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Bruno contributes to *osk* mRNA localization during *Drosophila* oogenesis

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Bruno contributes to *osk* mRNA localization during *Drosophila* oogenesis

by

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Thesis

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Arts

The University of Texas at Austin

August 2011

Dedication

To Papa God and my family

Acknowledgements

I thank my adviser Paul M. Macdonald first and foremost. I thank him especially for his patience and dealing with me for so long. I thank the members of the Macdonald lab past and present that have crossed paths with me. I thank my family for trusting me in all I do in the midst of everything. Lastly, I thank my savior Jesus Christ for meeting me, loving me, and encouraging me til the very end.

Abstract

Bruno contributes to *osk* mRNA localization during *Drosophila* oogenesis

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Oskar is a body pattern and germ cell determining protein in *Drosophila melanogaster*. This protein must be properly expressed to ensure correct formation of the posterior pole of the animal. Bruno is a RNA binding protein known to regulate *osk* mRNA translation during *Drosophila* oogenesis. *In vitro* work has implicated Bru in oligomerizing *osk* mRNA into silencing particles and thereby preventing *osk* mRNA from accessing translational machinery and inhibiting translation. In attempts to further investigate Bru mediated translational regulation, reporter transcripts with Bruno binding regions from the *osk* mRNA were analyzed for translation and localization of the transcript. Localization of these reporter transcripts has shown the first *in vivo* evidence for the Bru mediated silencing particle assembly model. In this thesis, I report on the distribution of reporter transcripts in the *Drosophila* egg chamber.

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Chapter 1: Introduction

OSKAR AND OOGENESIS

Embryonic body patterning in *Drosophila* relies on a small number of localized cytoplasmic determinants. These proteins are expressed at specific positions within the developing oocyte or embryo, and provide the spatial cues that allow formation of the different body parts at the appropriate places. The Oskar (Osk) protein is one such determinant, responsible for posterior body patterning and germ cell formation. Loss of Osk activity leads to loss of abdominal segments and posterior structures on the *Drosophila* larva (Lehmann and Nüsslein-Volhard 1986). Conversely, having too much Osk activity, or Osk activity in the wrong places, is also detrimental. This type of misexpression results in loss of the anterior structures and produces a mirror image of the posterior structures (Ephrussi and Lehmann 1992; Smith *et al*. 1992). Therefore, the expression of Osk protein is highly regulated to ensure that it only appears at the appropriate position and at the appropriate level.

The *Drosophila* oocyte develops within an egg chamber. An egg chamber consists of both somatic and germline cells. The somatic follicle cells surround the 16 germline cells, which include 15 nurse cells and the oocyte. The germline cells are connected via ring canals, which form as a consequence of incomplete cytokinesis. The nuclei of the nurse cells undergo polytenization and produce materials for the oocyte development; these materials are then are transported into the oocyte through the ring canals.

Osk first appears at the posterior pole of the oocyte during stage 9 of oogenesis. This extreme restriction on the distribution of Osk protein is achieved by controlling the localization and translation of the *osk* mRNA. *osk* mRNA is transcribed in the nurse cells

of egg chambers beginning early in oogenesis. The mRNA is translationally repressed in the nurse cells and prior to its localization to the posterior pole of the oocyte (Kim-Ha et al. 1991; Ephrussi et al. 1991). Once localized to the posterior pole during stage 9 of oogenesis, the *osk* mRNA becomes translationally active, producing Osk protein (Kim-Ha et al., 1995; Rongo et al., 1995; Markussen et al., 1995).

OSK MRNA AND BRUNO

osk mRNA contains a short 5' untranslated region (UTR) of 15 nucleotides and a long (approximately 1 kb without the poly(A) tail) 3' UTR. Within the 3'UTR are regions known to regulate translation, the AB and C regions. The AB region is located near the coding region of the *osk* mRNA. The C region is near the poly(A) tail. Within these regions are Bruno binding sites. Mutating the Bru binding sites in both the AB and C regions on the *osk* mRNA leads to loss of Bruno mediated translational repression (Kim-Ha *et al*., 1995; Webster *et al*., 1997). A subset of Bruno binding sites, those in the C region, are also important for translational activation (Reveal *et al*., 2010). (Figure 1)

Bruno binding sites bind to Bruno (Bru), a RNA binding protein, and mediate translational regulation of *osk* mRNA. There are three types of Bru binding sites in the AB and C regions defined by their sequence. The Bru response element (BRE) has the strongest affinity with a core sequence of U(G/A)U(A/G)U(G/A)U. The type II (UUGUCC) and type III (UAAAGUCUUCUA) are sequences without the core sequence. Bru binds to the AB region with stronger affinity than the C region (Kim-Ha *et al*., 1995; Webster *et al*., 1997;Reveal *et al*., 2010). (Figure 1)

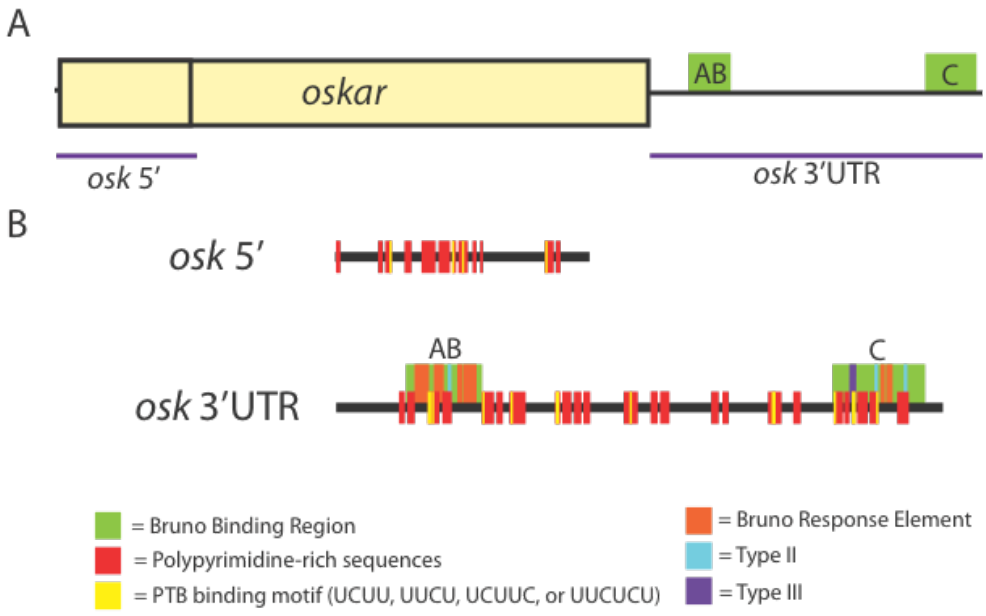

Figure 1: **Diagram of the *osk* mRNA** A. Schematic of the full *osk* mRNA. The coding region is in light yellow and the untranslated regions are depicted as lines. The *osk* 5' region includes the 5' UTR with approximately 400 nt of the coding region. B. Binding sites within the *osk* 5' and 3'UTR. The mRNA and the binding sites are drawn to scale.

Two models for Bru mediated repression have been proposed. In both models, translation is repressed by inhibiting initiation. In the first model, Bru, on the *osk* 3'UTR, recruits an ovarian protein Cup. Cup binds to eIF4E, the cap binding protein, and prevents it from interacting with eIF4G. If the molecule of eIF4E bound by Cup is that bound to the same molecule of *osk* mRNA, then translation in blocked since the eIF4E/eIF4G interaction is essential for initiation (Nakamura et al. 2004).

A second model for Bru mediated translational repression involves the formation of silencing particles. Recombinant Bru oligomerizes RNAs bearing 2 copies of the AB region *in vitro*, suggesting that Bru performs the same function with *osk* mRNA *in vivo*. The RNP particles – silencing particles - formed by oligomerization of multiple copies of

osk mRNA could exclude components of the translation machinery and thereby prevent translation. Previously, it was reported that Bru binding sites are not sufficient to associate different molecules of *osk* mRNA in vivo (see below), raising doubts about this model (Chekulaeva *et al*. 2006). (Figure 2)

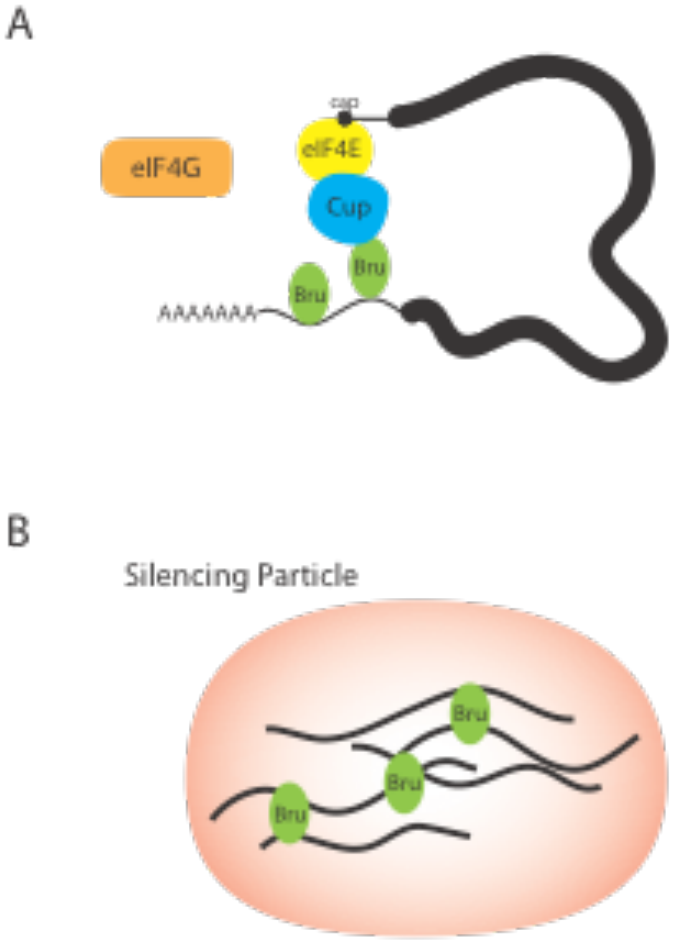

Figure 2: **Bru mediated translational repression of *osk* mRNA** A. Bru mediating translational repression of *osk* mRNA (black line) via Cup interaction B. Bru can oligomerizes *osk* mRNA (black lines) and form silencing particles preventing the translational machinery from accessing *osk* mRNA

OSK MRNA AND PTB

Polypyrimidine track-binding protein (PTB) (also known as heterogeneous nuclear ribonucleoprotein (hnRNP1)) is a protein involved in multiple forms of post-transcriptional regulation. These include alternative splicing (Valcarcel and Gebauer 1997; Spellman *et al*. 2005), translational initiation via internal ribosome entry site (IRES) (Stoneley and Willis 2004; Jang 2006; Semler and Waterman 2008), and mRNA localization (Cote *et al*. 1999; Ma *et al*. 2007).

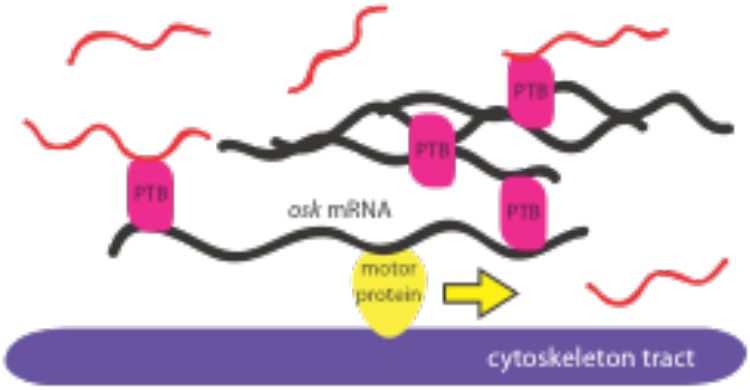

Figure 3: Diagram of PTB mediated Piggybacking

In *Drosophila* oogenesis, PTB localize to the posterior pole with *osk* mRNA. PTB contributes to translational repression of *osk* mRNA, as PTB mutants show ectopic expression of Osk protein. Another defect of the PTB mutants is a dramatic reduction in piggybacking. Piggybacking is a phenomenon in which localization-competent *osk* mRNA confers posterior localization on other mRNAs that do not localize on their own. For piggybacking to occur, the mRNAs must contain the *osk* 3' UTR. While the 3'UTR on the reporter transcript is not sufficient for posterior localization, it does support interaction with the 3' UTR of the intact *osk* mRNA. (Figure 3) This interaction is mediated by PTB, which binds to multiple sites dispersed throughout the *osk* 3'UTR as

well as a 5' region of the *osk* mRNA. The precise positions of the PTB binding sites are not known. However, *Drosophila* PTB is expected to bind to short U/C-rich sequences (the characterized binding preference for mammalian PTB), and such sequences can be found throughout the regions shown to bind PTB *in vitro* (Singh *et al*. 1995; Perez *et al*. 1997; Besse *et al* 2008). (Figure 1)

Expressing a GFP reporter gene with the *osk* 3' UTR in a wild type background, Besse *et al*. showed the reporter mRNA localizing to the posterior pole of the oocyte with the endogenous *osk* mRNA. However, mutating PTB greatly decreases the localization of the GFP reporter gene to the posterior pole of the oocyte while localization of the endogenous *osk* mRNA is not affected (Besse *et al*. 2008).

BRUNO AND PIGGYBACKING

If Bru does form silencing particles, in the mechanism proposed to explain how Bru represses *osk* mRNA translation, these particles would be expected to support piggybacking since mRNAs with *osk* 3' UTRs (the site of the Bru binding sites) should be bound to one another. However, in the paper demonstrating the role of PTB in piggybacking it was claimed that BREs are neither necessary nor sufficient for piggybacking. This work was cited as data not shown, (Besse *et al*. 2008) and therefore the experiments that support this claim are unknown. If correct, this would argue against the silencing particles model of translational repression by Bru.

Current work in the Macdonald lab includes experiments with GFP reporter mRNAs to study the role of the various Bru binding sites in translational repression and activation. For each mRNA the distribution of GFP protein provides information about where that mRNA is repressed and where it is activated. To interpret these data, it is essential to know where the mRNA is itself distributed, so as to distinguish between

patterns of expression due to translational regulation and patterns due to mRNA localization. In this thesis I characterize the distributions of a set of such reporter mRNAs. Notably, regions of the *osk* mRNA bearing Bru binding sites support one phase of mRNA localization, the transport from the nurse cells into the oocyte. Multiple lines of evidence argue that this transport is due to Bru-dependent piggybacking, providing the first *in vivo* evidence for the Bru mediated silencing particle model of *osk* mRNA translational regulation. I discuss future experiments that can further test this model.

Chapter 2: Results

UAS-GFP AND *UAS-OSK::GFP*

Two different classes of reporter transgenes were used for this study. One class is based on *UAS-GFP*, which contains the GFP coding region under UAS/GAL4 transcriptional control. The other class is based on *UAS-osk::GFP*, which differs only by inclusion of the first 434 bases of the *osk* mRNA fused to the to the 5' end of the GFP coding region. Both classes have a truncated fs(1)*k10* 3' UTR (lacking the K10 mRNA localization signal) from the vector. Between the coding region and the *K10* 3' UTR the AB region, the C region, or a combination of both AB and C region were attached, and the transgenes characterized. (Figure 4) The *mat-alpha-GAL4-VP16* transgene was used as the source of GAL4 to drive expression of the transgenes. This GAL4 driver is active only in the female germ line.

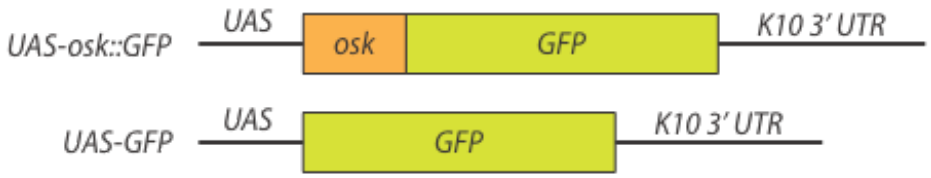

Figure 4: Diagram of GFP reporter transgenes

It is necessary to use reporters that expressed mRNA at similar levels. This is to control for effects due to the amount of mRNA and observe the effects caused by the mRNA characteristics. Younghee Ryu in the Macdonald lab determined the mRNA

expression levels using the RNase protection assay. (Table 1) Lines of similar expression levels were chosen for further characterization by fluorescence *in situ* hybridization.

Reporter \ *osk* 3'UTR region	none	AB	C	ABC	AB mut	C mut
UAS-osk::GFP	2.10±0.2	1.49±0.2	2.38±0.4	1.25±0.2	2.16±0.0	2.77±0.5

Table 1: **Relative mRNA levels for *UAS-osk::GFP*** RNase Protection assay results of *UAS-osk::GFP* transgenes. Values have been standardize using the rp49 mRNA.

THE PRESENCE OF THE AB OR C REGIONS LEAD TO OOCYTE ENRICHMENT OF REPORTER MRNAS

The *UAS-GFP* and *UAS-osk::GFP* mRNAs displayed an uniform distribution throughout the nurse cells and the oocyte during the previtellogeneic stages as well as later stages of oogenesis. No hybridization signal was detected in the somatic follicle cells, demonstrating that the assay specifically detected the GFP reporter transgene mRNAs. (Figure 5A,A' and Figure 6A,A')

The addition of the AB region conferred strong oocyte enrichment of the reporter mRNAs, while the C region conferred weaker oocyte enrichment. The combination of the AB and C regions displayed oocyte enrichment comparable to the AB region alone. (Figure 5 B,C and Figure 6B, C)

In later stages the oocyte enrichment is lost for transgenes with AB or C regions. Unlike transgenes with the complete *osk* 3' UTR, there is no posterior localization in stage 9 and later egg chambers. The reporter transgenes with the AB and C regions did show very weak posterior localization. The absence of posterior localization suggests

PTB-dependent piggybacking does not occur. Instead, I hypothesize that there is a Bru-dependent mRNA transport mechanism to the oocyte, which is distinct from the posterior localization within the oocyte. (Figure 5)

BRU BINDING SITES ARE INVOLVED IN OOCYTE ENRICHMENT OF REPORTER MRNA

To test the hypothesis of Bru-dependent mRNA transport to the oocyte, I compared the distribution of *UAS-osk::GFP*-AB and *UAS-osk::GFP-C* mRNAs to transgene mRNAs that differ only by mutation of all the Bru binding sites. Mutating these sites in either the AB or the C regions disrupted the transient oocyte enrichment seen in the wild type versions. Thus, the oocyte enrichment of the reporter mRNA is dependent on Bru binding sites. This further implicates Bru to mediate oocyte transport of *osk* mRNA. (Figure 5)

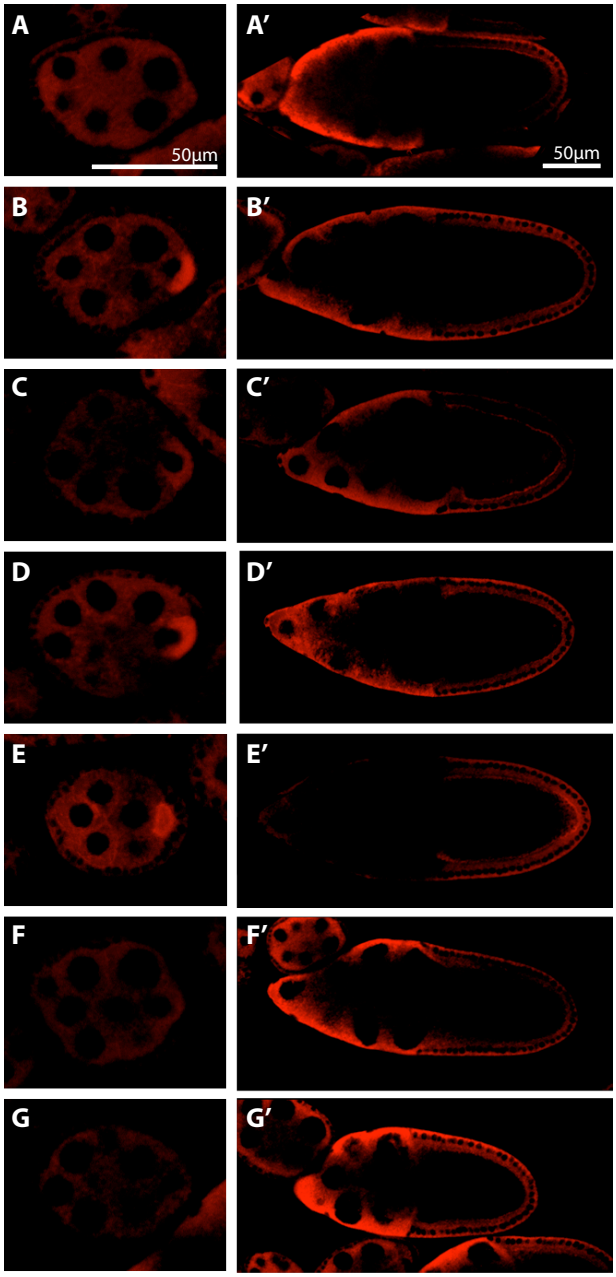

Figure 5: ***In situ* hybridizations of *UAS-osk::GFP* egg chambers** Stage 5 (A-G); Stage 10 egg chambers. (A'-G') Gain was increased in later stage egg chambers to observe any weak posterior localization. *UAS-osk::GFP* (A, A') *UAS-osk::GFP-AB* (B, B') *UAS-osk::GFP-C* (C, C') *UAS-osk::GFP-ABC* (D, D') *UAS-osk::GFP-osk 3'UTR* (E, E') *UAS-osk::GFP-AB mutant* (F, F'), and *UAS-osk::GFP-C mutant* (G, G') Bru binding sites were mutated for the AB and C region mutants.

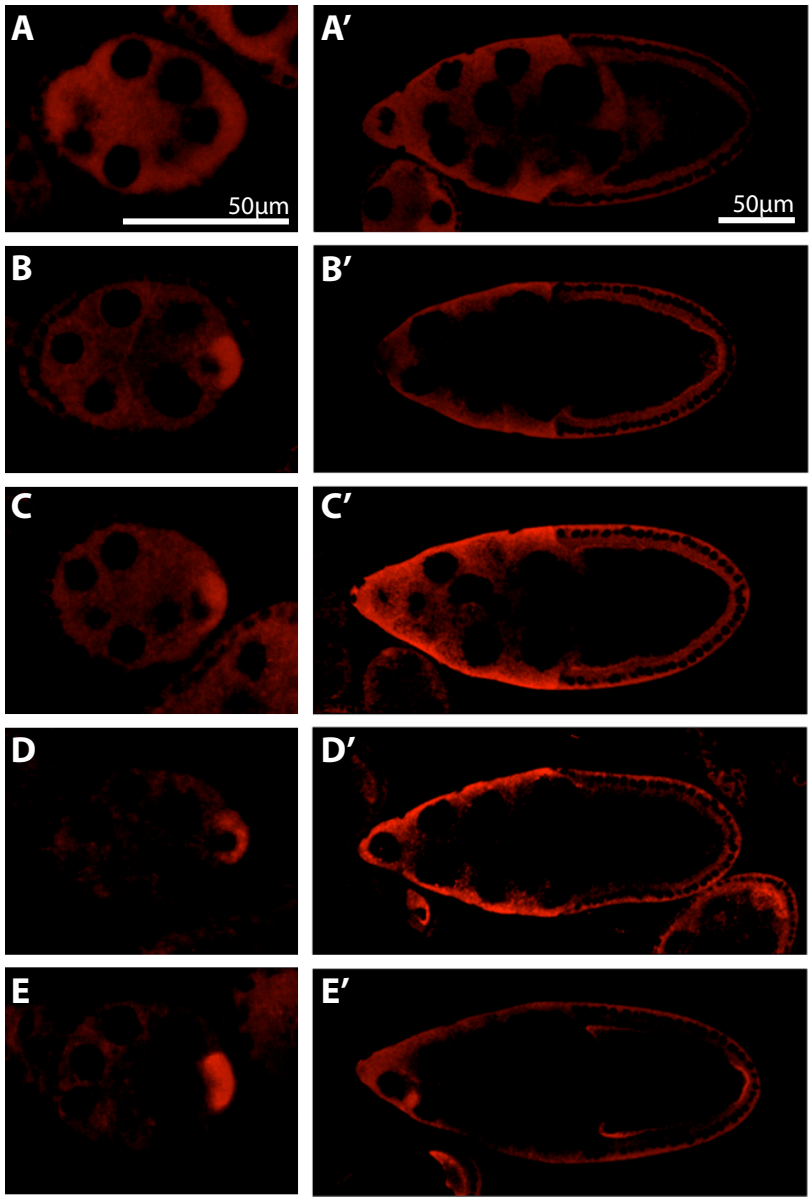

Figure 6: **In situ hybridizations of *UAS-GFP* egg chambers** Stage 5 (A-E); Stage 10 egg chambers. (A'-E'*) UAS-GFP* (A, A') *UAS-GFP-AB* (B, B') *UAS-GFP-C* (C, C') *UAS-GFP-ABC* (D, D') *UAS-GFP-osk 3'UTR* (E, E')

Chapter 3: Discussion

BRU-DEPENDENT OOCYTE TRANSPORT OF MRNA

In situ hybridization of reporter mRNAs results showed that the *osk* 3' UTR AB and C regions confer a form of mRNA localization - transport from the nurse cells to the oocyte - on reporter mRNAs. The AB and C regions differ in the degree of oocyte enrichment conferred on the reporter mRNAs. The AB region reporter transgenes had stronger oocyte enrichment than the C region alone. This is expected if the enrichment is Bru dependent. Bru binds to the AB with higher affinity than the C region, therefore more mRNA molecules with the AB region will be able to transport to the oocyte compared to those with the C region.

Another line of evidence for Bru-dependent mRNA transport to the oocyte is the disruption of oocyte enrichment when mutating the Bru binding sites in the AB or C regions. This argues that the ability to bind Bru is what is responsible for the transport to the oocyte of mRNAs with the AB and C regions.

OOCYTE TRANSPORT OF THE REPORTER MRNAS IS NOT PTB-DEPENDENT

As shown in Figure 7, putative PTB binding sites as well as large U/C rich regions are spread throughout the AB and C regions. The mutations in the Bru binding sites of the *UAS-osk::GFP-AB* transgene do no affect any of the putative PTB binding sites. The mutations in the Bru binding sites of the *UAS-osk::GFP-C* transgene alter two of the three putative PTB binding sites. However, the large U/C rich region which may bind PTB remains intact.

None of our reporter transgenes showed localization of the mRNA to the posterior pole of stage 9/10 oocytes. PTB is known to support posterior mRNA localization by

piggybacking. (Besse *et al*. 2008) Lack of such localization is evidence against PTB being the mediator.

BRU IS NOT REQUIRED BUT MAY BE INVOLVED IN *OSK* MRNA TRANSPORT

Previous work in the lab by Mark Snee showed that Bru is not required for *osk* mRNA transport. Using genomic *osk* transgenes, he expressed wild type *osk* mRNA or an *osk* mRNA with all the Bru binding sites mutated. The experiments were done in an *osk* mRNA null background, such that the only *osk* mRNA present was from the transgene, and thus no piggybacking on wild type *osk* mRNA was possible. He found that mutation of all the Bru binding sites did not interfere with transport of the mRNA into the oocyte. This brings up two possibilities. Either Bru is sufficient but not required for *osk* mRNA transport, or Bru mediates piggybacking rather than intrinsically being able to transport *osk* mRNA. Piggybacking is consistent with known properties of Bru such as oligomerization of RNA molecules.

Further experiments to test the Bru mediated piggybacking model can be done. One is to ask if endogenous *osk* mRNA is required for the observed transport to the oocyte of the *UAS-osk::GFP-AB* and *UAS-osk::GFP-C* reporter mRNAs. To do this experiment the reporter mRNAs can be expressed in *osk* RNA null flies. Although such mutants arrest oogenesis at stage 7, the oocyte transport can be detected at earlier stages. If oocyte enrichment is lost, this would support the piggybacking model. On the contrary, if the oocyte enrichment of the reporter mRNA is maintained in the *osk* mRNA null, this invokes a novel role for Bru in *osk* mRNA localization. One possible model of Bru mediated localization may involve Cup. Cup, which binds to Bru, is necessary for the posterior localization of *osk* mRNA and Barentsz (Btz). (Wilhelm *et al*. 2003) Btz is necessary for the kinesin mediated transport and posterior localization of *osk* mRNA.

(van Eeden *et al*. 2001) Therefore, we can speculate that an auxiliary role of Bru is to mediate *osk* mRNA localization via Cup and Btz. Expressing these transgenes in weak Bru mutants will clarify the involvement of Bru.

Another experiment is to ask if PTB is required for transport of the reporter mRNAs to the oocyte. *UAS-osk::GFP-AB mutant* and *UAS-osk::GFP-C mutant* reporters argue for the involvement of Bru, but cannot rule out PTB involvement. Expressing *UAS-osk::GFP-AB* and *UAS-osk::GFP-C* transgenes in a PTB mutant can test for the involvement of PTB.

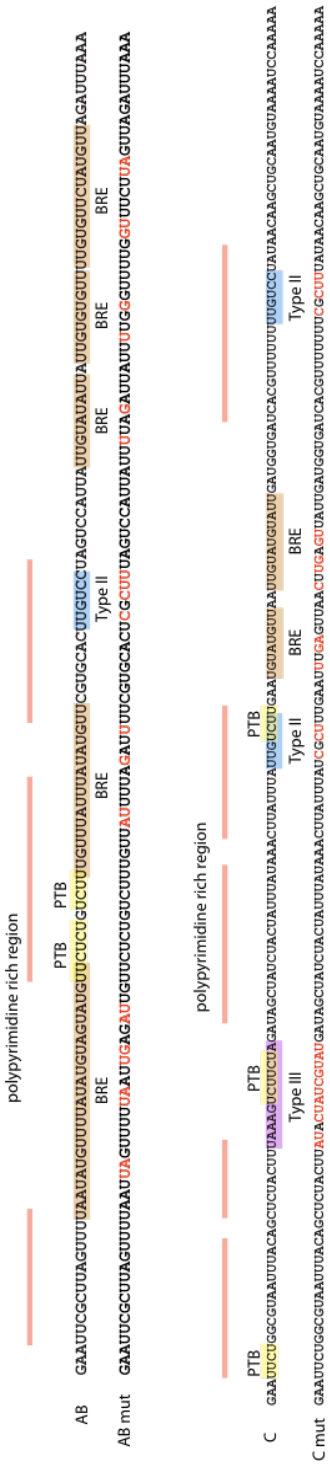

Figure 7: Bruno binding sequences and PTB binding sites in the AB and C regions

Chapter 4: Materials and Methods

FLUORESCENCE IN SITU HYBRIDIZATION

Ovaries were dissected in 1XPBS and fixed in 4% paraformaldehyde. Then treated with 20μg/ml of Proteinase K. Probe detecting the GFP made with digoxigenin-UTP (DIG RNA labeling Mix, 10X conc. Roche), Anti-Digoxigenin-POD (Fab fragments Roche), and the TSA^{TM} Plus Cyanine 5 Kit (PerkinElmer) were used to label the reporter mRNA.

FLY STOCKS

UAS-GFP lines are as described in Reveal et al. 2010. *UAS-osk::GFP* has the first 434 nucleotide sequence added to the 5' end of mGFP6 in a pUASp vector. AB (2669-2795) and C (3397-3555) regions of the *osk* 3'UTR were added as BamHI and BglII fragments into the BamHI in the pUASp vector. *UAS-GFP-osk 3'UTR* is as describe in Besse et al. 2008.

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Vita

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