Expression of and regulation by *nanos* in *Drosophila* early embryos

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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2015

**Abstract**

Three categories of cytoplasmic post-transcriptional regulation that modulate mRNA stability, subcellular localization, and translation are coordinated to precisely control when and where a protein is expressed. In *Drosophila* oocytes and early embryos, subcellular mRNA localization and local translation play fundamental roles in determining the body axes and specifying the germline. Nanos protein is one of the determinants whose subcellular localization is accomplished through local translation of its transcripts in the posterior germ plasm. I quantified Nanos protein in early embryos at closely spaced stages and revealed that *nanos* mRNA is indeed translated at the posterior pole of embryos. I then tested the role of an RNA-binding protein, Staufen, as a *nos* translational activator competitive binding with the RNA-binding protein Smaug. I further started to uncover the role of Nanos in target transcript regulation as a co-factor together with the RNA-binding proteins Pumilio and Brain Tumor.
Acknowledgments

I would first like to thank my supervisor, Dr. Howard Lipshitz for his patience and guidance. Thank you Howard for your support and the memorable experience in your lab. I would also like to thank John Laver, Wenxi Cao, and Xiao Li for their tremendous help in my report and presentation preparation. I would thank Zhifei Zhu and Yuan Yao for their help in solving mathematical questions. Many thanks to Hua Luo, Angelo Karaiskakis, Kristin Ancevicius, and Alexander Marsolais for their support in my experiments and training in the Lipshitz lab. Thanks to Dr. Craig Smibert for his help in my background knowledge enrichment. I thank the Smibert lab for constructs and reagents, and my supervisory committee members, Dr. Craig Smibert, Dr. Timothy Hughes, and Dr. Freda Miller for their valuable comments and guidance. Most of all I thank my family for their constant support.
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Chapter 1: Introduction

1.1 Post-transcriptional regulatory mechanisms

In eukaryotes, transcription and translation occur in separate compartments. This allows eukaryotes to carry out extensive post-transcriptional regulation (PTR) in both the nucleus and the cytoplasm, thereby providing additional layers of gene regulation. Three categories of PTR act on mRNAs in the cytoplasm to modulate their stability, localization, and translation. These three processes are coordinated to ensure that proteins are produced in subcellular regions where they are required and to ensure that proteins are absent at times and places when and where they may be deleterious.

mRNA decay rate is a major determinant of mRNA steady-state level within a cell. Mechanisms of eukaryotic mRNA decay can be grouped into two categories: those that are dependent on poly(A) tail removal (e.g. deadenylation-dependent) and those that are independent of poly(A) removal (as reviewed by Day and Tuite, 1998; Semotok and Lipshitz, 2007). Deadenylation is often the first step in mRNA turnover, (as reviewed by Day and Tuite, 1998; Semotok and Lipshitz, 2007). Subsequently, most deadenylated transcripts are decapped (i.e. their 5’ 7-methylguanosine cap structure is removed), after which the transcript is subjected to 5’-to-3’ exonucleolytic decay (as reviewed by Day and Tuite, 1998; Semotok and Lipshitz, 2007). Transcripts may also undergo 3’-to-5’ exonucleolytic degradation subsequent to deadenylation as a minor pathway (as reviewed by Semotok and Lipshitz, 2007). In addition, degradation can be initiated by endoribonucleases through deadenylation-independent mechanisms (as reviewed by Semotok and Lipshitz, 2007).

Translational regulation provides an energy-saving and rapid mechanism to control subcellular gene expression. Two general modes of translational control have been identified and studied: first, global control of common translation steps, which is accomplished by modifying core translation initiation and elongation factors (as reviewed by Day and Tuite, 1998); and second, specific controls applied to distinct mRNAs, which occurs via specific RNA binding proteins (RBPs) recognizing particular cis-elements present in their target mRNAs (as reviewed by Gebauer and
Hentze, 2004).

Cells can localize their mRNAs to particular subcellular regions through a variety of mechanisms. For example, mRNAs can be localized co-translationally to the endoplasmic reticulum (ER) via an RNA-protein complex termed “signal recognition particle” (SRP) (as reviewed by Blower, 2013). mRNA localization can also be accomplished independent of translation via cis-acting sequences and the trans-acting factors (TAFs) that bind these cis-elements (as reviewed by Liao et al., 2015). This second method of localization can occur via active transport or diffusion and trapping (as reviewed by Liao et al., 2015).

The three types of cytoplasmic PTR described above often function together on the same mRNA. For example, mRNA subcellular localization and translation are coordinated to accomplish precise spatial expression of a protein. ZBP1 is an RBP that localizes its target mRNA, \(\beta\)-actin, and is also involved in translational repression of \(\beta\)-actin mRNA by blocking translation initiation (Huttelmaier et al., 2005). Phosphorylation of ZBP1 by the Src tyrosine kinase reduces the affinity of ZBP1 for \(\beta\)-actin mRNA, thereby derepressing \(\beta\)-actin translation (Huttelmaier et al., 2005).

mRNA stability and translational regulation can also be coupled in space and time. For example, poly(A)-binding protein (PABP) both protects transcripts from hydrolysis by exonucleases and stimulates mRNA translation (Munroe and Jacobson, 1990). Similarly, a number of maternal mRNAs that are translationally repressed are degraded during early embryogenesis (as reviewed by Semotok and Lipshitz, 2007). There are also examples of translation leading to mRNA decay, and of translationally repressed mRNAs being stable (as reviewed by Semotok and Lipshitz, 2007).

mRNA degradation can also be linked to mRNA localization. Research in *Drosophila* has shown that mRNA localization to the germ plasm can be achieved by generalized decay of transcripts in the bulk cytoplasm and protection from decay in the germ plasm. The first identified mRNA localized through this degradation-and-protection mechanism is *Hsp83* mRNA, which is ubiquitously distributed throughout the oocyte and early embryo (Ding et al., 1993). Upon egg activation, degradation of *Hsp83* transcripts in the bulk cytoplasm is triggered by
Smaug RBP, while Hsp83 transcripts present in the germ plasm are protected from degradation (Bashirullah et al., 1999; Semotok et al., 2005; Tadros et al., 2007).

1.2 Subcellular RNA localization and localized translation

In the simplest model, sites of mRNA localization and protein synthesis correspond (as reviewed by Liao et al., 2015). This “RNA localize and translate” model (Figure 1-1) is a straight-forward mechanism, in which patterns of protein expression are generated by translation of patterned mRNAs and, consequently, show a similar expression pattern. Research has shown that the majority of mRNAs are localized and that this localization closely correlates with the distribution of their protein products (Lécuyer et al., 2007).

Usually, however, a more complex mechanism exists, in which localization is inefficient, and the unlocalized transcripts are translationally repressed and/or destabilized. In this mechanism, protein-coding mRNAs may be distributed throughout the cell or be localized at multiple sites in the cell, whereas translation occurs at only one specific site (Figure 1-1) (Liu-Yesucevitz et al., 2011). This is likely due to the presence of localized translational activators or the depletion of translational repressors (Colegrove-Otero et al., 2005; Yasuda et al., 2013; Kim-Ha et al., 1995). For example, in fibroblasts, $\beta$-actin mRNA localization is achieved by Rho-regulated transport along actin filaments by the myosin II motor (Kislauskis et al., 1994). During transport, the transcripts are bound by RBP, ZBP1 (zipcode binding protein 1), which represses translation (Kislauskis et al., 1994). After $\beta$-actin mRNA reaches its destination, ZBP1 binding is attenuated via phosphorylation and $\beta$-actin is translated (Kislauskis et al., 1994).

The transcripts encoding the actin polymerization nucleator, the Arp2/3 complex, are also regulated through the mRNA localization and local translation (Mingle et al., 2005). There are seven different subunits for the Arp2/3 protein complex. All seven mRNAs are localized in primary chicken embryo fibroblasts (CEFs) and primary human foreskin fibroblasts (HFFs) (Mingle et al., 2005). Localization of these seven
mRNAs is also regulated by Rho and myosin II (Mingle et al., 2005). Moreover, the translational repressor ZBP1 binds these mRNAs in vitro (Mingle et al., 2005). Whether this interaction occurs in vivo is unclear.

In neurons, a large number of mRNAs are specifically targeted to dendrites and axons. The localization and subsequent local translation of these mRNAs are important for dendrite growth, synaptic plasticity, and neural functions (Jung et al., 2013). The RBP RNG105 (RNA granule protein 105) forms RNA granules together with mRNAs that localize to dendrites and encode CaMKIIα, BDNF, DREB, MAP2, and TrkB (Shiina et al., 2005). RNG105 represses the translation of targets during transport into the dendrites of hippocampal neurons (Shiina et al., 2005). The synaptic protein, BDNF, then dissociates the RNA granules and releases mRNAs for local translation in dendrites (Shiina et al., 2005).

mRNA localization and localized translation control also happen in other organisms. In the frog (Xenopus laevis), several translational regulators are localized to an electron-dense structure, termed the “mitochondrial cloud” at the vegetal pole of oocytes (Heasman et al., 1984). In this vegetal cap structure, several mRNAs are present and under local translational control (Forristall et al., 1995; Hudson and Woodland, 1998). For example, Vg1 mRNA is present in both the bulk cytoplasm and the mitochondrial cloud. In the vegetal pole, Vg1 protein is synthesized whereas, elsewhere Vg1 is repressed by the frog ELAV protein, ElrB (Colegrove-Otero et al., 2005).

In mouse fibroblasts, the RBP Fus/TLS (fused in sarcoma/translocated in liposarcoma) is involved in the local translational regulation of tumor-suppressor protein APC (adenomatous polyposis coli) target mRNAs in APC-mRNP granules (Yasuda et al., 2013). Mutations altering Fus/TLS subcellular localization lead to ectopic translation of APC-mRNP mRNAs (Yasuda et al., 2013).

1.3 Role of subcellular mRNA localization and localized translation in Drosophila

In Drosophila, subcellular mRNA localization and local translation occur during
Figure 1-1. Subcellular mRNA localization and localized translation. Top is the mRNA localization and translation mechanism in which mRNA is exclusively localized to subcellular sites and translated. Bottom shows more complicated but more usual mechanism in localized translation, in which the unlocalized transcripts are kept repressed and/or destabilized.
oogenesis and early embryogenesis and play fundamental roles in determining the body axes and specifying the germline.

For example, oskar (osk) mRNA is localized to, and translationally activated at, the posterior pole of the oocyte. The local synthesis of OSK protein at the posterior plays a central role in germ plasm assembly (Ephrussi et al., 1991). osk mRNA localization requires polarization of the microtubule cytoskeleton, which depends on the recruitment of PAR-1 to the posterior cortex in response to a signal from follicle cells, where it induces an enrichment of microtubule plus ends (Morais-de-Sá et al., 2014). osk mRNA localization and local translation at the posterior generates a positive feedback loop in which OSK recruits PAR-1 to the posterior to increase polarization of the microtubule cytoskeleton, which in turn directs the localization of more osk mRNA (Zimyanin et al., 2007). At the posterior pole, STAU protein is also required for localization of osk mRNA. Full-length STAU protein has five double-stranded RNA binding domains (dsRBDs). STAU lacking a proline-rich insertion in dsRBD2 is able to associate with osk mRNA and activate its translation, but fails to localize the RNA to the posterior (Micklem et al., 2000). STAU lacking dsRBD5 localizes osk normally, but does not activate its translation (Micklem et al., 2000). Thus, dsRBD2 is required for microtubule-dependent localization of osk, and dsRBD5 is required for osk translation, once localized (Micklem et al., 2000). osk localization is also regulated by its own protein products: long-OSK protein is translated at the posterior pole of oocytes and anchors short-OSK encoding mRNAs and short-OSK protein. Short-OSK recruits other pole plasm components, such as Vasa, and thus plays an important role in formation of the germ plasm (Markussen et al., 1995).

To ensure that OSK protein only accumulates at the posterior pole, Bruno (BRU) RBP represses unlocalized osk mRNA by binding to Bruno regulatory elements in the osk 3’ UTR (Kim-Ha et al., 1995). Dimerization of BRU in the germ plasm acts to alleviate translational repression (Kim et al., 2015).

In contrast to osk, bicoid (bcd) mRNA is localized to, and translated at, the anterior pole of the early embryo. Localization of bcd mRNA during oogenesis is a
multi-step process that requires *exuperantia* (*exu*), *swallow* (*swa*), and *staufen* (*stau*). The EXU and SWA proteins function in *bcd* mRNA transport along the cytoskeleton to the anterior cortex of oocyte (Schnorrer *et al.*, 2000). STAU is required to anchor *bcd* mRNA at the anterior (Ferrandon *et al.*, 1994).

After fertilization, translation of *bcd* mRNA at the anterior is followed by diffusion of BCD protein to establish a BCD protein gradient emanating from the anterior (Drocco *et al.*, 2012). BCD is a homeodomain-containing transcription factor, that transcriptionally activates or represses target genes in the anterior to establish the body pattern. One direct target of BCD is the *hunchback* (*hb*) gene (Schaeffer *et al.*, 2000).

HB protein is also encoded in the anterior half of early embryos by maternal transcripts that are distributed throughout the early embryo. Maternal *hb* mRNA is translationally repressed and destabilized in the posterior (Wreden *et al.*, 1997) by the Pumilio/Nanos/Brain Tumor (PUM/NOS/BRAT) ternary complex (Chagnovich and Lehmann, 2001; Loedige *et al.*, 2014).

### 1.4 nanos subcellular mRNA localization and localized translation

Nanos (NOS) is a posteriorly localized zinc-finger-type RNA-binding protein that functions as part of the PUM-BRAT-NOS translational repressor. NOS is vital for anterior-posterior body patterning, abdominal segmentation, germ cell migration and cell cycle control (Irish *et al.*, 1989; Asaoka-Taguchi *et al.*, 1999). As mentioned above, in early embryos, NOS represses translation of *hb* mRNA at the posterior together with PUM and BRAT (Chagnovich and Lehmann, 2001; Loedige *et al.*, 2014). Later, NOS translationally represses *cycB* in primordial germ cells, controlling their migration and cell division (Asaoka-Taguchi *et al.*, 1999). The latter functions require concentration of NOS protein in the germ plasm and germ cells.

*nos* mRNA is inefficiently localized to the posterior of late stage oocytes and early embryos (Bashirullah *et al.*, 1999; Forrest and Gavis, 2003) (Figure 1-2 A). Quantitative analysis has shown that 96% of *nos* transcripts are unlocalized in the
bulk cytoplasm (Bergsten and Gavis, 1999). Upon egg activation, posterior enrichment of *nos* mRNA is enhanced by degradation of unlocalized transcripts in the bulk cytoplasm and protection from degradation in the posterior germ plasm (Bashirullah *et al.*, 1999) (Figure 1-2 B).

Spatially restricted NOS protein synthesis is accomplished by translational repression of unlocalized *nos* mRNA together with translational activation of posteriorly localized *nos* (Smibert *et al.*, 1999; Bergsten and Gavis, 1999) (Figure 1-2 C). *nos* is translationally repressed by two RBPs: Glorund (GLO), which interacts specifically with a stem-loop structure in the *nos* 3’ UTR (Kalifa *et al.*, 2006); and Smaug (SMG), which binds to two similar stem-loops (Smibert *et al.*, 1996) in the *nos* 3’ UTR, known as Smaug Recognition Elements (SREs) (Smibert *et al.*, 1999).

Translation of *nos* mRNA at the posterior pole begins during late oogenesis and generates posteriorly localized NOS protein products (Forrest *et al.*, 2004) (Figure 1-2 D). It has, however, not been shown unambiguously that NOS synthesis continues in the early embryo. Immunohistochemistry (IHC) has shown that NOS protein is detectable at the posterior end in 0.5 to 1 hour old embryos, while in 1 to 1.5 hours old embryos, the IHC signal was present throughout the posterior half (Wang *et al.*, 1994). This temporal expression pattern is consistent with NOS synthesis in the embryo. However, this analysis was not quantitative and could not exclude the possibility that NOS protein did not change in total amount but was transported or diffused away from the posterior.

As introduced above, *hb* mRNA is the only well-characterized NOS target in early embryos where it is translationally repressed by the NOS/PUM/BRAT ternary complex in the posterior half of the embryo (Chagnovich and Lehmann, 2001; Loedige *et al.*, 2014). NOS is the only posteriorly localized component of this ternary complex (Macdonald, 1992; Sonoda and Wharton, 2001) (Figure 1-2 C, E, and F), suggesting that the posterior specificity of *hb* regulation is determined by NOS. Whether NOS always contributes to PUM and/or BRAT regulation in the posterior of the embryos is not known.

cycB mRNA has been reported to be translationally repressed by NOS during
Figure 1-2. The expression pattern of *nos* mRNA, NOS protein, PUM protein, and BRAT protein.

*nos* expression in (A) 0.5-1 hour embryos; (B) 1.5-2 hour embryos. NOS expression in (C) early embryos; (D) Stage 14 oocytes (green). (E) PUM expression in early embryos. (F) BRAT expression in early embryos. Modified from: (A,B) from Bashirullah *et al.* (1999); (C) from Dahanukar and Wharton (1996); (D) from Kalifa *et al.* (2006); (E) from Macdonald (1992); (F) from Sonoda and Wharton (2001).
primordial germ cell migration (Asaoka-Taguchi et al., 1999). However, PUM also represses cycB translation in the anterior of the embryo, where NOS is absent (Vardy and Orr-Weaver, 2007). Thus, PUM also likely acts in a NOS-independent manner.

1.5 Goals of this thesis

The main goal of my thesis was to test whether nos mRNA is translated at the posterior pole of the Drosophila early embryo. To do so, I first optimized a quantitative IF method for detection of NOS protein and then quantified NOS protein in embryos at closely spaced stages during the first 90 minutes of development. My quantitative IF and Western blot experiments revealed that nos mRNA is indeed translated at the posterior pole of the embryo.

To test the mechanism underlying NOS synthesis at the posterior, the role of a candidate nos mRNA translation activator, Staufen (STAU), was tested. I found that nos mRNA is enriched in STAU immunoprecipitates in a RNA-immunoprecipitation experiment (RIP) experiment and that this interaction was negatively regulated by SMG, suggesting a competitive binding of SMG and STAU to nos mRNA.

Finally, I assessed whether NOS is an obligatory binding partner of PUM and BRAT by asking if posterior-localized targets of BRAT and/or PUM are enriched in NOS RIP experiments.

Together my data show that nos mRNA is translated at the posterior pole of early embryos, and begin to uncover the role of STAU as an activator of nos translation, as well as the role of NOS itself in the targets regulation of its binding partners.
Chapter 2: Materials and Methods

2.1 Fly stocks

Fly strains were raised on standard medium at 25 °C. Wild-type stocks were \textit{W}^{1118} (Tadros et al., 2007). The \textit{nos} mutant line used was \textit{nos}^{BN}, which is an RNA-null allele (Wang et al., 1994). The \textit{nos} over-expression line used was 6x \textit{gfp-nos} (Forrest et al., 2004).

2.2 Embryo and oocyte collections

Embryos were collected on apple juice agar plates after a two-hour pre-lay. For ovary dissection, females that were just hatched were collected and kept in bottles containing yeast and males for 3-5 days, allowing ovary development. Ovaries were then dissected in Ringer’s solution and Stage 14 oocytes were collected.

2.3 Western blots

Stage 14 oocytes and early embryos were collected and homogenized in 1.5 ml microfuge tubes in the presence of 1.5x urea sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 10 mM DTT, 20% glycerol, 5 M urea) and protease inhibitor (Roche Complete Mini) buffer that was pre-heated in a boiling water bath prior to homogenization. Supernatant extracts were resolved on an 8% SDS-PAGE gel. Protein was then transferred to PVDF membrane. Primary antibodies were: Guinea pig anti-NOS 1:1000 (gift of Hua Luo, Lipshitz lab) and mouse anti-Tubulin (Sigma). The primary anti-NOS antibody was pre-absorbed by incubating with embryos from the \textit{nos} RNA-null strain prior to incubation with the membrane. Secondary antibodies were: goat anti-guinea pig horseradish peroxidase (HRP) and goat anti-mouse HRP. All secondary antibodies were used at 1:5000. Membranes were imaged using ChemiDoc MP System (Bio-Rad).
2.4 Immunofluorescence (IF) staining and microscopy

Embryos were collected and fixed for 20 minutes on a nutator in fixative (4% formaldehyde, 1x PBS and heptane). The solution was then removed and embryos were dehydrated in methanol. Embryos were then washed in PBST and blocked in 10% BSA solution. Primary antibodies were: mouse anti-GFP 1:500 (Roche bimonalclonal anti-GFP antibody) and guinea pig anti-NOS 1:1000 (gift of Hua Luo, Lipshitz lab). Secondary antibodies were: Alexa 488 goat anti-mouse and Cyanine 3 conjugated to donkey anti-guinea pig. All secondary antibodies were used at 1:150. DAPI staining was performed to label nuclei. Embryos were stored in the solution containing DABCO (Sigma) in 75% glycerol-PBST. For quantitative comparison of the IF signal between two different genetic backgrounds, I stained embryos from the two backgrounds in the same tube, physically separated by a nylon mesh (Figure 2-1). Embryos were spread by pipetting onto slides and mounted.

A spinning disk confocal microscope (Leica DMIRE2; Olympus IX81) was employed for imaging. For each embryo, I photosliced a 50 μm thick section containing the entire posterior germ plasm region by confocal microscopy (Figure 2-2 A). Images for each embryo were merged and exported to generate a single image containing all IF signal in the posterior half of the embryo.

2.5 Imaging processing and analyses

Regular image rotation and cropping was performed to obtain a final image of 100 μm by 200 μm of the embryos’ posterior pole approximately centred on its anterior-posterior axis (Figure 2-2 B). These 200 μm x 100 μm regions were further divided into four 50 μm x 100 μm regions of interest (ROIs) aligning 0-50, 50-100, 100-150, 150-200 μm from the posterior end (termed “ROI 0-50”, “ROI 50-100”, “ROI 100-150”, “ROI 150-200” respectively) (Figure 2-2 C). IF signal intensity was measured for each ROI using ImageJ. Background cut-off was applied by setting the average level in the ROI 150-200 at nuclear cycle 1 (NC1) to zero. ROI 0-50 area adjustment was carried out by dividing the IF signal by the fraction of the embryo’s area relative to
Figure 2-1. Two groups of embryos were stained in the same tube for quantitative comparison.
One group of embryos was caged in an inner tube, which cap and bottom were a layer of nylon mesh. Embryos from the two backgrounds were then stained in the same tube and were spread by pipetting onto different slides for confocal microscopy.
Figure 2-2. Image processing and data analysis.
(A) A 50 μm thick section containing the entire posterior germ plasm region was photosliced by confocal microscopy. (B) The original image was processed to obtain a 200 μm x 100 μm image for analysis. (C) The 200 μm x 100 μm image was further divided into four 50 μm x 100 μm regions of interest (ROIs). Signals in these ROIs were measured. (D) Background cut-off and area adjustment were applied. (E, F) The area under the intensity curve was integrated to show the total amount of the protein.
the total area in ROI 0-50 images. For each NC, between 5 to 15 embryos were imaged. NCs 1 and 2, 3 and 4, 5 and 6, 7 and 8 were combined and signal intensity curves were plotted using the mean intensity for each ROI along the anterior-posterior axis (Figure 2-2 D). The area under the intensity curve was then integrated (Figure 2-2 E and F).

For IF analyses using anti-NOS polyclonal antibody, the background was measured by quantifying the non-specific signal from nosRN mutant embryos in each ROI. Then the background was subtracted from the anti-NOS signal measured in wild-type embryos for each ROI and NC.

2.6 Synthetic antibody expression and purifications

Expression followed the protocol described in Laver et al (2012). Briefly, synthetic antibody expression plasmids were transformed into BL21 cells and expression was induced using IPTG (Sigma). Cells were harvested and lysed using the B-PER bacterial protein extraction reagent (Thermo) and protease inhibitors (Roche). Fabs were then purified by loading bacterial lysate onto a Protein A beads (Roche) column. Beads were washed and Fabs were eluted. The eluates were dialysed overnight at 4 °C and stored at -20 °C.

2.7 RNA co-immunoprecipitations (RIPs)

RIP followed the protocol described in Laver et al (2012). Briefly, anti-FLAG M2 beads (Sigma) were washed and pre-coated with specific Fabs overnight at 4 °C. Embryo lysates were prepared and diluted. The upper clear layer of embryo lysate was transferred into a 600 μl tube with the pre-coated anti-FLAG beads. RIP was carried out at 4 °C for 2 hours. Beads were then washed and Fabs and associated RNAs were eluted with FLAG peptides (Sigma).

2.8 RNA extractions

Trizol reagent (Invitrogen) was used for RNA extraction according to the manufacturer's instructions. One additional phenol extraction step was applied after
phenol:chloroform extraction. 50 μl homogenized embryo lysates were used for total RNA extraction. For RIPed samples, approximately 15 μl of eluate was used for each sample.

2.9 cDNA reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

1 μg of total RNA was used for cDNA synthesis with random hexamers. For RIPed samples, 4 μl of purified RNA was used. The SuperScript II or SuperScript VILO kit was used for cDNA preparation (Invitrogen). Primers for 15 candidate target mRNAs were synthesized by IDT (Table 2-1). Sybr green mix was used for cDNA amplification. 4 μl of RTed cDNA was used for each qPCR assay. A Bio-Rad Real-time PCR detection system (CFX384 Real-Time System; C1000 Touch Thermal Cycler) was employed to amplify and detect target enrichment. Real-time data were analyzed with Bio-Rad software (CFX Manager, version 2.1).
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pgc</td>
<td>GGAGTAGCCCTTTGCTTGT AGTCGCACATTTCCGGGCTCT</td>
</tr>
<tr>
<td>osk</td>
<td>TGCTGAGCCACGCCCAGAATG CGCTGACTAGCAGCGTACA</td>
</tr>
<tr>
<td>dm</td>
<td>ACGGAAACTAGATGTTTACGCAAGTACGGATCAGAGCGGTAAAGG</td>
</tr>
<tr>
<td>RasGAP1</td>
<td>ACGCCACTGGAACCGATTGTTGTCGACTG</td>
</tr>
<tr>
<td>pum</td>
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</tr>
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</tr>
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</tr>
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</tr>
<tr>
<td>mei-P26</td>
<td>AGAGAGAGCAGCAGACATTT GCCATGCAATTGGACGACA</td>
</tr>
</tbody>
</table>

Table 2-1 primers for anti-NOS RT-qPCR assays
Chapter 3: Results

3.1 Localized translation of nanos mRNA in Drosophila early embryos

3.1.1 nos is translated in Drosophila early embryos

It is currently unknown whether nos mRNA is translated in early embryos (see Introduction). To begin addressing this issue, I used Western blots to compare protein levels between stage 14 oocytes (mature oocytes before fertilization and egg laying), 0-0.5 hour embryos, and 0.5-1 hour embryos using an anti-NOS polyclonal antibody. First, I analyzed a Western blot of wild-type (Figure 3-1, lane 2-4) and nos RNA-null strains (Figure 3-1, lane 5-7). A band was present in wild-type samples but absent in nos mutant samples at a molecular weight (Figure 3-1, red arrow) consistent with that reported in a previous study (Gavis and Lehmann, 1994). Second, my time course data showed that the total amount of NOS protein increased during the first hour of embryogenesis (Figure 3-2, lane 3-5). These observations strongly suggest that NOS protein is synthesized during early embryonic development.

3.1.2 NOS protein accumulates at the posterior of embryos carrying 8x nos mRNA

It is known that nos mRNA is present both in the bulk cytoplasm and in the germ plasm during early embryogenesis. I next asked where NOS is synthesized, employing quantitative immunofluorescence confocal microscopy (see Materials and Methods).

To increase the NOS signal-to-noise ratio, I carried out this experiment using transgenic flies that carry six copies of an otherwise wild-type nos transgene that expresses NOS protein tagged at its N-terminus with GFP (Forrest et al., 2004). Expression of the GFP-NOS fusion protein in this nos over-expression strain (6x gfp-nos) was then detected with an anti-GFP antibody.

My data showed that GFP-NOS signal at the posterior end was detectable just after egg laying in nuclear cycle 1 (NC1) embryos (Figure 3-3, NC1), consistent with the observation that maternal NOS protein is synthesized at the posterior during
Figure 3-1. Polyclonal antibody detects NOS on Western blot.
Lysates were made from 0-1 hour wild-type (*w*1118, lanes 2-4) and *nos* RNA-null mutant (*nosBN*, lanes 5-7) embryos. Lysates from equal numbers of embryos were loaded for the *w*1118 and *nosBN* groups (lanes 2 and 5). To avoid signal saturation during imaging, serial dilutions were carried out (lanes 3 and 4, lanes 6 and 7). One specific band from *w*1118 sample but not *nosBN* sample (red arrow) represents endogenous NOS protein. Bottom panel shows the same membrane reprobed with anti-TUB antibody to show the loading amount of total lysate in each lane. Lane 1: M, protein marker.
Figure 3-2. NOS is synthesized in early embryos.
Western blot experiment with polyclonal anti-NOS antibody. Lysates were prepared from nos$^{BN}$ mutant embryos (lane 2), wild-type stage 14 oocytes (lane 3), and wild-type embryos at different development stages (lanes 4 and 5). Lysates from equal numbers of embryos or oocytes were loaded. NOS was present in late stage oocytes (lane 3) and increased during embryonic development (lanes 4 and 5). No NOS signal was detected in the mutant sample (lane 2). Bottom panel shows the same membrane stripped and reprobed with anti-TUB antibody to show the loading amount of total lysate in each lane. Lane 1: M, protein marker.
oogenesis (Forrest et al., 2004). As embryos developed to NC9, GFP-NOS signal at the posterior pole appeared to increase (Figure 3-3). To quantify GFP-NOS fusion protein accumulation along the anterior-posterior body axis, I measured the immunofluorescence in 50 μm “regions of interest” (ROIs), 0-50, 50-100, 100-150, 150-200 μm from the posterior end (Figure 3-4; see Material and Methods for details). Consistent with my qualitative observations in Figure 3, GFP-NOS protein showed enrichment in ROI 0-50 through all stages whereas increases in ROI 50-100 and ROI 100-150 started at NC 5-6, and increases in ROI 150-200 increased at NC 7-8. This sequential increase of GFP-NOS signal from the posterior end to the more anterior regions is consistent with the hypothesis that nos mRNA translation is restricted to the posterior pole of early embryos, and GFP-NOS protein product diffuses to anterior ROIs after synthesis (Smibert et al., 1996).

To quantify the total amount change of GFP-NOS during early embryonic development, I integrated the area under the intensity curve after setting a background cutoff (see Material and Methods). The integrated GFP-NOS signal rose continuously from NC 1-2 to NC 7-8, and gave an approximately 2.5 fold increase in total GFP-NOS levels during these early embryonic stages (Figure 3-5).

I also quantified GFP-NOS fusion protein at the posterior end (ROI 0-50). GFP-NOS levels in ROI 0-50 showed significant increases from NC 1-2 embryos to NC 7-8 embryos (Figure 3-6).

I conclude that GFP-NOS protein is actively synthesized in nos over-expression embryos and accumulates at the posterior end of the embryo. However, it was possible that SMG and/or GLO levels in the 6x gfp-nos strain were not sufficient to repress the translation of gfp-nos transcripts at the posterior pole, leading to a leaky expression of GFP-NOS in early embryos. In order to eliminate this possibility, it was necessary to detect endogenous NOS translation in the wild-type background.
Figure 3-3. GFP-NOS accumulates at the posterior pole in early embryos. Quantitative immunofluorescence (IF) experiment using anti-GFP antibody was performed to detect GFP-NOS spatial and temporal expression pattern. Embryos from 6x gfp-nos overexpression strains were collected for this experiment and staged by nuclear cycle (NC). During early embryonic development, GFP-NOS protein is accumulated at the posterior end. The two images in the same panel were taken from the same embryo but using different channels and magnifications. In each panel, the image on the left side is DAPI, which was used to stage the embryo by NC. The image on the right shows the posterior half of the same embryo for GFP-NOS accumulation. Scalebars: 50 μm. In each panel, embryo posterior is to the right and dorsal to the top of the page.
Figure 3-4. Quantification of GFP-NOS intensity along the anterior-posterior axis. Sample size is over 10 in each group. x axis shows the distance to the posterior pole of the four sub-regions from which I measured the signal. y axis shows the absolute intensity of GFP signal measured by ImageJ software. Plotted lines with different colors reflect the GFP-NOS protein distribution along anterior-posterior axis at different NCs. Details are in Materials and Methods.
Figure 3-5. Total GFP-NOS levels in early embryos. Anti-GFP signal was integrated to measure the total amount of GFP-NOS fusion protein in 6x gfp-nos embryos. After background cutoff, the area under the GFP signal intensity line was calculated for each embryo. GFP-NOS protein increased 2.5-fold between NC1-2 and NC7-8. Mann-Whitney rank sum test. * p<0.05; ** p<0.01. For details see Materials and Methods.
Figure 3-6. GFP-NOS levels at the posterior pole of 6x gfp-nos embryos. After background cutoff, anti-GFP signal in the posterior region ROI 0-50 was calculated and compared across NCs in 6x gfp-nos embryos. GFP-NOS increased at the posterior pole between NC1-2 and NC7-8 of embryonic development. Mann-Whitney rank sum test. * p<0.05; ** p<0.01; *** p<0.001.
3.1.3 The NOS polyclonal antibody is suitable for quantitative IF analysis.

Before detecting endogenous NOS translation in the wild-type background, I needed to assess whether our polyclonal anti-NOS antibody quantitatively detects GFP-NOS fusion protein.

To do so, I carried out an IF experiment using pre-absorbed anti-NOS antibody. GFP-NOS posterior enrichment and expression level increases were observed (Figure 3-7). However, the anti-NOS IF showed relatively high background, thus preventing me from observing maternal pre-loaded NOS protein at the posterior (Figure 3-7, NC1 confer Figure 3-3, NC1). This high background is consistent with my Western blot data which showed that several non-specific bands were detected in nos RNA-null mutant embryos (Figure 3-1). I, therefore, measured anti-NOS signal in nos mutant embryos along their body axis, and used this value as the background to subtract from the anti-NOS signal measured in wild-type embryos (see Material and Methods).

These results are plotted in Figure 3-8 and 3-9. My data showed that GFP-NOS protein accumulated until NC 5-6, and was then maintained at a stable level at NC 7-8 (Figure 3-9). The total anti-NOS signal in embryos showed approximately 5-fold enrichment at NC 5-6 and NC 7-8 compared with NC 1-2 (Figure 3-9). Similarly, quantitative analysis showed an increase in GFP-NOS in ROI 0-50 at NC 5-6 and NC 7-8 of 2.5 to 3 fold compared with NC 1-2 (Figure 3-10).

These observations are consistent with my anti-GFP data, demonstrating that gfp-nos mRNA is translated in the posterior germ plasm during early embryonic development. However, my GFP data showed an increase between NC 5-6 and NC 7-8, whereas after NC 5-6 the anti-NOS level decreased. If the GFP antibody recognizes only mature GFP molecules, and GFP maturation time takes approximately 27 minutes (Heim et al., 1995), then the increases observed with anti-GFP antibody would be delayed relative to those observed with anti-NOS antibody. Besides, the increase of GFP-NOS detected by anti-NOS antibody was greater than that detected by anti-GFP antibody (5-fold versus 3-fold). This may be due to the fact that there are two endogenous copies of nos; thus the total amount of
Figure 3-7. Anti-NOS IF of 6x gfp-nos embryos.
Quantitative IF experiment using anti-NOS antibody was performed to detect the NOS spatial and temporal expression pattern. Embryos from 6x gfp-nos strains were collected and staged by NC. The two images in each panel were taken from the same embryo but using different channels and magnifications: on the left is DAPI, which was used to stage the nuclear number and pattern; on the right is NOS. Scalebars: 50 μm. In each panel, the embryo’s posterior is to the right and dorsal to the top of the page.
Figure 3-8. Quantification of NOS in 6x gfp-nos embryos using the anti-NOS antibody. Sample size was over 15 in each group. x axis shows the distance to the posterior pole of the four sub-regions from which I measured the signal. y axis shows the absolute intensity of NOS signal measured using ImageJ software. Plotted lines with different colors reflect the NOS protein distribution along the anterior-posterior axis at different NCs.
Figure 3-9. Total GFP-NOS levels in 6x gfp-nos early embryos assayed via anti-NOS IF.

Anti-NOS signal was integrated to measure the total amount of NOS protein in 6x gfp-nos embryos. After background correction, the area under the NOS signal intensity line was calculated for each embryo. NOS protein increased 5-fold between NC1-2 and NC5-6. Mann-Whitney rank sum test. ns p>=0.05; * p<0.05; ** p<0.01. For details see Materials and Methods.
Figure 3-10. NOS levels at the posterior pole of 6x gfp-nos embryos. After background correction, anti-NOS signal in the posterior region ROI 0-50 was calculated and compared across NC stages in 6x gfp-nos embryos. NOS protein increased at the posterior pole between NC1-2 and NC7-8. Mann-Whitney rank sum test. ns p>=0.05; * p<0.05; ** p<0.01.
GFP-NOS and NOS via anti-NOS detection is 1.33 times that of GFP-NOS alone via anti-GFP detection (8 copies versus 6 copies).

I conclude that the polyclonal NOS antibody reliably detects NOS protein by IF.

3.1.4 **Endogenous NOS protein accumulates at the posterior pole in w^{1118} strains.**

To assess whether endogenous NOS protein is synthesized during early embryonic development in a wild-type background, I performed IF experiments using the optimized method in a w^{1118} strain. I found that endogenous NOS protein is enriched at the posterior pole of the embryo, and that the amount of NOS increased at the posterior end during embryonic development from NC 1 to NC 6 (Figures 3-11 to 3-12).

Unlike my anti-NOS IF results from 6x gfp-nos embryos, the NOS signal in all ROIs was much weaker presumably because there are four times more NOS transcripts in nos over-expression embryos compared with w^{1118} embryos. However, the measured fold increase of endogenous NOS in w^{1118} embryos was over 30-fold between NC 1-2 and NC 5-6 (Figure 3-13), which is much higher than the measured fold change in the 6x gfp-nos strain. This difference of NOS accumulation between the wild-type and nos over-expression background may be due to instability of GFP-NOS fusion proteins since my preliminary anti-GFP Western blot experiments detected minimal amounts of full-length GFP-NOS protein from 6x gfp-nos strains.

Analysis focusing on ROI 0-50 showed an increase of NOS protein signal at the posterior end during early embryonic development (Figure 3-14). The endogenous NOS protein increased by more than 5-fold at NC 5-6 and NC 7-8 compared with NC 1-2, which is also higher than the observation from the 6x gfp-nos over-expression strain. A discrepancy exists, in which NOS is exclusively synthesized at the posterior pole but the fold increase in ROI 0-50 (5-fold) was much smaller than the fold increase in the posterior half of embryos (30-fold). My explanation for this difference in fold increase is that NOS protein is synthesized at the posterior pole and is transported/diffused to adjacent anterior regions (see Discussion and Future Directions).
Figure 3-11. Endogenous NOS accumulates at the posterior of early w^{118} embryos. Quantitative IF experiment using anti-NOS antibody was performed to detect the NOS spatial and temporal expression pattern. Embryos from w^{118} were collected and staged by NC. At NC1-2, NOS protein at the posterior could not be distinguished from background. However, at later NC stages, NOS clearly accumulates at the posterior. The two images in each panel were taken from the same embryo: the images on the left is DAPI, which was used to stage by nuclear number and pattern; the image on the right is anti-NOS. Scalebars: 50 μm. In each case, the embryo’s posterior is to the right and dorsal to the top of the page.
Figure 3-12. Quantification of NOS in w^{1118} embryos.
Sample size was over 15 in each group. x axis shows the distance to the posterior end of the four sub-regions from which I measured the signal. y axis shows the absolute intensity of NOS signal measured using ImageJ software. Plotted lines with different colors reflecting the NOS protein distribution along anterior-posterior axis at different NCs.
Figure 3-13. Total NOS levels in early $w^{1118}$ embryos. Anti-NOS signal was integrated to measure the total amount of NOS protein in $w^{1118}$ embryos. After background correction, the area under the NOS signal intensity line was calculated for each embryo. NOS protein increased between NC1-2 and NC5-6. Mann-Whitney rank sum test. ns p>=0.05; * p<0.05; ** p<0.01. For details see Materials and Methods.
Figure 3-14. NOS levels at the posterior pole of \( w^{118} \) embryos.
After background correction, anti-NOS signal in the posterior region ROI 0-50 was calculated and compared across NCs. NOS protein increased at the posterior pole between NC1-2 and NC5-6. Mann-Whitney rank sum test. * p<0.05; ** p<0.01.
Taking all of my data together, I conclude that endogenous *nos* mRNA is translated during the first hour of embryogenesis. Specific translation of *nos* mRNA at the posterior pole is likely to be achieved by a combination of translational repression mediated by SMG and possibly GLO in the bulk cytoplasm and relief of this repression in the germ plasm (Smibert *et al.*, 1999).

### 3.2 Evidence that STAU may regulate translation of *nos* mRNA in the germ plasm

It is clear that *nos* is translationally repressed in the bulk cytoplasm by SMG, but how *nos* is actively translated in the germ plasm is not clear (see Introduction). The double-stranded RNA-binding protein, STAU, is a known translational activator in the germ plasm (Micklem *et al.*, 2000). Furthermore, *nos* mRNA co-purifies with GFP-STAU (Laver *et al.*, 2013). A simple hypothesis is that STAU and SMG compete for binding to *nos* mRNA in the germ plasm. If so, then STAU binding to *nos* mRNA should increase in *smg* mutants.

To test this model, I performed RIP with anti-STAU antibody and identified STAU-associated mRNAs via RT-qPCR. The known STAU target *bcd* mRNA was highly enriched in anti-STAU immunoprecipitates compared to negative control (Figure 3-15). No *nos* mRNA enrichment was detected from the *w^{118}* (wild-type) sample (Figure 3-16, red bar). However, I detected 2-fold enrichment of *nos* transcripts when SMG was removed (Figure 3-16, blue bar). This result is consistent with the competitive-binding model.

### 3.3 mRNAs bound by NOS in *Drosophila* early embryos

#### 3.3.1 Anti-NOS synthetic antibodies immunoprecipitate endogenous NOS protein

To date, the NOS RBP has had only one well characterized target, *hb* mRNA (Chagnovich and Lehmann, 2001). I received four synthetic antibodies (Fabs) that may specifically recognize NOS from Hong Na and John Laver: anti-NOS G1, G2, F11, and F12. To test if these Fabs immunoprecipitate NOS, I expressed and purified
RT-qPCR showing that anti-STAU co-IP a STAU target mRNA from early embryo lysate.

RT-qPCR experiments were performed to detect STAU targets from anti-STAU RIP eluates. Two group of samples, from wild-type and smg mutant embryos respectively, were tested in this RIP experiment. Relative enrichment of the known STAU target, *bcd*, was assessed. Anti-hEGFR RIP was performed in parallel as a negative control, and *RpL32* was used as an internal non-target control. Target expression was normalized to *RpL32*, which was set as '1' (dashed line). *bcd* showed more than 10-fold enrichment in both samples.
Figure 3-16. Co-IP of nos mRNA with STAU increased in smg mutants. RT-qPCR was performed to detect STAU interacting RNAs from anti-STAU RIP eluates. Two group of samples, from wild-type and smg mutant embryos respectively, were tested in this RIP experiment. Relative enrichment of the candidate target, nos, was calculated by normalizing to the control antibody hEGFR, followed by normalization to Rpl32 RNA, which was set as '1' (dashed line).
these Fabs in *E. coli* (Figure 3-17). To confirm that the purified anti-NOS Fabs could IP NOS protein, I performed anti-NOS IP and detected NOS on a Western blot using our polyclonal anti-NOS antibody. Both anti-NOS G1 and F11 Fabs immunoprecipitated NOS protein (Figure 3-18, lane 3 and 4).

These data also confirmed that the band observed in my previous Western blot represents endogenous NOS protein (Figure 3-1).

### 3.3.2 Anti-NOS RIP-RT-qPCR shows that NOS associates with *hb* mRNA.

To confirm that the selected two Fabs (anti-NOS G1 and F11) could pull-down NOS protein together with its associated RNAs, I carried out anti-NOS RIP experiments and used RT-qPCR to test if the known NOS target *hb* mRNA was enriched. I found that both anti-NOS Fabs could IP *hb* mRNA (Figure 3-19) and that Fab G1 worked better than Fab F11, consistent with my observation from the anti-NOS IP-Western experiment (Figure 3-18), in which Fab G1 IPed more NOS protein than Fab F11. I detected 3-fold and 2.5-fold enrichment of *hb* mRNA, compared to negative control immunoprecipitations from anti-NOS Fab G1 and F11, respectively (Figure 3-19).

*CycB* mRNA is also an established target of PUM (Asaoka-Taguchi *et al.*, 1999) and was detected in a PUM RIP-Chip experiment carried out by John Laver in the Lipshitz and Smibert labs (Laver *et al.*, 2015). However, no enrichment of *cycB* was detected by anti-NOS RIP (Figure 3-19). This observation is consistent with the finding that *cycB* mRNA is repressed by PUM outside of the spatial domain of NOS expression (Vardy and Orr-Weaver, 2007). Alternatively, since *cycB* RNA becomes enriched in the posterior pole plasm and disappears from the bulk cytoplasm (Ding and Lipshitz, 1993), the mRNA may not be abundant enough for detection.

### 3.3.3 Tests of candidate NOS targets from the PUM and BRAT RIP-Chip dataset

John Laver used RIP-Chip to identify mRNAs bound by PUM alone, BRAT alone, or co-bound by BRAT and PUM (Laver *et al.*, 2015). I used RIP-RT-qPCR to assess the role of NOS in binding these targets. From the original lists (consisting of
Figure 3-17. Expression and purification of anti-NOS Fabs. Four synthetic antibodies (Fabs), anti-NOS G1, G2, F11, and F12, were expressed and purified from *E. coli*. All four Fabs showed the correct size on a Coomassie stained SDS-PAGE gel. In lanes with odd numbers, DTT was added to reduce the disulfide bond, and therefore each Fab gave two bands slightly above 25 kDa. In lanes with even numbers, DTT was not added, and Fabs ran near 55 kDa.
Figure 3-18. Western experiments showed that the anti-NOS G1 and F11 Fabs IP NOS protein from early embryo lysates.
Anti-NOS Western blot was performed on eluates from anti-NOS RIP experiments. Four anti-NOS Fabs (lanes 3-6) were tested. Synthetic antibody hEGFR (lane 2) was used as a negative control RIP. Eluate from IP beads not coated with synthetic antibody was used as a blank control (lane 1).
Figure 3-19. RT-qPCR showing that anti-NOS G1 and F11 Fabs co-IP a NOS target mRNA from early embryo lysate.

RT-qPCR experiments were performed to detect NOS targets from anti-NOS RIP eluates. Two anti-NOS Fabs (G1 and F11) were tested. Relative enrichment of the known NOS target, *hb*, and a candidate target, *cycB*, was assessed. Anti-hEGFR RIP was performed in parallel as a negative control, and *RpL32* was used as an internal non-target control. Target expression was normalized to *RpL32*, which was set as '1' (dashed line). *hb* (black bars) showed 2- to 3- fold enrichment, *cycB* (grey bars), did not.
200 PUM-and-BRAT common targets, 997 BRAT-not-PUM targets, and 441 PUM-not-BRAT targets), I selected several with more than two-fold enrichment based on the RIP-Chip data, that were expressed during the first 1.5 hours of embryonic development, and were enriched at the posterior pole based on the Fly-FISH database (http://fly-fish.ccbr.utoronto.ca/). Four candidates from the PUM-and-BRAT common target list (pgc, osk, dm, and RasGAP1, termed “PUM and BRAT”), five candidates from the BRAT-not-PUM target list (ovo, aret, Bsg25D, orb, and gcl, termed “BRAT-only”), and four candidates from the PUM-not-BRAT targets list (pum, pbl, tlk, and nos, termed “PUM-only”) were selected and tested in my RT-qPCR experiments. As a negative control, I selected two genes that are neither bound by PUM nor by BRAT (eIF4G and mei-P26, termed “non-targets”). These two negative controls are also expressed in early embryos and are enriched at the posterior.

My data (Figure 3-20) showed that two out of four candidates from the PUM-and-BRAT group gave more than three-fold enrichment in NOS IPs; the enrichment for candidates from the PUM-only group were all less than two-fold; and the candidates from the BRAT-only group showed more than two-fold enrichment in NOS interaction. Neither of the two selected non-targets showed more than two-fold enrichment (Figure 3-20). I also tested another anti-NOS Fab F11 and found a similar pattern of enrichment as for G1, but with less absolute enrichment (Figure 3-21), consistent with the finding that F11 pulls down less NOS than G1.

These data are very preliminary and will require additional replicates before any conclusion can be drawn regarding NOS’s role in PUM-and-BRAT, PUM-only and BRAT-only complexes.
Figure 3-20. Relative enrichment of PUM and/or BRAT targets after anti-NOS G1 RIP.

RT-qPCR was performed to detect putative targets in anti-NOS G1 RIP eluates. 15 candidate targets showing a posterior mRNA pattern were selected as described in the text. These 15 candidates consisted of 4 mRNAs from the PUM-and-BRAT common list (black bars), 4 mRNAs from the PUM-only list (dark grey bars), 5 mRNAs from BRAT-only list (light grey bars), and 2 posteriorly localized mRNAs that are not bound by PUM or BRAT (white bars). anti-hEGFR RIP was performed in parallel as a negative control, and ribosomal protein coding mRNA RpL32 was used as an internal non-target control. Target expression were normalized to RpL32, which was set as '1' (dashed line).
Figure 3-21. Relative enrichment of PUM and/or BRAT targets after anti-NOS F11 RIP.
RT-qPCR was performed to detect putative NOS targets in anti-NOS F11 RIP eluates. The same 15 candidate targets shown in Figure 19 were assayed using the methods and colour codes described in the Figure 19 legend.
Chapter 4: Discussion and Future Directions

4.1 NOS protein is synthesized in late oocytes and early embryos

Prior to my studies, evidence had been presented for NOS synthesis and localization during oogenesis (Forrest et al., 2004), but there were no definitive data regarding synthesis in the embryo. If the maternally pre-loaded NOS at the posterior pole is sufficient for anterior-posterior axis formation and is transported into germ cells later on, NOS protein does not need to be synthesized in the early embryo. My Western blot and quantitative IF experiments have shown that NOS protein is synthesized during early embryonic development in Drosophila. Therefore, there are two phases of NOS protein accumulation. The first phase is during late oogenesis; the second phase is in the early embryo.

As SMG is synthesized de novo in the early embryo (Benoit et al., 2009), my IF data suggested that GLO protein still represses nos translation in the bulk cytoplasm after egg laying until SMG is synthesized and represses nos translation. As maternal NOS protein accumulates starting from late oogenesis at the posterior pole, and late oogenesis takes only a few hours before egg laying, it is likely that nos mRNA localizes in late oocytes and begins translation by overcoming GLO’s repression in the germ plasm. Then this translation simply continues after fertilization but the repressor in the bulk cytoplasm is changed to SMG after NC 3.

To test the model that embryonic nos translation is a continuous process that starts at late oogenesis rather than starts by egg activation, future experiments would assess whether NOS is synthesized in embryos that do not undergo egg activation. Stage 14 oocytes would be dissected and fixed at closely spaced stages (e.g. every 10 minutes, which is consistent with the time period for each NC). NOS protein in these oocyte samples with different development time after dissection would be assessed by quantitative IF and microscopy. If NOS synthesis is a continuous process starting at late oogenesis, I would observed an increase in NOS levels in these post-dissected oocytes with a similar accumulation rate to my data in early embryos. If embryonic
NOS synthesis is triggered by egg activation, NOS levels would not increase in these post-dissected oocytes.

4.2 Evidence for transportation/diffusion of NOS protein from its site of synthesis in the germ plasm

My quantitative IF assays have shown that NOS protein first accumulates at the posterior pole of the early embryo and, later, more anteriorly.

The mechanism of NOS protein movement from the posterior pole is unknown. It may diffuse as is the case of BCD from its site of synthesis at the anterior pole (Drocco et al., 2012). Initial mathematical modeling of BCD protein movement showed that BCD proteins follow the cytoplasm flow and diffuse to the more posterior regions after being synthesized at the anterior pole (Drocco et al., 2012). However later analysis found that BCD protein diffusion could not fully explain the speed of BCD protein movement, and introduced mRNA diffusion rate as another factor that influences BCD protein movement (Dilão, 2014).

In the equation calculating BCD protein concentration in early embryos Drocco et al. (Equation 1) assumed that BCD protein undergoes one-dimensional diffusion with uniform degradation.

\[
\frac{\partial c(x, t)}{\partial t} = s(x, t) + D \nabla^2 c(x, t) - k(t)c(x, t) \quad (1)
\]

For the case of NOS protein, no measurement of NOS degradation has been reported. Therefore, I omitted the degradation function in the polynomial and kept the source function and diffusion function in my equation. I set parameters as reported (Diffusion rate = 4 \( \mu \text{m}^2/\text{s} \); major axis \( a = 250 \mu \text{m} \); minor axis \( b = 90 \mu \text{m} \)) (Drocco et al., 2011). Moreover, I set the starting NOS amount at the posterior pole equal to 1
unit and the total NOS amount at t=60 minutes equal to 30 units (measured from Figure 3-13, in which NOS total amount increased by 30-fold between NC1-2 and NC 5-6). Then, I calculated my equation (Equation 2) using numerical integration imposing the Neumann condition at the posterior end of the embryo as Drocco et al. (2012) did.

\[
\frac{\partial c(x, t)}{\partial t} = s(x, t) + D \nabla^2 c(x, t) \tag{2}
\]

I briefly estimated the diffusion frontier by time: NOS diffuses \( \sim 75 \) \( \mu \)m from the posterior end by 10 minutes after egg laying; \( \sim 130 \) \( \mu \)m by 20 minutes; \( \sim 200 \) \( \mu \)m by 30 minutes; \( \sim 250 \) \( \mu \)m by 40 minutes (Figure 4-1). Based on this prediction, NOS protein would diffuse beyond ROI \(_{150-200}\) by NC3-4, which is roughly the same as, but slightly faster than, my observation (confer Figure 3-12, in which the increase in NOS levels is observed by NC3-4 but not statistical significant until NC 5-6). This may be due to the fact that I did not take NOS protein degradation into consideration or other possibilities affecting NOS transport/diffusion rate.

For the case of NOS protein movement outside the posterior germ plasm, future experiments would need to measure the flow rate of cytoplasm in the Drosophila early embryo’s posterior regions. Since the germ plasm is a cytoplasmic domain distinct from the bulk cytoplasm at the posterior end, the diffusion rate at the posterior may be quite different from the measured rate at the anterior.

4.3 STAU may regulate nos translation through a competitive binding mechanism

Because the translational repressor, SMG, is concentrated in the germ plasm (Smibert et al., 1999; Siddique et al., 2012) together with nos mRNA, the translation of nos at the posterior pole may be caused by relief of repression by SMG.

Based on the results from my preliminary experiment, active translation of nos in the germ plasm occurs in unfertilized eggs. Therefore, nos translation is triggered by
Figure 4-1. NOS protein diffusion frontier is estimated.
(A, A’, A1-6) A 3D graph is generated showing the protein accumulation at, and diffusion from, the posterior pole. The height of the spots on the graph represent protein concentration. The posterior pole was set at the middle of the surface. (A) At t=0, very little amount of NOS is localized at the posterior pole (1 unit). (A’) a zoom-in view of the middle part of (A). (A1-6) NOS protein amount (peak height) is increased at the and diffused out (the height adjacent areas) from the posterior pole. Scale: A, A1-6: 400 x 400 μm²; A’: 4 x 4 μm². (B, B’, B1-6) density map from the posterior view of the embryo. Similar scales and time points were shown. (C, C’, C1-6) density map from the lateral view of the embryo. NOS diffusion from the posterior pole is observed.
egg activation rather than fertilization. Since there is no zygotic transcription in unfertilized eggs, maternally encoded proteins must be relieved of SMG-dependent repression.

STAU is a candidate translational activator for NOS posterior-specific translation. First, STAU protein is enriched in the germ plasm during early embryogenesis (St Johnston et al., 1991). Second, STAU’s presence at the posterior temporally overlaps with my data, showing that nos is actively translated during the first hour after egg laying (NC 1 to 6). Third, STAU is a known translational activator of bcd and osk mRNA (Micklem et al., 2000). Fourth, nos mRNA co-IPs with STAU-GFP, indicating that it may be a target of STAU (Laver et al., 2013). Fifth, the predicted secondary structures that encompass the first SRE in the nos 3’ UTR show a “Y” shaped stem structure that is similar to a Type III STAU-recognized structure (SRS) (Laver et al., 2013). The possible spatial overlap of SMG and STAU binding sites led to the hypothesis that, in the germ plasm, STAU may compete with SMG for binding to the nos 3’ UTR, thus derepressing translation (Figure 4-2 A). My data showing increased binding of STAU to nos mRNA in smg mutants is consistent with this hypothesis. However, unpublished data from the Lipshitz lab suggests that there is excess germ plasm in smg mutants, which may be an alternative explanation.

Future experiments to test the competitive binding model would start with a gel shift assay to assess whether STAU can bind the SRS-like structure in vitro. If so, nos 3’ UTR 1-185 nt regions carrying different mutations, which are predicted to block either SMG or STAU binding (Figure 4-2 B), would be inserted into a GFP or luciferase reporter to assess the effects of binding in S2 cells. Then transgenic flies with these reporters carrying SMG and/or STAU binding mutations would be generated to further test the model in vivo. As GLO protein also represses nos translation in the early embryos, and the STAU binding site also spatially overlaps the GLO binding site, future experiments with the same idea in assessing STAU-SMG competitive binding would be used to assess STAU-GLO competitive binding. Experiments would start with assessing nos mRNA and STAU interaction in glo mutant background. Then in vitro gel shift assay and in vivo reporters would be
Figure 4-2. STAU-SMG competitive binding model.

(A) The possible spatial overlap of SMG and STAU binding sites was graphed. In the bulk cytoplasm, nos translation is repressed by SMG, whereas in the germ plasm, STAU may compete with SMG for binding to the nos 3' UTR, thus derepressing translation. (B) Mutations that shorten the stem-structure that STAU may recognize will block STAU binding to the nos 3' UTR, but will not affect SMG binding. Mutations alter the motif sequence that SMG recognizes will block SMG binding, but will not change STAU recognizing the nos 3’ UTR.
STAU-GLO competitive binding model.

4.4 In vivo targets of NOS

Recent data have shown that both BRAT and PUM are ubiquitously distributed RNA-binding proteins (Macdonald, 1992; Sonoda and Wharton, 2001) and that, while they co-regulate certain mRNAs, many of their targets are not shared (Laver et al., 2015). NOS is a third component of this complex and the only one that is localized (to the germ plasm and posterior regions of the early embryo). I carried out RIP-RT-qPCR to assess whether NOS binds to PUM-BRAT co-targets, PUM-only targets and/or BRAT-only targets. I selected targets in each category that are known to be posterior-localized. My data are too preliminary to draw any conclusion.

Future experiments would include anti-PUM or anti-BRAT RIP-Chip in nos mutant background. If NOS is a co-factor for PUM and BRAT posterior function, the enrichment of PUM and/or BRAT posterior targets should be altered when NOS is absent, whereas PUM and/or BRAT targets in the bulk cytoplasm would show limited change. Then RIP-Chip or RIP-Seq would be carried out to identify NOS targets genome-wide followed by comparison to lists of BRAT-PUM co-targets, BRAT-only targets, and PUM-only targets. Analyses of the posterior localized subsets of each category with NOS-bound mRNAs would be a first test of NOS’s role. Furthermore, these experiments might permit computational identification of NOS’s binding site, which has remained mysterious (Loedige et al., 2014).
REFERENCES


