been identified as constituents of other cytoplasmic RNP granules (Kanai et al. 2004; Jain et al. 2016; Hubstenberger et al. 2017). This overlap in core RBPs raises the question of whether vegetal transport RNPs might represent a class cytoplasmic RNP granules.

RNP granules have traditionally been classified in accordance with their cellular context and their presumed cellular function. In this way, germline specifying granules (such as germ plasm in *Drosophila*, P-granules in *C. elegans*, Balbiani body, and nuage) have been considered distinct from other classes of cytoplasmic RNP granules (such as stress granules, p-bodies, and neuronal transport granules) (reviewed in Buchan 2014). Studying these structures independently has revealed much about their biological activity and identified many of the protein and RNA components involved in RNP granule assembly, maintenance, and function. Yet, while differences have emerged, fundamental commonalities between different RNP granules are also apparent. Granules are distinct cellular compartments that are not bound by membranes but are still capable of concentrating protein and RNA components, maintaining and regulating their structures, and controlling their composition to modulate biochemical activities (reviewed in Banani

et al. 2017). In all cases, they contain translationally repressed mRNAs that are capable of (re)-entering translation in response to appropriate signals (Hüttelmaier et al. 2005; Brengues et al. 2005; Bhattacharyya et al. 2006; Nagamori et al. 2011). And perhaps most intriguing, mRNP granules display broad overlap in protein composition (reviewed in Buchan 2014).

While a number of proteins are directly conserved amongst different granule types a broader compositional trend is observed in the enriched of both multivalent RBPs and proteins that contain extended stretches of low sequence complexity, termed intrinsically disorder regions (IDRs) (Kedersha et al. 2013; Buchan 2014). Such multivalent proteins, consisting of multiple modular domains (Li et al. 2012; Lin et al. 2015) and IDRs with repeated interaction motifs (Kato et al. 2012; Elbaum-Garfinkle et al. 2015; Nott et al. 2015; Protter et al. 2018) are thought to work in combination with RNA species that contain multiple protein-binding sites (Li et al. 2012; Han et al. 2012) to drive polymerization-based phase separations (Brangwynne et al. 2015). In such structures, granule components are held in a condensed liquid or gel-like phase in the granule and dispersed phase in the cytoplasm (Weber and Brangwynne 2012; Toretzky and Wright 2014).

Work in *C. elegans* has demonstrated that P granules form through such liquid-liquid demixing from the surrounding cytoplasm in response to developmental polarity protein gradients (Brangwynne et al. 2009; Saha et al. 2016). The viscosity of P granules, believed to control the intra-granule dynamics of protein constituents (Wei et al. 2017), is developmentally regulated and relies on DEAD-box helicases to prevent it from polymerizing into a nondynamic solid (Hubstenberger et al. 2013; Elbaum-Garfinkle et al. 2015; Hubstenberger et al. 2015). Post-translational modifications, in the form of

phosphorylation events, leads to the disassembly of the granule during late embryogenesis, offering a mechanism whereby germline mRNA are released from translational repression (Wang et al. 2014).

Like P granules, stress granules are liquid-like RNP granules that function in the control of gene expression (Patel et al. 2015). However, unlike P granules, stress granules are not regulated by developmental cues designed to restrict gene expression to a specific spatial pattern, but rather are distributed cytoplasmic bodies that transiently form in response to cellular stresses such as heat shock, amino acid starvation, hypoxia, and chemical perturbation (Decker and Parker 2012). Stress granules contain a liquid-like shell that surrounds more stable cores (Jain et al. 2016). The dynamics and assembly of these structures are mediated through the activity of ATP consuming complexes, with chaperone proteins and DEAD-box helicase family members featuring prominently in stress granule maintenance (Jain et al. 2016). Interestingly, the self-assembly capabilities of RNA are believed to modulate partitioning of mRNAs into stress granules, and may play a structural role beyond complexing protein-protein interactions (Van Treeck et al. 2018).

Using a core set of underlying principles, phase separated RNP granules are capable of regulating gene expression in diverse biological settings. Yet, the mechanisms by which phase separation affects granule function remain poorly understood. Vegetal transport complexes in *Xenopus laevis* oocytes represent a unique class of mRNPs, displaying features of both germ and somatic granule types. Capable of acting as long-term storage sites for specific translationally repressed mRNA, vegetal transport RNPs are governed by developmental cues. Additionally, they require motor-mediated transport over an extended distance and interact extensively with the cellular cytoskeleton.

In this study, we find that vegetal transport granules are unexpectedly large, containing multiple vegetally-localized mRNAs, and are surrounded by microtubules and molecular motors. Biochemical isolation and proteomic analysis of vegetal RNP transport granules reveals a high degree of similarity to other classes of cytoplasmic RNP granules. In addition to two-thirds of the proteins being directly conserved amongst cytoplasmic granules, the over-representation of multivalent RBPs and proteins containing IDRs provides a compositional link between the vegetal RNP transport granule and an emerging class of phase separated mRNPs. In vegetal RNP transport granules, mobility of constituent proteins and RNAs *in vivo* differs distinctly; constituent proteins are dynamic while localized RNAs are immobile. Our results reveal the *Xenopus* vegetal transport RNP as a new multiphase cytoplasmic granule, composed of a non-dynamic mRNA-containing substructure enmeshed in a dynamic liquid-like protein layer.

Results

During stages II-III of *Xenopus laevis* oogenesis, *vg1* mRNA is enriched in the vegetal oocyte cytoplasm (Fig. 3.1A), as visualized by fluorescence *in situ* hybridization (FISH). At higher magnification, individual foci of *vg1* mRNAs can be visualized in large clusters (Figure 3.1B). *VegT* mRNA is also localized to the vegetal cortex during stages II-III of oogenesis (Zhang and King 1996) (Fig. 3.1 A'). At higher magnification, individual foci of *vg1* mRNAs can be visualized in surprisingly large clusters (Figure 3.1 B), which also contain *vegT* mRNA (Figure 3.1B'). These clusters, comprised of multiple *vg1* and *vegT* foci, average between 5-10 μm (Figure 3.1 B,B'). By contrast, a non-localized mRNA, *gapdh*, is uniformly distributed in the oocyte cytoplasm (Figure 3.1 C') and is not enriched in *vg1* mRNA clusters (Figure 3.1 D,D'). Our results suggest that multiple copies of vegetally localized mRNAs are packaged together during vegetal localization and we hypothesized that these large mRNA clusters may represent vegetal RNP transport granules

In order to characterize the vegetally localized *vg1* RNPs, we carried out gel filtration chromatography using lysates prepared from stage II-III *Xenopus* oocytes. We found that *vg1* mRNA (Figure 3.1E), as well as the *vg1* RNA-binding protein Vera (Igfbp3; Figure 3.1F), chromatograph as large complexes in the void volume. After treatment of oocyte lysate with RNase prior to gel filtration chromatography, Vera is no longer present in the void volume and is instead found in smaller complexes (Figure 3.1F). This is in contrast to tubulin, which chromatographs in later fractions regardless of RNase treatment (Figure 3.1F). Co-immunoprecipitation analyses using pooled fractions (fractions 2-5, Figures 3.1E,F) from the void volume after gel filtration chromatography demonstrated

that well-characterized *vg1* RNA binding-proteins (RBPs), Vera (Havin et al. 1998; Deshler et al. 1998), Staufen (Yoon and Mowry 2004), and hnRNPAB (40LoVE; Czaplinski et al. 2005) all co-immunoprecipitate with one another (Figure 3.1G). Importantly, these *vg1* RBPs also co-immunoprecipitate *vg1* mRNA from the large RNP pool (Figure 3.1H). These results suggest that *vg1* mRNA is transported to the vegetal cortex in a large RNP.

We have previously shown that vegetal RNA transport relies on microtubule motor proteins, Kinesin and Dynein (Messitt et al. 2008; Gagnon et al. 2013). To determine whether the large *vg1* RNPs are associated with microtubules and motor proteins, we performed FISH-IF for *vg1* mRNA and Tubulin (Figure 3.1I,J), Kinesin (Figure 3.1I') and Dynein (Figure 3.1J'). The large *vg1* RNPs appear to be encased in microtubule networks (Figure 3.1I,J), which are decorated with both Kinesin and Dynein motors (Figure 3.1I',J'), suggesting that the large *vg1* mRNA complexes are transport RNPs.

Assembly into large RNPs is required for vegetal RNA localization

Studies of vegetal RNA transport in *Xenopus* oocytes have previously relied on microinjection of fluorescently-labeled RNA to probe the localization pathway. In particular, a vegetal localization element (VLE) from the 3'UTR of *vg1* mRNA is faithfully localized to the vegetal cortex after microinjection into stage II-III *Xenopus* oocytes and accumulates at the vegetal cortex after time in culture (Gautreau et al. 1997; Lewis et al. 2008). During the process of vegetal localization, exogenous VLE RNA is enriched in the vegetal cytoplasm (Figure 3.2A), and is both colocalized with endogenous *vg1* mRNA in the vegetal cytoplasm (Figure 3.2A') and incorporated into the large *vg1* RNP complexes

(Figure 3.2B,B'). By contrast, a mutated version of *VLE* RNA (Lewis et al. 2004) that is not capable of vegetal localization (Figure 3.2C) is not present the large *vg1* RNP complexes (Figure 3.2D,D').

Incorporation of microinjected RNA into large RNP complexes was further examined by gel filtration chromatography (Figure 3.2E). Both endogenous *vg1* mRNA (black) and microinjected *VLE* RNA (green) displayed enrichment within large complexes that chromatographed in the void volume, while microinjected non-localized RNAs, *mut-VLE* (red) and β -globin (blue) did not (Figure 3.2E). Because simultaneous detection of the microinjected RNAs required tagging of the RNAs with unique bar codes, we tested whether the bar codes used for *VLE* RNA (B-A) or *mut-VLE* RNA (B-B) could affect the chromatography of microinjected β -globin RNA complexes. Neither B-A, nor B-B tagged β -globin RNA were incorporated into large RNP complexes, (Figure 3.2F). Taken together, these results suggest that incorporation into the large *vg1* RNP complexes (termed vegetal transport RNPs) is important for vegetal RNA localization.

Purification of vegetal transport RNPs

To analyze the vegetal transport RNPs, we developed a multi-step biochemical approach to isolate the vegetal transport RNPs from *Xenopus* oocytes (Figure 3.3A), as follows: (1) Manual sorting of whole stage II-III oocytes with crosslinking to reduce the possibility of re-association of proteins and RNAs after oocyte lysis (Mili and Steitz 2004). (2) Generation of yolk-cleared oocyte lysate. (3) Enrichment of large RNP complexes by size-exclusion chromatography. (4) Enrichment of specific vegetal RNP transport

complexes via immunoprecipitation with antibodies specific for proteins known to interact with vegetally transported RNA. For the final step, we carried parallel immunoprecipitation with two known *vg1* RBPs, rather than a single marker, because the known *vg1* RBPs, Staufen, hnRNPAB, PTB, and Vera, have multiple roles in RNA biogenesis and are not solely involved in mRNA localization (Yisraeli 2005; Snedden et al. 2013; Heraud-Farlow and Kiebler 2014) (Snedden et al. 2013). However, we reasoned that a combination of two *vg1* RBPs would give high confidence that proteins associated with both would represent constituents of vegetal transport RNPs. Of the known *vg1* RBPs, we chose Staufen1 and hnRNPAB for the following reasons: (1) hnRNPAB is highly enriched in *vg1* transport RNPs (Figure 3.3B,C); (2) Staufen1 is enriched in *vg1* transport RNPs (Figure 3.3D,E); (3) Staufen1 is suggested to confer vegetal targeting for *Xenopus* maternal RNPs (Snedden et al. 2013); (4) High-quality, affinity-purified, antibodies are available for both hnRNPAB and Staufen1; (5) Both Staufen1 and hnRNPAB chromatograph as large complexes in the void volume after gel filtration chromatography (Figure 3.3F), coincident with the fractions containing *vg1* mRNA (Figure 3.1E).

After size exclusion chromatography (Figures 3.1E-F, Figures 3.3A,F), we pooled the fractions containing *vg1* RNA, hnRNPAB and Staufen1 (“pool”; Figures 3.1A,F). In order to specifically isolate the large RNP complexes for further analysis, we performed three parallel immunoprecipitations from the pool (Figure 3.3A) using Staufen1 and hnRNPAB antibodies, with IgG as a negative control. Immunoblot analysis confirmed that each of these *vg1* RNA binding proteins co-immunoprecipitated with the other, but not with IgG (Figure 3.1G). In addition, RIP-qRT-PCR, demonstrated that *vg1* mRNA was significantly enriched in the RBP-IPs compared to IgG (Figure 3.1H).

The Vegetal Transport RNP Proteome

Next, we performed mass spectrometry, in biological quadruplicates, on each eluate from the three immunoprecipitations. We considered a candidate protein to be a vegetal RNP transport granule component if the Stauf1 and hnRNPAB immunoprecipitates had identified peptides in at least three of the four replicates, and if these peptides counts were at least two-fold enriched over those in IgG in each replicate. We identified a set of 86 proteins that interact with both Stauf1 and hnRNPAB (Figure 3.3G). Among the identified proteins in the hnRNPAB/Stauf1 dataset (Figure 3.3I) are known *vg1* RNA binding proteins, Elav1, Elav2, hnRNPAB, Igf2bp3 (Vera), Ptb1 (PTB), Stau1, and Stau2 (Havin et al. 1998; Deshler et al. 1998; Cote et al. 1999; Yoon and Mowry 2004; Allison et al. 2004; Colegrove-Otero et al. 2005; Czapinski et al. 2005; Arthur et al. 2009; Snedden et al. 2013). In addition, we note the presence of both Dynein (Dync1l1 and Dync1l2) and Kinesin (Kif3b) subunits. Both Kinesins and Dynein have previously been identified as motors responsible for active transport of *vg1* mRNA along microtubules (Betley et al. 2004; Messitt et al. 2008; Gagnon et al. 2013). The presence of proteins that are already known to interact with *vg1* mRNA increased confidence that the additional 76 proteins are likely to represent newly identified components of the vegetal transport RNP.

We validated potential vegetal transport RNP components by both FISH-IF (Figure 3.4 A-N) and gel filtration chromatography (Figure 3.4O). As shown in Figure 3.4, Cpeb1 (A), Ddx1 (C), Ddx3 (E), Lsm14A (G), Lsm14B (I), Ybx1 (K), and Ybx2 (M) all show significant enrichment at the vegetal cortex and in the vegetal cytoplasm, in a distribution similar to known *vg1* RBPs (Figure S3.1), Elav1 (A), Elav2 (C), PTB (E) and Vera (G). Viewed at higher magnification (Figure 3.4), Cpeb1 (B), Ddx1 (D), Ddx3 (F), Lsm14A (H),

Lsm14B (J), Ybx1 (L), and Ybx2 (N) each co-localize with *vg1* mRNA (B' , D' , F' , H' , J' , L' , N') in large RNP structures surrounded by microtubule networks (B'' , D'' , F'' , H'' , J'' , L'' , N''). Analysis of Cpeb1, Ddx1, Ddx3, Lsm14A, Lsm14B, Ybx1, and Ybx2 by gel filtration chromatography shows that each of these proteins chromatographs in the void volume (Figure 3.4O) in large RNP complexes that are sensitive to RNase treatment (not shown). Using this approach, we have validated 43 of the potential vegetal RNP transport granule components listed in Figure 3.3I (Table S3.1).

Gene ontology (GO) analysis (Figure 3.3H) revealed that four of the five top GO-categories are related to nucleic acid binding, with the top category, perhaps not surprisingly, being RNA binding. We also found a significant enrichment of ATP binding, in part contributed by the four DDX helicases identified in the vegetal transport RNP (Figure 3.3H), which may act as RNP remodeling enzymes through their ability to unwind RNA and alter RNA-protein interactions (Gilman et al. 2017).

The Vegetal Transport RNP is a New Type of Cytoplasmic Granule

A striking feature of the vegetal transport RNP proteome is the abundance of proteins that are known components of previously-described cytoplasmic RNP granules, including stress granules, processing bodies, neuronal granules, and germ granules (designated S, P, N, and G, respectively, in Figure 3.3I). Remarkably, approximately two-thirds of the identified vegetal transport RNP components are present in at least one other RNP granule type (Figure 3.3J,K), with the remaining third of the identified proteins representing novel components of the vegetal transport RNP (Figure 3.3J).

Based on the prevalence of low-complexity domain and intrinsically disordered regions in the proteomes of known cytoplasmic RNP granules (Uversky 2017), we also examined the prevalence of such domains in the vegetal transport RNP components. Using, SLIDER (Peng et al. 2014), a predictive tool for intrinsically disordered domains, we found that nearly 75% of vegetal transport RNP components contained a putative intrinsically disordered domain, and 60% of the novel identified components fall into this category (Figure 3.5A). This is a significant enrichment compared to that of the *Xenopus* proteome. Prion-like domains represent a more specific category of intrinsically disordered regions, and also exhibit overrepresentation in the vegetal transport proteome. Vegetal transport RNPs are enriched 6-fold in prion-like domains compared to the entire *Xenopus* proteome, as determined with the predictor PLAAC (Lancaster et al. 2014)(Figure 3.5B).

Low complexity and prion-like domains are, by definition, rich in particular amino acids. Intrinsically disordered domains are overrepresented with arginine and glycine, and prion-like domains are rich in glutamine and asparagine (Kaganovich 2017). We therefore investigated overrepresented motifs in our vegetal transport granules, using the online-suite, MEME (Bailey and Elkan 1994). We found an enrichment of phenylalanine-glycine repeats (Figure 3.5C). These motifs are classically found in nuclear pore proteins where they have shown the ability to form hydrogels and act as molecular sieves (Frey et al. 2006). Taken together, these results suggest that the vegetal transport RNPs may represent a new class of cytoplasmic RNP granule.

Vegetal Transport Granules Exhibit a Mesh-like Substructure that Co-localizes with Vegetally-localized RNAs

Because prion-like domains are over-enriched in the vegetal transport granule proteome, we next tested whether the vegetal transport RNPs contained amyloid like structures. We stained oocyte sections with Thioflavin S (thio-S), a dye that selectively fluoresces in the presence of cross-beta strands, characteristic of amyloid structures (Guntern et al. 1992), as well as structured RNAs (Xu et al. 2016). As shown in Figure 3.5, the vegetal oocyte cytoplasm stains richly with thio-S (3.5D), revealing structures that resemble vegetal transport granules (3.5E) with a mesh-like substructure evident at high magnification (3.5F). Combined FISH and thio-S staining (Figure 3.5G-J) confirms that thio-S stains transport granules, as three localized mRNAs, *vgl* (3.5G), *vegT* (3.5H), and *trim36* (3.5I; Cuykendall and Houston 2010) are highly enriched. By contrast, non-localized *gapdh* mRNA is not enriched (Figure 3.5J). It is notable that the vegetal mRNAs appear to be highly coincident with the mesh-like staining, perhaps suggesting an RNA contribution to the vegetal transport granule substructure.

Dynamics of Vegetal Transport Granule RNAs and Proteins Differ in vivo

Other types of cytoplasmic RNP granules have been described as phase-separated bodies, with constituent proteins and mRNAs exhibiting liquid-like behavior in some granules and amyloid-like properties in others (Banani et al. 2017; Shin and Brangwynne 2017). To determine the dynamics of vegetal transport granules *in vivo*, we marked the transport granules by microinjection of fluorescently-labeled VLE RNA into stage II-III *Xenopus*

oocytes expressing mCherry-tagged proteins. Protein mobility was assessed by FRAP (fluorescence recovery after photobleaching) in live oocytes (Figure 3.6A-D). In these experiments, the ROI was positioned to bleach only half of an individual transport granule in order to evaluate intra-granule mobility. We tested three previously known vegetal transport granule proteins, hnRNPAB, Staufen1 and Vera, as well as a newly-identified constituent, Ybx1. Each of these proteins contain at least two RNA-binding domains and hnRNPAB and Ybx1 additionally contain intrinsically disordered regions (IDRs). The four RBPs exhibited reduced mobility in the transport granule, as compared to mCherry alone, with hnRNPAB showing the highest mobile fraction (85.4%) and Vera having the lowest (52.4%) (Figure 3.6B). These data indicate that protein constituents of vegetal transport granules are relatively dynamic within the transport granule. By contrast, mobility of vegetally localized RNAs within transport granules is far lower (Figure 3.6E-F). *VLE* RNA is highly immobile, with a mobile fraction of 4.4% (Figure 3.6F), and *VegTLE* RNA is similarly immobile (not shown). Non-localized RNAs, however, are more mobile; the mobile fractions for *mut-VLE* RNA (Figure 3.2C') and *gapdh* mRNA (Figure 3.1G') are 60.9% and 55.7%, respectively (Figure 3.6F,H). The low mobility of localized RNAs combined with the dynamic behavior of constituent proteins may suggest at least two distinct phases within vegetal transport granules, with localized RNA as a potentially solid- or gel-like element.

Discussion

Here, we present evidence that *Xenopus laevis* vegetal mRNA localization proceeds through the formation of a large multiphase messenger ribonucleoprotein (mRNP) granule, bearing tremendous compositional and functional overlap with other classes of cytoplasmic mRNP granules. The maternally loaded *vg1* mRNA is observed to accumulate in large bodies in the vegetal hemisphere of stage II-III oocytes. These large RNP complexes, hereafter termed granules, are encapsulated by a dense network of microtubules, with potential points of intercalation apparent throughout (Figure 3.1I-J). In contrast with some other types of transport complexes, where mRNA are packaged singly (Batish et al. 2012; Buxbaum et al. 2014; Little et al. 2015), multiple *vg1* transcripts are contained within individual granules (Figure 3.1B). Additional vegetally localized mRNA species, *vegT* and *trim38* (Zhang and King 1996; Cuykendall and Houston 2009), are also incorporated into *vg1* granules, with these mRNAs appearing evenly distributed throughout the body (Figures 3.1B, 3.5H-I). Interestingly, both dynein and kinesin motor types, which have previously been shown to play a role in *vg1* mRNA localization (Betley et al. 2004; Messitt et al. 2008; Gagnon et al. 2013), appear to localize to the microtubules associated with the granule, suggesting that they interact with the broader granule structure rather than with individual mRNPs (Figure 3.1I-J).

The large size of vegetal transport granules, in excess of 10 μ M at their widest point, serves as the basis for a novel isolation strategy. Through size exclusion chromatography of stage II-III oocyte lysate, large *vg1*-containing RNPs can be isolated from fractions eluting in the void volume (Figures 3.1E, 3.3F). Further purification using interactions with the known *vg1* RNA binding proteins (RBPs), Staufen1 and hnRNPAB (Figures 3.1G,

3.3A), allows for confidence in the specificity of vegetal transport granule capture. After mass spectrometry, 86 putative vegetal transport granule proteins were identified (Figure 3.3G,I). Interestingly, the composition of the vegetal transport granule shows a high degree of similarity to other classes of cytoplasmic RNP granules (Figure 3.3J,K). In addition to two-thirds of the proteins being directly conserved amongst cytoplasmic granules, the overrepresentation of multivalent RBPs and proteins containing intrinsically disordered regions (IDRs) provides a compositional link between the vegetal transport granule and an emerging class of phase separated mRNPs (Banani et al. 2017; Shin and Brangwynne 2017) (Figures 3.3J,K and 3.5A,B).

Characterization of the composition of vegetal transport granules using both *in vivo* and fixed imaging strategies, reveals that the structures contain both nondynamic scaffold components as well as dynamic client proteins that are both enriched over and actively exchanging with the surrounding cytoplasm (Figures 3.3B-D, 3.4A-N, 3.6A-D). This multiphase body composition parallels findings in P granules (Wang et al. 2014) and stress granules (Jain et al. 2016) and may reflect a fundamental aspect of mRNP granule organization. Interestingly, within the dynamic phase, protein mobility may correlate with the degree of resident valency, as Vera which harbors multiple RNA-binding domains shows reduced mobility relative to lower valency RBPs and a non-valent mCherry control (Figure 3.6A). This is in fitting with a model of mRNP granule composition that is specified by heterotypic multivalent interactions (Banani et al. 2016), wherein the intra-granular behavior of a client protein is dictated by its potential to interact within the granule environment.

Within vegetal transport granules, localized mRNAs are nondynamic, demonstrating no intra-granule redistribution upon photobleaching (Figure 3.6E-G). This suggests a material distinction between the RNA containing portion of the granule and a dynamic protein phase. Interestingly, while nonlocalized mRNAs are not concentrated within the granule, they are also not excluded (Figures 3.1C-D, 3.2C-D). Nonlocalizing mRNAs appear to remain in dynamic exchange with the surrounding cytoplasm (Figure 3.6E,F,H) and fail to establish a concentration bias. Incorporation into the vegetal transport granule relies on sequence-encoded features of mRNA (Figure 3.2C; Lewis et al., 2004) and likely represents a capture event of freely diffusing mRNA. This is supported by the observation that exogenously expressed transcripts are recruited into preexisting granules, rather than packaged independently (Figure 3.2A). Importantly, the observation that sequence mutations within the *vg1* vegetal localization element that are known to ablate localization (Lewis et al. 2004) also block granule incorporation (Figure 3.2C), functionally links the two processes and establishes the vegetal transport granule as a key mediator of *vg1* mRNA localization.

A clear substructure is apparent upon staining with Thioflavin (Figure 3.5F), a dye most commonly thought to stain amyloid-like aggregates formed by protein β -sheet interactions (Nilsson 2004). Interestingly, while exhaustive efforts to identify granule protein components with both structured patterns of granule localization and nondynamic intra-granule motility failed to provide insights into the composition of the mesh-like substructure, evidence of a more direct role for RNA-RNA interactions is tantalizing. The near complete overlap of Thioflavin staining with localized mRNA (Figure 3.5G-I) opens

the possibility that RNA self-assembly may drive the observed multiphase behavior of the vegetal mRNP transport granule. In this model, the interactions of localized mRNAs promotes RNA-gelation within mRNPs. Previous research demonstrating that depletion of *vegT* mRNA, a component of the vegetal mRNP granule, leads to the mislocalization of *vg1* mRNA (Heasman et al. 2001), suggests that heterotypic mRNA interactions may be fundamental to this process. While RNA has traditionally been viewed as a multivalent platform, capable of mediating intermolecular interactions between *trans*-acting proteins, recent studies have challenged this ancillary role for RNA in granule organization, and offered new roles for RNA-RNA interactions in defining the composition of mRNP granules (Jain and Vale 2017; Van Treeck et al. 2018; Langdon et al. 2018; Niepielko et al. 2018; Yamazaki et al. 2018). Intriguingly, Thioflavin has also been demonstrated to recognize dense networks of RNA structure (Xu et al. 2016), raising the possibility that RNA may be the source of Thioflavin staining observed in vegetal transport granules.

A unifying theme of cytoplasmic mRNP granules is that they all contain translationally repressed mRNAs that are capable of (re)-entering translation in response to environmental cues (Buchan 2014). This paradigm extends to the *Xenopus* vegetal transport granule, where resident *vg1* mRNA remains translationally quiescent throughout localization (stages I-III) (Tannahill and Melton 1989). This repression is relieved in later oogenesis when *vg1* mRNA is restricted to the vegetal cortex (Tannahill and Melton 1989), and appears to be coincident with the phosphorylation-mediated remodeling of the *vg1* mRNP complex by the developmentally regulated Erk2 MAP kinase during oocyte maturation (Git et al. 2009). In light of this, it is tempting to speculate that the vegetal transport granule may have a direct function in translational control. Indeed, more than

20% of proteins contained in the vegetal transport granule have roles in translational regulation, as determined by gene ontology analysis (Mi et al. 2017).

Phase separated granules have been proposed to aid in the translational repression of mRNA by concentrating reactants to shift the equilibrium of interacting proteins towards associated states (Buchan 2014). Translational control of *vg1* mRNA is in part mediated through the direct binding of the 3'UTR by ELAVL2 (Otero et al. 2001; Colegrove-Otero et al. 2005), which is also present in the vegetal transport granule. Interestingly, while Elavl2 remains present in the oocyte beyond maturation, it is no longer observed to bind *vg1* mRNA (Otero et al. 2001; Colegrove-Otero et al. 2005). The presence of the poly(A)-dependent translational repressor, CPEB1 within the vegetal mRNP granule (Figures 3.3I, 3.4A-B), suggests an additional level of translational control for localized mRNA. CPEB, known to act as a translational repressor during oogenesis (Richter 2007), is recruited to nearly all types of cytoplasmic mRNP granules (Buchan 2014), where it has been shown to both block initiation complex formation and recruit deadenylase enzymes to maintain translationally limiting short poly(A) tail lengths (Richter 2007). A more prominent role for deadenylation as a tool for post-transcriptional regulation in granules is supported by the recent finding that deadenylase activity can be accelerated by more than two orders of magnitude within phase separated bodies (Sheu-Gruttadauria and MacRae 2018).

By defining the physical cargo of vegetal transport in *Xenopus* oocytes as an mRNP granule, regulation of distinct transport and developmental steps can be understood as a reflection of the unique physical properties of phase-separated structures. Cytoplasmic mRNP granule components have been observed to transition among diffuse, liquid-like, or

more solid states in response to environmental cues that regulate coassembly, viscosity, and demixing specificity within granules (Hubstenberger et al. 2013; Elbaum-Garfinkle et al. 2015; Hubstenberger et al. 2015; Jain et al. 2016). In this way, the innately reversible interactions that underlie body composition allow for fine-tuning of granule dynamics, which can be exploited as a regulatory mechanism. Future studies are likely to build upon these themes, allowing research into the mechanisms of granule-mediated mRNA localization to provide insights into more global patterns of cellular organization and post-transcriptional gene regulation.

Methods

Oocyte isolation and culture

Oocytes were harvested and isolated from *Xenopus laevis* females (NXR) as described in (Newman et al. 2018), with the following exceptions. Oocytes were enzymatically defolliculated by manual rocking in collagenase solution (3 mg/ml Collagenase I, 0.1 M KPO₃) followed by washes in MBSH (Neil and Mowry 2018). Stage II and III oocytes were manually sorted and cultured overnight (16-18 hours) at 18°C in XOCM (Jeschonek and Mowry, 2018).

RNA in vitro transcription and microinjection

Fluorescently-labeled-RNAs were generated using the MEGAscript T7 transcription kit (Ambion) according to manufacturer's instructions, in the presence of 2.5 µl of CyTM 3- or 5-UTP (GE Healthcare) as in (Jeschonek and Mowry 2018). The concentration of the resuspended RNA was assessed using a NanoDrop microvolume spectrophotometer (ThermoFisher) and diluted to a stock concentration of 1 µM.

Microinjection was performed as in (Newman et al. 2018; Agüero et al. 2018). Cy-labeled wildtype (Gautreau et al. 1997) and nonlocalizing mutant (Lewis et al. 2004) VLE RNAs were prepared from linearized plasmids. For vegetal localization element (VLE) wildtype versus mutant RNA incorporation into endogenous granule experiments (Figure 3.2) RNA was injected at 500 nM. For barcode labeling experiments, RNA was injected at 1.25 nM. In all cases oocytes were incubated overnight (16-24 hrs) following injection.

Construct Preparation

Barcode RNAs were generated from PCR products using pSP64TSNRLMCS:XBM5 (Mowry 1996), pSP73:VLE (Gautreau et al. 1997), and pSP73:mutVLE (Lewis et al. 2004) plasmids as templates. Primer sequences were as follows:

VLE_barcodeA forward 5'-
GAAATTAATACGACTCACTATAGGGTCAAGCGGAGCAGGATCCCCATTTCTAC
-3'
mutVLE_barcodeB forward 5'-
GAAATTAATACGACTCACTATAGGGCGAGATAGATAGTCAGGATCCCCCAATA
CAAC-3'
VLE(mut_wt)_ Reverse 5'-GATCCGTATATCATCGATGAATTCCAGT-3'
XBM5_barcodeA forward 5'-
GAAATTAATACGACTCACTATAGGGTCAAGCGGAGCAGGATCCAAGCTCAGAA
TAAACGCTCAAC-3'
XBM5_barcodeB forward 5'-
GAAATTAATACGACTCACTATAGGGCGAGATAGATAGTCAGGATCCAAGCTCA
GAATAAACGCTCAAC-3'
XBM5_barcodeC forward 5'-
GAAATTAATACGACTCACTATAGGGGAGAGTTGAACTTGTAGCATCCAAGCTCAG
AATAAACGCTCA-3'
XBM5_ Reverse 5'-
GATCCGTATATCATCGATGAATTCGGATCCACATGTAGGGTCTC-3'

Fluorescence in situ hybridization and immunofluorescence

Oocytes were treated for 1 hour at 22°C with FTG fixative (80 mM K PIPES, pH6.8, 1 mM MgCl₂, 5 mM EGTA, 0.2% Triton X-100, 3.7% formaldehyde, 0.25% glutaraldehyde, 0.5 µM paclitaxel), followed by post-fixation in 100% methanol overnight at 22°C as in (Becker and Gard 2006). After re-hydration in TBS (10 mM Tris, pH 7.4, 155 mM NaCl), oocytes were incubated overnight at 4°C in 100 mM NaBH₄ in TBS (Becker and Gard 2006). After washing 2× in TBSN (0.1% NP-40 in TBS), followed by 2 washes in TBSNB (0.2% ultrapure BSA in TBSN) oocytes were equilibrated stepwise in TBSNB-30S (30% sucrose in TBSNB) and after equilibration in an embedding mold for 30 minutes at 22°C, TBSNB-

30S was replaced with O.C.T. compound (Fisher 23-730). Cryosectioning and fluorescence in situ hybridization (FISH) was performed as in (Neil and Mowry 2018), with the *vg1* Quasar 570 and/or *gapdh*, *trim26*, and *vegT* Quasar 670, probe sets (LGC Biosearch Technologies) applied at 500 nM. For assessment of incorporation of injected *VLE* RNA into endogenous granules, a modified *vg1* probe set (Quasar 670) was engineered to exclude probes that recognized the endogenous *VLE* sequence. For combined FISH-IF, slides were subsequently washed twice in TBSNB, incubated for 3 hours at 22°C in TBS-plus (5% v/v normal goat serum, 2 mg/ml ultrapure BSA in TBSN), and incubated overnight at 4°C with primary antibodies at 20 µg/ml in TBS-plus. Primary antibodies [C14orf166 (Abcam ab188326), Cct6A (Novus NBP2-33583), Cct8 (Novus NBP1-88285), Cnot1 (Novus NBP2-31892), CPEB1 (ProteinTech 13274-1-AP), Ddx1 (Abcam ab151962), Ddx3 (Abcam ab151965), Ddx5 (Acris AP17277PU-N), Dhx9 (ThermoFisher PA5-55754), Dynein Intermediate chain 1 (Abcam ab23905), Elavl1/Hur (Santa Cruz sc-5261), Elavl2/Hub (Novus NBP2-38012), Fam98A (Abcam ab68625), Fam98B (Novus NBP1-83474), hnRNPAB/40LoVe (Novus NBP2-16865), hnRNP D/Auf1 (Abcam ab168281), hnRNP Q/Syncrin (Abcam ab10687), Ilf3 (Abcam ab89100), G3bp2 (Novus NBP1-82977), Kif5B/kinesin (Abcam ab28060), Lsm2 (Novus NBP2-38093), Lsm14A (Abcam ab89038), Lsm14B (LifeSpan LS-C201608), Nudt16 (ProteinTech 12889-1-AP), PABPC1/EPABp (antibodies-online.com ABIN108538), Padi2 (Abcam ab16478), PTB (Kress et al. 2004), RACK1 (Novus H00005725-M01), RPS6 (Abcam ab137826), RUVBL1 (Novus NBP1-84914), Staufen1 (Abcam ab73478), Tia1 (Novus NBP1-33452), α Tubulin (Sigma-Aldrich T9026), Vera (Gagnon et al. 2013), Ybx1 (Novus NBP1-84339), and Ybx2 (ThermoFisher

PA5-30614)] were directly labeled with Alexa-405, Alexa-546, or Alexa-647 using Zenon labeling kits (ThermoFisher). After four 10-minute washes in TBSNB at 22°C, slides were mounted with ProLong Gold Antifade.

For Thioflavin S staining, following cryosectioning slides were rehydrated in PBS, followed by successive washes in 70% ethanol and 80% ethanol. Slides were incubated in filtered (0.22 μ m syringe filter) Thioflavin S solution (1% solution in 80% ethanol) for 20 minutes at room temperature. Slides were then washed with 80% ethanol and 70% ethanol (with nutation) for 5-minute intervals. Slides were then washed quickly with distilled water 4x. Following staining, slide were either mounted in Prolong Gold Antifade or processed for FISH as in (Neil and Mowry 2018) with probe sets restricted to Quasar 670 to avoid overlap with the Thioflavin S fluorescence.

Imaging was performed on a Zeiss LSM 800 Confocal Laser Scanning Microscope.

Fluorescence Recovery After Photobleaching (FRAP)

Fluorescently - labeled RNA was injected into stage II oocytes (2nl of 200nM labeled RNA) and allowed to localize for 48 hours. Imaging was performed on an Olympus FV3000 confocal microscope with a UPLSAPO 30x oil objective (1.05NA). A 10 μ m² ROI was bleached using a 561nm laser at 100% for 2 seconds. Recovery was monitored at five second intervals for 100 iterations. FRAP calculations were done as previous described (Gagnon et al. 2013). Curve fitting was done in GraphPad Prism 7 using $Y=Y_0 + (Plateau-Y_0)*(1-\exp(-K*x))$.

Oocyte lysate preparation

Approximately 2500 stage II-III oocytes were washed with PBS, followed by crosslinking (in 0.1% formaldehyde in PBS) for 10 minutes at 22°C. The reaction was quenched with 0.25 M Glycine (in 25 mM Tris pH 7.4,) for 5 minutes at 22°C. Oocytes were washed in column running buffer [CRB; 0.05% NP-40, 1 mM DTT, 10 mM Hepes 7.4, 100 mM KOAc, 3 mM MgOAc, 5 mM EGTA, 100 mM sucrose, 2 nU/ml Ribolock RNase Inhibitor, 1× HALT Protease Inhibitors (ThermoFisher)] and homogenized in CRB (~20 µl/oocyte). The lysate was clarified twice by centrifugation at 10,000×g for 10 minutes at 4°C and adjusted to a final protein concentration of 50 mg/ml in CRB.

Size Exclusion Chromatography

Oocyte lysate was chromatographed on Sephacryl S400 HR resin (GE Healthcare) in CRB. Column fractions were analyzed by qRTPCR for *vg1* mRNA and by immunoblot for proteins of interest. The fractions containing the *vg1* RNA peak were pooled for further analysis. For RNase treatment of oocyte lysates prior to S400 chromatography, Ribolock RNase Inhibitor was omitted from all buffers. After crosslinking, lysis, and clarification, RNase A (Sigma-Aldrich) was added to a final concentration of 0.1 µg/µl and incubated for 10 minutes at 37°C.

Immunoprecipitation and Immunoblotting

Antibodies [Stau1 (Abcam, ab73478), hnRNPAB (Abcam, ab19974), and rabbit IgG (EMD, NI01)] were covalently crosslinked to Dynabead M270 Epoxy Magnetic Beads (Invitrogen, #14311D). Antibody-coupled Dynabeads were washed in 0.1% BSA for 5

minutes at 22°C, blocked for 1-hour in RNA blocking solution (0.05% CHAPS, 160 µg torula RNA, PBS), and washed three times in CRB. S400 *vg1* RNA peak “pool” was incubated for 6 hours at 4°C with antibody-coupled Dynabeads in PIP buffer (50 mM Tris pH 7.4, 0.1% NP40, 0.05 mM MgCl₂, 2 nU/ml Ribolock RNase Inhibitor, and 1× Protease Inhibitors) for protein co-IP or RIP buffer (25 mM Tris pH 7.4, 0.5% NP40, 0.5 mM DTT, 150 mM KCl, 5 mM EDTA, 2 nU/ml Ribolock RNase Inhibitor, 1× HALT Protease Inhibitors) for RNA co-IP. For mass spectrometry, bound proteins were eluted with (50 mM Tris pH 8.0, 0.2% SDS, 0.1% Tween-20) twice for 7 minutes at 25°C, followed by incubation at 70°C for 1 hour to reverse the crosslinking. Tween-20 was removed from the eluents using HiPPR columns (ThermoFisher, #88305), and eluted into PBS. Antibodies for immunoblotting were as follows: C14orf166 (Abcam ab188326) 1:1000, CPEB1 (ProteinTech 13274-1-AP) 1:1000, Ddx1 (Abcam ab151962) 1:1000, Ddx3 (Abcam ab151965) 1:1000, Ddx5 (Acris AP17277PU-N) 1:200, Decr2 (Thermofisher PA5-30503) 1:1000, eIF4A1 (Abcam ab137556) 1:1000, eIF4G (Novus H00001981) 1:1000, hnRNPAB/40LoVe (Novus NBP2-16865) 1:1000, hnRNPR (Proteintech 15018-1-AP) 1:1000, Ilf2 (Novus NBP1-82586) 1:1000, Ilf3 (Abcam ab89100) 1:1000, G3bp2 (Novus NBP1-82977) 1:1000, Lsm14A (Abcam ab89038) 1:1000, LSM1 (Abcam 1b167194) 1:1000, Lsm14B (LifeSpan LS-C201608) 1:1000, Nudt16 (ProteinTech 12889-1-AP) 1:1000, PABPC1/EPABp (antibodies-online.com ABIN108538) 1:1000, PRKR (Proteintech 10771-1-AP) 1:1000, PTB (Kress et al. 2004) 1:2000, Staufen (Yoon and Mowry 2004) 1:2000, Tubulin (Sigma-Aldrich T9026) 1:500, Vera (Gagnon et al. 2013) 1:2000, Ybx1 (Novus NBP1-84339) 1:100, and Ybx2 (ThermoFisher PA5-30614) 1:1000. Secondary antibodies,

Goat anti-Rabbit IgG (Abcam ab97200) or Goat Anti-Mouse IgG (Abcam ab97265) were used at 1:10,000.

Mass Spectrometry

Mass spectrometry was performed at the COBRE Proteomics Core Facility affiliated with Brown University on a Q-Exactive Plus LC-MS/MS mass spectrometer. Peptide counts were generated by the core facility. Downstream analysis was performed in-house using R. For Stauf1 and hnRNPAB IPs, only those proteins with peptides in at least 3 replicates were considered. Enrichment over IgG was determined if the sum of peptides across all four biological replicates was at least two-fold greater than the sum of the peptides in all four IgG experiments. For unannotated protein hits (i.e.: LOC, MGC, Xelaev, Xetrov delimiters), peptides were BLAST against the *Xenopus laevis* JGI 9.1v1.8.3.2 annotation database, available from xenbase.org. Area proportional venn diagrams were produced using the VennDiagram package for R.

RNA Quantitation

After crosslink reversal, 2.5 pg of *luciferase* RNA (Promega #L4561) was added each RNA sample to control for extraction efficiency. After addition of TriZol (Invitrogen), RNA was extracted using the Direct-Zol RNA MicroPrep kit (Genesee #11-300MB) with on-column DNase I digestion, as per manufacturer's protocol. cDNA was prepared from 10 µl of RNA using the iScript cDNA synthesis kit (BioRad, #1708891) as per manufacturer's protocols.

For analysis of *vg1* mRNA distribution across fractions, 1-step q-RT-PCR was performed using Luna One-Step qPCR (NEB), as per manufacturer protocol. For RNA-

IPs, two step qRT-PCR was performed using SybrGreen Powerup MasterMix (ABI), as per manufacturer protocols.

Forward *vg1* primer: 5'- GGTATCTCCTCCTCCTGTCCCT-3'.

Reverse *vg1* primer: 5'- TTGGGTGGATGTCATCGGAGT-3'.

Forward *luciferase* primer: 5'- GGATACCGGGAAAACGCTGG-3'.

Reverse *luciferase* primer: 5'- ATCAAGGCGTTGGTCGCTTC-3'.

Absolute copy numbers of *vg1* and *luciferase* were determined by standard curve. To normalize *vg1* expression and account for any differences in extraction efficiency, *vg1* copy number was divided by *luciferase* copy number per fraction.

For analysis of barcode-labeled RNA distribution across fractions, 1-step q-RT-PCR was performed using Luna One-Step qPCR (NEB), as per manufacturer protocol.

barcodeA forward 5'-TCAAGCGGAGCAGGATCC-3'

barcodeB forward 5'-CGAGATAGATAGTCAGGATCC-3'

barcodeD forward 5'-AGAGTTGAACTTGTCATCC-3'

barcodeC reverse 5'-GATCCGTATATCATCGATGAATTC-3'

barcodeB forward 5'-CGAGATAGATAGTCAGGATCC-3'

barcodeC reverse 5'-GATCCGTATATCATCGATGAATTC-3'

For RNA-IP analysis, LinRegPCR (Ramakers et al. 2003) was used to calculate primer efficiency for both *vg1* and *luciferase* primers. The geometric mean was determined for primers across experiments. Relative fold enrichment was calculated using the Pfaffl (Pfaffl 2001) method (an assumption-free version of the commonly misused $\Delta\Delta C_t$ calculation), and the geometric mean efficiency of *vg1* and *luciferase* primers as determined

by LinRegPCR. Log2 fold enrichment of *vg1* in RBP-IPs was calculated relative to IgG and normalized to *luciferase*. A Mann-Whitney U test was performed for statistical significance (De Neve et al. 2013).

Gene Ontology

GO analysis was determined using DAVID (Huang et al. 2009b, 2009a), using *Xenopus tropicalis* as background.

Intrinsically Disordered and Prion-Like Domain Analysis

Identification of putative intrinsically disordered domains were determined using SLIDER (Peng et al. 2014), which predicts long disordered segments (>30 consecutive disordered residues), against the hnRNPAB/Stau1 dataset or the *Xenopus laevis* J-strain 9.2 genome available at xenbase.org. Only those proteins with a SLIDER score greater than 0.55 were considered likely to have an IDR. Prion-like domains were calculated using PLAAC (Lancaster et al. 2014) with default settings. Only those proteins with a COREscore greater than zero were considered to have a prion-like domain. Statistics were performed in R using the Fisher's exact test.

Motif Enrichment Analysis

Motif analysis was performed with MEME in Classic Mode, allowing for one or more occurrences per sequence (Bailey and Elkan 1994).

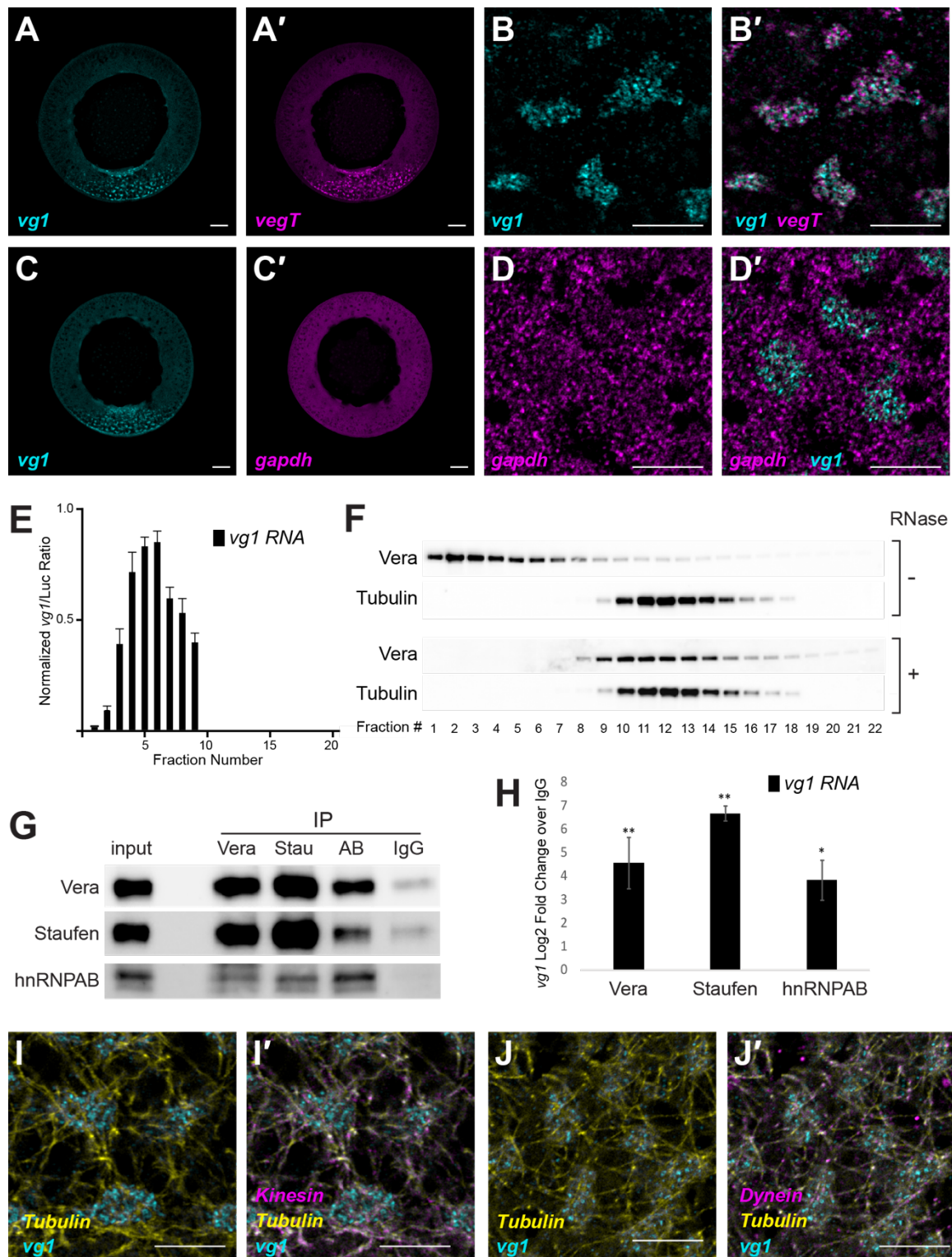


Figure 3.1. *vg1* mRNA is part of a large RNP complex during vegetal RNA localization.

(A) A cryosection of a stage II oocyte probed by FISH for *vg1* mRNA (cyan) and *vegT*

mRNA (A', magenta) is shown, with the vegetal cortex at the bottom. Scale bar=50 μ m. (B) Higher magnification view of *vg1* RNA in the vegetal cytoplasm of a stage II oocyte probed by FISH for *vg1* mRNA (cyan), shown merged in B' with *vegT* mRNA (magenta). Scale bar=10 μ m. (C) A cryosection of a stage II oocyte probed by FISH for *vg1* mRNA (cyan) and *gapdh* mRNA (C', magenta) is shown, with the vegetal cortex at the bottom. Scale bar=50 μ m. (D) Higher magnification view of *vg1* RNA in the vegetal cytoplasm of a stage II oocyte probed by FISH for *vg1* mRNA (cyan), shown merged in D' with *gapdh* mRNA (magenta). Scale bar=10 μ m. (E) *vg1* mRNA levels were quantitated in S-400 column fractions (1-8) by qRT-PCR, normalized to Luc control RNA. Error bars represent standard error of the mean ($n=11$ columns). (F) Oocyte lysate was treated with RNase A prior to gel filtration chromatography, followed by immunoblot analysis with anti-Vera and anti-tubulin antibodies. S-400 column fractions from untreated (-) lysate are shown at the top and column fractions from RNase-treated (+) lysate are shown at the bottom. (G) Pooled S-400 fractions (2-5) were immunoprecipitated using anti-Vera, anti-Staufen1, anti-hnRNPAB, and IgG. After SDS-PAGE, co-immunoprecipitation of Vera, Staufen1, and hnRNPAB were confirmed by immunoblotting with anti-Vera (top), anti-Staufen1 (middle), and anti-hnRNPAB (bottom). (H) Pooled S-400 fractions (2-5) were immunoprecipitated using anti-Vera, anti-Stau1, anti-hnRNPAB, and IgG. Following isolation of bound RNAs, *vg1* RNA was detected by qRT-PCR, with normalization to a *luciferase* control. Shown is log2 fold enrichment for Vera (left), Staufen1 (middle), and hnRNPAB (right) over IgG. $n=5$ and error bars represent standard error of mean. ** indicates $p < 0.01$, * indicates $p < 0.05$. (I) FISH-IF is shown for *vg1* mRNA (cyan) and

anti-tubulin (yellow), merged in I' with anti-kinesin (magenta). Scale bar=10 μm . (J)

FISH-IF is shown for *vg1* mRNA (cyan) and anti-tubulin (yellow), merged in J' with anti-dynein (magenta). Scale bar=10 μm .

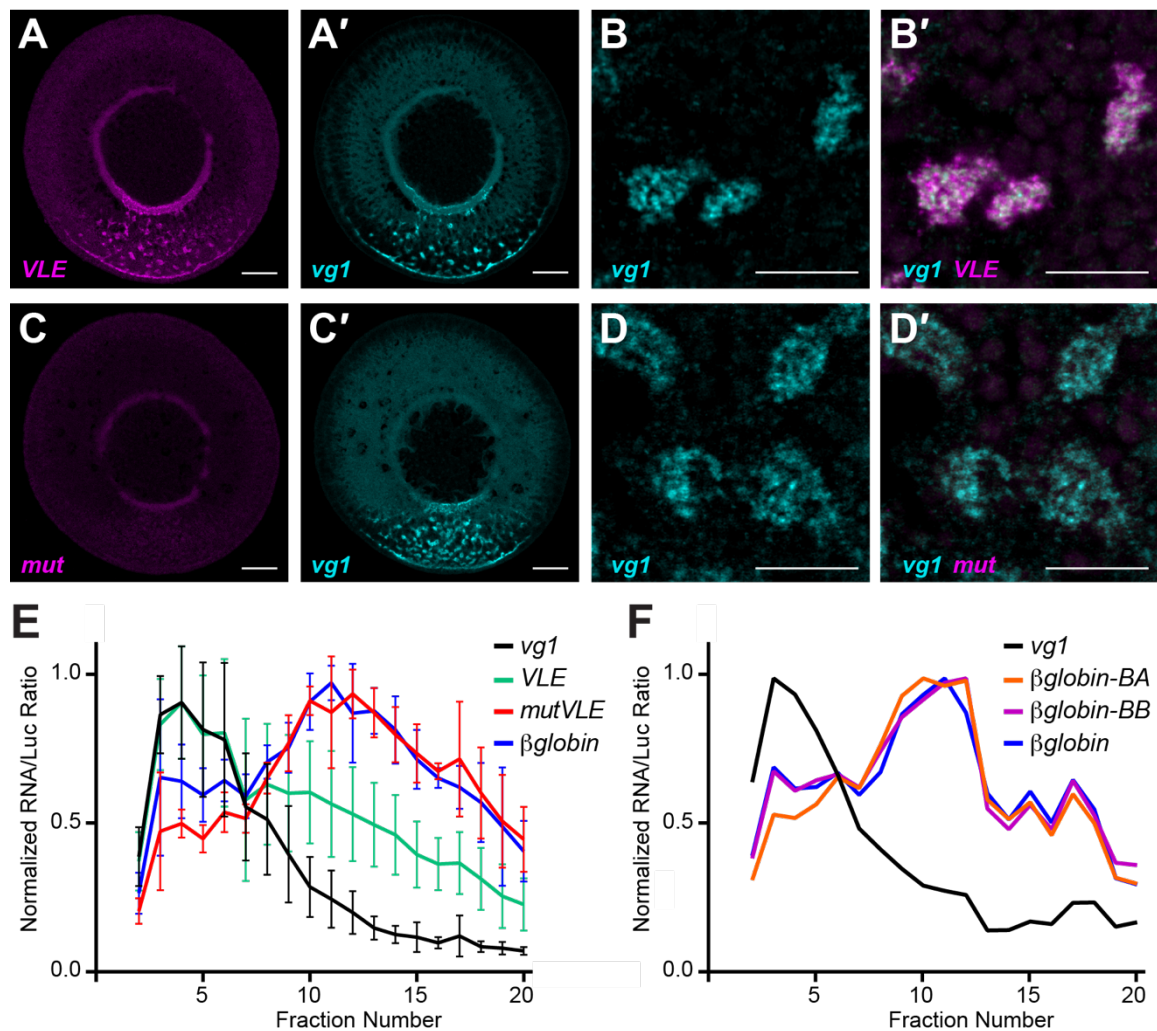
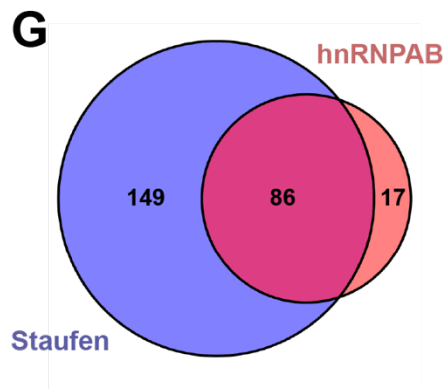
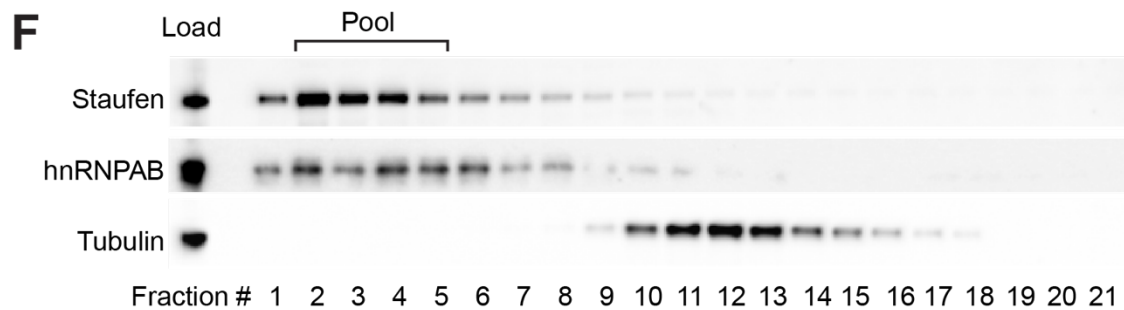
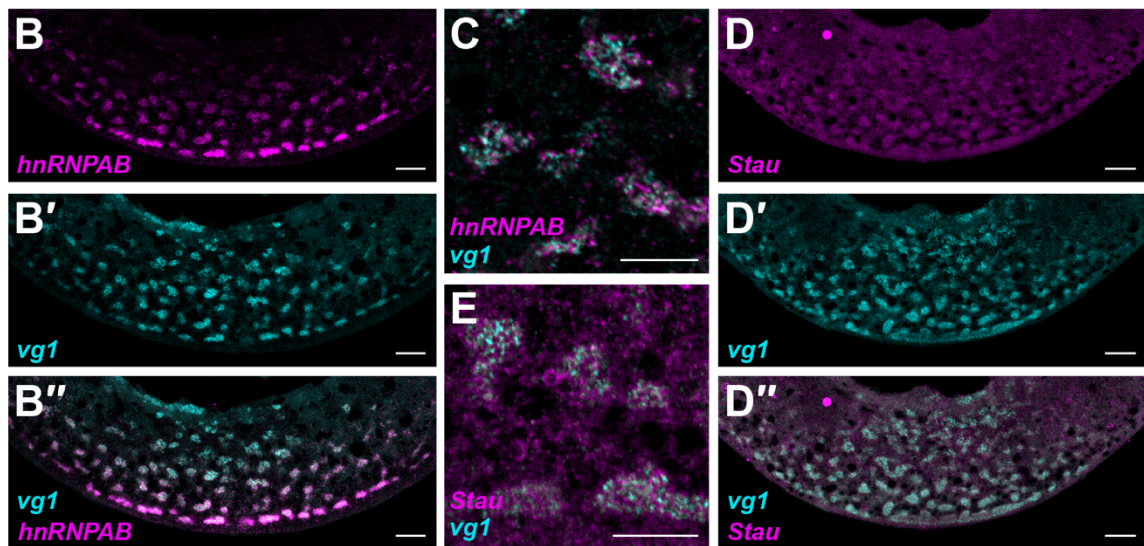
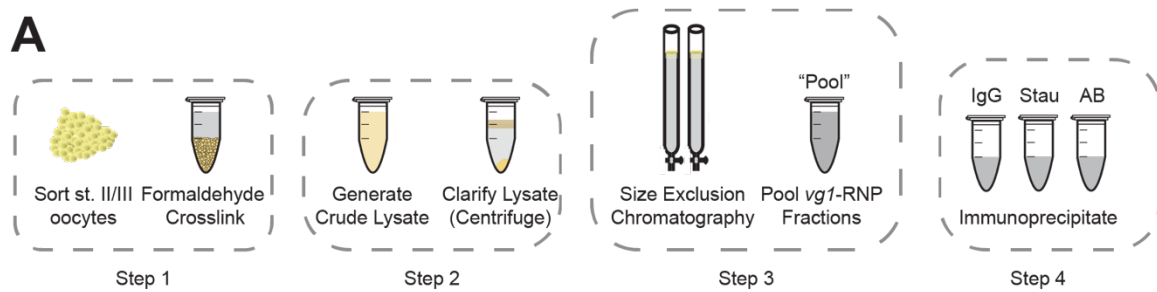


Figure 3.2. Assembly into large RNPs is required for vegetal localization. (A) A cryosection of a stage II oocyte is shown, with microinjected fluorescently-labeled *VLE* RNA in magenta and endogenous *vg1* mRNA (A') detected by FISH in cyan is shown. The vegetal cortex at the bottom, and the scale bar=50 μ m. (B) High magnification view of the vegetal cytoplasm of a stage II oocyte with endogenous *vg1* mRNA (cyan) detected by FISH and microinjected *VLE* RNA shown in magenta (merged in B'). Scale bar=10 μ m. (C) A cryosection of a stage II oocyte is shown, with microinjected fluorescently-labeled mutant *VLE* RNA (*mut*) in magenta and endogenous *vg1* mRNA (C') detected by FISH in cyan. The vegetal cortex at the bottom and the scale bar=50 μ m. (D) High magnification view of

the vegetal cytoplasm of a stage II oocyte with endogenous *vgl* mRNA (cyan) detected by FISH and microinjected mutant *VLE* RNA (*mut*) shown in magenta (merged in D'). Scale bar=10 μ m. (E) Stage II-III oocytes were microinjected with *VLE* RNA (green), non-localizing *mut-VLE* RNA (red), and non-localizing β -globin RNA (blue). Oocyte lysates were subjected to gel filtration chromatography and the levels of the microinjected RNAs and endogenous *vgl* mRNA (black) were quantitated in S-400 column fractions by qRT-PCR, normalized to Luc control RNA. Bar codes for PCR detection were as follows: B-A (*VLE* RNA), B-B (*mut-VLE* RNA), and B-C (β -globin RNA). Error bars represent standard error of the mean ($n=4$ columns). (F) Stage II-III oocytes were injected with β -globin RNA (blue, as in E, above), β -globin RNA tagged with bar code B-A (orange), and β -globin RNA tagged with bar code B-B (magenta). Oocyte lysates were subjected to gel filtration chromatography and the levels of the microinjected RNAs and endogenous *vgl* mRNA (black) were quantitated in S-400 column fractions by qRT-PCR, normalized to Luc control RNA.



H

Molecular Function - Gene Ontology	Count	Percentage	P-value
RNA binding	27	36	2.20E-16
nucleotide binding	18	24	3.70E-12
ATP binding	15	20	3.27E-02
nucleic acid binding	10	13.3	1.62E-02
mRNA binding	6	8	3.17E-05
mRNA 3'-UTR binding	4	5.3	1.07E-03
ATP-dependent RNA helicase activity	4	5.3	7.81E-03
double-stranded RNA binding	4	5.3	3.69E-04
helicase activity	3	4	2.01E-02
poly(A) binding	3	4	1.16E-03
poly(U) RNA binding	2	2.7	2.25E-02
mRNA 5'-UTR binding	2	2.7	3.72E-02

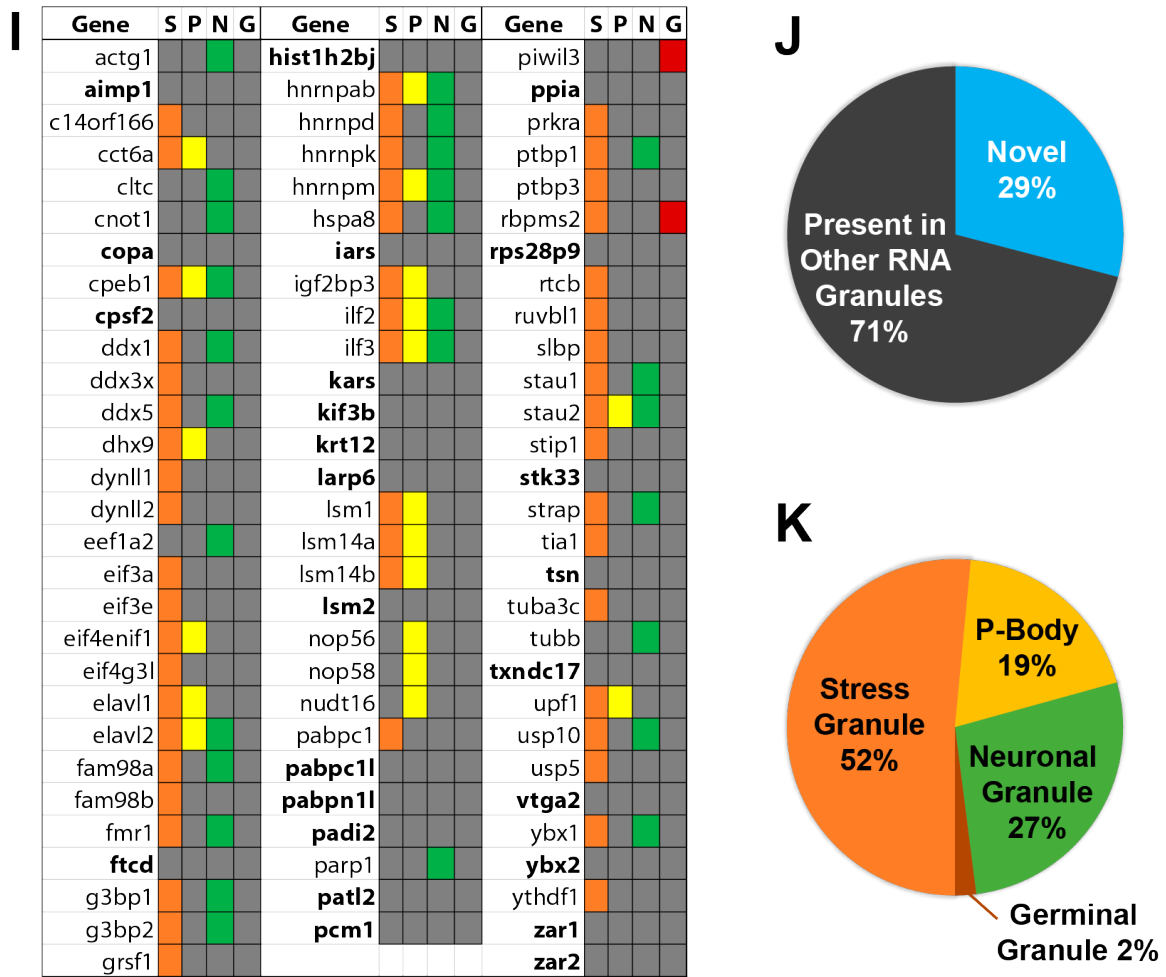
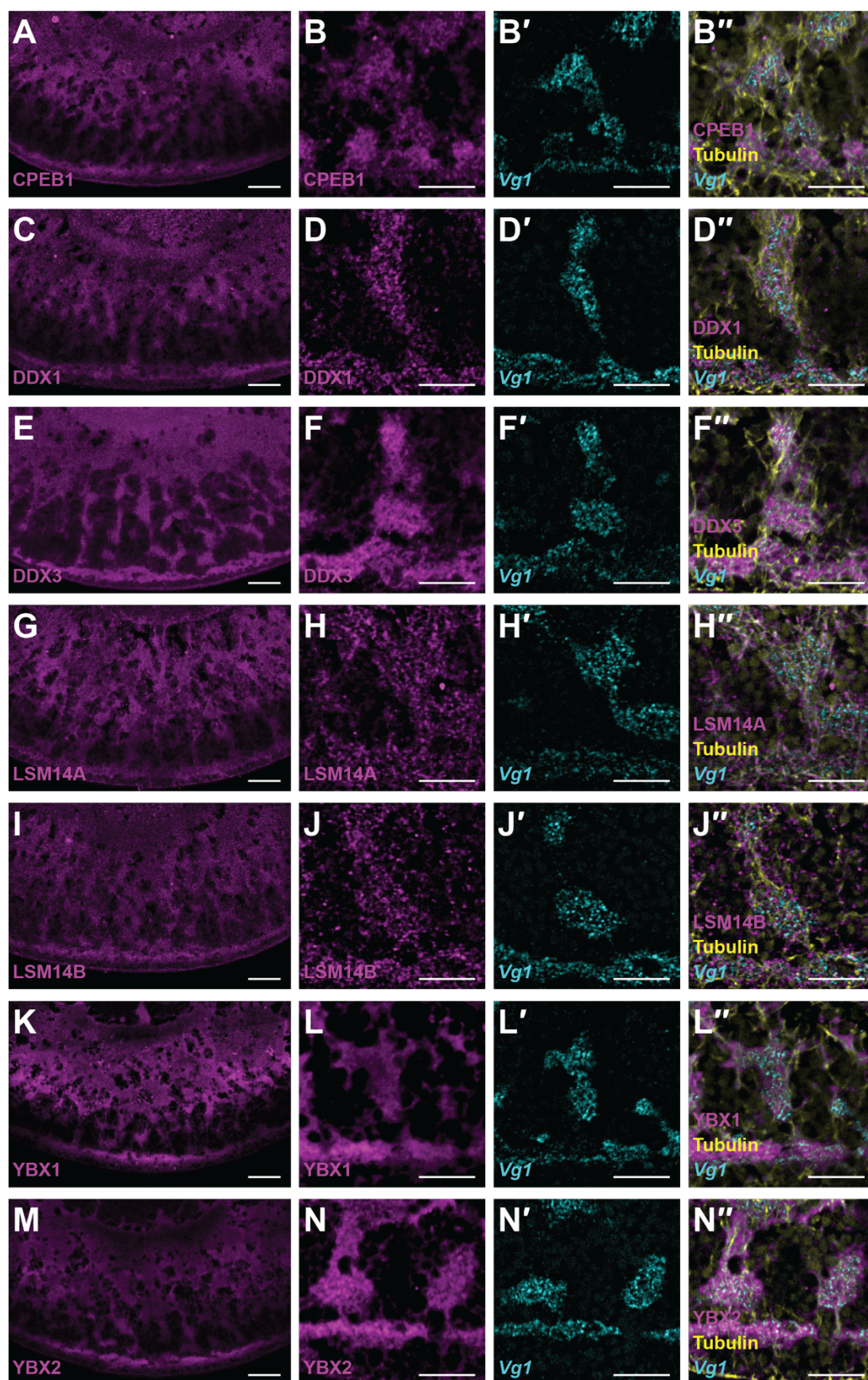


Figure 3.3. Isolation and characterization of *vg1* transport mRNPs. (A) Schematic of the *vg1* RNP isolation procedure. Stage II/III oocytes were crosslinked, homogenized, clarified by centrifugation, and fractionated by Sephacryl S-400 size-exclusion chromatography. Fractions containing *vg1* mRNA were pooled and divided equally for immunoprecipitations using anti-Staufen1 and anti-hnRNPAB antibodies, with IgG as a negative control. (B) A cryosection of a stage II oocyte is shown with the vegetal cortex at the bottom. Combined FISH-IF was used to detect hnRNPAB (B, magenta) and *vg1* mRNA (B', cyan) The merge is shown in B''. Scale bar=20 μ m. (C) High magnification view of the vegetal cytoplasm of a stage II oocyte showing colocalization of *vg1* mRNA (cyan) and

hnRNPAB (magenta). Scale bar=10 μ m. **(D)** A cryosection of a stage II oocyte is shown with the vegetal cortex at the bottom. Combined FISH-IF was used to detect Staufen1 (D, magenta) and *vg1* mRNA (D', cyan) The merge is shown in D''. Scale bar=20 μ m. **(E)** High magnification view of the vegetal cytoplasm of a stage II oocyte showing colocalization of *vg1* mRNA (cyan) and Staufen1 (magenta). Scale bar=10 μ m. **(F)** Immunoblot analysis of S-400 column fractions probed with anti-Staufen1, anti-hnRNPAB, and anti- α tubulin. The input (load) is at the left and fraction numbers are shown at the bottom. Staufen1 and hnRNPAB chromatograph primarily in the void volume; fractions 2-5, which were pooled for further purification. **(G)** Mass spectrometry was performed on parallel immunoprecipitations (IPs) using anti-Staufen1, anti-hnRNPAB, and IgG. A Venn diagram is shown for proteins enriched in hnRNPAB and Stau1 IPs over IgG control. **(H)** Gene ontology (GO) analysis of the proteins enriched in *vg1* transport RNPs. The Count column indicates the number of proteins identified in (G) with a given molecular function gene ontology category. The Percentage column indicates the percent of proteins in (G) containing a given gene ontology category. The P-value column represents the significance of a measure of overrepresentation of each of these categories, as calculated by DAVID. **(I)** Protein constituents of *vg1* transport RNPs share constituents with stress granules (S, orange), p-bodies (P, yellow), neuronal granules (N, green), and germ granules (G, red). Proteins found in *vg1* transport RNPs but not reported in other granules, are bolded. **(J)** Overlap (gray) of *vg1* transport RNPs constituents with other cytoplasmic granules; unique proteins are indicated by blue. **(K)** Overlap of *vg1* transport RNPs constituents with stress granules (orange), p-bodies (yellow), neuronal granules (green), and germ granules (red).



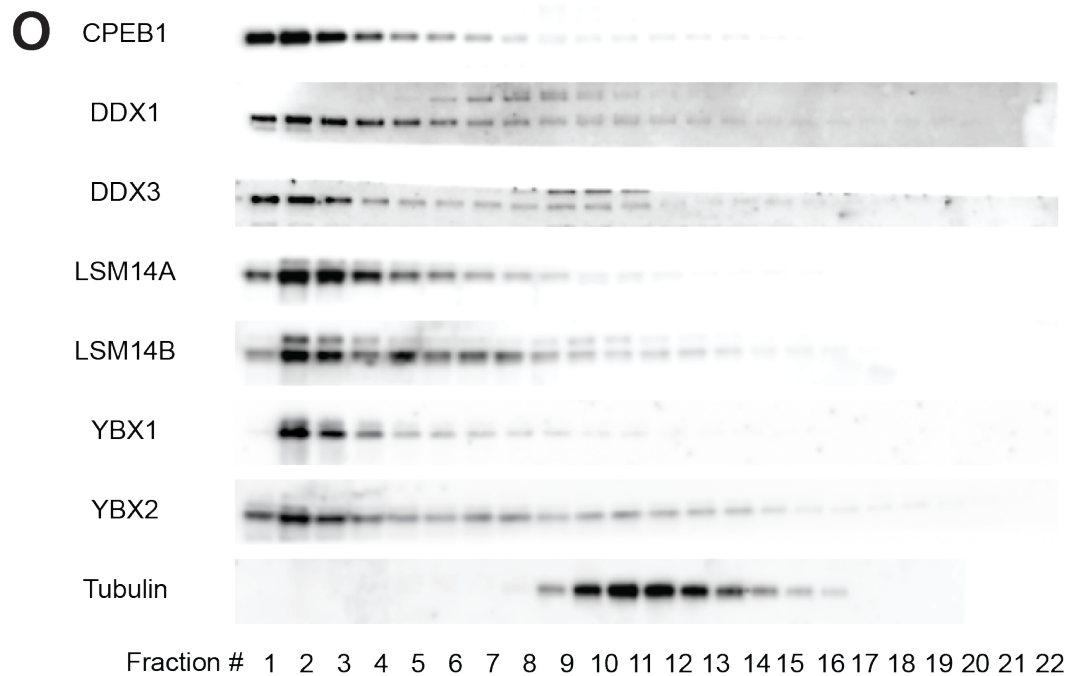


Figure 3.4. Validation of vegetal transport RNP constituents. (A) Immunofluorescence (IF) was performed on cryosectioned stage II-III oocytes with anti-Cpeb1 antibodies. The vegetal cortex at the bottom and the scale bar=20 μ m. (B) High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Cpeb1 (B, magenta), *vg1* mRNA (B', cyan) and merged in B'' with anti-tubulin (yellow). Scale bar=10 μ m. (C) IF was performed on cryosectioned stage II-III oocytes with anti-Ddx1 antibodies. The vegetal cortex at the bottom and the scale bar=20 μ m. (D) High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Ddx1 (D, magenta), *vg1* mRNA (D', cyan) and merged in D'' with anti-tubulin (yellow). Scale bar=10 μ m. (E) IF was performed on cryosectioned stage II-III oocytes with anti-Ddx3 antibodies. The vegetal cortex at the bottom and the scale bar=20 μ m. (F) High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Ddx3 (F, magenta), *vg1* mRNA (F', cyan) and merged in F'' with anti-tubulin (yellow). Scale bar=10 μ m. (G) IF was performed on cryosectioned

stage II-III oocytes with anti-Lsm14A antibodies. The vegetal cortex at the bottom and the scale bar=20 μm . **(H)** High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Lsm14A (H, magenta), *vg1* mRNA (H', cyan) and merged in H'' with anti-tubulin (yellow). Scale bar=10 μm . **(I)** IF was performed on cryosectioned stage II-III oocytes with anti-Lsm14B antibodies. The vegetal cortex at the bottom and the scale bar=20 μm . **(J)** High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Lsm14B (J, magenta), *vg1* mRNA (J', cyan) and merged in J'' with anti-tubulin (yellow). Scale bar=10 μm . **(K)** IF was performed on cryosectioned stage II-III oocytes with anti-Ybx1 antibodies. The vegetal cortex at the bottom and the scale bar=20 μm . **(L)** High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Ybx1 (L, magenta), *vg1* mRNA (L', cyan) and merged in L'' with anti-tubulin (yellow). Scale bar=10 μm . **(M)** IF was performed on cryosectioned stage II oocytes with anti-Ybx2 antibodies. The vegetal cortex at the bottom and the scale bar=20 μm . **(N)** High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Ybx2 (N, magenta), *vg1* mRNA (N', cyan) and merged in N'' with anti-tubulin (yellow). Scale bar=10 μm . **(O)** Immunoblot analysis of S-400 column fractions probed using anti-Cbeb1, anti-Ddx1, anti-Ddx3, anti-Lsm14A, anti-Lsm14B, anti-Ybx1, anti-Ybx2, and anti- α tubulin antibodies. The fraction numbers are shown at the bottom.

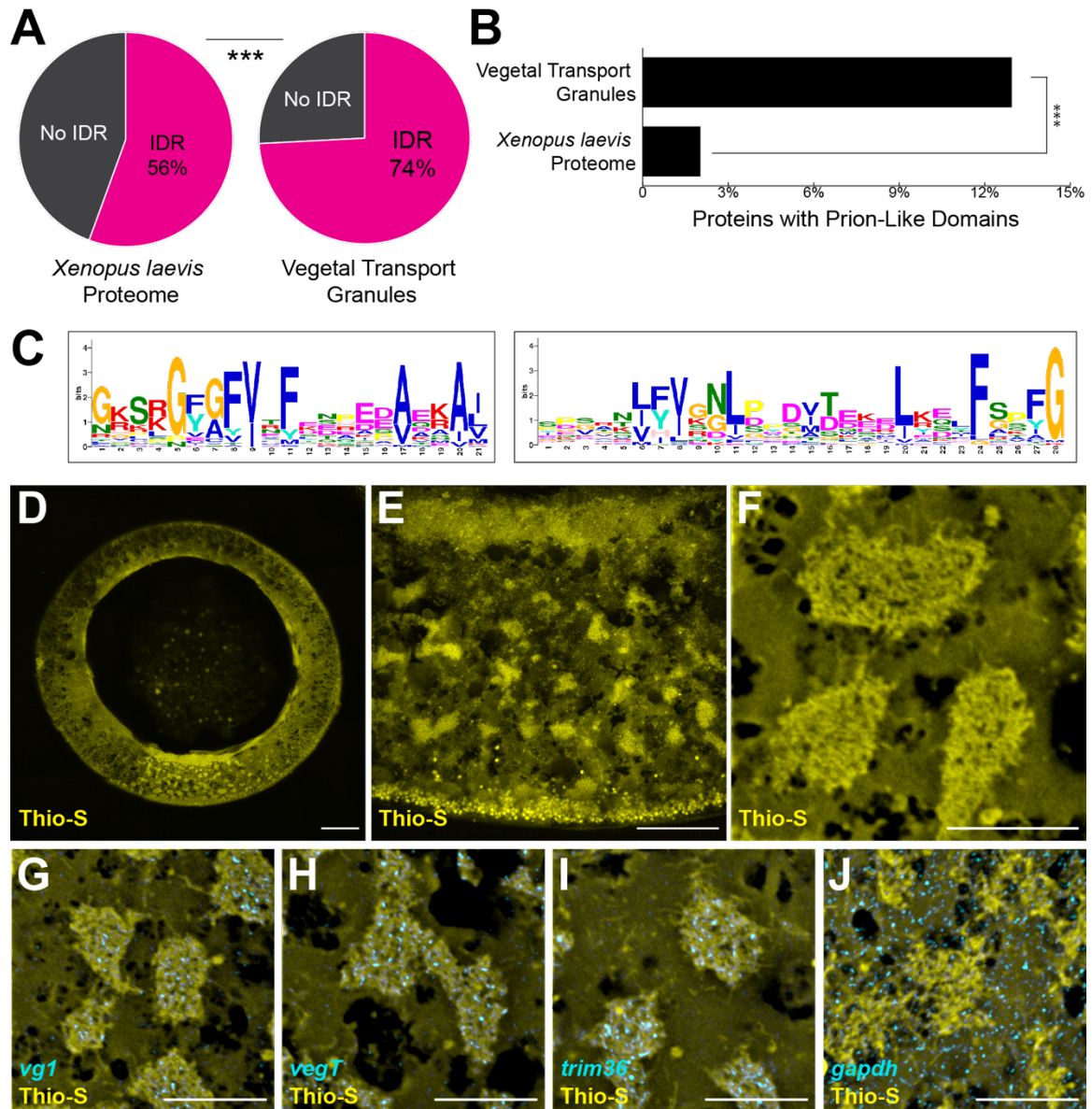


Figure 3.5. Vegetal RNP transport granules display amyloid-like characteristics. (A) Comparison by SLIDER analysis for long stretches of intrinsically disordered regions (IDR) for the *X. laevis* proteome vs. the vegetal RNP transport granule proteome. *** indicates $p < 0.001$ (B) Comparison of the percentage of prion-like domains in the vegetal RNP transport granule proteome compared to the entire *Xenopus* proteome, as determined with the predictor PLAAC. *** indicates $p < 0.001$ (C) MEME motif analysis of vegetal RNP transport granule proteome reveals enrichment of FG motifs. (D) A stage II oocyte stained

with thioflavin-S (thio-S) is shown, with the vegetal cortex at the bottom. Scale bar=50 μ m. (E) A cryosection of the vegetal cytoplasm of a stage II oocyte stained with thio-S is shown, with the vegetal cortex at the bottom. Scale bar=20 μ m. (F) High magnification view of the vegetal cytoplasm of a stage II oocyte stained with thio-S. Scale bar=10 μ m. (G-J) High magnification views of the vegetal cytoplasm are shown; scale bars=10 μ m. Thio-S staining was combined with FISH for detection of the following RNAs: (G) *vgl* RNA, (H) *vegT* RNA, (I) *trim36* RNA, (J) *gapdh* RNA.

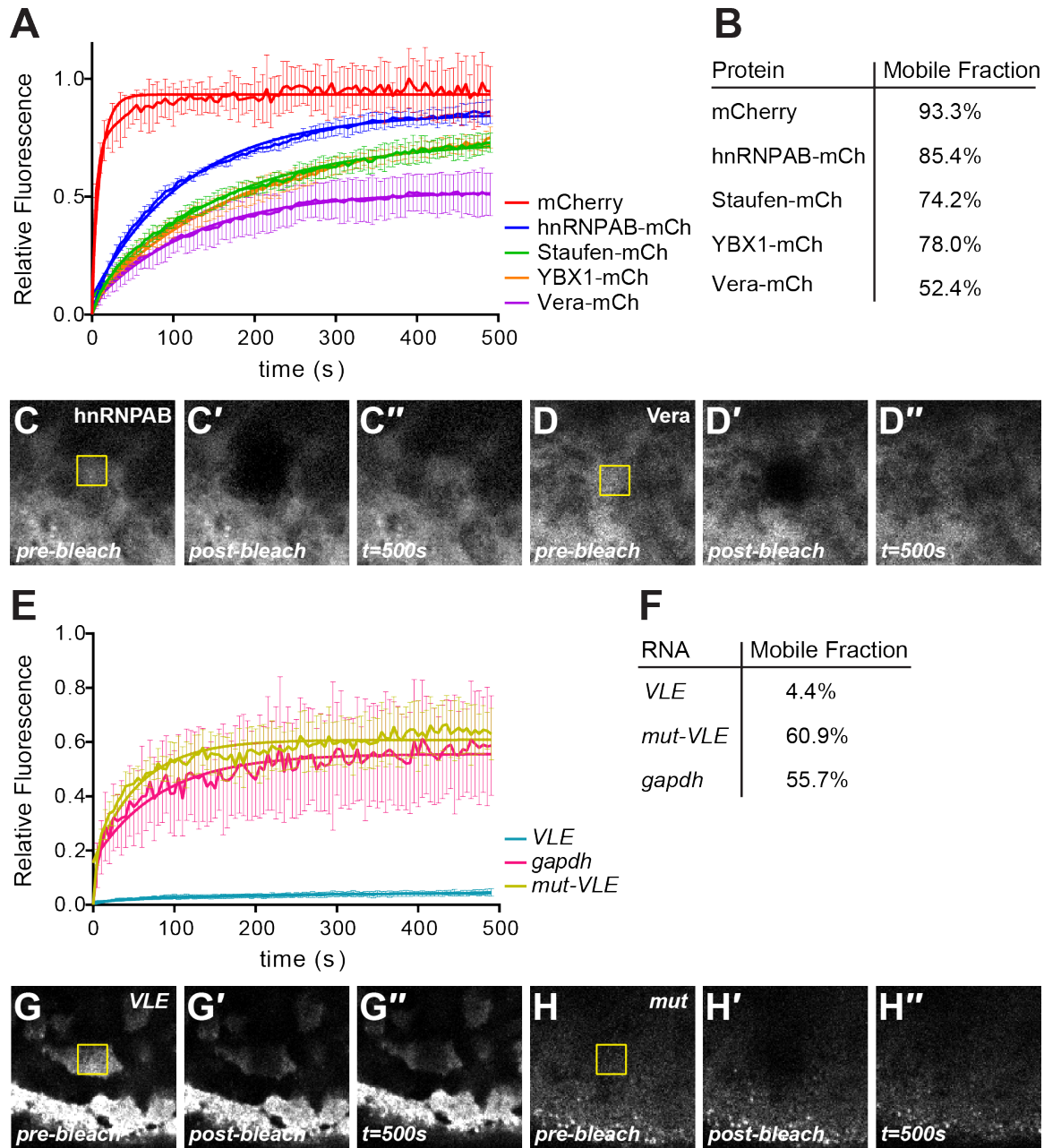


Figure 3.6. Vegetal RNP transport granules display amyloid-like characteristics. (A)

Stage II oocytes expressing mCherry, or the following mCherry-tagged proteins: hnRNPAB-mCh, Staufen1-mCh, Ybx1-mCh, Vera-mCh, were microinjected with Cy5-labeled *VLE* RNA to mark transport granules. Recovery curves are shown for mCherry (red), hnRNPAB-mCh (blue), Staufen1-mCh (green), Ybx1-mCh (orange), and Vera-

mCh (purple). **(B)** The mobile fraction for each protein, as determined by FRAP. **(C)** An image of the vegetal cytoplasm of an oocyte expressing hnRNPAB-mCh is shown, with a $10\ \mu\text{m}^2$ ROI marked in the pre-bleach image. C' and C'' show the post-bleach and 500 second (s) timepoints, respectively. **(D)** An image of the vegetal cytoplasm of an oocyte expressing Vera-mCh is shown, with a $10\ \mu\text{m}^2$ ROI marked in the pre-bleach image. D' and D'' show the post-bleach and 500 second (s) timepoints, respectively. **(E)** Stage II oocytes were microinjected with Cy3-labeled *VLE* RNA to mark transport granules and co-injected with the following Cy5-labeled RNAs for FRAP analysis: *VLE* RNA, *mut-VLE* RNA, or *gapdh* RNA. Recovery curves are shown for *VLE* RNA (cyan), *mut-VLE* RNA (magenta), or *gapdh* RNA (yellow). **(F)** The mobile fraction is shown for each RNA, as determined by FRAP. **(G)** An image of the vegetal cytoplasm of an oocyte microinjected with Cy5-labeled *VLE* RNA is shown, with a $10\ \mu\text{m}^2$ ROI marked in the pre-bleach image. G' and G'' show the post-bleach and 500 second (s) timepoints, respectively. **(H)** An image of the vegetal cytoplasm of an oocyte with Cy3-labeled *mut-VLE* RNA is shown, with a $10\ \mu\text{m}^2$ ROI marked in the pre-bleach image. H' and H'' show the post-bleach and 500 second (s) timepoints, respectively.

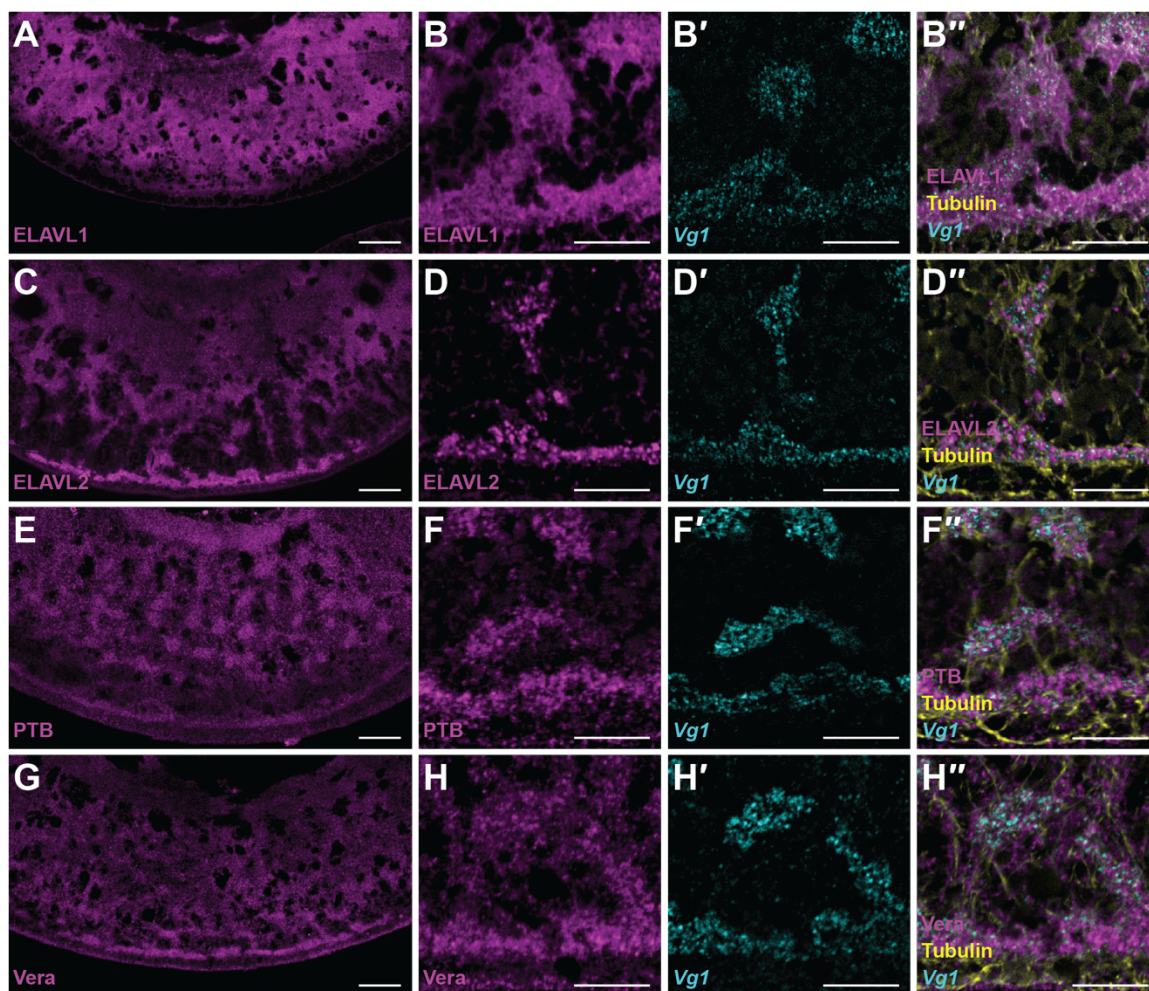


Figure S3.1. Combined FISH-IF analysis of previously-identified *vg1* RBPs. (A) Immunofluorescence (IF) was performed on cryosectioned stage II-III oocytes with anti-Elav1 antibodies. The vegetal cortex at the bottom and the scale bar=20 μ m. (B) High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Elav1 (B, magenta), *vg1* mRNA (B', cyan) and merged in B'' with anti-tubulin (yellow). Scale bar=10 μ m. (C) Immunofluorescence (IF) was performed on cryosectioned stage II-III oocytes with anti-Elav2 antibodies. The vegetal cortex at the bottom and the scale bar=20 μ m. (D) High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Elav2 (D, magenta), *vg1* mRNA (D', cyan) and merged in D'' with anti-tubulin

(yellow). Scale bar=10 μm . **(E)** Immunofluorescence (IF) was performed on cryosectioned stage II-III oocytes with anti-PTB antibodies. The vegetal cortex at the bottom and the scale bar=20 μm . **(F)** High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for PTB (F, magenta), *vg1* mRNA (F', cyan) and merged in F'' with anti-tubulin (yellow). Scale bar=10 μm . **(G)** Immunofluorescence (IF) was performed on cryosectioned stage II-III oocytes with anti-Vera antibodies. The vegetal cortex at the bottom and the scale bar=20 μm . **(H)** High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Vera (H, magenta), *vg1* mRNA (H', cyan) and merged in H'' with anti-tubulin (yellow). Scale bar=10 μm .

Protein	FISH-IF	Chromatography
C140rf166	+	+
Cct6	+	na
Cct8	+	na
Cnot1	+	na
Cpeb1	+	+
Ddx1	+	+
Ddx3	+	+
Ddx5	+	+
Decr2	na	+
Dhx9	+	na
Dynein	+	na
eIF4A1	na	+
eIF4G	na	+
Elavl1 / Hur	+	na
Elavl2 / Hub	+	na
Epabp	+	+
Fam98a	+	na
Fam98b	+	na
hnRNPAB / 40LoVe	+	+
hnRNPD / Auf1	+	na
hnRNPR	na	+
hnRNPQ / Syncrip	+	na
Ilf2	na	+
Ilf3	+	+
G3bp2	+	+
Kif5b / Kinesin I	+	na
Lsm1	na	+
Lsm2	+	na
Lsm14a	+	+
Lsm14b	+	+
Nudt16	+	+
Padi2	+	na
PRKR	na	+
Ptbp1 / PTB	+	+
Rack1	+	na
Rps6	+	na
Ruvbl1	+	na
Staufen1	+	+
Tia1	+	na
Tubulin	+	+
Vera	+	+
Ybx1	+	+
Ybx2	+	+

Table S3.1. Validation of potential vegetal transport granule constituents. Proteins

listed in Figure 3.3I, for which antibodies were available, were validated by two approaches:

1) Combined immunofluorescence and FISH for *vg1* RNA (FISH-IF). 2) Chromatography as large RNP complexes using gel filtration chromatography and immunoblot analysis.

References

- Aguero T, Newman K, King ML. 2018. Microinjection of *Xenopus* oocytes. *Cold Spring Harb Protoc*.
- Allison R, Czaplinski K, Git A, Adegbenro E, Stennard F, Houliston E, Standart N. 2004. Two distinct Staufen isoforms in *Xenopus* are vegetally localized during oogenesis. *RNA*.
- Arthur PK, Claussen M, Koch S, Tarbashevich K, Jahn O, Pieler T. 2009. Participation of *Xenopus* Elr-type proteins in vegetal mRNA localization during oogenesis. *J Biol Chem* **284**: 19982–19992.
- Bailey TL, Elkan C. 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proceedings Int Conf Intell Syst Mol Biol* **2**: 28–36.
- Banani SF, Lee HO, Hyman AA, Rosen MK. 2017. Biomolecular condensates: organizers of cellular biochemistry. *Nat Rev Mol Cell Biol* **18**: 285–298.
- Banani SF, Rice AM, Peeples WB, Lin Y, Jain S, Parker R, Rosen MK. 2016. Compositional control of phase-separated cellular bodies. *Cell* **166**: 651–663.
- Batish M, van den Bogaard P, Kramer FR, Tyagi S. 2012. Neuronal mRNAs travel singly into dendrites. *Proc Natl Acad Sci U S A* **109**: 4645–50.
- Becker BE, Gard DL. 2006. Visualization of the cytoskeleton in *Xenopus* oocytes and eggs by confocal immunofluorescence microscopy. *Methods Mol Biol* **322**: 69–86.
- Betley JN, Heinrich B, Vernos I, Sardet C, Prodon F, Deshler JO. 2004. Kinesin II mediates *vg1* mRNA transport in *Xenopus* oocytes. *Curr Biol* **14**: 219–24.
- Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. 2006. Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells. *Cold Spring Harb Symp Quant Biol* **71**: 513–21.
- Birsoy B, Kofron M, Schaible K, Wylie C, Heasman J. 2006. Vg1 is an essential signaling molecule in *Xenopus* development. *Development* **133**: 15–20.
- Blower MD. 2013. Molecular insights into intracellular RNA localization. *Int Rev Cell Mol Biol* **302**: 1–39.
- Blower MD, Feric E, Weis K, Heald R. 2007. Genome-wide analysis demonstrates conserved localization of messenger RNAs to mitotic microtubules. *J Cell Biol* **179**: 1365–73.

- Brangwynne CP, Eckmann CR, Courson DS, Rybarska A, Hoege C, Gharakhani J, Julicher F, Hyman AA. 2009. Germline P granules are liquid droplets that localize by controlled dissolution/condensation. *Science* **324**: 1729–1732.
- Brangwynne CP, Tompa P, Pappu R V. 2015. Polymer physics of intracellular phase transitions. *Nat Phys* **11**: 899–904.
- Bregues M, Teixeira D, Parker R. 2005. Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. *Science* **310**: 486–9.
- Buchan JR. 2014. mRNP granules. Assembly, function, and connections with disease. *RNA Biol* **11**: 1019–30.
- Buxbaum AR, Haimovich G, Singer RH. 2015. In the right place at the right time: visualizing and understanding mRNA localization. *Nat Rev Mol Cell Biol* **16**: 95–109.
- Buxbaum AR, Wu B, Singer RH. 2014. Single β -actin mRNA detection in neurons reveals a mechanism for regulating its translatability. *Science* **343**: 419–422.
- Cajigas IJ, Tushev G, Will TJ, tom Dieck S, Fuerst N, Schuman EM. 2012. The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging. *Neuron* **74**: 453–66.
- Chin A, Lécuyer E. 2017. RNA localization: Making its way to the center stage. *Biochim Biophys Acta* **1861**: 2956–2970.
- Colegrove-Otero LJ, Devaux A, Standart N. 2005. The *Xenopus* ELAV protein ElrB represses *vgl* mRNA translation during oogenesis. *Mol Cell Biol* **25**: 9028–39.
- Cote CA, Gautreau D, Denegre JM, Kress TL, Terry NA, Mowry KL. 1999. A *Xenopus* protein related to hnRNP I has a role in cytoplasmic RNA localization. *Mol Cell* **4**: 431–437.
- Cuykendall TN, Houston DW. 2010. Identification of germ plasm-associated transcripts by microarray analysis of *Xenopus* vegetal cortex RNA. *Dev Dyn*.
- Cuykendall TN, Houston DW. 2009. Vegetally localized *Xenopus trim36* regulates cortical rotation and dorsal axis formation. *Development*.
- Czaplinski K, Köcher T, Schelder M, Segref A, Wilm M, Mattaj IW. 2005. Identification of 40LoVe, a *Xenopus* hnRNP D family protein involved in localizing a TGF-beta-related mRNA during oogenesis. *Dev Cell* **8**: 505–15.

- De Neve J, Thas O, Ottoy JP, Clement L. 2013. An extension of the Wilcoxon-Mann-Whitney test for analyzing RT-qPCR data. *Stat Appl Genet Mol Biol* **12**: 333–346.
- Decker CJ, Parker R. 2012. P-bodies and stress granules: possible roles in the control of translation and mRNA degradation. *Cold Spring Harb Perspect Biol* **4**: a012286.
- Deshler JO, Highett MI, Abramson T, Schnapp BJ. 1998. A highly conserved RNA-binding protein for cytoplasmic mRNA localization in vertebrates. *Curr Biol* **8**: 489–96.
- Deshler JO, Highett MI, Schnapp BJ. 1997. Localization of *Xenopus* *vg1* mRNA by Vera protein and the endoplasmic reticulum. *Science* **276**: 1128–1131.
- Eberwine J, Belt B, Kacharina JE, Miyashiro K. 2002. Analysis of subcellularly localized mRNAs using *in situ* hybridization, mRNA amplification, and expression profiling. *Neurochem Res* **27**: 1065–77.
- Elbaum-Garfinkle S, Kim Y, Szczepaniak K, Chen CC-H, Eckmann CR, Myong S, Brangwynne CP. 2015. The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics. *Proc Natl Acad Sci USA* **112**: 7189–94.
- Eliscovich C, Buxbaum AR, Katz ZB, Singer RH. 2013. mRNA on the move: the road to its biological destiny. *J Biol Chem* **288**: 20361–8.
- Frey S, Richter RP, Görlich D. 2006. FG-rich repeats of nuclear pore proteins form a three-dimensional meshwork with hydrogel-like properties. *Science* **314**: 815–7.
- Gagnon JA, Kreiling J a, Powrie EA, Wood TR, Mowry KL. 2013. Directional transport is mediated by a Dynein-dependent step in an RNA localization pathway. *PLoS Biol* **11**: e1001551.
- Gautreau D, Cote C a, Mowry KL. 1997. Two copies of a subelement from the *vg1* RNA localization sequence are sufficient to direct vegetal localization in *Xenopus* oocytes. *Development* **124**: 5013–20.
- Gilman B, Tijerina P, Russell R. 2017. Distinct RNA-unwinding mechanisms of DEAD-box and DEAH-box RNA helicase proteins in remodeling structured RNAs and RNPs. *Biochem Soc Trans* **45**: 1313–1321.
- Git A, Allison R, Perdiguero E, Nebreda AR, Houliston E, Standart N. 2009. Vg1RBP phosphorylation by Erk2 MAP kinase correlates with the cortical release of *vg1* mRNA during meiotic maturation of *Xenopus* oocytes. *RNA* **15**: 1121–33.

- Guntern R, Bouras C, Hof PR, Vallet PG. 1992. An improved thioflavine S method for staining neurofibrillary tangles and senile plaques in Alzheimer's disease. *Experientia* **48**: 8–10.
- Han TW, Kato M, Xie S, Wu LC, Mirzaei H, Pei J, Chen M, Xie Y, Allen J, Xiao G, et al. 2012. Cell-free formation of RNA granules: bound RNAs identify features and components of cellular assemblies. *Cell* **149**: 768–79.
- Havin L, Git A, Elisha Z, Oberman F, Yaniv K, Schwartz SP, Standart N, Yisraeli JK. 1998. RNA-binding protein conserved in both microtubule- and microfilament-based RNA localization. *Genes Dev* **12**: 1593–8.
- Heasman J, Wessely O, Langland R, Craig EJ, Kessler DS. 2001. Vegetal localization of maternal mRNAs is disrupted by *vegT* depletion. *Dev Biol* **240**: 377–386.
- Heraud-Farlow JE, Kiebler MA. 2014. The multifunctional Staufen proteins: Conserved roles from neurogenesis to synaptic plasticity. *Trends Neurosci* **37**: 470–479.
- Holt CE, Schuman EM. 2013. The central dogma decentralized: New perspectives on RNA function and local translation in neurons. *Neuron*.
- Huang DW, Sherman BT, Lempicki RA. 2009a. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37**: 1–13.
- Huang DW, Sherman BT, Lempicki RA. 2009b. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**: 44–57.
- Hubstenberger A, Cameron C, Noble SL, Keenan S, Evans TC. 2015. Modifiers of solid RNP granules control normal RNP dynamics and mRNA activity in early development. *J Cell Biol* **211**: 703–716.
- Hubstenberger A, Courel M, Bénard M, Souquere S, Ernoult-Lange M, Chouaib R, Yi Z, Morlot J-BB, Munier A, Fradet M, et al. 2017. P-body purification reveals the condensation of repressed mRNA regulons. *Mol Cell* **68**: 144–157.e5.
- Hubstenberger A, Noble SL, Cameron C, Evans TC. 2013. Translation repressors, an RNA helicase, and developmental cues control RNP phase transitions during early development. *Dev Cell* **27**: 161–173.
- Hüttelmaier S, Zenklusen D, Lederer M, Dictenberg J, Lorenz M, Meng X, Bassell GJ, Condeelis J, Singer RH. 2005. Spatial regulation of *beta-actin* translation by Src-dependent phosphorylation of ZBP1. *Nature* **438**: 512–5.

- Jain A, Vale RD. 2017. RNA phase transitions in repeat expansion disorders. *Nature* **546**: 243–247.
- Jain S, Wheeler JR, Walters RW, Agrawal A, Barsic A, Parker R. 2016. ATPase-modulated stress granules contain a diverse proteome and substructure. *Cell* **164**: 487–98.
- Jambhekar A, Derisi JL. 2007. *cis*-acting determinants of asymmetric, cytoplasmic RNA transport. *RNA* **13**: 625–42.
- Jeschonek, SP, Mowry KL. 2018. Whole-mount immunofluorescence for visualizing endogenous protein and injected RNA in *Xenopus* Oocytes. *Cold Spring Harb Protoc* **5**: prot097022.
- Kaganovich D. 2017. There is an inclusion for that: Material properties of protein granules provide a platform for building diverse cellular functions. *Trends Biochem Sci* **42**: 765–776.
- Kanai Y, Dohmae N, Hirokawa N. 2004. Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. *Neuron* **43**: 513–25.
- Kato M, Han TW, Xie S, Shi K, Du X, Wu LC, Mirzaei H, Goldsmith EJ, Longgood J, Pei J, et al. 2012. Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. *Cell* **149**: 753–67.
- Kedersha N, Ivanov P, Anderson P. 2013. Stress granules and cell signaling: more than just a passing phase? *Trends Biochem Sci* **38**: 494–506.
- Kress TL, Yoon YJ, Mowry KL. 2004. Nuclear RNP complex assembly initiates cytoplasmic RNA localization. *J Cell Biol* **165**: 203–11.
- Lancaster AK, Nutter-Upham A, Lindquist S, King OD. 2014. PLAAC: A web and command-line application to identify proteins with prion-like amino acid composition. *Bioinformatics* **30**: 2501–2502.
- Langdon EM, Qiu Y, Ghanbari Niaki A, McLaughlin GA, Weidmann CA, Gerbich TM, Smith JA, Crutchley JM, Termini CM, Weeks KM, et al. 2018. mRNA structure determines specificity of a polyQ-driven phase separation. *Science* **360**: 922–927.
- Lécuyer E, Yoshida H, Parthasarathy N, Alm C, Babak T, Cerovina T, Hughes TR, Tomancak P, Krause HM. 2007. Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. *Cell* **131**: 174–87.
- Lewis RA, Kress TL, Cote CA, Gautreau D, Rokop ME, Mowry KL. 2004. Conserved and clustered RNA recognition sequences are a critical feature of signals directing RNA localization in *Xenopus* oocytes. *Mech Dev* **121**: 101–9.

- Lewis RA, Gagnon JA, Mowry KL. 2008. PTB/hnRNP I is required for RNP remodeling during RNA localization in *Xenopus* oocytes. *Mol Cell Biol* **28**: 678–86.
- Li P, Banjade S, Cheng H-C, Kim S, Chen B, Guo L, Llaguno M, Hollingsworth J V., King DS, Banani SF, et al. 2012. Phase transitions in the assembly of multivalent signalling proteins. *Nature* **483**: 336–40.
- Lin Y, Protter DSW, Rosen MK, Parker R. 2015. Formation and maturation of phase-separated liquid droplets by RNA-binding proteins. *Mol Cell* **60**: 208–19.
- Little SC, Sinsimer KS, Lee JJ, Wieschaus EF, Gavis ER. 2015. Independent and coordinate trafficking of single *Drosophila* germ plasm mRNAs. *Nat Cell Biol* **17**: 558–68.
- Medioni C, Mowry K, Besse F. 2012. Principles and roles of mRNA localization in animal development. *Development* **139**: 3263–3276.
- Melton DA. 1987. Translocation of a localized maternal mRNA to the vegetal pole of *Xenopus* oocytes. *Nature* **328**: 80–2.
- Messitt TJ, Gagnon JA, Kreiling JA, Pratt CA, Yoon YJ, Mowry KL. 2008. Multiple kinesin motors coordinate cytoplasmic RNA transport on a subpopulation of microtubules in *Xenopus* oocytes. *Dev Cell* **15**: 426–36.
- Mi H, Huang X, Muruganujan A, Tang H, Mills C, Kang D, Thomas PD. 2017. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic Acids Res* **45**: D183–D189.
- Mili S, Moissoglu K, Macara IG. 2008. Genome-wide screen reveals APC-associated RNAs enriched in cell protrusions. *Nature* **453**: 115–9.
- Mili S, Steitz JA. 2004. Evidence for reassociation of RNA-binding proteins after cell lysis: implications for the interpretation of immunoprecipitation analyses. *RNA* **10**: 1692–4.
- Mowry KL. 1996. Complex formation between stage-specific oocyte factors and a *Xenopus* mRNA localization element. *Proc Natl Acad Sci U S A* **93**: 14608–13.
- Nagamori I, Cruickshank VA, Sassone-Corsi P. 2011. Regulation of an RNA granule during spermatogenesis: acetylation of MVH in the chromatoid body of germ cells. *J Cell Sci* **124**: 4346–55.
- Neil CR, Mowry K. 2018. Fluorescence *in situ* hybridization of cryosectioned *Xenopus* oocytes. *Cold Spring Harb Protoc* **2018**: pdb.prot097030.

- Newman K, Agüero T, King ML. 2018. Isolation of *Xenopus* oocytes. *Cold Spring Harb Protoc* **2018**: 86–91.
- Niepielko MG, Eagle WVI, Gavis ER. 2018. Stochastic seeding coupled with mRNA self-recruitment generates heterogeneous *Drosophila* germ granules. *Curr Biol* **28**: 1872–1881.e3.
- Nilsson MR. 2004. Techniques to study amyloid fibril formation *in vitro*. *Methods* **34**: 151–60.
- Nott TJ, Petsalaki E, Farber P, Jervis D, Fussner E, Plochowietz A, Craggs TD, Bazett-Jones DP, Pawson T, Forman-Kay JD, et al. 2015. Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles. *Mol Cell* **57**: 936–947.
- Otero LJ, Devaux A, Standart N. 2001. A 250-nucleotide UA-rich element in the 3' untranslated region of *Xenopus laevis* *vg1* mRNA represses translation both *in vivo* and *in vitro*. *RNA* **7**: 1753–67.
- Patel A, Lee HO, Jawerth L, Maharana S, Jahnel M, Hein MY, Stoyanov S, Mahamid J, Saha S, Franzmann TM, et al. 2015. A liquid-to-solid phase transition of the ALS protein FUS accelerated by disease mutation. *Cell* **162**: 1066–77.
- Peng Z, Mizianty MJ, Kurgan L. 2014. Genome-scale prediction of proteins with long intrinsically disordered regions. *Proteins Struct Funct Bioinforma* **82**: 145–158.
- Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.
- Protter DSW, Rao BS, Van Treeck B, Lin Y, Mizoue L, Rosen MK, Parker R. 2018. Intrinsically disordered regions can contribute promiscuous interactions to RNP granule assembly. *Cell Rep* **22**: 1401–1412.
- Ramakers C, Ruijter JM, Deprez RHL, Moorman AFM. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* **339**: 62–6.
- Rebagliati MR, Weeks DL, Harvey RP, Melton DA. 1985. Identification and cloning of localized maternal RNAs from *Xenopus* eggs. *Cell* **42**: 769–77.
- Richter JD. 2007. CPEB: a life in translation. *Trends Biochem Sci* **32**: 279–285.

- Saha S, Weber CA, Nousch M, Adame-Arana O, Hoege C, Hein MY, Osborne-Nishimura E, Mahamid J, Jahnelt M, Jawerth L, et al. 2016. Polar positioning of phase-separated liquid compartments in cells regulated by an mRNA competition mechanism. *Cell* **166**: 1572–1584.e16.
- Schisa JA. 2012. New insights into the regulation of RNP granule assembly in oocytes. *Int Rev Cell Mol Biol* **295**: 233–89.
- Sheu-Gruttadauria J, MacRae IJ. 2018. Phase transitions in the assembly and function of Human miRISC. *Cell* **173**: 946–957
- Shin Y, Brangwynne CP. 2017. Liquid phase condensation in cell physiology and disease. *Science* **357**.
- Snedden DD, Bertke MM, Vernon D, Huber PW. 2013. RNA localization in *Xenopus* oocytes uses a core group of *trans*-acting factors irrespective of destination. *RNA* **19**: 889–95.
- Tannahill D, Melton DA. 1989. Localized synthesis of the Vg1 protein during early *Xenopus* development. *Development* **106**: 775–85.
- Toretsky JA, Wright PE. 2014. Assemblages: functional units formed by cellular phase separation. *J Cell Biol* **206**: 579–88.
- Uversky VN. 2017. Intrinsically disordered proteins in overcrowded milieu: Membraneless organelles, phase separation, and intrinsic disorder. *Curr Opin Struct Biol* **44**: 18–30.
- Van Treeck B, Protter DSW, Matheny T, Khong A, Link CD, Parker R. 2018. RNA self-assembly contributes to stress granule formation and defining the stress granule transcriptome. *Proc Natl Acad Sci U S A* **115**: 2734–2739.
- Wang JT, Smith J, Chen B-C, Schmidt H, Rasoloson D, Paix A, Lambrus BG, Calidas D, Betzig E, Seydoux G. 2014. Regulation of RNA granule dynamics by phosphorylation of serine-rich, intrinsically disordered proteins in *C. elegans*. *Elife* **3**: e04591.
- Weber SC, Brangwynne CP. 2012. Getting RNA and protein in phase. *Cell*.
- Weeks DL, Melton DA. 1987. A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF-beta. *Cell* **51**: 861–7.
- Wei M-T, Elbaum-Garfinkle S, Holehouse AS, Chen CC-H, Feric M, Arnold CB, Priestley RD, Pappu R V., Brangwynne CP. 2017. Phase behaviour of disordered proteins underlying low density and high permeability of liquid organelles. *Nat Chem* **9**: 1118–1125.

- Xu S, Li Q, Xiang J, Yang Q, Sun H, Guan A, Wang L, Liu Y, Yu L, Shi Y, et al. 2016. Thioflavin T as an efficient fluorescence sensor for selective recognition of RNA G-quadruplexes. *Sci Rep* **6**: 24793.
- Yamazaki T, Souquere S, Chujo T, Kobelke S, Chong YS, Fox AH, Bond CS, Nakagawa S, Pierron G, Hirose T. 2018. Functional domains of *NEAT1* architectural lncRNA induce paraspeckle assembly through phase separation. *Mol Cell* **70**: 1038–1053.
- Yisraeli JK. 2005. VICKZ proteins: a multi-talented family of regulatory RNA-binding proteins. *Biol Cell* **97**: 87–96.
- Yoon YJ, Mowry KL. 2004. *Xenopus* Staufin is a component of a ribonucleoprotein complex containing *vg1* RNA and kinesin. *Development* **131**: 3035–45.
- Zhang J, King ML. 1996. *Xenopus vegT* RNA is localized to the vegetal cortex during oogenesis and encodes a novel T-box transcription factor involved in mesodermal patterning. *Development*.

Chapter Four: Discussion, Conclusions, and Future Directions

The work presented in this thesis characterizes *Xenopus laevis* vegetal mRNA localization as proceeding through the formation of a large multiphase messenger ribonucleoprotein (mRNP) granule (Figure 4.1), bearing tremendous compositional and functional overlap with other classes of cytoplasmic mRNP granules. While previous work within the Mowry laboratory has explored the mechanisms of motor-driven vegetal transport, both the molecular and physical nature of the RNP cargo remained largely unknown. In identifying the vegetal transport granule as a phase separated body, my work provides insights into not only the developmentally programmed transport of maternally loaded mRNA, but also increases the understanding of broader mechanisms that govern post-transcriptional gene regulation.

***Vg1* mRNA Localizes to a Large RNP Granule**

Using a new cryosection-based imaging strategy in conjunction with single-molecule fluorescent *in situ* hybridization (smFISH) (Chapter 2), the maternally loaded *vg1* mRNA is observed to accumulate in large bodies in the vegetal hemisphere of stage II-III oocytes. These large RNP complexes, hereafter termed granules, are encapsulated by a dense network of microtubules, with potential points of intercalation apparent throughout (Chapter 3, Figure 3.1I-J). In contrast with some other types of transport granules, where mRNA are packaged singly (Batish et al. 2012; Buxbaum et al. 2014; Little et al. 2015), multiple *vg1* transcripts are contained within individual granules (Chapter 3, Figure 3.1B). Additional vegetally localized mRNA species believed to rely on common *cis*- and *trans*-acting elements (Bubunencko et al. 2002; Snedden et al. 2013) are also incorporated into *vg1*

granules, with these mRNAs appearing evenly distributed throughout the body (Chapter 3, Figure 3.1B', 3.5G-I). Interestingly, both dynein and kinesin motors, which have previously been shown to play a role in *vg1* mRNA localization (Messitt et al. 2008; Gagnon et al. 2013), appear to localize to the microtubules associated with the granule, suggesting that they interact with the broader mRNP structure rather than with individual mRNPs (Chapter 3, Figure 3.1I',J', Figure 4.1).

The large size of vegetal mRNP granules, often in excess of 10 μ M at their widest point, served as the basis for a novel isolation strategy. Through size exclusion chromatography of stage II-III oocyte lysates, large *vg1*-containing RNPs can be isolated from fractions eluting in the void volume (Chapter 3, Figure 3.1, 3.3). Further purification using interactions with the known *vg1* RNA binding proteins (RBPs), Staufen1 and hnRNPAB, allows for high confidence in the specificity of vegetal transport granule capture. This multi-step biochemical purification process represents one of the most stringent mRNP granule capture protocols employed in any system (Kanai et al. 2004; Elvira et al. 2006; Jain et al. 2016). After mass spectrometry, 86 putative vegetal transport granule proteins were identified. Interestingly, the composition of the vegetal transport granule shows a high degree of similarity to other classes of cytoplasmic RNP granules (Chapter 3, Figure 3.3J-K). In addition to two-thirds of the proteins being directly conserved amongst cytoplasmic granules, the over representation of multivalent RBPs and proteins containing intrinsically disordered regions (IDRs) provides a compositional link between the vegetal transport granule and an emerging class of phase separated mRNPs (Banani et al. 2017; Shin and Brangwynne 2017) (Chapter 3, Figure 3.3I-K, 3.5A-B).

Through extensive characterization of the composition of vegetal transport granules using both *in vivo* and fixed imaging strategies, as well as traditional cell and molecular biology approaches, the work contained within this thesis presents mRNA localization in *Xenopus laevis* oocytes as proceeding through the formation of a large multiphase mRNP granule (Chapter 3, Figure 4.1). This structure contains both nondynamic scaffold components as well as dynamic client proteins that are both enriched over and actively exchanging with the surrounding cytoplasm (Chapter 3, Figure 4.1). I further propose the vegetal mRNP granule to be an active mediator of mRNA localization, with changes in composition and dynamics being controlled over both physical space within the oocyte and over developmental time. Phase transition behavior provides unique regulatory potential in mRNA translational control, cytoskeletal association, and transport. Intriguingly, the presence of a non-dynamic RNA-sieve core challenges the notion that protein-protein interactions are the predominant driver of mRNP granule dynamics, arguing instead that the phase behavior of RNA itself may play a role in structuring phase separated bodies.

The Dynamic *Xenopus laevis* Vegetal Transport Granule

A hallmark of phase separated mRNP granules is the ability to concentrate proteins and RNA into discrete cellular compartments for extended periods, while remaining in dynamic exchange with the surrounding medium. Multivalent molecules, consisting of proteins with multiple modular domains (Li et al. 2012; Lin et al. 2015) or stretches of low sequence complexity with repeated interaction motifs, termed intrinsically disordered regions (IDRs) (Kato et al. 2012; Elbaum-Garfinkle et al. 2015; Nott et al. 2015; Protter et al. 2018) in combination with RNA species that contain multiple protein-binding sites (Li

et al. 2012; Han et al. 2012; Yamazaki et al. 2018) are thought to drive the polymerization-based phase separations that maintain such dynamic structures. Interestingly, while many of the components of various mRNP granules are known, general principles that define body composition are lacking. Efforts to tease apart such interactions using synthetic multivalent molecules have revealed that both the composition and dynamics of mRNP granules are highly sensitive to environmental cues (Aumiller and Keating 2016; Banani et al. 2016; Wei et al. 2017), providing insights into both the function of, and possible points of modification for, these structures.

Multivalent Interactions as the Foundation for Vegetal Transport Granule Composition

The enrichment of RBPs, typified by high levels of multivalency (Castello et al. 2012), and proteins containing IDRs within vegetal transport granules suggests that their body composition may rely on conserved principles of phase separation. Interestingly, phase separation through polymerization does not demand a dramatic enrichment of macromolecular constituents relative to the surrounding medium (Wei et al. 2017). This is in fitting with the observed colocalization of both established *vg1* mRNA interacting proteins and newly characterized vegetal transport granule components identified in Chapter 3 (Figure 3.3I). While several of these proteins demonstrated specific association with the microtubule network, the majority showed diffuse localization throughout the vegetal transport granule, with only minimal enrichment and no apparent substructuring (Figure 4.1). Photobleaching experiments revealed all tested proteins to be in dynamic exchange with their surroundings (Chapter 3, Figure 3.3). However, differences in the relative mobilities of individual proteins within vegetal transport granules were observed

(Chapter 3, Figure 3.6). The multivalent RBP Vera, known to bind directly to *vg1* mRNA (Deshler et al. 1998), had a lower percent mobile fraction relative to proteins with reduced RNA-binding capabilities (Chapter 3, Figure 3.6A). This is in fitting with a model of phase separated body composition where client partitioning is dependent on valency levels, and more specifically with the ability to interact with less-dynamic scaffold components (Banani et al. 2016). In addition to its own capacity to mediate multiple intermolecular interactions, Vera requires the binding of multiple proximal *cis*-elements to drive vegetal mRNA localization (Lewis et al. 2004). Proteins lacking the degree of modular domain-mediated multivalency of Vera, but possessing more extensive IDRs, such as hnRNPAB and YBX1, also demonstrated restricted motion in the vegetal mRNP granule relative to a control protein (mCherry). Together these results suggest that both domain and IDR-mediated multivalent interactions may contribute to the intrabody dynamics of the vegetal transport granule.

While work has been done both *in vitro* and *in vivo* to characterize how changes in the valency of synthetic molecules affects the dynamics of artificial phase separated structures, termed droplets (Li et al. 2012; Majumdar et al. 2012; Protter et al. 2018), the *Xenopus laevis* vegetal transport granule offers the unique opportunity to perform such experiments in an endogenous mRNP granule using known RBP components. Building on the photobleaching assay in Chapter 3 (Figure 3.6), exogenously expressed mCherry-labeled RBPs harboring functional-ablating mutations within their RNA binding domains can be used to probe the contribution of multivalency to the inter- and intra-body exchange of protein components. PTB constructs with known inactivating mutations in each of the four individual RNA binding domains (Kafasla et al. 2011) and multiple higher-order

combinations thereof, generated in Appendix A, can be used to test how degrees of multivalency affect endogenous *in vivo* granule dynamics. Additionally, domain analyses of IDR containing RBPs, such as hnRNPAB and YBX1, can be used to distinguish the contributions of modular domains and IDRs in regulating diffusive properties, and generate a new understanding of how granule composition contributes to the function and physical properties of a complex endogenous mRNP structure.

Coordination of Granule Composition and Dynamics

Cytoplasmic mRNP granule components have been observed to transition among diffuse, liquid-like, or more solid states in response to environmental cues that regulate coassembly, viscosity, and demixing specificity within granules (Hubstenberger et al. 2013; Elbaum-Garfinkle et al. 2015; Hubstenberger et al. 2015; Jain et al. 2016). In this way, the innately reversible interactions that underlie body composition allow for fine-tuning of granule dynamics, which can be exploited as a regulatory mechanism. mRNP modification and remodeling pathways feature prominently in controlling the material states of specific components of mRNP granules (Hubstenberger et al. 2013; Wang et al. 2014; Elbaum-Garfinkle et al. 2015; Hubstenberger et al. 2015; Aumiller and Keating 2016), and represent likely mediators of granule function.

Exploiting a Dynamic Mesh

One predicted hallmark of phase separated structures is that they generate an effective mesh which determines the size scale at which droplet properties impact molecular diffusion and permeability (Wei et al. 2017). The size of this effective mesh is

dependent on the molecular interactions that underlie granule organization, and can serve as an indicator of relative viscosity (Wei et al. 2017). Probing *in vivo* size exclusion barriers using fluorescently labeled dextrans of varying sizes, provides a means of both qualitatively and quantitatively assessing dynamic behavior (Updike et al. 2011; Wei et al. 2017). In this way, differences in the permeability of endogenous granules to a standardized set of labeled dextrans can be used to monitor changes in dynamics/viscosity moving both positionally, within an individual oocyte, and temporally, through oogenesis and development, without the need for a more complete understanding of a potentially changing composition. Such an assay could identify potential points of regulation for vegetal transport granules.

RNA DEAD-box Helicases as Mediators of Functional Dynamics

The material properties of mRNP granules are widely observed to be dependent on the activity of ATP-driven remodeling events (Brangwynne et al. 2011; Hubstenberger et al. 2013; Jain et al. 2016). DEAD-box RNA helicase family members, conserved amongst both somatic and germ mRNP granule types (Linder and Jankowsky 2011; Voronina et al. 2011; Protter and Parker 2016), are important mediators of the assembly and maintenance of dynamic liquid-like bodies (Hubstenberger et al. 2015; Elbaum-Garfinkle et al. 2015; Jain et al. 2016). Identification and subsequent characterization of the DEAD-box RNA helicases DDX1, DDX3, DDX5, and Dhx9 as components of the vegetal transport granule (see Chapter 3 and Appendix B), suggest an attractive scenario through which remodeling events could control both granule dynamics and RNP composition as a means of mediating specific granule functions. Indeed, previous work in both stress and P granules has demonstrated similar roles for RNA helicases, particularly DDX3, in transiently organizing

translationally repressed mRNA in liquid-like structures, as well as facilitating their release upon granule dissolution (Hilliker et al. 2011; Elbaum-Garfinkle et al. 2015). Vegetal mRNA localization in *Xenopus* oocytes proceeds through a number of coordinated steps where mRNP remodeling could potentially function. Indeed, remodeling of the Vera-*vg1* mRNA interaction is proposed to be an early or initiating event in *vg1* mRNA transport (Lewis et al. 2008). Through the nonprocessive unwinding of double stranded RNA, helicases can control access to *cis*-acting RNA elements and mediate granule assembly and composition (Linder and Jankowsky 2011). In this way, RNA helicases can control broad features of granule dynamics by adjusting the valency of the RNA species that serve as a platform for higher-order assemblies.

Beyond the RNA helicases identified through vegetal transport granule isolation, DDX6 displays a particularly interesting subcellular localization. Believed to promote more fluid-like behavior in mRNP granules (Hubstenberger et al. 2013), in *Xenopus* oocytes DDX6 accumulates at the base of microtubule networks extending towards the vegetal cortex (see Appendix Figure B.1H). Cortical staining of some granule components often shows a dramatic enrichment relative to transport granules in the vegetal cytoplasm, suggesting a compositional and/or material distinction between the two physical locations within the oocyte. Further analysis of cortical protein dynamics could offer insights into both mechanisms of remodeling and cortical anchoring.

Using DEAD-box helicase constructs that I developed in Appendix B, which have been engineered to act as dominant negative mutants (Tanner et al. 2003; Cordin et al. 2004), the *in vivo* function of RNA helicases can be further investigated. Assessing the effect of exogenously expressed wildtype versus mutant DDX1, DDX3, DDX5, and DDX6

proteins on the localization of fluorescently labeled-*vg1* mRNA and the dynamic properties of vegetal mRNP granule components using established imaging strategies (Chapter 3), offers the potential to identify unique steps during vegetal localization as being mediated by remodeling of a functionally dynamic RNP granule.

Post-Translational Modifications Control Granules through Developmental Programs

Post-translational modification of a protein can alter its interaction potential and significantly change the nature of its association with phase separated bodies (Aumiller and Keating 2016; Banani et al. 2016). Synthetic *in vitro* systems have demonstrated that modifications to both modular RNA or protein interacting domains as well as IDRs can reduce the valency of a protein and cause it to be partitioned out of an mRNP body (Aumiller and Keating 2016; Banani et al. 2016). Interestingly, such modifications can reorganize the composition of a phase separated structure on a time scale of seconds to minutes, increasing their utility as a potential mediator of mRNP granule function (Banani et al. 2016). Beyond controlling internal body composition, post-translational modification of less dynamic structural components can cause phase separated bodies to rapidly disassemble (Wang et al. 2014; Banani et al. 2016). In *C. elegans*, P granule assembly and disassembly are controlled by the developmentally regulated activity of the kinase MBK-2/DYRK and the phosphatase PP2A (Wang et al. 2014). While dephosphorylation of the MEG-1 and 3 proteins promotes granule assembly, phosphorylation causes rapid demixing and subsequent release of germline specifying mRNA (Wang et al. 2014).

Similar mechanisms of developmental regulation may exist for *Xenopus* transport granules. Indeed, phosphorylation of Vera by Erk2 MAP kinase during oocyte maturation

has previously been shown to promote its release from the vegetal cortex, coincident with a corresponding redistribution of *vg1* mRNA (Git et al. 2009). By contrast, *Staufen*, which is also phosphorylated at this time, remains cortically anchored (Git et al. 2009), suggesting that the vegetal mRNP granule either undergoes compositional change or is disassembled. Using engineered proteins with post-translational mimics, the effect of post-translational modifications on granule dynamics can be further examined *in vivo* using the photobleaching assay developed in Chapter 3 (Figure 3.5). In combination with studies profiling endogenous protein modifications, this data could provide a new picture of the vegetal transport granules as a developmentally regulated body.

Understanding Vegetal Transport Granules as a Functional Structures: Mediators of Post-Transcriptional Gene Regulation

A unifying theme of cytoplasmic mRNP granules is that they all contain translationally repressed mRNAs that are capable of (re)-entering translation in response to environmental cues (Buchan 2014). This paradigm extends to the *Xenopus* vegetal transport granule, where resident *vg1* mRNA remains translationally quiescent throughout localization (stages I-III) (Tannahill and Melton 1989). Repression is relieved in later oogenesis when *vg1* mRNA is restricted to the vegetal cortex (Tannahill and Melton 1989), and appears to be coincident with the previously proposed cortical remodeling (Git et al. 2009). In light of this, it is tempting to speculate that the vegetal mRNP granule may have a direct function in translational control. Indeed, more than 20% of proteins contained in the vegetal transport granule have roles in translational regulation, as determined by gene ontology analysis (Mi et al. 2017).

Translational control of *vg1* mRNA is in part mediated through an AU-rich 250 nucleotide sequence in its 3'UTR, termed the vegetal translational control element (VTE) (Otero et al. 2001). Direct binding of the VTE in stage I-III oocytes by the identified granule protein Elavl2 is required for inhibition, as antibody interference experiments resulted in ectopic translation of *vg1* mRNA (Otero et al. 2001; Colegrove-Otero et al. 2005). Interestingly, while Elavl2 remains present within the oocyte beyond stage III, it is no longer observed to bind the VTE (Otero et al. 2001; Colegrove-Otero et al. 2005). Phase separated granules have been proposed to aid in the translational repression of mRNA by concentrating reactants to shift the equilibrium of interacting proteins towards associated states (Buchan 2014). Similar mechanisms may operate in the vegetal transport granule, where local concentration differences in *vg1* mRNA and Elavl2 between repressed and active states could dictate binding-mediated inhibition.

Ribosomal inhibition has also been proposed as a conserved mechanism by which phase separated granules control translation (Buchan 2014). By either physically occluding ribosomal subunits and/or assembly factors, as is the case with stress granules and processing bodies (Sonenberg and Hinnebusch 2009), or sterically restricting ribosomal activity, as has been observed in both neurons (Buxbaum et al. 2014) and *Drosophila* germ granules (Chekulaeva et al. 2006), mRNP granules can prevent the processive movement of translationally competent ribosomal complexes along mRNA. Interestingly, only small ribosomal proteins were identified in the proteomic analysis of the vegetal mRNP granule (see Chapter 3), suggesting that the structure may affect ribosomal assembly by limiting the availability of large ribosomal proteins. This model is particularly attractive as in addition to small ribosomal proteins, the vegetal mRNP granule contains members of the

eIF4E-eIF4G cap-binding complex, which facilitates recruitment of translation initiation complexes (Sonenberg and Hinnebusch 2009), potentially priming mRNA for translation upon granule disassembly. Within the granule, the dynamic properties of ribosomal proteins have not yet been examined but could be further tested using the photobleaching assay developed in Chapter 3 to provide additional evidence for ribosomal inhibition as a means of translational control for vegetally localized mRNAs.

The inclusion of the poly(A)-dependent translational repressor, CPEB1 within the vegetal mRNP granule (Chapter 3), suggests an additional level of translational control for localized mRNA. CPEB, known to act as a repressor during oogenesis (Richter 2007), is recruited to nearly all cytoplasmic mRNP granules (Buchan 2014), where it has been shown to both block initiation complex formation and recruit deadenylase enzymes to maintain translationally limiting short poly(A) tail lengths (Richter 2007). A more prominent role for deadenylation as a tool for post-transcriptional regulation in granules is supported by the recent finding that deadenylase activity can be accelerated by more than two orders of magnitude within phase separated bodies (Sheu-Gruttadauria and MacRae 2018).

Vegetal RNP Granules and the Cytoskeleton: Sites for Organization and Connection

vg1 mRNA localization is known to depend upon motor-mediated transport along microtubules (Betley et al. 2004; Messitt et al. 2008; Gagnon et al. 2013). Work presented within this thesis (Chapter 3) identifies the vegetal RNP granule as the physical cargo upon which dynein and kinesin motors act to transport maternal mRNA to the vegetal cortex in *Xenopus* oocytes (Figure 4.1). While much work has been done to characterize both the RNA sequences and motor requirements necessary for intracellular transport in *Xenopus*

oocytes, the relationship between phase separated bodies and the cytoskeleton remains poorly understood in this or any other system. Evidence is emerging that through mechanisms of compositional control, mRNP granules may be able to both facilitate connections with the surrounding microtubule network as well as impact its overall organization (Lin et al. 2016; Hernández-Vega et al. 2017). This presents a model for mRNA localization wherein the role for vegetal mRNP granules may extend beyond passive cargo and assume a more direct role in mediating vegetal transport.

Recent work in neurons has demonstrated that phase separated bodies have the capacity to promote the local formation of microtubule bundles (Hernández-Vega et al. 2017). By concentrating tubulin, these structures promote microtubule polymerization, and effectively shape the surrounding cytoskeletal environment (Hernández-Vega et al. 2017). The identification of not only tubulin (Tuba3c), but also the cytoskeletal mediator clathrin heavy chain (Chc) within vegetal mRNP granules (Chapter 3, Figure 3.3I) suggests that transport granules could extend this model and actively organize the microtubule network to promote localization. In *Drosophila* oocytes Chc is essential for the organization of microtubule polarity, specifying the accumulation of microtubule plus ends at the posterior cortex (Vazquez-Pianzola et al. 2014). This microtubule asymmetry is paralleled in *Xenopus* oocytes, where a similar subpopulation of microtubules are oriented with their plus ends directed towards the vegetal cortex (Messitt et al. 2008). These microtubules allow for bidirectional transport using both dynein and kinesin motors and are essential for *vg1* mRNA localization (Gagnon et al. 2013). By controlling both the extension and polarity of the microtubule network, vegetal mRNP granules could exert tremendous influence over their ultimate localization.

Recent work exploring the role of the dipeptide repeat proteins polyGly-Arg (GR_n) and polyPro-Arg (PR_n) in stabilizing protein and RNA-mediated interactions within mRNP granules, has uncovered a novel link between phase separated bodies and cytoskeletal elements (Lee et al. 2016; Lin et al. 2016). In addition to general recruitment to phase separated mRNP bodies, PR_n poly-dipeptides specifically accumulate along polymeric forms of IDRs located within intermediate protein filaments (Lin et al. 2016). This, in addition to the observed liquid-like properties of intermediate filament structures (Lin et al. 2016), supports the possibility of a direct interaction between mRNP granules and intermediate filaments. This interaction is particularly interesting within the context of vegetal transport granules as *Xenopus* cytokeratin intermediate filaments display a vegetal distribution pattern (Godsave et al. 1984) that bears remarkable similarity to that observed for *vg1* mRNA (Chapter 3). How microtubule networks are able to interact with dynamic mRNP granules is an open question, but intermediate filaments have been proposed as a potential mediator (Lin et al. 2016). The presence of both extended IDRs and a conserved structured domain make intermediate filaments well suited to serve as adaptor proteins (Kornreich et al. 2015). The identification of the cytokeratin Krt12 as a component of the vegetal mRNP granule (Chapter 3, Figure 3.3I), provides a tractable entry point to further explore the relationship between intermediate filaments, microtubules, and vegetal transport in *Xenopus* oocytes.

RNA-RNA Interactions: Mediators of Recruitment, Phase Separation, and Granular Substructuring

Within the context of phase separated body composition, RNA has traditionally been viewed as a multivalent platform, capable of mediating intermolecular interactions between *trans*-acting proteins (Banani et al. 2016). In this way, interaction with RNA promotes protein-mediated phase separation by lowering the saturation concentration needed for demixing to occur (Molliex et al. 2015; Zhang et al. 2015; Lin et al. 2015; Saha et al. 2016). By controlling the threshold concentration for phase separation in this manner, RNA can nucleate droplet formation at specific cellular sites (Clemson et al. 2009; Dundr 2013; Saha et al. 2016) as well as dictate the overall protein composition and dynamics of higher-order assemblies (Elbaum-Garfinkle et al. 2015; Zhang et al. 2015). However, recent studies have challenged this ancillary role for RNA in granule organization, and offered new roles for RNA-RNA interactions in defining the composition of mRNP granules (Jain and Vale 2017; Van Treeck et al. 2018; Langdon et al. 2018; Niepielko et al. 2018; Yamazaki et al. 2018).

Within vegetal transport granules, localized mRNAs are nondynamic, demonstrating no intra-granule redistribution upon photobleaching (Chapter 3, Figure 3.6). This suggests a material distinction between the RNA containing portion of the granule and the observed dynamic protein components of the granule (Figure 4.1). Upon staining with Thioflavin (Chapter 3, Figure 3.5), a dye most commonly thought to stain amyloid-like aggregates formed by protein β -sheet interactions (Nilsson 2004), a clear substructure is apparent (Chapter 3, Figure 3.5F). Interestingly, while exhaustive efforts to identify granule protein components with both structured patterns of granule localization and nondynamic intra-granule motility failed to provide insights into the composition of

the mesh- or gel-like substructure, evidence of a more direct role for RNA-RNA interactions was observed (Chapter 3, Figure 3.5G-I). The near complete overlap of localized mRNA with the Thioflavin staining opens up the possibility that RNA self-assembly may drive the observed multiphase behavior of the vegetal mRNP transport granule. In this model, the interactions of localized mRNAs promotes RNA-gelation within mRNPs. Previous research demonstrating that depletion of *vegT* mRNA, a component of the vegetal mRNP granule, leads to the mislocalization of *vg1* mRNA (Heasman et al. 2001), suggests that heterotypic mRNA interactions may be fundamental to this process. In other phase separated bodies, such RNA-RNA interactions are found to be mediated by mRNA secondary structure (Langdon et al. 2018). Within vegetal mRNP granules a particularly attractive candidate for this structural motif is RNA G-quadruplexes, which are predicted to form in both *vg1* and *vegT* mRNAs (Kikin et al. 2006). Recent work has demonstrated that Thioflavin, in addition to recognizing protein β -sheet interactions, also recognizes dense networks of RNA G-quadruplexes (Xu et al. 2016), raising the possibility that RNA may be the source of Thioflavin staining observed in vegetal transport granules. Additionally, several of the identified components of vegetal mRNP granules have been demonstrated to preferentially interact with G-quadruplexes, including the conserved helicase Dhx9 (Chakraborty and Grosse 2011) and key neuronal granule protein FMR1/FMRP (Darnell et al. 2001) (Chapter 3). While direct RNA-RNA phase transitions have been observed in trinucleotide repeat expansions linked to neurological disease (Jain and Vale 2017) and are suggested to contribute to stress granule

assembly (Van Treeck et al. 2018), a role for RNA phase separation as a scaffold for mRNP granule assembly has never before been demonstrated.

In addition to potential structural roles in vegetal mRNP granules, RNA-RNA interactions likely play a role in mediating recruitment of mRNA. Incorporation into the vegetal transport granule relies on sequence-encoded features of mRNA and represents a capture event of freely diffusing mRNA, as evidenced by the recruitment of exogenously expressed transcripts into preexisting vegetal mRNP granules (Chapter 3, Figure 3.2B). This model is conceptually similar to the mRNA recruitment observed in *Drosophila* germ granules, where RNA self-association drives germ granule mRNA localization (Niepielko et al. 2018). Interestingly, germ granules are thought to be initially seeded by a piRNA-Piwi adhesion trap present within the nascent granule (Vourekas et al. 2016). As the Piwi protein Piwil3 has been identified as a component of the vegetal mRNP granule (Chapter 3, Figure 3.3I), a conserved mechanism could potentially initiate mRNA localization in *Xenopus*.

Phase Separation May Organize *Xenopus* Oocytes for Long Term Viability

Beyond vegetal mRNP granule phase separation (Chapter 3), evidence suggests that *Xenopus laevis* oocytes may use phase structuring as a global mechanism of cytoplasmic organization. Indeed, the vegetal wedge in which mRNP granules localize displays semi-solid features and can be manually removed from the bulk of the cytoplasm using tweezers (data not shown). Recent work from the Mitchison laboratory, demonstrating that *Xenopus* Balbiani body is organized into amyloid-like hydrogels, has been used to propose phase separation as a conserved mechanism for the long-term protection of germline

determinants (Boke et al. 2016). Our observations would extend this theory beyond specific substructures and propose that widespread control over the material properties of oocytes supports their function as long-lived germ cells. This mechanism in many ways parallels the behavior of the bacterial cytoplasm, where in the absence of metabolic activity the cytoplasm assumes a less dynamic state which facilitates dormancy (Parry et al. 2014). Such control over global phase behavior may be an evolutionarily conserved means of promoting latency for cells that are environmentally responsive.

Conclusion

Building on the extensive research into the mechanisms that drive motor-based transport of maternal mRNAs that preceded my time in the Mowry laboratory, the work within this thesis provides a new context for understanding mRNA localization. By defining the physical cargo of vegetal transport as a phase separated mRNP granule (Figure 4.1), regulation of distinct transport and developmental steps can be understood as a reflection of the unique physical properties of liquid-like structures. Changes in granule composition and dynamics are controlled by a core set of underlying molecular interactions, which are themselves responsive to intracellular cues. By furthering our understanding of the principles that define the body composition of the vegetal transport granule as it proceeds through oogenesis, future work will not only provide insights into mRNA localization but will enhance our understanding of regulated gene expression through cellular organization.



Figure 4.1 Vegetal mRNA localizes in a multiphase ribonucleoprotein transport granule. Localized mRNAs accumulate in large bodies in the vegetal hemisphere of stage II-III *Xenopus laevis* oocytes. These large RNP granules are encapsulated by a dense network of microtubules (schematically represented as yellow lines) with which the molecular motors dynein and kinesin associate. Vegetal transport granules are heterotypic and contain multiple copies of each localized mRNA (schematically represented as black lines). While mRNA mobility is restricted within the granule, protein components (schematically represented as colored dots) are dynamic, showing rapid inter- and

intragranular exchange (represented by dashed arrows). The physical properties of phase separated structures allows vegetal transport RNPs to generate a local enrichment of *trans*-acting proteins relative to the surrounding cytoplasm.

References

- Aumiller WM, Keating CD. 2016. Phosphorylation-mediated RNA/peptide complex coacervation as a model for intracellular liquid organelles. *Nat Chem* **8**: 129–37.
- Banani SF, Lee HO, Hyman AA, Rosen MK. 2017. Biomolecular condensates: organizers of cellular biochemistry. *Nat Rev Mol Cell Biol* **18**: 285–98.
- Banani SF, Rice AM, Peeples WB, Lin Y, Jain S, Parker R, Rosen MK. 2016. Compositional control of phase-separated cellular bodies. *Cell* **166**: 651–63.
- Batish M, van den Bogaard P, Kramer FR, Tyagi S. 2012. Neuronal mRNAs travel singly into dendrites. *Proc Natl Acad Sci U S A* **109**: 4645–50.
- Betley JN, Heinrich B, Vernos I, Sardet C, Prodon F, Deshler JO. 2004. Kinesin II mediates *vg1* mRNA transport in *Xenopus* oocytes. *Curr Biol* **14**: 219–24.
- Boke E, Ruer M, Wühr M, Coughlin M, Lemaitre R, Gygi SP, Alberti S, Drechsel D, Hyman AA, Mitchison TJ. 2016. Amyloid-like self-assembly of a cellular compartment. *Cell* **166**: 637–50.
- Brangwynne CP, Mitchison TJ, Hyman AA. 2011. Active liquid-like behavior of nucleoli determines their size and shape in *Xenopus laevis* oocytes. *Proc Natl Acad Sci U S A* **108**: 4334–39.
- Bubunencko M, Kress TL, Vempati UD, Mowry KL, King ML. 2002. A consensus RNA signal that directs germ layer determinants to the vegetal cortex of *Xenopus* oocytes. *Dev Biol* **248**: 82–92.
- Buchan JR. 2014. mRNP granules. Assembly, function, and connections with disease. *RNA Biol* **11**: 1019–30.
- Buxbaum AR, Wu B, Singer RH. 2014. Single β -actin mRNA detection in neurons reveals a mechanism for regulating its translatability. *Science (80-)* **343**: 419–22.
- Castello A, Fischer B, Eichelbaum K, Horos R, Beckmann BM, Strein C, Davey NE, Humphreys DT, Preiss T, Steinmetz LM, et al. 2012. Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell* **149**: 1393–406.
- Chakraborty P, Grosse F. 2011. Human DHX9 helicase preferentially unwinds RNA-containing displacement loops (R-loops) and G-quadruplexes. *DNA Repair (Amst)* **10**: 654–65.

- Chekulaeva M, Hentze MW, Ephrussi A. 2006. Bruno acts as a dual repressor of *oskar* translation, promoting mRNA oligomerization and formation of silencing particles. *Cell* **124**: 521–33.
- Clemson CM, Hutchinson JN, Sara SA, Ensminger AW, Fox AH, Chess A, Lawrence JB. 2009. An architectural role for a nuclear noncoding RNA: *NEAT1* RNA is essential for the structure of paraspeckles. *Mol Cell* **33**: 717–26.
- Colegrove-Otero LJ, Devaux A, Standart N. 2005. The *Xenopus* ELAV protein ElrB represses *vg1* mRNA translation during oogenesis. *Mol Cell Biol* **25**: 9028–39.
- Cordin O, Tanner NK, Doère M, Linder P, Banroques J. 2004. The newly discovered Q motif of DEAD-box RNA helicases regulates RNA-binding and helicase activity. *EMBO J* **23**: 2478–87.
- Darnell JC, Jensen KB, Jin P, Brown V, Warren ST, Darnell RB. 2001. Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. *Cell* **107**: 489–99.
- Deshler JO, Highett MI, Abramson T, Schnapp BJ. 1998. A highly conserved RNA-binding protein for cytoplasmic mRNA localization in vertebrates. *Curr Biol* **8**: 489–96.
- Dundr M. 2013. Nucleation of nuclear bodies. *Methods Mol Biol* **1042**: 351–64.
- Elbaum-Garfinkle S, Kim Y, Szczepaniak K, Chen CC-H, Eckmann CR, Myong S, Brangwynne CP. 2015. The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics. *Proc Natl Acad Sci USA* **112**: 7189–94.
- Elvira G, Wasiak S, Blandford V, Tong X-K, Serrano A, Fan X, del Rayo Sánchez-Carbente M, Servant F, Bell AW, Boismenu D, et al. 2006. Characterization of an RNA granule from developing brain. *Mol Cell Proteomics* **5**: 635–51.
- Gagnon JA, Kreiling JA, Powrie EA, Wood TR, Mowry KL. 2013. Directional transport is mediated by a Dynein-dependent step in an RNA localization pathway. *PLoS Biol* **11**: e1001551.
- Git A, Allison R, Perdiguero E, Nebreda AR, Houliston E, Standart N. 2009. Vg1RBP phosphorylation by Erk2 MAP kinase correlates with the cortical release of *vg1* mRNA during meiotic maturation of *Xenopus* oocytes. *RNA* **15**: 1121–33.
- Godsave SF, Wylie CC, Lane EB, Anderton BH. 1984. Intermediate filaments in the *Xenopus* oocyte: the appearance and distribution of cytokeratin-containing filaments. *J Embryol Exp Morphol*.

- Han TW, Kato M, Xie S, Wu LC, Mirzaei H, Pei J, Chen M, Xie Y, Allen J, Xiao G, et al. 2012. Cell-free formation of RNA granules: bound RNAs identify features and components of cellular assemblies. *Cell* **149**: 768–79.
- Heasman J, Wessely O, Langland R, Craig EJ, Kessler DS. 2001. Vegetal localization of maternal mRNAs is disrupted by *vegT* depletion. *Dev Biol* **240**: 377–86.
- Hernández-Vega A, Braun M, Scharrel L, Jahnel M, Wegmann S, Hyman BT, Alberti S, Diez S, Hyman AA. 2017. Local nucleation of microtubule bundles through Tubulin concentration into a condensed Tau phase. *Cell Rep* **20**: 2304–12.
- Hilliker A, Gao Z, Jankowsky E, Parker R. 2011. The DEAD-box protein Ded1 modulates translation by the formation and resolution of an eIF4F-mRNA complex. *Mol Cell* **43**: 962–72.
- Hubstenberger A, Cameron C, Noble SL, Keenan S, Evans TC. 2015. Modifiers of solid RNP granules control normal RNP dynamics and mRNA activity in early development. *J Cell Biol* **211**: 703–16.
- Hubstenberger A, Noble SL, Cameron C, Evans TC. 2013. Translation repressors, an RNA helicase, and developmental cues control RNP phase transitions during early development. *Dev Cell* **27**: 161–73.
- Jain A, Vale RD. 2017. RNA phase transitions in repeat expansion disorders. *Nature* **546**: 243–47.
- Jain S, Wheeler JR, Walters RW, Agrawal A, Barsic A, Parker R. 2016. ATPase-modulated stress granules contain a diverse proteome and substructure. *Cell* **164**: 487–98.
- Kafasla P, Lin H, Curry S, Jackson RJ. 2011. Activation of picornaviral IRESs by PTB shows differential dependence on each PTB RNA-binding domain. *RNA* **17**: 1120–31.
- Kanai Y, Dohmae N, Hirokawa N. 2004. Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. *Neuron* **43**: 513–25.
- Kato M, Han TW, Xie S, Shi K, Du X, Wu LC, Mirzaei H, Goldsmith EJ, Longgood J, Pei J, et al. 2012. Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. *Cell* **149**: 753–67.
- Kikin O, D’Antonio L, Bagga PS. 2006. QGRS Mapper: a web-based server for predicting G-quadruplexes in nucleotide sequences. *Nucleic Acids Res* **34**: W676–82.
- Kornreich M, Avinery R, Malka-Gibor E, Laser-Azogui A, Beck R. 2015. Order and disorder in intermediate filament proteins. *FEBS Lett* **589**: 2464–76.

- Langdon EM, Qiu Y, Ghanbari Niaki A, McLaughlin GA, Weidmann CA, Gerbich TM, Smith JA, Crutchley JM, Termini CM, Weeks KM, et al. 2018. mRNA structure determines specificity of a polyQ-driven phase separation. *Science* **360**: 922–27.
- Lee K-H, Zhang P, Kim HJ, Mitrea DM, Sarkar M, Freibaum BD, Cika J, Coughlin M, Messing J, Molliex A, et al. 2016. C9orf72 dipeptide repeats impair the assembly, dynamics, and function of membrane-less organelles. *Cell* **167**: 774–88.
- Lewis RA, Kress TL, Cote CA, Gautreau D, Rokop ME, Mowry KL. 2004. Conserved and clustered RNA recognition sequences are a critical feature of signals directing RNA localization in *Xenopus* oocytes. *Mech Dev* **121**: 101–09.
- Lewis RA, Gagnon JA, Mowry KL. 2008. PTB/hnRNP I is required for RNP remodeling during RNA localization in *Xenopus* oocytes. *Mol Cell Biol* **28**: 678–86.
- Li P, Banjade S, Cheng H-C, Kim S, Chen B, Guo L, Llaguno M, Hollingsworth J V., King DS, Banani SF, et al. 2012. Phase transitions in the assembly of multivalent signalling proteins. *Nature* **483**: 336–40.
- Lin Y, Mori E, Kato M, Xiang S, Wu L, Kwon I, McKnight SL. 2016. Toxic PR polypeptides encoded by the C9orf72 repeat expansion target LC domain polymers. *Cell* **167**: 789–802.
- Lin Y, Protter DSW, Rosen MK, Parker R. 2015. Formation and maturation of phase-separated liquid droplets by RNA-binding proteins. *Mol Cell* **60**: 208–19.
- Linder P, Jankowsky E. 2011. From unwinding to clamping - the DEAD box RNA helicase family. *Nat Rev Mol Cell Biol* **12**: 505–16.
- Little SC, Sinsimer KS, Lee JJ, Wieschaus EF, Gavis ER. 2015. Independent and coordinate trafficking of single *Drosophila* germ plasm mRNAs. *Nat Cell Biol* **17**: 558–68.
- Majumdar A, Cesario WC, White-Grindley E, Jiang H, Ren F, Khan MR, Li L, Choi EM-L, Kannan K, Guo F, et al. 2012. Critical role of amyloid-like oligomers of *Drosophila* Orb2 in the persistence of memory. *Cell* **148**: 515–29.
- Messitt TJ, Gagnon JA, Kreiling JA, Pratt CA, Yoon YJ, Mowry KL. 2008. Multiple kinesin motors coordinate cytoplasmic RNA transport on a subpopulation of microtubules in *Xenopus* oocytes. *Dev Cell* **15**: 426–36.
- Mi H, Huang X, Muruganujan A, Tang H, Mills C, Kang D, Thomas PD. 2017. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic Acids Res* **45**: 183–89.

- Molliex A, Temirov J, Lee J, Coughlin M, Kanagaraj AP, Kim HJ, Mittag T, Taylor JP. 2015. Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. *Cell* **163**: 123–33.
- Niepielko MG, Eagle WVI, Gavis ER. 2018. Stochastic seeding coupled with mRNA self-recruitment generates heterogeneous *Drosophila* germ granules. *Curr Biol* **28**: 1872–81.
- Nilsson MR. 2004. Techniques to study amyloid fibril formation *in vitro*. *Methods* **34**: 151–60.
- Nott TJ, Petsalaki E, Farber P, Jervis D, Fussner E, Plochowietz A, Craggs TD, Bazett-Jones DP, Pawson T, Forman-Kay JD, et al. 2015. Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles. *Mol Cell* **57**: 936–47.
- Otero LJ, Devaux A, Standart N. 2001. A 250-nucleotide UA-rich element in the 3' untranslated region of *Xenopus laevis* *vg1* mRNA represses translation both *in vivo* and *in vitro*. *RNA* **7**: 1753–67.
- Parry BR, Surovtsev I V., Cabeen MT, O'Hern CS, Dufresne ER, Jacobs-Wagner C. 2014. The bacterial cytoplasm has glass-like properties and is fluidized by metabolic activity. *Cell* **156**: 183–94.
- Protter DSW, Parker R. 2016. Principles and properties of stress granules. *Trends Cell Biol* **26**: 668–79.
- Protter DSW, Rao BS, Van Treeck B, Lin Y, Mizoue L, Rosen MK, Parker R. 2018. Intrinsically disordered regions can contribute promiscuous interactions to RNP granule assembly. *Cell Rep* **22**: 1401–12.
- Richter JD. 2007. CPEB: a life in translation. *Trends Biochem Sci* **32**: 279–85.
- Saha S, Weber CA, Nusch M, Adame-Arana O, Hoege C, Hein MY, Osborne-Nishimura E, Mahamid J, Jahnel M, Jawerth L, et al. 2016. Polar positioning of phase-separated liquid compartments in cells regulated by an mRNA competition mechanism. *Cell* **166**: 1572–84.
- Sheu-Gruttadauria J, MacRae IJ. 2018. Phase transitions in the assembly and function of human miRISC. *Cell* **173**: 946–57.
- Shin Y, Brangwynne CP. 2017. Liquid phase condensation in cell physiology and disease. *Science* **357**.

- Snedden DD, Bertke MM, Vernon D, Huber PW. 2013. RNA localization in *Xenopus* oocytes uses a core group of *trans*-acting factors irrespective of destination. *RNA* **19**: 889–95.
- Sonenberg N, Hinnebusch AG. 2009. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* **136**: 731–45.
- Tannahill D, Melton DA. 1989. Localized synthesis of the Vg1 protein during early *Xenopus* development. *Development* **106**: 775–85.
- Tanner NK, Cordin O, Banroques J, Doère M, Linder P. 2003. The Q motif: a newly identified motif in DEAD box helicases may regulate ATP binding and hydrolysis. *Mol Cell* **11**: 127–38.
- Updike DL, Hachey SJ, Kreher J, Strome S. 2011. P granules extend the nuclear pore complex environment in the *C. elegans* germ line. *J Cell Biol* **192**: 939–48.
- Van Treeck B, Protter DSW, Matheny T, Khong A, Link CD, Parker R. 2018. RNA self-assembly contributes to stress granule formation and defining the stress granule transcriptome. *Proc Natl Acad Sci USA* **115**: 2734–39.
- Vazquez-Pianzola P, Adam J, Haldemann D, Hain D, Urlaub H, Suter B. 2014. Clathrin heavy chain plays multiple roles in polarizing the *Drosophila* oocyte downstream of Bic-D. *Development* **141**: 1915–26.
- Voronina E, Seydoux G, Sassone-Corsi P, Nagamori I. 2011. RNA granules in germ cells. *Cold Spring Harb Perspect Biol* **3**.
- Vourekas A, Alexiou P, Vrettos N, Maragkakis M, Mourelatos Z. 2016. Sequence-dependent but not sequence-specific piRNA adhesion traps mRNAs to the germ plasm. *Nature* **531**: 390–94.
- Wang JT, Smith J, Chen B-C, Schmidt H, Rasoloson D, Paix A, Lambrus BG, Calidas D, Betzig E, Seydoux G. 2014. Regulation of RNA granule dynamics by phosphorylation of serine-rich, intrinsically disordered proteins in *C. elegans*. *Elife* **3**: e04591.
- Wei M-T, Elbaum-Garfinkle S, Holehouse AS, Chen CC-H, Feric M, Arnold CB, Priestley RD, Pappu R V., Brangwynne CP. 2017. Phase behaviour of disordered proteins underlying low density and high permeability of liquid organelles. *Nat Chem* **9**: 1118–25.
- Xu S, Li Q, Xiang J, Yang Q, Sun H, Guan A, Wang L, Liu Y, Yu L, Shi Y, et al. 2016. Thioflavin T as an efficient fluorescence sensor for selective recognition of RNA G-quadruplexes. *Sci Rep* **6**: 24793.

- Yamazaki T, Souquere S, Chujo T, Kobelke S, Chong YS, Fox AH, Bond CS, Nakagawa S, Pierron G, Hirose T. 2018. Functional domains of *NEAT1* architectural lncRNA induce paraspeckle assembly through phase separation. *Mol Cell* **70**: 1038–53.
- Zhang H, Elbaum-Garfinkle S, Langdon EM, Taylor N, Occhipinti P, Bridges AA, Brangwynne CP, Gladfelter AS. 2015. RNA controls PolyQ protein phase transitions. *Mol Cell* **60**: 220–30.

Appendix A:
Polypyrimidine Tract-Binding Protein (PTB) –
A Multivalent Tool for Studying Endogenous
Granule Dynamics

Introduction

hnRNP I/Polypyrimidine Tract Binding-protein (PTB), is a heterogeneous nuclear ribonucleoprotein (hnRNP), that functions in alternative splicing, polyadenylation, mRNA stability, internal ribosomal entry site-mediated translation, and mRNA localization (reviewed in Dreyfuss et al. 2002; Auweter and Allain 2008). PTB contains four RNA recognition motifs (RRMs) separated by three linkers (Figure A.1A; Auweter and Allain 2008). Each RRM of PTB is capable of sequence specific binding of RNA molecules, with mutational analyses revealing conserved mechanisms of mRNA binding (Figure A.1B; Yuan et al. 2002; Simpson et al. 2004).

In *vg1* mRNA localization, PTB associates with *vg1* mRNA in the oocyte nucleus as part of a core RNP complex (Kress et al. 2004), and is subsequently colocalized with the RNA to the vegetal cortex (Cote et al. 1999). PTB is essential to *vg1* mRNA localization, as mutations in its cognate binding sites within the VLE results in a complete loss of localization (Cote et al. 1999; Lewis et al. 2004). While a core *vg1* RNP complex is assembled in the nucleus it subsequently undergoes a variety of alterations within the cytoplasm, including the addition, modification, and rearrangement of protein factors (King et al. 2005). Of particular interest is the PTB-mediated remodeling of the Vera-VLE interaction that distinguished nuclear and cytoplasmic *vg1* RNP complexes, and is required for *vg1* localization (Lewis et al. 2008). Formation of a localization-competent RNP is believed to be an early event in the *vg1* localization pathway and may be initiated by such remodeling. Interestingly, PTB has been shown to promote higher-order assembly of RNP structures in *Drosophila* embryos (Besse et al. 2009).

Recently, the ability of RNA-binding proteins to engage in multiple intra- and inter-molecular interactions has been shown to be critical for the formation of phase separated structures (reviewed in Chapter 1; Banani et al. 2016; Shin and Brangwynne 2017). PTB represents a unique source of multivalency, with well resolved binding capabilities mediated exclusively through modular RRM domains and an established capacity to phase separate (Li et al. 2012; Kato et al. 2012). Mutational analyses limiting RRM binding capabilities can be used to directly assess the role of multivalency in controlling protein recruitment and dynamics within an endogenous phase separated mRNP granule (see Chapter 4). While much emphasis has been placed on the role of such multivalency in phase separation, experimentation has been limited to synthetic macromolecules and *in vitro* phase separation assays (Li et al. 2012; Kato et al. 2012). A putative model for the body composition of mRNP granules has emerged in which client partitioning is dependent on valency levels, and more specifically with the ability to interact with less-dynamic scaffold components (Banani et al. 2016). However, to date this model has not been tested in complex endogenous mRNP granules. *Xenopus* vegetal transport granules, due to their size and known composition are ideally positioned to directly test this model, using photobleaching assays in conjunction with mutational strategies.

Methods

Cloning

PTB RRM mutant constructs were generated using pSP64TSNRLMCS:PTB-FLAG construct as template DNA for QuikChange II mutagenesis kit (Agilent Technologies), which were performed per the manufacturers protocol (Figure A.1B-C). Primer sequences were as follows:

quikPTB_RRM1_forward

5'-TACATCCAGTACATTCTTCACAAAGAAGCAAAGACGGACGAC-3'

quikPTB_RRM1_reverse

5'-GTCGTCCGTCTTTGCTTCTTTGTGAAGAATGTACTGGATGTA-3'

quikPTB_RRM2_forward

5'-ACGGTGCTGAAAATATCAACTGCAACATCAAACAATCAGTTT-3'

quikPTB_RRM2_reverse

5'-AAACTGATTGTTTGSTGTTGCAGTYGATATTTTCAGCACCGT-3'

quikPTB_RRM3_forward

5'-GATGTGCACAGAGTGTCAATAGCATCAAACAAGAAAGAAAAT-3'

quikPTB_RRM3_reverse

5'-ATTTTCTTTCTTGTTAAAGAGTATTTTCACTCTGTGCACATC-3'

quikPTB_RRM4_forward

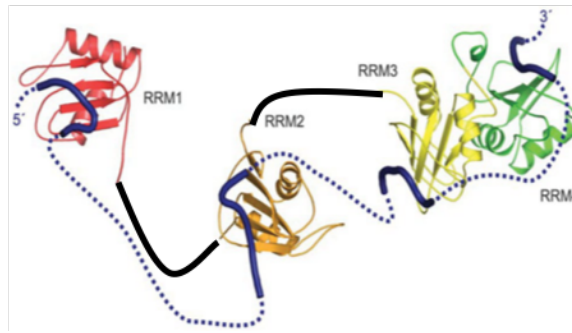
5'-AATCATCACCATGCAGTGTCCCTTTATTGCATCCACAATTTAA-3'

quikPTB_RRM4_reverse

5'-TTAAATTGTGGATGCAATAAAGGACACTGCATGGTGATGATT-3'

Higher order combinations of RRM mutants were generated using lesser mutant constructs as templates for additional site mutation (Figure A.2).

A



B

RRM mutated	Mutations	Location
■ RRM1	S131I – N132L – L136A	β4
■ RRM2	I214S – F216A – K218S	β2
■ RRM3	K368S – L370A – F371S	β2
■ RRM4	R523A – S527I – K528A	β4

C

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VgRBP60 MEGIVQDITVGTGRGSDLEFS-CVTNGPFIMSNAAAGENLYSGNGNDSKKFKGDGRSVAGSRVHLRLKLPDVTAEVVISLGLPFQKVTNLLMLKGNQ 99
hnRNP I MDGIVFDIAVGTGRGSDLEFSTCVTNGPFIMSSNSAS----AANGNDSKKFKGDGRSAGVPSRVHLRLKLPDVTAEVVISLGLPFQKVTNLLMLKGNQ 96

VgRBP60 AFLENSTEEAANTHISYTTNVPVLRGQPIYIQENKKTDDSPNQARAQAALQAVNSVSGTTALSASAAAVDVGIMSQQSPVLRIVENLFPVT 199
hnRNP I AFLENSTEEAANTHISYTTNVPVLRGQPIYIQENKKTDDSPNQARAQAALQAVNSVSGTNLALASAAAVDAGAGAGQSPVLRIVENLFPVT 196

VgRBP60 LDVLQQIFSKFGTVLKEIFKQKQFQALLQYQDVPVSAQHAKLSLDGQNIYNACCTLRIDFSKLTSLNVKYNNDKSRDVTTPDLPSGDQSPSLDQTI-AA 298
hnRNP I LDVLHQIFSKFGTVLKEIFKQKQFQALLQYQDVPVSAQHAKLSLDGQNIYNACCTLRIDFSKLTSLNVKYNNDKSRDVTTPDLPSGDQSPSLDQTHAAA 296

VgRBP60 FGAPGLISANPYAGAGFHPAFAIPQ--G-----VHG-LAPLTLSAAAAAGRLGHLGLIPGNSVLLVSNLNPVTPQCLFIFGVYGDVHRK 390
hnRNP I FGAPGLISASPYAGAGFPTFAIPQAAGLSVPNVHGALAPLAIPS-AAAAAGRIAPGLAGAGNSVLLVSNLNPVTPQCLFIFGVYGDVHRK 395

VgRBP60 LFKKENALVQMADGNQAQLAMSHLNGQLHKGKPLRTTVSEHQTVQLPREGQEDQGLTKDYSTSLHFFKKPGSKNFQNIFFPSATLHLSNIPPSVSEED 490
hnRNP I LFKKENALVQMADGNQAQLAMSHLNGHLKGKPIRITLSEHQNVQLPREGQEDQGLTKDYGNSPLHFFKKPGSKNFQNIFFPSATLHLSNIPPSVSEED 495

VgRBP60 LKILFSNNGYAVKGFKKFKDKFMALIQMGSVVEEAIESLIEHNDMGENHHHSTSEKTTI• 552
hnRNP I LKVLFSNNGGVVKGFKFKDKFMALIQMGSVVEEAVQALIDLHNDLGENHHHSTSEKTTI• 557

```

Figure A.1 Mutational strategy for ablation of PTB RNA-binding capabilities.

(A) Polypyrimidine tract-binding protein (PTB) contains four RNA Recognition motifs (RRMs), depicted as unique colored domains, whose association with RNA, depicted as a blue line, is schematically represented. (B) *in vitro* mutational assays

have identified functionally ablating mutations for each of PTB's four RRM's (Kafasla et al. 2011). (C) Mutational sites are highly conserved between human hnRNP I and *Xenopus laevis* PTB/VgRBP60.

	RRM(s) mutated
Single Mutants	 RRM1
	 RRM2
	 RRM3
	 RRM4
Double Mutants	RRM1/RRM2
	RRM3/RRM4
Full Mutant	RRM1/RRM2/RRM3/RRM4

Figure A.1 List of FLAG-tagged PTB RRM mutants. Mutations within each of Polypyrimidine tract-binding protein's (PTB) four individual RNA Recognition motifs (RRMs), as well as higher-order combinations thereof, were developed for *in vivo* expression within *Xenopus* oocytes.

References

- Auweter SD, Allain FH-T. 2008. Structure-function relationships of the polypyrimidine tract binding protein. *Cell Mol Life Sci* **65**: 516–27.
- Banani SF, Rice AM, Peeples WB, Lin Y, Jain S, Parker R, Rosen MK. 2016. Compositional control of phase-separated cellular bodies. *Cell* **166**: 651–63.
- Besse F, López de Quinto S, Marchand V, Trucco A, Ephrussi A. 2009. *Drosophila* PTB promotes formation of high-order RNP particles and represses *oskar* translation. *Genes Dev* **23**: 195–207.
- Cote C a., Gautreau D, Denegre JM, Kress TL, Terry N a., Mowry KL. 1999. A *Xenopus* protein related to hnRNP I has a role in cytoplasmic RNA localization. *Mol Cell* **4**: 431–37.
- Dreyfuss G, Kim VN, Kataoka N. 2002. Messenger-RNA-binding proteins and the messages they carry. *Nat Rev Mol Cell Biol* **3**: 195–205.
- Kafasla P, Lin H, Curry S, Jackson RJ. 2011. Activation of picornaviral IRESs by PTB shows differential dependence on each PTB RNA-binding domain. *RNA* **17**: 1120–31.
- Kato M, Han TW, Xie S, Shi K, Du X, Wu LC, Mirzaei H, Goldsmith EJ, Longgood J, Pei J, et al. 2012. Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. *Cell* **149**: 753–67.
- King M Lou, Messitt TJ, Mowry KL. 2005. Putting RNAs in the right place at the right time: RNA localization in the frog oocyte. *Biol Cell* **97**: 19–33.
- Kress TL, Yoon YJ, Mowry KL. 2004. Nuclear RNP complex assembly initiates cytoplasmic RNA localization. *J Cell Biol* **165**: 203–11.
- Lewis RA., Kress TL, Cote CA., Gautreau D, Rokop ME, Mowry KL. 2004. Conserved and clustered RNA recognition sequences are a critical feature of signals directing RNA localization in *Xenopus* oocytes. *Mech Dev* **121**: 101–09.
- Lewis RA, Gagnon JA, Mowry KL. 2008. PTB/hnRNP I is required for RNP remodeling during RNA localization in *Xenopus* oocytes. *Mol Cell Biol* **28**: 678–86.
- Li P, Banjade S, Cheng H-C, Kim S, Chen B, Guo L, Llaguno M, Hollingsworth J V., King DS, Banani SF, et al. 2012. Phase transitions in the assembly of multivalent signalling proteins. *Nature* **483**: 336–40.
- Shin Y, Brangwynne CP. 2017. Liquid phase condensation in cell physiology and disease. *Science* **357**.

- Simpson PJ, Monie TP, Szendrői A, Davydova N, Tyzack JK, Conte MR, Read CM, Cary PD, Svergun DI, Konarev P V., et al. 2004. Structure and RNA Interactions of the N-Terminal RRM Domains of PTB. *Structure* **12**: 1631–43.
- Yuan X. 2002. Chemical shift mapping of RNA interactions with the polypyrimidine tract binding protein. *Nucleic Acids Res* **30**: 456–62.

Appendix B:
Conserved RNA Helicases in Vegetal
Transport Granules

Introduction

DEAD-box helicases perform diverse cellular functions in virtually all steps of RNA metabolism. Through their well-established capacity to nonprocessively unwind double-stranded RNA, DEAD-box helicases can promote duplex formation, remodel protein-RNA interactions, and serve as assembly platforms for higher-order ribonucleoprotein (RNP) complexes (Putnam and Jankowsky 2013). Such ATP-dependent processing has been functionally linked to a diversity of large RNP structures, with a core set of DEAD-box helicases being conserved amongst an emerging class of phase-separated mRNP granules (Chapter 2; Linder and Jankowsky 2011; Voronina et al. 2011; Protter and Parker 2016). Here, helicase activity mediates the assembly, maintenance, and physical properties of mRNP granules (Hubstenberger et al. 2013, 2015; Elbaum-Garfinkle et al. 2015; Jain et al. 2016).

Multivalent molecules, consisting of proteins with multiple modular domains (Li et al. 2012; Lin et al. 2015) or stretches of low sequence complexity with repetitive interaction motifs, termed intrinsically disordered regions (IDRs) (Kato et al. 2012; Elbaum-Garfinkle et al. 2015; Nott et al. 2015; Protter et al. 2018) in combination with RNA species that contain multiple protein-binding sites (Li et al. 2012; Han et al. 2012; Yamazaki et al. 2018) are thought to drive the polymerization-based phase separations that maintain phase separations. RNA helicases represent a potential mediator of RNA valency, as their activity holds the potential to either create or obstruct access to *cis*-acting sequence elements and thus bias the overall composition of mRNP complexes. Additionally, the identification of IDRs within helicases has also suggested a more direct role in mediating phase separation (Elbaum-Garfinkle et al. 2015).

Identification and subsequent characterization of the DEAD-box RNA helicases Ddx1, Ddx3, Ddx5, and Ddx9 as components of the vegetal transport granule (see Chapter 3 and Appendix B), suggest an attractive scenario through which remodeling events could control both granule dynamics and RNP composition as a means of mediating specific granule functions. Indeed, previous work in both stress and P granules has demonstrated similar roles for RNA helicases, particularly Ddx3 and Ddx6, in transiently organizing translationally repressed mRNA in liquid-like structures, as well as facilitating their release upon granule dissolution (Hilliker et al. 2011; Elbaum-Garfinkle et al. 2015). In neurons both Ddx1 and Ddx3 are affiliated with motile granules, while association with Ddx6 containing P-bodies at neuronal processes is thought to be an important regulator of translational control (Miller et al. 2009). Vegetal mRNA localization in *Xenopus* oocytes proceeds through a number of coordinated steps where mRNP remodeling could potentially operate in a similar helicase-mediated manner to control granule function.

Results/Discussion

Following isolation of vegetal transport granules through chromatography and affinity purification, mass spectrometry identified four conserved granule RNA helicases as enriched within the RNP (Chapter 3). Each of Ddx1, Ddx3, Ddx5, and Ddx9 have been previously identified as components of other RNP granules, with defined roles in the regulation of the material properties of phase separated structures (Hilliker et al. 2011; Buchan 2014; Elbaum-Garfinkle et al. 2015). Ddx6 was also selected as a candidate RNA helicase for characterization as a potential interactor with *Xenopus* vegetal transport

granules, due to its conserved role in mediating granule disassembly in developmental mRNPs (Elbaum-Garfinkle et al. 2015).

Ddx1, Ddx3, Ddx5, and Ddx9 each showed enrichment within vegetal transport granules by combined fluorescence *in situ* hybridization and immunofluorescence (FISH-IF)(Figure B.1A-F, I-J), as predicted by proteomic analysis. The presence of Ddx3 is particularly interesting, as it has been suggested to mediate granule assembly in both stress and P granules (Hilliker et al. 2011; Elbaum-Garfinkle et al. 2015). Ddx6 showed no apparent enrichment in vegetal transport granules, however, discrete puncta of Ddx6 staining were observed at the vegetal cortex and appeared coincident with points where microtubule networks extend from vegetal transport granules to the cortical microtubule network (Figure B.1G,H). Cortical staining of some granule components often shows a dramatic enrichment relative to transport granules in the vegetal cytoplasm, suggesting a compositional and/or material distinction between the two physical locations within the oocyte. (Figure 3.4, S3.1). As Ddx6 has previously been shown to promote more liquid-like properties in P granules in response to developmental cues (Hubstenberger et al. 2015; Elbaum-Garfinkle et al. 2015), it represents an attractive candidate for mediating a cortical remodeling of *vg1* mRNP granules.

Expression profiles over oogenesis suggest that helicase activity may be controlled throughout development, with possible modifications observed for both Ddx1 and Ddx6 (Figure B.2). Similarly, Ddx5 seems to show changes in expression level through oogenesis (Figure B.2). Helicase activity has been shown to dictate distinct developmental functions in germ granules, with control of granule viscosity being directly tied to Helicase expression

(Hubstenberger et al. 2015). It is tempting to speculate that similar mechanisms of control could factor in mRNP regulation in *Xenopus* oocytes.

By constructing Ddx protein constructs that harbor functionally ablating mutations (Figure B.3), a role for RNA helicases in vegetal transport granule regulation can be further probed *in vivo*. Mutations within the DEAD-box motif of Ddx proteins blocks the ability of these proteins to couple ATP-hydrolysis to the unwinding and subsequent release of double stranded RNA (Putnam and Jankowsky 2013). This creates a mutant that is capable of competing with endogenous helicases for RNA binding but is unable to mediate potential remodeling events. Similar constructs have been used as dominant negative mutants in other systems (Wu and Brosh 2010) and could potentially operate similarly within *Xenopus* oocytes. Assessing the effect of exogenously expressed wildtype versus mutant DDX1, DDX3, DDX5, and DDX6 proteins on the localization of fluorescently labeled-*vg1* mRNA and the dynamic properties of vegetal mRNP granule components using established imaging strategies (Chapter 3), offers the potential to identify unique steps during vegetal localization as being mediated by remodeling of a functionally dynamic RNP granule.

Methods

Oocyte isolation and culture

Oocytes were harvested and isolated from *Xenopus laevis* females (NXR) as described in (Newman et al. 2018), with the following exceptions. Oocytes were enzymatically defolliculated by manual rocking in collagenase solution (3 mg/ml Collagenase I, 0.1 M KPO₃) followed by washes in MBSH (Neil and Mowry 2018). Stage II and III oocytes were manually sorted and cultured overnight (16-18 hours) at 18°C in XOCM (Jeschonek and Mowry, 2018).

Immunoblotting

Staged oocyte lysates were prepared as in (Yoon and Mowry 2004), with one oocyte equivalent of S10 lysate per lane resolved by SDS-PAGE gel. Antibodies for immunoblotting were as follows: Ddx1 (Abcam ab151962) 1:1000, Ddx3 (Abcam ab151965) 1:1000, Ddx5 (Acris AP17277PU-N) 1:200, and Ddx6 (Abcam ab103308) 1:1000. Secondary antibodies, Goat anti-Rabbit IgG (Abcam ab97200) or Goat Anti-Mouse IgG (Abcam ab97265) were used at 1:10,000.

Fluorescence in situ hybridization and immunofluorescence

Oocytes were treated for 1 hour at 22°C with FTG fixative (80 mM K PIPES, pH6.8, 1 mM MgCl₂, 5 mM EGTA, 0.2% Triton X-100, 3.7% formaldehyde, 0.25% glutaraldehyde, 0.5 µM paclitaxel), followed by post-fixation in 100% methanol overnight at 22°C as in (Becker and Gard 2006). After re-hydration in TBS (10 mM Tris, pH 7.4, 155 mM NaCl), oocytes were incubated overnight at 4°C in 100 mM NaBH₄ in TBS (Becker and Gard 2006). After

washing 2× in TBSN (0.1% NP-40 in TBS), followed by 2 washes in TBSNB (0.2% ultrapure BSA in TBSN) oocytes were equilibrated stepwise in TBSNB-30S (30% sucrose in TBSNB) and after equilibration in an embedding mold for 30 minutes at 22°C, TBSNB-30S was replaced with O.C.T. compound (Fisher 23-730). Cryosectioning and fluorescence in situ hybridization (FISH) was performed as in (Neil and Mowry 2018), with the *vg1* Quasar 570 applied at 500 nM. Slides were subsequently washed twice in TBSNB, incubated for 3 hours at 22°C in TBS-plus (5% v/v normal goat serum, 2 mg/ml ultrapure BSA in TBSN), and incubated overnight at 4°C with primary antibodies at 20 µg/ml in TBS-plus. Primary antibodies [Ddx1 (Abcam ab151962), Ddx3 (Abcam ab151965), Ddx5 (Acris AP17277PU-N), Ddx6 (Abcam ab103308), Dhx9 (ThermoFisher PA5-55754), and αTubulin (Sigma-Aldrich T9026)] were directly labeled with Alexa-405 or Alexa-647 using Zenon labeling kits (ThermoFisher). After four 10-minute washes in TBSNB at 22°C, slides were mounted with ProLong Gold Antifade. Imaging was performed on a Zeiss LSM 800 Confocal Laser Scanning Microscope.

Cloning

cDNA was prepared from staged *Xenopus* oocyte lysate (stages II-III) using iScript™ cDNA Synthesis Kit (Bio-Rad) in accordance with the manufacturers protocol. Proteins were cloned into pSP64TSNRLMCS:FLAG vectors for expression in *Xenopus* oocytes. Primer sequences were as follows:

Ddx1_forward 5'-CATGAGCCATGGATGGCGGCCTTCTCAGAAAT-3'

Ddx1_reverse 5'- CTCATGGGGCCCCGAAGCTTCGGAAAAGCTGATTA-3'

Ddx3_forward 5'- CATGAGCTCGAGATG GTCATGTGGCCGTGG-3'

Ddx3_reverse 5'- CTCATGCTCGAGGC GTTGCCCCACCAGTCAAC-3'

Ddx5_forward 5'- CATGAGAGATCTATGCCAGGATTTAACGACAGG-3'

Ddx5_reverse 5'- CTCATGCTCGAGGCTTGGGGGTAGGATGTTGGC-3'

Ddx6_forward 5'- CATGAGAGATCTATGAGCACCGCCAGAACAG-3'

Ddx6_reverse 5'- CTCATGGGGCCCCAGGTTTGTCTTCCCCAGATTC-3'

DEAD-box helicase mutant constructs were generated using wildtype pSP64TSNRLMCS:Ddx-FLAG constructs as template DNA for QuikChange II mutagenesis kit (Agilent Technologies), which were performed per the manufacturers protocol. Primer sequences were as follows:

quikDDX1DQAD_forward

5'-CCGTTTCCTGGTACTTGATCAAGCTGATGGTTTACTTTC-3'

quikDDX1DQAD_reverse

5'-GAAAGTAAACCATCAGCTTGATCAAGTACCAGGAAACGG-3'

quikDDX3DQAD_forward

5'-CAAGTATTTGGTACTAGATCAAGCAGACAGAATGCTTGACATG-3'

quikDDX3DQAD_reverse

5'-CATGTCAAGCATTCTGTCTGCTTGATCTAGTACCAAATACTTG-3'

quikDDX5DQAD_forward

5'-GCACATATTTGGTGCTTGATCAAGCAGACAGAATGCTTG-3'

quikDX5DQAD_reverse

5'-CAAGCATTCTGTCTGCTTGATCAAGCACCAAATATGTGC-3'

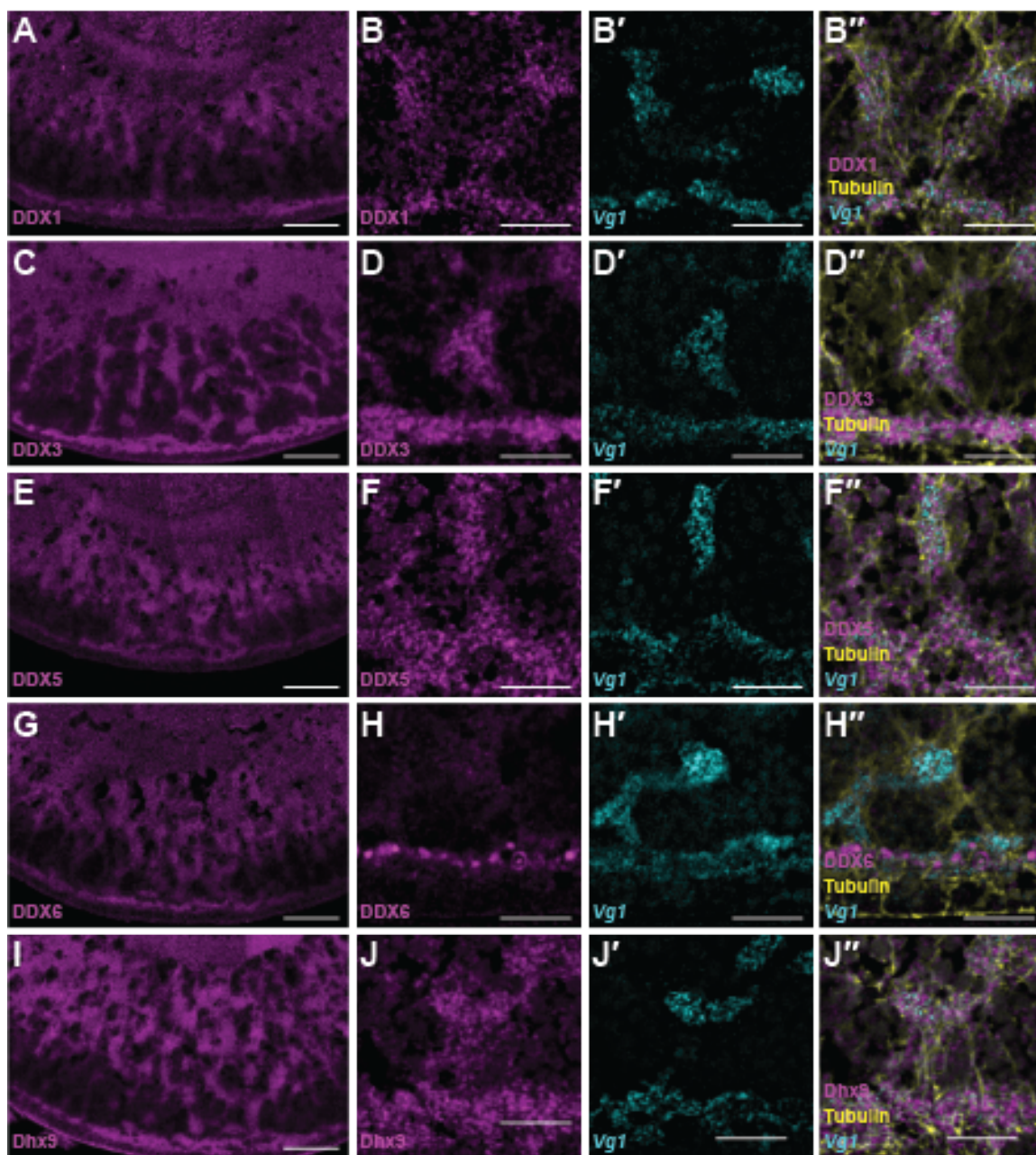


Figure B.1 Combined FISH-IF analysis of RNA helicases. (A) Immunofluorescence (IF) was performed on cryosectioned stage II-III oocytes with anti-Ddx1 antibodies. The vegetal cortex at the bottom and the scale bar=20 μ m. (B) High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Ddx1 (B, magenta), *vg1* mRNA (B',

cyan) and merged in B'' with anti-tubulin (yellow). Scale bar=10 μ m. (C) Immunofluorescence (IF) was performed on cryosectioned stage II-III oocytes with anti-Ddx3 antibodies. The vegetal cortex at the bottom and the scale bar=20 μ m. (D) High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Ddx3 (D, magenta), *vg1* mRNA (D', cyan) and merged in D'' with anti-tubulin (yellow). Scale bar=10 μ m. (E) Immunofluorescence (IF) was performed on cryosectioned stage II-III oocytes with anti-Ddx5 antibodies. The vegetal cortex at the bottom and the scale bar=20 μ m. (F) High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Ddx5 (F, magenta), *vg1* mRNA (F', cyan) and merged in F'' with anti-tubulin (yellow). Scale bar=10 μ m. (G) Immunofluorescence (IF) was performed on cryosectioned stage II-III oocytes with anti-Ddx6 antibodies. The vegetal cortex at the bottom and the scale bar=20 μ m. (H) High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Ddx6 (H, magenta), *vg1* mRNA (H', cyan) and merged in H'' with anti-tubulin (yellow). Scale bar=10 μ m. (I) Immunofluorescence (IF) was performed on cryosectioned stage II-III oocytes with anti-Dhx9 antibodies. The vegetal cortex at the bottom and the scale bar=20 μ m. (J) High magnification view of the vegetal cytoplasm of a stage II oocyte showing FISH-IF for Dhx9 (J, magenta), *vg1* mRNA (J', cyan) and merged in J'' with anti-tubulin (yellow). Scale bar=10 μ m.

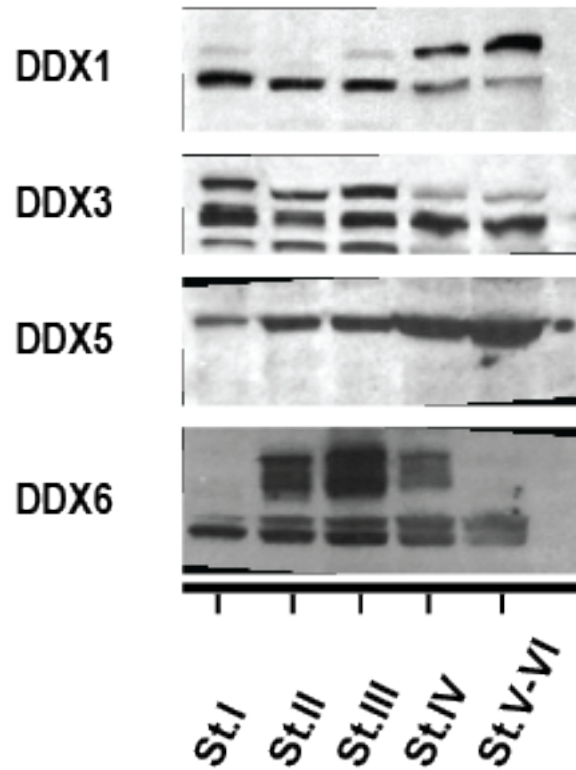
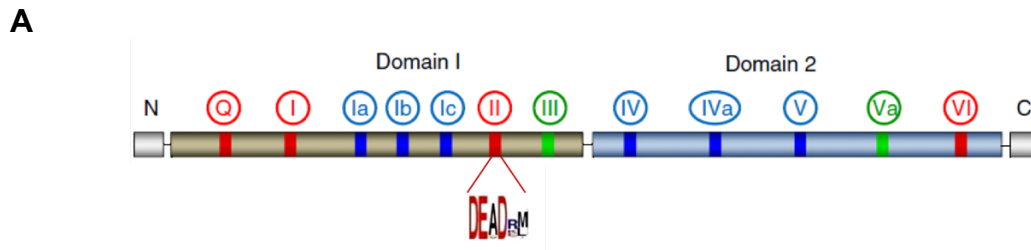


Figure B.2 DEAD-box RNA helicase protein expression through *Xenopus* oogenesis.

Western blot analyses of Ddx1, Ddx3, Ddx5, and Ddx6 proteins were performed using staged *Xenopus* oocyte lysate spanning oogenesis.



B

Construct	Details
pSP64TSNRLMCS:DDX1 wildtype-FLAG	Cloned from Stage II-III <i>Xenopus laevis</i> oocyte cDNA
pSP64TSNRLMCS:DDX1 mutant-FLAG	Functionally blocking DQAD mutation inserted into DEAD-box motif.
pSP64TSNRLMCS:DDX3 wildtype-FLAG	Cloned from Stage II-III <i>Xenopus laevis</i> oocyte cDNA
pSP64TSNRLMCS:DDX3 mutant-FLAG	Functionally blocking DQAD mutation inserted into DEAD-box motif.
pSP64TSNRLMCS:DDX5 wildtype-FLAG	Cloned from Stage II-III <i>Xenopus laevis</i> oocyte cDNA
pSP64TSNRLMCS:DDX5 mutant-FLAG	Functionally blocking DQAD mutation inserted into DEAD-box motif.
pSP64TSNRLMCS:DDX6 wildtype	Cloned from Stage II-III <i>Xenopus laevis</i> oocyte cDNA
pSP64TSNRLMCS:DDX6 mutant-FLAG	Functionally blocking DQAD mutation inserted into DEAD-box motif.

Figure B.3 DEAD-box RNA helicase constructs. (A) Schematic of the conserved primary structure of the DEAD-box helicase core with two RecA-like helicase domains. Circled numbers indicate the location of characteristic sequence motifs, colored according to their function (red, ATP binding and hydrolysis; blue, RNA binding, green, coordination between RNA and ATP binding). (B) Table of DEAD-box helicases cloned from cDNA

generated from stage II-III *Xenopus* oocyte lysate into an *in vivo* expression vector (pSP64TSNRLMC), and DEAD-box motif mutants generated using QuikChange Site-Directed Mutagenesis (Agilent).

References

- Becker BE, Gard DL. 2006. Visualization of the cytoskeleton in *Xenopus* oocytes and eggs by confocal immunofluorescence microscopy. *Methods Mol Biol* **322**: 69–86.
- Buchan JR. 2014. mRNP granules. Assembly, function, and connections with disease. *RNA Biol* **11**: 1019–30.
- Elbaum-Garfinkle S, Kim Y, Szczepaniak K, Chen CC-H, Eckmann CR, Myong S, Brangwynne CP. 2015. The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics. *Proc Natl Acad Sci USA* **112**: 7189–94.
- Han TW, Kato M, Xie S, Wu LC, Mirzaei H, Pei J, Chen M, Xie Y, Allen J, Xiao G, et al. 2012. Cell-free formation of RNA granules: bound RNAs identify features and components of cellular assemblies. *Cell* **149**: 768–79.
- Hilliker A, Gao Z, Jankowsky E, Parker R. 2011. The DEAD-box protein Ded1 modulates translation by the formation and resolution of an eIF4F-mRNA complex. *Mol Cell* **43**: 962–72.
- Hubstenberger A, Cameron C, Noble SL, Keenan S, Evans TC. 2015. Modifiers of solid RNP granules control normal RNP dynamics and mRNA activity in early development. *J Cell Biol* **211**: 703–16.
- Hubstenberger A, Noble SL, Cameron C, Evans TC. 2013. Translation repressors, an RNA helicase, and developmental cues control RNP phase transitions during early development. *Dev Cell* **27**: 161–73.
- Jain S, Wheeler JR, Walters RW, Agrawal A, Barsic A, Parker R. 2016. ATPase-modulated stress granules contain a diverse proteome and substructure. *Cell* **164**: 487–98.
- Kato M, Han TW, Xie S, Shi K, Du X, Wu LC, Mirzaei H, Goldsmith EJ, Longgood J, Pei J, et al. 2012. Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. *Cell* **149**: 753–67.
- Li P, Banjade S, Cheng H-C, Kim S, Chen B, Guo L, Llaguno M, Hollingsworth J V., King DS, Banani SF, et al. 2012. Phase transitions in the assembly of multivalent signalling proteins. *Nature* **483**: 336–40.
- Lin Y, Protter DSW, Rosen MK, Parker R. 2015. Formation and maturation of phase-separated liquid droplets by RNA-binding proteins. *Mol Cell* **60**: 208–19.
- Linder P, Jankowsky E. 2011. From unwinding to clamping - the DEAD box RNA helicase family. *Nat Rev Mol Cell Biol* **12**: 505–16.

- Miller LC, Blandford V, McAdam R, Sanchez-Carbente MR, Badeaux F, DesGroseillers L, Sossin WS. 2009. Combinations of DEAD box proteins distinguish distinct types of RNA: Protein complexes in neurons. *Mol Cell Neurosci* **40**: 485-95.
- Neil CR, Mowry K. 2018. Fluorescence *in situ* hybridization of cryosectioned *Xenopus* oocytes. *Cold Spring Harb Protoc* **2018**: pdb.prot097030.
- Newman K, Agüero T, King ML. 2018. Isolation of *Xenopus* oocytes. *Cold Spring Harb Protoc* **2018**: 86–91.
- Nott TJ, Petsalaki E, Farber P, Jervis D, Fussner E, Plochowietz A, Craggs TD, Bazett-Jones DP, Pawson T, Forman-Kay JD, et al. 2015. Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles. *Mol Cell* **57**: 936–47.
- Protter DSW, Parker R. 2016. Principles and properties of stress granules. *Trends Cell Biol* **26**: 668–79.
- Protter DSW, Rao BS, Van Treeck B, Lin Y, Mizoue L, Rosen MK, Parker R. 2018. Intrinsically disordered regions can contribute promiscuous interactions to RNP granule assembly. *Cell Rep* **22**: 1401–12.
- Putnam AA, Jankowsky E. 2013. DEAD-box helicases as integrators of RNA, nucleotide and protein binding. *Biochim Biophys Acta* **1829**: 884–93.
- Voronina E, Seydoux G, Sassone-Corsi P, Nagamori I. 2011. RNA granules in germ cells. *Cold Spring Harb Perspect Biol* **3**.
- Wu Y, Brosh RM. 2010. Helicase-inactivating mutations as a basis for dominant negative phenotypes. *Cell Cycle* **9**: 4080-90.
- Yamazaki T, Souquere S, Chujo T, Kobelke S, Chong YS, Fox AH, Bond CS, Nakagawa S, Pierron G, Hirose T. 2018. Functional domains of *NEAT1* architectural lncRNA induce paraspeckle assembly through phase separation. *Mol Cell* **70**: 1038–53.
- Yoon YJ, Mowry KL. 2004. *Xenopus* Staufén is a component of a ribonucleoprotein complex containing *vg1* RNA and kinesin. *Development* **131**: 3035–45.