COMPARTMENTALIZATION OF RNA REGULATION IN THE DROSOPHILA GERM PLASM:
FOUNDER GRANULE ASSEMBLY AND FUNCTION

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ABSTRACT

Posttranscriptional gene regulation plays an important role in the specification of cell fates, particularly during early embryogenesis. Formation of large ribonucleoprotein (RNP) granules serves to organize RNA molecules on the basis of their regulatory requirements. RNP granules are a prominent feature of the *Drosophila* germ plasm, a specialized cytoplasm at the posterior of the embryo harboring factors that are required for establishing germline fate. At least two types of granules can be distinguished. The first, which we term founder granules, contains oskar (*osk*) mRNA along with Staufen protein. Founder granules provide for the local production of Osk protein, which recruits additional protein and mRNA components to assemble the second type of granule, called germ granules. Germ granules are incorporated into the primordial germ cells (pole cells) as they form at the posterior of the embryo and are required for germline development. In contrast, founder granules remain largely outside of the pole cells. Aberrant targeting of *osk* to pole cells by packaging in germ granules impedes germline development, demonstrating the importance of segregating *osk* to founder granules. The work presented herein aims to characterize the assembly of *osk* into founder granules and to determine how this segregation prevents incorporation of excess *osk* in pole cells and thus protects germline development. We found that cis elements in the *osk* mRNA mediate the packaging of *osk* in founder granules, to form RNP granules that are compositionally distinct from germ granules. We show that this segregation allows for the compartmentalized degradation of *osk* mRNA in founder granules leading up to and during pole cell formation, thereby preventing uptake of excess *osk* by pole cells. Our results illustrate how segregation of mRNAs within the same subcellular location to discrete RNP granules allows for their differential regulation during subsequent stages of development.
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CHAPTER 1: INTRODUCTION
1.1 Posttranscriptional Regulation of Gene Expression

The human body comprises over 200 cell types that differ dramatically in morphology and function. For example, intestinal epithelial cells are specialized cells with structures called villi that protrude into the interior of the intestine to absorb nutrients during digestion (Figure 1.1 A). Nociceptors are a type of sensory neuron that spans from the central nervous system to more peripheral locations and are important for sensing pain (Figures 1.1 B). Macrophages function as part of the immune system to engulf and destroy pathogens (Figure 1.1 C), and adipocytes contain special compartments for storing fat (Figure 1.1 D). Despite their differences, all cells in the body are derived from a single fertilized egg and most contain the same set of genetic information. How such variety can stem from a single cell lies in the extensive control over gene expression. There are various opportunities in the progression from DNA to RNA to protein at which gene expression can be controlled (Figure 1.2).

Transcriptional regulation is the earliest step in the gene expression process, primarily relying on the presence of transcriptional activators and repressors and their ability to bind to specific regulatory sequences in DNA that control associated genes. Activators generally function by recruiting or activating RNA polymerase, whereas repressors prevent RNA polymerase from transcribing the mRNA. Furthermore, the structure of chromatin, in which the DNA is packaged, can be modulated to control access of transcription factors and polymerase to the DNA. Together these controls allow for activation and repression of specific genes based on cellular demand. However, transcriptional regulation alone can fall short in the response rate to stimuli and especially in the spatial control of gene expression.

Posttranslational regulation can help overcome the shortcomings of translational regulation mainly through posttranslational modifications (PTMs), which are small molecule additions to proteins after they are translated, such as phosphate groups. PTMs can determine whether a protein is active, where it localizes in the cell, and when it is degraded. The quick addition or removal of PTMs to existing proteins aids the cell in rapidly responding to stimuli rather than beginning the response at the transcriptional level. In addition, localizing proteins to a specific
Figure 1.1: Illustrations of diverse cell type examples

(A) Intestinal epithelial cell. (B) Nociceptor. (C) Macrophage. (D) Adipocyte.
region of the cell can be an effective strategy for restricting or enriching protein activity to that location. However, regulation at the posttranslational level can be inefficient and not all proteins can be stored in an inactive form.

There has been increasing appreciation of the importance of posttranscriptional regulation, which in combination with transcriptional and posttranslational regulation allows for precise spatiotemporal control of gene expression. mRNAs contain regulatory elements called untranslated regions (UTRs) at the beginning (5′ UTR) and end (3′ UTR) of the mRNA sequence. While regulatory elements can be found in the coding sequence (CDS) of the mRNA, most mRNA regulatory elements are found in the 5′ and 3′ UTRs. These elements are recognized by RNA binding proteins (RBPs) based on either the nucleotide sequence of the element, the structure into which the element is folded, or both. There are many ways that RBPs can regulate their mRNA targets, such as alternative splicing to produce different mRNAs from the same gene, addition or removal of the 5′ cap and poly(A) tail as described below to affect stability of the mRNA, selective mRNA export from the nucleus, localization the mRNA to specific subcellular regions, control of the mRNA’s translational status, and degradation of the mRNA. Mechanisms of mRNA localization, translational control, and mRNA stability and degradation are described in more detail below.

**mRNA Localization**

Localization of mRNAs to subcellular regions is key to generating asymmetry or polarity of cellular morphology and function through local protein production. Since one mRNA molecule can be translated repeatedly to produce many proteins, this mode of posttranscriptional regulation is an economical way to enrich proteins where they are needed. Furthermore, translational activation of a localized pool of mRNA based on extrinsic cues ensures a swift and spatially limited response to stimuli. For example, *Arc* mRNA is localized to the dendrites of neurons where extrinsic signals activate its translation and the locally produced protein functions in long term potentiation (Bramham and Wells, 2007; Nikolaenko et al., 2018; Panja et al., 2014). Similarly, *gurken (grk)* mRNA localizes to the dorsal anterior corner of *Drosophila melanogaster* oocytes where the locally produced protein begins a signaling cascade that establishes the dorsal-ventral axis of the future
Figure 1.2: Selected mechanisms for regulating gene expression

DNA

Transcription

mRNA

Translation

Protein

Transcriptional Regulation:
- Chromatin structure
- Transcriptional activators & repressors

Posttranscriptional Regulation:
- Alternative splicing
- mRNA localization
- mRNA stability
- Translational activators & repressors

Posttranslational Regulation:
- Protein modification
- Protein localization
- Protein stability
fly. However, if Grk protein is not restricted to the dorsal side, dorsal-ventral patterning is lost (Neuman-Silberberg and Schupbach, 1993; Wieschaus et al., 1978). mRNA localization also plays an important role in cell differentiation. For example, in the budding yeast *Saccharomyces cerevisiae*, ASH1 mRNA is enriched in the daughter cell during division to prevent mating type switching specifically in that cell, while mating type switching is uninhibited in the mother cell (Long et al., 1997; Takizawa et al., 1997).

The first discoveries of subcellular mRNA localization were made in studies of maternally supplied mRNAs in Xenopus (Rebagliati et al., 1985) and Drosophila (Berleth et al., 1988; Frigerio et al., 1986) oocytes. Since then, mRNA localization has been extensively studied in the contexts of Drosophila oogenesis, embryogenesis, and neuron morphogenesis among other systems. Over 70% of mRNAs in early Drosophila embryos are localized to distinct domains (Lecuyer et al., 2007). Studies of localized mRNAs in Drosophila have been instrumental in deciphering mRNA localization mechanisms as described below.

mRNAs can be actively transported by motor proteins of the Kinesin, Dynein, or Myosin families via specific adaptor proteins that bind directly to the mRNA or to RBPs. For example, in the Drosophila oocyte, bicoid (*bcd*) mRNA, which is necessary for establishing the anterior-posterior axis, is linked to Dynein for active transport towards the anterior on microtubules (Figure 1.3 A) (Berleth et al., 1988; Duncan and Warrior, 2002; Januschke et al., 2002; Meng and Stephenson, 2002; Pokrywka and Stephenson, 1991; Schnorrer et al., 2000; St Johnston et al., 1989; Well et al., 2006). Another example of mRNA localization by active transport occurs in the Drosophila class IV dendritic arborization neurons. Dynein-mediated active transport of the mRNA of the translational repressor Nanos (*nos*) on microtubules promotes the dendritic localization of the mRNA where its local translation is required for proper dendrite morphogenesis (Brechbiel and Gavis, 2008).

Active transport of mRNAs to their target destinations depends on an appropriately organized cytoskeleton. However, cytoskeletal tracks are not always organized in a way that supports transport to any subcellular location. For example, whereas the cytoskeleton of the
Figure 1.3: Mechanisms of mRNA localization

(A) Active transport of *bicoid* mRNA towards the anterior by Dynein on microtubules. (B) *nanos* mRNA localization by diffusion and entrapment by a protein ensemble anchored to the actin cytoskeleton. (C) Posterior enrichment of *hsp83* mRNA by local protection from degradation.
Drosophila oocyte during mid-oogenesis is organized with the microtubule minus ends concentrated at the anterior of the oocyte, the cytoskeleton is reorganized during later stages of oogenesis resulting in loss of that bias (Theurkauf et al., 1992). To localize mRNAs after this reorganization, the oocyte depends on a diffusion and entrapment mechanism in which mRNAs circulate through the cytoplasm and those that reach the target destination become anchored in place. This mechanism is used for localization of nos mRNA to the oocyte posterior. The mRNA is swept through the oocyte by cytoplasmic flows, and upon reaching the posterior is captured by posterior-specific protein ensembles that are anchored to the actin cytoskeleton (Figure 1.3 B) (Forrest and Gavis, 2003).

Another mechanism for the enrichment of mRNAs in a specific region is local protection from degradation. This mechanism relies on the degradation of mRNAs throughout most of the cell and their protection from degradation in the region where their function is needed. For example, in early Drosophila embryos, hsp83 mRNA is protected from degradation at the posterior of the embryo, while hsp83 mRNA is degraded throughout the rest of the embryo leading to an enrichment of hsp83 mRNA at the posterior (Figure 1.3 C) (Bashirullah et al., 2001).

While our understanding of mRNA localization has primarily arisen from studies of neurons, oocytes, and early embryos of different model organisms, the concepts apply to widespread cellular functions. For instance, in migrating fibroblasts, β-actin mRNA is localized to the leading edge of the cell, where it is translated. The resulting high local concentration of β-actin protein subunits promotes polymerize of actin filaments that help generate the cellular protrusions necessary for migration (Lawrence and Singer, 1986). In addition to cytosolic locations, mRNAs localize to organelles. mRNAs encoding mitochondrial proteins that are transcribed in the nucleus are localized to the mitochondria and translated there. For example, the ATP2 mRNA, whose protein product is required for respiration, is localized to the mitochondria (Corral-Debrinski et al., 2000; Egea et al., 1997; Liu and Liu, 2007; Margeot et al., 2005). In addition, mRNAs are localized to the endoplasmic reticulum (ER) independent of the canonical ER-targeting mechanism for translation of integral membrane and secreted proteins. There are different purposes for localizing mRNAs to the ER. In S. cerevisiae, for example, asymmetric ASH1 mRNA partitioning involves association of
the mRNA with the ER membrane (Schmid et al., 2006). In addition, ER-associated mRNAs can escape the translational repression imparted on cytosolic mRNAs that are sequestered and translationally repressed under stressful conditions (Unsworth et al., 2010). Localization of mRNAs to the centrosome is thought to be important for centrosome function and assembly (Lerit et al., 2013). In addition, similarly to the ER, mRNA association with centrosomes helps to distribute mRNA during cell division. This occurs during early development of Drosophila germ cells when mRNAs required for directing germ cell development associate with the astral microtubules emanating from the centrosomes of the future germ cell precursors (Lerit and Gavis, 2011).

**mRNA Stability, Degradation, and Translational Control**

During transcription, RNA polymerase copies the DNA sequence to produce a pre-mRNA; an immature mRNA message. During and immediately following transcription, the pre-mRNA is processed to produce a mature mRNA (Figure 1.4). mRNA processing can influence the identity of the encoded protein through alternative splicing and by affecting mRNA stability can dictate how long protein is produced from the mRNA. A modified nucleotide, 7-methylguanosine (m7G) is added to the 5’ end of the mRNA, and a poly-adenosine (poly(A)) tail is also added to the 3’ end. Poly(A) polymerases add the poly(A) tail by polymerizing a long stretch of A’s at a specific site in the 3’UTR. In addition to affecting the nuclear export and translational activity of the mRNA, the 5’ m7G cap and 3’ poly(A) tail protect the mRNA from the activities of exonucleases in the cytoplasm, thereby preventing mRNA degradation and promoting mRNA stability. When an mRNA must be degraded, specific RBPs collectively referred to as degradation factors bind to cis-elements usually located in the 3’UTR and recruit the degradation machineries.

The general pathway of mRNA degradation begins with removal of the poly(A) tail by one of three types of deadenylase complexes: CCR4-NOT, PAN2-PAN3, or PARN. Associated factors then recruit the decapping machinery including the decapping enzyme Dcp2, the Dcp2 activator Dcp1 (Ling et al., 2011), and several other cofactors that vary among different organisms (Coller and Parker, 2004; Franks and Lykke-Andersen, 2008a; Li and Kiledjian, 2010). After decapping, the mRNA is vulnerable to the action of the 5’ to 3’ exonuclease Xrn1 (Meyer et al., 2004), which
Figure 1.4: pre-mRNA processing

DNA

Transcription

Introns

pre-mRNA

5' UTR

Exons

3' UTR

pre-mRNA Processing

mRNA

5' m⁷G Cap

5' UTR

CDS

3' UTR

poly(A) tail
is recruited to the mRNA by components of the decapping machinery. In Drosophila, for example, the Xrn1 nuclease Pacman (Pcm) is recruited by Dcp1 (Braun et al., 2012; Nissan et al., 2010). A less common pathway of mRNA degradation begins with deadenylation followed by degradation via the 3’ to 5’ exonuclease activity of the 10-subunit exosome complex (Chlebowski et al., 2013). The 5’ cap is then removed from the short mRNA fragment by a homodimer of the scavenger decapping enzyme DcpS (Chen et al., 2005; Liu et al., 2002). While, 5’ to 3’ mRNA degradation is considered the predominant mechanism, the two pathways can partially compensate for one another, suggesting that they may work together to degrade mRNAs (Garneau et al., 2007). (Fig 1.5)

Translation involves many factors beyond the ribosome and is highly regulated. Modulation of the general translation machinery has a widespread impact on the translation of most mRNAs in a cell. However, translational control is often specific to one or a subset of mRNAs, with different classes of mRNAs experiencing different translational regulatory events simultaneously. Targeted translational control is accomplished through modulation of RBPs that bind to cis-elements in the mRNA and primarily dictate whether translation initiation factors can interact with the mRNA. The cis-elements are normally located in the 3’UTR and the regulatory RBP interacts with factors at the 5’ m7G cap effectively circularizing the mRNA (Wells et al., 1998). For example, poly(A) binding protein (PABP) binds to the poly(A) tail and PABP-interacting protein 1 (PAIP1), and interaction between PABP on the 3’ poly(A) tail with the initiation factor eIF3 at the 5’ m7G cap stimulates translation (Figure 1.6 A) (Martineau et al., 2008). However, binding of PAIP2 to PABP causes it to release the poly(A) tail, decircularizing the mRNA and inhibiting translation (Figure 1.6 B) (Karim et al., 2006). In addition to affecting translation initiation factors, mRNA circularization is hypothesized to help ribosomes reinitiate on the same mRNA after termination to further promote translation (Nelson and Winkler, 1987).

Translation regulation is closely coupled to mRNA degradation to ensure that mRNAs that are no longer required are eliminated, thus guaranteeing a halt to translation. It also prevents translation of mRNAs during their degradation so that potentially toxic truncated proteins are not produced. mRNA regulation by the RBP Tristetraprolin (TTP) is a good example of the link between
Figure 1.5: mRNA degradation mechanisms

A

RBP bound to 3'UTR recruits deadenylase.

Poly(A) tail is removed. Dcp1 & 2 are recruited.

5' m\(^7\)G cap is removed. 5' to 3' exonuclease is recruited.

5' to 3' mRNA degradation.

B

RBP bound to 3'UTR recruits deadenylase.

Poly(A) tail is removed. Exosome is recruited.

3' to 5' mRNA degradation. 5' m\(^7\)G cap is removed.

(A) 5' to 3' mRNA degradation. (B) 3' to 5' mRNA degradation.
Figure 1.6: Examples of translational control mechanisms.

(A) PAIP1-mediated translational activation. (B) PAIP2-mediated translational repression. (C) TTP-mediated translational repression coupled to mRNA degradation.
translational repression and mRNA degradation. TTP binds to AU-rich elements in the 3'UTR, which are signals that destabilize mRNAs (Barreau et al., 2005; Shirai et al., 2014). The bound TTP recruits the protein eIF4E2 to the 5’ m7G cap to block translation initiation (Sanduja et al., 2011) and recruits the CCR4-NOT deadenylation machinery to trigger the degradation of the mRNA target (Fig 1.6 C) (Tao and Gao, 2015).

Translational regulation is also tightly coupled to mRNA localization. Consider, for example, the complex regulation of the mRNA oskar (osk) during Drosophila oogenesis. Osk protein activity at the posterior of the oocyte is essential for patterning the future embryo, but ectopic activity is toxic to development (Ephrussi and Lehmann, 1992; Smith et al., 1992). Therefore, unlocalized osk mRNA is translationally repressed, while osk mRNA enriched at the posterior of the oocyte is translationally active. See section 1.3 below for a detailed description of osk localization, translational regulation, and degradation in the context of Drosophila oogenesis and early embryogenesis.

1.2 RIBONUCLEOPROTEIN GRANULES

TYPES AND FUNCTIONS

Ribonucleoprotein granules (RNPs) are non-membrane bound organelles consisting of RNA and RBPs. mRNAs are ubiquitously packaged in RNPs based on their regulatory requirements. Thus, RNPs vary extensively in composition and function from the small neuronal mRNA transport RNPs to the large translationally repressive stress granules (Van Treeck and Parker, 2018). The general functions of RNPs include the compartmentalization of regulatory activities such that mechanisms that could interfere with one another are separated, promotion of efficient mRNA regulation by co-regulating related mRNAs, and promotion of reactions by concentrating factors required for multistep processes. As expected from their key function in mRNA regulation, RNPs play important roles in many processes including the cellular stress response (Buchan et al., 2008; Kedersha et al., 2016), storage of maternal mRNAs during oogenesis (Schisa, 2014), and synaptic plasticity (Sudhakaran et al., 2014), as well as disease...
related processes such as tumorigenesis (El-Naggar and Sorensen, 2018; Grabocka and Bar-Sagi, 2016), and neurodegeneration (Li et al., 2013; Neumann et al., 2006; Zhang et al., 2018).

P-bodies and stress granules are examples of RNPs that have been extensively studied. In addition to their mRNA contents, P-bodies contain proteins involved in translational repression and 5’ to 3’ mRNA degradation (Decker and Parker, 2012; Parker and Sheth, 2007). Based on their content, p-bodies were originally considered sites of mRNA degradation, however it was shown that p-bodies are not required for mRNA degradation (Decker et al., 2007; Eulalio et al., 2007). In addition, mRNAs can exit p-bodies and re-enter the translation cycle (Brengues et al., 2005), which would not be expected of mRNAs that are being actively degraded. Therefore, an alternative hypothesis was proposed that p-bodies are storage sites of translationally repressed mRNAs and inactive degradation machineries (Franks and Lykke-Andersen, 2008b; Parker and Sheth, 2007). Stress granules share many components with p-bodies and as their name suggests, form under stressful environmental conditions such as extreme temperatures or oxidative stress. Also like p-bodies, stress granules harbor translationally repressed mRNAs. However, unique to stress granules is the inclusion of translation initiation factors (Stoecklin and Kedersha, 2013). Therefore, stress granules are thought to be temporary sites for mRNA storage under stressful conditions that safeguard mRNAs while also preventing unwanted translation as the cell undergoes its stress response.

Another extensively characterized type of RNPs are the germ granules. Germ granules are found in the germline; the lineage of cells that ultimately gives rise to the sperm or oocytes. In some organisms including most common model organisms, germ granules are present in all germline stages except for mature sperm. In contrast, in the mammalian germline, only the newly specified primordial germ cells in the embryo contain germ granules. However, despite this difference, germ granules are necessary for germline function in all animals and play various roles such as in the specification and differentiation of germ cells, protection of germline genomic integrity, and even organization of chromatin during cell division (Voronina et al., 2011).
The class of germ granules has been further divided based on their various characteristics in different organisms or at different stages of germline development. One of these, the chromatoid body has been primarily studied in mammals. During spermatogenesis, the chromatoid body is in close contact with the nucleus at a site enriched for nuclear pores (Fawcett, 1970), and regulates mRNAs following their nuclear export (Soderstrom and Parvinen, 1976). Another type, the P granules, are present in the C. elegans embryo from the one cell stage and through sequential asymmetric cell divisions are inherited by a single precursor cell that will ultimately give rise to the germline (Strome and Wood, 1982). Like P granules, the Drosophila germ granules contain mRNAs required for germline specification (Illmensee and Mahowald, 1974). As described in chapter 1.3, these germ granules form at the posterior of the oocyte where they are maintained and during embryogenesis direct formation of the primordial germ cells.

RNP Assembly

By definition, RNPs form due to interactions between RNA and RBPs. Large RNPs that contain many mRNAs are considered biomolecular condensates (Banani et al., 2016) and are thought to form in a process called liquid-liquid phase separation (LLPS) in which interactions between the RNA and protein contents result in a phase with different characteristics from the surrounding cytoplasm. These condensates can be dynamic and liquid-like with the abilities to exchange contents with the cytoplasm and fuse with one another (Brangwynne et al., 2009; Shin and Brangwynne, 2017) or they can be less dynamic and more solid or gel-like (Woodruff et al., 2017). It is important that RNP dynamics are carefully controlled as becoming too stable can trigger the formation of pathological aggregates that lead to neurodegenerative diseases as described below (Ambadipudi et al., 2017; Patel et al., 2015; Ramaswami et al., 2013; Wegmann et al., 2018).

RNP properties and assembly are influenced by many protein-protein interactions. Commonly, interactions between intrinsically disordered regions (IDRs) or low complexity domains (LCDs) of proteins drive LLPS (Decker et al., 2007; Kato et al., 2012; Reijns et al., 2008). Specific interactions between structured and often multivalent protein interaction domains also contribute to RNP assembly (Li et al., 2012; Ling et al., 2008; Tourriere et al., 2003).
mRNAs also contribute to RNP assembly and properties (Buchan et al., 2008; Kedersha et al., 2016; Kedersha et al., 1999; Liu et al., 2005; Pillai et al., 2005; Sheth and Parker, 2003; Teixeira et al., 2005). One way in which they can do this is by acting as a scaffold on which RBPs bind. When RBPs on different mRNAs interact, the network of interactions contributes to LLPS. Likewise, the mRNAs can interact with each other through both base pairing or base stacking (Zanchetta et al., 2008). Interestingly, the mRNA domain associated with the RNP can extend beyond the protein domain, thereby facilitating interactions with smaller incoming RNPs (Moon et al., 2019). Taken together, RNP granules form when the combination of local protein-protein, protein-RNA, and RNA-RNA interactions reach a threshold level that favors their assembly.

Protein modifications can have a significant impact on RNP properties and assembly (Hofweber, 2018). RBP PTMs can increase or decrease the strength of molecular interactions and can influence the molecular contents of RBPs by either recruiting or excluding factors (Itakura et al., 2018; Rhoads et al., 2018). Consistent with PTMs playing an important role in RNP structure, the multivalent interaction motifs and IDRs that help drive RNP assembly are commonly posttranslationally modified (Bah and Forman-Kay, 2016; Chong and Forman-Kay, 2016; Tompa et al., 2014; Xie et al., 2007). PTMs can affect the charge or steric properties of amino acids, which can either strengthen or weaken interactions. The positively charged amino acid arginine and the aromatic amino acid tyrosine are two of the amino acid residues that play key roles in driving RBP LLPS (Wang et al., 2018). Phosphorylation of tyrosine adds a negative charge that can either strengthen or weaken interactions depending on the interacting partner (Wang et al., 2018), and arginine methylation affects its charge distribution, hydrophobicity, and hydrogen bonding ability and increases its bulkiness (Evich et al., 2016). Thus, these commonly modified residues present an opportunity to regulate RNP formation through PTMs.

While factors promoting RNP disassembly are not as well understood as those promoting assembly, several events have been implicated including translational activation of mRNAs, mRNA degradation, PTMs, protein turnover, and autophagy (Buchan, 2014).
RNP CONNECTION TO DISEASE

Considering their many critical functions in mRNA regulation, it is not surprising that RNP dysfunction in the form of both RNP hyper- and hypo-assembly is linked to disease (Shukla and Parker, 2016). Diseases caused by RNP hyper-assembly have attracted much attention. The RNP dysfunction in these diseases arise from two types of events. One of these events is the expression of mRNAs with abnormally long expansions of repetitive sequences, which leads to the formation of RNPs that sequester RBPs so that they are not available to regulate neuronal functions. In myotonic dystrophy type 1, this is seen in the expansion of CUG repeats in the DMPK mRNA (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). This results in the sequestration of RBPs in nuclear RNPs (Miller et al., 2000; Philips et al., 1998; Timchenko et al., 1996) which leads to splicing defects (Jiang et al., 2004; Kanadia et al., 2003). Amyotrophic lateral sclerosis (ALS) pathogenesis can involve repeat expansion as well as protein-driven hyper-assembly. Specifically, mutation of different RBPs such as TDP-43 can cause β-amyloid formation, which causes toxic stabilization of stress granules in neurons leading to ALS symptoms (Guo et al., 2011; Kim et al., 2013; Li et al., 2013).

Disease can also be caused by RNP hypo-assembly. For example, in Constant spring α-thalassemia, the α-globin gene has a mutated stop codon. This allows the ribosome to progress into the 3'UTR resulting in ejection of a group of proteins from the 3'UTR called the mRNA stability complex, thereby at least partially disassembling the α-globin RNP and leading to degradation of the mRNA. This leads to reduced α-globin protein in red blood cells which causes hemoglobin deficiency and thalassemia (Waggoner and Liebhaber, 2003; Weiss and Liebhaber, 1995).

1.3 EEARLY DROSOPHILA DEVELOPMENT AND OSKAR REGULATION

DROSOPHILA OOGENESIS AND OSKAR POSTTRANSCRIPTIONAL REGULATION

The Drosophila oocyte develops with 14 morphologically distinct stages in a structure called an egg chamber that also contains 15 polyploid cells referred to as nurse cells (Spradling, 1993). The nurse cells and oocyte are connected by cytoplasmic bridges called ring canals through
which the nurse cells provide organelles and macromolecules including maternal mRNAs to the transcriptionally silent oocyte. The egg chambers mature in an assembly line arrangement in substructures of the ovary called ovarioles. As the egg chamber matures, the oocyte and egg chamber grow, and the oocyte occupies a progressively larger fraction of the egg chamber as it receives material from the nurse cells and accumulates yolk.

Regulation of the maternally-supplied mRNA oskar (osk) during oogenesis has been extensively studied (Figure 1.7). Accumulation of osk mRNA in the oocyte begins very early in oogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991). Nurse cell to oocyte transport of osk is mediated by Dynein on microtubules and requires a stem loop structure in the 3’UTR called the oocyte entry signal (OES), which is linked to Dynein through the RBP Egalitarian (Dienstbier et al., 2009; Jambor et al., 2014). At stage 7 of oogenesis, the oocyte microtubules rearrange so their minus ends are concentrated at the anterior with a slight bias of their plus ends oriented towards the posterior (Theurkauf et al., 1992; Zimyanin et al., 2008). By stage 8, osk mRNA begins to accumulate at the posterior via Kinesin-mediated active transport towards the plus ends of microtubules (Brendza et al., 2000; Khuc Trong et al., 2015; Parton et al., 2011; St Johnston, 2005; Zimyanin et al., 2008). This process continues through stage 10a. Several factors are required for osk localization during mid-stages of oogenesis. One required protein is the RBP Staufen (Stau), which is a member of the osk RNP throughout oogenesis (Martin et al., 2003). However, the role of Stau in the osk localization process is unclear. In addition to Stau, osk localization during mid-oogenesis requires the splicing dependent deposition of the Exon Junction Complex (EJC) adjacent to a stem-loop structure in the osk CDS called the Spliced oskar Localization Element (SOLE), the formation of which also depends on splicing (Ghosh et al., 2012; Hachet and Ephrussi, 2004; Simon et al., 2015). An isoform of Tropomyosin (Tm1-C) acts as a cargo adaptor linking the osk 3’UTR to Kinesin-1 which is activated by the EJC and SOLE (Gaspar et al., 2017; Veeranan-Karmegam et al., 2016). Also, a sequence at the tip of the OES called the dimerization domain (DD) promotes higher order packaging of osk mRNAs in transport RNPs which increases localization efficiency (Jambor et al., 2011).
Figure 1.7: *Drosophila* oogenesis and *oskar* mRNA localization

St 6: Translationally repressed *oskar* mRNA is transported from the nurse cells to the oocyte.

St 8: Microtubule rearrangement in the oocyte allows early posterior enrichment of *oskar* mRNA by active transport. Localized *oskar* mRNA is translated.

St 10: Local translation continues as more *oskar* mRNA is transported to the posterior.

St 12: Microtubules reorganize around the oocyte periphery. More *oskar* mRNA is localized by diffusion and entrapment and maintained there by an actin-based anchor.

St 14:

Legend:
- *oskar*
- nucleus
- ring canal
- microtubule
- actin
For osk localization to effectively limit Osk protein activity to the posterior, which is essential for development (Smith et al., 1992), the unlocalized mRNA must be translationally repressed and the localized mRNA must be translationally activated. The primary translational repressor of osk is the RBP Bruno (Bru), which binds to multiple regions in the osk 3'UTR referred to as Bruno Response Elements (BREs) (Kim-Ha et al., 1995; Snee et al., 2008). Bru represses osk translation through two mechanisms. In one mechanism, Bru recruits Cup to interfere with binding of translation initiation factors at the 5' m\(^7\)G cap (Kinkelin et al., 2012; Nakamura et al., 2004; Wilhelm et al., 2003). In the other mechanism, Bru promotes oligomerization of unlocalized osk mRNAs into silencing particles that are inaccessible to the translation machinery (Chekulaeva et al., 2006). Furthermore, Polypyrmididine tract binding protein (PTB) also binds the 3'UTR to promote assembly of the multicopy translationally silent osk RNPs (Besse et al., 2009). Translational activation of osk at the posterior of the oocyte is less well understood, but is known to involve Stau (Micklem et al., 2000). One hypothesis is that relief from translational repression at the posterior could be due to an insufficient amount of Bru at the posterior to repress the relatively high osk concentration at the posterior (Kanke and Macdonald, 2015; Smith et al., 1992). Surprisingly, Bruno has also been implicated in the translational activation of osk (Kanke and Macdonald, 2015). Specifically, one of the BREs in the 3'UTR (BRE-C) is involved in translational activation. Some have hypothesized that this function of Bru could be activated by phosphorylation (Reveal et al., 2010; Yoshida et al., 2004). In addition, sequences at the 3' end of the 3'UTR close to the polyadenylation signal are required for osk translational activation, which is consistent with involvement of the cytoplasmic poly-A binding protein Orb and PABP in activating osk translation (Castagnetti and Ephrussi, 2003; Chang et al., 1999; Vazquez-Pianzola et al., 2011).

At stage 10b of oogenesis, the microtubules reorganize into parallel bundles around the cortex and initiate the churning of the oocyte cytoplasm, called ooplasmic streaming, to mix the oocyte contents (Dahlgaard et al., 2007; Serbus et al., 2005; Theurkauf et al., 1992). Since osk is anchored at the posterior, its localization is maintained during ooplasmic streaming, which continues into late stages of oogenesis. At stage 11, the nurse cells contract and extrude their contents into the oocyte in a process called “nurse cell dumping”, and the nurse cells ultimately die.
Figure 1.8 Diagram of oskar mRNA and selected regulatory factors

Abbreviations: AUG = Start codons, EJC = Exon junction complex, SOLE = Spliced oskar localization element, OES = Oocyte entry signal, DD = Dimerization domain.
From stages 11 through 14 more osk is localized to the posterior. This late phase of osk localization occurs by diffusion and entrapment. While the cis-elements in the osk mRNA that are required for the late phase of osk localization have not yet been identified, the RBP Rumplestiltskin (Rump), its cofactor Lost, as well as the actin cytoskeleton and locally translated Osk protein are required (Sinsimer et al., 2011; Snee et al., 2007; Suyama et al., 2009). Accumulation of Osk protein at the posterior is reinforced by translation of the newly localized osk mRNA.

By stage 14 of oogenesis, all of the nurse cells are gone, an egg shell has formed, and the oocyte is prepared for egg activation and subsequent fertilization.

**GERM PLASM FUNCTION AND ASSEMBLY**

The *Drosophila* germ plasm is a specialized cytoplasm containing many factors necessary for directing development of the primordial germ cells (pole cells). It is also necessary for anterior-posterior patterning of the embryo. The germ plasm forms at the posterior of the oocyte in a complex hierarchy of protein-protein, protein-mRNA, and mRNA-mRNA interactions and is nucleated by the locally translated Osk protein. Translation of osk produces two isoforms due to alternative translation start sites; Long Osk, which influences the actin cytoskeleton to anchor the germ plasm to the posterior cortex and Short Osk, which recruits other factors to the germ plasm (Markussen et al., 1995; Rongo et al., 1997; Rongo et al., 1995; Tanaka et al., 2011; Vanzo et al., 2007; Vanzo and Ephrussi, 2002). Short Osk recruits Vasa (Vas) and Tudor (Tud), which are also required for germ plasm assembly (Boswell and Mahowald, 1985; Dehghani and Lasko, 2015). Vas is an RNA helicase thought to be involved in the translational activation of mRNAs in the germ plasm (Lasko and Ashburner, 1988). Tud is the founding member of the family of proteins that contain the eponymous tudor domain. Tud contains 11 tudor domains (Arkov et al., 2006) and binds to symmetrically dimethylated arginines (sDMAs) on another germ plasm protein, Aubergine (Aub) (Liu et al., 2010). Tud is expected to bind other sDMA modified germ plasm proteins as well (Ren et al., 2014; Thomson and Lasko, 2004) acting to form the protein ensembles of germ granules. Consistent with this, the sDMA methyltransferase Capsuleen and its cofactor Valois are also recruited to the germ plasm by Short Osk (Anne et al., 2007; Gonsalvez et al., 2006). In addition to
Osk, Vas, and Tud, which are crucial to germ plasm establishment, the germ plasm contains many other proteins as well as mitochondria.

Over 100 mRNAs are also enriched in the germ plasm (Jambor et al., 2015; Lecuyer et al., 2007). Since the germ plasm directs the development of the future germ cells, it is critical to the survival of the species. As might be expected of such an important group of molecules, mRNAs in the germ plasm are carefully organized for proper regulation. Among these, nanos (nos), polar granule component (pgc), and germ cell-less (gcl) have been well studied. The nos, pgc, and gcl mRNAs arrive at the posterior in single mRNA-copy RNPs and are then assembled into the germ granules, which ensure their faithful incorporation in pole cells as described below. Interestingly, despite the uniform distribution of proteins such as Vas, Osk, Tud, and Aub in germ granules, the mRNAs assemble into distinct sub-granular homotypic clusters (Little et al., 2015; Trcek et al., 2015). This homotypic cluster formation is a two-step process beginning with recruitment of one mRNA to the granule and subsequent recruitment of like mRNAs. Independent signals in the 3'UTRs mediate the initial targeting to germ granules and subsequent self-assembly of homotypic clusters (Eagle et al., 2018; Niepielko et al., 2018). In the germ plasm, osk mRNA also forms large RNPs together with Stau, but these are distinct from germ granules (Little et al., 2015; Trcek et al., 2015; Vanzo et al., 2007).

The nos, pgc, and gcl mRNAs all localize by a similar diffusion and entrapment mechanism guided by their 3'UTRs (Forrest and Gavis, 2003; Ganguly et al., 2012; Little et al., 2015; Rangan et al., 2009; Trcek et al., 2015). They are then maintained in the germ plasm by persistent microtubule-mediated trafficking of germ granules towards the posterior cortex (Sinsimer et al., 2013). Despite their common localization pattern and mechanism of localization, these mRNAs are translationally activated at dramatically different times. Translational activation of nos occurs as soon as it is localized to the posterior of the oocyte (Clark et al., 2000). This results in a high local concentration of Nos protein available at the onset of embryogenesis that will translationally repress hunchback mRNA for proper anterior-posterior patterning (Curtis et al., 1997; Sonoda and Wharton, 1999). Then, when pole cells form, Nos will maintain translational repression of many mRNAs, which is essential for PGC development and function (Hayashi et al., 2004; Sato et al., 2007).
Translation of \textit{gcl} does not occur until embryogenesis when it is required for directing pole cell formation (Cinalli and Lehmann, 2013; Jongens et al., 1994; Jongens et al., 1992). PGC, which is translated after \textit{gcl}, contributes to the transcriptional quiescence of the early pole cells (Hanyu-Nakamura et al., 2008).

**EARLY DROSOPHILA EMBRYOGENESIS AND THE MATERNAL TO ZYGOTIC TRANSITION**

For about the first 2 hours after fertilization, the \textit{Drosophila} embryo develops as a syncytium; a large multi-nucleate cell. The nuclei undergo 13 semi-synchronous rounds of divisions (nuclear cycles) before they cellularize. From nuclear cycle 1 to 7 the zygotic genome is transcriptionally silent and the maternally provided factors from oogenesis have complete control over embryogenesis. Because there is no transcription during this period, all changes to protein or mRNA levels must be controlled by posttranscriptional mechanisms. At nuclear cycle 8, there is minor activation of zygotic transcription, but development is still mostly directed by maternal gene products.

After nuclear cycle 9, the primordial germ cells (pole cells) begin to form at the site of the germ plasm under direction of germ granule factors. Pole cell formation depends on actin-based protrusions at the posterior of the embryo which form pole buds and subsequent contraction of basal actin rings (Warn et al., 1985). Initial actin remodeling is triggered by the arrival of centrosomes and their astral microtubules, which reach the posterior in association with the future pole cell nuclei (Raff and Glover, 1989; Raff et al., 1990). The centrosomes trigger the release of germ granules from the actin cytoskeleton. The germ granules are then actively transported by Dynein along the astral microtubules, resulting in their enrichment in pole cells (Lerit and Gavis, 2011). Gcl promotes both pole bud formation and cellularization (Robertson et al., 1999) through spatial regulation of the centrosomes (Lerit et al., 2017) and by blocking somatic fate specification (Pae et al., 2017).

High level zygotic genome activation (ZGA) begins at nuclear cycle 13, and is required for cellularization of the somatic nuclei at nuclear cycle 14 (Foe and Alberts, 1983) marking the mid-blastula transition (MBT) (Tadros and Lipshitz, 2009). To prepare for the switch from maternal to
Figure 1.9: *Drosophila* embryogenesis and pole cell formation

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<tr>
<th>Time after Fertilization</th>
<th>Nuclear Cycle</th>
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<td>0 min</td>
<td>NC1</td>
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<tr>
<td>30 min</td>
<td>NC2</td>
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<tr>
<td>MZT 1 hr</td>
<td>NC3</td>
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<tr>
<td>1 hr 30 min</td>
<td>NC4</td>
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<td>2 hr 30 min</td>
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- **nucleus**
- **germ granule**
- **microtubule**
- **centrosome**
zygotic control of development, there is widespread degradation of maternal mRNAs in a process called the maternal to zygotic transition (MZT) that lasts about 3 hours. This degradation begins about 1 hour after fertilization (Laver et al., 2015a) and involves many trans-acting factors that recruit degradation machineries to specific maternal mRNAs. Among these trans-acting factors are the RBPs Smaug (Smg), Brain Tumor (Brat), Pumilio (Pum), and Aub as well as microRNAs (Barckmann et al., 2015; Laver et al., 2015a; Tadros et al., 2007; Thomsen et al., 2010). Even though their unlocalized counterparts are degraded during the MZT, germ plasm-localized mRNAs are spared to ensure their enrichment in pole cells, whose MZT and ZGA processes occur about 2 hours after they do in the soma (Siddiqui et al., 2012).

Towards the end of the MZT, about 1 hour after somatic cellularization, gastrulation begins. Complex cellular migrations during gastrulation transform the embryo from a single layer of cells into a larva that will hatch from the egg after about 20 hours. Among the many events of gastrulation, the pole cells migrate from the posterior pole to the interior of the embryo and coalesce in the somatic gonadal niche, where they will eventually give rise to the sperm or eggs of the adult fly.

1.4 MOTIVATION: IMPORTANCE OF DISTINCT GERM PLASM RNP GRANULES

A collaboration between the Gavis and Wieschaus labs published in 2015 (Little et al., 2015) raised interesting questions regarding the regulation of osk mRNA in the germ plasm. The authors found that in contrast to germ granule mRNAs such as nos, osk does not extensively colocalize with germ granule protein markers or germ granule mRNAs. Instead, osk colocalizes strongly with one of its primary regulatory RBPs, Stau, in the germ plasm forming RNP granules containing up to 250 osk mRNAs. This shows that osk is segregated from germ granule mRNAs and is packaged in a distinct type of RNP granule. Since osk is the founding member of the germ plasm, we later named these RNPs founder granules. The authors also showed that germ granules and founder granules display different localization patterns when pole cells form, with exclusive enrichment of germ granules in the pole cells. They further demonstrated the functional relevance
of segregating osk from germ granules with the result that ectopic incorporation of osk in germ granules and subsequent uptake in pole cells hinders early pole cell development.

These results led us to question how osk is excluded from pole cells. The work presented in this thesis addresses this question in two parts investigating first how osk is sequestered away from germ granules and targeted specifically for packaging in founder granules and second how founder granules prevent enrichment of osk in pole cells.
CHAPTER 2: ASSEMBLY OF OSKAR mRNA IN FOUNDER GRANULES
2.1 INTRODUCTION

Enrichment of osk mRNA and its translation at the posterior of the Drosophila oocyte is required for proper embryonic patterning and formation of the germ cell progenitors, known as pole cells (Ephrussi et al., 1991; Lehmann and Nusslein-volhard, 1986; Smith et al., 1992). Multiple cis-acting elements in the osk mRNA together with many trans-acting factors contribute to the multi-step process of osk localization throughout oogenesis. Despite extensive studies of osk localization over the past 30 years, there are still gaps in our understanding of the factors involved. (See section 1.3 for a description of Drosophila oogenesis.)

osk is transcribed in the nurse cells and is enriched in the oocyte from stage 1 onward (Kim-Ha et al., 1991). In the nurse cells, osk is found in small RNPs containing 1 to 2 osk mRNAs (Little et al., 2015). Transport of osk from the nurse cells to the oocyte relies on a stem loop structure in the 3'UTR called the oocyte entry signal (OES) that is linked to Dynein by the RBP Egalitarian (Dienstbier et al., 2009; Jambor et al., 2014). In the oocyte, the osk RNPs multimerize, resulting in RNPs containing up to 4 osk mRNAs (Little et al., 2015).

From stage 8 to 10, osk is specifically localized to the posterior of the oocyte via Kinesin-mediated active transport (Brendza et al., 2000; Ephrussi et al., 1991; Kim-Ha et al., 1991; Zimyanin et al., 2008). Kinesin is recruited to osk by the Tropomyosin isoform Tm1C (Veeranan-Karmegam et al., 2016), which binds directly to the osk 3'UTR. In addition to Kinesin, many proteins have been identified that contribute to this localization process by promoting transport and/or translation of the localized mRNA, which positively feeds back to reinforce osk mRNA localization (Markussen et al., 1995; Rongo et al., 1995). One of these is the double stranded RBP (dsRBP) Staufen (Stau), which associates with osk RNPs upon their entry to the oocyte and throughout the rest of oogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991; Little et al., 2015; St Johnston et al., 1991). Two Stau dsRBDs, dsRBD2 and dsRBD5, contribute respectively to osk active transport and osk translational activation at the posterior (Micklem et al., 2000).

Among other proteins, components of the exon junction complex (EJC) including eIF4AIII, Y14, Mago Nashi, and Barentsz were all found to contribute to osk localization during mid-stages
of oogenesis (Hachet and Ephrussi, 2001; Mohr et al., 2001; Newmark and Boswell, 1994; Newmark et al., 1997; Palacios et al., 2004; van Eeden et al., 2001). The EJC is deposited on mRNAs 20-24 nucleotides upstream of exon-exon junctions in a splicing dependent manner, and is involved in mRNA quality control through nonsense-mediated mRNA decay (Fribourg et al., 2003; Gehring et al., 2003; Kataoka et al., 2001; Kim et al., 2001; Le Hir et al., 2001; Lykke-Andersen et al., 2001). Consistent with splicing dependent deposition of the EJC, it was found that osk localization requires splicing and that the 3'UTR is not sufficient for active transport of the mRNA to the posterior as initially thought (Hachet and Ephrussi, 2004). However, the 3'UTR does contribute to osk localization through an mRNA-mRNA interaction between a short sequence at the tip of the OES called the dimerization domain (DD) that promotes higher order osk RNP formation (Jambor et al., 2011). While, the osk 3'UTR allows the localization of a lacZ-osk3'UTR transgenic reporter RNA through this "hitchhiking" interaction, the osk 3'UTR is not required for osk localization and is not sufficient in the absence of endogenous osk.

Even though the osk gene contains 3 introns, deposition of the EJC is only required at the first exon-exon junction for osk localization (Hachet and Ephrussi, 2004). Splicing of the first intron allows the formation of a stem loop structure between the last 18 nucleotides of the first exon and first 10 nucleotides of the second. This stem loop structure was named the Spliced oskar Localization Element (SOLE) and acts in conjunction with the adjacent EJC and the 3'UTR to mediate osk localization during mid oogenesis (Ghosh et al., 2012). Live imaging studies showed that the EJC/SOLE activates Kinesin motility leading to posterior osk enrichment (Gaspar et al., 2017).

Following stage 10, the nurse cells initiate a cell death program and contract to extrude their contents into the oocyte. Just prior to this event, the microtubules reorganize into parallel bundles around the cortex of the oocyte allowing mixing of the oocyte and nurse cell contents in a process called ooplasmic streaming (Dahlgaard et al., 2007; Serbus et al., 2005; Theurkauf et al., 1992). During this time, osk mRNA is maintained at the posterior through an actin-based anchor that requires Osk protein, and more osk mRNA accumulates at the posterior (Sinsimer et al., 2011; Snee et al., 2007; Suyama et al., 2009). Localized osk RNPs grow in number and size and can
contain up to 250 osk mRNAs (Little et al., 2015). Other mRNAs and proteins also continue to localize to the posterior to complete the formation of the germ plasm, which contains the determinants for pole cell development. In the germ plasm, mRNAs that will become enriched in pole cells are packaged in well-characterized granules called germ granules. In contrast, osk mRNA is packaged in compositionally distinct granules that we have named founder granules, since osk is the founding member of the germ plasm. The late phase of osk mRNA localization occurs by a diffusion and entrapment mechanism similar to and concurrent with localization of germ granule mRNAs such as nanos (nos) (Glotzer et al., 1997; Sinsimer et al., 2011). Localization of both osk and nos during late oogenesis requires the RBP Rumplestiltskin (Rump), which binds directly to both osk and nos. Rump’s cofactor Lost is also required for localization of both osk and nos during late oogenesis (Sinsimer et al., 2011).

It is currently unclear how these mRNAs that localize simultaneously by a similar diffusion and entrapment process and involve common factors are effectively segregated to distinct RNP granules in the germ plasm. The work presented in this chapter addresses this question with two aims. The first aim investigates whether the EJC/SOLE and osk 3’UTR function in the late phase of osk localization. Involvement of an osk-specific localization element could explain the distinction in granule targeting. The second aim tests whether the 3’UTRs of osk and nos are sufficient for targeting the mRNAs to their respective RNP granules in the germ plasm.

2.2 Results

Late Phase Oskar mRNA Localization Does Not Require the EJC

To determine whether elements involved in the active transport of osk during mid-oogenesis also function in osk localization during late oogenesis, we generated flies expressing an osk transgene tagged with superfolder gfp (sfgfp) and ty1 epitope sequences (osk-ty1-sfgfp-osk3’UTR), as well as versions with either a deletion of the first two introns to disrupt splicing-dependent deposition of the EJC adjacent to the SOLE (oskΔi1,2-ty1-sfgfp-osk3’UTR), replacement of the osk 3’UTR by the inert tub 3’UTR (osk-ty1-sfgfp-tub3’UTR), or both (oskΔi1,2-ty1-sfgfp-tub3’UTR) (Figure 2.1 A). The sfgfp sequence tag allows for detection of the transgenic
Figure 2.1: The EJC is not required for late phase oskar mRNA localization

(A) Diagrams and abbreviations of sfgfp-tagged transgenes. Grey = osk sequences. White = introns. Green = sfgfp sequences. Black = tub 3'UTR sequences. (B-E) Confocal z projections of sfgfp smFISH signal of stage 8 egg chambers expressing transgenes described in (A). Yellow dashed lines indicate the oocyte-nurse cell boundary. (F-I) Confocal z projections of all germ plasm localized sfgfp smFISH signals of osk 3'UTR transgenes described in (A) at stages 10 (F, H) and 13 (G, I). (J) Quantification of total localized sfgfp smFISH signal intensities. n = 10 oocytes each. Values are mean ± standard deviation. * p < 0.05, ** p < 0.01, *** p < 0.001
mRNA in the context of endogenous osk, which is necessary for initial germ plasm establishment, since the Δi1,2 and tub 3′UTR transgenic mRNAs are not expected to localize in mid-oogenesis.

Unexpectedly, we found that the tub 3′UTR transgenic mRNAs were not stably expressed. Levels of both the osk-ty1-sfgfp-tub3′UTR and oskΔi1,2-ty1-sfgfp-tub3′UTR mRNAs in the nurse cells (Figure 2.1 D, E) and oocyte were dramatically lower than both the osk-ty1-sfgfp-osk3′UTR and oskΔi1,2-ty1-sfgfp-osk3′UTR mRNAs (Figure 2.1 B, C). This indicates that the osk 3′UTR is important for promoting osk mRNA stability, and precluded us from using these transgenes to determine whether the osk 3′UTR functions in late phase osk localization.

Quantification of the total localized sfgfp smFISH signal intensity at stage 10 showed that both the osk-ty1-sfgfp-osk3′UTR and oskΔi1,2-ty1-sfgfp-osk3′UTR mRNAs could localize during mid-oogenesis (Figure 2.1 F, H). However, localization of the oskΔi1,2-ty1-sfgfp-osk3′UTR mRNA was less efficient than the osk-ty1-sfgfp-osk3′UTR mRNA (Figure 2.1 J). This is consistent with the finding that without EJC deposition on osk mRNA, transport efficiency of osk RNPs is reduced by 83% (Ghosh et al., 2012) and suggests that the oskΔi1,2-ty1-sfgfp-osk3′UTR mRNA only localizes by hitchhiking with endogenous osk using the DD in the 3′UTR (Jambor et al., 2011).

By stage 13, the localized signal intensities of the osk-ty1-sfgfp-osk3′UTR and oskΔi1,2-ty1-sfgfp-osk3′UTR mRNAs increased by 34% and 43%, respectively (Figure 2.1 J). Therefore, the EJC is not required for late phase osk localization. Furthermore, since the EJC promotes the activity of Kinesin associated with osk mRNA (Gaspar et al., 2017), the more efficient localization of the oskΔi1,2-ty1-sfgfp-osk3′UTR mRNA compared to the osk-ty1-sfgfp-osk3′UTR transgenic mRNA suggests that the EJC may partially antagonize posterior osk enrichment during late oogenesis. The EJC could continue to promote the association of some osk mRNA with microtubules through Kinesin activation. This association would remove the mRNA from the pool of mRNAs streaming through the oocyte in late oogenesis and thus decrease the efficiency of osk mRNA localization.

**Late Phase oskar Localization Requires a SOLE UA-rich Sequence**

While the oskΔi1,2-ty1-sfgfp-osk3′UTR mRNA is not bound by the EJC at the first exon-exon junction, it can form an intact SOLE structure. To test whether the SOLE influences late phase
osk localization independent of the EJC, we generated a version of the osk-ty1-sfgfp-osk3'UTR transgene with a substitution of SOLE proximal stem sequence (Figure 2.2 A) with lacZ sequence (SOLE\textsuperscript{PS-Lz}) that was previously shown to disrupt SOLE localization activity during mid-oogenesis (Ghosh et al., 2012). As expected, the SOLE\textsuperscript{PS-Lz} mRNA localized to the posterior of the oocyte by stage 10 in the presence of endogenous osk, although with reduced efficiency compared to the osk-ty1-sfgfp-osk3'UTR (SOLE\textsuperscript{WT}) control mRNA (Figure 2.2 B-D). This is consistent with the reduced localization efficiency at stage 10 of the osk\textDelta{i1,2}-ty1-sfgfp-osk3'UTR mRNA. However, in contrast to the osk\textDelta{i1,2}-ty1-sfgfp-osk3'UTR mRNA, the SOLE\textsuperscript{PS-Lz} mRNA was not further enriched by stage 13 (Figure 2.2 B, I). Therefore, the SOLE is required for late phase osk localization, and this role is independent of the EJC.

One possibility for how the SOLE can act independently of the EJC during late phase osk localization is by switching from cooperation with the EJC to the hnRNP F/H homolog Glorund (Glo). Glo serves in diverse roles during oogenesis including in late phase osk localization (Kalifa et al., 2009; Tamayo, 2018). Glo has two distinct mRNA binding modes: one for binding UA-rich sequences and one for binding short G-tract sequences (Tamayo et al., 2017). Glo binds osk mRNA in vivo and specifically binds the UA-rich proximal stem of the SOLE and a nearby G-tract in vitro (Tamayo, 2018) (Figure 2.2 A). Glo mutants that disrupt its ability to bind UA-rich and G-tract sequences both affect late phase osk localization (Tamayo, 2018). To directly test whether these sequences are required for late phase osk localization, we generated versions of the osk-ty1-sfgfp-osk3'UTR transgene with either the G-tract (SOLE\textsuperscript{GAG}) or the UA-rich sequence (SOLE\textsuperscript{UA-mut}) disrupted. Like the SOLE\textsuperscript{PS-Lz} mRNA, the SOLE\textsuperscript{UA-mut} mRNA showed reduced localization efficiency at stage 10 in the context of endogenous osk and failed to enrich further at stage 13 (Figure 2.2 B, E, J). A compensatory mutation of SOLE\textsuperscript{UA-mut} that restored the proximal stem structure, but not the UA-rich sequence restored localization efficiency at stage 10, but did not restore late phase localization (Figure 2.2 B, F, K). Therefore, the UA-rich sequence of the proximal stem, but not the structure alone is required for late phase osk localization. This is in contrast to the early phase, which relies on the structure, but not the sequence of the proximal stem (Ghosh et al., 2012). In combination with previous Glo mutant analyses, this result suggests that Glo
Figure 2.2: The SOLE is required for late phase oskar mRNA localization

(A) Diagram of the SOLE and adjacent G-tract region. Red box = UA-rich proximal stem. Blue box = G-tract. (B) Quantification of total localized sfgfp smFISH signal intensities. n = 8 oocytes each. Values are mean ± standard deviation. * p < 0.05, ** p < 0.01, *** p < 0.001. (C-L) Confocal z projections of all germ plasm localized sfgfp smFISH signals of SOLE-WT (C,H), SOLE-PS-Lz (D,I), SOLE-UA-mut (E,J), SOLE-UA-mut-comp (F,K), and SOLE-GAG (G,L) transgenes at stage 10 (C-G) and 13 (H-L).
binding at the UA-rich proximal stem mediates late phase osk localization. The $SOLE^{GAG}$ mRNA showed normal localization at both stage 10 and stage 13 (Figure 2.2 B, G, L). Therefore, although Glo can bind this SOLE proximal G-tract in vitro, this G-tract is not required for late phase osk localization in vivo.

**THE OSKAR AND NANOSES 3’UTRs DIRECT GRANULE TARGETING**

Germ granule mRNAs are enriched in germ granules in a two-step process: targeting and clustering. In the targeting step, a single mRNA associates with a preformed germ granule protein ensemble. Then, this mRNA acts as a seed that recruits more mRNAs of the same type to form homotypic clusters (Niepielko et al., 2018). Analysis of the germ granule mRNA *polar granule component (pgc)* showed that distinct localization elements in the 3’UTR independently mediate these targeting and clustering steps (Eagle et al., 2018). Separate targeting and clustering elements have also been identified in the nos 3’UTR (Niepielko and Gavis, unpublished).

To determine whether the osk 3’UTR similarly contains localization elements that specifically mediate assembly of founder granules, we generated flies expressing osk and nos transgenes with their 3’UTRs exchanged and asked whether they were targeted to germ granules or founder granules (Figure 2.3 A). To allow detection by smFISH in the context of endogenous osk and nos, the osk and nos transgenes were tagged with *sfgfp* and *egfp* sequences, respectively. We performed dual smFISH and immunofluorescence to observe the transgenic mRNAs labeled with *sfgfp-647* or *egfp-647* smFISH probes, germ granules labeled with *cycB-565* smFISH probes, and founder granules marked by Stau-488 immunofluorescence. Because the transgenes were expected to produce GFP-labeled proteins that could associate with germ granules, we defined germ granule-association as 647 signal (transgenic mRNA) colocalized with any 565 signal (*cycB*). We defined founder granule association as 647 signal colocalized with 488 signal (Stau) that was not associated with 565 (*cycB*) which should represent Stau only.

Based on the previous finding that fewer than 25% of osk particles in the germ plasm colocalize with germ granules (Little et al., 2015), we set a threshold for granule association at 25%
Figure 2.3: The *oskar* and *nanos* 3'UTRs are sufficient for granule targeting

(A) Diagrams and abbreviations of transgenes. Blue = *oskar* sequences. Green = *sfgfp* or *egfp* sequences. Orange = *nos* sequences. (B-I) Confocal z projections of *sfgfp* or *egfp* smFISH signal in the germ plasm of early embryos with *cycB* smFISH signal marking germ granules (B-E) or Stau IF signal marking founder granules (F-I). Note that *cycB* is associated with about 50% of germ granules (Little et al., 2015). (J) Quantification of colocalization of transgenic mRNAs with germ granules or founder granules. n = 5 embryos each. Values are mean ± standard deviation.
of sfAFP or egFP particles colocalized with germ granule or founder granule signals as described above. The control osk-ty1-sfgfp-osk3'UTR and egfp-nos-nos3'UTR mRNAs behaved as expected. About 45% of osk-ty1-sfgfp-osk3'UTR particles colocalized with founder granules (Figure 2.3 F, J), whereas colocalization with germ granules was below the 25% cutoff (Figure 2.3 B, J). Conversely, about 50% of egfp-nos-nos3'UTR particles colocalized with cycB-containing germ granules, (Figure 2.3 C, J), similarly to the behavior of nos (Little et al., 2015). In contrast, colocalization with founder granules was below the 25% cutoff (Figure 2.3 G, J). Thus, the granule preference of the 3'UTR exchanged transgenic mRNAs was determined by the 3'UTRs. Like egfp-nos-nos3'UTR, about 50% of osk-ty1-sfgfp-nos3'UTR particles were colocalized with germ granules (Figure 2.3 D, J), whereas fewer than 25% colocalized with founder granules (Figure 2.3 H, J). Furthermore, similarly to osk-ty1-sfgfp-osk3'UTR, about 55% of egfp-nos-osk3'UTR particles colocalized with founder granules (Figure 2.3 I, J), whereas fewer than 25% colocalized with germ granules (Figure 2.3 E, J). Taken together, these results indicate that the osk and nos 3'UTRs are sufficient to target mRNAs to founder granules and germ granules, respectively.

The oskar Coding Sequence or 5'UTR Mediates Enrichment in RNP Granules

We noticed that the egfp-nos-osk3'UTR mRNA did not appear to become enriched in the germ plasm as well as the osk-ty1-sfgfp-nos3'UTR, osk-ty1-sfgfp-osk3'UTR, and egfp-nos-nos3'UTR mRNAs (Figure 2.4 A-D). To investigate this observation, we quantified the fluorescence intensities of the transgenic mRNA puncta colocalized with their respective granules relative to fluorescence intensities of the unlocalized transgenic mRNA particles and normalized to granule size as determined by the colocalized cycB or Stau relative intensities. Whereas the egfp-nos-nos3'UTR puncta associated with germ granules were not significantly different in size from the osk-ty1-sfgfp-osk3'UTR puncta associated with founder granules, the egfp-nos-osk3'UTR puncta associated with founder granules were significantly smaller than the osk-ty1-sfgfp-osk3'UTR particles (Figure 2.4 E). The ability of the egfp-nos-osk3'UTR mRNA to colocalize with founder granules at least as well as osk-sfgfp-osk3'UTR but not enrich within them indicates the presence of a targeting element in the osk 3'UTR and an element that further mediates enrichment in founder
Figure 2.4: The oskar CDS or 5'UTR mediates enrichment in RNP granules

(A-D) Confocal z projections of sfgfp or egfp smFISH signal in the germ plasm of nuclear cycle 3-5 embryos expressing transgenes described in Fig. 2.3 A. (E) Quantification of size of germ plasm localized particles relative to unlocalized particle intensity and normalized to granule size. n = 5 embryos each. Values are mean ± standard deviation. * p < 0.05, ** p < 0.01, *** p < 0.001
granules in the osk CDS or 5'UTR. Interestingly, the osk-ty1-sfgfp-nos3'UTR particles associated with germ granules were significantly larger than granule associated particles of all the other transgenic mRNAs indicating enhanced enrichment activity (Figure 2.4 E). The result that osk-ty1-sfgfp-nos3'UTR particles did not show significant association with founder granules indicates that the osk CDS and 5'UTR do not contain a targeting signal. Rather, these data combined suggest the presence of an additional element in the osk CDS or 5'UTR that mediates accumulation in RNP granules in the germ plasm.

2.3 DISCUSSION

Although the germ granule mRNA nos and the founder granule mRNA osk both localize to the germ plasm during late oogenesis by a diffusion and entrapment mechanism and rely on common factors for this localization such as Rump and Lost (Glotzer et al., 1997; Sinsimer et al., 2011), they are specifically assembled into distinct RNP granules in the germ plasm (Little et al., 2015). In this chapter, we show that granule targeting elements in the osk and nos 3'UTRs determine the granule into which the mRNAs are incorporated. Furthermore, our results suggest that the CDS or 5'UTR of the osk mRNA contribute to enrichment of osk mRNA in RNP granules. Together with the finding that the SOLE is required for late phase localization, these results suggest that the SOLE and osk 3'UTR both contribute to osk mRNA enrichment in RNP granules. However, colocalization of osk-ty1-sfgfp-nos3'UTR mRNA only with germ granules, suggests that the potential founder granule-targeting activity of the SOLE cannot overcome the germ granule-targeting activity of the nos 3'UTR. Therefore, we expect the contribution of the SOLE to enrichment of osk in RNP granules to be relatively weak.

The reduced enrichment of egfp-nos-osk3'UTR is also consistent with the presence of a clustering element within the osk CDS or 5'UTR, however further analyses are necessary to test for clustering of osk mRNA in founder granules and the presence of an osk clustering element. Super resolution imaging can be used to determine whether osk mRNA clusters in founder granules like nos and pgc do in germ granules. It would also be worthwhile to narrow down the locations or
exact sequences of the osk targeting and clustering elements, as this could provide additional insight into how founder granules and germ granules assemble side by side as distinct structures.

We also assessed the function of the early acting EJC/SOLE complex in late phase osk localization. We found that the EJC is not required for late phase osk localization, but that the SOLE is. In contrast to its earlier role in osk localization before stage 10 of oogenesis that relied only on the structure of the SOLE proximal stem (Ghosh et al., 2012), the UA-rich sequence of the proximal stem is important for localization after stage 10 of oogenesis. Combined with the previous findings that Glo can bind the SOLE in vitro and that its corresponding UA-rich sequence binding activity is necessary for osk localization (Tamayo, 2018), our results suggest that the SOLE switches from partnering with the EJC for early phase osk localization to partnering with Glo for late phase osk localization. Glo G-tract binding ability is also necessary for osk localization (Tamayo, 2018). However, we found that osk localization does not require the SOLE proximal G-tract. Thus, Glo may bind a different G-tract in the osk mRNA, or the Glo G-tract binding activity may be indirectly required for osk mRNA localization.
CHAPTER 3: RNP GRANULE MAINTENANCE
3.1 INTRODUCTION

RNP structure and dynamics must be carefully tuned to ensure proper mRNA regulation and prevent diseases associated with hyper and hypo RNP formation as described in Chapter 1.2 (Shukla and Parker, 2016). Different strategies exist for controlling RNP stability. RNP assembly is driven by the summation of many weak protein-protein, protein-mRNA, and mRNA-mRNA interactions (Van Treeck and Parker, 2018). These interactions can be affected positively or negatively by local protein and mRNA concentration, PTMs, translational status of the mRNA, and both mRNA and protein turnover (Buchan, 2014; Hofweber, 2018).

Once RNP granules form, they may require prolonged stabilization to achieve their purpose. For example, Drosophila germ granules formed during oogenesis are maintained at the posterior cortex until they are needed to direct pole cell development during embryogenesis. Under standard conditions, the time from the onset of germ granule formation in the oocyte to the onset of pole cell formation in the embryo is about 10 hours, but can take days when egg laying is postponed due to suboptimal conditions such as lack of food or other stressors. During this time, germ granules are stably anchored at the posterior cortex by an actin network (Forrest and Gavis, 2003).

Like germ granules, founder granules are maintained at the posterior during the oocyte to embryo transition. The likely purpose of founder granule maintenance is investigated in Chapter 4 and further discussed in Chapter 5. Since founder granules have not been extensively characterized, little is known about factors influencing their stability. We made the serendipitous discovery in mutants of the Drosophila CCR4 deadenylase twin, that founder granules are smaller than normal in terms of number of osk mRNAs in late stage oocytes (Figure 3.1) and set out to further characterize this phenotype as described below.
Figure 3.1: Founder granules are small in *twin* mutant oocytes

(A,B) Confocal z projections of *oskar* smFISH signal in the germ plasm of wild type (A) and *twin* mutant (B) stage 13 oocytes. (C) Quantification of the average size of *osk* particles in the germ plasm of wild type and *twin* mutant stage 13 oocytes. n = 4 - 5 oocytes each. Values are mean ± standard error of the mean. * p < 0.05. ** p < 0.01, *** p < 0.001
3.2 RESULTS

REDUCED OSKAR mRNA LEVELS DO NOT AFFECT FOUNDER GRANULE SIZE

We found that there was an approximate 50% reduction in the amount of osk mRNA localized to the germ plasm in twin mutants (Figure 3.2 A). To determine whether this reduction caused the smaller founder granules observed in twin mutants, we measured founder granule sizes in osk<sup>A87/+</sup> oocytes. The osk<sup>A87</sup> allele is an RNA null (St Johnston unpublished), and in a heterozygous background there is about 50% less mRNA produced (Figure 3.2 B) and about half the amount localized to the germ plasm than in wild-type oocytes (Figure 3.2 A). However, founder granule size was unaffected in osk<sup>A87/+</sup> oocytes (Figure 3.2 C). Therefore, the reduction of localized osk in twin mutants does not explain the small founder granule size. Furthermore, osk mRNA instability does not explain the decrease in founder granule size or osk mRNA localization as twin mutant oocytes contain wild-type total osk mRNA levels (Figure 3.2 B).

RNPs ARE DESTABILIZED IN TWIN MUTANT OOCYTES

Interestingly, looking earlier in oogenesis, we found that founder granules in stage 10 twin mutant oocytes were wild-type in size (Figure 3.3 A), indicating that they are destabilized between stages 10 and 13 of oogenesis. To determine whether this destabilization was specific to founder granules, we asked whether unlocalized osk RNPs were also destabilized. Interestingly, unlocalized osk RNPs in twin mutant stage 10 nurse cells, stage 10 oocytes, and stage 12 oocytes are wild-type in size, but are small in twin mutant stage 13 oocytes (Figure 3.3 B). This suggests that Twin functions in stabilizing both localized and unlocalized osk RNPs in late stage oocytes. Furthermore, we found that this role is not specific to osk RNPs, since germ granules are also destabilized between stages 12 and 13 in twin mutant oocytes as indicated by a reduction in the number of cyclin B (cycB) mRNAs in germ granules (Figure 3.3 C). However, in contrast to osk, there were also fewer cycB mRNAs in germ granules in stage 12 twin mutant oocytes than in wild-type oocytes. This is consistent with the reduction in localized osk in twin mutants leading to reduced recruitment of germ granule components, which is known to reduce the size of germ granules (Niepielko et al., 2018). Therefore, RNP destabilization is a general effect of twin deficiency.
Figure 3.2: Decreased oskar mRNA level does not affect founder granule size

(A) Quantification of total localized osk smFISH signal intensities in wild type, twin mutant, and osk<sup>ABT</sup>/+ stage 13 oocytes. n = 5 - 15 oocytes each. (B) Quantification of the total amount of osk mRNA relative to rpl7 mRNA by RT-qPCR in wild type, twin mutant, and osk<sup>ABT</sup>/+ stage 13-14 oocytes. n = 3 biological replicates each. (C) Quantification of average size of osk particles in the germ plasm of wild-type, twin mutant, and osk<sup>ABT</sup>/+ stage 13 oocytes. n = 5 - 7 oocytes each. All values are means ± standard deviation. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 3.3: RNPs are destabilized in twin mutant oocytes after stage 12

(A) Quantification of average size of osk particles in the germ plasm and twin mutant stage 10 and 13 oocytes. n = 4 – 5 oocytes each. Values are mean ± standard error of the mean. (B) Quantification of average size of unlocalized osk particles in wild-type and twin mutant stage 10, 12, and 13 oocytes. n = 4 – 7 oocytes each. Values are mean ± standard deviation. (C) Average size of cycB particles in the germ plasm of wild-type and twin mutant stage 12 and 13 oocytes. n = 5 – 6 oocytes each. Values are mean ± standard error of the mean. * p < 0.05, ** p < 0.001, *** p < 0.001.
3.3 Discussion

RNPs in the germ plasm form during oogenesis and serve important functions in embryogenesis. Therefore, these RNPs must be stably maintained during the oocyte to embryo transition and through the early stages of embryogenesis until their functions are required. In the work presented here we show that RNPs in the germ plasm as well as smaller unlocalized RNPs are destabilized in twin mutant oocytes indicating that a general RNP stabilizing condition is disrupted in the absence of Twin function.

Further studies are necessary to determine why RNPs are destabilized in the twin mutant background. Presumably poly(A) tail shortening is important to RNP stabilization. This is counterintuitive since deadenylation is generally associated with mRNA destabilization, which in turn should destabilize RNPs. Whether the RNP destabilization in twin mutants is a direct effect of the poly(A) status of the mRNAs packaged in them or an indirect effect altering the abundance or activity of a stabilizing or destabilizing trans-acting factor is unknown. Future experiments addressing changes to the transcriptome and proteome in twin mutant oocytes could provide interesting candidates for follow up studies. Prior to these experiments the phenotypic impact of this RNP destabilization should be addressed to determine the biological significance of RNP stabilization specifically in late stage oocytes. Since we see a reduction in germ granule size, it is likely that fewer pole cells form during embryogenesis, which could affect fertility.
CHAPTER 4: FOUNDER GRANULE FUNCTION IN OSKAR REGULATION
4.1 INTRODUCTION

By allowing precise spatiotemporal regulation, posttranscriptional regulation of gene expression provides a level of control beyond what transcriptional regulation alone can achieve. This is especially important under conditions when transcription is inactive, which is the case during the earliest stages of embryogenesis for all organisms. With its relatively late zygotic genome activation (ZGA), approximately 2 hours after fertilization, and consequent reliance on maternal mRNAs for its early developmental program, Drosophila has provided a valuable model for studying posttranscriptional mRNA regulation. Mechanisms including mRNA localization, localized translation, and mRNA degradation all contribute to the spatial and temporal regulation of maternal mRNAs necessary to establish the body axes, specify germline fate, and transition from maternal to zygotic gene expression.

The transcriptionally quiescent oocyte is supported by transcriptionally active cells called nurse cells, which deposit mRNAs and proteins in the oocyte via cytoplasmic bridges. Many of these maternally supplied mRNAs become enriched in specific subcellular locations in the oocyte for local protein production. For example, 16% and 4% the maternally supplied mRNA of the genes oskar (osk) and nanos (nos) are localized to the posterior of the oocyte (Bergsten and Gavis, 1999) where they are translationally activated, while the unlocalized populations remain translationally repressed.

Locally produced Osk protein nucleates formation of a specialized cytoplasm called the germ plasm that contains hundreds of mRNAs involved in germline development during embryogenesis. Many of these mRNAs are packaged in at least two types of ribonucleoprotein (RNP) granules in the germ plasm. RNP granules are non-membrane bound organelles consisting of RNA and protein and influence all aspects of mRNA regulation. RNPs serve to compartmentalize regulatory events thereby segregating potentially antagonistic reactions. They also promote efficient mRNA regulation through the co-regulation of related mRNAs. Of the RNPs in the Drosophila germ plasm, the germ granules have been well-characterized. Germ granules are necessary for the development and function of the germ cell precursors (pole cells) (Hanyu-
Nakamura et al., 2008; Jongens et al., 1994; Kobayashi et al., 1996; Mahowald, 2001). The only known mRNA component of the other germ plasm RNP granule is osk. Because germ plasm formation begins with osk localization to the posterior of the oocyte (Mahowald, 2001), we refer to the osk-containing RNPs as founder granules.

The *Drosophila* embryo develops as a syncytium that undergoes 13 rounds of semi-synchronous nuclear divisions (nuclear cycles) before cellularization. During nuclear cycle 9, approximately one hour after fertilization, a subset of nuclei reach the germ plasm and the pole cells form under direction of germ granule factors. As the pole cells form, germ granules become enriched in them and continue to direct germline development (Lerit and Gavis, 2011). About 2 hours after fertilization the rest of the embryo cellularizes and ZGA begins. Concurrently, there is widespread clearance of maternal mRNAs to switch control of development from maternally provided factors to zygotically produced gene products. Maternal mRNAs that have been sequestered in the pole cells are not subject to this wave of mRNA degradation, but instead are degraded by a later acting MZT that is specific to the pole cells (Siddiqui et al., 2012). Gastrulation begins at about 3 hours after fertilization. During gastrulation, the pole cells collectively migrate inside the embryo and ultimately coalesce in the gonads where they will give rise to either the sperm or the eggs in the adult fly. (Figure 4.1)

Previously, we have shown that, unlike germ granules, founder granules are not enriched in pole cells. We demonstrated the functional relevance of this distinction by targeting osk to germ granules by swapping its 3’ untranslated region (UTR) with that of nos. The subsequent uptake of osk in pole cells impaired germline development, resulting in formation of fewer pole cells, defective pole cell migration during gastrulation, and poor coalescence in the gonad (Little et al., 2015). This highlights the importance of separating and precisely regulating these two populations of mRNAs that both occupy the germ plasm during pole cell formation.

In the work presented here, we examine the mechanism that prevents uptake of germ plasm-enriched osk mRNA in pole cells. Our results show that leading up to pole cell formation, founder granules associate with mRNA degradation machineries. The subsequent degradation of
Figure 4.1: Events of early *Drosophila* embryogenesis

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germ plasm-localized osk results in founder granule destabilization and ultimately prevents uptake of excess osk in pole cells. This degradation of osk in the germ plasm occurs by a distinct mechanism from the degradation of unlocalized osk during the MZT.

4.2 RESULTS

INCORPORATION OF UNTRANSLATABLE OSKAR mRNA INTO POLE CELLS IMPAIRS GERMLINE DEVELOPMENT

The adverse effects on pole cell development caused by forcing osk into germ granules (Little et al., 2015) could be due to osk mRNA itself or to an excess of Osk protein in the pole cells. To distinguish between these possibilities, we targeted an untranslatable osk transcript to pole cells. osk mRNA is translated to produce two isoforms, Long Osk and Short Osk, using two alternative start codons. We engineered mutations that change both start codons to arginines (M1, 139R) in the oskΔ1,2-nos3′UTR (oΔn) transgene, which expresses osk mRNA carrying the nos 3′UTR for germ granule targeting instead of its own localization elements (Figure 4.2 A). Both oΔn and oM1,139RΔn transcripts were expressed at comparable levels (Figure 4.2 B), but only oΔn produced Osk protein (Figure 4.2 C, D). Like oΔn mRNA, oM1,139RΔn colocalized with germ granules and was incorporated into pole cells (Figure 4.3).

Like embryos from females expressing oΔn, embryos from females expressing oM1,139RΔn formed fewer pole cells than wild-type embryos (Figure 4.4 A-C, F). The oM1,139RΔn embryos also showed pole cell migration defects similar to those of oΔn embryos, with many pole cells failing to reach the gonad (Figure 4.4 D, E, G). Thus, mis-incorporation of osk mRNA itself in germ granules interferes with germline development, independently of Osk protein production. Moreover, regulation of osk at the mRNA level is required in the embryonic germ plasm to prevent its potential threat to germline development.
**Figure 4.2: Transgene design and expression**


(B) Quantification of transgenic mRNA levels by RT-qPCR. n = 3 biological replicates each. Values are mean ± standard deviation.  

(C) Transgenic protein expression by immunoblot with Kinesin heavy chain serving as a loading control.  

(D) Quantification of transgenic protein in the germ plasm by total localized Oskar IF intensity. n = 11 embryos each. Values are mean ± standard deviation. Note that the transgenes are expressed in a wild type *oskar* background.  

* p < 0.05, ** p < 0.01, *** p < 0.001.
(A-F) Confocal Z projections of oskar and nanos smFISH signal in the germ plasms of wild type (A,B), oΔn (C,D), and o^{M1.139R}Δn (E,F) embryos before (A,C,E) and after (B,D,F) pole cell formation.
Figure 4.4: Incorporation of oskar mRNA in pole cells hinders germline development independent of translation

(A-D) Confocal z projections of pole cells marked by Vasa IF in wild-type (A) and transgenic (B,C) embryos. (D) Example of an embryo expressing oskΔi-nos3'UTR with no lost pole cells. (E) Example of an embryo expressing oskΔi-nos3'UTR with >4 pole cells lost during migration. Arrows: pole cells in gonads. Arrow head: pole cells that have failed to reach the gonad. Note that gonads are populated by fewer pole cells in (E) as compared to (D). (F) Box and whisker plot showing quantification of number of pole cells per embryo. n = 15 embryos each. (G) Quantification of pole cells lost during migration. n = 91 - 136 embryos. * p < 0.05, ** p < 0.01, *** p < 0.001.
FOUNDER GRANULES HAVE LIMITED MOTILITY AND DO NOT DISPERSE DURING POLE CELL FORMATION

Our results suggest that segregation of osk from germ granule mRNAs by its packaging into founder granules prevents enrichment of osk in pole cells, where it impairs their development. Germ granules become enriched in the pole cells by virtue of their accumulation at centrosomes associated with posterior nuclei. This accumulation occurs by Dynein-mediated transport on astral microtubules and triggers the budding of the plasma membrane around the nuclei and associated germ granules to form the pole cells (Lerit and Gavis, 2011). During mid-oogenesis, osk RNPs are transported to the posterior of the oocyte by Kinesin (Brendza et al., 2000; Gaspar et al., 2017). Thus, in contrast to the behavior of germ granules, founder granules might be dispersed by Kinesin-mediated transport away from the pole cell nuclei, preventing their accumulation in pole cells.

To begin to address this possibility, we compared the behavior of founder granules marked with Stau-GFP and germ granules marked with Vas-mCherry in live embryos. As previously observed (Lerit and Gavis, 2011), germ granules exhibited directed motility toward embryonic nuclei at the onset of pole cell formation. By contrast, most founder granules appeared to jiggle in place. Tracking of both germ granules and founder granules showed that founder granules moved more slowly, with smaller displacements, and with fewer linear trajectories than germ granules (Figure 4.5 A-C). Whereas germ granules became clustered around nuclei as a result of their directed motility (Figure 4.5 D, F-H), the less motile founder granules remained uniformly distributed in the germ plasm (Figure 4.5 E-H). However, founder granules can be detected in pole cells, indicating that they can be passively incorporated into them (Figure 4.5 I).

GERM PLASM-LOCALIZED OSKAR IS DEGRADED DURING POLE CELL FORMATION

Although founder granules did not disperse, they appeared to decrease in number as pole cells formed, suggesting they were destabilized. To further investigate this behavior, we visualized respective founder granule and germ granule constituent mRNAs, osk and nos, before, during, and after pole cell formation by quantitative fluorescence in situ hybridization (FISH). As measured by total fluorescence intensity, the amount of osk mRNA in the germ plasm decreased by 98% from
Figure 4.5: Founder granules have limited motility and do not disperse

(A-C) Quantification of average track speed (A), linearity (B), and displacement (C) of germ and founder granules before and during pole cell formation. n = 3 movies with 491 – 3221 tracks. (D,E) Maximum projections of the first and last 25 sec of a 2-min movie of pole cell formation showing positions of germ (D) and founder (E) granules at the beginning and end of the movie. White dot indicates the center of the nucleus. (F,G) Plotted positions of germ and founder granules at the onset of (F) and 2.5 min into pole cell formation (G). Red and orange dots indicate approximate positions of nuclei in and out for the frame, respectively. Plots were divided into 4 regions of equal area to quantify the distribution of granules in each region with region 4 being the closest to the cortex (n = 108 – 248 particles) (H). (I) Fixed confocal z-series projection of osk smFISH signal in the pole cells of a nuclear cycle 10 embryo. Yellow dashed lines outline pole cells. Values are mean ± standard error of the mean. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 4.6: oskar mRNA is degraded during pole cell formation

(A-F) Confocal z-series projections showing germ plasm localized smFISH signals of osk (A-C) and nanos (D-F) at nuclear cycles 2, 9, and 12. (G) Quantification of total localized signal intensities of osk and nos. n = 5 – 10 embryos. Values show are mean ± standard deviation.
before pole cell formation to after pole cell formation (Figure 4.6 A-C, G). Consistent with previous studies showing that germ granule mRNAs are maintained in the germ plasm throughout pole cell formation (Bashirullah et al., 2001), there was no significant change in the amount of nos (Figure 4.6 D-G). This result is most consistent with degradation of osk mRNA in the germ plasm during pole cell formation.

mRNA degradation typically involves removal of the 5' cap followed by exonuclease digestion. In Drosophila, decapping is carried out by Decapping Protein 2 (Dcp2) and its cofactor Decapping Protein 1 (Dcp1) (Siwaszek et al., 2014). To confirm that osk is degraded in the germ plasm, we visualized Dcp1 by immunofluorescence together with osk by FISH in embryos from nuclear cycle 2 to nuclear cycle 12. Within the germ plasm, the frequency of detecting a particle containing one or more osk transcripts colocalized with Dcp1 began to increase at nuclear cycle 5 and continued to increase through pole cell formation (Figure 4.7 A-C). Consistent with the differential stability of osk and germ granule mRNAs in the germ plasm, we did not detect colocalization of Dcp1 with a representative germ granule mRNA, cycB (Figure 4.7 D). In addition, we found that 77% and 49% of osk particles that colocalized with Dcp1 also colocalized with the decapping complex associated protein Me31B (Figure 4.8 A, B) and the 5' to 3' exonuclease Pacman (Pcm) (Figure 4.8 C, D), respectively. Thus, degradation machinery is specifically recruited to osk in the germ plasm. We conclude that osk degradation minimizes the incorporation of osk in pole cells and ensures that any passively engulfed mRNA does not persist.

In addition to osk mRNA levels, the sizes of founder granules, as measured by the number of osk mRNAs they contain (see Methods), decreased over the course of pole cell formation (Figure 4.9 A). By contrast, germ granule size distributions, measured by the number of nos transcripts they contain, were unchanged (Figure 4.9 B). These data suggest that founder granules are selectively destabilized and osk mRNA in the germ plasm is degraded during pole cell formation.

**OSKAR mRNA IS DEGRADED WITHIN FOUNDER GRANULES**

We next considered what accounts for the specific vulnerability of osk in the germ plasm. Two scenarios are consistent with the observed decrease in founder granule size. In one, founder
Figure 4.7: oskar mRNA associates with Dcp1 in the germ plasm

(A,B) Confocal z-series projections showing osk smFISH signal and Dcp1 IF signal in the germ plasm of nuclear cycle 2 (A) and 8 (B) embryos. (C) Quantification of % of osk particles colocalized with Dcp1 from nuclear cycle 2 to 12. n = 6 – 9 embryos. Values are mean ± standard deviation. The first significant increase from nuclear cycle 2 is at nuclear cycle 5. (D) Quantification of the percent of osk or cycB particles colocalized with Dcp1 from nuclear cycle 2 to 12. n = 6 – 7 embryos. Values are mean ± standard deviation.
Figure 4.8: oskar mRNA associates with Me31B and Pcm in the germ plasm

(A) Confocal z-series projection of osk smFISH, Me31B IF, and Dcp1 IF signals in the germ plasm of nuclear cycle 7 embryos. A') Image in (A) masked by the Dcp1 channel such that only osk smFISH and Me31B IF signals overlapping with Dcp1 IF signal are visible. (B) Quantification of the percent of Dcp1-colocalized osk particles that are colocalized with Me31B or not. n = 5 embryos. (C) Confocal z-series projection of osk smFISH, Pcm IF, and Dcp1 IF signals in the germ plasm of nuclear cycle 7 embryos. (C') Image in (C) masked by the Dcp1 channel such that only osk smFISH and Pcm IF signals overlapping with Dcp1 IF signal are visible. (D) Quantification of the percent of Dcp1-colocalized osk particles that are colocalized with Me31B or not. n = 7 embryos. Values are mean ± standard deviation.
Figure 4.9: Founder granules are destabilized during pole cell formation

(A,B) Size distributions of osk (A) and nos (B) particles in the germ plasm in terms of the number of mRNAs per particle. n = 4 – 28 embryos.
granules disassemble, leaving osk accessible to the degradation machinery. Other mRNAs that remain packaged in RNP granules, like those in germ granules, would remain protected from the degradation machinery in the germ plasm. Alternatively, founder granules could serve as the site of osk degradation, thereby compartmentalizing the activity of the degradation machinery to preserve germ granule and other mRNAs that reside in the germ plasm.

To distinguish whether founder granule disassembly occurs before or after osk degradation, we first determined whether Dcp1 associates with intact founder granules containing both osk mRNA and Stau. Stau and Dcp1 were detected by immunofluorescence together with osk mRNA by FISH and the frequencies of colocalization were quantified. This analysis revealed that 35.0 ± 5.9% of Stau particles that colocalized with osk were also colocalized with Dcp1 at nuclear cycle 8, and of the Dcp1 particles colocalized with Stau, 90.0 ± 5.4% were also colocalized with osk. Thus, Dcp1 associates with intact founder granules. We also analyzed the sizes of osk particles that colocalized with Dcp1. If founder granules disassemble prior to osk degradation, Dcp1 should preferentially colocalize with small osk particles. However, we found that Dcp1 associated with osk particles of all sizes (Figure 4.10 A, B). Together, these results are consistent with osk degradation occurring within founder granules.

In a second approach, we compared the timing of: 1) osk association with degradation machineries; 2) changes in total osk levels; and 3) changes in founder granule composition from nuclear cycle 2 to 12. As described above, colocalization of osk with Dcp1 was first detected at nuclear cycle 5 (Figure 4.7 C). The amount of osk in the germ plasm decreased subsequently at nuclear cycle 7 (Figure 4.11 A), and the average size of osk particles associated with Stau declined sharply at nuclear cycle 9 (Figure 4.11 B). The amount of Stau in founder granules, measured by immunofluorescence intensity, began to decrease at nuclear cycle 10 (Figure 4.11 C) along with a dramatic decrease in the percentage of Stau particles that remained associated with osk (Figure 4.11 D). This order of events is consistent with association of the degradation machineries with founder granules leading to osk degradation, followed by the destabilization of founder granules. In addition, the percentage of osk particles colocalized with Stau remained unchanged until nuclear cycle 10, when there was a small decrease in colocalization (Figure 4.11 E). Therefore, osk mRNA...
Figure 4.10: Dcp1 associates with all sizes of oskar particles in the germ plasm

(A) Size distributions of oskar particles in the germ plasm in terms of the number of mRNAs per particle colocalized with Dcp1 (A) and of all oskar particles in the germ plasm (B). n = 6 – 9 embryos.


(A) Quantification of total fluorescence intensity of localized osk from nuclear cycle 2 to 12 relative to the average intensity at nuclear cycle 3. n = 5 – 13 embryos. (B) Quantification of the average size of osk particles colocalized with Stau in terms of the number of mRNAs from nuclear cycle 2 to 12. n = 4 – 10 embryos. (C) Quantification of the percent of Stau particles colocalized with osk from nuclear cycle 2 to 12. n = 4 – 10 embryos. (D) Quantification of the percent of osk particles colocalized with Stau from nuclear cycle 2 to 12. n = 4 – 10 embryos. (E) Quantification of the average size of Staufen particles in the germ plasm from nuclear cycle 3 to 12. n = 3 – 8 embryos each. Values in (A), (C), and (D) are mean ± standard deviation; values in (B) and (E) are mean ± standard error of the mean. T-tests compared each nuclear cycle to nuclear cycle 2 (A-D) or nuclear cycle 3 (E). * p < 0.05, ** p < 0.01, *** p < 0.001.
is not released from founder granules prior to the onset of degradation. Taken together these data support a model in which osk degradation occurs within founder granules.

**OSKAR mRNA DEGRADATION IN THE GERM PLASM IS DISTINCT FROM DEGRADATION OF UNLOCALIZED OSKAR**

Widespread clearance of maternal mRNAs occurs during the MZT and is controlled by both maternal and zygotic degradation machineries. The maternal decay machinery is set in motion by egg activation whereas the zygotic pathway is engaged following zygotic genome activation (Laver et al., 2015b). Genome-wide microarray analysis showed that osk is degraded during the MZT by both maternal and zygotic pathways (Thomsen et al., 2010). However, because only 16% of osk mRNA in the embryo is localized to the germ plasm and packaged in founder granules (Bergsten and Gavis, 1999; Little et al., 2015), this analysis predominantly measured the unlocalized population of osk mRNA in the bulk embryonic cytoplasm. Moreover, previous studies differ in their estimates of the onset of maternal mRNA degradation (Despic and Neugebauer, 2018; Laver et al., 2015b). Therefore, we sought to determine how degradation of osk in the germ plasm relates to degradation of osk during the MZT.

As a first step, we measured the change in the amount of osk per volume of germ plasm and the change in the amount of osk per volume of bulk cytoplasm in the same embryo from nuclear cycles 2 to 12 (Figure 4.12). The first significant decrease in germ plasm localized osk per volume occurred at nuclear cycle 7, consistent with our analysis of total localized osk (Figure 4.11 A). For unlocalized osk, the first significant decrease occurred at nuclear cycle 11. Thus, degradation of osk in the germ plasm begins well in advance of degradation of osk during the MZT.

Next, we asked whether osk degradation in the germ plasm is initiated by the same factors that act later during the MZT. The RNA-binding proteins Aubergine (Aub) and Brain tumor (Brat) have been shown to direct clearance of maternal mRNAs including osk in the bulk cytoplasm during the MZT (Barckmann et al., 2015; Laver et al., 2015a). We therefore asked whether these proteins also regulate degradation of osk in the germ plasm. In brat mutant embryos, degradation of osk in the germ plasm occurred similarly to that in wild-type embryos (Figure 4.13 A, C-E, F-H) indicating
Figure 4.12: *oskar* mRNA is degraded earlier in the germ plasm than in the bulk cytoplasm

(A) Quantification of *osk* smFISH fluorescence intensity per volume in the germ plasm and bulk cytoplasm from nuclear cycle 2 to 12 relative to nuclear cycle 2. The first significant changes from nuclear cycle 2 in the germ plasm and bulk cytoplasm occur at nuclear cycle 7 and 12, respectively. n = 5 embryos each. Values are mean ± standard deviation. (B-G) Confocal z-series projections of *osk* smFISH signal in the germ plasm (B,D,F) and bulk cytoplasm (C,E,G) at nuclear cycle 3 (B,C), 7 (D,E), and 12 (F,G).
Figure 4.13: Aubergine is required for oskar mRNA degradation in the germ plasm

(A,B) Quantification of localized oskar fluorescence intensity in wild type and aubergine mutant
and brain tumor mutant embryos (n = 16 – 23 embryos) (A) and wild type fertilized and
unfertilized embryos (n = 3 – 10 embryos) (B). C-K) Confocal Z projections of oskar smFISH
signal in the germ plasm of wild type and aubergine mutant and brain tumor mutant embryos.
Values in (A) are mean ± standard error; values in (B) are mean ± standard deviation. * p <
0.05, ** p < 0.01, *** p < 0.001.
that Brat is selective for osk in the bulk cytoplasm. However, osk was stabilized in the germ plasm of aub mutant embryos (Figure 4.13 A, I-K). Because most aub mutant embryos arrest development prior to pole cell formation, we assessed osk degradation in wild-type unfertilized eggs and found that osk was degraded independently of progression through embryogenesis (Figure 4.13 B). Thus, the delay in osk degradation in aub mutants is not simply a consequence of the developmental arrest. Moreover, we found that Dcp1 and osk do not colocalize in the germ plasm of aub mutant embryos (Figure 4.14 A-C). These results suggest a role for Aub in recruitment of Dcp1, resulting in decapping and subsequent degradation of osk. In addition, osk particle sizes were stabilized in the germ plasm of aub mutants (Figure 4.14 D, E). These data support the conclusion that founder granule destabilization is not a prerequisite for osk degradation and are consistent with osk degradation causing founder granule destabilization.

4.3 DISCUSSION

During oogenesis, mRNAs in the Drosophila germ plasm are sorted into germ granules and founder granules based on their fates in the embryo. Germ granules contain mRNAs necessary for germline development such as nos, pgc, and cycB and promote the enrichment of these mRNA in pole cells (Lerit and Gavis, 2011). Founder granules contain osk mRNA, which is necessary for germ plasm formation and whose accumulation in pole cells is detrimental to germline development (Little et al., 2015). Here we show that founder granules prevent enrichment of osk in pole cells by recruiting factors for compartmentalized osk degradation in the germ plasm.

Founder granule-associated osk degradation is distinct from degradation of unlocalized osk during the MZT temporally and mechanistically. Degradation of osk in the germ plasm occurs well in advance pole cell formation whereas the bulk of MZT-associated mRNA degradation occurs after pole cells have formed. osk can be passively incorporated in pole cells, which remain transcriptionally quiescent longer than the soma (Siddiqui et al., 2012), creating an additional need for an early, MZT-independent mechanism in the germ plasm. osk degradation in the germ plasm still occurs in unfertilized eggs, indicating that it is mediated solely by maternal factors whereas osk degradation in the bulk cytoplasm relies on both maternal and zygotic machineries
Figure 4.14: Founder granules are stabilized when oskar mRNA is stabilized

(A) Quantification of colocalization of osk and Dcp1 in wild type and aub mutant embryos. n = 5 – 44 embryos each. (B,C) Confocal z-series projections of osk and Dcp1 in wild-type (B) and aub mutant (C) germ plasm of age matched embryos. (D,E) Size distributions of founder granules in terms of the number of osk mRNAs per particle in wild-type (D) and aub mutant (E) germ plasm. n = 4 – 28 embryos each. Values are mean ± standard deviation * p < 0.05, ** p < 0.01, *** p < 0.001.
The mechanistic difference in osk degradation in the germ plasm compared to that during the MZT could be attributed to the difference in structure of the large localized osk RNPs compared to the smaller unlocalized osk RNPs (Little et al., 2015). Another possibility could be the greater need for specificity in the germ plasm. Unlocalized osk and nos are degraded with the same kinetics (Thomsen et al., 2010) and degradation factors (Barckmann et al., 2015; Laver et al., 2015a) during the MZT, suggesting a shared degradation mechanism. However, germ granule mRNAs are not vulnerable to degradation in the germ plasm while osk is being degraded.

This leads us to question how germ granule mRNAs are protected from degradation in the germ plasm. Germ granule structure may be organized such that the mRNAs are held inaccessible to and thus protected from the degradation machineries. This is consistent with the concept of mRNA regulons in which mRNAs contributing to common functions are assembled in RNPs together for efficient co-regulation. Along these lines, it is tempting to speculate that founder granules could harbor other mRNAs in the germ plasm with potentially harmful effects on pole cell development. Interestingly, in a sensitized genetic background, retroelement mRNAs localize to the germ plasm using the same localization machinery as osk (Tiwari et al., 2017). Perhaps a function of founder granules is to sequester and destroy retrotransposon mRNAs in the germ plasm in addition to osk to prevent their reinsertion into the genome and thus protect genome integrity.

However, mRNAs in the germ plasm do not have to be assembled in large RNPs to protect them from degradation. For example, hsp83 mRNA is stably enriched in the germ plasm through pole cell formation, but not incorporated in germ granules (Eagle and Gavis, unpublished). This further suggests a need to compartmentalize mRNA degradation in the germ plasm and emphasizes the relevance of sequestering degradation activity in founder granules to preserve mRNAs like hsp83 in the germ plasm.

In addition to investigating how germ granule mRNAs are protected from degradation in the germ plasm, this work raises the questions of what triggers osk degradation in the germ plasm and when events earlier than those described here occur including deadenylation and the trigger
of degradation. It is possible that these events are initiated by changes during the oocyte to embryo transition or even during late oogenesis.

In combination with previous work in the field, our results support a model in which distinct RNPs package classes of mRNAs that require specific regulatory events in the crowded germ plasm environment. This molecular organization is achieved in the oocyte and is based on mRNA fates in the embryo. It ensures that mRNAs necessary for germline development are protected and faithfully incorporated in pole cells and mRNAs sharing the same subcellular location but that could be detrimental to germline development are destroyed.
CHAPTER 5: OUTLOOK AND CONCLUDING REMARKS
5.1 CONTEXT AND OVERVIEW

Posttranscriptional control of mRNA allows for precise spatiotemporal regulation of gene expression. Throughout their lifetimes mRNAs are assembled into RNPs in association with proteins that control all aspects of their regulation. For example, RNPs control the localization of mRNA to specific subcellular domains. mRNA localization is an effective and efficient means to asymmetrically distribute protein activities in order to polarize cellular functions and morphologies. In the context of mRNA localization, RNP proteins can influence the translational status of the mRNA to prevent translation before it is localized and activate translation at the appropriate time. They can also mediate association with motor proteins for transport, and anchor the mRNA at its destination. As the regulatory needs of the mRNA changes, RNP composition can change with the recruitment of new proteins and the release of those that are no longer needed. Thus, the complement of proteins associated with the RNP is dynamic and highly choreographed to achieve precise mRNA regulation.

The RNPs of the Drosophila germ plasm include at least two distinct types: germ granules and founder granules. The founder granules contain osk mRNA, whose posterior localization and translation are required for germ plasm establishment. The germ granules contain mRNAs such as nos and gcl, which are required for pole cell development. Despite their shared subcellular location and partial overlap in localization mechanism during oogenesis, these granules are physically and behaviorally distinct with only germ granules becoming enriched in the pole cells that form at the site of the germ plasm during embryogenesis. Aberrant incorporation of osk in germ granules and subsequent uptake by pole cells hinders germine development, highlighting the importance of the segregation of germ granule and founder granule contents within the germ plasm.

The goal of the work presented in this thesis was to determine how osk is excluded from pole cells by addressing the formation of founder granules as structures distinct from germ granules and the function of founder granules in embryogenesis to prevent osk enrichment in pole cells. We ultimately conclude that founder granules mediate the specific degradation of osk in the embryonic
germ plasm. The remainder of this chapter will focus on discussion of models describing events examined in this thesis, experimental challenges, and potential follow-up analyses.

5.2 Challenges of Oskar Transgene Analysis

The cloning of sequence-tagged *osk* transgenes for use in the experiments described in Chapter 2 was difficult and exposed a potential gap in the knowledge of cis elements that contribute to *osk* regulation. Initially, we designed constructs (not shown in this thesis) that used the *egfp* CDS as a sequence tag at the 3’ end of the *osk* CDS followed by approximately 200 nucleotides of *osk* 3’ genomic sequences. These transgenes were under control of the UASp promoter to drive specific expression in the female germline using the Gal4-UAS system. However, expression of the control *UASp-osk-egfp-osk3’UTR* transgene revealed issues with both phases of *osk* mRNA localization. While the transgenic mRNA was enriched at the posterior by stage 10, we often observed ectopic patches of the mRNA. This could be explained by overexpression of the transgene. However, by stage 13, less transgenic mRNA was present at the posterior than at stage 10 indicating that the mRNA was not adequately anchored at the posterior before the onset of ooplasmic streaming. Since the transgene was expressed in a wild-type *osk* background, a lack of Osk protein at the posterior was not the cause of the maintenance failure.

We eventually cloned a version of the transgene that behaved like endogenous *osk*. This construct used the *sfgfp* CDS as the sequence tag at the 3’ end of the *osk* CDS and included a short linker between the *osk* and *sfgfp* sequences encoding the TY1 epitope tag. This *osk-ty1-sfgfp-osk3’UTR* transgene was under control of the *osk* promoter and included about 2.5 kb of 5’ *osk* genomic sequences and about 4 kb of 3’ *osk* genomic sequences. Transgenic mRNA of this transgene displayed normal localization patterns at stages 10 and 13 and was used as a control in the experiments in Chapter 2 as well as the basis for cloning the other *osk* transgenes used in Chapter 2. Besides the different sequence tag and inclusion of the *ty1* sequence and extensive 5’ and 3’ genomic sequences, the *UASp-osk-egfp-osk3’UTR* and *osk-ty1-sfgfp-osk3’UTR* transgenes differed only in a short sequence at the 5’ end of the 3’UTR. The first 11 bases in the 3’UTR of
UASp-osk-egfp-osk3’UTR (included in the fly base annotation) were replaced with a different sequence of 6 bases in the 3’UTR of osk-ty1-sfgfp-osk3’UTR.

While it is unclear why osk-ty1-sfgfp-osk3’UTR transgenic mRNA behaves like endogenous osk and UASp-osk-egfp-osk3’UTR does not, there are some possible explanations based on the different construct designs. For one, egfp and sfgfp have significantly different sequences. It is possible that some structure within the egfp sequence or between the egfp and osk sequences could affect regulation of the UASp-osk-egfp-osk3’UTR mRNA. Another possibility is that the 5’ and/or 3’ genomic sequences could influence late phase localization in an event associated with transcription, perhaps through mediating the deposition of an important late phase localization factor. Whatever the cause, the behaviors of these transgenes show that osk regulatory elements exist that are yet to be discovered.

5.3 RNP Stability during Late Oogenesis

During a brief analysis of twin (CCR4) mutants described in Chapter 3, we found that RNPs become destabilized between stages 12 and 13 of oogenesis indicating that Twin-mediated mRNA deadenylation stabilizes RNPs in late oocytes. Interestingly a study of P-bodies in Drosophila S2 cells addressing whether large P-body structures are necessary for mRNA degradation found that inhibiting mRNA decay by depleting NOT1 of the CCR4-NOT deadenylase complex to prevent deadenylation caused P-bodies to disperse (Eulalio et al., 2007). The authors ultimately concluded that P-body assembly is a consequence and not a cause of mRNA decay. Their finding was most likely the result of a different scenario than what we observed with Twin depletion in oocytes, which in addition to founder granules affected germ granules, which are sites of stable mRNA storage. Nonetheless, it is tempting to speculate that there could be some similarity in why deadenylation may be important for P-body stability as well as general RNP stability.

Further analyses are required to understand why RNPs are destabilized in twin mutant late stage oocytes. Interpretation of such analyses are expected to be challenging due to probable pleiotropic effects of Twin depletion, since Twin is a general mRNA decay factor. Despite the challenge, careful phenotypic analysis of germline development could be fruitful. Furthermore,
proteomic and transcriptomic analyses may help pinpoint interesting candidates to follow up on Twin’s role in RNP stability.

Since founder granules are destabilized in twin mutant oocytes, it is possible that founder granule composition and/or structure beyond granule size is affected. Consistent with this, we found that germ plasm localized osk mRNA was stabilized in twin mutant embryos by about 3 nuclear cycles. This could indicate that overall founder granule structure is important to the efficient degradation of osk mRNA in the germ plasm and supports that founder granules function in osk mRNA degradation.

5.4 Challenges of Stabilizing oskar mRNA

One unanswered question following from our model that founder granule-mediated osk mRNA degradation safeguards pole cell development is whether stabilizing osk mRNA in founder granules would affect pole cell development to a similar extent as targeting osk mRNA to germ granules. Unfortunately, we have been unable to address this question. While osk mRNA and founder granules are stabilized in the germ plasm of aub mutants, these embryos arrest early in embryogenesis, often before pole cells form. Therefore, they cannot be used to study pole cell development.

Attempts at stabilizing osk mRNA by depleting different factors of the general mRNA degradation machineries have also failed. Although twin mutant embryos showed stabilized osk mRNA levels in the germ plasm for about 3 nuclear cycles, these also had the additional phenotype that founder granules were destabilized in late stage oocytes. Therefore, we could not use this background to stabilize founder granules. We also attempted to use RNAi to deplete pcm. However, out of 5 pcm RNAi lines tested, none gave sufficient knockdown in the early embryo to prevent osk degradation in the germ plasm.

Future attempts at osk mRNA and founder granule stabilization could include analysis of pcm mutants. We can also assess founder granules in dcp1 mutants. Dcp1 is required for osk mRNA localization during mid stages of oogenesis (Lin et al., 2006). However, a specific point mutation disrupts Dcp1’s role in mRNA decapping independent of its role in osk mRNA localization.
(Lin et al., 2006). This mutation would allow us to bypass the earlier role of Dcp1 in osk mRNA regulation, making it a good candidate to attempt to stabilize osk mRNA in the germ plasm during pole cell formation.

**5.5 The Future of Founder Granule Analysis**

Because we lack a picture of founder granule composition beyond osk mRNA and Stau, we are limited in our basic understanding of founder granules as well as in our ability to study them. Therefore, an important next step in founder granule analysis will be to identify additional founder granule components. One method to achieve this is BioID (Roux et al., 2013; Roux et al., 2012). In this strategy, Stau is fused to the promiscuous biotin ligase, BirA, which biotinylates proteins that it contacts for enough time. The biotinylated proteins can then be isolated by streptavidin pull down and identified by mass spectrometry.

Stau BioID can also be used to explore how the composition of founder granules changes over time. For example, by comparing the complement of proteins associated with founder granules in oocytes and embryos, we may be able to identify the initial trigger of osk mRNA degradation in founder granules. In addition to adding to the model of osk mRNA degradation in the germ plasm, identifying the trigger of osk mRNA degradation would provide new strategies for stabilizing osk mRNA in founder granules to assess the phenotypic consequences of osk mRNA persistence in the germ plasm without targeting the mRNA to germ granules as discussed in Chapter 5.4.

**5.6 A Model: Distinct RNPs for Distinct mRNA Fates**

mRNAs in the Drosophila germ plasm can be divided into at least two categories based on their functions in germline development. The mRNAs populating these categories are assembled into two distinct types of RNP granules during oogenesis dictated by specific cis elements lying in different regions of the mRNAs. Germ granules contain mRNAs necessary for directing early pole cell formation and function. They stably maintain these mRNAs in the germ plasm and ensure their faithful inheritance by the germline as pole cells form. Founder granules harbor osk mRNA, which must be carefully regulated. Local osk translation in the oocyte is essential for germ plasm
assembly. However, germ plasm localized osk must not persist at high levels during pole cell formation. We propose that founder granules mediate both of these critical events by enriching osk mRNA in the germ plasm during oogenesis and degrading osk mRNA in the germ plasm during early embryogenesis. The segregation of osk mRNA from germ granules and its subsequent degradation ensure that osk does not persist in pole cells, which would negatively impact germline development. Compartmentalization of osk degradation in founder granules rather than freely in the germ plasm prevents aberrant degradation of the germ plasm mRNAs that may not be protected by compartmentalization in larger RNP granules and contributes to limiting the exposure of germ granule mRNAs to degradation factors.
CHAPTER 6: MATERIALS AND METHODS
6.1 CHAPTER 2 DROSOPHILA STRAINS

Genotype                                      Shorthand
attP40[w+, osk-ty1-sfgfp-osk3’UTR]             OS134
attP40[w+, oskΔi1,2-ty1-sfgfp-osk3’UTR]        OS135
attP40[w+, osk-ty1-sfgfp-tub3’UTR]             CRS143
attP40[w+, oskΔi1,2-ty1-sfgfp-tub3’UTR]        CRS144
attP40[w+, osk-ty1-sfgfp-nos3’UTR]             CRS145
attP40[w+, oskΔi1,2-ty1-sfgfp-nos3’UTR]        CRS146
attP40[w+, egfp-nos-osk3’UTR]                 OS137
p[w+, egfp-nos]² / CyO                        OS144
attP40[w+, osk-SOLE⁸AG-ty1-sfgfp]             JT1
attP40[w+, osk-SOLE⁸UA-mut-ty1-sfgfp]          JT2
attP40[w+, osk-SOLE⁸G/A-UA-mut-ty1-sfgfp]     JT3
attP40[w+, osk-SOLE⁸UA-mut-Comp-ty1-sfgfp]    JT4
attP40[w+, osk-SOLE⁸PS-Lz-mut-ty1-sfgfp]      JT5

6.2 CHAPTER 3 DROSOPHILA STRAINS

Genotype                                      Shorthand
y¹ w67c23                                      YW
yw; P[w+, twin²GS12209] / TM3 Sb Ser           OS77
yw; P[w+, twin²GS8113] / TM3 Sb Ser           OS78
osk⁸A87 ru st e ca / TM3 Sb                  OS79

6.3 CHAPTER 4 DROSOPHILA STRAINS

Genotype                                      Shorthand
attP40[w+, UASp-oskM₁,M₁39RΔi1,2-nos3’UTR]   CRS103
attP40[w+, UASp-oskΔi1,2-nos3’UTR]            OS22
pVas-mCherry / CyO ; Stau-GFP / TM6 3         OS70
**6.4 ANTIBODIES**

IF = Immunofluorescence, IB = Immunoblot, IHC = Immunohistochemistry

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Use</th>
<th>Source</th>
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<tr>
<td>Rabbit α Twin</td>
<td>IF 1:1000</td>
<td>Simonelig lab</td>
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<tr>
<td>Rat α Cup #219</td>
<td>IF 1:250</td>
<td>Smibert lab</td>
</tr>
<tr>
<td>Mouse α Dcp1</td>
<td>IF 1:1000</td>
<td>Siomi lab</td>
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<tr>
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<td>IF 1:500; IB 1:1,000</td>
<td>Newbury lab</td>
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<td>IF 1:2,000; IB 1:1,000</td>
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<td>Sigma clone DM1A ascites</td>
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<td>GE Healthcare Life Sciences</td>
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<td>GE Healthcare Life Sciences</td>
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<td>HRP Donkey α Rabbit</td>
<td>IB 1:2,000</td>
<td>GE Healthcare Life Sciences</td>
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Alexa-568 Goat α Rabbit IF 1:1,000 Molecular Probes
Alexa-488 Goat α Rabbit IF 1:1,000 Molecular Probes
Alexa-568 Goat α Mouse IF 1:1,000 Life Technologies
Alexa-488 Goat α Mouse IF 1:500 Life Technologies
Alexa-647 Goat α Mouse IF 1:1,000 Life Technologies
Alexa-568 Goat α Rat IF 1:1,000 Molecular Probes
Alexa-488 Goat α Rat IF 1:1,000 Molecular Probes

6.5 Generation of Germline Clones

FRT40 brat^{G1} / CyO, Act-GFP; UASmed8:GFP, UAS-NLS- lacZ virgin females were crossed to y¹, w^{67c23}, hs-FLP+/+; p[w⁺, ovo^{D^{2La}}, p[w⁺, ovo^{D^{2La}}, p[ry⁺, FRT]^{F0A} / CyO, hs-hid males and allowed to seed bottles for 2 to 3 days in a 25°C incubator. Adults were removed and the seeded bottles were aged for 1 day. The bottles were heat shocked for 3 hours in a 37°C water bath on the second and third days after the adults were removed. Non-CyO female progeny were used for embryo collections.

6.6 Embryo Collection, Fixation, and Storage

Embryos were collected on apple juice agar plates at room temperature for experiments staged by nuclear cycle and in a 25°C incubator for experiments staged by collection timing. The embryos were transferred to a wire mesh basket, rinsed, dechorionated in 50% bleach, and rinsed again. Then, the embryos were fixed in a glass scintillation vial at the interface between 2 mL of EM grade 4% paraformaldehyde (PFA) (3:1 1x phosphate buffered saline (PBS):methanol-free EM grade 16% PFA, Polysciences) and 8 mL of heptane for 20 min at room temperature with gentle shaking. The PFA was removed and replaced with 10 mL of methanol and the vial was shaken vigorously by hand for 2 min to remove the vitelline membrane. Devitellinized embryos were allowed to sink to the bottom of the vial for 30 sec, transferred to a 1.5 mL eppendorf tube and washed 3 times with methanol. Fixed and devitellinized embryos were stored in methanol at -20°C for no more than 1 month.
Embryos used for RNA, DNA, or protein extraction were dechorionated as described above, transferred to a 1.5 mL eppendorf tube containing PBS, flash frozen in liquid nitrogen, and stored at -80°C.

6.7 SINGLE MOLECULE FLUORESCENCE IN SITU HYBRIDIZATION (smFISH)

Dechorionated embryos were transferred into PBS with 0.1% Tween-20 (Sigma-Aldrich) (PBST) by 3 min sequential incubations in solutions of 7:3, 1:1 and 3:7 methanol:PBST and finally rinsed twice with PBST. Rehydrated embryos were then equilibrated in 500 μL of smFISH wash buffer (4x SSC buffer, 35% deionized formamide, 0.1% tween-20) for 10 min with rocking. (Formamide (Thermo Fisher Scientific) was deionized using 5 g of ion exchange resin (BioRad) per 100 mL of formamide by stirring at room temperature for about 30 min, filtered to remove resin and stored at -20°C.) The smFISH wash buffer was removed, and replaced with smFISH oligo probes diluted in 100 μL of hybridization buffer (10% w/v dextran sulfate, 100 μg/mL salmon sperm DNA, 2 mM vanadyl ribonucleoside, 20 μg/mL bovine serum albumen (NEB), 4x SSC buffer, 0.1% tween-20, 35% deionized formamide). (The amount of smFISH oligo probes used depended on the experiment, with 1 μL of probes per 100 μL of hybridization buffer used for quantification of total localized fluorescence intensity and 3 μL of probes per 100 μL of hybridization buffer used for colocalization analyses and particle intensity analyses.) The tube was flicked gently to suspend the embryos in the hybridization buffer. The embryos were incubated in the dark at 37°C overnight without rocking. The next day embryos were washed twice with 500 μL of pre-warmed smFISH wash buffer in the dark at 37°C without rocking. The embryos were then rinsed twice with 500 μL of PBST, incubated in 1.25 μg/mL DAPI in PBST and rinsed 4 times with PBST. The embryos were then mounted under #1.5 glass coverslips (VWR) in Vectashield Mountant (Vector Laboratories) for quantification of total localized fluorescence intensity and Prolong Diamond Antifade Mountant (Thermo Fisher Scientific) for colocalization analyses and particle intensity analyses.
6.8 smFISH Oligo Probes

smFISH probes of 20 nt oligonucleotides with 2 nt spacing were designed with Stellaris Probe Designer and produced by Biosearch Technologies. Probe sets complementary to osk (CG10901; 99 oligos), nos (CG5637; 63 oligos), cycB (CG3510; 48 oligos), or egfp (32 oligos) were conjugated to Atto 647N dye (Sigma-Aldrich) or Atto 565 dye (Sigma-Aldrich) and purified by HPLC as previously described (Raj et al., 2008). smFISH probes of 20 nt oligonucleotides with 2 nt spacing for sfgp (31 oligos) were designed with Stellaris Probe Designer and purchased from Biosearch Technologies conjugated to the Quasar 670 fluorophore.

6.9 Immunofluorescence

Embryos were rehydrated stepwise into PBST (PBS, 0.1% Tween-20 [Sigma-Aldrich]) and then blocked in Image it FX (Thermo Fisher Scientific) for 30 min at room temperature followed by washing 3 × 15 min in PBHT (PBS, 0.1% Tween-20, 0.25 mg/ml heparin [Sigma-Aldrich], 50 µg/ml tRNA [Sigma-Aldrich]). Embryos were then incubated in primary antibody diluted in PBHT overnight at 4°C with rocking. Embryos were washed sequentially for 5, 10 and 20 min in PBHT at room temperature, then incubated with the appropriate secondary antibody diluted in PBHT in the dark for 2 hr at room temperature with rocking. Embryos were washed 3 × 10 min in PBST at room temperature with rocking followed by 2 min incubation in 1.25 µg/ml DAPI in PBST and rinsed 4 times with PBST. The embryos were then mounted as described above. For double immunofluorescence/smFISH experiments, embryos were refixed in 4% PFA for 30 min at room temperature with rocking and rinsed 4× with PBST before proceeding with smFISH as described above.

6.10 Quantification of Fluorescence Intensity

Confocal imaging was performed using a Leica SP5 laser scanning microscope with a 63× 1.4 NA oil immersion objective and GaAsP “HyD” detectors in standard detection mode. All imaging parameters were kept identical within each experiment. For quantification of total intensity, z-series with a 2 µm step size were used to capture the germ plasm-localized signal. Image processing and
analysis were done in FIJI. Z-projections were made with the “sum slices” function and the threshold adjusted so the entire localized signal was included. The total fluorescence intensity of the localized signal (integrated density function in FIJI) was then measured. Average intensities were normalized to the average initial intensity for each experiment. Experiments were performed in triplicate and data displayed as mean ± standard error of the mean unless otherwise indicated.

To quantify the fluorescence intensity of RNA within the germ plasm (localized) vs RNA within the bulk cytoplasm (unlocalized), two images were captured for each embryo: one at lower laser power optimized for the brighter germ plasm RNA signal and one at higher laser power optimized for the unlocalized RNA signal. Image processing and analysis were done in FIJI. Z-projections spanning 12 µm (6 µm above and below the mid-point of the germ plasm in the z-axis) were made with the “sum slices” function. A ROI of approximately 1600 µm$^3$ within the germ plasm and a ROI of approximately 83100 µm$^3$ within bulk cytoplasm was selected for each embryo. A large ROI was chosen for the bulk cytoplasm to minimize variation in signal due to interference from yolk. The integrated density of the ROI region was divided by the corresponding volume.

### 6.11 Quantification of Particle Size and Colocalization

Confocal imaging was performed using a Leica SP5 laser scanning microscope with a 63× 1.4 NA oil immersion objective and GaAsP “HyD” detectors in photon counting mode. An optical zoom of 1.52× was applied to achieve a pixel size of 72 × 72 nm. An approximate 5 µm z-series was taken of the posterior third of the embryo with a step size of 340 nm. The fluorescence intensities of individual particles of osk, nos, and Stau and colocalization between osk and Dcp1, Me31B, Pcm, or Stau and between cycB and Dcp1 were quantified as previously described (Niepielko et al., 2018), with unlocalized nos and osk particles assumed to contain an average of 1 and 2 transcripts, respectively as previously determined (Little et al., 2015). With these assumptions, the average intensities of unlocalized particles were used to determine the number of transcripts in germ plasm localized particles of the same image. In addition to allowing the determination of the number of transcripts in RNP granules, this method internally normalizes each image allowing direct comparisons. Approximately 500 to 1500 particles per channel were analyzed per embryo.
6.12 Live Imaging and Particle Tracking

Live imaging of founder granules and germ granules was performed on a Nikon Ti-E with Yokogawa spinning disc module (CSU-21) with a 60× 1.4 NA oil immersion objective. Embryos were dechorionated and mounted in halocarbon oil 95 (Halocarbon Products Corporation) under a #1.5 glass coverslip (VWR) on a Lumox dish (Sarstedt), oriented with the dorsal side closest to the coverslip. Movies were acquired at 100 ms exposure in two channels with a frame rate of 5 frames per second. Particles were tracked using the Autoregressive Motion algorithm of Imaris version 9.1.2 (Bitplane).

6.13 Immunohistochemistry

Embryos were rehydrated stepwise into PBST, then blocked by incubation in BBT (PBST supplemented with 0.1% globulin free BSA [Sigma-Aldrich]), 5 × 25 min at room temperature with rocking. The embryos were then incubated with primary antibody diluted in BBT overnight at 4°C with rocking. The embryos were then washed for 10, 20 and 30 min with BBT at room temperature with rocking. The embryos were blocked again for 30 min with BBT + 2% normal goat serum (NGS), then incubated with biotin secondary antibody in BBT + 2% NGS for 2 hr at room temperature with rocking. The embryos were washed for 10, 20, and 2 × 30 min in PBSTx (PBS, 0.1% TritonX100 [Sigma-Aldrich]) at room temperature with rocking. Secondary antibody amplification was performed with Vectastain reagent according to the manufacturer's instructions (Vectastain Elite PK6100 ABC kit; Vector Labs) and detected with peroxidase immunohistochemistry. Embryos were mounted in 80% glycerol and imaged using Nomarski optics.

6.14 Immunoblotting

Embryos were thawed on ice and homogenized in 2 volumes of boiling 2× sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 5 M urea, 0.1 M DTT, 0.01% bromophenol blue) and boiled for 5 minutes. Samples were spun for 15 min at 4°C. The supernatant was run on a 10% SDS-PAGE gel and proteins were transferred to a nitrocellulose membrane. The membrane was blocked 3 × 10 min in Blotto (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20, 5% nonfat dry milk).
The membrane was cut and incubated at 4°C overnight in primary antibody diluted in Blotto. After washing 3 × 10 min in Blotto at room temperature, the membrane was incubated in HRP secondary antibody diluted in Blotto for 1 hr at room temperature. The membrane was washed 3 × 10 min in TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20). Antibodies were detected using the Lumi-Light Plus Western Blotting Substrate (Roche).

6.15 RT-qPCR

RNA was extracted from dechorionated embryos using the RNeasy kit (Qiagen). 1 µg total mRNA was used to generate cDNA using the Quantitect RT kit (Qiagen). 2 µl cDNA was combined with 25 µl 2× TaqMan Gene Expression Master Mix (Thermo Fisher Scientific), 2.5 µl of 20× TaqMan Gene Expression Assay (Thermo Fisher Scientific, osk custom – Dm 02134535, 4351372 or rpl7 Dm 01817653, 4351372), and 20.5 µl of nuclease free H₂O. qPCR was performed on an Applied Biosystems 7900HT standard 96-well qPCR instrument. Three biological replicates were performed with three technical replicates each, all using a CT threshold of 0.6613619. Technical replicates were averaged and copy number determined using previously established standard curves (Eagle et al., 2018). Copy number of the three biological replicates was normalized to the rpl7 control and presented as mean ± standard deviation.
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