Pumilio-mediated Repression of mRNAs in the Early *Drosophila melanogaster* Embryo

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Post-transcriptional regulation plays an important role in governing various processes in all organisms. The development of the early embryo of *Drosophila melanogaster* is governed solely by post-transcriptional mechanisms; therefore, further insights into post-transcriptional regulation can be gained by studying the *Drosophila* embryo. This thesis addresses the actions of the translational repressor, Pumilio, in regulating two mRNAs during early embryogenesis. First, we examined the ability of Pumilio to regulate the mRNA stability of *bicoid*, a gene required for *Drosophila* head development. *bicoid* mRNA contains the canonical Pumilio recognition site, termed the Nanos response element (NRE), within the 3'UTR. Interestingly, we show that Pumilio binds to the NRE both in vitro and in vivo; however, no physiological significance is associated with this interaction. Furthermore, in *pumilio* mutant embryos *bicoid* mRNA stability and translation are unaltered, demonstrating that Pumilio does not regulate *bicoid* mRNA. Second, Pumilio has been shown to negatively regulate *Cyclin B*, the cyclin necessary for mitotic entry, in the somatic cytoplasm of the embryo and this repression is alleviated by the PNG Kinase complex through currently unidentified mechanisms. We further investigated the actions of Pumilio in regulating *Cyclin B* and discovered that the canonical partner of Pumilio, Nanos, is not involved in repressing somatic *Cyclin B*. Furthermore, we show that the 3'UTR of *Cyclin B* is not required for the regulation by Pumilio and the PNG Kinase complex. Lastly, through genetic

analyses, we conclude that Pumilio may actually act upstream of the PNG Kinase complex to regulate *Cyclin B*.

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This dissertation is dedicated to my mother, Lily.

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1. Introduction

1.1 Post-transcriptional regulation of early *Drosophila* embryogenesis

Post-transcriptional mechanisms of regulation are implemented in myriad organisms ranging from *E. coli*, *S. cerevisiae*, *C.elegans*, *D. melanogaster*, *M. musculus* and *H.sapiens*. Post-transcriptional control is an important mode of regulation of numerous processes such as cancer formation, virus defense, embryonic development, and stem cell division. In furthering our knowledge of the many aspects of post-transcriptional regulation we can increase our understanding of various processes important to the health of all organisms.

The *Drosophila melanogaster* embryo is an ideal system to study post-transcriptional regulation. During the rapid nuclear divisions of the first two hours of *Drosophila* embryogenesis, no transcription occurs, causing the embryo to rely only on post-transcriptional mechanisms for development. All of the early embryonic processes such as mitosis, pattern formation, cell movement, and the establishment of germ cell identity are governed solely by maternal RNAs and proteins that are deposited into the oocyte during oogenesis. By using the *Drosophila* embryo we can gain insights into the overall biology of an organism as well as post-transcriptional mechanisms of control.

Drosophila has developed a multitude of post-transcriptional methods to ensure embryogenesis proceeds both correctly and efficiently. Post-transcriptional control allows for temporal regulation by guiding the timing of mRNA degradation and translation, whereas spatial regulation occurs via mRNA localization and translational repression.

In my graduate work, I have focused on the study of two aspects of post-transcriptional control: the regulation of mRNA stability and translational repression. In order to gain insights into these mechanisms, I have further examined the role of two known translational repressors, Nanos (Nos) and Pumilio (Pum), in the regulation of two maternal mRNAs crucial to embryogenesis: *bicoid* (*bcd*) and *Cyclin B* (*CycB*).

1.2 The Roles of Nanos and Pumilio in early *Drosophila* embryogenesis

The *nos* and *pum* genes were initially identified in a forward genetic screen conducted by Christianne Nusslein Volhard and colleagues to isolate genes involved in *Drosophila* segment polarity (Nusslein-Volhard, Frohnhofer et al. 1987). A group of nine maternal genes are necessary for the posterior development of the embryo, including *nos* and *pum*. In contrast to wildtype larvae that develop a head region, three thoracic segments, and eight abdominal segments, embryos derived from *nos* or *pum* mutant mothers (from here on denoted as *nos* or *pum* embryos) fail to complete embryogenesis and are completely void of an abdomen (Nusslein-Volhard, Frohnhofer et al. 1987). During further analysis of *nos* and *pum* embryos, it was discovered that the protein expression pattern of another maternal effect gene, *hunchback* (*hb*), is altered (Struhl 1989; Hulskamp, Pfeifle et al. 1990). Hb acts in the anterior as both a transcriptional activator where it promotes anterior gene expression and as a transcriptional repressor, inhibiting the expression of posterior group genes in the anterior (Kraut and Levine 1991; Papatsenko and Levine 2008). In wildtype embryos, *hb* mRNA is found throughout the entire embryo, but *hb* protein accumulates only in the anterior region of the embryo, forming an anterior gradient of Hb (Tautz 1988). However, in *nos* and *pum* embryos, *hb* mRNA distribution remains the same, but Hb accumulates throughout the embryo, causing the embryos to develop without an abdomen (Struhl 1989; Struhl, Struhl et al. 1989; Hulskamp, Pfeifle et al. 1990; Barker, Wang et al. 1992) (Fig 1).

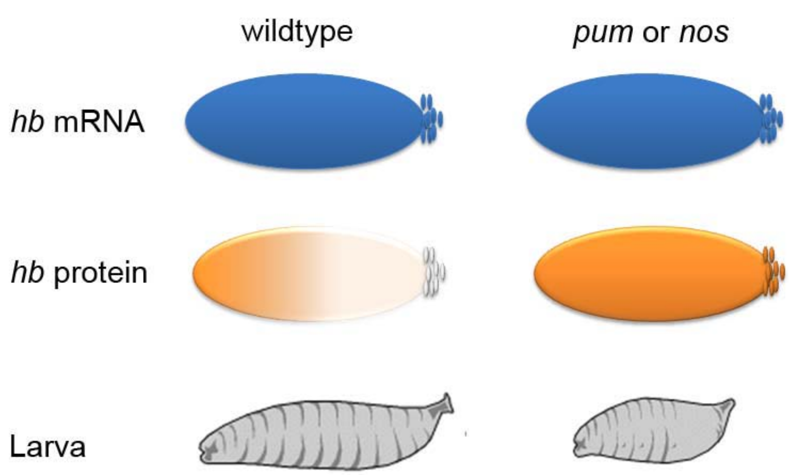

Figure 1: Hb accumulation in the posterior of *nos* or *pum* embryos inhibits abdomen formation

hb mRNA (blue) is distributed ubiquitously throughout wildtype, *pum*, and *nos* embryos. In wildtype embryos, *hb* protein (gold) forms an anterior gradient. In *nos* and *pum* embryos, *hb* protein accumulates throughout the embryo causing no abdominal segments to develop.

The aberrant expression of *hb* protein in *nos* mutant embryos, coupled with the finding that Nos protein is only present in the posterior of the embryo, where Hb is not found, suggested a role for the *nos* gene product as a repressor of *hb*. Based on the *pum* phenotype, it was also surmised that the *pum* gene product is a translational repressor of *hb*, but since Pum protein is found ubiquitously throughout the embryo, a clear mechanism for repression could not initially be determined (Barker, Wang et al. 1992; Macdonald 1992).

nos and *pum* do not only play roles in abdomen formation, but are also involved in the regulation of the germline precursor cells of the *Drosophila* embryo. In *nos* and *pum* mutant embryos, germline precursor cells form, but due to embryonic lethality, functionality of the germline precursor cells could not be determined. In order to circumvent this dilemma, Kobayashi and colleagues transplanted *nos* germline precursor cells into wildtype embryos and showed that *nos* germline precursor cells fail to migrate to the somatic gonad, resulting in sterility (Kobayashi, Yamada et al. 1996). Moreover, wildtype germline precursor cells are mitotically quiescent as they migrate towards the somatic gonad; however, *nos* and *pum* germline precursor cells enter mitosis prematurely (Asaoka, Sano et al. 1998; Asaoka-Taguchi, Yamada et al. 1999). Early entry into mitosis of *nos* and *pum* germline precursor cells is due to the accumulation of CycB, the cyclin necessary for entry into mitosis after DNA replication (Asaoka, Sano et al. 1998; Asaoka-Taguchi, Yamada et al. 1999) (Fig 2). CycB is normally absent in wildtype germline precursor cells as they migrate to the somatic gonad. The discovery of the aberrant expression of CycB in *nos* and *pum* germline precursor cells identified Nos and Pum as translational repressors of *CycB*.

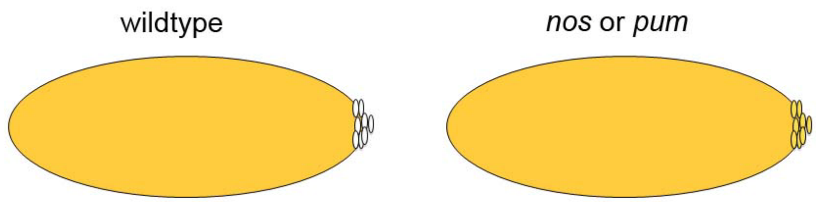

Figure 2: *CycB* protein accumulation in wildtype embryos compared to *nos* or *pum* mutant embryos

Schematic of wildtype versus *nos* or *pum* mutant embryos. The small circles at the psoterior represent the germline precursor cells (PGCs). *CycB* protein is represented as yellow shading. Take note that *CycB* protein is not present in the PGCs of wildtype embryos but accumulates in the PGCs of *pum* or *nos* mutant embryos.

1.3 The Mechanism of Nanos and Pumilio Regulation

pum and *nos* mutant embryos demonstrate the same loss of abdomen phenotype and the germline precursor cells in mutant embryos of both genotypes enter mitosis prematurely. Through the study of the mechanism of repression of *CycB* and *hb*, Pum and Nos proteins were found to be partners in the translational repression of *hb* and *cycB* mRNA.

1.3.1 The Mechanism of *hunchback* mRNA Repression

The location of sequences in *hb* mRNA important for repression in the posterior was narrowed to the 3' untranslated region (3'UTR) (Struhl 1989; Hulskamp, Pfeifle et al. 1990; Barker, Wang et al. 1992). Discrete sequences, termed Nos Response Elements (NREs), within the 3’UTR are both necessary and sufficient for the repression of *hb* by Nos (Wharton and Struhl 1991). Once the *cis*-acting elements required for *hb* mRNA repression were identified, only the *trans*-acting factors involved in this repression remained to be determined. Through biochemical assays, two molecules of Pum were discovered to bind to each *hb* NRE (Murata and Wharton 1995; Gupta, Lee et al. 2009). The interaction between Pum and the *hb* NRE is physiologically relevant to the repression of *hb* (Murata and Wharton 1995). In finding that Pum binds to the NRE, the first link between the genetic data and a physical mechanism was shown, demonstrating that a direct relationship between Pum and *hb* exists.

Pum, the founding member of the Puf RNA binding family, contains a novel C-terminal RNA binding domain, which is sufficient for the translational control of *hb* mRNA (Zamore, Williamson et al. 1997; Wharton, Sonoda et al. 1998). Furthermore, the Pum RNA binding domain recognizes a motif present in the NRE: UGUA. *hb* mRNA contains two NRE sequences, each bearing a UGUA motif (Wharton and Struhl 1991). Pum recognizes and binds to the UGUA sites and recruits Nos to the mRNA forming a ternary complex (Sonoda and Wharton 1999). Another translational repressor, Brain Tumor (Brat), joins the Pum/Nos/NRE complex, resulting in the translational repression of *hb* via both poly(A)-dependent and poly(A)-independent mechanisms (Chagnovich and Lehmann 2001; Sonoda and Wharton 2001) (Fig 3).

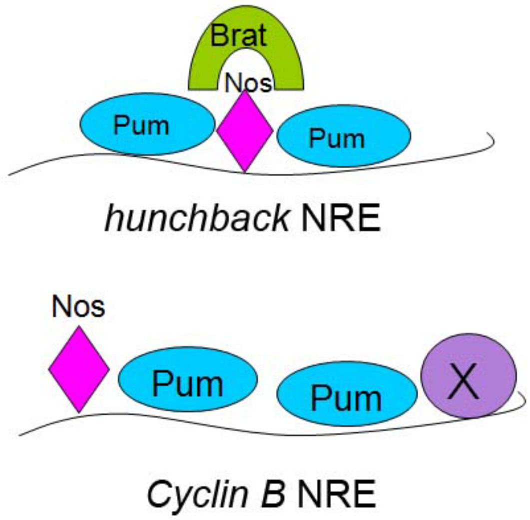

Figure 3: Repressor complexes on the *hb* and germline *CycB* 3'UTRs

Schematic of Pum and Nos complexes on the *hb* and *CycB* 3'UTRs at the NRE. In the anterior, two Pum molecules bind to the *hb* NRE and recruit Nos and Brat to the mRNA. In the germline precursor cells (PGCs), two Pum proteins bind to the *CycB* NRE and recruit Nos. Currently, another unidentified germline co-repressor (protein X) is thought to bind to the *CycB* NRE in the germline precursor cells and aid in its repression.

1.3.2 The Mechanism of *Cyclin B* mRNA Repression in the Germline Precursor Cells

Once it was discovered that Pum recognizes the NRE in the *hb* 3'UTR, one method of identifying the *CycB* NRE involved searching for Pum binding sites within the 3UTR. Kadyrova and colleagues utilized this strategy in their quest to locate the *CycB* NRE, by scanning large regions of the 3'UTR to look for Pum binding sites (Kadyrova, Habara et al. 2007). Pum binds to three regions in the *CycB* 3'UTR, but the NRE was narrowed to a 50 nucleotide segment that proved to be both necessary and sufficient for *CycB* repression in the germline precursor cells by Pum and Nos. Within the 50 nucleotide segment, there are two Pum binding sites and a putative Nos recognition site (Kadyrova, Habara et al. 2007). Furthermore, if Nos is tethered to *CycB* mRNA in the absence of Pum, repression still occurs, suggesting that the role of Pum is primarily to recruit Nos. Unlike the regulation of *hb*, Brat does not play a role in the repression of *CycB* in the germline precursor cells. Instead, a currently unidentified germline corepressor acts in concert with Nos and Pum to impede the translation of *CycB* (Kadyrova, Habara et al. 2007) (Fig. 3).

1.3.3 Evolutionary Conservation of Pumilio and Nanos

Studies of *nos* and *pum* originated in *Drosophila*; however, *nos* and/or *pum* homologs have been identified in numerous organisms such as *S. cerevisiae, C.elegans, D. rerio, M. musculus* and *H. sapiens*. Mice and humans have two *pum* homologs: *pum1* and *pum2* and two *nos* homologs: *nos1* and *nos2* (Spassov and Jurecic 2002; Spassov and Jurecic 2003). The RBD of human Pum is 80% identical to the Drosophila Pum RBD (Spassov and Jurecic 2002). Pum2 is present in human embryonic stem cells as well as human germ cells, suggesting a role for Pum2 in human development and regulation of the human germline, as in flies (Moore, Jaruzelska et al. 2003). Pum2 and Nos1 have been found to physically interact in human germ cells, demonstrating the conservation of the Pum-Nos complex from flies to humans (Jaruzelska, Kotecki et al. 2003).

Furthermore, human Pum2 has been shown to interact with RNAs, suggesting a conserved mechanism of action of Pum (Fox, Urano et al. 2005; Spik, Oczkowski et al. 2006). In addition, human Pum2 interacts with DAZL, a member of a gene family required for human fertility and resulting deletions in the members of the DAZ gene family causes azoospermia, suggesting a role for human Pum in fertility (Moore, Jaruzelska et al. 2003; Fox, Urano et al. 2005). Currently, in humans, no correlation has been made between infertility and mutations in Pum2; however, Pum2 knockout mice have smaller testes with degenerating seminiferous tubules (K. Kusz 2007; Xu, Chang et al. 2007). Overall, Pum and Nos are conserved throughout evolution and maintain their roles in regulating both germline and somatic development.

1.4 Nanos-Independent Regulation of mRNAs by Pumilio

The binding of Pum to *hb* or germline *CycB* is not sufficient for regulation in *Drosophila*. Furthermore, with the evidence that Pum and Nos work together from *hb* and *CycB* studies, it was thought that Pum did not regulate mRNAs throughout the embryo due to the restriction of Nos to the posterior region. However, in *S.cerevisiae*, where no Nos ortholog has been identified, Pum is sufficient for the regulation of targeted mRNAs. In the budding yeast, two members (Mpt5 and Puf4) of the Pum protein family (Puf) interact with Pop2, a subunit of the CCR4/Pop2/NOT deadenylase complex (Goldstrohm, Hook et al. 2006; Hook, Goldstrohm et al. 2007). Mpt5 or Puf4 recruit the CCR4/Pop2/Not deadenylase complex to the 3'UTR of the mRNA and the mRNA becomes destabilized (Goldstrohm, Seay et al. 2007).

Recent evidence supports the idea that Pum can act to repress mRNAs independently of Nos in *Drosophila*. First, bioinformatic analysis suggests that Pum may globally regulate maternal mRNAs (Gerber, Luschnig et al. 2006; Tadros, Goldman et al. 2007). Second, Pum interacts with the CCR4/Pop/NOT complex in the repression of *CycB* in the germline precursor cells, suggesting a method of potential regulation of other mRNAs without Nos (Kadyrova, Habara et al. 2007). Third, it has been shown that Pum regulates *bcd* mRNA stability in the anterior of the embryo, where Nos is not present (Gamberi, Peterson et al. 2002). Fourth, the repression of maternal *CycA*, *CycB,* and *smaug* in the somatic cytoplasm of the embryo is Pum-dependent and assumed to be Nos-independent (Tadros, Goldman et al. 2007; Vardy and Orr-Weaver 2007; Vardy, Pesin et al. 2009). Fifth, Pum and Nos mediate different processes in the neuromuscular junction of Drosophila *larvae* (Menon, Andrews et al. 2009). These findings demonstrate that Pum can potentially regulate mRNA independently of Nos; however, it is still currently unknown if Pum is sufficient for the regulation of mRNAs in *Drosophila* or needs the assistance of currently unidentified co-factors for repression.

1.4.1 The Regulation of *bicoid* in the Anterior of the *Drosophila* Embryo by Pumilio

An NRE was also identified in the 3'UTR of *bcd*, an anterior determinant essential for head development (Wharton and Struhl 1991). However, it was thought that Nos and Pum did not regulate *bcd* for two reasons. First, no overall changes in *bcd* protein accumulation occur in *nos* or *pum* mutants (Gamberi, Peterson et al. 2002). Second, *bcd* mRNA localizes to the anterior of the embryo where Nos is not detected (Berleth, Burri et al. 1988). Nevertheless, the *bcd* NRE was shown to be functional: ectopic Nos expressed in the anterior of the embryo represses *bcd* translation and the embryo fails to develop a head and thorax. However, *bcd* mRNA lacking the NRE is not repressed by ectopic Nos in the anterior (Wharton and Struhl 1991). Based on the finding that *bcd* mRNA is only regulated in the anterior by Nos under artificial conditions, the NRE was thought to have no endogenous role in regulating *bcd* mRNA.

Years later, Gamberi and colleagues explored the mechanism by which *bcd* stability, not translation, is governed (Gamberi, Peterson et al. 2002). They discovered that in *pum* embryos, *bcd* mRNA stabilizes. Normally, *bcd* mRNA cannot be detected by 120'after egg lay (AEL) at 25°; however, in *pum* embryos *bcd* mRNA does not disappear until approximately 2.8-3.3 hours AEL (Gamberi, Peterson et al. 2002) . Furthermore, Gamberi and colleagues demonstrated that Pum acts via the NRE to control the timing of *bcd* mRNA degradation (Gamberi, Peterson et al. 2002). The finding that Pum can govern *bcd* stability independently of Nos, was the first assertion that Pum and Nos do not always work in concert to regulate target mRNAs. In addition, with the recent evidence demonstrating the Nos-independent regulation of mRNAs by Pum occurs, we wished to re-examine the molecular mechanism of Pum-mediated regulation of *bcd*.

1.4.2 The Regulation of *Cyclin B* by Pumilio in the Somatic Cytoplasm of the *Drosophila* Embryo

Rapid nuclear divisions occur throughout the first two hours of embryogenesis and CycB is essential for progression from S phase to nuclear division. Orr-Weaver and colleagues discovered three maternal effect mutants that halt the cells cycle after S phase and cause the development of large polyploid nuclei (Shamanski and Orr-Weaver 1991; Renault, Zhang et al. 2003) . In all three mutants, *pan gu* (*png*), *plutonium* (*plu*), and *giant nuclei* (*gnu*), *CycB* protein levels are barely detectable (Fenger, Carminati et al. 2000; Zhang, Axton et al. 2004).

The Png Kinase complex does not regulate the transcription of *CycB* or mRNA stability; therefore, it remained unclear if the PNG Kinase complex governs *CycB* translation or protein stability (Lee, Van Hoewyk et al. 2003). However, Orr-Weaver and colleagues concluded that the PNG Kinase complex activates *CycB* translation by discovering that Pum plays a role in the regulation of *CycB* in the somatic cytoplasm (Vardy and Orr-Weaver 2007). In *png* mutants, CycB levels are low and in contrast CycB levels are restored to almost wildtype amounts in *png*; *pum* double mutants. Based on this finding, Orr-Weaver and colleagues hypothesized that Pum acts to stop *CycB* translation and the PNG Kinase complex acts antagonistically on Pum to relieve *CycB* repression. This counteraction by the PNG Kinase complex then allows *CycB* to be translated during embryogenesis. Currently, the method by which Pum represses *CycB* in the soma is not well-understood and a focus of my research is to further elucidate the mechanism of this two-tiered control of *CycB* expression in the somatic cytoplasm of the early *Drosophila* embryo.

1.5 Introduction to Thesis Research

In order to shed light on post-transcriptional control we utilized the early embryo of *Drosophila melanogaster* whose first few hours of development are regulated only via post-transcriptional mechanisms. We focused our post-transcriptional studies on the translational repressor, Pum, because with the recent findings demonstrating the ability of Pum to regulate mRNAs independently of Nos, questions remained regarding a novel mechanism of Pum-mediated post-transcriptional control. We explored the actions of Pum by studying the processes by which Pum governs *bcd* mRNA stability and *CycB* translation in concert with the Png Kinase Complex.

In this work, we wished to further understand the role that Pum plays in *bcd* mRNA stability. Through our investigations and much to our surprise, we discovered that *bcd* mRNA stabilization is not controlled by Pum. Furthermore, we also witnessed no role for the *bcd* NRE in *bcd* mRNA regulation.

Next, we investigated the repression of *CycB* in the somatic cytoplasm of the embryo. Ultimately, we determined that the 3'UTR is not responsible for the repression of *CycB* in the somatic cytoplasm. Moreover, we also demonstrated that Pum may actually act upstream of the PNG Kinase complex. These results show that Pum may regulate *CycB* through a novel mechanism and argues against the hypothesis that Pum may directly repress *CycB* mRNA translation.

2. Pumilio-mediated Regulation of *bicoid* mRNA Stability

2.1 Introduction

The maternal effect gene, *bcd*, is essential for embryonic head formation and anterior development of the *Drosophila* embryo (Frohnhofer and Nusslein-Volhard 1986). *bcd* is a member of the *Hox3* gene family and is only found in higher dipteran species (Stauber, Jackle et al. 1999; McGregor 2005). Embryos derived from *bcd* mutant mothers develop no head or thorax and fail to hatch into larvae (Frohnhofer and Nusslein-Volhard 1986). *bcd* mRNA localizes to the anterior of the oocyte through the binding of specific proteins to the *bcd* localization sequence present within the *bcd* 3'UTR (Macdonald and Struhl 1988; Arn, Cha et al. 2003; Snee, Arn et al. 2005; Irion and St Johnston 2007). *bcd* localization begins via active transport and is maintained by anchoring to the anterior cortex in an actin-dependent manner (Weil, Forrest et al. 2006). The poly-A tail of *bcd* mRNA extends upon egg activation, initiating the translation of *bcd* (Salles, Lieberfarb et al. 1994). The translation of the anteriorly localized *bcd* results in a *bcd* protein gradient emanating from the anterior pole of the embryo (Driever and Nusslein-Volhard 1988). Bcd, a DNA- and RNA-binding protein, can then activate the transcription of genes necessary for head development such as zygotic *hunchback* (*hb*) and *orthodenticle* (*oc*) as well as repress the translation of *caudal* mRNA (Driever and Nusslein-Volhard 1989; Hoch, Seifert et al. 1991; Liaw and Lengyel 1993; Rivera-

Pomar, Lu et al. 1995; Dubnau and Struhl 1996; Gao and Finkelstein 1998; Niessing, Blanke et al. 2002). This dual activation and repression by Bcd facilitates the development of the head and thoracic regions of the embryo.

Interestingly, a sequence similar to the *hb* NRE is present in the *bcd* 3'UTR (Fig 4).

hb NRE 1 UAAUCGUUGUCCAAUUGUAUAUAUUCGUAGCAUAAGUUUUCCA
hb NRE 2 UUUGUUGUCGAAAAUUGUACAUAAGCCAAUUAAGCCGCUAAUUC
bcd NRE CUGUUGUUCCUGAUUGUACAAAUACCAAGUGAUUGUAGAUA

Figure 4: *hb* and *bcd* NRE sequences

Conserved sequences of the two *hb* NREs and the *bcd* NRE. Each *hb* NRE contains one UGUA sequence; whereas, the *bcd* NRE has two UGUA sequences.

However, unlike the *hb* NRE, no function of the *bcd* NRE was initially determined. Deletion of the *bcd* NRE results in no noticeable phenotype and with the evidence that *bcd* mRNA does not overlap with Nos protein in the embryo, it was thought that the *bcd* NRE does not play a role in regulating *bcd* (Wharton and Struhl 1991) . Only under the artificial circumstances when Nos is ectopically expressed in the anterior, a role for the NRE was found (Wharton and Struhl 1991). In the anterior, if Nos is present, *bcd* mRNA is not translated. However, if the *bcd* NRE is deleted, *bcd* repression does not occur, demonstrating that the repression is NRE-dependent. Nevertheless, an endogenous role of the *bcd* NRE was not discovered, suggesting that the *bcd* NRE does not act during early *Drosophila* embryogenesis, and may just be an evolutionarily drifted sequence.

bcd mRNA is only found during a short window of time during embryonic development and is degraded by approximately 120' AEL at 25 ºC (Driever and Nusslein-Volhard 1988). Gamberi and colleagues speculated that ubiquitous Pum may act independently of Nos to regulate *bcd* temporally via an NRE-dependent manner. To this end, Gamberi and colleagues showed that in *pum*^{13} (a temperature-sensitive allele) embryos at 20ºC, *bcd* mRNA perdures longer than in wildtype embryos via Northern blot (Gamberi, Peterson et al. 2002). In wildtype embryos, *bcd* mRNA cannot be detected via Northern blot by 120' AEL (25°), but Gamberi and colleagues stated that *bcd* mRNA can even be seen an hour later in *pum*^{13} embryos. Not only does *bcd* mRNA perdure in *pum*^{13} embryos, but *bcd* protein also is more stable, presumably due to the continued translation of the stabilized mRNA (Gamberi, Peterson et al. 2002). Gamberi and colleagues observed a *bcd* protein perdurance in *pum*^{13} embryos compared to wildtype embryos via Western blot. Furthermore, Nos was found not to be involved in the regulation of *bcd* mRNA and protein perdurance, demonstrating for the first time that Pum may act independently of Nos in *Drosophila* (Gamberi, Peterson et al. 2002).

The *bcd* NRE was identified as the *cis*-acting sequence within the *bcd* 3'UTR necessary for the temporal degradation of *bcd* mRNA (Gamberi, Peterson et al. 2002). Point mutations in the two Pum recognition sequences within the *bcd* NRE result in the perdurance of *bcd* protein. Moreover, embryos expressing *bcd* transgenes bearing NRE mutations hatch into larvae, but develop abnormal mouthooks, lacking the ventral portion of the mouthook (Gamberi, Peterson et al. 2002).

Recently, new evidence demonstrates that Pum can regulate mRNAs independently from Nos, supporting the finding that Pum can regulate *bcd* mRNA without the help of Nos. Much of this work stems from the Orr-Weaver laboratory and their study of the *cyclins A* and *B* in *Drosophila*. Orr-Weaver and colleagues showed that Pum represses *CycA* and *CycB* in the somatic cytoplasm of the embryo, suggesting no Nos involvement due to posterior restriction of the translational repressor (Vardy and Orr-Weaver 2007; Vardy, Pesin et al. 2009). The method by which Pum regulates these mRNAs (*CycA*, *CycB*, *smaug*, and *bcd*) has not been further investigated, and we wished to decipher the mechanisms by which Pum-mediated regulation of *bcd* mRNA occurs in the anterior of the embryo.

2.2 Materials and Methods

Drosophila **strains and culture**

All flies were raised on standard cornmeal sucrose agar at 25°C. bcd^{6} flies were used as our *bcd* mutants, pum^{MSC}/pum^{FC8} transheterozygotes as our pum mutants, and nos^{BN} flies. *nos-bcd* flies were constructed in the Wharton lab by Anupama Danahakar. Wild type flies are w^{1118}. $pum^{MSC}/TM3$ *flies* were crossed to $pum^{FC8}/TM3$ flies to create pum^{MSC}/pum^{FC8} transheterozygotes. The *bcd*-NRE transgenes on the second chromosome were crossed into a bcd^{6} background in order to assess Bcd protein produced from the transgenes and to document embryo hatching and a larval mouthook phenotype. The *bcd*-NRE transgenes on the second chromosome were crossed to double the transgene copy number in order to assess any dominant effects of the transgenes. *nos-bcd* flies bearing the transgene on the third chromosome were crossed with flies bearing *bcd-NRE* transgenes on the second chromosome. The *bcd-CycB half-site* transgenes on the X and second chromosomes were crossed to *bcd* ^{6} flies and Bcd protein produced from the transgenes was examined along with percentage of hatched embryos. The *bcd-CycB half-site* transgenes on the X and second chromosome were crossed to double the transgene copy number. *nos-bcd* flies bearing the transgene on the third chromosome were crossed with flies bearing *bcd-CycB half-site* transgenes on the second chromosome.

RNA in situ hybridization

Bcd, *eve*, and *oc* mRNA were detected using standard *in situ* hybridization methods using digoxigenin-labeled DNA probes (Roche) and visualized with a Zeiss Axiophot microscope using Nomarski optics and a Q Imaging digital camera.

Immunohistochemistry

The embryos were fixed in 4% formaldehyde. Rabbit anti-Bcd: sc-66818 (1:100) was ordered from Santa Cruz Biotechnology, Inc and the 2B8 monoclonal anti-grasshopper Eve (1:50) developed by Kai Zinn was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa. Secondary antibodies (1:200) were from Jackson

ImmunoResearch Laboratories. The embryos were visualized with a Zeiss 510 confocal microscope.

bcd NRE mutant transgene generation

An Nhe1 site was inserted approximately 10 base pairs upstream of the Hpa1 site in the first 60 base pairs of the *bcd* NRE. NRE mut 1, NRE mut 2, and ΔNRE mutations were inserted into the *bcd* NRE as annealed oligos into the Nhe1 site and the endogenous Mlu1 site. The *bcd* genomic fragments were cloned into a pCasper4 derivative and injected into w^{1118} embryos.

Electromobility gel shift assay

Electromobility gel shift assays were performed as described in (Murata and Wharton 1995) except that each 10 µl reaction contained purified protein, reaction buffer [10 mM HEPES (pH 7.4), 20 mM KCl, 1 mM DTT, 0.2 mg/ml heparin, 0.05 mg/ml poly(U), 5% glycerol], and heat-denatured labeled RNA (10,000 cpm). RNA for all experiments was prepared by transcription of derivatives of the plasmid R4685. 32P-labeled RNA was prepared using T7 MEGAShortscript (Ambion). A plasmid encoding the Drosophila Pum RNA-binding domain was constructed as described in (Kadyrova, Habara et al. 2007).

Larval cuticle prep

Larval cuticle prep was prepared by mounting hatched larvae according to (Nusslein-Volhard and Wieschaus 1980).

SDS-PAGE and Western blotting

0-1 hour embryos were collected and aged according to create a timeline ranging from 0-1, 1-2, 2-3, 3-4, 4-5, and 5-6 hours. For the wildtype versus *pum* Western blot and the *bcd* transgenic Western blot, the embryos were fixed in methanol:heptane, stained with DAPI, and sorted to remove contaminating unfertilized eggs as in (Edgar, Sprenger et al. 1994). For the wildtype embryo versus unfertilized egg and the NRE mutants transgene Western blots the embryos were not fixed nor stained with DAPI. Next, embryos were squashed in 2X SDS buffer without bromophenol blue and lysate concentrations were determined via Bradford assay. Lysates were run on a 7% SDS-PAGE gel and transferred onto nitrocellulose and probed using standard Western blotting conditions. Bcd was detected using Rabbit anti-Bcd: sc-66818 (1:500) ordered from Santa Cruz Biotechnology, Inc. Secondary antibodies (1:2000) were from Jackson ImmunoResearch Laboratories.

Quantitative RT PCR

0-1 hour embryos were collected and aged according to create a timeline ranging from 0-1, 1-2, 2-3, 3-4, 4-5, and 5-6 hours. Embryos were then squashed in TRIzol (Invitrogen) and total RNA was isolated. Preparation of cDNA and qPCR reactions were performed according to the manufacturer (Applied Biosystems) measuring the level of *bcd* (or *nos* as a control) mRNA relative to the level of *sop* mRNA using assays DM02148187-g1, DM02134535-g1, and DM02361142-s1, respectively.

2.3 Results

2.3.1 *bicoid* protein stability in *pum*^{MSC}/*pum*^{FC8} embryos

Much of the focus of our laboratory revolves around dissecting the actions of Pum during early *Drosophila melanogaster* embryogenesis. Gamberi and colleagues demonstrated that Pum controls *bcd* mRNA stability and we wished to further investigate the involvement of Pum in *bcd* mRNA regulation. In Gamberi et al, increased *bcd* protein stability was observed in *pum*^{13} embryos; however, *pum*^{13} is a temperature-sensiitve allele and the experiments must be conducted at 20°C. We wished to examine *bcd* protein stability in a strong, well-characterized *pum* background. We assessed *bcd* protein perdurance via Western blot in *pum*^{MSC}/*pum*^{FC8} transheterozygotes versus wildtype embryos.

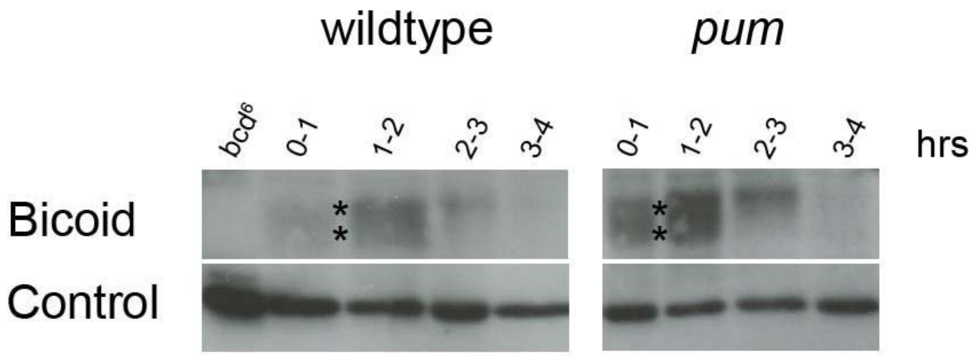

Figure 5: *bcd* protein disappears by 3-4 hours AEL in wildtype and *pum* embryos

Western blot of wildtype and pum^{MSC}/pum^{FC8} embryonic extract at hourly intervals shows that *bcd* protein appears during the first hour AEL in both wildtype and *pum* mutants. *bcd* protein is present as both an unphosphorylated and phosphorylated version and travels as a doublet (asterisk denotes the two forms of *bcd* protein) until only phosphorylated Bcd remains at 2-3 hrs AEL. By 3-4 hrs AEL, no *bcd* protein can be detected in both wildtype and pum mutant embryos. The two Western blots above originate from one blot.

Much to our surprise, there is no significant difference in the timing of *bcd* protein disappearance in pum^{MSC}/pum^{FC8} embryos versus wildtype embryos. As shown in Fig 5, *bcd* protein is present at 0-1 hour, becomes phosphorylated during the 1-2 hour timepoint as evidenced by the appearance of a slower mobility Bcd band. During 2-3 hours, only the phosphorylated version of Bcd remains, which is no longer present by 3-4 hours. This trend is observed in both wildtype and pum^{MSC}/pum^{FC8} embryos.

2.3.2 *bicoid* mRNA and protein perdurance in *pum*^{MSC}/*pum*^{FC8} embryos

Due to our surprising results we further analyzed *bcd* perdurance in wildtype versus *pum*^{MSC}/*pum*^{FC8} embryos. We wished to examine *bcd* mRNA and protein perudrance in finer detail throughout embryogenesis; therefore, we assessed *bcd* mRNA distribution over time in *pum*^{MSC}/*pum*^{FC8} and wildtype embryos by RNA in situ hybridization. As an additional control we analyzed *bcd* mRNA perdurance in *nos*^{BN} (null) embryos because Nos is restricted to the posterior and has not been implicated in *bcd* regulation. In order to stage the embryos, we simultaneously hybridized embryos with probes for *bcd* and *even-skipped* (*eve*) (Fig. 6).

No significant difference in *bcd* mRNA perdurance is apparent in wildtype, *pum*^{MSC}/*pum*^{FC8}, and *nos*^{BN} embryos. *bcd* mRNA is present at the beginning of embryogenesis and is localized to the anterior in all three genotypes and disappears throughout embryogenesis. Furthermore, no *bcd* mRNA is detected by cellularization (nuclear cycle 14) of the embryo in all three genotypes, which is consistent with previous reports.

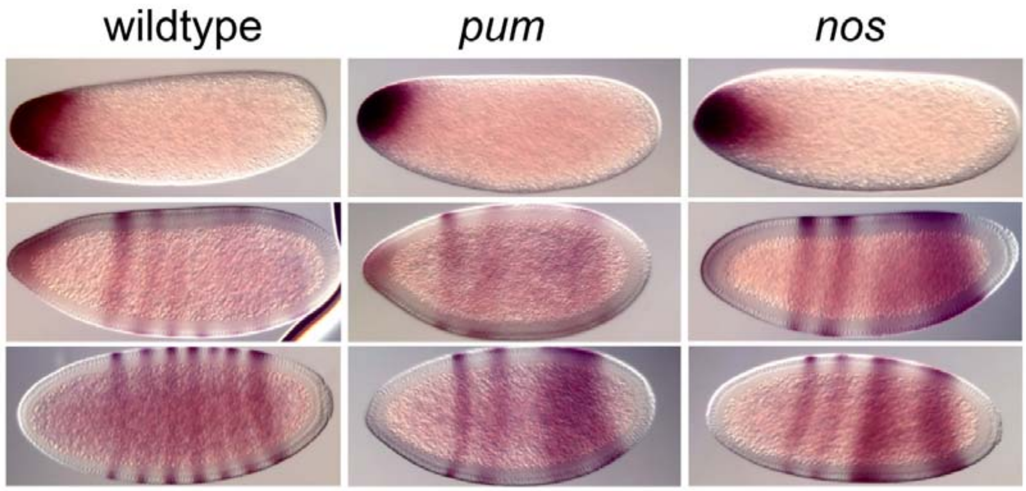

Figure 6: *bcd* mRNA perdurance in wildtype, *pum*, and *nos* embryos

In situ hybridization of *bcd* (localized to the anterior) and *eve* mRNAs in embryos over time derived from mothers of the genotypes: wildtype (w^{1118}), pum^{MSC}/pum^{FC8}, and nos^{BN}. Embryos in the lower panels are older. Embryos oriented with anterior to the left and dorsal at the top.

Next we asked if potentially *pum* may regulate *bcd* translation. To this end, we assessed *bcd* protein accumulation in pum^{MSC}/pum^{FC8} embryos compared to wildtype and nos^{BN} embryos. We performed immunohistochemistry using antibodies against Bcd and Eve and assessed *bcd* protein distribution through the beginning stages of embryogenesis via confocal microscopy. As with *bcd* mRNA, no significant difference was found in both the level or perdurance of *bcd* protein in all three genotypes. We found that only a slight amount of *bcd* protein is present by the very beginning of gastrulation, approximately 190-200’ AEL (Fig. 7 last panel) in all three genotypes. Furthermore, the loss of *bcd* protein occurs at the same rate in *pum* mutant embryos as in wildtype and *nos*^{BN} embryos. These findings are consistent with the idea that Pum does not play a role in destabilizing *bcd* mRNA.

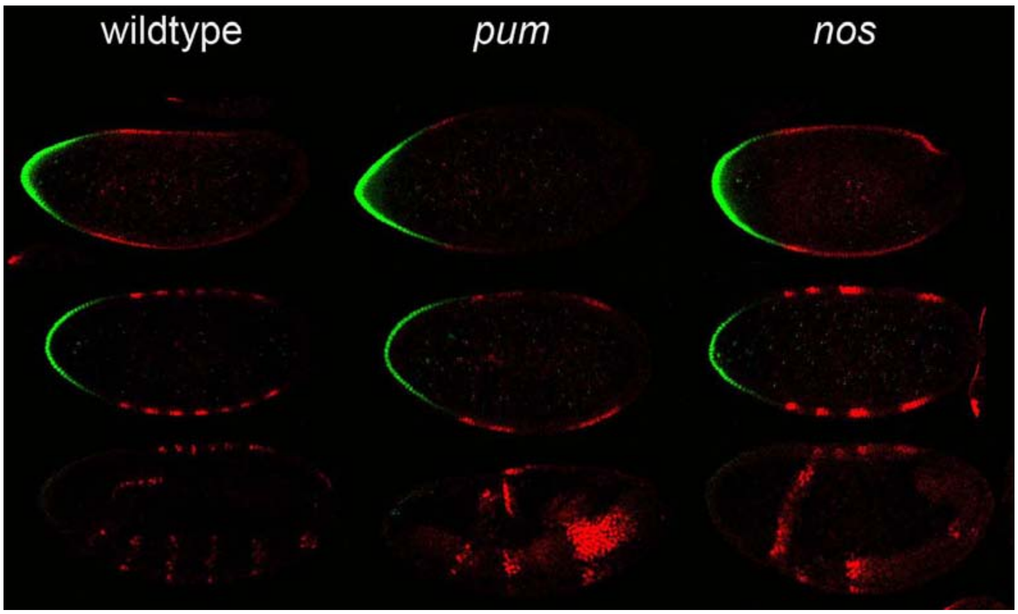

Figure 7: *bcd* protein perdurance in wildtype, *pum*, and *nos* embryos

Immunohistochemistry detecting Bcd (green) and Eve (red) in embryos over time derived from mothers of the genotypes: wildtype (*w*^{1118}), *pum*^{MSC}/*pum*^{FC8}, and *nos*^{BN}. Embryos in the lower panels are older. Embryos oriented with anterior to the left and dorsal at the top.

Finally, we analyzed if Pum may act indirectly to regulate *bcd* by assessing the distribution of a downstream transcriptional target of Bcd, *ocelliless* (*oc*, also known as *orthodenticle*). *oc* mRNA forms a band in the anterior of the embryo. In embryos where excess *bcd* protein is present, the *oc* band widens. In contrast, embryos with less *bcd* protein, form a thinner *oc* band (Gao and Finkelstein 1998). We found no significant difference in the *oc* stripe among *pum* embryos versus wildtype embryos (Fig. 8), suggesting that Pum does not indirectly regulate *bcd* in the anterior of the embryo.

Figure 8: *oc* mRNA pattern in wildtype and *pum* embryos

In situ hybridization of *oc* mRNA of embryos derived from wildtype (w^{1118}) or pum^{MSC}/pum^{FC8}. Embryos oriented with anterior to the left and dorsal at the top.

2.3.3 The NRE does not mediate *bicoid* mRNA stability

Although, Pum, as a *trans-acting* factor, does not govern *bcd* mRNA stability or translation, the Pum high affinity binding sites within *bcd* NRE have been implicated in regulating Bcd perdurance (Gamberi, Peterson et al. 2002). By analyzing samples from pooled embryos on blots, Gottlieb and colleagues concluded that mutations in the *bcd* NRE prolong accumulation of *bcd* mRNA and encoded protein in otherwise wild type embryos (Gamberi, Peterson et al. 2002). We wished to further investigate the involvement of the Pum binding sites and the NRE in the increased stability of *bcd* protein in vivo. To this end, we created three transgenes, two of which bear point mutations in the two high affinity Pum binding motifs within the *bcd* NRE (Fig. 9) and another transgene with the entire NRE deleted (Δ NRE) (Wharton and Struhl 1991). In the *bcd*-NRE mut 1 transgene we changed UG→AC and in *bcd*-NRE mut 2 we converted UA→ GC in both high affinity Pum binding sites as shown in Fig. 9.

```
mut 1                        AC                            AC
wt    CUGUUGUUCCUGAUUGUACAAAUACCAAGUGAUUGUAGAUA
mut 2                           GC                            GC
```

Figure 9: Pum binding site point mutations

bcd NRE sequence. Wt: wildtype *bcd* NRE sequence, mut1: *bcd* NRE with base pair changes (UG→AC), mut2: *bcd* NRE with base pair changes (UA→GC).

We wished to test if these NRE point mutations eliminate Pum binding both in vitro and in vivo. We examined Pum binding in vitro via an electromobility gel shift assay (EMSA). The RNA binding domain of Pum forms a complex with the wildtype *bcd* NRE with the same high affinity as a site within the *CycB* NRE previously reported to be bound by Pum with high affinity (Kadyrova, Habara et al. 2007) (Fig. 10). However, the Pum RNA binding domain does not bind to the two mutant forms of the *bcd* NRE (Fig. 10), demonstrating that these point mutations abolish Pumilio binding in vitro.

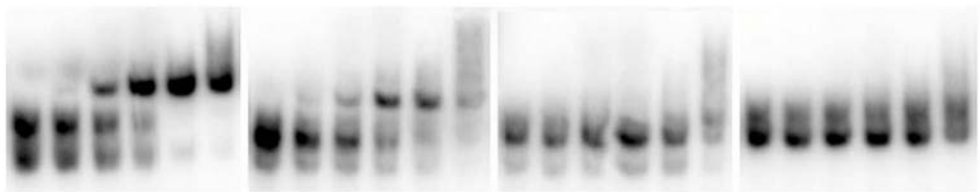

Figure 10: Point mutations in the *bcd* NRE abolish Pum binding

Electromobility gel shift shift experiments using the PumRBD and radiolabeled RNA bearing *CycB* Pum binding sequence, NRE wildtype, NRE mut 1, or NRE mut 2.

Next, we assessed the ability of Pum to bind to these mutant NREs in vivo by a Nos repression assay. We ectopically expressed Nos in the anterior of the embryo by replacing the 3'UTR of *nos* with the *bcd* 3'UTR (*nos-bcd*), which contains an anterior localization signal (Macdonald and Struhl 1988). When Pum binds to the *bcd* NRE, Nos is recruited to the mRNA and no *bcd* translation occurs; the embryo develops a bicaudal body plan. *bcd*-ΔNRE mRNA is resistant to Pum- and Nos-dependent repression: expression of such a mRNA suppresses the bicaudal phenotype, restoring anterior segmentation even in the presence of ectopic Nos (Fig. 11) (Wharton and Struhl, 1991). We surmised that if the Pum binding site mutants prevent Pum from binding to the *bcd* NRE, Nos will not be able to repress *bcd*, suppressing the bicaudal phenotype. In the presence of ectopic Nos, maternal expression of either *bcd*-NRE mut1 (Fig. 11) or *bcd*-NRE mut2 (not shown) mRNA also suppresses the bicaudal phenotype, consistent with the idea that the mut1 and mut2 NREs do not bind Pum appreciably in vivo, as is the case in vitro.

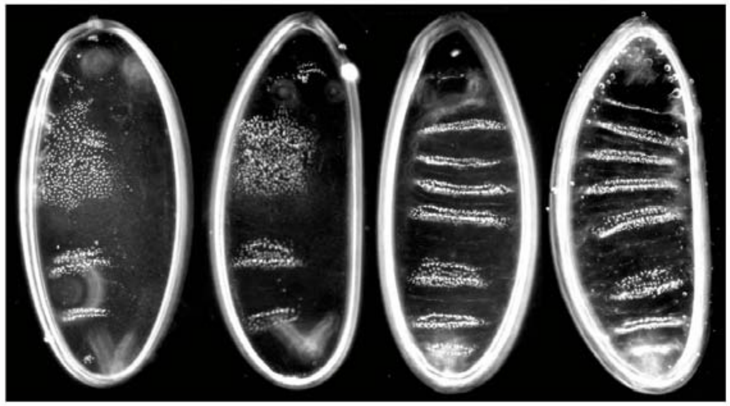

Figure 11: *bcd* NRE point mutations suppress the *bicaudal* phenotype

Darkfield photomicrographs of embryos from *nos-bcd*, wtNRE; *nos-bcd*, NRE mut1; *nos-bcd*, and ΔNRE; *nos-bcd* females. The *bicaudal* phenotype is suppressed in embryos expressing *bcd* NRE mut1 or *bcd* Δ NRE transgenes as witnessed by the presence of thoracic segments and head structures.

We then analyzed the effect of the NRE mutations on *bcd* protein perdurance via immunohistochemistry. The transgenes were expressed in a bcd^6 mutant background; therefore, all of the detected protein is a result of transgene expression. We used antibodies against Bcd and Eve and showed that *bcd* protein derived from a *bcd*-NRE mut 1 transgene disappears at the same time as the *bcd* protein derived from a wildtype *bcd* transgene (Fig. 12). Very minimal protein can be detected at the start of gastrulation in both transgenic lines. We confirmed this result with the other *bcd* NRE mutant transgenes (NRE mut 2 and Δ NRE-data not shown). Therefore, we conclude that *bcd* mRNA stability is not prolonged due to mutations in the NRE that abolish Pumilio binding.

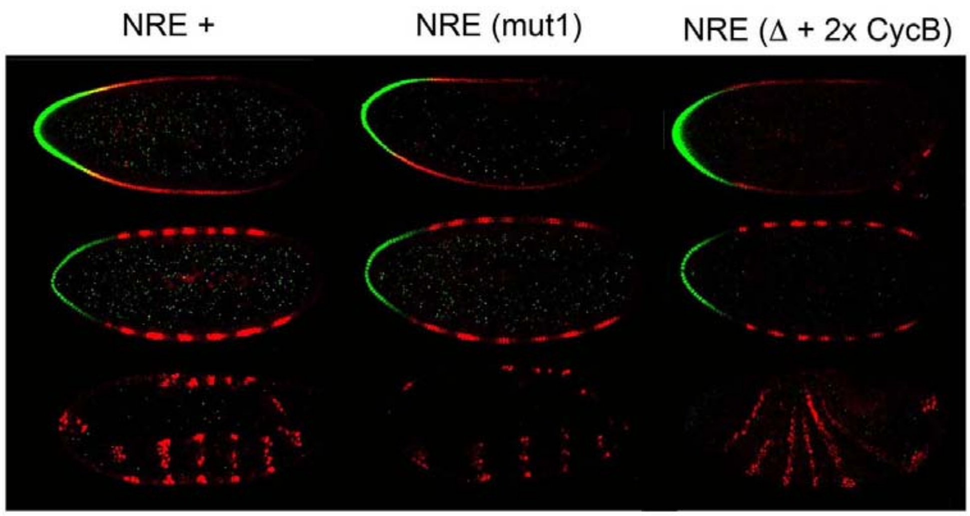

Figure 12: *bcd* protein stability is not different when derived from *bcd* NRE wildtype, *bcd* NRE mutant, or *bcd* (Δ +2x CycB) transgenes

Immunohistochemistry detecting Bcd (green) and Eve (red) in embryos expressing *bcd* –NRE+, *bcd*-NRE mut1, or *bcd*-**(**Δ +2x *CycB)* transgenes in a *bcd* mutant background. Embryos in the lower panels are older. Embryos oriented with anterior to the left and dorsal at the top.

Not only did we analyze *bcd* protein perdurance via immunohistochemistry, but we also determined whether the NRE mutants altered *bcd* protein stability via Western blot. We collected embryos from mothers bearing one of the following *bcd* transgenes in a *bcd* mutant background: *bcd*-wildtype NRE, *bcd*-NRE mut 1, and *bcd*-Δ NRE. We collected embryos every hour and aged them appropriately, creating a time line ranging from 0-1 hours and ending at 5-6 hours (Fig. 13). *bcd* protein displays reduced motility at approximately 2-3 hours AEL because of Torso-mediated phosphorylation (Ronchi, Treisman et al. 1993), which is shown in Fig. 13. *bcd* protein levels decrease by 3-4 hours AEL and only trace levels are seen at 4-5 hours and all protein is gone by 5-6 hours for all three transgenic lines. Again, these results suggest that *bcd* mRNA stability is not dependent on the NRE.

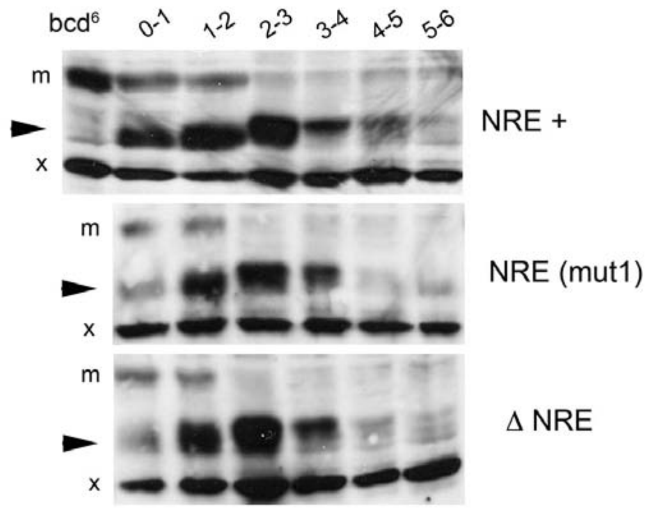

Figure 13: *bcd* protein does not perdure when translated from NRE point mutants

Western blots of *bcd* protein (arrowhead) produced from wildtype, NRE mut1, and Δ NRE transgenes expressed in a *bcd*^{6} mutant background over time. “m” denotes a maternal band recognized by the anti-Bcd antibody and “x” highlights another background band recognized by the anti-Bcd antibody and is used as a loading control. Only a trace of *bcd* protein is present in hour 4-5 in all three transgenic extracts.

In summary, all of these data demonstrate that the Pum binding sites within the *bcd* NRE do not play a role in regulating *bcd* protein stability.

2.3.4 The *bicoid* NRE has no physiological function in the embryo

Thus far our work has shown no role for Pum or the NRE in regulating *bcd* mRNA stability in the early embryo. In contrast, Gamberi and colleagues identified both Pum and the NRE to be involved in governing *bcd* perdurance (Gamberi, Peterson et al. 2002). Since our work is at odds, we further investigated the possibility that Pum might regulate *bcd* in some manner. First, we replaced the *bcd* NRE with two copies of a high affinity Pum binding site derived from *CycB* mRNA (Kadyrova, Habara et al. 2007). The bcd-ΔNRE + 2X *CycB* transgene rescues the bcd^6 phenotype and Bcd protein derived from the bcd-ΔNRE + 2X *CycB* transgene does not perdure longer than wildtype *bcd*. This results demonstrates that Pum binding is not sufficient to alter *bcd* mRNA stability or function.

Second, Gamberi and colleagues reported embryonic head development defects associated with mutant *bcd* NREs. Namely, embryos expressing *bcd* mRNA with mutant NREs experience severe head involution defects as well as malformation of the ventral mouthook (Gamberi, Peterson et al. 2002). However, in our examination of the head structures, we did not observe any significant differences in head structures from embryos expressing a wildtype *bcd* transgene in a *bcd* mutant background versus any of the *bcd* NRE mutant transgenes in a *bcd* mutant background (Fig.14). We viewed numerous *bcd* NRE lines (wildtype, mut 1, mut2, and Δ NRE) and saw no difference in the head structures. Furthermore, the ventral mouth hook formed normally in all of the embryos (Fig. 14).

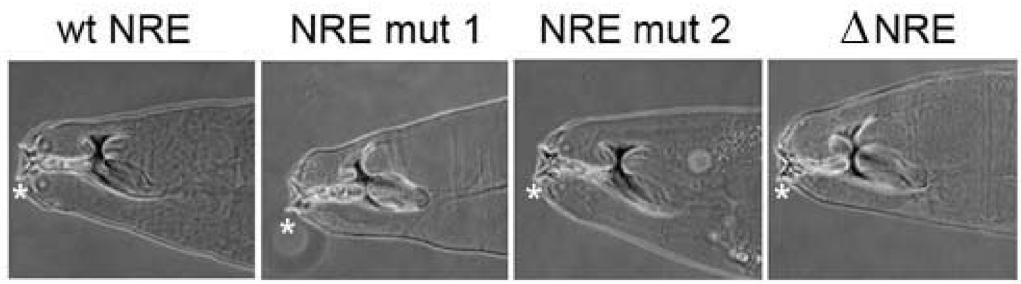

Figure 14: No significant difference in head development among larvae expressing wildtype or mutant NREs

Head cuticles of larvae expressing wt NRE, NRE mut1, NRE mut 2, or NRE delta transgenes in a *bcd* mutant background.

Next, we examined the hatching efficiency of embryos from mothers possessing either the *bcd* wildtype transgenes or *bcd* NRE mutant transgenes. We documented the number of eggs that hatch over three separate occasions for each transgene, calculated the percentage of hatched embryos, and have shown the average of the three experiments in Table 1. Approximately, the same amount of embryos hatched containing a wildtype transgene or a mutant transgene, suggesting that the mutant NRE transgenes act the same as a wildtype transgene to rescue the *bcd* mutant phenotype embryonic death. We further analyzed if the *bcd*-NRE mutant transgenes induced the dominant head phenotype documented by Gamberi and colleagues (Gamberi, Peterson et al. 2002).

Table 1: Hatching Efficiency of NRE Mutants

Genotype	**% hatched**
bcd	0%
wtNRE; bcd	80%
Mut 1NRE;bcd	71%
Mut 2NRE; bcd	87%
ΔNRE; bcd	87%

We did not observe any differences in the head structures of embryos containing two mutant NRE trangenes in a bcd$^+$ background, compared to embryos expressing wildtype transgenes (Fig. 15) and no abnormal head structures were identified.

Figure 15: No dominant head defects presentin larvae expressing NRE mutants

Head cuticles of larvae expressing wt NRE, NRE mut1, NRE mut 2, or NRE delta transgenes in a wildtype *bcd* background.

2.3.5 The prolonged accumulation of *bicoid* mRNA and protein in unfertilized eggs

In the course of performing the analysis of *bcd* protein perdurance via Western blot, we observed variability in *bcd* protein disappearance. The differences in the perdurance of *bcd* protein may be dependent upon the age of the females; older females lay a higher proportion of unfertilized eggs compared to younger females. This finding may potentially explain the observation by Gamberi and colleagues that *bcd* mRNA and protein perdures in *pum* mutant embryos. Furthermore, in support of this idea, Surdej and Jacobs-Lorena demonstrated that *bcd* mRNA destabilizes at the maternal-to-zygotic-transition, approximately 2 hours AEL, but remains stable in unfertilized eggs (Surdej and Jacobs-Lorena 1998).

We examined *bcd* mRNA and protein perdurance in embryos versus activated, unfertilized eggs by quantitative RTPCR and Western blotting, respectively. In embryos, *bcd* mRNA dramatically decreases by 2-3 hours AEL and reaches levels near zero by the 3rd hour of embryogenesis (Fig. 16)

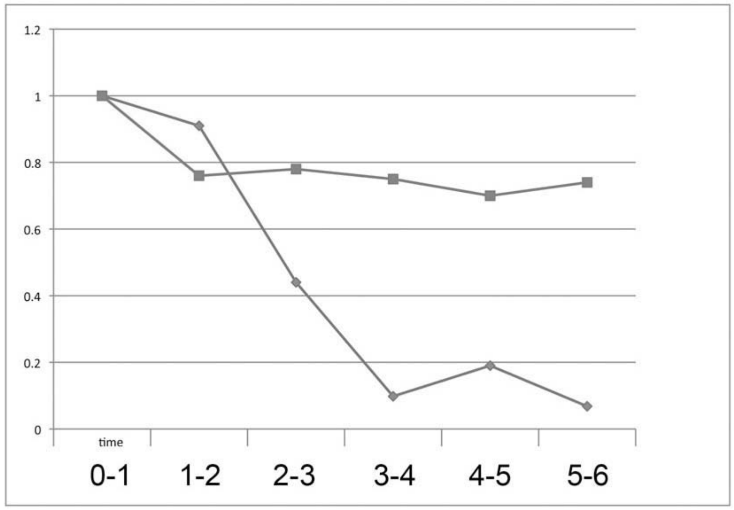

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Figure 16: *bcd* mRNA perdurance in fertilized embryos versus unfertilized eggs

qRT-PCR analysis of *bcd* expression in wildtype embryos (diamond) relative to unfertilized eggs (square) of the ages 0-1 hour, 1-2 hour, 2-3 hour, 3-4 hour, 4-5 hour, and 5-6 hour. Data were analyzed by relative quantification and were normalized to *sop* mRNA.

In contrast, *bcd* mRNA levels remain high and stable into the fifth hour AEL. The same trend is witnessed for *bcd* protein in embryos compared to unfertilized eggs (Fig. 17). In unfertilized eggs, *bcd* protein levels remain high and stable at the later time points, whereas *bcd* protein in the embryos decreases over time resulting in only a slight amount of *bcd* protein present at five-six hours AEL.

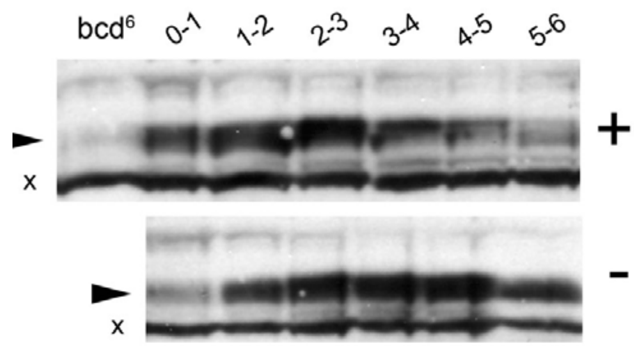

Figure 17: *bcd* protein perdurance in fertilized embryos versus unfertilized eggs

Western blot of *bcd* protein expression in wildtype embryos (+) relative to unfertilized eggs (-) of the ages 0-1 hour, 1-2 hour, 2-3 hour, 3-4 hour, 4-5 hour, and 5-6 hour. Arrowhead represents *bcd* protein and the X represents a background band recognized by the anti-Bcd antibody and is used as a loading control.

2.4 Discussion

The assertion that *bcd* stability is governed by Pum in a Nos-independent manner shifted the view in the *Drosophila* translational regulation field that Pum and Nos may not always act together to regulate target mRNAs. This idea of Pum-mediated repression of mRNAs has been further supported with the work in yeast, in which no Nos homolog exists, that demonstrates that Pum governs mRNA stability and translation. Additionally, studies from Orr-Weaver and colleagues suggest that Pum governs the translation of *CycB*, *CycA*, and *smaug* mRNAs independently of Nos in the somatic cytoplasm of the

Drosophila embryo (Tadros, Goldman et al. 2007; Vardy and Orr-Weaver 2007; Vardy, Pesin et al. 2009).

Our studies demonstrate that the binding of Pum is insufficient to regulate *bcd* mRNA stability. Furthermore, we have shown that the NRE is not involved in governing *bcd* mRNA. We analyzed *bcd* mRNA and protein perdurance in vivo in wildtype, *pum*, and *nos* embryos and observed no significant differences in the disappearance of *bcd* mRNA and protein among the three genotypes. Also, we created point mutants in the *bcd* NRE that abolish Pum binding, which did not have an effect on *bcd* protein perdurance. Finally, no head defects such as mouthook malformation or aberrant head involution were detected in larvae that expressed the *bcd*-NRE mutant transgenes.

The presence of a nonfunctional NRE within the *bcd* 3'UTR remains puzzling. Answers as to why an NRE exists within the *bcd* 3'UTR may be found by examining the evolution of *bcd* among dipterans. *bcd* is a derivative of the *Hox3* gene and is present only in higher dipterans such as *Drosophila*, where *bcd* is localized to the anterior region of the embryo (McGregor 2005). Interestingly, in lower dipterans a *Hox3* gene exists and in the lower dipteran, *Clogmia albipunctata* embryo, *Hox3* mRNA is not localized to the anterior; instead, it is found ubiquitously throughout the embryo (Stauber, Prell et al. 2002). This finding suggests that during the period in evolution when higher dipterans developed, *bcd* evolved from *Hox3* and became localized to the anterior. Therefore, a plausible explanation for the presence of the NRE in the *bcd* 3'UTR exists in that Nos and Pum may have repressed *bcd* mRNA translation in the posterior of lower dipteran embryos; before the localization machinery evolved. Further support that a Nos and Pum-like mechanism of repression exists in *Clogmia* is based on expression data demonstrating that *hb* mRNA is found throughout the embryo, but *hb* protein is present only in the anterior of the *Clogmia* embryo, much like the *Drosophila* embryo (Rohr, Tautz et al. 1999).

Another putative explanation for the occurrence of the NRE in the *bcd* 3'UTR exists. In the lower dipteran, *Clogmia albipunctata*, *Hox3* acts both maternally and zygotically (Stauber, Prell et al. 2002). However, in higher dipterans like *Drosophila*, the *Hox3* gene duplicated, creating a zygotic active gene (*zen*) and a maternal-effect gene (*bcd*) (Stauber, Jackle et al. 1999). Potentially, Nos and Pum may have been active zygotically in lower dipterans where it repressed *Hox3*.

Understanding the mechanisms of regulation of *bcd* in *Drosophila melanogaster* may shed light on post-transcriptional regulation in all organisms. The discovery that Pum binding to a target mRNA is not sufficient for repression, suggests that a more complicated mechanism of control exists in the *Drosophila* embryo. This finding impacts a recent study by Gerber and colleagues, who showed by co-immunoprecipitation that the RBD of Pum binds to numerous mRNAs in the early *Drosophila* embryo (Gerber, Luschnig et al. 2006). Gerber and colleagues assumed in vivo significance of Pum binding to these target mRNAs, which may not be accurate. In addition, human Pum2 has been shown to interact with numerous mRNAs; however, physiological relevance has been inferred but not experimentally shown (Fox, Urano et al. 2005); (Spik, Oczkowski et al. 2006). The finding that the Pum-*bcd* interaction occurs with no phenotypic consequence, suggests that great rigor must be taken in studying mouse and human Pum.

3. The Regulation of *Cyclin B* in the Somatic Cytoplasm of the Drosophila Embryo

3.1 Introduction

Nos and Pum regulate cell cycle progression during *Drosophila* embryogenesis. Germline precursor cells (pole cells) are initially located at the posterior pole of the embryo, but then migrate towards the somatic gonad during embryogenesis. Just before their migration, the pole cells cease dividing and only resume once they have contacted the gonad. Pole cells mutant for *nos* or *pum* form (Kobayashi, Yamada et al. 1996), but fail to migrate properly to the somatic gonad and exit their mitotic quiescence during their migration (Asaoka-Taguchi, Yamada et al. 1999). Entry into mitosis during migration in these mutants is due to the accumulation of CycB, which is normally translationally repressed in wild-type pole cells by Pum and Nos (Asaoka-Taguchi, Yamada et al. 1999). Kadyrova and colleagues identified the NRE in the *Cyclin B* 3'UTR to which Pumilio and Nanos bind and demonstrated that this sequence is necessary for *CycB* repression in the pole cells (Kadyrova, Habara et al. 2007). Both Nos and Pum are required for *CycB* translation inhibition; however, Brat does not play a role in the repression of *CycB* in the pole cells (Sonoda and Wharton 2001; Kadyrova, Habara et al. 2007). Furthermore, if Nos is tethered to the *CycB* mRNA in the absence of Pum repression still occurs, insinuating that the role of Pum is to primarily recruit Nos (Kadyrova, Habara et al. 2007).

Until recently, it was thought that Pum did not regulate mRNAs throughout the somatic cytoplasm for mainly one reason: Nos protein is only present in the posterior half of the embryo, in a gradient emanating from the posterior pole (Gavis and Lehmann 1992; Wang, Dickinson et al. 1994). Therefore, because Nos is considered to be the canonical partner of Pum, and Nos localization is restricted to the posterior, Pum was not thought to regulate mRNAs throughout the embryo. However, recent evidence has suggested that Pum may act independently of Nos in the somatic cytoplasm. First, bioinformatic analysis suggests that Pum may globally regulate the stability of maternal mRNAs (Tadros, Goldman et al. 2007). Second, it has been shown that members of the Pum protein family (Puf), including Pumilio, interact with Pop2, a subunit of the CCR4/Pop2/NOT deadenylase complex (Goldstrohm, Seay et al. 2007; Kadyrova, Habara et al. 2007). This direct interaction suggests that Pum has the ability to employ a mechanism of regulating mRNA stability in the absence of Nos. Third, Orr-Weaver and colleagues showed that the translation of *CycB* and *CycA* mRNA in the soma of the *Drosophila* embryo is dependent on the activity of the Pan Gu Kinase (PNG) complex (Fenger, Carminati et al. 2000) (Vardy, Pesin et al. 2009). Interestingly, the PNG complex activates *CycB* translation by relieving Pum repression of *CycB* mRNA in the somatic cytoplasm through a currently unknown mechanism (Vardy and Orr-Weaver 2007).

In early embryos the cell cycle is composed of only the replication phase (S) and mitosis (M). In embryos derived from mothers lacking any of the components of the

PNG complex, the nuclei replicate without dividing, suggesting that the complex is necessary for nuclear division (Freeman, Nusslein-Volhard et al. 1986; Shamanski and Orr-Weaver 1991). The level of CycB, the cyclin necessary for mitotic entry, is decreased in PNG complex component mutant embryos, demonstrating that the PNG complex regulates CycB levels (Fenger, Carminati et al. 2000; Vardy and Orr-Weaver 2007). The PNG kinase complex is composed of three proteins: Pan Gu, Plutonium (Plu), and Giant Nuclei (Gnu). The Pan Gu protein has strong homology to Serine/Threonine kinases and contains only a catalytic domain (Fenger, Carminati et al. 2000). The regulatory subunits of the kinase complex are Plutonium, a protein with ankyrin-like repeats and Giant Nuclei, which encodes a novel protein (Axton, Shamanski et al. 1994) (Renault, Zhang et al. 2003). Orr-Weaver and colleagues propose that Gnu acts to bring together Plu and Pan Gu in order to form an active complex (Lee, Van Hoewyk et al. 2003). And together, these components, release *CycB* from its repression by Pumilio in the somatic cytoplasm.

In my thesis work, I investigated the relationship between the PNG kinase complex and Pum in the somatic cytoplasm of the embryo to uncover the regulatory mechanism behing *CycB* repression and the progression of the cell cycle.

3.2 Materials and Methods

Drosophila strains and culture

All flies were raised on standard cornmeal sucrose agar at 25°C. nos^{BN} flies were used as our *nos* mutants and pum^{ET3}/pum^{MSC} transheterozygotes or pum^{ET7}/pum^{MSC} as our *pum* mutants. The PNG complex mutant flies were a gift from Terry Orr-Weaver: plu^{3}, gnu^{305}, png^{1058} (strong allele) and png^{3318} (weak allele). $CycB^{2}/CycB^{3}$ transheterozygotes are *CycB* mutants. Wild type flies are w^{1118}. The plu^{3} allele was recombined onto chromosomes carrying either the $CycB^{2}$ or $CycB^{3}$ alleles. The *CycB* 3'UTR deletion transgenes (created by Y Habara in the Wharton laboratory) on the third chromosome were crossed into a $CycB^{2}$ *plu*/$CycB^{3}$ *plu* or a *png* background in order to assess *CycB* protein accumulation.

SDS-PAGE and Western blotting

0-2 hour embryos were collected and squashed in 2X SDS buffer without bromophenol blue and lysate concentrations were determined via Bradford assay. Lysates were run on an 8% SDS-PAGE gel, transferred onto nitrocellulose and probed using standard Western blotting conditions. F2F4 monoclonal anti-CycB concentrate (1:8000) , A12 monoclonal anti-CycA (1:10), 9E10 monoclonal anti-c-myc were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa. Sigma monoclonal anti-tubulin (1:10,000) was used. Rabbit anti-Pumilio antibody was created by the Wharton laboratory and used at a concentration of 1:2000. Secondary antibodies (1:2000) were from Jackson ImmunoResearch Laboratories.

Electromobility gel shift assay

Electromobility gel shift assays were performed as described in (Murata and Wharton 1995)except that each 10 µl reaction contained purified protein, reaction buffer [10 mM HEPES (pH 7.4), 20 mM KCl, 1 mM DTT, 0.2 mg/ml heparin, 0.05 mg/ml poly(U), 5% glycerol], and heat-denatured labeled RNA (10,000 cpm). RNA for all experiments was prepared by transcription of derivatives of the plasmid R4685. 32P-labeled RNA was prepared using T7 MEGAShortscript (Ambion). A plasmid encoding the Drosophila Pum RNA-binding domain was constructed as described in Kadyrova, Habara et al. 2007

Phophatase Treatment

0-2 hour embryos expressing a Pum-C-terminus transgene were collected, dechorionated and squashed in RIPA buffer. The embryonic lysates were spun at 2000g for 5' and the protein concentration determined via Bradford assay. Ten ugs of total protein were incubated with λ phosphatase for 1 hour at 25°C. 2X SDS was added to the lysate and then run on a 15% Anderson gel (Riechmann and Ephrussi 2004).

3.3 Results

3.3.1 The Regulation of *Cyclin B* in the Somatic Cytoplasm is Nos-Independent

Orr-Weaver and colleagues demonstrated that Pum represses *CycB* in the somatic cytoplasm of the embryos and this repression is relieved by the PNG Kinase Complex (Vardy and Orr-Weaver 2007). The restriction of Nos to the posterior of the embryo led Orr-Weaver to hypothesize the existence of a Nos-independent mechanism of repression of *CycB* in the somatic cytoplasm. We wished to definitively show if Nos regulates somatic *CycB*. To this end, we created *plu; nos* and *png; nos* double mutant flies. If Nos regulates somatic *CycB* then in the *plu; nos* and *png; nos* double mutants, *CycB* protein levels should be higher than in *plu* or *png* single mutants. As shown in Figure 18, *CycB* protein levels are low in both the double and single mutants, demonstrating that Nos does not function in regulating somatic *CycB*.

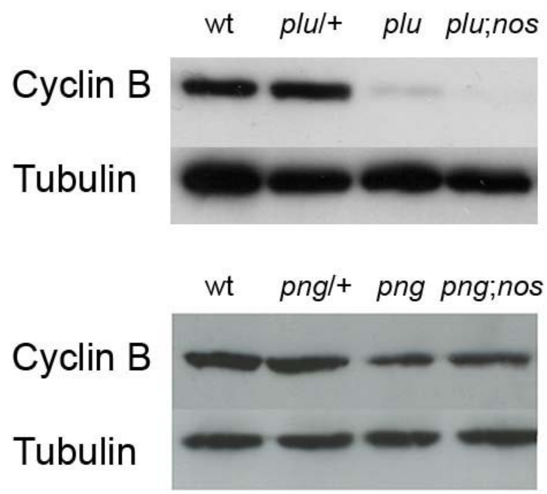

Figure 18: Somatic CycB levels are not restored by the absence of Nos

Western blots (upper panels) of *CycB* protein in wildtype, *plu*^{3}/+, *plu*^{3}, and *plu*^{3}*; nos*^{BN} embryos with Tubulin as a loading control. CycB levels are not significantly different in *plu*^{3} versus *plu*^{3}*; nos*^{BN} embryos. Western blots (lower panels) probed for CycB and Tubulin as a loading control in wildtype, png^{3318}/+, *png*^{3318}, and *png*^{3318}*; nos*^{BN} embryos. No differences are observed in CycB protein accumulation in *png*^{3318} and *png*^{3318}*; nos*^{BN}embryos.

3.3.2 The 3'UTR of *Cyclin B* is Not Reguired for Regulation via the PNG Kinase Complex

In addition to the historical evidence demonstrating that Pum represses mRNAs via the 3'UTR, Vardy and Orr-Weaver observed that a luciferase reporter fused with the *CycB* 3'UTR is regulated in a png-dependent manner. Based on these findings, we focused on the *CycB* 3'UTR to identify the *cis-acting* sequence that mediates regulation by the PNG Kinase complex.

3.3.2.1 The Identification of Pum Binding Sites within the *Cyclin B* 3'UTR

We wished to identify the site (Pum Response Element, PRE) within the *CycB* mRNA that confers regulation by Pum and the PNG Kinase complex in the somatic cytoplasm of the embryo. Pum has been found to bind to numerous sites within the *CycB* 3'UTR (Lyuda). More specifically, Pum binds to three regions within the *CycB* 3'UTR; however, only a 50 nucleotide (nt) segment is necessary and sufficient to repress *CycB* in the pole cells by Pumilio and Nanos. The other two regions are not involved in *CycB* regulation in the pole cells and are strong candidates for the PREs that confer *CycB* repression in the somatic cytoplasm. We have further narrowed the regions to which Pum binds within these segments. For these experiments, we conducted electromobility shift assays using radiolabeled RNA and the purified recombinant RNA binding domain of Pum (Fig 19). Pum binds only to the first 93 nts (Segment 1) and the last 70 nts in Region F. Both of these segments contain the canonical Pum binding motif: UGUA. To further define the exact binding sites of Pum within these segments, we performed site-directed mutagenesis at these Pum binding motifs. Altering the UGUA motif abolishes the ability of Pum to bind these particular segments of the *CycB* 3'UTR (Fig 19).

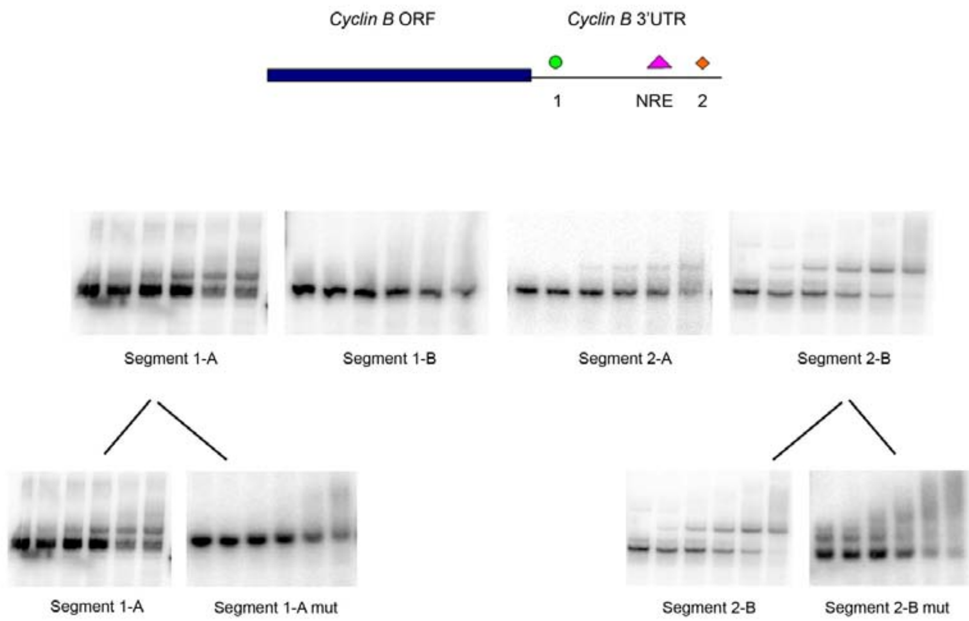

Figure 19: Pum binds two segments in the *CycB* 3'UTR in addition to the NRE

Representation of the *CycB* ORF and the three Pum binding sites within the *CycB* 3'UTR in the upper panel. There are three Pum binding sites within the *CycB* 3'UTR: segment 1 at the 5' proximal region of the 3' UTR (green circle), the NRE (pink triangle), and segment 2 at the 3' proximal region of the 3'UTR (orange diamond). We further defined the regions of Pum binding within segments 1 and 2 via electromobility gel shifts assays utilizing the Pum RBD. Pum binds to the first half of segment 1, termed 1-A, and not to the second half of segment 1-B. This interaction is specific: a mutation in the UGUA sequence within segment 1A abolishes Pum binding (Segment 1-A mut). Pum binds strongly to segment 2B and weakly to segment 2-A. Mutation of the UGUA within segment 2B (2-B mut) abolishes Pum binding.

3.3.2.2 The In Vivo Significance of Pum Binding Sites within the *Cyclin B* 3'UTR

We identified two Pum binding sites in addition to the NRE within the *CycB* 3'UTR. In order to determine if these PREs function in vivo to confer regulation of *CycB* via Pum and the PNG Kinase complex, we utilized different *CycB* 3'UTR deletion transgenes. We crossed these *CycB* transgenes into a *plu CycB* mutant background and a *CycB* background as a control; therefore only transgenic *CycB* protein is detected. If a region of the *CycB* 3'UTR necessary for regulation is deleted, we expect *CycB* protein levels to remain the same in both the *plu CycB* and *CycB* backgrounds.

Much to our surprise, the replacement of the entire *CycB* 3'UTR with the *tubulin* 3'UTR still allowed for the regulation of *CycB* via the PNG Kinase Complex. *CycB* protein levels are lower in the *plu CycB* mutant background when translated from either a wildtype *CycB* transgene or a *CycB-tubulin* transgene compared to a *CycB* mutant background (Fig 20). The ability of the PNG Kinase complex to regulate *CycB* mRNA lacking the *CycB* 3'UTR demonstrates that the *CycB* regulatory elements are not present within the *CycB* 3'UTR or that somatic *CycB* may not be a direct target of Pum and the PNG Kinase complex.

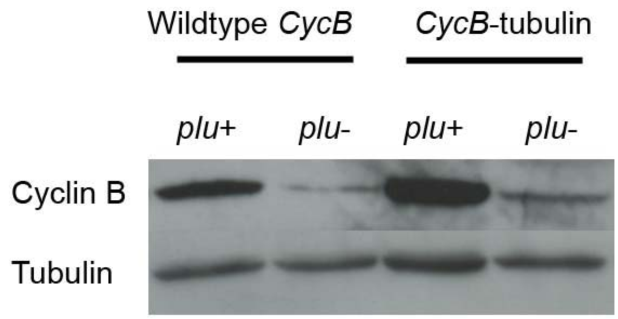

Figure 20: The 3'UTR of *CycB* is not required for regulation

Western blots (upper panel) of *CycB* protein and Tubulin as a loading control. Wildtype and *CycB-tubulin* transgenes were crossed into $CycB^2plu^3/CycB^3plu^3$ or $CycB^2/CycB^3$ mutant backgrounds; therefore, the only CycB detected is translated from the two transgenes. *CycB* protein levels remain low in a *plu* mutant background when translated from either a wildtype *CycB* transgene or a *CycB-tubulin* transgene.

The result demonstrating the *CycB* 3'UTR is not required for repression in a *plu* mutant background was very surprising. Although, Png and Plu are thought to exist in a complex to regulate *CycB*, we considered the possibility that *CycB* may be regulated differently by the two proteins. Therefore, we wished to confirm if a similar result occurs in a *png* mutant background. As shown in Fig 21, we observed the same result when the *CycB-tubulin* transgene is crossed into *png* mutant background as in a *plu* background.

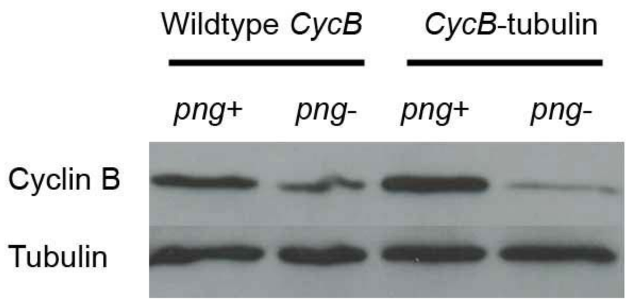

Figure 21: *CycB-tubulin* is sufficient to confer regulation in *png* mutant embryos

Western blots (upper panel) of *CycB* protein and Tubulin as a loading control. Wildtype and *CycB-tubulin* transgenes were crossed into *png/+* or *png-* mutant backgrounds. *CycB* protein levels remain low in a *png-* mutant background when translated from either a wildtype *CycB* transgene or a *CycB-tubulin* transgene.

3.3.3 The PNG Kinase Complex does not Regulate Pumilio Levels

Orr-Weaver and colleagues hypothesized that Pum represses *CycB* via the 3'UTR and the PNG kinase complex relieves this repression by Pum, allowing *CycB* translation to occur. Potentially, the PNG Kinase complex may regulate *pum* protein levels. For example, in *png* complex mutant embryos, Pum levels may raise, causing increased repression of *CycB*. We examined Pum levels in png^{3318} and plu^{3} embryos and observed no alteration of *pum* protein levels in png^{3318} and plu^{3} embryos in comparison to wildtype embryos (Fig 22).

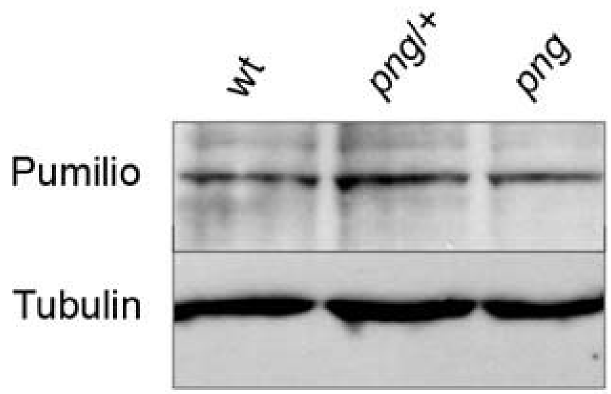

Figure 22: Pum levels are unchanged in *png* embryos

Western blot of Pum with Tubulin as a loading control in wildtype (w^{1118}), *png*/+, and *png* embryos. Pum levels are similar in all three genotypes.

3.3.4 Pumilio is Phosphorylated

The PNG Kinase complex does not regulate *pum* protein levels; however the kinase may phosphorylate Pum, thereby affecting the ability of Pum to repress *CycB*. Furthermore, the C-terminus of Pumilio, including the RNA binding domain (RBD) is both necessary and sufficient to confer repression of *hb* in the posterior and *CycB* in the pole cells of the early embryo; therefore, the RBD alone may be able to regulate *CycB* in the somatic cytoplasm.

We first examined if the Pum RBD is phosphorylated by observing mobility shifts of the protein on a polyacrylamide gel after Western blotting. We treated embryonic extract expressing myc-Pum RBD with λ phosphatase. As a positive control for λ phosphatase activity, we assessed the phosphorylation status of Cyclin A (CycA), which is known to be phosphorylated. As shown in Fig 23, CycA exists as a doublet. The reduced mobility of the upper band is due to phosphorylation: when treated with λ phosphatase, the upper band shifts downward and travels with a faster mobility. This shift in CycA mobility after treatment with λ phosphatase confirms our phosphorylation detection method. The migration of myc-Pum RBD changed after incubation with λ phosphatase, suggesting that myc-Pum RBD is phosphorylated.

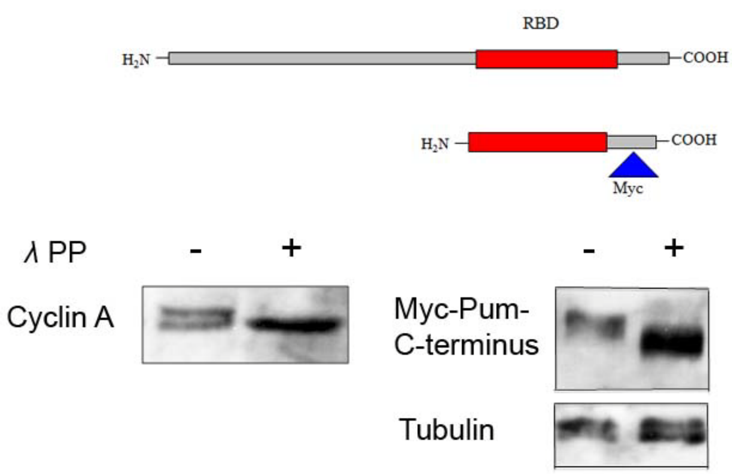

Figure 23: The Pum RBD is phosphorylated

Upper image is a schematic of full-length Pum protein. The red box represents the Pum RBD. The transgene utilized in our experiments consists primarily of the Pum RBD and has a myc tag (blue triangle) in the C-terminus (bottom image). Protein extracts were either treated with λ phosphatase (λPP) as denoted by the + symbol or not incubated with λ phosphatase as denoted by the – symbol and migratory changes were observed via Western blotting. CycA was used as a positive control, the myc-tagged C-terminus of Pum was our experimental blot as noted by their changes in mobility. Tubulin was used as both a loading control and negative control.

3.3.5 Genetic Interactions Demonstrate that Pum Acts Upstream of the PNG Kinase Complex

Based on our findings demonstrating no involvement of the *CycB* 3'UTR in the regulation of somatic *CycB* and no alteration in Pum levels in *png* mutants, we reconsidered the initial hypothesis of the PNG Kinase complex acting upstream of Pum to alleviate the repression of *CycB*. In order to decipher the *png-pum* genetic pathway, we utilized both the strong (1058) and weak (3318) alleles of *png*. Vardy and Orr-Weaver created *png*^{3318}*; pum* double mutants, restoring *CycB* protein levels to approximately wild-type levels. However, the use of the weak allele allows for three potential interpretations of the results (Fig 24). First, the PNG Kinase complex may act positively to promote *CycB* translation, whereas Pum represses *CycB* translation in a separate regulatory pathway. Second, the PNG Kinase may act upstream to repress Pum; thereby, allowing *CycB* translation to occur. Third, Pum may act upstream of the PNG Kinase complex to repress the complex and ultimately cause the translation of *CycB*.

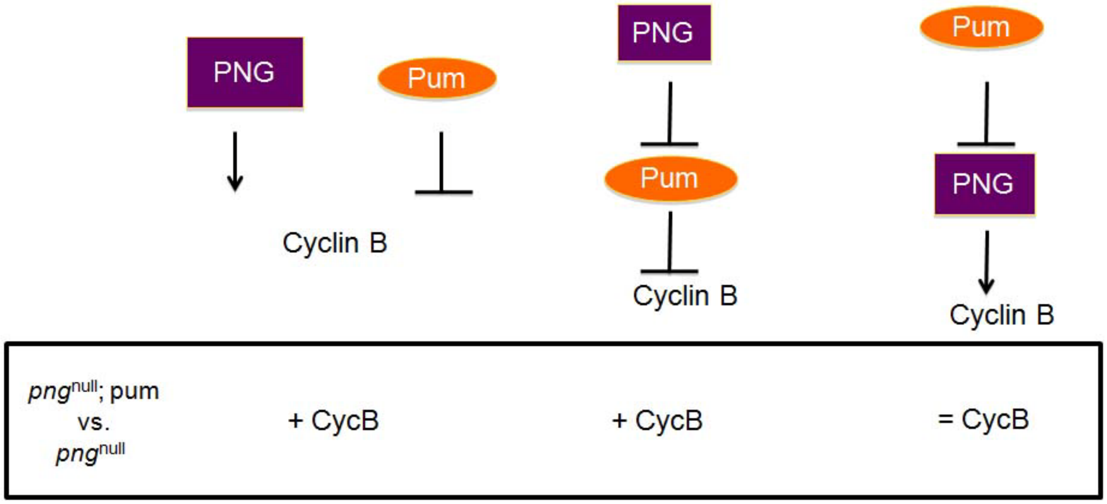

Figure 24: Potential PNG and Pum regulatory pathways

Above are the three putative interactions between the PNG Kinase complex and Pum in the regulation of *Cyclin B.* In order to differentiate between the three pathways we can compare *CycB* protein accumulation between the *png*^{null} single mutant and the *png*^{null}; *pum* double mutant. Greater CycB levels in *png*^{null}; *pum* embryos compared to *png*^{null} embryos are denoted by + CycB. Equal levels of *CycB* protein in *png*^{null}; *pum* and *png*^{null} embryos are denoted by = CycB.

We differentiated between these three possibilities by assessing *CycB* protein levels in *png* single mutants versus *png*; *pum* double mutants, when using either the weak or strong alleles of *png*. If Pum and the PNG Kinase complex act in two separate pathways or if the PNG Kinase complex acts upstream of Pum, *CycB* levels will be higher in *png* ^{3318}; *pum* and *png* ^{1058}; *pum* than in the *png* single mutants. However, if Pum is upstream of the PNG Kinase complex, then *CycB* levels will be the same in *png* ^{1058}; *pum* embryos compared to *png* ^{1058} embryos. We found that *CycB* protein levels are the same in *png* ^{1058}; *pum* and *png* ^{1058} embryos (Fig 25), suggesting that Pum acts upstream of the PNG

Kinase complex to regulate *CycB* protein levels in the somatic cytoplasm of the early embryo.

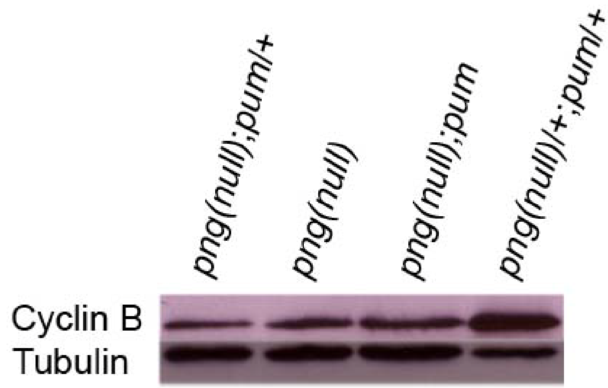

Figure 25: *CycB* protein levels are not restored in *png(null)*; *pum* double mutants

Western blot of *CycB* protein with Tubulin as a loading control in *png(null);pum*//+, *png(null), png(null); pum*, and *png(null*)/+; *pum*/+ embryos. Note that CycB levels are not significantly different in *png(null)* versus *png(null); pum* embryos.

3.4 Discussion

The regulation of *CycB* in the somatic cytoplasm of the *Drosophila* embryo by Pum was initially assumed to be Nos-independent due to the restriction of Nos to the posterior of the embryo. We have definitively shown that Nos does not repress *CycB* in the somatic cytoplasm. Furthermore, we demonstrated that although the 3'UTR of *CycB* contains multiple Pum binding sites, the 3'UTR does not confer the repression of *CycB.* If Pum acts directly on the *CycB* mRNA to prevent translation in the somatic cytoplasm, this finding is the first instance showing that Pum does not repress via a sequence within the 3'UTR of a target mRNA.

The intial hypothesis describing the potential regulatory mechanism of *CycB* in the somatic cytoplasm entailed the PNG Kinase complex acting upstream of Pum, which regulates *CycB* mRNA directly. Instead, we have shown genetically, that Pum may actually act upstream of the PNG Kinase complex to regulate *CycB*.

Currently, much remains to be elucidated on the interactions between the PNG Kinase complex and Pum in the regulation of *CycB* and the cell cycle. For example, the possibility still exists that a pontentially unidentified factor may be the link between the PNG Kinase complex, Pum and *CycB*. However, our studies suggest the potential for new direct targets (*png*, *plu*, and *gnu*) of Pum to be discovered.

4. Conclusions, Future Directions, and Significance

In my doctoral research, I have focused on deciphering the mechanisms of Pum-mediated regulation in the early *Drosophila* embryo. Specifically, I have shown that Pum does not regulate *bcd* mRNA stability. Additionally, I have increased our understanding of how Pum and the PNG Kinase complex act to regulate *CycB* in the somatic cytoplasm of the embryo. In furthering our knowledge of post-transcriptional regulation in *Drosophila*, we can shed insights on post-transcriptional mechanisms of control in higher organisms.

4.1 Regulation of *bicoid* Stability

In Chaper 2, we reported that Pum is not involved in regulating *bcd* mRNA stability. This finding has generated numerous interesting questions that can be followed up in *Drosophila* as well as other organisms. First, Pum does not temporally regulate *bcd* and promote *bcd* degradation; therefore, the proteins truly involved in regulating *bcd* mRNA remain to be identified. The timing of *bcd* mRNA disappearance coincides nicely with the maternal-zygotic transition during embryogenesis, when the majority of maternal mRNAs are degraded so that zygotic transcripts can begin to regulate the later stages of embryogenesis. Potentially, *bcd* mRNA stability may be regulated along with the other maternal mRNAs by Smaug, a regulator of maternal mRNA destabilization (Tadros, Goldman et al. 2007).

Another sequence located directly upstream of the *bcd* stop codon within the *bcd* 3'UTR (*bcd* instability element-BIE) has been implicated in regulating *bcd* mRNA stability during early embryogenesis (Surdej and Jacobs-Lorena 1998). When the BIE is deleted from the *bcd* 3'UTR, *bcd* mRNA persists into 3-4 hours AEL; whereas, wildtype *bcd* disappears before 2 hours AEL. Furthermore, the BIE is sufficient to destabilize *bcd* because when inserted into a stable mRNA, the mRNA destabilizes (Surdej and Jacobs-Lorena 1998). The *cis-acting* element that regulates *bcd* mRNA has been identified; therefore, we can isolate potential BIE-binding *trans-factors* by multiple methods. First, we can apply a bioinformatics approach by assessing if any known RNA-binding proteins have a target sequence similar to the BIE sequence. Next, we can screen for a potential BIE interactor by two different approaches such as the RNA-affinity purification assay or the yeast3hybrid assay. Once candidate BIE-interacting factors have been identified, we can utilize *Drosophila* genetics to assign significance to these interactions.

4.2 The Regulation of Somatic *Cyclin B* by the PNG Kinase and Pumilio

In Chapter 3, we determined that Pum acts upstream of the PNG Kinase complex to regulate *CycB* protein accumulation in the embryo. Interestingly, the *png* and *plu* mRNAs each have two sites that align nicely with the Pum binding sites within the *hb* and *CycB* NREs as shown in Table 2. This finding is encouraging, but we further need to definitively demonstrate if Pum regulates any of the PNG Kinase complex components.

Table 2 Predicted Pum binding sites Exist in *plu* and *png*

CycB	UGUAauuu
hb 1	UGUAuaua
hb 2	UGUAcaua
plu 1	UGUAauaa
plu 2	UGUAaaaa
png 1	UGUAcuua
png 2	UGUAuauu

Curiously, one of the mRNAs co-immunoprecipitated by Gerber and colleagues in their screen to identify Pum target mRNAs is *plu* (Gerber, Luschnig et al. 2006). In an effort to assess Pum binding of *plu* mRNA, I co-immunoprecipitated mRNAs associated with myc-Pum RBD and used the 3'UTR of *plu* as bait in a yeast3hybrid assay. Although, Gerber and associates observed *plu* mRNA to be associated with Pum, we could not recapitulate this finding via the co-immunoprecipitation experiment nor the yeast3hybrid (data not shown).

We next wished to determine whether Pum governs PNG Kinase complex components biochemically. To this end, we assessed Png, Gnu, and Plu levels in pum^{MSC}/pum^{FC8}embryos. Currently, the antibodies for Png and Plu do not seem to be reliable; therefore, we can circumvent this problem by assessing the protein amounts of tagged versions of these proteins. However, I believe that even though Pum may regulate the levels of Png, Plu, or Gnu, we may not witness a change in protein accumulation in *pum* mutants alone. Much as we needed to create *png*; *pum* mutants to observe the involvement of Pum in the regulation of *CycB* in the somatic cytoplasm, we may need to assess Png, Plu and Gnu levels in double mutant embryos. The reasoning behind this hypothesis stems from the finding by Orr-Weaver and colleagues that Plu and Gnu levels are low in *png* mutants, similar to *CycB* protein, suggesting a potential positive feedback mechanism exists between Png and its two regulatory subunits (Lee, Van Hoewyk et al. 2003).

The sites within the *CycB* mRNA that confer regulation via Pum and PNG Kinase complex, if any exist, must be identified. As an extra control to further definitively demonstrate that the CycB 3'UTR does not contain the sequence necessary for CycB regulation via Pum and the PNG Kinase complex, we can replace the *CycB* 3'UTR with the *bcd* 3'UTR, which has been shown not to confer regulation via the PNG Kinase complex (Tadros, Houston et al. 2003). Additionally, as a method to achieve the goal of identifying a *CycB* sequence that is regulated via the PNG Kinase complex, we created the following GFP heterologous reporters: *CycB* 5'UTR-GFP ORF-*tubulin* 3'UTR, *tubulin* 5'UTR-GFP ORF-*CycB* 3'UTR, *tubulin* 5'UTR-GFP ORF-*tubulin* 3'UTR, and *tubulin* 5'UTR-CycBGFP-*tubulin*-3'UTR. We will cross these different reporter transgenes into a *png* mutant background and if GFP levels are lower in a *png* mutant background, it will suggest that either the 5'UTR, 3'UTR, and/or CycB ORF are sufficient for regulation. Once a tentative region that confers regulation is identified, we will further define the site necessary for regulation by the PNG Kinase complex and Pum.

Furthermore, it has not escaped our attention that *CycB* is potentially not regulated on a translational level, but instead *CycB* protein stability is affected by the PNG Kinase complex. In order to investigate this possibility, we tagged the *CycB* genomic construct with Dronpa, a photoswitchable fluorescent protein (Habuchi, Ando et al. 2005; Habuchi, Dedecker et al. 2006). We plan to observe the stability of CycB in wildtype versus *png* mutant backgrounds by turning Dronpa "on and "off".

Lastly, we hypothesized that the interactions between the PNG Kinase complex, Pum and *CycB* may be very direct; however, all of the data thus far are purely genetic. Therefore, it is feasible that another currently unidentified factor or factors may be involved in regulating *CycB* in the somatic cytoplasm. If our biochemical data do not support a direct link between the PNG Kinase complex, Pum and the regulation of *CycB*, we may start investigating whether other factors are involved and pursue the identification of these unknown factors.

4.3 Implications of Thesis Work on Pumilio-mediated Regulation in *Drosophila*

Interestingly, we demonstrated that Pum binds to *bcd* mRNA via the NRE in vivo; however, this interaction is not sufficient to confer regulation. In yeast, the binding of a Pum homolog to a target mRNA is sufficient for degradation (Goldstrohm, Hook et al. 2006; Hook, Goldstrohm et al. 2007). Thus far in *Drosophila*, Pum has not been shown to be sufficient to repress target mRNAs and may possibily need a partner protein such as

Nos or another currently unidentified partner to act. Potentially, Pum may regulate other mRNAs besides *hb* and *CycB* in the embryo and a modified version of the Nos recruitment assay may be used, in which we can screen for potential Pum-interacting proteins.

Pum has also been implicated in regulating other processes during other points of *Drosophila* development. For instance, Pum and Nos have been shown to function at the neuromuscular junction (NMJ) of the *Drosophila* larvae. Curiously, although Pum and Nos both act at the NMJ, they have divergent functions, suggesting that Pum and Nos act independently of one another (Menon, Andrews et al. 2009). More specifically, Pum has been shown to directly repress eIF4E and the glutamate receptor IIA at the NMJ (Menon, Sanyal et al. 2004; Menon, Andrews et al. 2009). Thus far, it is unclear if Pum is sufficient to regulate these mRNAs; however, further research can be conducted to uncover the mechanism by which Pum acts in the NMJ. Pum also plays a role in learning and memory of Drosophila: *pum* mutant flies have defective memory and Pum is highly expressed in adult fly mushroom bodies, the neuronal center that regulates learning and memory (Dubnau, Chiang et al. 2003). The exact role of Pum in learning and memory is yet to be uncovered. Dubnau and colleagues have predicted potential neuronal Pum targets based on the *hb* and *bcd* NRE sequences via a bioinformatics approach(Chen, Li et al. 2008). My work suggests that the presence of an NRE or Pum binding sites in general do not necessarily imply Pum represses translation of the target mRNA. Results from computational predictions, in vitro binding assays, and in vivo expression experiments alone cannot be the determining factor in identifying a Pum target. Further genetic studies must be conducted in vivo in order to demonstrate the significance of Pum binding.

4.4 Significance of Thesis Work

Post-transcriptional regulatory mechanisms intricately govern various developmental processes among all organisms. By utilizing the powerful genetically tractable organism, *Drosophila melanogaster*, we can further our understanding and knowledge of the fundamental workings of post-transcriptional regulation. In my thesis work, I focused on uncovering the mechanisms by which Pum regulates *bcd* and *CycB* mRNAs.

Discovering that Pum does not regulate *bcd* mRNA demonstrates that Pum-mediated regulation is more complicated than simply binding to a recognition sequence within the mRNA. Specifically, Pum may need to interact with co-factors in order to function as a translational repressor. With the knowledge that Pum homologs exist in higher organisms, we can apply this information to other systems and identify unknown Pum partners and open the door to new modes of Pum-mediated repression.

Thus far no vertebrate orthologs of the PNG kinase complex have been found; however, Pan Gu and Plutonium both have conserved functional domains: a serine/threonine kinase domain and ankyrin-like repeat domain, respectively, and may therefore play a role in the processes of higher organisms (Elfring, Axton et al. 1997;

Fenger, Carminati et al. 2000). More specifically, *Drosophila* Pan Gu Kinase acts similarly to the vertebrate specific Serine/Threonine kinase, Mos, which is a regulator of meiosis and known to affect the stability of *CycB* protein in the mouse (O'Keefe, Kiessling et al. 1991; Verlhac, Kubiak et al. 1996; Fenger, Carminati et al. 2000).

Interestingly, *c-mos* is a proto-oncogene and *CycB* is upregulated in various types of cancer cells (de Foy, Gayther et al. 1998; Winters, Hunt et al. 2001; Aaltonen, Amini et al. 2009). Potentially, in cancer cells, the misregulation of *CycB* may be due to the actions of *c-mos* and furthering our knowledge of the regulation of *CycB* may give us insight into cancer formation and patient prognosis. By studying the regulation of *CycB* by the PNG Kinase complex in *Drosophila*, we may uncover novel, yet conserved mechanisms of regulation that directly apply to the regulation of *CycB* by *c-mos* in higher organisms.

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Biography

Krystle Nomie was born in New York, New York on October 3, 1981. She was raised in Allentown, Pennsylvania and graduated from Allentown Central Catholic High School in 1999. She attended Boston University and graduated in 2003 with a B.A. in Biology. After graduation, she began entered Duke University through the Cell and Molecular Biology program. She affiliated with the Department of Cell Biology after joining the laboratory of Dr. Haifan Lin. After Dr. Lin accepted a position at Yale University, she began working with Dr. Robin Wharton.

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