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Post-transcriptional Regulation of the Drosophila Anterior Determinant, Bicoid

John McLaughlin
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POST-TRANSCRIPTIONAL REGULATION OF THE DROSOPHILA ANTerior DETERMINANT, BICOID

by

John M. McLaughlin

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York

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Abstract

POST-TRANSCRIPTIONAL REGULATION OF THE DROSOPHILA ANTERIOR DETERMINANT, BICOID

By John M. McLaughlin
Adviser: Diana P. Bratu

In a wide variety of biological contexts, messenger RNA (mRNA) is known to have a complex and dynamic life cycle. In particular, the localization and translational control of mRNA are essential for proper development in eukaryotes. The fly Drosophila melanogaster is an excellent model for studying these processes. During D. melanogaster oogenesis, several mRNAs are trafficked and localized within the developing egg chamber, and regulated at the translational level to enable embryo patterning. One such mRNA, bicoid, is localized at the anterior of the oocyte and translated in the early embryo, where its encoded protein directs formation of the fly’s head and thoracic segments.

In this thesis, we investigated several aspects of bicoid’s post-transcriptional regulation that may impact its stability and translational timing. First, we demonstrate that bicoid mRNA is present in Processing Bodies (P-bodies), cytoplasmic organelles implicated in mRNA storage and decay. Perturbing P-body formation/structure, via manipulation of the mRNA decay pathway, affects the levels of bicoid and additional maternal transcripts. We next explored the possibility that the microRNA (miRNA) pathway regulates the translational timing of bicoid. We find that in cell culture experiments, a bicoid reporter gene is translationally repressed by miR-305; we also demonstrate that miR-305 is expressed in ovaries. However, loss of miR-305 is not sufficient to alter ectopic bicoid mRNA translation
in the egg chamber. To determine if any genes are singly required for *bicoid* translational repression in the egg chamber, we used GFP-tagged transgenes to express *bicoid* mRNA *in vivo*. Although we do not yet identity any candidate genes in this small screen, we show that overexpression of *bicoid* mRNA results in its translation in the egg chamber, suggesting that one or more factors normally act in its translational repression.

Overall, our work points to several plausible avenues of investigation into processes that regulate the translational timing of *bicoid* mRNA during oogenesis. Moreover, our findings are also relevant to a general understanding of the complex, multifaceted problems surrounding mRNA post-transcriptional regulation.
Acknowledgments

First and foremost I would like to thank my mentor, Diana Bratu, for accepting me into the lab when I was in a troubled position, and tolerating me over the years. I have been very lucky to train under her mentorship, which has shaped my intellectual development and will leave a lasting impact, regardless of my career path. She has instilled in me a certain discipline, ethos, and appreciation for the aesthetic qualities of my work; I hope that these personal attributes occasionally come across, even if I don’t always live up to them. I thank the current Bratu lab members Livia, Irina, Omar, and Katie for their advice, support, and especially the fun memories during my time in the lab. I would like to acknowledge the undergraduates who have helped with my research, Daniel, Hadiya, and Abigail, as well as the many former Bratu lab members. I would like to thank my family, friends, and especially my fiancée Emily for their support during my years in the program. Finally, I would like to thank my committee members, the Hunter College Biology Department, and the Graduate Center for their advice and support.

“The value of having for a time rigorously pursued a rigorous science does not rest especially in its results: for in relation to the sea of worthy knowledge, these will be but a negligible little drop. But it brings forth an increase of energy, of deductive ability, of persistence; one has learned to gain one’s purpose purposefully. To this extent, in respect to all one does later, it is very laudable to have once been a scientific man [or woman.]”

Friedrich Nietzsche, Human, All Too Human
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Chapter 1

RNA post-transcriptional regulation

1.1 Background and Context

In the last several decades, the importance of post-transcriptional regulation of RNA has become increasingly apparent. In a wide variety of organisms and biological contexts, the regulation of RNA is essential for proper development. Messenger RNA (mRNA) in particular has been the focus of much of this research. An mRNA is subjected to processing events during and immediately following its transcription, for example via RNA splicing and regulated nuclear export. Once exported to the cytoplasm, an mRNA undergoes a variety of other regulatory events throughout its life cycle, including transport and localization, translational repression or activation, and decay. *D. melanogaster* oogenesis has been an instrumental paradigm for understanding the roles of localized and translationally controlled mRNAs in development, and dissecting the mechanisms by which they are regulated.

1.2 Post-transcriptional regulation of messenger RNA

Following transcription and nuclear export, mRNAs form complex, higher-order particles containing multiple proteins and RNA species (Fig 1.1). Some mRNAs, such as *oskar* mRNA in *D. melanogaster*, can dimerize via base-pairing interactions within the 3' untranslated region (3'UTR), forming large particles (Marchand et al, 2012). Trans-acting factors, such as RNAs or proteins, are also capable of interacting with an mRNA. RNA-binding proteins
can include members of the exon junction complex (EJC), translational repressors and/or activators, or other sequence-specific RNA-binding proteins. Additional trans-acting factors may include translation initiation factors and adaptor proteins that couple the mRNA to the microtubule (MT) or actin cytoskeletons.

Figure 1.1: Generic depiction of higher-order messenger ribonucleoprotein (mRNP). The illustration depicts two mRNA molecules bound by RNA-binding proteins and dimerizing via their 3'UTRs. Listed below are cis and trans-acting factors that can mediate post-transcriptional regulation.

Through the assembly of mRNAs into particles, cells can exert finer temporal and spatial control over the stability and translational efficacy of mRNA transcripts. This regulation is especially important in the context of development. The morphology of certain cell types, such as neurons, causes the sites of protein expression to be very distant from the cell nucleus, making post-transcriptional gene regulation such as mRNA localization and translation control especially important. Cells which require a rapid response to environmental stimuli will also rely more heavily on post-transcriptional regulation in order to quickly adjust their translational profiles.
1.2.1 mRNA localization

Subcellular localization of mRNA transcripts confers several benefits. It aids the spatial compartmentalization of gene products within the cell, a requirement for eukaryotic cells which are relatively large and complex. It also allows for rapid changes in cellular gene expression; the translation of an already localized transcript can occur more quickly than \textit{de novo} synthesis and transport of an mRNA. These processes are especially important in the context of development. A large, morphologically complex tissue like the \textit{D. melanogaster} egg chamber relies heavily on the localization of mRNA. The developing fly embryo then makes use of these maternally localized transcripts, and their protein products, to efficiently pattern new tissues as cells divide and differentiate.

One of the best characterized cases of mRNA localization is that of \textit{ASH1} mRNA in the budding yeast \textit{S. cerevisiae}. During budding, this mRNA is transported to the yeast daughter cell, where it is translated and Ash1 protein localizes to the nucleus and acts as a transcriptional repressor. The mother cell, which is depleted of Ash1 protein, can activate expression of the \textit{HO} endonuclease gene, which allows for mating type switching. Thus, the localization of \textit{ASH1} mRNA in this system serves a critical functional role.

The localization of \textit{ASH1} mRNA depends on the actin cytoskeleton and actin-based motor proteins. Depolymerizing actin, by using drugs such as latrunculin-A, results in mislocalized \textit{ASH1} mRNA (Long et al, 1997). Proper localization of \textit{ASH1} mRNA is also dependent on four ‘zipcode’ sequences present in both the open reading frame (ORF) and 3’UTR of the transcript. Each sequence element is sufficient to localize a heterologous reporter transcript to a budding daughter cell, however the presence of all four elements confers the most efficient localization (Chartrand et al, 2002). Proteins required for \textit{ASH1} mRNA transport and localization include She1p, a type V myosin motor, as well as She2p and She3p, a zipcode RNA-binding protein and adaptor protein, respectively (Bohl et al, 2000).
Neurones — another important paradigm for mRNA localization — contain many mRNA species that are localized in dendrites or axons, cellular processes which are relatively autonomous and distant from the nucleus. B-actin is another well studied mRNA that is localized in a variety of systems including Xenopus laevis, chicken, mice, and humans. In neurons, B-actin mRNA is localized to dendrites and its local translation plays a key role in regulating synapse strength during processes such as learning and memory. Transport and localization of B-actin mRNA requires both the MT and actin cytoskeletons (Condeelis et al, 2005). A 54 nucleotide cis-element in the B-actin 3’UTR, termed the ‘zipcode’, is necessary and sufficient for proper localization of the mRNA (Chao et al, 2009). This sequence is recognized by the protein ZBP1 (zipcode-binding protein 1), which functions in a protein complex that localizes B-actin mRNA (Ross et al, 1997). Following its initial identification, ZBP1 became the founding member of a family of RNA-binding proteins that regulate various mRNAs in different species (Yisraeli, 2005).

Many of the themes underlying ASH1 and B-actin mRNA localization recur in different organisms and biological contexts. The presence of cis-acting sequences and/or secondary structure motifs, often within an mRNA’s 3’UTR, is essential for linking an mRNA to specific RNA-binding proteins (RBPs). These RBPs in turn may couple a specific mRNA to different microtubule or actin-based motor proteins. RBPs and cis-elements can also play roles in assembling mRNAs into larger particles that aid in transport and/or translational repression of the transcripts.
1.2.2 mRNA translational control

A subset of post-transcriptional regulation is dedicated to controlling where and when an mRNA is translated into a protein within the cell. Translation of mRNA is understood in terms of the ‘closed-loop’ model, according to which, circularization of the mRNA — by bridging the 5’ and 3’ ends of the transcript — helps promote an efficient rate of translation and recycling of ribosomes on the transcript. This closed-loop configuration is achieved by several core eukaryotic translation initiation factors (eIFs), whose loading on the mRNA is a prerequisite for translation initiation (Fig. 1.2).

In flies, the 5’ methyl-guanosine (m$^7$G) cap structure of an mRNA is first bound by eukaryotic translation initiation factor E (eIF4E). A critical factor for circularization of the mRNA and translation initiation is the large scaffolding protein eIF4G. By binding eIFs at the 5’ end of the mRNA (eIF4E and the DEAD-box RNA helicase eIF4A), as well as poly-A binding protein (PABP) at the poly-A tail, eIF4G aids in the 5’ to 3’ looping of the transcript. In addition, it also interacts with eIF3, an eIF which binds the 40S ribosomal subunit. Thus, eIF4G helps to both circularize the mRNA and to begin unwinding and scanning of the 5’UTR by the 40S ribosomal subunit. The three critical eIFs — eIF4E, eIF4A, and eIF4G — are collectively referred to as eIF4F once they are bound to the mRNA.

A great deal of translational regulation is thought to occur prior to the ‘initiation’ phase. Because eIF4E is considered a limiting factor for translation initiation, translational repression can be achieved primarily by sequestration of eIF4E, or by preventing its binding to the 5’ cap or eIF4G. Illustrating this concept, there is a widely conserved family of eIF4E-binding proteins (4E-BPs) in eukaryotes; in flies, eIF4E-transporter (4E-T) and Cup are two such proteins. Through the use of a consensus amino acid motif shared among 4E-BPs, these proteins can abolish or decrease rates of translation initiation by competing with eIF4G for binding of eIF4E (Sonenberg and Hinnebusch, 2009).
Figure 1.2: Simplified schematic of eukaryotic translation initiation factors (eIFs). Depicted are the cap-binding protein eIF4E, the RNA helicase eIF4A, and scaffold protein eIF4G which bridges the complex to poly-A binding protein (PABP). This is thought to circularize the mRNA and enhance the rate of translation initiation.

1.3 Transport and localization of an mRNA: the case of bicoid

*bicoid* (*bcd*) mRNA is one of the well studied and classic patterning transcripts of the *D. melanogaster* egg chamber. Because of the long history of studies (since 1988) on *bcd*, the mechanism of its localization within the oocyte is fairly well characterized. The *bcd* gene is the anterior patterning determinant of the *D. melanogaster* embryo; the mRNA is synthesized in nurse cell nuclei, transported along the MT cytoskeleton into the oocyte, and finally localized and anchored at the oocyte anterior cortex.

During early embryonic development, the previously localized *bcd* mRNA is then translated and Bcd protein diffuses to form an anterior-posterior (AP) gradient within the embryo. Bcd protein is a homeodomain transcription factor that activates the expression of several gap genes in the embryo, the most notable being *hunchback* (*hb*). Bcd’s cascade of target genes are responsible for patterning the head and thoracic structures of the embryo. In the
absence of Bcd, embryonic head and thoracic structures do not form and posterior segments are duplicated at the anterior.

Some of the early research on *bcd* mRNA transport focused on identifying regions within the transcript which are required for its proper localization. A localization signal was eventually narrowed to a 625 base pair sequence within the *bcd* 3’UTR (Fig. 1.3), which is necessary for proper *bcd* mRNA localization and is also sufficient to confer anterior localization on a heterologous transcript (Macdonald and Struhl, 1988).

**Figure 1.3: The structured 3’UTR of *bicoid* mRNA.** Depicted is a simplified *bcd* 3’UTR, organized into five ‘domains’, with critical secondary structures and their functions indicated. This diagram is adapted from a predicted RNA structure based on enzymatic probing experiments (Brunel and Ehresmann, 2004).
There are two distinct and genetically separable phases of bcd mRNA transport during oogenesis, the first being its transport from the nurse cell to oocyte compartment of the egg chamber, and the second is bcd mRNA’s tight localization and anchoring at the oocyte anterior cortex. Several trans-acting protein factors are required for these separate phases of bcd mRNA transport and localization (Fig. 1.4). One of the early-acting protein factors required for proper bcd mRNA localization is Exuperantia (Exu). A technically ingenious study demonstrated that the formation of bcd mRNA/Exu particles in the nurse cell cytoplasm is required for bcd mRNA’s eventual anterior cortical localization (Cha et al, 2001). A fluorescent, synthetic bcd mRNA directly injected into the oocyte cytoplasm localizes towards any cortical surface, but not specifically at the anterior. But injection of the synthetic bcd mRNA first into the nurse cell cytoplasm, followed by removal and re-injection into the ooplasm rescues anterior bcd mRNA localization. However, this rescue does not occur if the first injection is performed on exu mutant egg chambers, demonstrating that Exu is critical in forming nurse cell particles that are competent for transport.

The later phase of bcd mRNA transport and localization within the oocyte is mediated by the RNA-binding protein Staufen (Stau). Staufen is known to bind different mRNA species in the D. melanogaster egg chamber and embryo (Laver et al, 2013), although it was initially identified as a maternal gene required for the correct localization of bcd mRNA. In stau mutant females, bcd mRNA is not efficiently anchored at the oocyte anterior cortex, resulting in a steeper Bcd gradient in the embryo and failure to activate Bcd target genes (St Johnston et al, 1989). Drosophila Stau is one of the first proteins in which the double-stranded RNA binding domain (dsRBD) was identified and characterized (St Johnston et al, 1992). Although Stau contains five dsRBDs, the third domain has been the most well characterized biochemically. From mutagenesis analysis, it is known that this domain is required for localization of both bcd and osk mRNAs (Ramos et al, 2000). A synthetic bcd 3’UTR injected into early D. melanogaster embryos can efficiently form particles with Stau protein, and this process requires an intact MT cytoskeleton (Ferrandon et al, 1994).
The association of \textit{bcd} mRNA with Stau is mediated through secondary structure motifs in the 3’UTR. The \textit{bcd} 3’UTR contains several long stem-loops which are required for Stau recognition (Ferrandon et al, 1997). In addition, intermolecular interactions between complementary loops are also necessary for efficient particle formation; \textit{bcd} mRNAs that cannot dimerize \textit{in vitro} are also incapable of forming \textit{bcd} mRNA/Stau particles \textit{in vivo} (Wagner et al, 2000).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{\textit{bicoid} mRNA localization in the egg chamber. Depicted are several trans-acting protein factors responsible for localization of \textit{bcd} mRNA in the nurse cell (Exuperantia: Exu; Swallow: Sww) or the oocyte (Staufen: Stau; Miranda: Mira) compartments of the egg chamber. Also shown are MTs and the MT motors Kinesin and Dynein. Either Miranda or an unknown adaptor protein is thought to link Staufen/\textit{bcd} mRNA complexes to the MT cytoskeleton.}
\end{figure}
1.4 The microRNA pathway

MicroRNAs (miRNAs) constitute a class of endogenous, highly conserved, small regulatory RNAs. They represent one of several classes of small silencing RNAs that are known to exist in eukaryotes. They function as negative regulators of gene expression at the mRNA level, either through the destruction or translational silencing of their target mRNA transcripts.

1.4.1 Discovery of microRNAs

The history of miRNA biology dates to the early 1990s, although the term itself would not come into use until roughly ten years later. The first identified miRNA, *C. elegans lin-4*, was reported in 1993 by two separate groups (Lee et al, 1993; Wightman et al, 1993). The expression of the *lin-4* gene is negatively correlated with protein levels of LIN-14; a decrease in LIN-14 levels is critical for beginning the first larval stage. Interestingly, upon examining the *lin-4* and *lin-14* sequences, researchers found that *lin-4* encodes small RNAs of roughly 22 and 61 nucleotides, which bear antisense complementarity to a sequence within the *lin-14* 3'UTR. This was the first indication that *lin-4* may mediate its negative regulatory effects via an antisense RNA-RNA interaction. In the following years, many additional *C. elegans* miRNAs would be cloned, as well as miRNAs across a wide variety of eukaryotes.

1.4.2 Canonical biogenesis of microRNAs in *D. melanogaster*

MiRNAs are typically encoded by individual gene loci or in clusters which can potentially generate several miRNAs. The canonical mode of miRNA biogenesis begins with transcription by RNA Polymerase II, to generate a primary miRNA (pri-miRNA) transcript of several hundred nucleotides. In the nucleus, the RNase III enzyme Drosha, in complex with the double-stranded RNA-binding domain (dsRBD) protein Pasha, cleaves the 5’ and 3’ single-stranded ends of the pri-miRNA to create a pre-miRNA stem-loop (Fig. 1.5). Following its export to the cytoplasm, the pre-miRNA is further processed by a separate RNase III enzyme, Dicer-1 (Dcr-1). Dcr-1, cooperating with Loquacious, cleaves the loop
end of the pre-miRNA, liberating a single-stranded RNA of 19-23 nt, the mature miRNA. Its complementary stand, the miR*, is usually degraded although in some contexts may be incorporated into an RNA-induced silencing complex (RISC) and participates in target silencing. The mature miRNA exists in complex with an Argonaute (Ago) family protein; in flies, Ago1 is dedicated to the miRNA pathway.

Figure 1.5: Simplified depiction of canonical microRNA biogenesis in D. melanogaster. A miRNA gene is transcribed by RNA Pol II to produce the primary miRNA (pri-miRNA) transcript. After processing by Drosha/Pasha, the pre-miRNA duplex is exported to the cytoplasm. Another processing event by Dicer-1/Loquacious liberates the mature miRNA strand, which is then loaded into Ago1 to form the microRISC.
1.4.3 Alternative microRNA biogenesis pathways

In addition to the canonical mode of miRNA biogenesis, there exist several ‘alternative’ biogenesis pathways, several of which were first characterized in flies. The ‘mirtron’ pathway processes miRNAs located in the introns of protein-coding genes. Instead of cleavage by Drosha to form the pre-miRNA duplex, as in the canonical pathway, the pre-miRNA duplex is liberated by the nuclear splicing machinery (Fig. 1.6). The pre-miRNA is then exported to the cytoplasm and cleaved by Dicer-1. Interestingly, there is also the case of one vertebrate miRNA, miR-451, which is matured in a Dicer-independent fashion (Cheloufi et al, 2010; Yang et al, 2012). The pre-miRNA is not processed by Dicer cleavage but rather resected by Ago2 to produce the mature miRNA. To date, vertebrate miR-451 is the only validated case of Dicer-independent miRNA biogenesis.

Figure 1.6: Alternative miRNA biogenesis: the mirtron pathway. A mirtron is liberated by the conventional splicing pathway to form a lariat structure. After debranching, the pre-miRNA is exported to the cytoplasm and processed as a conventional miRNA.

1.4.4 Argonaute proteins and mechanisms of microRNA silencing

A wide range of eukaryotes are known to express miRNAs. In many of these organisms, including Drosophila, miRNAs play major roles in various developmental processes, such as tissue patterning, maintenance of cell identity, morphogenesis, metabolism, and immune function. The explosion of miRNA research over the last decade has uncovered roles for miRNAs in virtually every aspect of cellular and developmental biology.
The core of miRNA activity resides in an Argonaute (Ago) family protein. Ago proteins are evolutionarily widespread and ancient; they are found in animals, plants, fungi (S. cerevisiae being a notable exception), and some bacteria and archae. *D. melanogaster* contains five Ago proteins, falling into two subfamilies: Ago1 and Ago2 in the Ago subfamily, and Piwi, Ago3, and Aubergine (Aub) in the PIWI subfamily. An Ago protein contains three characteristic domains: PAZ, MID, and PIWI. The PAZ (Piwi-Argonaute-Zwille) domain is located at the protein’s N-terminus, and is responsible for anchoring the 3’ end of its bound small RNA (Meister, 2013), while the MID (middle) domain anchors the 5’ end of the small RNA. The PIWI domain contains a ‘catalytic triad’ (DDX) motif that bears the endonuclease slicer activity of the Ago protein. *D. melanogaster* Ago2 functions in the siRNA pathway and therefore possesses a robust slicer activity for cleavage of perfectly complementary RNA targets, while Ago1 possesses a weak slicer activity. A small RNA duplex is sorted on the basis of its structural features into one of several RNA silencing pathways, and thus into different Ago proteins. An siRNA duplex of perfect complementarity is processed by Dicer-2, which then aids in loading into Ago2, whereas a miRNA duplex of incomplete complementarity is processed by Dicer-1 and then loaded into Ago1.

In the vast majority of examined cases, miRNAs act as negative regulators of mRNA stability and/or translation. In plants, the Watson-Crick base pairing between a miRNA and its target is perfectly complementary, in most cases resulting in target mRNA destruction. In animals, the base pairing is partially complementary, with the most important miRNA sequence region required for function being the ‘seed,’ nucleotides two through eight on the mature miRNA. Disruption of seed binding in a canonical seed-matched target typically abolishes the miRNA’s activity. In miRNA/target pairs lacking a perfect seed match, base pairing with the 3’ end of the miRNA can compensate (Fig. 1.7).
Plant miRNAs are often perfectly complementary to their target mRNA. In animals, miRNAs without perfect seed matches can compensate with additional basepairing at their 3’ end.

The silencing activity of a miRNA/Ago1 complex is mediated via accessory protein factors that interact with Ago1 (Fig. 1.8). One such protein is GW182, which associates with Ago1 via its N-terminal glycine-tryptophan (GW) repeats (Behm-Ansmant et al, 2006). The GW182 C-terminus binds several other protein factors, including poly-A binding protein (PABP) and the Not1 subunit of the CCR4-Not deadenylase complex. Presumably, recruitment of the CCR4-Not deadenylase complex to an mRNA target results in poly-A tail shortening and either translational silencing or initiation of target RNA decay. Previous studies suggest that GW proteins play a major role as effectors of RNA silencing. Depletion of GW182 results in derepression of miRNA targets, and directly tethering GW182 to an mRNA reporter causes reporter silencing, even in the absence of an Ago protein (Behm-Ansmant et al, 2006).

A complete mechanistic understanding of miRNA-mediated repression is still being pursued, and silencing mechanisms may vary substantially between organisms or cellular contexts. One active area of investigation is determining the relative contributions of pure trans-
Figure 1.8: MiRNA function in *D. melanogaster*. MiRNAs act as negative regulators of mRNA stability and/or translation. In flies, miRNAs base pair with partial complementarity within the 3’UTR of a target mRNA. Ago1, in complex with the miRNA, can recruit protein factors such as GW182, which help to mediate the downstream translational repression of the target mRNA.

1.4.5 Identifying microRNA targets

One of the major goals of miRNA research programs is the identification of miRNA targets. These investigations often begin *in silico* with computational target prediction tools. Algorithms for target prediction can take into account and weight several different variables, including the evolutionary conservation of the target site or miRNA family, the free energy of miRNA binding to its target, or the mRNA’s secondary structure. One commonly used miRNA target prediction tool is TargetScan (www.targetscan.org), which has prediction options for human, mouse, fly, fish, and worms (Lewis et al, 2005; Kheradpour et al, 2007). TargetScan incorporates into its predictions both evolutionary conservation of the target site within the mRNA sequence, and the conservation of the miRNA family.
Plant miRNA target prediction is simplified by the fact that these miRNAs possess perfect base pair complementarity with their mRNA targets. In animals, the miRNA seed region is often perfectly complementary to the target, but not in every case. Moreover, the lack of perfect seed binding can be compensated by more extensive base pairing at the 3’ end of the miRNA. These nuances make reliable miRNA target prediction in animal species more difficult. Target prediction in Drosophila is aided by the robust collection of genome sequence data available; TargetScan Fly uses the sequenced genomes of 12 Drosophila species in its miRNA target prediction.

1.4.6 MicroRNA annotation and community resources

When miRNA sequences began accumulating in significant quantities, the research community established databases for their recording and annotation in different model systems. The most popular example, the online database ‘miRbase’, is a comprehensive collection of freely available and downloadable miRNA sequences from a variety of species (Griffiths-Jones, 2004; Kozomara and Griffiths-Jones, 2014). In addition to the classic model systems such as mouse, human, fly, Arabidopsis, and worm, the database collects, annotates, and assists in naming miRNAs from many non-model species in a variety of biological taxa. For flies, miRbase provides information on the pre-miRNA duplex and mature miRNA sequence, including references of its first cloning and experimental characterization, for example whether its expression has been confirmed by Northern blotting and/or deep sequencing. To date, there are 256 mature miRNA sequences reported for D. melanogaster, with 60% of them considered ‘high confidence’ miRNAs (miRbase.org).

1.4.7 Genetic tools for studying microRNA function

The rapid advancement of miRNA research in D. melanogaster is owed in great part to its advanced repertoire of genetic tools. The most useful tools have combined existing fly technology — the Gal4/UAS system in particular — with transgenic techniques geared towards miRNA biology. Some notable tools among these include the miRNA sensor, miRNA
sponge, and miRNA promoter reporter transgenes, as well as engineered miRNA deletion alleles.

A **miRNA sensor** consists of a fluorescent transgene with binding sites for a specific miRNA of interest within its 3'UTR (Fig. 5.16). This sensor can be expressed ubiquitously or driven in specific tissues. When the sensor is co-expressed with its cognate miRNA, levels of the fluorescent reporter are decreased. By combining the miRNA sensor with mutant alleles for the miRNA of interest or miRNA pathway mutants, tissue-specific patterns of miRNA activity can be uncovered (Dai and Lai, 2012).

The **miRNA sponge** utilizes a similar principle of operation, however — in contrast to a sensor — its purpose is to sequester miRNA from its endogenous target (Loya et al, 2009). Therefore, a sponge typically contains more binding sites for its cognate miRNA than a miRNA sensor (10-20 versus 2-3). Sponges can be used to mimic a miRNA hypomorph or null allele in a tissue-specific fashion, by driving expression of the sponge with tissue-specific Gal4 lines. This gives the researcher a greater degree of control than a traditional mutant allele of a miRNA.

A **miRNA promoter reporter** can be used to report tissue-specific expression of a specific miRNA. The known or predicted promoter of a miRNA is linked to a fluorescent or histological tag (e.g. GFP or lacZ), allowing one to visualize tissues in which a miRNA is endogenously expressed (Dai and Lai, 2012). This strategy has been used successfully in flies and vertebrates, including mice.

**Conditional miRNA expression** is especially simple in flies, due to the well established Gal4/UAS technology. In order to uncover signaling pathways in which specific miRNAs may participate, a Gal4-inducible miRNA transgene can be expressed ectopically or at higher levels in particular tissues of interest (Bejarano et al, 2012).
The use of **miRNA deletions** is increasing, due to the easy availability of gene targeting technology. One recent publication reported the systematic targeted deletion of 80 *D. melanogaster* miRNAs (Chen et al, 2014), which in combination with already existing mutants cover over 99% of fly miRNAs.

### 1.4.8 Studies on microRNA function during *D. melanogaster* oogenesis

*D. melanogaster* has been one of the main workhorses for elucidating the miRNA pathway and identifying functions for individual miRNAs in a variety of developmental contexts. There has been relatively less work on miRNA function during oogenesis, but to date a number of informative publications exist on this system. Some of the earliest studies on oogenesis demonstrated that Dicer-1 is required in germline stem cells for their self-renewal ability, suggesting that miRNA activity is essential for proper function of this cell type (Jin and Xie, 2007). Around the same time as these studies was the first systematic screen of miRNA function during early embryonic development. In this study antisense oligonucleotides, targeting 46 different miRNAs, were injected into early embryos to assay the effects of miRNA knock-down on downstream development. Substantial patterning defects were observed for roughly half of the knock-downs, indicating that various miRNAs play key roles in early embryonic patterning and development (Leaman et al, 2005).

Several years later, the topic of miRNA function in germline cells of the egg chamber was first addressed (Reich et al, 2009). GFP transgenes were engineered with or without binding sites for miR-312, a miRNA previously demonstrated to be expressed at high levels in 0-1 hour embryos, before the onset of zygotic transcription. This GFP reporter indicated high levels of miR-312 activity in the nurse cells. Following this landmark paper, a number of studies followed up by examining miRNA function in both germline cells and follicle cell signaling pathways. For instance, an engineered knock-out of miR-184 helped demonstrate
the necessity of this miRNA for dorsal-ventral patterning of the embryo (Iovino et al, 2009). More recent work has taken advantage of an increasing number of targeted miRNA knock-out lines. Border cell migration, an important event required for formation of the embryonic micropyle, requires miR-989 (Kugler et al, 2013). And miR-318, which is highly expressed in follicle cells, is required for dorsal-ventral axis specification and chorion formation; mutants lacking miR-318 produce thin eggshells resulting in a reduced hatching rate (Ge et al, 2015).
Chapter 2

Processing body function in the *Drosophila* egg chamber

2.1 Background and Context

Processing bodies (P-bodies) are membrane-less cytoplasmic domains that exist in a wide range of eukaryotes, and are known to be involved in the post-transcriptional regulation of mRNA stability and translation. Over the last decade, research has uncovered homologous P-body components in a variety of model organisms, from mammals to yeast. As basic knowledge of P-body structure and function has grown, these organelles have also been implicated in neurodegenerative disorders which stem from the aggregation of RNA/protein complexes in the cytoplasm (Shukla and Parker, 2016).

2.2 Discovery and characterization of P-bodies

In 1997, the first mammalian cDNA encoding a 5’-3’ RNA exonuclease (Xrn1) was isolated and its encoded protein characterized. In mouse cells, Xrn1 was distributed as discrete cytoplasmic particles throughout the cytoplasm (Bashkirov et al, 1997). Although unknown at the time, this represented one of the first studies in the field of P-body research that would unfold over the following two decades.
The coining of the term ‘P-body’ dates to a 2003 Science article which characterized components of the yeast mRNA decapping and 5'-3' RNA decay pathways, and found them to localize together in discrete cytoplasmic foci (Sheth and Parker, 2003). This study made several observations which suggested that P-bodies are sites of RNA decay. P-bodies contain mRNA decapping factors as well as mRNAs. They also respond to perturbations of mRNA translation: when translation elongation was inhibited by trapping mRNA in polysomes, P-bodies decreased in size, whereas inhibiting translation initiation caused P-bodies to increase in size. These dual observations suggest that P-bodies normally contain a pool of translationally inactive/repressed mRNA (Sheth and Parker, 2003). Interestingly, components of the yeast RNA exosome did not localize to these structures, suggesting that they are dedicated specifically to the 5'-3' RNA decay pathway.

Since then, further support accumulated for the hypothesis that these structures represent sites of translationally repressed/inactive mRNA (Figure 2.1). First, P-body formation requires the presence of RNA; inhibition of transcription or treatment of yeast cells with RNase abolished the formation of P-bodies (Sheth and Parker, 2003). Additionally, translation initiation factors (with the exception of eIF4E) and ribosomal proteins are absent from P-bodies (Brengues et al, 2005). More recent immuno-electron microscopy (immuno-EM) experiments in the D. melanogaster egg chamber have reinforced the understanding of P-bodies as electron-dense cytoplasmic regions from which ribosomes are excluded (Weil et al, 2012). The list of protein factors that are associated with P-bodies has also grown substantially (Table 3.1).
2.3 Evolving terminology: P-bodies, GW-bodies, and sponge bodies

Although ‘P-body’ is now the preferred term for describing these cytoplasmic domains, as research in the field initially progressed, new terminology proliferated to refer to these structures in different organisms and tissues. As homologous protein components were identified in different model systems, their large degree of functional overlap was revealed, and the field eventually settled on the term ‘P-body’.

The term ‘sponge body’ was coined in a 1997 study on *D. melanogaster* egg chambers, in reference to cytoplasmic, electron-dense, ‘sponge-like’ structures containing maternal mRNA and protein (Wilsch-Brauninger et al, 1997). A follow-up study helped confirm the idea that sponge bodies and P-bodies refer in large part to the same structures (Snee and Macdonald, 2009). Many of the protein factors described as sponge body markers, such as Me31B, Dcp1/2, and eIF4E, had already been well characterized as central components of P-bodies (Jain and Parker, 2013). Although no longer in common use, the term ‘sponge body’ has persisted when referring to a subset of P-body like structures specific to germ cells (Kloc et al, 2014).

‘GW-bodies’ have been studied since 2002, when a human autoimmune antigen was identified in patients suffering from sensory neuropathy. Named ‘GW182’ for its molecular weight and glycine-tryptophan (GW) repeats, the protein localized in discrete cytoplasmic speckles also containing mRNAs, which became known in the literature as ‘GW-bodies’ (Eystathioy et al, 2002). Subsequent studies identified GW182 orthologues in *C. elegans*, *D. melanogaster*, and vertebrates species; interestingly, GW orthologues do not exist in plants or fungi (Eulalio et al, 2009a). GW proteins are essential for miRNA-mediated gene silencing, a fact that was first demonstrated in *D. melanogaster* cells (Rehwinkel et al, 2005). As components of GW and P-body were identified and characterized, it was discovered that
they have close compositional similarity and the term ‘P-body’ became the settled usage. Although, one recent exception to this general usage rule is a study characterizing the degree of overlap between P-body and GW-body components in the early *D. melanogaster* embryo (Patel et al, 2016).

Figure 2.1: Simplified depiction of P-body function. P-bodies are membrane-less cytoplasmic domains that harbor translationally repressed and/or decaying mRNA. It is thought that a subset of mRNAs can traffic reversibly in and out of P-bodies, transitioning from a translationally repressed to activated state.
2.4 Conservation of P-body components in different model systems

P-body research originated by identifying homologies between translational repressors in *Drosophila* and decapping activators in budding yeast (Coller et al, 2001). It is fitting that since this initial study, dozens of P-body associated proteins have been characterized in different model systems. The best characterized systems, *D. melanogaster*, *C. elegans*, *S. cerevisiae*, and human cells, share many homologous factors that localize to P-bodies. A unifying theme for P-bodies in different organisms has been the presence of proteins involved in different aspects of RNA metabolism: mRNA decapping activators, translational repressors, and RNA exonucleases. However, there are a few notable differences among organisms with respect to P-body composition. In *S. cerevisiae* and mammals, the CCR4-Not deadenylase complex is associated with P-bodies, while it is notably absent in *D. melanogaster*. And because GW182 and its homologues only exist in metazoans, this protein is not found in yeast P-bodies.

2.5 Structure of P-bodies in the *D. melanogaster* egg chamber

In *D. melanogaster*, P-bodies contain members of the mRNA decapping and 5'-3' RNA decay pathways, as well as some proteins that were originally identified as germline translational repressors (Table 3.1). A core component of fruit fly P-bodies is ‘maternal expression at 31B’ (Me31B), a DEAD-Box RNA helicase first identified as a gene essential for oogenesis (de Valoir et al, 1991). Roughly a decade later after its cloning, Me31B became implicated in the translational regulation of several maternally expressed mRNA transcripts (Nakamura et al, 2001). This study demonstrated that Me31B forms cytoplasmic particles with *osk, bcd, Bicaudal D (BicD), nanos (nos), oo18 RNA-binding protein (orb), polar granule component (pgc), and germ cell-less (gcl)* mRNAs in the egg chamber. It also demonstrated a physical interaction between Me31B and Exu, a protein already implicated in the localization of *bcd*.
mRNA; this interaction was RNase-sensitive, suggesting that the proteins exist in a larger RNA-rich complex in the cytoplasm.

Table 2.1: Protein factors associated with *Drosophila* P-bodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me31B</td>
<td>decapping activator/translational repressor</td>
</tr>
<tr>
<td>Trailer Hitch (Tral)</td>
<td>translational repressor</td>
</tr>
<tr>
<td>Dcp1/2</td>
<td>decapping proteins</td>
</tr>
<tr>
<td>Pacman</td>
<td>5’-3’ exonuclease</td>
</tr>
<tr>
<td>eIF4E</td>
<td>translation initiation factor</td>
</tr>
<tr>
<td>Edc3</td>
<td>decapping activator</td>
</tr>
<tr>
<td>GW182</td>
<td>miRNA function</td>
</tr>
<tr>
<td>Ge-1</td>
<td>decapping activator</td>
</tr>
<tr>
<td>Pat1</td>
<td>decapping activator</td>
</tr>
<tr>
<td>Staufen</td>
<td>mRNA localization</td>
</tr>
</tbody>
</table>

Fluorescence microscopy of egg chamber P-body components has demonstrated a morphology ranging from punctate foci to more sponge-like, reticulated structures which are distributed throughout the cytoplasm of both nurse cell and oocyte compartments (Figure 2.2). More recently, immuno-EM has allowed for high resolution and fine-grained imaging of P-bodies in the nurse cells and oocyte (Weil et al, 2012; Davidson et al, 2016). This type of analysis has confirmed that ribosomes are excluded from P-bodies, and revealed that maternal mRNAs exhibit differential levels of association with the P-body ‘core’ and exterior regions.
Figure 2.2: P-body morphology in the *D. melanogaster* egg chamber.

(A) Fluorescence images of fly egg chambers, demonstrating mutual co-localization of P-body components Me31B, Pacman, Dcp1, and Dcp2. Images adapted from Lin et al, 2008.

(B) Immuno-EM image of a cytoplasmic area within an oocyte, with electron-dense P-body regions (outlined with broken blue line) excluding ribosomes (black particles). Scale = 200 nm, image adapted from Weil et al, 2012.
2.6 P-bodies, cellular stress, and stress granules

P-bodies are dynamic structures that respond to changing cellular conditions such as stress. In *D. melanogaster*, stressors that can induce a P-body response include starvation/nutrient deprivation, high temperature, and extended virginity (in females). When exposed to stress conditions, P-bodies can drastically increase in size, transitioning from a punctate to a more reticulated morphology. For example, flies exposed to protein-poor nutrient conditions (without yeast paste) rapidly undergo a starvation response, one effect of which is large cytoplasmic aggregates of P-bodies in the egg chamber. This response is mediated by insulin-like peptide signaling via the follicle cells (Burn et al, 2014). Abrogation of the P-body response drastically reduces survival rates in flies under starvation conditions, which suggests that P-bodies perform essential functions for cell survival, perhaps by storing non-translating mRNA for later use (Burn et al, 2014).

Structures called ‘stress granules’ (SG) have been studied in parallel to P-bodies and display considerable overlap in their protein composition. For this reason, they are considered a similar but distinct type of cytoplasmic mRNP granule. A main difference between SGs and P-bodies is the SG’s association with translation initiation factors, including eIF4G and eIF4A (Table 3.2). SGs also lack members of the mRNA decapping complex, Dcp1 and Dcp2 (Decker and Parker, 2012). Therefore, SGs are thought to harbor translationally active mRNAs, or mRNAs that must be rapidly shifted into active translation in response to cellular conditions.

Table 2.2: Protein factors uniquely associated with stress granules

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PABP</td>
<td>poly-A tail binding protein</td>
</tr>
<tr>
<td>eIF3</td>
<td>translation initiation factor</td>
</tr>
<tr>
<td>eIF4G</td>
<td>translation initiation factor</td>
</tr>
<tr>
<td>eIF4A</td>
<td>translation initiation factor</td>
</tr>
<tr>
<td>40S ribosomal subunit</td>
<td>translation</td>
</tr>
</tbody>
</table>

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2.7 Links between P-bodies and small RNA silencing pathways

A number of studies have explored links between P-bodies and the miRNA and siRNA pathways; in 2005, two separate publications were the first to report interactions between P-bodies, GW182, and the miRNA/siRNA pathways in human cells. The first study demonstrated that Ago1 and Ago2 co-localize with the P-body component Dcp1 and physically interact with both Dcp1 and Dcp2 (Liu et al., 2005a). Importantly, Ago2’s siRNA-binding domain was required for its localization to P-bodies, suggesting that its function in siRNA-mediated silencing is responsible for its P-body association. The link between miRNA/siRNA activity and P-bodies was explored further through use of the MS2 RNA aptamer system for live imaging of cellular mRNA. MS2-YFP reporter genes were engineered to contain artificial miRNA or siRNA binding sites. These reporter genes localized to P-bodies when co-expressed with their cognate miRNA/siRNA, but failed to localize in the absence of their targeting small RNA. Collectively, these data presented a first link between the activity of small RNA-mediated gene silencing pathways and mRNA localization to P-bodies.

In the second study, again using human cell lines, interactions between GW182, Ago proteins, and P-bodies were examined (Liu et al., 2005b). The study described physical interactions between GW182 and both Ago1 and Ago2, and found that depletion of GW182 from cells reduced the silencing efficacy of miRNA or siRNA-targeted reporter genes. Furthermore, repression of the reporter genes required the Ago protein’s localization to P-bodies, even when the reporter was directly tethered to an Ago protein. This study further reinforced the notion of P-bodies as sites of small RNA-mediated gene silencing.

Subsequent studies have demonstrated that even in the absence of microscopically detectable P-bodies, the miRNA pathway functions normally (Eulalio et al., 2007a). P-bodies may be spatially correlated with small RNA activity but are not themselves required for
small RNA-mediated silencing. However, global loss of miRNA function results in P-body
disassembly, suggesting that miRNA-targeted mRNAs may represent a large fraction of all
translationally repressed mRNPs in the cytoplasm (Eulalio et al, 2007b).

2.8 P-bodies and RNA decay

Degradation of mRNA is accomplished via specialized cellular pathways, with separate path-
ways dedicated to 5'-3' or 3'-5' decay. It is important to note that, in most examined cases, P-bodies have been associated exclusively with the 5'-3' decay pathway; components of the 3'-5' decay pathway, such as the RNA exosome, do not localize to P-bodies. This highlights the notion that RNA decay is highly regulated and compartmentalized within the cell.

A necessary precursor event for 5'-3' decay is the removal of the mRNA poly-adenosine
(poly-A) tail (Fig. 2.3). Poly-A tail removal is performed mainly by the CCR4-Not complex,
a cytoplasmic multi-subunit protein complex. In flies, this complex contains two catalytic
subunits, Ccr4 and Pop2, which physically remove adenosine nucleotides from the 3' end of
the tail. The Not1 protein is a scaffolding subunit required for formation of the complex.
Once the poly-A tail is shortened below a critical threshold of 10-12 adenosines, poly-A
binding protein (PABP) can no longer bind the tail and the mRNA becomes susceptible to
removal of the 5' methylguanosine (m7G) cap structure. The Dcp1/2 proteins which per-
form this function are localized to P-bodies; Dcp2 harbors the enzymatic activity while Dcp1
functions as a decapping ‘co-activator’. Once decapping occurs, an mRNA rapidly becomes
a substrate for the 5'-3' decay pathway. The enzyme Pacman (*Drosophila* homolog of Xrn1)
is the 5'-3' exonuclease which performs this function.

A large body of evidence has established that P-bodies represent sites of RNA decay, in
addition to their roles in mRNA translational repression and storage. They contain mem-
bers of the mRNA decapping and 5'-3' decay pathway: Dcp1, Dcp2, and Pacman. P-bodies
also respond in both size and number to manipulations of the 5'-3' RNA decay pathway.
Preventing early precursor steps of RNA decay, by blocking CCR4 activity, causes P-bodies to diminish in number. Conversely, blocking the decay process at a later stage, for example via removal of Xrn1, increases the size and number of P-bodies. These findings indicate that by decreasing or increasing the pool of deadenylated/non-translating mRNAs, P-bodies respond by decreasing or increasing in size and number, respectively.

With respect to RNA decay and its relationship to P-bodies, studies on *D. melanogaster* egg chambers have demonstrated similarities to those in yeast and other organisms. Fly P-bodies respond to both alterations in mRNA translation rates and compromised RNA decay activity. For example, in both *dcp2* and *pacman* mutant egg chambers, with compromised decapping and 5’-3’ decay activities respectively, P-bodies increase in size, suggesting that deadenylated mRNAs accumulate in P-bodies (Lin et al, 2008). Conversely, treatment of egg chambers with cycloheximide, which traps actively translating mRNA in polysomes, greatly reduces formation of P-bodies, as would be expected owing to a decreased cytoplasmic pool of non-translating RNA (Lin et al, 2008).
Figure 2.3: Deadenylation followed by RNA decay in P-bodies. An mRNA is first deadenylated in the cytoplasm by the CCR4-Not complex, followed by recruitment into a P-body (broken line). The 5′-3′ decay of mRNA is one activity that can occur in P-bodies.
Chapter 3

*bicoid* mRNA association with P-bodies

### 3.1 Introduction

P-body function in the *Drosophila* egg chamber has been examined in a limited number of studies (Lin et al, 2008). However, previous work has demonstrated that several maternally localized transcripts, including *oskar, bicoid*, and *gurken*, each display some degree of localization to P-bodies. Building on preliminary studies which were spatially restricted to the anterior of the oocyte, we began by examining the degree of *bcd* localization to P-bodies throughout oogenesis.

### 3.2 Presence of *bicoid* mRNA in nurse cell and oocyte P-bodies

Previous work on *bcd* mRNA’s association with P-bodies was restricted to the oocyte anterior. We examined *bcd*’s presence in P-bodies at varying stages of oogenesis, in both nurse cell and oocyte compartments of the egg chamber. To visualize P-bodies in the egg chamber, we made use of GFP and YFP protein-trapped fly lines, labeling the *Drosophila* P-body components Me31B (YFP label) and Trailer Hitch (Tral) (GFP label). Each protein-trapped line displays a punctate fluorescent signal consistent with P-body morphology observed by antibody staining (Lin et al, 2008). We performed single molecule fluorescence *in situ* hybridization (smFISH) using *bcd*-specific Stellaris probes, and imaged *bcd* mRNA in the Me31B-YFP and Tral-GFP egg chambers, respectively. Using a spinning disc confocal
microscope, we observed extensive co-localization during early and mid-late oogenesis, in both the nurse cell and oocyte compartments of the egg chambers (Fig. 3.1).

**Figure 3.1:** *bcd* mRNA associates with P-bodies at different developmental stages. (A) Early and (B) mid-to-late stage Me31B-YFP and Tral-GFP (green) egg chambers were used for smFISH for *bcd* mRNA (red). The merge (yellow) panels define areas where *bcd* mRNA co-localizes with P-body components. Images are composites of 3-4 optical Z slices (Z step of 0.5 μm). Scale bar = 25 μm, with 40X magnification. Representative images from at least three independent experiments.
At higher magnification, the co-localization of \( bcd \) mRNA with Me31B-YFP is more apparent in the egg chamber’s nurse cells, as well as P-body morphology which varies from punctate foci to more reticulated structures (Fig. 3.2).

Figure 3.2: \( bcd \) mRNA associates with P-bodies in the nurse cells. (A) Stage 6 and (B) stage 8 egg chambers expressing Me31B-YFP (green) and used for smFISH for \( bcd \) mRNA (red). The merge (yellow) panels define areas where \( bcd \) mRNA co-localizes with P-body components. Images are composites of 3-4 optical Z slices (Z step of 0.5 \( \mu m \)). Scale bar = 25 \( \mu m \), with 63X magnification. Representative images from at least three independent experiments.

3.3 Function of the CCR4-Not deadenylase complex and 5’-3’ RNA decay pathway

One of the precursor events necessary for sequestration of mRNAs into P-bodies is the shortening of the 3’ poly-adenosine (poly-A) tail. In flies, this function is performed mainly by the CCR4-Not deadenylase complex, a multi-subunit protein complex (Fig. 3.3). Of the several members of the full complex, we examined the two catalytic protein subunits which degrade the poly-A tail, Ccr4 and Pop2, and the scaffolding subunit, Not1, which is required for efficient formation of the complex.
Figure 3.3: Schematic of the CCR4-Not complex. Pop2 and Ccr4 are the catalytic subunits bearing deadenylase activity. Not1 serves as a scaffold for the entire complex.

It is thought that following entry into a P-body, an mRNA is capable of becoming a substrate for the 5′-3′ RNA decay pathway. Following removal of the 5′ methylguanosine (m7G) cap by the Dcp1/2 proteins, the mRNA is decayed from the 5′ end by the 5′-3′ exonuclease, Pacman (Fig. 3.4). With the goal of modulating P-body size and observing downstream effects on maternal mRNA levels, we used transgenic RNAi fly lines (TRiP) to deplete different components of the CCR4-Not complex and 5′-3′ decay pathway. We used TRiP lines targeting *pacman, ccr4, not1,* and *pop2* (Table 3.1). Our experimental setup was as follows: for each knock-down, changes in P-body morphology were observed by visualizing Me31B-YFP and Tral-GFP, respectively. In parallel, ovarian total RNA was isolated from each knock-down and used for qPCR to measure the levels of three maternally localized transcripts, *bcd, osk, nos,* as well as three ‘reference’ genes, *rp49, cdk9,* and *HetA* (Table 3.2). The use of *HetA,* a germline-specific transposable element, as a reference gene allowed
Figure 3.4: Schematic of mRNA decapping and 5’-3’ decay. Following 5’ decapping by Dcp1/2, an mRNA can serve as a substrate for the 5’-3’ exonuclease Pacman.

Table 3.1: Members of the *Drosophila* CCR4-Not deadenylase and 5’-3’ mRNA decay pathways

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>pacman</td>
<td>5’-3’ exonuclease</td>
</tr>
<tr>
<td>not1</td>
<td>scaffolding subunit</td>
</tr>
<tr>
<td>ccr4</td>
<td>3’-5’ exonuclease subunit</td>
</tr>
<tr>
<td>pop2</td>
<td>3’-5’ exonuclease subunit</td>
</tr>
</tbody>
</table>

3.4 Effect of pacman knock-down on P-body size

In pacman KD egg chambers, P-bodies increased in size and intensity (Me31B-YFP and Tral-GFP signal) (Fig. 3.5, A), consistent with previous observations in pacman mutant egg chambers (Lin et al, 2008). However, there was not a substantial change in maternal...
Table 3.2: mRNAs measured by qPCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Expression</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>bicoid</td>
<td>germline</td>
<td>target gene</td>
</tr>
<tr>
<td>oskar</td>
<td>germline</td>
<td>target gene</td>
</tr>
<tr>
<td>nanos</td>
<td>germline</td>
<td>target gene</td>
</tr>
<tr>
<td>rp49</td>
<td>germline &amp; somatic</td>
<td>reference gene</td>
</tr>
<tr>
<td>cdk9</td>
<td>germline &amp; somatic</td>
<td>reference gene</td>
</tr>
<tr>
<td>HetA</td>
<td>germline</td>
<td>reference gene</td>
</tr>
</tbody>
</table>

transcript levels between the control (mCherry) and pacman knock-downs (Fig. 3.5, B).

3.5 Effect of not1 knock-down on P-body morphology

In knock-downs of not1, the scaffolding subunit, P-body morphology was drastically changed, from the typical punctate to a more diffuse morphology (Fig. 3.6, A). There was also a substantial decrease in bcd, nos, and to a lesser extent osk mRNA levels when compared to the control knock-down (Fig. 3.6, B).

3.6 Effects of ccr4 and pop2 knock-downs on P-body morphology

We next examined knock-downs of ccr4 and pop2, both exonuclease subunits of the deadenyylase complex. The ccr4 knock-downs did not display a substantial change in P-body morphology (Fig. 3.7, A) or maternal transcript levels (Fig. 3.7, B). However, similar to not1, knock-down of pop2 resulted in a diffuse P-body morphology (Fig. 3.8, A) and the levels of maternal mRNAs were significantly decreased (Fig. 3.8, B).
Figure 3.5: *pacman* KD increases P-body size. (A) Control (*mCherry*) and *pacman* knock-downs in Me31B-YFP and Tral-GFP (green) egg chambers, respectively. Images are composites of 3-4 optical Z slices (Z step of 0.5 µm). Scale bar = 25 µm, with 63X magnification. Representative images from at least three independent experiments. (B) qPCR quantification of mRNA levels. The colored legend indicates the *ccr4* and control (*mCherry*) knock-downs. The x-axis indicates the mRNA being quantified. The y-axis indicates the relative mRNA level as a percentage of the control knock-down. N = 9 (combined three technical replicates from three independent experiments). Error bars = SD (in some samples, error bars are concealed by the colored box).
Figure 3.6: *not1* KD causes diffuse P-body distribution. (A) Control (*mCherry*) and *not1* knock-downs in Me31B-YFP and Tral-GFP (green) egg chambers, respectively. Images are composites of 3-4 optical Z slices (Z step of 0.5 µm). Scale bar = 25 µm, with 63X magnification. Representative images from at least three independent experiments. (B) qPCR quantification of mRNA levels. The colored legend indicates the ccr4 and control (*mCherry*) knock-downs. The x-axis indicates the mRNA being quantified. The y-axis indicates the relative mRNA level as a percentage of the control knock-down. N = 9 (combined three technical replicates from three independent experiments. Error bars = SD (in some samples, error bars are concealed by the colored box).
Figure 3.7: ccr4 KD alters P-body distribution. (A) Control (mCherry) and ccr4 knock-downs in Me31B-YFP and Tral-GFP (green) egg chambers, respectively. Images are composites of 3-4 optical Z slices (Z step of 0.5 µm). Scale bar = 25 µm, with 63X magnification. Representative images from at least three independent experiments. (B) qPCR quantification of mRNA levels. The colored legend indicates the ccr4 and control (mCherry) knock-downs. The x-axis indicates the mRNA being quantified. The y-axis indicates the relative mRNA level as a percentage of the control knock-down. N = 9 (combined three technical replicates from three independent experiments. Error bars = SD (in some samples, error bars are concealed by the colored box).
Figure 3.8: *pop2* KD causes diffuse P-body distribution. (A) Control (*mCherry*) and *pop2* knock-downs in Me31B-YFP and Tral-GFP (green) egg chambers, respectively. Images are composites of 3-4 optical Z slices (Z step of 0.5 µm). Scale bar = 25 µm, with 63X magnification. Representative images from at least three independent experiments. 

(B) qPCR quantification of mRNA levels. The colored legend indicates the *ccr4* and control (*mCherry*) knock-downs. The x-axis indicates the mRNA being quantified. The y-axis indicates the relative mRNA level as a percentage of the control knock-down. N = 9 (combined three technical replicates from three independent experiments. Error bars = SD (in some samples, error bars are concealed by the colored box).
3.7 Synopsis and Discussion

In this chapter, we report that bcd mRNA co-localizes with two P-body markers, Me31B and Tral, at different stages of oogenesis, in both the nurse cell and oocyte compartments of the egg chamber. We examined the distribution of egg chamber P-bodies using RNAi lines targeting different members of the CCR4-Not deadenylase complex. Depleting not1 and pop2, and presumably reducing cytoplasmic deadenylase activity, resulted in a dramatic change in P-body distribution, from a punctate to a more diffuse cytoplasmic morphology.

Both Ccr4 and Pop2 are catalytic subunits of the deadenylase machinery, yet the ccr4 knock-down did not produce the same dramatic phenotype of pop2 KD. One possible explanation for this finding is tissue-specific activities of Ccr4 and Pop2. Previous work has shown that in Drosophila S2 cells, the activity of one subunit predominates. Similarly, it is possible that in the ovary the activity of Pop2 is more essential for deadenylase function, with Ccr4 playing a minor role.

Overall, our findings seem to suggest that cytoplasmic P-bodies act to stabilize maternal transcripts, which appears to contradict the notion that RNA decay is one of their major activities. Under normal cellular conditions, it is unlikely that RNA decay plays a major role in the regulation of the maternal patterning transcripts in the egg chamber. The fact that mature Drosophila eggs - which no longer perform transcription - are still capable of being fertilized for over one week after maturation suggests that the localized transcripts are highly stable in vivo.

One interesting observation from previous work on oocyte P-bodies is that shortly following egg activation, bcd mRNA dissociates from Me31B foci in the egg. During this developmental time period, bcd mRNA translation is initiated. This correlation might suggest that bcd’s removal from P-bodies is required for its translation, although a causal link
has not been established. The authors of this study observed Me31B foci disassembly following egg activation, suggesting that egg activation has a more global effect on P-body morphology, which is perhaps linked to the global changes in protein translation that are known to accompany egg activation.

An obvious question raised by our findings, as well as by previous work, is the mechanism by which bcd mRNA is localized to P-bodies in the first place. To our knowledge, there has not been an exhaustive description of cellular mRNAs regarding their localization to P-bodies, similar to the large-scale screen for localized embryonic mRNAs performed by the Krause lab, University of Toronto. Therefore, the field currently lacks an estimate of the fraction of cellular mRNAs which are sequestered in P-bodies. However, super-resolution fluorescence imaging as well as electron microscopy have definitively shown that maternal transcripts differ in the degree of their compartmentalization within P-bodies (Weil et al, 2012), suggesting that this process must be regulated at some level. The mechanism by which this compartmentalization is controlled is also unknown.
Chapter 4

MiR-305 expression and predicted targeting of *bicoid* mRNA

4.1 Introduction

In model systems such as yeast and mammalian cell culture, P-bodies have been implicated in the activity of small regulatory RNA pathways, such as the siRNA and miRNA pathways (Liu et al, 2005). Both the 5’ and 3’ untranslated regions (UTR) of an mRNA can serve important roles with respect to translational regulation. A collection of previous work on *bcd* mRNA suggests that the 3’UTR may be the main mediator of its translational repression. First, replacing the *osk* 3’UTR with that of *bcd* causes it to adopt *bcd*’s translational timing. Second, it has been shown that the *bcd* 5’UTR is not required for its translational repression during oogenesis, suggesting that the 3’UTR region may be more important for this function (Driever et al, 1990). A survey of the literature led us to hypothesize that *bcd*’s presence in egg chamber P-bodies may be indicative of regulation by one or more miRNAs. With the importance of *bcd*’s 3’UTR in mind, we explored this possibility by using computational methods to identify possible miRNA binding sites in the *bcd* transcript.
4.2 Computational microRNA target prediction

Investigations into miRNA targeting of an mRNA typically begin with a computational prediction of candidate miRNAs. Various softwares are now available for this purpose, which vary in their prediction methods. Some algorithms place an emphasis on evolutionary conservation of the miRNA site within the target, while others use mRNA secondary structure to predict single-stranded regions that would be amenable to miRNA binding. We used the free online miRNA prediction software TargetScan (http://www.targetscan.org) to query the \textit{bcd} 3’UTR. TargetScan uses the evolutionary conservation of the miRNA binding site and conservation of the miRNA family as criteria for identifying likely target sites. An alignment of the \textit{bcd} 3’UTR sequence from twelve \textit{Drosophila} species identified a single highly conserved, predicted binding site for miR-305 (Fig. 4.1).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.1.png}
\caption{Partial sequence alignment of \textit{bcd} 3’UTR containing predicted miR-305 site. The predicted seed-binding region in the \textit{bcd} 3’UTR is highlighted in red. The top of the alignment indicates the nucleotide number following the stop codon.}
\end{figure}

4.3 Design of \textit{bicoid} 3’UTR reporter assay

Reporter gene assays are often used to test the repressive effects of a miRNA on a putative mRNA target. To examine the capacity for miR-305 to repress translation via the
bcd 3’UTR, we used the pSiCheck reporter gene assay (Promega) in Drosophila S2 cells (Fig. 4.2). The full bcd 3’UTR was cloned downstream of the Renilla luciferase coding sequence. On the same vector, a firefly luciferase transgene served as an internal control for transfection efficiency. S2 cells were then co-transfected with the Luciferase-bcd 3’UTR vector and a DsRed-miRNA or empty DsRed plasmid. The assay’s ability to robustly measure target-specific miRNA-mediated silencing was demonstrated by co-expression of mirtron-2 and its “perfect target” transgene. Mirtron-2 strongly repressed its target reporter but had no effect on the bcd 3’UTR reporter, as expected (Fig. 4.3).

**Figure 4.2: MiRNA Reporter Assay: Principle of Operation.** Assay components: (1) Renilla Luciferase reporter containing the 3’UTR of interest. (2) A DsRed fluorescent reporter encoding a pri-miRNA of interest in its 3’UTR. The two vectors are co-transfected into Drosophila S2 cells, followed by luminescence measurements.

**Figure 4.3: Proof of principle: mirtron-2 repression of a perfect target.** For each floating box, the pSiCheck vector is indicated in the color-coded legend. The co-transfected DsRed vector is indicated on the x-axis. Relative luciferase (Renilla/Firefly) is indicated on the y-axis. N = 8 (combined four technical replicates from each of two independent experiments). Error bars = SD.
4.4 The *bicoid* 3’UTR contains a Nanos Response Element

The *bcd* 3’UTR contains a motif known as the Nanos Response Element (NRE), a bipartite motif which has been previously implicated in regulating *bcd* mRNA stability during early embryogenesis (Gamberi et al, 2002). A homologous NRE found in the *hunchback* (*hb*) 3’UTR serves as a binding site for the translational repressor Pumilio, and there is evidence that Pumilio can also bind the *bcd* NRE (Gamberi et al, 2002). The *bcd* NRE consists of one “Box A” and two “Box B” motifs, and partially overlaps with the predicted miR-305 binding site (Fig. 4.4). Interestingly, several lines of evidence, drawn from studies published in the last few years, suggest a role for Pumilio as an accessory component of the miRNA machinery in different cellular contexts. First, sequence analysis of mRNAs has demonstrated that Pumilio binding sites are significantly enriched in the vicinity of high-confidence miRNA binding sites (Galgano et al, 2008). In human cells, Pumilio binding has been shown to enhance miRNA recognition of different target mRNAs (Kedde et al, 2010; Miles et al, 2012). Second, the *C. elegans* Pumilio homolog, FBF, as well as mammalian PUM-2, physically associate with Ago1 and eEF1A to attenuate translation elongation (Friend et al, 2012).

![Figure 4.4: Organization of the *hunchback* and *bicoid* Nanos Response Elements.](image)

The *hb* 3’UTR contains two full NREs, while *bcd* contains “1.5” NREs comprised of one Box A and two Box B motifs.
To determine whether an intact NRE is required for repression by miR-305, we employed a mutagenesis scheme which allows for the separation of miR-305 and NRE-mediated repression on bcd (Fig. 4.5). The dinucleotide mutations introduced into each Box have been previously demonstrated to abolish Pumilio binding to the hb NRE (Wharton and Struhl, 1991).

Figure 4.5: Mutagenesis of bicoid Nanos Response Element. Mutations indicated in purple above WT Box A (red) and Box B (blue) sequences. MiR-305 seed region is boxed.

Table 4.1: NRE Box sequences and mutations

<table>
<thead>
<tr>
<th>NRE Box</th>
<th>WT sequence</th>
<th>Mutated sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Box A</td>
<td>guugu</td>
<td>caugu</td>
</tr>
<tr>
<td>Box B1</td>
<td>auugua</td>
<td>auacua</td>
</tr>
<tr>
<td>Box B2</td>
<td>auugua</td>
<td>auacua</td>
</tr>
</tbody>
</table>

4.5 The upstream and downstream NRE Boxes are not required for repression by miR-305

We performed reporter assays to test miR-305’s ability to repress the bcd 3’UTR in the context of each dinucleotide mutation, both singly and in combination. The miR-305 seed binding region was also separately mutated such that the altered nucleotides do not overlap with any NRE Box sequence. Mutation of the miR-305 seed binding region abolished miR-305’s ability to repress, as expected (Fig. 4.6). We then tested the necessity for miR-305 repression of the upstream Box A and Box B2, which falls downstream of the miR-305 binding site. Mutation of either sequence alone had no effect on miR-305’s ability to repress translation of the target (Figs. 4.7 - 4.8).
Figure 4.6: Seed region mutation abolishes repression by miR-305. For each floating box, the pSiCheck vector is indicated in the color-coded legend. The co-transfected DsRed vector is indicated on the x-axis. Relative luciferase (Renilla/Firefly) is indicated on the y-axis. *** = p < 0.001; ns = not significant. N = 12 (combined four technical replicates from each of three independent experiments). Error bars = SD.

Figure 4.7: NRE Box A is not required for miR-305 mediated repression. For each floating box, the pSiCheck vector is indicated in the color-coded legend. The co-transfected DsRed vector is indicated on the x-axis. Relative luciferase (Renilla/Firefly) is indicated on the y-axis. **** = p < 0.0001; *** = p < 0.001; ns = not significant. N = 12 (combined four technical replicates from each of three independent experiments). Error bars = SD.
Figure 4.8: NRE Box B2 is not required for miR-305 mediated repression. For each floating box, the pSiCheck vector is indicated in the color-coded legend. The co-transfected DsRed vector is indicated on the x-axis. Relative luciferase (Renilla/Firefly) is indicated on the y-axis. **** = p < 0.0001; *** = p < 0.001; ns = not significant. N = 12 (combined four technical replicates from each of three independent experiments). Error bars = SD.

4.6 The bicoid NRE Box B1 directly overlaps with the miR-305 binding site

The Box B1 sequence in the bcd NRE directly overlaps with the predicted binding region for miR-305 (Fig. 4.5). To determine if miR-305 can repress in the context of a mutated Box B1, we introduced compensatory mutations into the pre-miR-305 hairpin (the compensatory mutant hairpin is termed miR-305*) such that miR-305* can bind, with an identical level of complementarity, to the mutant NRE sequence (Fig. 4.9). MiR-305* was able to repress both the bcd NRE Box B1 mutant reporter (Fig. 4.10), and a reporter bearing combined mutations in all three NRE Boxes (Fig. 4.11). Collectively, these observations demonstrate that an intact NRE sequence is not required for miR-305 mediated repression.
Figure 4.9: Schematic of pre-miR-305 compensatory mutations. The pre-miR-305 hairpin (top) with the mature miR (boxed) and miR* sequences indicated in green. The compensatory mutant pre-miR-305 hairpin (bottom) with mutated nucleotides indicated in red.

Figure 4.10: NRE Box B1 is not required for miR-305 mediated repression. For each floating box, the pSiCheck vector is indicated in the color-coded legend. The co-transfected DsRed vector is indicated on the x-axis. Relative luciferase (Renilla/Firefly) is indicated on the y-axis. **** = p<0.0001; *** = p<0.001; ns = not significant. N = 12 (combined four technical replicates from each of three independent experiments). Error bars = SD.
4.7 Detection of ovarian miR-305 expression by qPCR

To determine if miR-305 is expressed in fly ovaries, consistent with a role in regulation of \textit{bcd} translation, we used a modified qPCR assay for the detection of specific mature miRNAs. The assay makes use of stem-loop forming reverse-transcription (RT) primers, whose secondary structure increases the affinity of the primer for the 3’ end of its specific mature miRNA (Fig. 4.12). Abundant levels of miR-305 were detected in ovarian small RNA lysate, along with several other miRNAs, while miR-280 served as a negative control (Fig. 4.13). In the time since these experiments were performed, several published studies have corroborated the maternal expression of miR-305 using RNA sequencing (Marco, 2015).
Figure 4.12: Stem-loop qPCR assay for detecting mature miRNAs. A stem-loop forming DNA primer, whose 3’ end is specific for a single mature miRNA, is added to a small RNA lysate. An RT reaction produces a cDNA, followed by a conventional qPCR reaction to quantify levels of the miRNA.

Figure 4.13: Quantitation of ovarian miR-305 levels. The raw qPCR amplification curves for miR-305 and other miRNAs (left). These curves were converted to histograms (right) based on absolute quantitation methods, and presented as log(copy number) per 250 ng of small RNA lysate.
4.8 Organization of the miR-305 locus and mutant alleles

The miR-305 gene is located on the left arm of chromosome 2, clustered with miR-275 and the non-coding RNA CR43857 (Fig. 4.14). We obtained a targeted knock-out of miR-275/305 (Stephen Cohen laboratory, Institute of Molecular and Cell Biology, Singapore), as well as the cuc<sup>1</sup> allele of CR43857(Bloomington Drosophila Stock Center), which abolishes its transcription (Table 4.2).

![Figure 4.14: Organization of the miR-305 gene locus. MiRs 275 and 305 are clustered and transcribed from the non-coding RNA CR43857.](image)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-275/305 KO</td>
<td>Targeted deletion of miR-275/305 cluster</td>
</tr>
<tr>
<td>cuc&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Abolishes transcription of CR43857</td>
</tr>
<tr>
<td>Df(2L) BSC189</td>
<td>Deletion spanning the CR43857 and miR-275/305 loci</td>
</tr>
</tbody>
</table>
To validate the miR-305 KO, these alleles were crossed in several combinations, followed by total ovarian RNA isolation and qPCR to detect levels of the miR-305 primary transcript. The miR-305 homozygous KO, as well as each allele in trans to a deficiency spanning the entire miR-275/305 locus (*Df(2L) BSC189*), reduced miR-305 expression to undetectable levels (Fig. 4.15).

**Figure 4.15: Validation of miR-305 KO alleles.** The miR-275/305 KO, *cuc*¹, and *Df(2L)* alleles were combined, ovarian RNA isolated, and pri-miR-305 levels measured by qPCR. Histogram represents combined samples from two independent experiments.
4.9 MiR-305 GFP sensor reports activity in the egg chamber

We used a miRNA sensor fly line (gift from Stephen Cohen’s lab) to determine if miR-305 is active in the germline cells of the egg chamber. The sensor contains a ubiquitously expressed GFP transgene tagged with an NLS, and two miR-305 binding sites in its 3’UTR (Fig. 4.16). The sensor line was crossed into the mutant alleles described above. Partial de-repression of the sensor was observed in each mutant background, compared to the WT (Fig. 4.17).

**Figure 4.16:** MiR-305 sensor: Principle of operation. It consists of a GFP transgene, driven by the Ubiquitin promoter, tagged with a nuclear localization sequence (NLS), and two optimal binding sites for miR-305 in its 3’UTR.

**Figure 4.17:** MiRNA sensor reports miR-305 activity in the egg chamber. The miR-305 GFP sensor was crossed into WT, miR-275/305 KO, Df(2L), and cuc1 backgrounds. De-repression of the sensor was observed in the nurse cells in comparison to the WT background. Image represents single optical Z slice. Scale = 25 µm, with 63X magnification. Representative images from three independent experiments.
4.10 Synopsis and Discussion

In this chapter, we demonstrated that the bcd 3’UTR contains a predicted target site for miR-305, and that miR-305 is expressed and active in ovaries. miR-305 is able to repress a bcd 3’UTR reporter gene in an S2 cell assay system. This repressive activity does not require an intact Nanos Response Element in the bcd 3’UTR.

While the bcd 3’UTR reporter experiments were performed in a cell culture model, they may be suggestive of miRNA regulation taking place in vivo. Repression of the bcd reporter was specific and dependent on both miR-305 and the seed binding region within the 3’UTR. Collectively, these data suggest that miR-305 is at least capable of regulating the bcd 3’UTR and is expressed and active during the appropriate developmental time period to do so.

Previous work has suggested possible interactions between Pumilio and the miRNA pathway, for instance that Pumilio binding is required to change the local secondary structure of an mRNA in order to allow for miRNA access. We explored a similar possibility in the bcd 3’UTR reporter assay by mutating NRE sequence motifs separately and in combination, to determine whether these motifs must be intact in order for miR-305 to repress the reporter. While Pumilio is expressed at high levels in S2 cells, Nanos protein is not present (Weidmann and Goldstrohm, 2012). It is possible that Nanos is required to mediate the repressive effects of Pumilio in S2 cells, although Pumilio has been shown to be capable of binding the NRE and repressing translation independently of Nanos (Weidmann and Goldstrohm, 2012).
Chapter 5

Design and expression of *bicoid* transgenes

5.1 Introduction

In order to study the timing of *bcd* translation *in vivo*, we constructed GFP-tagged *bcd* transgenes for expression during oogenesis. These transgenic fly lines were then manipulated using RNAi or mutant alleles to investigate the genetic requirements for *bcd* translational repression in the egg chamber.

5.2 Design of *gfp-bcd* transgene for *in vivo* expression

In order to recapitulate *bcd*’s native expression levels as closely as possible, we constructed a GFP-tagged transgene bearing the endogenous *bcd* promoter, coding sequence, introns, 5’ and 3’ regulatory elements (Fig. 5.1). Using the ΦC31 site-specific integration system, the transgene was inserted into the X chromosome. The *gfp-bcd* fly line is viable as a homozygous insertion. To confirm correct expression of the transgenic *bcd*, early stage embryos were visualized for GFP expression. We observed the expected anterior gradient of GFP-Bcd, indicating that the localization and translational regulation of *bcd* mRNA is intact (Fig. 5.2).
Figure 5.1: Design of \textit{gfp-bcd} transgene. \textit{gfp-bcd} consists of the \textit{bcd} promoter, CDS, introns, 5’ and 3’ UTRs, with an N-terminal GFP tag.

Figure 5.2: Embryonic expression of \textit{gfp-bcd} transgene. The GFP-tagged Bcd protein correctly localizes to the anterior of the embryo, forming an A-P concentration gradient. Image is a composite of 47 optical Z slices (Z step of 0.5 \( \mu \text{m} \)) and stitched from multiple Z stacks. Scale = 25 \( \mu \text{m} \), with 40X magnification.

5.3 Effect of miR-305 KO on \textit{bicoid} mRNA translation in the egg chamber

To determine if miR-305 activity is required for the repression of \textit{bcd} translation, we introduced the \textit{gfp-bcd} transgene into a miR-305 homozygous KO background. The \textit{gfp-bcd}; miR-305 KO/KO and \textit{gfp-bcd} ovaries were indistinguishable with respect to GFP signal, suggesting that the absence of miR-305 has no detectable effect on the timing of \textit{bcd} translation (Fig. 5.3).
Figure 5.3: A miR-305 KO background does not cause premature *gfp-bcd* translation. The *gfp-bcd* transgene was crossed into a homozygous miR-305 KO background, and ovaries examined for GFP signal. Images are composites of 3-4 optical Z slices (Z step of 0.5 µm). Scale = 25 µm, with 63X magnification.

5.4 High levels of *bicoid* mRNA result in its translation in the egg chamber

We decided to test the idea that by driving high levels of *bcd* mRNA expression in the egg chamber, the repressive factors, whether protein or RNA, could be titrated out of the system, resulting in Bcd protein expression. In order to accomplish this, a new transgene was designed, identical to *gfp-bcd* except for the *bcd* promoter replaced with a UAS for Gal4-inducible expression (Fig. 5.4).

Figure 5.4: Design of UAS-*gfp-bcd* transgene. UAS-*gfp-bcd* is identical to *gfp-bcd*, with the exception of the *bcd* promoter replaced by a 5X UAS sequence.
To compare the mRNA expression levels of each transgenic line, ovarian RNA was isolated and the levels of \textit{bcd} mRNA analyzed. A homozygous \textit{gfp-bcd} line expressed roughly two times the level of \textit{bcd} mRNA as the WT, as expected, whereas the \textit{UAS-gfp-bcd} line, driven by maternal Gal4, displayed ovarian \textit{bcd} mRNA levels approximately 15-fold higher than the WT (Fig. 5.5).

![Figure 5.5](image)

**Figure 5.5: Ovarian \textit{bicoid} mRNA expression levels of \textit{gfp-bcd} transgenic lines.** Ovaries from the indicated lines were dissected, total RNA isolated, and mRNA levels quantified by qPCR. Histograms represent samples combined from two independent experiments.

To determine if the high levels of \textit{bcd} mRNA correlated with Bcd protein expression, ovaries from each fly line were used for immunofluorescence using a Bcd antibody. In the \textit{UAS-gfp-bcd} ovaries, robust expression of Bcd protein was detected, localized to nurse cell nuclei as expected (Fig. 5.6). Bcd was not detected in the WT and \textit{gfp-bcd} egg chambers, suggesting that just two additional copies of \textit{bcd} are not sufficient to cause detectable levels of protein. We also constructed an identical \textit{UAS-gfp-bcd} transgene bearing a mutation in the miR-305 seed binding site. When expression of the WT and seed mutant \textit{UAS-gfp-bcd} transgenes were driven with Gal4 in the egg chamber, a modest increase in GFP signal in the seed mutant egg chambers was observed (Fig. 5.7).
Figure 5.6: Ovarian protein expression of *gfp-bcd* transgenes. Ovaries from the indicated lines were dissected, fixed, and probed with anti-Bcd antibody. Images are composites of 3-4 optical Z slices (Z step of 0.5 µm). Scale = 25 µm, with 63X magnification.
Figure 5.7: Quantitation of GFP expression from *UAS-gfp-bcd* transgenes. 

(A) Ovaries from the indicated lines were dissected, fixed, and then imaged. Representative images from the indicated genotypes. Images are composites of 3-4 optical Z slices (Z step of 0.5 µm). Scale = 25 µm, with 63X magnification. (B) Nuclear GFP fluorescence levels of each genotype were measured across 10 Z slices using ImageJ software, and plotted according to developmental stage. N = three independent experiments. Error bars = SD.
5.5 Pumilio is expressed in the germarium

Because bcd mRNA contains an NRE, we examined whether Pumilio is expressed during oogenesis. Ovaries from a protein-trapped GFP-Pumilio line (Kyoto DGRC) were fixed and imaged; consistent with previously published findings (Parisi and Lin, 1999), we observed Pumilio puncta exclusively in the terminal filament and GSCs (Fig. 5.8). Their different spatial expression patterns renders it less plausible that Pumilio regulates bcd mRNA translation during oogenesis.

Figure 5.8: GFP-Pumilio is expressed in the germarium. GFP-Pumilio expression in the terminal filament and germline stem cells of the germarium (indicated by broken line). Image represents a single optical Z slice. Scale = 25 µm, with 63X magnification. Simple schematic of the germarium with terminal filament and germline stem cells indicated (left panel).

5.6 RNAi screen for repressors of bicoid mRNA translation

Model organism RNAi screens can be used to identify genes that mediate different biological processes. One assumption built into the design of our screen is that the removal of individual repressor proteins would be sufficient for premature/ectopic translation of bcd mRNA in the egg chamber (Fig. 5.9). We used this knock-down approach to screen genes falling into several classes: miRNA pathway members, P-body components, general translational repressors, genes specifically implicated in bcd localization/regulation (Table 5.1). For all of the knock-downs analyzed, we did not observe any substantial indication that the gfp-bcd
transgene is prematurely translated, either from observing raw GFP signal or after probing with an anti-Bcd antibody.

![Diagram of RNAi screen for repressors of bicoid mRNA translation]

Figure 5.9: Design of RNAi screen for repressors of bicoid mRNA translation. The gfp-bcd transgene is introduced into the background of a maternal Gal4 driver. This fly is then mated with a female bearing an RNAi transgene targeting a chosen gene, and the progeny of interest (indicated by blue box) are analyzed.

Table 5.1: Genes screened for repressors of bicoid translation

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<td>bruno</td>
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<td>eIf4E-T</td>
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5.7 Synopsis and Discussion

In this chapter, we outlined the design strategy for two GFP-tagged bcd transgenes which drive different expression levels in the egg chamber. We demonstrated that loss of miR-305
activity in the egg chamber is not sufficient to cause premature translation of bcd, suggesting either that 1) miR-305 does not play a role in bcd translational repression, or 2) miR-305 cooperates with additional, redundant mechanisms. However, after driving the expression of bcd at sufficiently high levels, we did observe Bcd protein in nurse cell nuclei. This finding suggests that oversaturation of ‘repressive’ factors may allow translation of bcd mRNA at a time period when it is normally translationally silent.

A recurring theme in miRNA research is the ‘fine-tuning’ of target mRNA levels or translation rates by a miRNA. There are very few examples of miRNA regulation in which a miRNA controls the translation of its target transcript in a binary manner. One such example, and incidentally one of the first miRNA-target pairs ever identified, is the case of the C. elegans lin-4 gene and its target lin-14. It is thought that, in the majority of cases, miRNAs act either singly or in combination to ‘tune’ mRNA levels depending on cellular conditions; on this view, miRNAs act to increase the robustness of gene expression.

Our observation that over-expression of bcd mRNA results in Bcd protein translation in the egg chamber may not seem surprising, but at a minimum it demonstrates that the mRNA is competent to be translated during oogenesis. One obvious explanation for this finding, which we favor, is that the high level of bcd mRNA saturates the ‘repressive’ factors in the germline, allowing some amount of the mRNA to access the translational machinery. The apparent high level of control over the translational timing of bcd mRNA also raises the question of its developmental and evolutionary significance. It is conceivable that ectopic translation of the mRNA during egg chamber development may result in premature activation of Bcd target genes in nurse cell nuclei, or interfere with proper formation of the Bcd protein gradient in the embryo. These questions can only be properly answered when bcd translational regulators are identified, which we attempted with our RNAi screen.
A major limitation of our RNAi screen for repressors of bcd mRNA translation is that it fails to detect contributions from multiple, partially redundant factors. This may be one reason that we could not identify any single protein factor as required for bcd mRNA translational repression. However, this is a general feature of most RNAi-based screens. One alternative possibility would be to perform the screen in a sensitized genetic background, for example a miR-305 KO/+ genotype. Of course, this assumes that miR-305 does contribute to bcd translational repression, which may not be the case. Other classically studied patterning transcripts, such as osk mRNA, are regulated by several well-characterized translational repressors, which cause strong phenotypic effects when mutated. The lack of any similarly validated repressors of bcd mRNA translation made interpretation of our screen more difficult, for lack of a good positive control.
Chapter 6

General Discussion and Future Directions

6.1 Evolutionary and functional significance of bicoid mRNA post-transcriptional regulation

The localization and translational regulation of a particular mRNA typically serves a functional role for the organism. For instance, osk mRNA is intricately regulated in order to limit Oskar protein production to the oocyte posterior beginning at stage 9 of oogenesis; this regulation is essential for the formation of germ plasm, and consequently germ cells in early embryos (Appendix). Is there likewise an evolutionary significance underlying bcd mRNA localization and translational silencing within the D. melanogaster egg chamber? Interestingly, the bcd gene is recently evolved and found in only a few species of higher flies outside the Drosophila genus, including the housefly Musca domestica and the blowfly genus Calliphora (Fig. 6.1). The bcd gene is thought to have originated as a duplication of hox3, resulting in two genes which diverged and specialized to produce zerknult (zen) and bcd on chromosome 3 (Shaw et al, 2001). The functional divergence of zen and bcd explains their different expression patterns in D. melanogaster: zen expressed zygotically and bcd maternally.

There are at least two different modes of embryonic patterning that exist in insects. In ‘long germ’ model insects, such as D. melanogaster and the wasp Nasonia vitripennis, almost the complete volume of the mature egg will develop into embryonic tissue. In contrast ‘short
Figure 6.1: Evolution of the \textit{bicoid} gene in Dipteran flies. This phylogenetic tree is adapted from Simpson, 2002. The red box outlines branches of the tree containing species with a \textit{bcd} gene. A possible origin point of the \textit{bcd} gene is also indicated.

short germ insects, such as the red flour beetle \textit{Tribolium castaneum}, dedicate only a small portion of the egg volume to eventual embryonic tissue (Fig. 6.2). This morphological constraint has two important implications for short germ insects. First, because of the large amount of extraembryonic tissue present at the anterior of the egg, they typically do not use a maternally localized anterior patterning center in the embryo. Second, the volume of the short germ embryo is small enough such that a posterior patterning center suffices for embryonic patterning and development.

It is obvious that the morphology of the \textit{D. melanogaster} embryo will dictate how sig-
Figure 6.2: Long versus short germ patterning in insects. Schematics describing the morphology of long and short germ insect embryos, with anterior (A) and posterior (P) ends indicated (top). Blue dots indicate extra-embryonic tissue. Long germ embryos have both anterior and posterior patterning centers (indicated by gold stars), whereas short germ embryos have only a posterior patterning center (bottom). H = head; Th = thorax; Ab = Abdomen. This figure is adapted from Rosenberg et al, 2009.

Signaling can proceed during early embryogenesis. Because it is a large syncytial blastoderm, proteins are free to diffuse along the long axis of the embryo. It is therefore well suited for an anterior patterning center that utilizes morphogen gradients to activate gene expression. According to the Synthesis-Diffusion-Degradation (SDD) model, Bcd protein is translated from a localized source of \( bcd \) mRNA at the embryo’s anterior pole, after which it diffuses towards the embryo posterior to form an A-P protein gradient. The rates of diffusion and subsequent protein degradation determine the distribution of Bcd along the embryo A-P axis (Drocco et al, 2012).

Why is there such tight temporal control over \( bcd \) mRNA translation? One obvious
explanation is that earlier translation of \textit{bcd} mRNA, for instance during egg chamber development, would interfere with the dynamics of Bcd protein diffusion in the embryo. This interference would likely occur due to nurse cell dumping, an event in late oogenesis during which the nurse cells begin apoptosis, transferring and mixing their cytoplasmic contents into the oocyte. Therefore, it would seem that the more energetically favorable strategy is one that \textit{D. melanogaster} employs: anchoring of \textit{bcd} mRNA to the oocyte anterior pole, followed by its regulated translation in the early embryo.

\textbf{6.2 Function of the \textit{bicoid} Nanos Response Element}

In cell reporter assays, we introduced mutations into the \textit{bcd} NRE to examine whether an intact NRE sequence is necessary for miR-305 mediated repression. Our reasoning for this experiment was based on previous work characterizing the NRE as an important secondary structure that can mediate access for miRNAs. For example, in both \textit{D. melanogaster} and human cells, Pumilio binding to the 3'UTR of the \textit{E2F3} oncogene enhances the repressive activity of miRNAs that bind in the local vicinity (Miles et al, 2012). Similarly, in human fibroblasts, binding of the \textit{p27} 3'UTR by Pumilio alters the local secondary structure, allowing access for miRs-221 and 222 (Kedde et al, 2010).

The most recent study to examine NRE functionality in the \textit{bcd} 3'UTR concluded that mutation of the NRE results in a modest increase in \textit{bcd} mRNA stability during early embryogenesis (Gamberi et al, 2002). Interestingly, more recent work from a PhD thesis determined that the \textit{bcd} NRE is bound by Pumilio both \textit{in vitro} and \textit{in vivo}, but plays no physiological role either in \textit{bcd} mRNA stability or levels of translation during embryogenesis (Nomie, 2009). These studies did not examine Pumilio or \textit{bcd} NRE function during oogenesis, however they are consistent with our finding that \textit{pum} mutant ovaries do not exhibit an altered pattern of \textit{bcd} mRNA translation. Furthermore, our observation that Pumilio and \textit{bcd} mRNA are not expressed during the same stages of oogenesis also supports the notion that Pumilio does not substantially contribute to regulation of \textit{bcd} mRNA \textit{in vivo}. 71
The fact that the bcd NRE is perfectly conserved among 12 Drosophila species suggested to us that it serves an important regulatory function, and our observation that the NRE directly overlaps with the predicted miR-305 binding site also strengthened this idea. Interestingly, the NRE is conserved in maternally expressed patterning transcripts from other insect species. The short germ insect Tribolium lacks bcd, and instead uses orthodenticle and hunchback to accomplish anterior patterning of the embryo. Tribolium otd-1 mRNA is supplied maternally and — in contrast to bcd — uniformly distributed throughout the cytoplasm; it also contains an NRE which causes its targeted decay in the posterior half of the embryo (Schroder, 2003). This suggests that the NRE has been evolutionarily conserved for the purpose of embryonic A-P patterning. Why, then, does bcd mRNA contain an NRE if its expression does not overlap with that of Nanos or Pumilio?

6.3 MicroRNA function in the Drosophila egg chamber

We have presented evidence that miR-305 can regulate bcd mRNA in cell culture and that it is expressed during oogenesis, consistent with a role in regulating bcd mRNA in vivo. Using a homozygous miR-305 KO fly, we did not find conclusive evidence that miR-305 impacts the translational repression of bcd mRNA in the egg chamber. Although it is inconclusive whether miR-305 plays any role in regulating bcd mRNA, it likely has other targets in the egg chamber. MiR-305 is a conserved miRNA, with 270 predicted conserved targets according to TargetScan Fly; 131 of these predicted targets are unnamed genes. Of the predicted named targets, several are expressed during oogenesis, including upf2 and slowmo. Previous work on miR-305 has its activity and possible targets in other fly tissues. In the fat body, miR-305 targets p53 in response to nutrient signaling (Barrio et al, 2014), and in the brain, miR-305 has been shown to be required for memory formation (Busto et al, 2015). The miR-305 homozygous knock-out (KO) fly is viable but it is reported to have a reduced life span, in addition to an extra bristle phenotype on the dorsal thorax (Foronda et al, 2014).
Additionally, our small RNAi screen for protein factors that impact \( bcd \) mRNA translation did not reveal any convincing hits in the miRNA pathway, such as Ago1 or GW182. There are several possible explanations for this outcome. First, it is possible that several protein or RNA factors cooperate in a partially redundant manner to repress translation of \( bcd \) mRNA during oogenesis. This would explain why no single factor produced a result in the screen. A second, but less likely, possibility is that the amount of Bcd protein produced by knock-down of a single protein factor was insufficient to detect by direct observation of GFP signal or immunofluorescence with anti-Bcd antibody. Thirdly, it is also possible that the miRNA pathway has no role in regulating \( bcd \) mRNA stability or translation.

New genetic engineering technologies have facilitated the creation of targeted knock-outs, particularly for miRNA genes which are small and thus underrepresented using traditional mutagenesis approaches; this has allowed for a deeper understanding of miRNA biology. A recent knock-out study of \( D. \ melanogaster \) miRNAs yielded 80 new miRNA mutants, with 80% of these mutants exhibiting at least one abnormal phenotype (Chen et al, 2015). The nature of miRNA/target interactions allows for an ample amount of redundancy; many different miRNAs can bind one transcript, and a single miRNA is capable of targeting many different transcripts. Most miRNA/target interactions are thought to be of the fine tuning variety, with the miRNA slightly decreasing the amount or translational efficiency of a targeted mRNA. Underscoring the idea of redundancy in miRNA regulatory networks, a systematic deletion screen of individual \( C. \ elegans \) miRNAs, covering 83% of all miRNA genes, revealed only a small fraction of mutants (7 out of 87) to display any abnormal phenotype (Miska et al, 2007).

In addition to the 3’UTR reporter assays examining \( bcd \) mRNA, we performed assays using 3’UTRs derived from the maternally expressed genes \( smaug, \ hunchback, \) and \( \text{string} \) (\( cdc25 \)) (Fig. 6.3). Based on TargetScan predictions, each gene contained predicted binding sites for one or more miRNAs for which we have demonstrated expression in ovaries: miR-
284, miR-8, and miR-956, respectively. Similar to the results from the bcd 3’UTR reporter assays, we found that each miRNA was capable of repressing its predicted target, and this regulation was abolished by mutation of the miRNA seed binding site. These results are merely suggestive of possible miRNA/target interactions that could occur in the egg chamber.
Figure 6.3: Maternally expressed transcripts are predicted microRNA targets. S2 cell luciferase reporter assays demonstrating translational repression of mRNAs by their predicted targeting miRNAs. For each floating box, the pSiCheck vector is indicated in the color-coded legend. The co-transfected DsRed vector is indicated on the x-axis. Relative luciferase (Renilla/Firefly) is indicated on the y-axis. **** = p < 0.0001; *** = p < 0.001; ** = p < 0.01; ns = not significant. N = 12 (combined four technical replicates from each of three independent experiments. Error bars = SD.
6.4 Do P-bodies regulate maternal mRNA stability?

We used transgenic RNAi to knock down key components of the mRNA deadenylase and 5'-3' decay pathways; loss of deadenylase activity, via not1 or pop2 KD, resulted in altered P-body morphology. Our results indicate that loss of P-body integrity is correlated with a decrease in the levels of some maternal mRNAs, including bcd and nos (Chapter 3.4-3.6). This finding seems paradoxical in light of the fact that P-bodies are well characterized as sites of RNA decay; one would expect an increase in maternal mRNA levels if P-body/RNA decay activity is compromised.

There are a few idiosyncrasies of D. melanogaster egg development that may partially explain our observations. First, bcd mRNA is known to be very stable over the course of oogenesis (Surdej and Jacobs-Lorena, 1998). It is therefore unlikely that RNA decay plays a substantial role in its normal life cycle during development of the mature egg. Second, the P-body component Me31B is already well known to exist in particles with many maternally expressed transcripts, including the localized mRNAs osk, bcd, and nos. Disruption of P-bodies, and consequently Me31B/RNA particles, may interfere with normal mRNP formation for several of these transcripts. Third, immuno-EM experiments on D. melanogaster egg chambers have revealed P-bodies to be structured and compartmentalized, with certain transcripts, such as bcd, retained in the ‘core’ and others positioned more closely to the P-body periphery (Davidson et al, 2016). It is possible that disruption of P-body formation interferes with this structuring, allowing components of the 5'-3' decay machinery close proximity to mRNAs to which they otherwise would not have access. Therefore, when either P-body formation or composition is compromised (e.g. through our RNAi KDs), these maternal transcripts undergo some form of unregulated RNA decay.

Consistent with this hypothesis is a recent publication reporting that in mutants of Drosophila Cup, an eIF4E-binding protein that is also considered a P-body component,
oskar mRNA levels are significantly decreased (Broyer et al, 2016). This finding suggests, that in some contexts, P-bodies can play a role in stabilizing maternal transcripts.

6.5 P-bodies and translational control of bicoid mRNA

In contrast to the other classic patterning transcripts of the D. melanogaster egg chamber, bcd mRNA is not translated until early embryogenesis. How this timing is achieved was one of the questions motivating this thesis. Previous work in this system has shown that disassembly of P-bodies after egg activation is tightly correlated with translation of bcd mRNA; this suggests that removal of bcd mRNA from P-bodies is a key precursor event to its translation (Weil et al, 2012). It is possible that incorporation of bcd mRNA into P-bodies is mediated by multiple protein factors, and that once incorporated into a P-body, bcd mRNA is effectively shielded from the translational machinery for the duration of oogenesis. This would explain why removal of miR-305 or knock-down of miRNA pathway factors had no detectable effect on the timing of bcd mRNA translation. However, this hypothesis is also countered by the fact that knock-down of me31b had no observable effect on translation of bcd in the egg chamber.

A possible mechanism for translational repression of bcd mRNA is via bcd’s sequestration in P-bodies, which exclude the translational machinery and ribosomes. Consistent with this possibility is our finding that overexpression of bcd with the UAS-gfp-bcd transgene results in Bcd protein translation in the egg chamber. It would be interesting to analyze the subcellular distribution of the transgenic bcd mRNA, to observe whether it is present outside of P-bodies. Perhaps oversaturation of P-bodies with high levels of bcd results in access of bcd mRNA to the translational machinery.

This raises the question of how bcd mRNA is sequestered within P-bodies during the normal course of its life cycle in the egg chamber. Because P-bodies are composed of many different proteins, the aggregation of many mRNA species and their trans-acting factors re-
sults in P-body formation. The fact that \textit{bcd} mRNA is present within the P-body ‘core’ may simply be a consequence of \textit{bcd}’s particular protein associations, rather than any active process of regulation. Overexpression of \textit{bcd} mRNA would likely interfere with the stoichiometry of mRNA/protein interactions, shifting the equilibrium towards \textit{bcd} mRNA being present outside of P-bodies. However, this outcome would also depend on whether P-body/\textit{bcd} associated proteins are in large excess or which are limiting factors in this interaction.

6.6 Future Directions

6.6.1 MiR-305 function in the egg chamber and embryo

Because miR-305 is deposited maternally, it can possibly function during early embryogenesis rather than oogenesis. It would be interesting to more closely analyze maternal and zygotic miR-305 mutant embryos for defects in patterning, for instance in the expression patterns of pair rule genes. It is also possible that miR-305 acts to degrade \textit{bcd} mRNA in the early embryo, after the developmental window for its translation has ended.

6.6.2 Screen for repressors of \textit{bicoid} mRNA translation

Our screen for repressors of \textit{bcd} mRNA translation was performed in a genetic background containing one copy of a \textit{gfp-bcd} transgene and two endogenous copies of \textit{bcd}. An alternate possibility would be to perform a similar screen in a sensitized background, for example containing only one WT copy of miR-305. This approach may be more likely to bypass redundant interactions that possibly occur in a WT physiological setting. Another possibility is a genetic background which is compromised for \textit{bcd} mRNA localization, for example in a \textit{stau} mutant. This approach may uncover interactions between proper \textit{bcd} mRNA localization and its translational repression.
6.6.3 Does bicoid exist in mRNPs with other mRNA species?

Several trans-acting protein factors that interact with bcd mRNA have been well characterized (Chapter 1.3), however a question that has gone almost completely unaddressed is the mRNA composition of bcd particles in the egg chamber. Our lab has acquired preliminary imaging data demonstrating that bcd and osk mRNAs co-exist in particles in the egg chamber nurse cells. One interesting avenue of investigation would be to examine the functional significance of bcd/osk particle formation. For example, is formation of these particles necessary for proper translational regulation of each transcript? This could be partly explored by overexpressing bcd mRNA — using the UAS-gfp-bcd transgene — and then examining whether bcd and osk mRNA co-localization is altered.

6.6.4 P-body morphology and compartmentalization of mRNPs

Previous work on egg chamber P-bodies suggests that P-bodies are structured, with certain mRNPs preferentially localized to discrete locations, such as the core or periphery. One possible avenue for investigating this phenomenon would be the use of immuno-EM imaging in combination with RNAi knock-down of different P-body components, to examine how compromising the activity of individual component proteins affects the overall morphology and distribution of transcripts within the P-body. These experiments would also help address the hypothesis we offered in Chapter 6.4 for explaining the decreased levels of maternal transcripts when P-body morphology is compromised.
Chapter 7

Materials and Methods

7.1 Fly lines

Table 7.1: Gal4-inducible RNAi lines from the Bloomington *Drosophila* Stock Center

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<td>33727</td>
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<td><em>ccr4</em> (<em>twin</em>) TRiP</td>
<td>32490</td>
</tr>
<tr>
<td><em>not1</em> TRiP</td>
<td>32836</td>
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<tr>
<td><em>pop2</em> TRiP</td>
<td>52947</td>
</tr>
<tr>
<td><em>thor</em> (<em>4E-T</em>) TRiP</td>
<td>36815</td>
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<td><em>staufen</em> TRiP</td>
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Table 7.2: Gal4 driver lines from the Bloomington *Drosophila* Stock Center

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<tr>
<td>Gal4 4442</td>
<td>y¹ w* ; P[GAL4-nos.NGT]40</td>
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<td>Gal4 7062</td>
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Table 7.3: Lines constructed in the lab

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Table 7.4: Lines from the Kyoto Drosophila Stock Center

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7.2 Luciferase reporter assays

Luciferase assays were performed as previously described (Okamura et al, 2007). Briefly, the DsRed-miRNA and pSiCheck vectors were co-transfected into Drosophila S2 cells using Effectene transfection reagent (Qiagen) and plated at a density of 1.1-1.2 x 10^6 cells/ml. Cells were incubated for three days at room temperature, lysed, and both Renilla and firefly luciferase levels measured by luminometer.

7.3 Cloning

UAS-gfp-bcd

A plasmid containing the complete bicoid locus bearing an N-terminal GFP tag was a gift from Thomas Gregor, Princeton University (Gregor et al, 2007). Primers spanning the bicoid 5’ UTR and 1 kb downstream of the annotated 3’ UTR were used to clone the bicoid transgene into the pENTR/D-TOPO vector (Gateway system). Using the Gateway system, this transgene was then recombined into a modified UASp vector (gift from Jennifer Zallen lab, Memorial Sloan Kettering Cancer Center) to produce UAS-gfp-bcd for expression in the
germline.

**gfp-bcd**

Primers spanning 200 nt of the *bcd* promoter sequence and 1 kb downstream of the annotated 3’ UTR were used to clone *bicoid* into pBID-G (Addgene # 35195), which was then transformed onto the X chromosome using the Phi-C31 Integrase system.

**pSiCheck-*bicoid* 3’ UTR**

The full *bcd* 3’ UTR was cloned into the pSiCheck 2 vector (Promega) using XhoI and NotI sites. Mutagenesis was performed by PCR using mismatched primers and verified by sequencing (Genewiz). Mutagenic primer sequences are included in the Methods section.

### 7.4 RNA isolation

Dissected ovaries were washed one time with 1X PBS and homogenized in Trizol. Total RNA was isolated according to the manufacturer’s instructions. Following extraction, the total RNA was washed twice in 75% ethanol and stored in H2O at -20 °C.

### 7.5 RT-qPCR

**Reverse transcription** reactions were performed as follows:

For miRNA amplification, 100 ng of small RNA was incubated with 2 pM RT primer at 70 °C for 5 minutes, cooled on ice for 5 minutes, followed by addition of RT enzyme and incubation at 42 °C for 1 hour. For amplification of mRNA targets, the protocol was identical except that 250 ng of RNA was incubated with 0.5 µg of dT or random hexamers primer.
qPCR reactions were performed in a Roche Lightcycler 480 as follows:
Each PCR reaction contained 1 µl of cDNA from RT reaction, 2 µl primer solution (10 µM forward and reverse), 2 µl dH2O, and 5 µl SYBR Green.
95 °C denaturation for 5 minutes, followed by 40 cycles of
95 °C for 20 seconds
58 °C for 15 seconds
58 °C for 15 seconds

7.6 Ovary dissection and fixation
Following dissection in 1X PBS, ovaries were washed in 1X PBS and then incubated for 15 minutes while rocking in 4% paraformaldehyde (PFA) in 1X PBS. Ovaries were then washed 4x 5 minutes in 1X PBS with 0.05% Triton X-100.

7.7 Fluorescence in situ hybridization
Ovaries were fixed, washed, and then smFISH was performed using bicoid-specific Stellaris RNA FISH probes (Biosearch Technologies). The protocol is described in Bayer et al, 2015.

7.8 Immunofluorescence
Dissected ovaries were fixed and then permeabilized in 1% Triton X-100, 1% BSA, with 1X PBS for 2 hours, then washed with 1X PBS. Primary antibodies were incubated overnight at room temperature or 4 °C in 0.3% Triton X-100, 0.1% BSA with 1X PBS, and then washed 4X in 0.05% Triton X-100, 0.1% BSA with 1X PBS. Secondary antibody was incubated at 1:1000 dilution for at least 2 hours at room temperature. Ovaries were then washed 4X in 1X PBS with 0.05% Triton X-100 and mounted with Prolong Gold media.
7.9 Confocal microscopy

Imaging was performed with a Leica DMI-4000B inverted microscope with a Yokogawa CSU 10 spinning disk head and Hamamatsu C9100-13 ImagEM EMCCD camera.

7.10 Image analysis

Image processing and analysis were performed using the NIH’s ImageJ software (https://imagej.nih.gov/ij).

7.11 Western Blotting

Ovaries were lysed mechanically with CytoBuster lysis buffer plus protease inhibitor cocktail. Protein samples were run on an 8% SDS-MES polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with appropriate primary antibodies overnight at 4 °C. Membranes were washed and then probed with fluorescent or HRP conjugated secondary antibodies, and imaged accordingly.

7.12 Oligonucleotide sequences and antibodies

Table 7.5: Oligo sequences for Gateway cloning

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Table 7.6: Oligo sequences for qPCR (FlyRnai.org “Fly Primer Bank”)

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Table 7.7: Oligo sequences for sequencing of \textit{gfp-bcd} transgenes

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Table 7.8: Oligo sequences for restriction digest cloning

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Table 7.9: Oligo sequences for mutagenesis by PCR

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Table 7.10: Oligo sequences for miRNA qPCR

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Table 7.11: Antibodies used for Immunofluorescence and Western Blotting

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Chapter 8

Appendix

Chapter 1

Drosophila melanogaster Oogenesis: An Overview

John M. McLaughlin and Diana P. Bratu

Abstract

The Drosophila melanogaster ovary has served as a popular and successful model for understanding a wide range of biological processes: stem cell function, germ cell development, meiosis, cell migration, morphogenesis, cell death, intercellular signaling, mRNA localization, and translational control. This review provides a brief introduction to Drosophila oogenesis, along with a survey of its diverse biological topics and the advanced genetic tools that continue to make this a popular developmental model system.

Key words Flp-FRT, Patterning, Follicle cells, Morphogenesis, Germ plasm, Mosaics, P element, RNAi, Drosophila, Oogenesis, Oocyte, mRNA localization, Gal4, Live, Imaging, CRISPR, Fluorescence

1 Ovary Structure in Insects and Higher Flies

The Drosophila genus, which includes D. melanogaster, is a member of the order Diptera, or the higher flies. As a holometabolous insect, Drosophila undergoes a complete metamorphosis, including a transition from larval to pupal form; the complete life cycle consists of four stages [1].

In the class Insecta, several ovarian morphologies have evolved, each of which utilizes a different organizational layout for oocyte development (Fig. 1). In the majority of examined cases, basal insects contain panoistic ovaries, in which oogonia, ensheathed by follicular cells, mature into oocytes in the absence of additional support cells [2]. In contrast, higher insects have meroistic ovaries in which support cells linked to the oocyte provide it with large amounts of mRNA, protein and other cellular material [3]. Meroistic ovarian morphology is further subdivided into two categories. Telotrophic meroistic oocytes maintain connections to support cells via a nutritive cord that extends anteriorly through the ovariole to the germarium. The support cells remain localized at the anterior end of the ovariole throughout oogenesis. In contrast, polytrophic meroistic oocytes are connected directly to
adjacent supporting nurse cells by cytoplasmic junctions (ring canals). In this system, the entire cyst moves as a unit through the ovariole; this is the strategy utilized by *Drosophila* species.

The basic unit of the ovary is the ovariole; there are 16–20 ovarioles per ovary, each being autonomous and containing its own stem cell populations and egg chambers at varying developmental stages. The ovariole can be divided into three principal regions (from anterior–posterior): the terminal filament, germarium, and vitellarium (reviewed in ref. 4). The terminal filament (TF) consists of a stack of 8–9 flattened cells which connect the germarium to the surrounding ovariole sheath and determine the orientation of ovariole development [5]. The germarium is divided into four regions (1, 2a, 2b, and 3) and is the site of germline stem cell (GSC) division, differentiation, and germline cyst formation. In germarium region 3, the germline cyst, containing nurse cells and oocyte, is ensheathed by a somatic cell layer (becoming an egg chamber) before being passed into the vitellarium [4].

---

**Fig. 1 Three distinct organizations of insect ovaries.** Schematic representation of ovarioles derived from panoistic, telotrophic meroistic, and polytrophic meroistic ovaries (anterior—*top*; posterior—*bottom*). The germarium region, somatic follicle cells, support/nurse cells, and the developing oocyte are indicated within each ovariole chain.
remaining development of the egg chamber, including vitellogenesis and choriogenesis, is completed in the vitellarium [4]. The entire process of oogenesis is thus divided into 14 morphologically distinct stages.

## 2 Stem Cell Compartmentalization Within the Germarium

Stem cells are essential in many adult organs to provide a localized renewed source of differentiated cells, thereby maintaining tissue homeostasis; they reside in “niches” in specific anatomical locations which contribute to their proper maintenance and function. The *D. melanogaster* ovary contains two main stem cell populations, the germline (GSC) and follicle stem cells (FSC), which collectively give rise to the nurse cells, oocyte, and follicle cells of the mature egg chamber. Each stem cell population resides in a unique, specialized niche containing several types of support cells (reviewed in ref. 6).

The niche microenvironment is essential for the regulated balance between stem cell self-renewal and differentiation. In the case of ovarian stem cell niches, this is achieved by both an array of secreted signaling molecules and direct adhesive connections between stem cells and their niche components ([7, 8] and reviewed in refs. 9, 10). The germline stem cell niche resides at the anterior tip of the germarium; it contains 2–3 GSCs and their support cells, the cap (CC) and TF cells [9] (Fig. 2a). GSCs can be reliably identified by their direct anchorage to CCs, and by the presence of an anteriorly localized spectrosome (also known as the fusome in cystocytes). In addition to CCs, the GSCs require escort cells (ECs, also known as inner germarial sheath cells) for their regulated differentiation; ECs are glial-like cells which surround germline cysts with cytoplasmic processes, preventing adjacent cysts from making direct contact [11, 12]. In the anterior half of the germarium, germline cysts are surrounded by ECs before they migrate posteriorly and become ensheathed by an epithelial follicle cell layer [12]. While CCs act to prevent GSC differentiation and promote self-renewal, the ECs support their differentiation [13]. Collectively, the CCs, TFs, and ECs constitute the complete niche for GSCs (reviewed in ref. 14).

Technical advances in live tissue culture have made it possible to visualize ovarian stem cells in their in vivo niche environments [15]. This has yielded insight into the dynamic interactions among stem cells and their niche components, which would otherwise be impossible to elucidate from fixed samples. For example, it was recently demonstrated by live imaging that ECs do not migrate with their encircled cyst, but rather use their cellular extensions to “pass” developing cysts posteriorly through the germarium [15].
Fig. 2 The germarium and mid-stage egg chamber: Structure and cell types. (a) A diagram of the cells housed in the germarium. They illustrate the initial GSC division, formation of the germline cyst, and its enclosure by a layer of follicle cells. Each cell type is denoted by its corresponding color in the key. (b) During stage 10, the oocyte encompasses about 50% of the egg chamber, with the follicle cells surrounding the oocyte having a columnar morphology, in contrast to the squamous follicle cells covering the nurse cells compartment. Ring canals (green) bridge the cytoplasm of adjacent nurse cells and the oocyte. At this stage, border cells (yellow) have completed their migration from the anterior of the egg chamber towards the oocyte. The oocyte nucleus (purple) is localized to the dorsal anterior quadrant, along with gurken mRNA (orange). bicoid (blue) and oskar (red) mRNAs are localized at the anterior and posterior cortex of the oocyte, respectively.
3  Cystoblast Division, Oocyte Differentiation, and Formation of the Egg Chamber

A differentiated GSC is termed a **cystoblast** (CB); this CB will divide a total of four times to produce 16 **cystocytes**. Each mitotic division is accompanied by incomplete cytokinesis, forming intercellular cytoplasmic bridges near the mitotic spindle called ring canals [16]. The invariant pattern of cystocyte division and ring canal formation was dissected in the 1960s through light and electron microscopy of sectioned ovaries [16]. Central to this process is the **fusome**, a specialized cytoplasmic organelle composed of skeletal membranous proteins, which helps form and maintain ring canals following each mitotic division (reviewed in refs. 17, 18). The nurse cell ring canals are composed of F-actin and accessory proteins [19], including the actin-binding protein Anillin, which is required for the earliest stages of ring canal development in the egg chamber [20]. Additional proteins are localized to the actin rings and necessary for later stages of nurse cell formation [21].

The determination of the oocyte from among the 16 cells of the cyst involves a series of “symmetry breaking” events beginning at the first CB division. The germarium is divided into discrete regions, which indicate the developmental stage of the nascent germline cyst and its oocyte. Region 1 of the germarium contains the GSC niche and germline cysts of 2, 4, or 8 cells; region 2a contains 16 cell germline cysts in which two pro-oocytes are determined [22]. At the time of egg chamber entry into region 2b of the germarium, the oocyte has been specified and can be distinguished by the presence of several mRNA and protein markers [23–25]. An intact, polarized microtubule (MT) cytoskeleton is also required for differentiation of the oocyte, as well as the localization of oocyte-specific markers. In the absence of an intact MT cytoskeleton, such as following colchicine treatment, the oocyte fails to differentiate and an egg chamber containing 16 nurse cells is formed. One model for oocyte differentiation proposes that the oocyte is always formed from the cystoblast inheriting the most fusome from the first mitotic division [26]. In region 3 of the germarium, the germline cyst (egg chamber) is almost completely ensheathed by an epithelial follicle cell layer. The budding of the egg chamber from the germarium marks stage 1 of oogenesis (reviewed in ref. 27).

4  Follicle Cell Development

The somatic follicle cells of the egg chamber originate from a pair of stem cell niches, positioned laterally on each side of the germarium between regions 2a and 2b [28]. These niches contain the follicle stem cells and make direct contact with ECs that are required for
maintenance of the FC niche [29]. There is regulatory overlap between the GSC and FSC niches, as FSCs also require secreted signaling molecules from the CCs to maintain their self-renewal capacity [30–32]. Three distinct types of follicle cell are formed during egg chamber development: polar cells, stalk cells, and epithelial follicle cells [33]. Polar cells are located at the anterior and posterior tips of each egg chamber, while stalk cells connect and bridge adjacent egg chambers; both populations originate from the same precursor follicle cell lineage [34]. By the end of stage 5, there are two polar cells at each egg chamber terminus; these cells, along with the TGF-α homolog Gurken protein, participate in signaling events that determine the egg chamber posterior pole and induce the reorganization of the MT cytoskeleton during mid-oogenesis [35].

The epithelial follicle cells ensheath the entire egg chamber and compose the bulk of the total follicle cell population; beginning at stage 1 of oogenesis, they can be distinguished on the basis of specific protein markers [36]. These cells are further subdivided into two categories: terminal follicular cells, which are contained within an area of ~10 cell diameters from each egg chamber pole, and mainbody follicular cells which cover the lateral surface area of the germline cyst (reviewed in ref. 33). The terminal follicle cells are specified by secreted signals from polar cells [37]. Until stage 6, the follicle cells proliferate by mitosis giving rise to a maximum number of ~1000 cells surrounding the egg chamber [38].

5 Cell Migration and Egg Chamber Morphogenesis

The development of a complex structure such as the egg chamber requires cell migratory and morphogenetic events; at various developmental stages, different cell populations within the egg chamber contribute to processes that are necessary for egg chamber development. One such migratory event is the long-range movement of border cells during mid-to-late oogenesis. Border cells are specified from a small population of terminal follicle cells at the egg chamber anterior, via secretion by adjacent polar cells of the Unpaired (Upd) ligand [39]. This population of 6–8 cells then detaches from their neighboring FCs, migrates between nurse cells towards the egg chamber posterior, and inserts into the dorsal side of the oocyte to form the micropyle [40]. Recent developments in ex vivo culturing techniques and live imaging have made border cell migration a popular model for the study of cell movement in general, as well as the related process of cancer metastasis [41].

During the same developmental time period as border cell migration, the epithelial follicle cells also undergo migration as well as morphological change. Before stage 9, most FCs are cuboidal in shape (reviewed in ref. 42). The mainbody follicular
7 cells migrate posteriorly to surround the oocyte, at the same time adopting a columnar morphology [37]. These cells then secrete eggshell components onto the underlying oocyte membrane [33]. The anterior terminal FCs undergo a “flattening” that creates a squamous morphology, covering the nurse cell compartment of the egg chamber; this flattening event involves a remodeling of existing cell junctions [43]. Following these migratory and cell shape changes during stages 9–10, there is a characteristic demarcation between squamous and columnar FC morphologies at the nurse cell-oocyte junction [42].

One additional and striking example of a large-scale morphogenetic change during oogenesis is the recently characterized mechanism by which the egg chamber progressively elongates along its A-P axis. Through live imaging, it was demonstrated that the follicular epithelium rotates circumferentially around the A-P axis of the egg chamber, in the process depositing a polarized matrix of collagen and other extracellular matrix proteins. This matrix acts as a “corset” which physically constricts the egg chamber and causes its elongation in the A-P direction ([44] and reviewed in refs. 45, 46).

6 Cell Cycle Regulation and Meiosis

The several distinct cell types of the ovary differ in the regulation of their cell cycles. However, one similarity between the nurse and follicle cells is their use of endocycles (also known as endoreduplication or endoreplication): DNA synthesis (S) and gap phases without an intervening mitosis or cell division (reviewed in refs. 47, 48). The resulting polyploidy allows cells to increase their quantity of mRNA and protein production, which is essential for oocyte growth and development. Follicle cells typically undergo six to eight endocycles, while nurse cells undergo 10–12 ([49] and reviewed in ref. 50). Beginning at stage 10b, the follicle cells surrounding the oocyte cease normal endocycles and begin gene amplification cycles (this event is termed the E/A switch). Four specific genomic loci, encoding genes involved in chorion (eggshell) and vitelline membrane synthesis, are amplified from 4 to 80-fold [47, 51]. This allows for the production of high levels of chorion-related proteins.

The oocyte undergoes both developmental maturation and meiosis throughout the course of oogenesis, and these processes are intimately linked. Meiotic double-stranded breaks must be repaired in order to maintain the integrity of the MT cytoskeleton and proper translational control of localized mRNAs [52, 53]. The balance of oocyte differentiation and progression through meiosis is achieved by two major meiotic checkpoints in oogenesis. Prophase I of meiosis begins early in egg chamber development, in
region 2a of the germarium, and is indicated by the presence of the synaptonemal complex in the two pro-oocytes (reviewed in refs. 54, 55). Beginning at stage 5, the oocyte arrests in diplotene stage of prophase I; this arrest lasts until roughly stage 13, at which point meiosis progresses to metaphase I [53]. A metaphase I arrest occurs at stage 14, and is maintained until egg activation triggers the resumption and completion of meiosis [53]. Egg activation in *Drosophila* occurs independently of fertilization, and is triggered instead by mechanical pressure on the oocyte during passage into the oviduct [56].

### mRNA Localization in the Egg Chamber

One of the well-studied processes occurring during *D. melanogaster* oogenesis is the localization and translational control of the key embryonic patterning transcripts. As a result of the pioneering work of Wieschaus and Nusslein-Volhard on the genetic control of *D. melanogaster* embryonic patterning [57], the late 1980s and early 1990s saw a flurry of publications on oocyte-localized mRNAs and their involvement in different aspects of germ plasm formation and embryonic development [58–60]. The mRNAs mainly responsible for patterning of the early embryo, *oskar*, *bicoid*, *gurken*, and *nanos*, are each localized to a distinct compartment of the oocyte before fertilization (reviewed in refs. 61–63) (Fig. 2b). The large size of the egg chamber’s nurse cell and oocyte compartments demands that mRNA is transported long distances. This process requires the microtubule and actin cytoskeletons as well as various trans-acting proteins that affect their transport, localization, and stability [64]. Advances in imaging technology have also made this an ideal system for investigating live trafficking of mRNAs [40, 65].

One example of a long-distance traveling transcript is *oskar* (*osk*) mRNA, which encodes the *D. melanogaster* germline determinant. *osk* was cloned in the early 1990s and shown to be localized as mRNA to the oocyte posterior pole [58, 59]. Genetic analyses conducted over the past 20 years have identified some of the protein factors required for transport and translational control of *osk* (and other mRNAs), including the RNA-binding protein Bruno [66, 67]. Live imaging studies of *osk* mRNA have shed light on the dynamic nature of the mRNA transport process [68, 69], and helped to refine models on how the localization of mRNA is achieved in large, complex tissues [70]. The oocyte MT cytoskeleton, and its interactions with mRNA and other cellular cargoes, has also been studied using live imaging techniques [71, 72]. One such study, using fluorescently labeled RNA injected into live oocytes, demonstrated the necessity of Exuperantia protein for the anterior transport of *bicoid* mRNA in the oocyte [73].
Formation and Function of Germ plasm

There are two main strategies for specifying germ cells during animal development: cytoplasmic inheritance of germline determinants (also known as preformation) or zygotic induction (epigenesis) of germ cell fate. Most insects use the inductive method, in which germ cells are specified during embryogenesis by signals from adjacent somatic cells [74]. In contrast, the holometabolous insects, including Drosophila species, employ the preformative method of germ cell specification. In this developmental mode, germline determinants, in the form of germ plasm (also known as pole plasm), are inherited maternally and specify germ cells during early embryonic development (reviewed in ref. 75).

The germ plasm is a specialized cytoplasm, assembled at the oocyte posterior pole; it has been studied for its role in germ cell determination in insects and other animals for over 100 years ([76] and reviewed in ref. 77). Germ plasm is characterized by the presence of polar granules, non-membranous electron-dense organelles containing mRNA, protein, ribosomes, and noncoding RNA, most notably the mitochondrial large ribosomal RNA (reviewed in refs. 78, 79). Three classes of proteins are highly represented in the Drosophila germ plasm: Tudor-domain containing proteins (e.g., Tudor), DEAD-box RNA helicases (e.g., Vasa), and Piwi family proteins (e.g., Aubergine) (reviewed in ref. 80). In addition, interactions between Tudor domain and Piwi proteins are important for assembly of the germplasm in Drosophila [81].

One of the critical upstream factors required for germ plasm formation is osk mRNA. Upon its localization to the oocyte posterior, osk is translated. Genetic manipulations or mutations causing mislocalization of osk to the oocyte anterior result in ectopic formation of germ cells at the anterior of the embryo ([82] and reviewed in refs. 83, 84). The use of two alternative translation start sites in osk mRNA produces two Oskar protein isoforms: Short Osk and Long Osk. The short isoform initiates formation of the germ plasm ([85] and reviewed in refs. 27, 78, 86), while Long Osk is required for the posterior anchoring of both Short Osk and osk mRNA. Loss of Long Osk from egg chambers causes dispersion of Short Osk from the oocyte posterior pole and a reduced number of pole cells formed during embryogenesis [87]. Classic transplantation experiments, in which a fraction of early embryonic germ plasm is transferred to the anterior pole of a separate embryo, first demonstrated the sufficiency of germ plasm for germ cell specification [88].

The germ plasm’s functional role begins ~1.5 h into embryonic development, at which point ~10 pole cells are formed from the cellularization of posteriorly localized nuclei and the surrounding germline determinants. These are the first cells formed in the
blastoderm embryo; they will be carried into the embryo during gastrulation and eventually migrate to form the primordial germ cells of the gonad (reviewed in ref. 84). The newly formed PGCs will differentiate into sperm or egg depending on the zygote’s sex, and participate in the formation of the next generation.

9 Approaches for Manipulating Gene Expression in the Ovary

One of the biggest strengths of D. melanogaster as a model system is its variety of advanced genetic tools. Below, we review a few of the most widely used tools for studying gene function and development in the ovary.

9.1 The Use of P elements for Mutagenesis and Gene Transfer in Drosophila

Forward genetic screens often involve the use of chemical agents or transposons to induce mutations in DNA sequence, which are then analyzed for their phenotypic effects on the organism. In D. melanogaster, the most popular chemical mutagen has been ethyl methanesulphonate (EMS), due to its ease of use and efficient induction of random point mutations (reviewed in ref. 89). An alternative to chemical mutagenesis is the use of mobile genetic elements (transposons) to transpose to new genomic locations and in the process disrupt gene function (reviewed in ref. 90). While many transposons exist in flies, the most commonly used has been the P element. A wild-type P element consists of a pair of terminal inverted repeats flanking the P transposase coding sequence, both of which are necessary for transposition. DNA sequences of interest can be placed between the P element inverted repeats and transformed into random locations in the fly genome; removing the transposase component, and instead supplying it in trans on a donor plasmid, allows control of the P element’s mobilization [90]. Since the first use of P elements for gene transfer in the early 1980s [91, 92], this transposon has become the main workhorse of D. melanogaster genetic engineering.

9.2 The Gal4-UAS System

9.2.1 General Principle of Operation

One of the more versatile and widely used genetic tools available in D. melanogaster is the Gal4-UAS system. This two-component system for inducible activation of gene expression originated in yeast, and was subsequently adapted to drive tissue-specific gene expression in flies [93]. The operating principle of this system is simple and consists of two parts: (1) the transcriptional activator protein Gal4, which selectively binds (2) upstream activation sequences (UAS) in DNA, thereby activating transcription of a downstream gene. The system as used in flies consists of a “driver” line, expressing a Gal4-encoding transgene under the control of tissue-specific promoter or enhancer elements, and a Gal4-responsive “UAS” line containing a gene of interest downstream of five or more tandem UAS sites (Fig. 3a). A simple genetic cross of these two lines will
Fig. 3 The Gal4-UAS and Flp-FRT systems: Principles of operation. (a) A female fly bearing a transgene under UAS control is crossed to a male fly containing a Gal4 transgene under the control of a tissue-specific promoter (in this example, a maternal promoter expressing at all stages of oogenesis). The progeny of the cross displays ovary-specific expression of the transgene, indicated by the orange signal. (b) Use of the Flp-FRT recombination system for creating homozygous mutant clones in the ovary. An original heterozygous mutant cell in G2 phase is shown. The induction of Flp recombinase activates recombination between homologous FRT sites of non-sister chromatids. Following mitosis and cell division, one daughter cell is homozygous for the mutation of interest (red asterisk) while the other daughter cell is homozygous WT (GFP). (c) Examples of germine (top) and follicle cell (bottom) clones in the ovary. The presence of a homozygous mutant clone [white asterisk (top); white broken line (bottom)] is indicated by loss of the GFP signal. Scale bar is 25 μm.
9.2.2 Germline-Specific Caveats of the Gal4-UAS System

Although its general principle of operation applies across all tissues, attention should be paid to a few specific details when using Gal4-UAS in the female germ line. The ability of Gal4-UAS to function in the germ line is a more recent improvement, following the discovery that the basal promoter, terminator, and 3′ UTR sequences of the UAS construct were critical for its proper expression [102]. The UAS vector modified for female germline expression, containing the P transposase promoter, K10 terminator, and 3′ UTR sequence, was named “UASp” [102]. This is in contrast to the standard “UAST” vector that is most commonly used for expression in somatic tissues. An extensive list of characterized Gal4 drivers for use in the female germ line has been recently assembled [103].

9.2.3 Gal4-Inducible RNA Interference (RNAi)

One of the more recently developed and powerful applications of Gal4-UAS is the inducible, tissue-specific activation of RNAi for knockdown of specific mRNA transcripts. This technology is especially useful for the study of genes that are highly pleiotropic or lacking in classical mutant alleles. Two popular consortia that have designed and stock RNAi lines are the Drosophila Transgenic RNAi Project (TRiP) [104] and the Vienna Drosophila RNAi Center (VDRC) [100]. While the TRiP has exclusively used a site-specific insertion strategy, via the ΦC31 integrase system, to generate its inducible RNAi lines [105], the VDRC carries both P element and ΦC31-mediated insertions [106]. The TRiP has created a few “generations” of vectors containing inducible RNAi transgenes. The most notable difference between these
vectors is their level of expression in somatic versus germline tissues. Therefore, the details of each type of vector must be examined carefully before choosing a fly line for a particular experiment; descriptions of each vector and its components are listed on the TRiP’s web page [104].

The large number of existing transgenic RNAi stocks has provided a platform for high-throughput loss of function screens in ovaries and other tissues [107–109]. Inducible RNAi is now a commonly used tool for studying gene function and development in *D. melanogaster* ovaries [110–112]. In principle, a specific mRNA transcript can be knocked down in any tissue(s) for which there is an appropriate Gal4 line. A myriad of additional applications of Gal4-UAS has been devised by using different combinations of recombinases and fluorescent markers ([113] and reviewed in ref. 114).

Mosaic analysis is an extremely valuable tool for studying gene function in a developing organism; it allows the production of homozygous mutant cells in a heterozygous mutant animal [115]. Traditionally, the use of mosaic analysis in *D. melanogaster* was a laborious and inefficient process. Ionizing radiation could be used to induce mitotic recombination between non-sister chromatids [116]; however, the efficiency of this process was very low (~1 %), the recombination events occurred at random locations, and the high levels of radiation often caused tissue damage to the fly. The later incorporation of a yeast site-specific recombination system, FLPase (Flp) and its FRT target sites (FLPase recombination targets), allowed the restriction of mitotic recombination to a single, known chromosome arm and greatly increased its efficiency [117].

Performing mosaic analysis with the Flp-FRT system typically involves crossing a mutation of interest, carried on an FRT chromosome, in *trans* to a homologous FRT chromosome bearing a fluorescent or histological marker (e.g., GFP or LacZ, respectively) (Fig. 3b). On a separate chromosome, either Gal4, a heat-shock promoter, or a tissue-specific promoter can drive the expression of Flp. In the progeny of this cross, mitotic recombination will occur between the two FRT sites; clones that are homozygous for the mutation of interest will be produced, and can be identified by their lack of visual marker. In the context of a specific tissue, the homozygous mutant clones will be surrounded by cells that are either WT homozygous (also descended from mitotically recombined cells) or heterozygous (Fig. 3c).

As egg chambers are multicellular structures containing a shared cytoplasm, a distinction must be made between transient and stem cell derived clones. Transient clones are produced when a mitotic recombination event takes place within the germline cyst after the first cystoblast division. In this case, the population of
nurse cells within one egg chamber will contain a mixture of genotypes: homozygous WT, homozygous mutant, and heterozygous. The visual marker protein, along with other gene products, can diffuse freely through ring canals and confound one’s ability to unambiguously determine the genotype of each nurse cell. Therefore only egg chambers that completely lack the visual marker in each germline cell, indicating that they are derived from a GSC recombination event, would be examined. Similarly, stem cell derived and transient clones can be generated in the FCs, which also contain ring canals that bridge small clusters of cells [118].

9.4 The Dominant Female-Sterile Method for Generating Germline Mosaics

One alternative version of germline mosaic analysis is the dominant female-sterile (DFS) technique: Instead of using visual markers to identify clonal cell populations carrying a mutation of interest, a dominant female-sterile allele is used to block the development of non-homozygous mutant egg chambers. There are several dominant female-sterile alleles available, yet the most commonly used is ovoD1 [119, 120]. The purpose of this technique is to exclusively permit the formation of egg chambers that are homozygous for the mutation of interest (i.e., cells that have lost ovoD1); egg chambers carrying cells that are heterozygous or homozygous for the ovoD1 allele degenerate early in development.

The ovoD1 allele was originally recovered on the X chromosome, and therefore the early use of this technique was limited to studying X-linked mutations. However, by engineering this allele into P element vectors and creating transformants on additional chromosomes, this system was later expanded to include analysis of autosomal mutations [121]. The main benefit of the DFS method, as opposed to using the fluorescent or histological markers described above, is for obtaining a uniform population of embryos that are maternally homozygous for a mutation of interest. In this way, the putative maternal effects of a gene can be analyzed by genetic or biochemical methods.

9.5 Emerging Technologies for Genome Engineering: The CRISPR/Cas System

A recent development in genome engineering technology, which has gained popularity in *Drosophila* and other model organisms, is CRISPR, for “Clustered Regularly Interspaced Palindromic Repeats”. These genomic repeats are the basis of a bacterial and archaeal RNA-based immune system through which organisms acquire the ability to recognize invading genomic material [122]. Adaptive immunity is built over time by incorporating small DNA repeats, captured from viral genomes or plasmids, into these specialized genomic clusters. CRISPR-associated (Cas) proteins, in complex with small guide RNAs transcribed from these clusters, are guided to homologous sequences to create double-stranded DNA (dsDNA) breaks. There are three known types of CRISPR systems, with the best characterized being Type II; these systems,
as well as the mechanistic details of CRISPR repeat acquisition and function, have been characterized (reviewed in refs. 122, 123).

CRISPR’s great value as a genome-engineering tool is its ability to generate dsDNA breaks at specific genomic locations in a variety of model systems [124]. These breaks then serve as an entry point for different genome modification protocols (non-homologous end joining, gene targeting with a dsDNA donor template, etc.). A template encoding a chimeric RNA (chiRNA), which combines the function of both guide and trans-activating CRISPR RNA (tracrRNA), is designed to target a specific DNA sequence. The chiRNA and Cas-expressing vectors are co-transfected or transformed, depending on the model system; the expression of both transgenes results in targeting of the desired DNA sequence (reviewed in ref. 125). The use of sequence-specific DNA breaks greatly improves the efficiency of knockout creation in mammalian cell culture, and organisms such as mice and flies. More recent technical improvements in the efficiency and specificity of guide RNA targeting have been applied in D. melanogaster (reviewed in ref. 126). This system is gaining popularity as a method for creating transgenic flies, with gene knockout lines having been generated using both non-homologous end joining (NHEJ) and homology-directed repair with a dsDNA donor template [127, 128]. One additional technical advance has been the creation of transgenic lines stably expressing germline-driven Cas9, dramatically increasing the efficiency of germline CRISPR targeting [128, 129]. Some of these lines are available from the Bloomington Drosophila Stock Center [99]. More information on the CRISPR system is available online, including the “CRISPR design tool” (based on [130]), discussion forum, FAQ, and troubleshooting [131].

10 Conclusion

Drosophila melanogaster oogenesis continues to serve as an important model system for understanding fundamental aspects of cell biology and development; in addition to its large variety of biological topics being actively investigated, it remains at the cutting edge of technological innovation. Techniques such as live imaging of cell migration, high-resolution light and electron microscopic imaging of mRNA trafficking, and genetic/genomic manipulations with site-specific recombinases and transgenic RNAi have all been applied, increasing the depth of our biological knowledge. Moving forward, the study of the Drosophila ovary is poised to further advance our understanding of basic mechanisms in eukaryotic biology.
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