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Organizing the oocyte: RNA localization meets phase separation

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Abstract

RNA localization is a key biological strategy for organizing the cytoplasm and generating both cellular and developmental polarity. During RNA localization, RNAs are targeted asymmetrically to specific subcellular destinations, resulting in spatially and temporally restricted gene expression through local protein synthesis. First discovered in oocytes and embryos, RNA localization is now recognized as a significant regulatory strategy for diverse RNAs, both coding and non-coding, in a wide range of cell types. Yet, the highly polarized cytoplasm of the oocyte remains a leading model to understand not only the principles and mechanisms underlying RNA localization, but also links to the formation of biomolecular condensates through phase separation. Here, we discuss both RNA localization and biomolecular condensates in oocytes with a particular focus on the oocyte of the frog, *Xenopus laevis*.

Keywords

RNA; RNA localization; oocytes; phase separation; biomolecular condensates; *Xenopus*

1. Introduction

In 1957, Francis Crick formulated the modern framework for molecular biology by coining the term “central dogma” and describing the flow of biological information from DNA to RNA to protein (Crick, 1958). Since that time, regulatory paradigms have been described for each of the steps of the central dogma. In particular, RNA localization has been found to exert precise control of gene expression both temporally and spatially. The first examples of localized RNAs were identified in the 1980’s, and in the intervening decades RNA localization has been described for a stunning number of RNAs in a wide range of cells types (Jeffery et al., 1983; Lawrence and Singer, 1986; Rebagliati et al., 1985). Notable examples are found in evolutionary distant organisms, with functions that include regulation of mating type switching in yeast, facilitation of motility in fibroblasts, and control of synaptic plasticity and axonal guidance in neurons (Mingle et al., 2005; Puthanveetil, 2013; reviewed in Singer-Krüger and Jansen, 2014). Although nearly ubiquitous in cells, RNA localization is perhaps best-characterized as a regulator of developmental patterning in vertebrate and invertebrate oocytes (reviewed in Houston, 2013; Medioni et al., 2012). However, asymmetric RNA localization is not restricted to mRNAs and is used to localize

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both coding and non-coding RNAs in a wide variety of cell types, suggesting that RNA localization provides a paradigm for organization of the cell throughout biology (reviewed in Cabili et al., 2015; Lécuyer et al., 2007; Weiß et al., 2015; Wilk et al., 2016).

Organization of the cytoplasm is also achieved through formation of biomolecular condensates, which are cellular subcompartments that form through phase separation and function to concentrate proteins and nucleic acids without the use of a membrane (reviewed in Banani et al., 2017). While now identified in an ever-growing set of cell types and subcellular localizations, the principles of phase separation were first described in oocytes and condensates have been found across many species in these cells (Brangwynne et al., 2009). Importantly, new insights into the biophysical basis for phase separation in cells coupled with intriguing links to RNA localization have reframed our view of both processes. Here, we review how studies in the oocyte model have established a basis for our understanding of the mechanisms directing RNA localization and the functional advantages it can confer. We then discuss recent advances in the field of phase separation and the range of biomolecular condensates found in oocytes, highlighting the intrinsic links between RNA localization and phase separation.

2. Functional advantages of RNA localization

In an mRNA-centric view, RNA localization provides numerous advantages to the cell over transport of protein products. Particularly in large cells such as oocytes, it has been hypothesized that it would be impossible to establish the gradients of factors and subcellular localization patterns required by the cell by merely transporting translated protein products (reviewed in Blower, 2013). However, as noted above, RNA localization is not only utilized in large, highly polarized cells like oocytes, but has been shown to be a general mechanism used in a wide variety of non-germline cells, including somatic cells, plant cells, and even unicellular organisms (Buskily et al., 2014; Cajigas et al., 2012; Okita and Choi, 2002; Singer-Krüger and Jansen, 2014; Sundell and Singer, 1991). In some of these examples, the advantages of RNA localization to the cell have not yet been defined. However, well-characterized examples of RNA localization have demonstrated a number of non-mutually exclusive advantages to this process, as detailed below (reviewed in Blower, 2013; Buxbaum et al., 2015; Du et al., 2007; Martin and Ephrussi, 2009; St Johnston, 2005).

First, asymmetric enrichment of mRNAs in a particular subcellular domain through RNA localization provides an energetically favorable mechanism to spatially and temporally decouple protein translation from mRNA transcription. Thus, cells can transport translation machinery and only a few copies of an mRNA to a particular site, often far from the nucleus, where multiple rounds of local translation can produce strong enrichment of the encoded protein. This energetic favorability is clearly advantageous in large cells such as oocytes and neurons, as well as in other polarized cells. For example, in fibroblasts mRNAs are localized to lamellipodia to allow for the locally high concentrations of cytoskeletal elements necessary for rapid motility (Kislauskis et al., 1997; Lawrence and Singer, 1986; Mardakheh et al., 2015). Second, mRNA localization, which generally transports translationally silenced mRNAs, can prevent ectopic protein activity during localization which may be toxic or deleterious to the cell. This is best-illustrated in germ cells, where developmental

determinants are often localized as mRNAs rather than proteins, preventing errors in embryonic patterning that can arise through spatially inappropriate protein activity (Ephrussi et al., 1991; Gavis and Lehmann, 1992). Third, mRNA localization and local translation allow for rapid response to stimuli by bypassing the need for signaling from the cytoplasm back to the nucleus, waiting for a transcriptional and translational response, and then transporting the protein product back to the appropriate site. This rapid response to external stimuli is particularly important in neurons where rapid translational responses far from the cell body are important for synaptic plasticity (reviewed in Martin et al., 2000). However, RNA localization is not restricted to protein-coding mRNAs. Localization of noncoding RNAs has been proposed to play a role in long-term memory formation by regulating the local translation and stability of these synapse localized mRNAs (reviewed in Mercer et al., 2008). Finally, an underappreciated advantage of localizing RNAs is that this process can bias macromolecular complex formation by creating intracellular regions of high of RNA and protein concentration. Traditionally, this process has been thought to facilitate the incorporation of nascent proteins into complexes with particular stoichiometries or differential binding partners depending on the site of translation (reviewed in Rodriguez et al., 2008). However, this inherent advantage of RNA localization can also be reconsidered in the context of biomolecular condensate formation. In this view, locally high concentrations of RNA and protein factors established by RNA localization can facilitate phase separation. This advantage may underlie the localization of non-coding RNAs where the RNA itself could be acting as a scaffold for macromolecular complex formation or as a regulator at the site of enrichment (Clemson et al., 2009; Kloc et al., 2005; Weiß et al., 2015).

While many advantages of mRNA localization coupled with local translation have been well described in the literature, the functions of localized non-coding RNAs are only recently emerging. In particular, functions for localized long non-coding RNAs (lncRNAs) have been identified both in the nucleus where lncRNAs can perform structural or gene regulatory roles, and in the cytoplasm, where localized lncRNAs can also function to regulate gene expression (Chen, 2016; Clemson et al., 2009; Noh et al., 2018). For example, the *neat1* lncRNA functions in the nucleus of mammalian cells as a necessary RNA scaffold for paraspeckle formation (Clemson et al., 2009). However, the functional advantages for localizing most lncRNAs, as well as many other classes of non-coding RNAs, remain to be determined. An exciting challenge for the field will be to not only identify the particular non-coding RNAs that are localized asymmetrically, but also to define how those RNAs function at their destinations.

3. Mechanisms of RNA localization

Traditionally, three primary mechanisms have been described to generate asymmetric RNA localization: diffusion and local entrapment, local stabilization and regulated degradation, and, most prominently in the literature, active transport by molecular motors (reviewed in Gagnon and Mowry, 2011; Martin and Ephrussi, 2009). More recently, formation of biomolecular condensates has emerged as a fourth mechanism for generating asymmetry in RNA distribution (reviewed in Langdon and Gladfelter, 2018). These mechanisms, schematized in Figure 1, have been observed in a wide array of somatic cells, germ cells, and even unicellular organisms and facilitate the localization of diverse RNAs.

The first mechanism of RNA localization, diffusion and local entrapment, is characterized by the accumulation of RNA at a subcellular destination through the capture of RNA molecules which are freely diffusing through the cell. For example, in *Drosophila* oocytes, *nanos* mRNA is maternally deposited at the anterior of the developing oocyte and freely diffuses until it is captured and stably anchored at the posterior pole of the oocyte (Forrest and Gavis, 2003). As shown in Figure 1A, the *Xenopus* homologue of *nanos* is enriched in the Balbiani body during early oogenesis via a diffusion and local entrapment mechanism (Chang et al., 2004). Interestingly, recent evidence has shown that both *Drosophila* germ granules and the *Xenopus* Balbiani body are biomolecular condensates, illustrating not only that many RNAs are localized in phase separated intermediates, but also suggesting that incorporation into phase separated structures may be a key mechanism for entrapment of diffusing RNAs (Boke et al., 2016; Kistler et al., 2018).

The second mechanism of RNA localization, local stabilization and regulated degradation, is characterized by the enrichment of RNAs in a subcellular destination through global degradation of non-localized RNA and selective protection of localized RNA, rather than the entrapment or transport of the RNA. For *nanos* mRNA in the *Drosophila* embryo, the diffusion and local entrapment process described above is highly inefficient and only ~4% of the total *nanos* mRNA is enriched at the posterior pole (Gavis and Lehmann, 1992). To create the robust localization necessary for proper anterior-posterior patterning, the fraction of the mRNA enriched at the posterior pole is stabilized, whereas the non-localized transcripts are targeted for degradation through recruitment of the CCR4-NOT deadenylase complex by Smaug, an RNA-binding protein (Zaessinger et al., 2006). The dual mechanisms for localization of *nanos* mRNA, coupling diffusion and local entrapment with local stabilization and selective degradation, highlight how a single transcript can utilize multiple mechanisms of RNA localization to achieve stringent enrichment at a particular location. As shown in Figure 1B, local stabilization and regulated degradation is also used in the *Drosophila* embryo to enrich *heat shock protein 83* (*hsp83*) mRNA in the posterior pole (Ding et al., 1993). Similarly to *nanos* mRNA, *hsp83* mRNA is degraded outside of the posterior pole by the binding of Smaug to elements in the 3' untranslated region (UTR) of the mRNA (Bashirullah et al., 2001; Chen et al., 2014; Semotok et al., 2008; Tadros et al., 2007).

The third mechanism of RNA localization, and most predominant in the literature, is active, motor-based transport of RNA along the cytoskeleton. Active transport allows for faster and more long-range RNA enrichment than simple diffusion and is essential for the localization of many RNAs. For example, as shown in Figure 1C, *ash1* mRNA, a regulator of mating type switching in budding yeast, is localized to the tip of the daughter cell along actin filaments in a myosin-dependent manner (Long et al., 1997; Takizawa and Vale, 2000). Motor-based transport is also important for the localization of RNAs in neurons, with numerous examples including the kinesin-based transport of β -actin mRNA to growth cones in immature neurons (reviewed in Das et al., 2019; Kiebler and Bassell, 2006; Zhang et al., 1999). Active transport of RNAs allows not only for rapid localization, but for exquisite control over the site of enrichment based on both biases in the orientation of cytoskeletal elements and by the activity of different molecular motors (reviewed in Gagnon and Mowry, 2011). This tight control of localization is critical for embryonic patterning, as illustrated by

the distinct localization patterns adopted by *bicoid*, *oskar*, and *gurken* RNAs in the *Drosophila* oocyte (reviewed in Weil, 2014). In this system, many mRNAs are synthesized in nurse cells and are transported through ring canals, or cytoplasmic bridges, into the oocyte by dynein (Clark et al., 2007). Once in the oocyte, continued motor-based transport of these RNAs allows them to adopt specific localization patterns (reviewed in Kugler and Lasko, 2009; St. Johnston, 2005). For example, *bicoid* mRNA localizes to the anterior of the oocyte via active transport by the minus end directed motor, dynein, along microtubules nucleated at the anterior cortex (Duncan and Warrior, 2002). Also transported by dynein is *gurken* mRNA, which is first transported, like *bicoid* mRNA, to the anterior of the oocyte (MacDougall et al., 2003). In a second step, *gurken* RNA is subsequently transported to the dorsal-anterior corner of the oocyte along microtubules nucleated by the oocyte nucleus (MacDougall et al., 2003). Localization of *oskar* mRNA to the posterior of the oocyte relies instead on the plus-end directed microtubule motor, kinesin (Brendza et al., 2000; Cha et al., 2002; Gáspár et al., 2017; Januschke et al., 2002; Zimyanin et al., 2008). As will be discussed in detail below, RNAs are also transported by molecular motors along microtubule arrays to the vegetal pole of the *Xenopus* oocyte (Gagnon et al., 2013; Messitt et al., 2008).

Finally, the most recently described mechanism of RNA localization is incorporation into biomolecular condensates (reviewed in Langdon and Gladfelter, 2018). Through this mechanism RNAs are enriched into non-membrane bound substructures based on the thermodynamic properties of the RNA and surrounding proteins, often in response to cellular cues. For example, as shown in Figure 1D, polyA⁺ mRNAs are sequestered during times of cellular distress to stress granules, which form by phase separation (reviewed in Kedersha et al., 2013). This mechanism of RNA localization allows for specific RNAs to be enriched within biomolecular condensates depending on the cellular environment and type of condensate. For example, as a means to reduce energy expenditure while allowing cells to repair damage, stress granules recruit RNAs encoding house-keeping genes, such as β -actin and GAPDH, restricting them from being actively translated, while excluding RNAs encoding proteins involved in stress-related repair such as heat shock proteins (reviewed in Fay and Anderson, 2018). While the mechanisms by which RNAs are incorporated into these structures is less well characterized, RNA has been shown to play a variety of roles in forming the biomolecular condensates in which they are enriched (reviewed in Van Treeck and Parker, 2018). Such roles include active structural roles in condensates, buffering phase separation, and regulation of the physical state of the condensates (Clemson et al., 2009; Jain and Vale, 2017; Langdon et al., 2018; Maharana et al., 2018; Van Treeck et al., 2018).

4. RNA localization in *Xenopus* oocytes

While RNA localization has been observed nearly universally across cell types and organisms, the developing oocyte of the African clawed frog, *Xenopus laevis*, has proven to be an ideal model system for the study of RNA localization in vertebrates. The *Xenopus* oocyte is an extremely large, highly polarized cell that uses a variety of mechanisms to localize both maternal mRNAs and non-coding RNAs during oogenesis, many of which are required for proper patterning of the embryo and specification of the germline (reviewed in Holt and Bullock, 2010; King et al., 2005; Medioni et al., 2012). The localization of these RNAs is developmentally coordinated during oogenesis, the stages of which are briefly

described below and provide a framework for the pathways of RNA localization that operate during *Xenopus* oogenesis.

4.1 *Xenopus* oogenesis

As depicted in Figure 2, *Xenopus* oogenesis proceeds through six morphologically distinct stages, designated I-VI, which are defined both by increasing size, yolk accumulation, and changes in external pigmentation (Dumont, 1972). During stage I of oogenesis, the oocyte is transparent with the germinal vesicle (GV) and Balbiani body, also termed the mitochondrial cloud, visible in the cytoplasm (Heasman et al., 1984). The Balbiani body is closely associated with the GV through a network of cytokeratin filaments and is the primary site of mitochondrial replication in the pre-vitellogenic oocyte. It also contains many classic germ line determinants, such as the *Xenopus* homologue of *nanos* mRNA and Vasa protein (reviewed in King et al., 2005). During stage II, the oocytes grow and become increasingly opaque, turning white in color and obscuring the GV from view (Dumont, 1972). In stage III, vitellogenesis, or the production of yolk, and pigmentation of the oocyte begin (Wallace and Dumont, 1968). These oocytes have nonpolarized dark pigment, turning the oocyte light brown in early stage III and black in late stage III. In stage IV, vitellogenesis continues and oocytes continue to rapidly expand in size; the largest yolk platelets accumulate in the vegetal hemisphere of the oocyte and the nucleus is displaced towards the animal hemisphere. Stage IV cells are marked by the beginning of pigment polarization, with the dark pigment enriched in the animal hemisphere of the oocyte. In stage V, vitellogenesis slows and distinct hemispheres are clearly visible with the pigment highly enriched in the animal hemisphere. Finally, stage VI oocytes have reached their full size and exhibit an unpigmented equatorial band, approximately 0.2 mm in diameter, dividing the animal and vegetal hemispheres of the oocyte; stage VI oocytes are fully mature and are ready for oviposition.

4.2 *Xenopus* RNA localization pathways

In addition to changes in size, pigmentation, and vitellogenesis, the distinct stages of *Xenopus* oogenesis are also marked by the developmentally staged localization of maternal RNAs. Localization of RNAs in the vegetal cytoplasm occurs through two different RNA localization pathways, the early, or METRO (messenger transport organizing center) pathway and the late pathway, with some RNAs displaying an intermediate phenotype (Forristall et al., 1995; King et al., 2005; Kloc and Etkin, 1995). Several other RNAs, including the *An1*, *2*, *3* and *4* RNAs, enrich in the animal cytoplasm of the oocyte through unknown mechanisms (Hudson et al., 1996; Rebagliati et al., 1985). The polarized distribution of RNAs along the animal-vegetal axis of the oocyte is critical for embryonic patterning, with the animal hemisphere giving rise to the ectoderm, the vegetal hemisphere giving rise to the endoderm, and the mesoderm induced via signaling from the vegetal blastomeres (Nieuwkoop, 1969; Sudarwati and Nieuwkoop, 1971). For a comprehensive list of known localized RNAs in *Xenopus* oocytes as well as the techniques used to identify them, please see Houston, 2013 (Houston, 2013).

As shown in Figure 3A, during stages I-II of oogenesis, several RNAs, including *nanos1/xcat-2* mRNA, the *Xenopus* homologue of *nanos*, are localized via the early localization

pathway. Generally, the early pathway is used to localize RNAs that function in germ cell determination, and RNAs localized by this pathway include mRNAs encoding germ plasm components and signaling molecules, such as *Xdazl* and *Xwnt11* mRNAs, as well as a family of non-coding RNAs called *Xlirts* (Forristall et al., 1995; Heasman et al., 1984; Houston et al., 1998; Kloc et al., 1993; Mosquera et al., 1993; Zearfoss et al., 2003). This pathway proceeds via a three step process in which RNAs are first exported from the GV into the cytoplasm, then enriched in the Balbiani body in stage I, and finally deposited onto the vegetal cortex in a ring-like pattern in stage II (Kloc and Etkin, 1995). As shown in Figure 1A, early localizing RNAs are enriched into the Balbiani body by the diffusion and local entrapment mechanism, as evidenced by the microtubule and microfilament independent, linear enrichment of microinjected, fluorescently-labeled RNA in the Balbiani body, with no degradation of the RNA in the surrounding cytoplasm (Chang et al., 2004). The mechanistic conservation between *Drosophila* and *Xenopus* oocytes for localization of germ line determining mRNAs highlights both the importance of the diffusion and local entrapment mechanism and the overall conservation of germplasm establishment from invertebrates to vertebrates (Kloc et al., 2004).

During stages II-IV of oogenesis, a second group of RNAs are enriched asymmetrically via the late localization pathway. Generally, the late pathway is used to localize RNAs, such as *vg1* and *vegT*, which are necessary for germ-layer patterning in the embryo (Birsoy et al., 2006; Joseph and Melton, 1998; Kofron et al., 1999; White and Heasman, 2008; Zhang et al., 1998). Importantly, spatially inappropriate expression of either *vg1* and *vegT* mRNAs, which encode, respectively, a TGF β growth factor family member and a T-box transcription factor, cause lethal embryonic phenotypes (Thomsen and Melton, 1993; Wallace and Dumont, 1968; Zhang and King, 1996). In stage I oocytes, while early localizing RNAs are enriched within the Balbiani body, late localizing RNAs are distributed ubiquitously in the cytoplasm, indicating a clear mechanistic distinction between the two pathways. During stages II-III, late pathway RNAs become restricted within the vegetal cytoplasm in a characteristic pattern: in the perinuclear cup region, in distinct, non-spherical cytoplasmic islands, and at the vegetal cortex (Figure 3A). By stage IV, the RNAs are restricted to the vegetal cortex, where they remain, translationally silenced, until expression is needed to pattern the embryo (Dale et al., 1989; Tannahill and Melton, 1989).

Like early pathway RNAs, initial enrichment of late pathway RNAs in the vegetal cytoplasm is independent of the cytoskeleton and may proceed through a diffusion and local entrapment mechanism (Yisraeli et al., 1990). However, the next step in late pathway RNA localization, transport to the vegetal cortex, relies on the cytoskeletal network and molecular motors (Gagnon et al., 2013; Messitt et al., 2008; Yisraeli et al., 1990; Yoon and Mowry, 2004). Interestingly, as in *Drosophila* oocytes, both plus end and minus end directed motors are employed by late localizing RNAs to reach their ultimate site of enrichment (Gagnon et al., 2013). The transport process has been best described for the *vg1* mRNA, which is transported in a multistep process from the perinuclear cup to the vegetal cortex using a mixed microtubule array (Messitt et al., 2008). As shown in Figure 3B, in the upper vegetal cytoplasm of the oocyte, *vg1* is transported unidirectionally from the perinuclear cup at the vegetal side of the GV towards microtubule minus ends by the molecular motor dynein (Gagnon et al., 2013). In the lower vegetal cytoplasm, *vg1* is transported bi-directionally

both towards the cortex and back towards microtubule plus ends in the upper vegetal cytoplasm by the molecular motor kinesin. This bidirectional transport continues until the *vg1* mRNA has been stably anchored at the vegetal cortex by an unknown mechanism likely involving interaction with the cytoskeletal network (Gagnon et al., 2013; Yisraeli et al., 1990). It is proposed that this bidirectional transport may provide a mechanism in which multiple iterations of transport enriches the RNA at the vegetal cortex even if the RNA is inefficiently captured by the anchoring process.

Although the early and late RNA localization pathways are clearly distinct in timing, mechanisms of RNA localization, and localization patterns, some RNAs exhibit characteristics of both pathways. These RNAs, which include *hermes* and *fatvg* mRNAs, are classified as localizing via an intermediate pathway as they are enriched in the Balbiani body during early oogenesis and to vegetal cytoplasm islands during mid-oogenesis (Chan et al., 2001, 1999; Zearfoss et al., 2004). However, it remains to be determined whether this represents a distinct pathway of localization or if these RNAs are using the established early and late pathways sequentially.

Interestingly, while these localization pathways have been viewed as important for localizing mRNAs for subsequent local translation, both *xlsirts* non-coding RNAs, which localize via the early pathway, and *vegT* mRNA, which localizes via the late pathway, have been shown to play structural roles in organizing the vegetal cytoplasm of the *Xenopus* oocyte. In addition to encoding a protein necessary for germ layer patterning, *vegT* mRNA is also thought to play a role as a RNA scaffold to nucleate cytoskeleton networks during oogenesis (Kloc et al., 2011, 2007, 2005). This role in cytoskeletal organization may explain why depletion of *vegT* mRNA, independent of its protein product, causes mislocalization of both early (*bicaudal-C* and *Xwnt11*) and late (*vg1*) mRNAs in the oocyte (Heasman et al., 2001). Vegetal cytoskeletal arrangement also relies on the localization of the *xlsirt* non-coding RNAs, as depletion of these transcripts also causes disruption of the cytoskeleton network, demonstrating a potential function of localizing these non-coding RNAs (Kloc et al., 2005).

5. RNPs and localization

In *Xenopus* oocytes, as well as all other cells, RNAs do not localize independently. Rather, RNAs are packaged with proteins to form ribonucleoprotein complexes (RNPs). These RNA-protein interactions, which are often required for the localization, translational silencing, and stability of the RNA are initiated by *cis*-acting sequences in the RNA called zip codes or localization elements (Kislauskis et al., 1994; reviewed in Singh et al., 2015). These *cis*-sequences can bind proteins based on their primary sequence, secondary structure, or both, and are most often found in the 3' UTR of mRNAs (reviewed in Jambhekar and Derisi, 2007). *Cis*-sequences are subsequently bound by *trans*-acting proteins to create a localization competent RNP (reviewed in Singh et al., 2015). A growing body of evidence suggests that perturbations to RNP assembly and subsequent RNA localization can lead to pathological phenotypes, particularly in neurodegenerative diseases (reviewed in Bovaird et al., 2018; Cody et al., 2013).

5.1 *Cis* sequences and “zip codes”

Not long after the asymmetric distribution of RNA was first observed, researchers began to identify *cis*-acting sequences that were required for proper localization of RNAs in a variety of organisms, including *Drosophila* oocytes, *Xenopus* oocytes, and fibroblasts (Kislauskis et al., 1994; Macdonald and Struhl, 1988; Mowry and Melton, 1992). Many of these early experiments identified minimal sequences which were sufficient for localization to their respective subcellular destinations, but those elements showed few shared features at the level of either primary sequence or secondary structure and varied in dramatically in length. For example, the localization element for *bicoid* mRNA in *Drosophila* oocytes was mapped to a 625 nucleotide (nt.) region of the 3' UTR which included extensive regions of predicted secondary structure (Macdonald and Struhl, 1988). Mutational analysis showed that both a non-sequence specific helical structure and an adjacent sequence specific recognition domain were both required for *bicoid* localization, highlighting the diverse ways in which *cis*-sequences can interact with *trans*-factors (Macdonald and Kerr, 1998). In contrast, the localization element for β -actin mRNA in chick embryonic fibroblasts was mapped to a bipartite RNA sequence: a primary 54-nt. region (termed the “zip code”) with a weaker 43-nt. element downstream in the 3' UTR (Kislauskis et al., 1994, 1993).

Cis-sequences directing localization have also been well characterized for *vg1* mRNA, a late pathway localizing RNA in *Xenopus* oocytes. A 340-nucleotide region of the 3' UTR of *vg1* mRNA, termed the vegetal localization element (*VLE*), is sufficient to drive late pathway-like RNA localization in *Xenopus* oocytes (Mowry and Melton, 1992). A second, distinct 250-nucleotide region of the *vg1* 3' UTR, termed the *vg1* translational element (*VTE*) has been identified as important for the maintenance of translational repression during transport (Otero et al., 2001). This AU-rich region is 118 nucleotides downstream of the *VLE* and binds to Elavl1 and Elavl2 to maintain translational repression (Colegrove-Otero et al., 2005). Similarly, the localization element of a second late pathway RNA, *vegT*, has also been identified and, while there is no overall primary sequence homology with the *vg1* *VLE*, repeated clusters of redundant *cis*-sequences, which bind *trans*-factors, does appear to be a conserved localization signal in *Xenopus* oocytes (Bubunenko et al., 2002). Subsequent studies have identified a number of *cis*-elements important for RNA localization in a wide range of other organisms, ranging in size from tens of nucleotides to 1 kb, but no universal patterns in sequence or structure have emerged (reviewed in Jambhekar and Derisi, 2007; Marchand et al., 2012; Van De Bor and Davis, 2004).

5.2 *Trans* factors

In most cases where the *cis*-acting localization elements have been identified, the full complement of RNA-binding proteins (RBPs), protein adapters, and molecular motors acting as *trans*-factors are not yet known. One exception is the well-characterized RNPs of pair-rule RNAs, such as *wingless*, *hairy*, and *ftz* mRNAs, which localize apically in the *Drosophila* blastoderm embryo (Davis and Ish-Horowicz, 1991). The localization of these RNPs, which is required for coordinating segmentation in the embryo, is dynein-dependent and directed towards the minus ends of microtubules (Bullock and Ish-Horowicz, 2001; Wilkie and Davis, 2001). Extensive studies have revealed both the *cis*-sequences required for localization of these mRNAs and the minimal set of *trans*-factors which are required to

create a localization competent RNP. mRNAs are directly bound by Egalitarian (Egl), which in turn binds to the adapter protein Bicaudal D (BicD), which then licenses recruitment of the dynein/dynactin motor complex (Bullock and Ish-Horowicz, 2001; Dienstbier et al., 2009; Wilkie and Davis, 2001). Interestingly, *cis*-acting sequences in the RNA cargoes promote the interaction of Egl with BicD, providing a mechanism for RNAs to regulate their own intracellular transport (McClintock et al., 2018).

Another well characterized example of *trans*-acting factors interacting with *cis*-sequences is found in the *vg1* RNP in *Xenopus* oocytes. Here, the *vg1* *VLE* interacts with several RBPs, including heterogeneous nuclear ribonucleoprotein AB (hnRNPAB/ 40LoVe), polypyrimidine tract binding protein (PTB/ hnRNPI), insulin-like growth factor 2 mRNA binding protein 3 (Igf2bp3/ Vg1 RBP /Vera), and Staufen1 (Cote et al., 1999; Czapinski et al., 2005; Deshler et al., 1998; Havin et al., 1998; Mowry, 1996; Yoon and Mowry, 2004). Binding of PTB and Vera to the *VLE* is required for *VLE* localization, as point mutations in their *cis*-binding sites abolish *VLE* localization (Cote et al., 1999; Deshler et al., 1998; Lewis et al., 2008, 2004). In the *vg1* RNP, both kinesin and dynein molecular motors drive vegetal localization, but the *trans*-factor adapters linking the motors to the RNP have not yet been identified (Gagnon et al., 2013; Messitt et al., 2008). Many *trans*-factors, including each of the above-mentioned *vg1* *VLE* RBPs, contain multiple RNA binding domains and are therefore capable of interactions either with a single RNA molecule at multiple sites or multiple RNA molecules at the same time (Cote et al., 1999; Deshler et al., 1998; Havin et al., 1998; Yoon and Mowry, 2004). This multivalency of interactions is important not only for RNP formation and subsequent RNA localization, but has also recently been characterized as a driver of phase separation (Lin et al., 2015).

6. Biomolecular condensates and phase separation

In 2009, the paradigm for understanding RNP complex structure, formation, and biology shifted with the first description of liquid-liquid phase separation in cells (Brangwynne et al., 2009). The observation of non-membrane bound compartments within the cell was not novel, as the membraneless nucleolus was first observed in the 1800s using the earliest forms of light microscopy (reviewed in Pederson, 2011). Rather, phase separation provided a new biophysical framework to describe how these compartments regulate their composition, concentrate specific biomolecules above their surroundings, and modulate internal biochemical activity without the use of a membrane. In the years since the first description of liquid-liquid phase separation, RNP granules (many of which have been revealed to be biomolecular condensates—also termed membraneless organelles) have been described in diverse cell types, including oocytes, and in a multitude of subcellular locations (reviewed in Banani et al., 2017). Notable examples include stress granules, processing bodies, and germ granules in several species (Brangwynne et al., 2009; reviewed in Decker and Parker, 2012; Saha et al., 2016; Voronina et al., 2011).

6.1 General principles of phase separation

The understanding of phase separation in biology is based on principles of thermodynamics applied within the cellular context (reviewed in Hyman et al., 2014). In this model,

molecules overcome entropy and form a dilute solution phase and a concentrated condensate phase based on the free energy of the solution and the chemical potential (reviewed in Banani et al., 2017). Thus, molecules are miscible in the cell until they reach a threshold concentration at which they undergo liquid-liquid demixing and phase separate. A defining feature of biomolecular condensates is their ability to exert control over their contents without a membrane, concentrating certain molecules and excluding others. One framework to understand this phenomenon is to define condensate components as either scaffolds or clients (Banani et al., 2016). Scaffold components, which are often only a small portion of the condensate components, drive condensate formation, while clients, which are often the majority of the condensate components, are selectively recruited into the scaffold complex without playing a significant role in condensate formation (reviewed in Ditlev et al., 2018).

While examples of biomolecular condensates vary in their biological functions, compositions, subcellular localizations, and size, the physical principles that drive their formation dictate certain similarities between them. As depicted in Figure 4, biomolecular condensates are often enriched for multivalent macromolecules that can drive phase separation through a multiplicity of interactions between protein-protein or protein-RNA interaction domains (Figure 4A), through transient interactions between protein intrinsically disordered regions (IDRs) with “sticker” domains (Figure 4B), or a combination of both types of interactions (Lin et al., 2015; Mittag and Parker, 2018; Van Treeck and Parker, 2018). Perhaps for this reason, many proteins capable of multivalent interactions are conserved components of biomolecular condensates, with diverse types of RNP granules exhibiting broad compositional overlap (Buchan, 2014; Cumberworth et al., 2013). In addition, RNA itself is multivalent, capable of interacting with multiple RBPs (reviewed in Van Treeck and Parker, 2018). Other similarities shared by biomolecular condensates include the reversibility of condensate formation and, in their liquid state, the capability of droplets to undergo fusion and fission, as well as distorting their spherical shape in response to shear forces (Brangwynne et al., 2011, 2009; Elbaum-Garfinkle et al., 2015; Weber and Brangwynne, 2015).

Biomolecular condensates do not exist solely in liquid-like states (reviewed in Alberti and Hyman, 2016). In a process thought to be driven by IDRs, biomolecular condensates can exist in a reversible continuum of decreasing dynamics from mixed liquids, to demixed liquid droplets, to gels, and finally to solid or fibrous aggregates (Han et al., 2012; Kato et al., 2012; Lin et al., 2015). The final transition to a solid aggregate is often irreversible and associated with pathology (Elbaum-Garfinkle and Brangwynne, 2015; Murakami et al., 2015). This continuum of phase transitions can be recapitulated *in vitro*, as many IDR-containing proteins form droplets which are initially liquid-like, but mature over time to a more solid-like state (Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015; Xiang et al., 2015; Zhang et al., 2015). Recent evidence in oocytes and other model systems demonstrates that *in vivo* many non-pathological phase separated bodies are heterogenous in phase, with particular elements or regions being more or less liquid-like (Feric et al., 2016; Putnam et al., 2019).

6.2 RNA localization and phase separation

As our understanding of both RNA localization and phase separation expands, several mechanisms by which the processes facilitate one another have emerged. For example, RNA localization and local translation can provide a mechanism for spatial and temporal control of phase separation in the cell. A recent study comparing the properties of proteins, which are either translated upon nuclear export and localized as proteins or transported as mRNAs and translated distally, demonstrated that the later class of mRNAs is significantly enriched for transcripts encoding proteins with IDRs (Weatheritt et al., 2014). Thus, the advantage of RNA localization as a means to prevent ectopic protein activity can also be viewed in the context of preventing ectopic phase separation by local translation of protein IDRs. RNA localization and local translation of IDR-containing proteins may also be advantageous to cells as it provides a mechanism for rapid phase separation in response to external stimuli. Furthermore, RNA localization may facilitate local phase separation by creating concentrations of both RNAs and proteins above the threshold concentration for phase separation of scaffolds. In this way, RNA localization can serve as an energetically-favorable biological means to regulate phase separation, as with phase separation of *Drosophila* germ granules, described below (Niepielko et al., 2018). Conversely, phase separation can facilitate RNA localization, as some biomolecular condensates, such as the *Xenopus* Balbiani body, contain localized RNAs (Boke et al., 2016). Indeed, RNA incorporation into biomolecular condensates itself can be described as a form of RNA localization. Finally, many of the *trans*-factors important for RNA localization are also highly enriched in biomolecular condensates, suggesting additional links between RNA localization and phase separation (Lin et al., 2015; reviewed in Mittag and Parker, 2018; Van Treeck and Parker, 2018).

7. Biomolecular condensates in oocytes and embryos

Just as the large, highly polarized oocyte was the source of many of the first examples of RNA localization, so too it has proved to be an excellent model for the identification and study of biomolecular condensates. Examples include phase separated germ granules, such as P-granules in *C. elegans*, the germ granules of *Drosophila* oocytes, and the Balbiani body in *Xenopus* oocytes, as well as other non-germ granule structures such as nucleoli, which have been studied in *Xenopus* oocytes. These examples of biomolecular condensates, which are detailed below, demonstrate the diversity of phase separated structures and the principles of phase separation that have been gleaned from work in these systems.

7.1 Nucleoli

Nucleoli are subdomains of the nucleus dedicated to ribosome biogenesis and, as mentioned previously, were the first membraneless compartment observed in the cell (reviewed in Pederson, 2011). Using the thermodynamic principles of liquid-liquid phase separation and the enormous nuclei of the stage V *Xenopus* oocyte (~0.4–0.5 mm), researchers quickly characterized the nucleolus as a biomolecular condensate with liquid-like properties (Brangwynne et al., 2011). Nucleoli contain subcompartments important to different stages of ribosome production, but until recently the mechanisms of subdomain formation were unclear (reviewed in Thiry and Lafontaine, 2005). Interestingly, the subcompartments of the

nucleolus were found to be co-existing, immiscible liquid phases within the larger biomolecular condensate, with differential biophysical properties and primary surface tension driving organization of the subcompartments (Feric et al., 2016). The large size of *Xenopus* oocyte nucleoli has made them the ideal model to study additional questions in phase separation, such as the role of ATP in phase separation and the role of transcription within condensates (Berry et al., 2015; Hayes et al., 2018).

7.2 P granules in *C. elegans*

P granules are germ granules in *C. elegans* that form perinuclearly in germ cells, become cytoplasmic in growing oocytes, and are subsequently enriched in the posterior of the one-cell embryo (reviewed in Seydoux, 2018). During cell division, P granules are ultimately asymmetrically inherited by the cells that will become the germ line. While originally observed in the 1980s, several decades later, P granules were the first biomolecular condensate to be described as forming via liquid-liquid phase separation (Brangwynne et al., 2009; Strome and Wood, 1982). Two scaffold proteins of the P granule, PGL-1 and PGL-3, are RBPs which bind RNA through RGG boxes and have dimerization domains that are required for condensate formation (Hanazawa et al., 2011; Kawasaki et al., 1998). The asymmetrical distribution of P granules in the posterior of the embryo depends not on these scaffold proteins, but on the anticorrelated gradients of MEG-3, an IDR-containing protein enriched in the posterior, and MEX-5, an RBP enriched in the anterior of the cell (Smith et al., 2016). This model for the spatial control of phase separation hypothesizes that phase transition is based on the availability of RNA, whereby MEX-5 RNA binding prevents MEG-3 from interacting with RNA, driving it to locally phase separate and leading to P granule assembly (Saha et al., 2016).

Much like nucleoli, individual P granules are surprisingly heterogeneous, as the proteins in assembled P granules are not uniformly distributed throughout the individual condensate and do not exhibit the same dynamics (reviewed in Updike and Strome, 2010). MEG-3 forms a gel-like assembly primarily at the periphery of the structure while PGL-3 is in a more dynamic phase primarily in the core, once again highlighting the diversity of biophysical states possible within a single condensate (Putnam et al., 2019). Although many of the biological functions of forming subcompartments within a larger condensate remain unclear, regulating the biophysical state of distinct subcompartments provides an attractive model for both stabilizing the structure against mechanical forces in the cell and tuning reaction kinetics (reviewed in Banani et al., 2017).

7.3 Germ granules in *Drosophila*

Similar to *C. elegans*, *Drosophila* germ line establishment occurs through asymmetrical inheritance of biomolecular condensates in the embryo (reviewed in Mahowald, 2001; Trcek and Lehmann, 2019). These condensates, termed germ granules, contain several localized RNAs, including *nanos* and *pgc*, and proteins required for germ line specification such as Vasa (reviewed in Trcek and Lehmann, 2019). The formation of germ granules is intrinsically linked to the polarization created by RNA localization and local translation of their protein products in the oocyte. Specifically, germ granule formation is nucleated by translation of the *oskar* mRNA, which is localized to the posterior pole by active transport

(Ephrussi et al., 1991; Niepielko et al., 2018). *Nanos* mRNA, which was localized to the posterior pole via a combination of diffusion and local entrapment and local stabilization and selective entrapment methods, is then incorporated into nascent germ granules, where *nanos* RNA acts to recruit more of the locally enriched *nanos* mRNA (Niepielko et al., 2018). In this way, three RNA localization mechanisms work in concert to produce the conditions and macromolecular gradients necessary for seeding and growth of a biomolecular condensate. Like nucleoli, P granules, and other biomolecular condensates, *Drosophila* germ granules display a range of biophysical states with both liquid-like and more solid or gel-like properties (Kistler et al., 2018).

7.4 The Balbiani body in *Xenopus* oocytes

Finally, the Balbiani body, the site of early pathway RNA localization during stage I of *Xenopus* oogenesis, and the site of germ granule formation has also been shown to be a biomolecular condensate (Boke et al., 2016). The Balbiani body is a non-membrane bound compartment which, in addition to localized RNAs and proteins, contains high numbers of membrane bound organelles, such as mitochondria and endoplasmic reticulum (Kloc et al., 2014). Self-assembly of this structure is driven by the N-terminal prion-like domain of Xvelo protein, which is highly enriched in the Balbiani body *in vivo* and able to form amyloid-like structures *in vitro* (Boke et al., 2016). Intriguingly, Xvelo displays characteristics of a scaffold *in vitro*, as phase separating Xvelo is able to recruit both mitochondria, through interactions with the N-terminal domain, and *nanos1* mRNA, through a putative C-terminal RNA-binding domain, as clients (Boke et al., 2016). Unlike many other biomolecular condensates, the Balbiani body does not appear to be liquid *in vivo* (Boke et al., 2016). Instead, the Balbiani body likely represents a more solid-like phase-separated structure, although it is not known if the initial formation proceeds through a liquid-like intermediate that eventually matures to a more solid-like state.

As *Xenopus* oocyte maturation occurs over months, a less dynamic condensate is an attractive model for stably maintaining factors in the proper subcellular location on an extended developmental timescale. The mechanism by which this solid-like structure disassembles and deposits the early pathway RNAs at the vegetal cortex during the transition to stage II of oogenesis is currently unclear. Nonetheless, the Balbiani body is an intriguing example of a solid-like biomolecular condensate that is not associated with a pathological state and the mechanisms by which it disassembles, once elucidated, may have important implications for disease-associated amyloid-like condensates. Additionally, while the biophysical state of the cytoplasmic islands in the *Xenopus* late RNA localization pathway has not yet been described, based on the similarities in function and timescale between the Balbiani body and the late-pathway vegetal RNA islands and the emergence of biomolecular condensates in RNA transport, it is an attractive hypothesis that late pathway RNAs also enrich within phase separated structures.

8. Conclusions

Over the decades since RNA localization was first described, it has become clear that this process plays a crucial role in subcellular organization. Recent technological advances have

enabled global studies highlighting the astonishingly complex combinations of *cis*-sequences and *trans*-factors that facilitate localization of RNAs. As the list of localized RNAs rapidly expands, it remains a challenge in the field to understand the mechanisms by which newly identified RNPs are localized and the cellular functions linked to their localization. This is particularly true of non-coding RNAs, as the mechanisms by which they are transported, the advantages this provides to the cell, and the functions of the localized transcripts are all much less well understood than for many mRNAs.

An exciting and new avenue for novel insights into RNA localization comes through appreciating the intrinsic linkage between RNA localization and phase separation. As both processes are emerging as means of creating asymmetry in a broad variety of cell types and organisms, it is becoming clear that these processes may be inherently linked and facilitate one another. This seems to be particularly true in oocytes as invertebrate and vertebrate germ granules alike have been shown to be structures in which both phase separation and RNA localization occur in concert. RNA localization can establish the local enrichment of factors necessary for phase separation, as in the case of *Drosophila* germ granules. Additionally, RNA localization, coupled with local translation can also allow for proteins with IDRs to phase separate only at particular subcellular destinations and in response to external cues, providing a mechanism for regulating this biophysical process within cells. In the coming years, an exciting challenge for the field will be to utilize many of the new techniques for studying both biomolecular condensates and RNA localization to inform our understanding of these processes.

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Figure 1: Mechanisms of RNA localization

Asymmetric RNA localization is achieved through four mechanisms. A. Diffusion and local entrapment leads to the enrichment of *nanos1* mRNA (magenta) in the Balbiani body in *Xenopus* oocytes. The Balbiani body is shown below the oocyte nucleus (GV), which is in the center of the oocyte. B. Local stabilization and regulated degradation leads to the enrichment of *hsp83* mRNA (magenta) in the posterior pole (right) of the *Drosophila* embryo. Degradation outside the posterior pole is directed by Smaug-RNA binding and recruitment of the CCR4-NOT complex (green). C. Active motor-based transport leads to the enrichment of *ash1* mRNA (magenta) at the tip of the daughter cell (lower right) in budding yeast. Transport is dependent on myosin (green) along actin filaments (black). D. Incorporation into biomolecular condensates enriches *actin* mRNAs (magenta) in stress granules in mammalian cells.

Figure 2: Schematic of *Xenopus* oogenesis

Xenopus oogenesis proceeds through six stages designated I-VI. Oocyte stages are defined by the increase in oocyte diameter, accumulation of yolk, and the onset and polarization of pigmentation. Oocytes are oriented along the animal-vegetal (AV) axis with the vegetal pole at the bottom. The white circle is the oocyte nucleus (GV) and the tan circle in stage I is the Balbiani body. Ranges in oocyte diameter are denoted below each stage, but individual oocytes are not to scale.

Figure 3: RNA localization in *Xenopus* oocytes

A. RNA localization in *Xenopus* oocytes proceeds through two primary pathways: early (or METRO) and late. Early pathway RNAs (magenta) are enriched in the Balbiani body in stage I oocytes and anchored in a ring-like pattern at the vegetal cortex in stage II. Late pathway RNAs (green) are ubiquitous throughout the cytoplasm in stage I and become enriched in the vegetal cytoplasm in stage II in a characteristic pattern: RNAs enrich in the perinuclear cup, cytoplasmic islands, and at the cortex. B. Late pathway RNAs (green) are transported (arrows) along microtubules in two discrete steps: unidirectional transport by dynein from the perinuclear cup towards to cortex in the upper vegetal cytoplasm (region 1) and bidirectional transport by kinesin motors in the lower vegetal cytoplasm (region 2).

Figure 4: Multivalent interactions mediate phase separation

A. Phase separation can be mediated by multivalent interactions between modular binding domains in proteins (green) interacting with protein or RNA binding partners (magenta). These interactions mediate condensate formation and form a dense phase (grey shaded circle), with networked complexes of interactions, surrounded by a dilute phase. B. Phase separation can also be mediated through multivalent interactions between “sticker” domains (magenta) in IDR containing proteins (green) which interact weakly and transiently with one another to drive biocondensate formation (grey shaded circle).