pumilio Regulates the Epithelial-Mesenchymal Transition and Gastrulation During Zebrafish Development

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DEDICATION

I would like to dedicate my dissertation thesis to my family. Whether by blood, or by years of friendship, my immediate and extended family have been my primary driving force to excel and a source of constant encouragement.

My parents are solely responsible for the person I am today: good, bad or indifferent. They allowed me the freedom to fly, the freedom to succeed and the freedom to fail. Without this faith in me, I would not have been able to become the accomplished individual I am today, and for that I love you and I thank you. For without failure, we cannot appreciate our successes. Ο τολμων νικα.

Just as important as the family I was born into, is the family I have created through years of friendships. They stuck right beside me through my darkest of times and offered me sunshine. Without their solace, jokes, and incredible knack to encourage and motivate in their own individual ways, I would have never made it this far. Each and every one has individually and communally contributed to my continual sanity over the decades. G, W, A: you guys are irreplaceable!

January 14th, 2011 was without a doubt the worst day of my life. I lost a great part of my life: my partner in crime, my little brother, my Schmo. TJ, in your passing you ushered in a new phase of my life, a stronger, more confident fireball surrounded by people who you touched, mentored, and greatly affected during your all-too-short 22 years with us. You always poked fun at me for being “smart” or “nerdy” and told me that the smarter I got, the stupider I got. I know that you have had and will always have my back; through thick and thin you are the greatest big little brother ever and will be forever missed greatly. I love you forever and always. I miss you Schmo and I’ll have one with you on the other side!

pumilio Regulates the Epithelial-Mesenchymal Transition and Gastrulation During Zebrafish Development

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2013

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The *pumilio* family of RNA binding proteins act as translational repressors to control developmental patterning events of invertebrates, germline stem cell maintenance, and neuronal growth through deadenylation, inhibition of translational elongation, and allow access of microRNAs to their targets. *pumilio* proteins have yet to be identified and functionally characterized in the zebrafish, *Danio rerio*. Here, three putative *pumilio* homologs in zebrafish are identified by their characteristic PUF (*pumilio* and FBF) binding

domains and found to be expressed during early development and enriched in the immature oocytes of the adult ovary. Loss of each homolog results in developmental defects, arising from improper formation of the shield and dorsal organizer structures during the first steps of gastrulation. Gastrulation events necessitate both proper patterns of gene signaling and cell motility, as gained by epithelial-to-mesenchymal transitions (EMT), to occur. In *pum1* morphants, both the presentation of wild-type signaling gradients and EMT events are inhibited. Through *in silico* and *in vivo* analysis, *acvr1b,* an Activin receptor, was identified as a target for *pum1*. *acvr1b* assists in patterning the gastrulating embryo and, through control of miR-200a expression, inhibits EMT events. Thus, *pum1* through its direct translational repression of *acvr1b* is able to modulate levels of miR-200a and control both patterning and EMT events in the early vertebrate embryo.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Neumann, J.C., Chandler, G.L., Damoulis, V.A., Fustino, N.J., Lillard, K., Looijenga, L., Margraf, L., Rakheja, D., and Amatruda, J.F. Mutation in the type IB bone morphogenic protein receptor Alk6b impairs germ-cell differentiation and causes germ-cell tumors in zebrafish. *PNAS.* **108**: 32 13153-13158 (2011).

Neumann, J.C., Lillard, K., Damoulis, V.A., and Amatruda, J.F. Zebrafish models of germ cell tumor. *Methods in Cell Biology*. **105** 3-24 (2011).

PRESENT PUBLICATIONS

Damoulis, V.A., Paruchuri, T., Shukla, A., Pearson, G., and Amatruda, J.F. *pumilio* Regulates Gastrulation and EMT via an *acvr1b*/miR-200a feedback loop during early development. (Manuscript in preparation).

LIST OF FIGURES

LIST OF DEFINITIONS

Bp- Base pair

DIG- Digoxigenin

Dpf- Days post fertilization

E3- Embryo water

EMT- Epithelial-to-mesenchymal transition

GCT- Germ cell tumor

GFP- Green fluorescent protein

Hpf- Hours post fertilization

MBT- Mid-blastula transition

PBE- *pumilio* binding element

PBS- Phosphate buffered saline

PCR- Polymerase chain reaction

PFA- Paraformaldehyde

PGC- Primordial germ cell

qPCR- Quantitative real-time PCR

RT-PCR- Reverse transcriptase PCR

CHAPTER ONE

General Introduction

Cancer is one of the most common causes of death worldwide and virtually all vertebrate species have the potential to develop cancer. By utilizing other vertebrate species as model organisms to study malignancy, we have enhanced our understanding of the mechanisms of human carcinogenesis and cancer progression (Goessling, North et al. 2007). As an established model of vertebrate development, *Danio rerio*, the zebrafish, is a model system that has been invaluable in the understanding of development, organogenesis, and guided cell migration events. More recent work has begun to use forward genetics, high efficiency transgenesis, targeted gene inactivation, and transplantation techniques to develop the zebrafish into a tool for studying various human pathologies, including cancer development and progression (Liu, Bhuiyan et al. 2010).

Zebrafish are advantageous for biological studies, as large transparent clutches of embryos can be obtained easily for experimentation. Additionally, the organogenesis processes of zebrafish are relatively similar to humans, making study of zebrafish molecular genetic pathways leading to cancer a novel way in which to model and research human cancer biology (Goessling, North et al. 2007). Zebrafish can develop almost any type of cancer and many of their tumors resemble human tumors by histological analysis (Amatruda, Shepard et al. 2002). In addition the tumors which zebrafish develop display characteristics similar to human tumors, such as genomic instability, invasiveness, transplantability, and the existence of cancer stem cells (Feitsma and Cuppen 2008). Studies over the past decades

have thus validated the zebrafish as a true model system in which to study the onset and progression of various human cancers.

Several key tumor suppressors and oncogenes in human disease have been shown to play similar roles in zebrafish disease. Two isolated zebrafish p53 mutants both show suppressed apoptosis and develop tumors at near one year of age, which were determined by histology to be malignant peripheral nerve sheath tumors (MPNSTs) (Berghmans, Murphey et al. 2005). Zebrafish that express c-myc in their lymphoid cells, under the *rag2* promoter, develop infiltrative T-cell leukemia (Langenau, Feng et al. 2005). The same *rag2* promoter can be used to drive *bcl2*, creating a model of B-cell leukemia (Langenau, Jette et al. 2005). Recent studies have introduced fusion oncoproteins through the creation of transgenic lines, such as the EWS-FLI1 fusion, the common chromosomal translocation in Ewing's sarcoma, and been able to recapitulate human disease (Leacock, Basse et al. 2012). The development of genetic models that develop disease or are predisposed to it allow for the search for new drugs through chemical screens. Utilizing these, and other models, in combination with growing small molecule compound libraries allows for *in vivo* analysis of drugs in studies of cancer prevention, toxicity, and development.

It is critical that embryonic development proceeds correctly for an animal to survive, thus changes which disrupt development too dramatically are rare in evolution (Roux and Robinson-Rechavi 2008). The dynamic processes of early vertebrate development are relatively similar between species, additionally tumors often reactivate these early developmental signaling networks and pathways for their own growth and development. Examples of pathways that are crucial, not only in development, but also in cancer

progression are the Wnt, Hedgehog, Notch, and BMP/TGFβ pathways. Alterations of these pathways have been found pathologically in many tumors, in which they recapitulate their developmental role. In tumors, these pathways play similar roles in selecting cell fates, cellular rearrangements, cytological context and morphologic design of the tumors are they do embryologically (Kelleher, Fennelly et al. 2006).

Wnt signaling, both canonically, through β-catenin, and non-canonical signaling, is important for diverse developmental processes in vertebrates (Verkade and Heath 2008). Specific work with zebrafish shows its necessity in determining the endocardial cell fate and regulating the cardiac valve formation (Hurlstone, Haramis et al. 2003). Truncation of the tumor suppressor APC (adenomatous polyposis coli) constitutively activates the Wnt pathway and is the primary molecular alteration in sporadic colon cancer. Zebrafish *apc* mutants recapitulate this phenotype, acting as bona fide models for studying digestive tract cancers (Haramis, Hurlstone et al. 2006).

Aberrations to the hedgehog pathway can also lead to cancers including esophageal, stomach, biliary tract, and pancreatic tumors. Secreted hedgehog ligands bind membrane receptor complexes called patched, activating GLI transcription factors and initiating target gene transcription (Kelleher, Fennelly et al. 2006). The generation of two zebrafish patched homolog mutants uncovered roles for hedgehog signaling somitogenesis, segmentation, and anterior-posterior axis patterning (Koudijs, den Broeder et al. 2008).

Notch signaling is highly conserved and is responsible for cell fate specification, stem cell maintenance, and initiation of differentiation in embryonic tissues (Kelleher, Fennelly et al. 2006). The best characterized role for Notch signaling is in hematopoiesis and

lymphopoiesis. A zebrafish model of T-cell acute lymphoblastic leukemia (ALL) shows that Notch signaling, independent of Myc, induces significant expansion of pre-leukemic clones (Blackburn, Liu et al. 2012). While this expansion could not directly induce leukemia, it was concluded that the primary role of Notch signaling was to expand the pre-malignant population, of which a subset could acquire transformations to become malignant (Blackburn, Liu et al. 2012). Notch also plays a role in solid tumors, including salivary gland tumors, neuroblastomas, medulloblastomas, lung cancer, and prostate cancer (Bailey, Singh et al. 2007). Angiogenesis and EMT events also involve Notch signaling, where it controls cardiac development in mice allowing endocardial cells to undergo Notch-induced EMT, invade a cell matrix, and form endocardium (Timmerman, Grego-Bessa et al. 2004).

TGFβ/BMP pathway members, including TGF-β, activins, and bone morphogenic proteins are a family of secreted ligands and cell surface receptors that participate in left-right asymmetry, limb development, mesoderm formation during gastrulation, differentiation, induction of cell migration and apoptosis (Kelleher, Fennelly et al. 2006). Several mouse knockout models are non-viable, illustrating the importance of this pathway for early developmental processes (Winnier, Blessing et al. 1995; Zhang and Bradley 1996). In addition, components of this pathway have been directly implicated in tumor formation, such as a zebrafish model of testicular germ cell tumors resulting from a truncation of a BMP type IB receptor, *alk6b* (Neumann, Chandler et al. 2011). TGFβ/BMP pathway is also implicated in metastasis of breast cancer, invasion of colorectal cancer cells into the epithelium, and other EMT events.

Aside from their necessity during developmental processes, these pathways each play a distinct role in tumor formation, progression, metastatic events, or the maintenance of a cancer stem cell population. These pathways, which are so critical to growth of organisms, can be later utilized by cancer cells, which are able to subtly alter some molecular components and use them to their own advantages. Interest and research into the fields of embryogenesis, development, and organogenesis provide not only insight into these processes directly, but also provide invaluable knowledge into the inner workings of cancer cells. The above pathways illustrate similarities between cancer cells and normal cells. Furthering our understanding of these pathways in an *in vivo* setting, especially one of active cell growth and fate changes, cell migration, EMT events, and differentiation, as is the case in the early zebrafish embryo, provides for an excellent tool with which to understand tumorigenesis and metastasis. Through work highlighting molecular regulators of developmental processes, such as those listed above, it will then be possible to draw parallels between the pathways which drive normal cells during embryogenesis, and cancer cells, which usurp these same pathways to support their own growth.

CHAPTER TWO:

pumilio is Critical in Early Developmental Processes

Introduction

The early development of metazoan embryos is characterized by dynamic, complex spatiotemporal patterns of gene expression, permitting precise control of cell fate and differentiation, morphogenic movements, and body axis determination. The genes that pattern these events are often similar between organisms (Domazet-Loso and Tautz 2010; Kalinka, Varga et al. 2010). Stringent control of these genes occurs transcriptionally and post-transcriptionally to support differentiation, patterning, and axis formation events necessary during the early stages or development.

A certain level of post-transcriptional control occurs at the mRNA level and is controlled by RNA-binding proteins (RBP). This protein class binds consensus RNA sequences and assists in regulating translation, splicing and RNA editing events (Spassov and Jurecic 2003; Lunde, Moore et al. 2007; Lukong, Chang et al. 2008). The broadly conserved PUF family of RBPs (PUMILIO and FBF proteins) can be defined by their characteristic RNA-binding domains consisting of approximately 8-alpha helical repeats necessary for binding consensus RNA sequences within 3' UTRs (Spassov and Jurecic 2003). These translational repressors act through a variety of mechanisms including attenuation of elongation, deadenylation, and facilitation of micro-RNA (miRNA) binding (Kedde, van Kouwenhove et al. 2010; Quenault, Lithgow et al. 2011; Friend, Campbell et al. 2012).

Studies of PUF family proteins in invertebrates have revealed roles in germline stem cell maintenance (Voronina, Paix et al. 2012), cell differentiation (Harris, Pargett et al. 2011), and embryonic patterning events (Murata and Wharton 1995; Schmitt-Engel, Cerny et al. 2012). Homologs have been widely identified within vertebrates (Spassov and Jurecic 2002; Spassov and Jurecic 2003; Fox, Urano et al. 2005; Kurisaki, Iwai et al. 2007; Lee, Hook et al. 2007; Ota, Kotani et al. 2011; Wang, Xu et al. 2012), however previous studies have not yet elucidated *in vivo* targets or molecular mechanisms of action of *pumilio* in these organisms (Galgano, Forrer et al. 2008). Identification of the PUF binding domain and its structure (Wang, McLachlan et al. 2002) allowed for identification of consensus *pumilio* binding sequences. Recent data have begun to elucidate the interactions between miRNAs and *pumilio* proteins in models ranging from *C. elegans* to human cells (Nolde, Saka et al. 2007; Galgano, Forrer et al. 2008; Kedde, van Kouwenhove et al. 2010; Leibovich, Mandel-Gutfreund et al. 2010; Miles, Tschop et al. 2012). This work has begun to reveal new targets and pathways in which *pumilio* plays a necessary role in gene regulation.

pumilio proteins have been implicated in *Drosophila* development and embryonic patterning (Gamberi, Peterson et al. 2002) through its regulation of *hunchback* (Murata and Wharton 1995). However, work as of yet, has not uncovered similar conserved role for identified *pumilio* homologs in vertebrate development. Murine knockout models of the orthologs *Pum1* and *Pum2* (Spassov and Jurecic 2003) are viable (Xu, Chang et al. 2007; Chen, Zheng et al. 2012), with these mice displaying fertility defects and small body size, suggesting a non-essential role in murine development. Additional work in mice has identified a role for *Pum2* in nervous system functioning (Siemen, Colas et al. 2011).

Recent work in the zebrafish model system has identified two PUF family members, including the identification and expression of a putative *pumilio2* homolog (Wang, Xu et al. 2012), as well as a PUF domain containing protein, *puf-A* (Kuo, Wang et al. 2009), which lacks a high degree of homology to other known *pumilio* proteins, but which further studies show plays a role in germ cell migration and regulating cellular response to genotoxic stress (Chang, Fan et al. 2011).

The role of *pumilio* in early vertebrate development is unknown. Here, three orthologs in zebrafish are identified and found to be expressed in early embryos. It is further shown, through loss of function experiments, that *pumilio* proteins are essential for early development, and *pumilio* may be acting in cooperation with p53. *pumilio* knockdown embryos exhibit gross morphological defects as well as impaired hematopoiesis and gonadal formation.

Materials and Methods

Phylogenetic Analysis. Multiple-sequence alignment of the *pumilio* homologs was performed using CLUSTAL-W and the BLOSUM matrix. The final tree diagram was generated as the N-J Tree with branch distance. Phylogenetic analysis was done at http://align.genome.jp.

RT-PCR. Total RNA from zebrafish embryos and isolated adult tissues was obtained using the TRIZOL reagent (Invitrogen, according to manufacturer's protocol). cDNA products

were amplified with specific primer sets for each gene of interest. Rpl13a was used as a loading control for the RT-PCR reactions. Semi-quantitative RT-PCR for embryonic and adult tissue expression was performed using 100ng of total isolated RNA per reaction and the Qiagen One-Step RT-PCR kit (According to manufacturer's protocol with the program: 50°C/ 30'; 95C°/15'; (95°C/1', 55°C/ 1', 72°C/ 1') x 27; 72°C/ 10').

Cloning of full-length cDNA, fragments, and riboprobe synthesis. Total RNA isolated from the ovary was used to amplify gene-specific cDNA fragments to use as riboprobes. The Qiagen One-Step RT-PCR kit (According to manufacturers protocol with the program: 50°C/ 30'; 95C°/15'; (95°C/1', 55°C/ 1', 72°C/ 1') x 35; 72°C/ 10') was used to amplify cDNA for TA cloning into the pCRII-TOPO TA vector (Invitrogen) according to manufacturers protocols. DIG labeled antisense riboprobes were synthesized via *in vitro* transcription using the Promega T7 and SP6 polymerases (according to manufacturers protocols).

Subcloning of full-length cDNA. Reverse transcription of total isolated ovary RNA was preformed using the Superscript III Reverse Transcriptase according to manufacturers protocols (Invitrogen). Gene specific cDNA products were then amplified by standard PCR (Table 2.1). PCR products were then TA cloned into the pGEMT-Easy vector (Fischer Scientific) according to manufacturers protocols. The T7 and SP6 mMessage machine kits (Ambion) were then used to synthesize RNA for injection, according to protocols.

***in situ* Hybridization on Embryos and Whole Ovaries.** Embryos were collected, staged, and fixed overnight in 4% paraformaldehyde buffered with 1x PBS at 4°C. Adult female fish were anesthetized with an over dose of Tricaine, heads were removed and the ventral side was cut open. Adults were fixed in 4% PFA/1x PBS overnight at 4°C. After fixation, ovaries were carefully removed and transferred to 100% MeOH at -20°C overnight. Embryos were also transferred to 100% MeOH at -20°C. *in situ* hybridization was performed as described (Thisse and Thisse 2008) with modifications as noted below. Embryos were prepared and probed overnight with gene specific probes at 500-1000ng/mL at 70°C. Whole ovaries were permeabilized with 50µg/mL Proteinase K at 37°C for 15' followed by a 20' 4% PFA/1xPBS fixation at room temperature. Whole ovaries were probed with gene specific probes at 1000ng/mL at 70°C overnight, washed and all *in situs* were visualized by an alkaline phosphatase reaction by BCIP/NBT coloration reaction.

***in situ* Hybridization on Sections of Paraffin Embedded Ovaries.** Adult female fish were anesthetized with an overdose of Tricaine, heads were removed, and the ventral side of the female was cut open. Fish were fixed in Larison's Fixative (30% EtOH, 10% Formalin, 0.05M Sodium Phosphate pH 7.2) for 3 days at room temperature. Fish were then transferred into Decalcification Solution (30% EtOH, 3% Formalin, 0.2M EDTA) for 4 days at room temperature, changing to fresh solution after 2 days. Samples were then paraffin embedded, cut by transverse sectioning, and mounted on slides. Prepared sections were deparaffinized in xylene, rehydrated, and processed for *in situ* hybridization, with some modifications (Thisse and Thisse 2008). Slides were treated with 40µg/mL Proteinase K followed by a 20' 4%

PFA/1xPBS fixation step. Slides were hybridized with gene specific riboprobes at 1000ng/mL at 70°C overnight, washed, and visualized by an alkaline phosphatase reaction by BCIP/NBT coloration reaction.

Morpholino Injections. Zebrafish embryos were obtained by mating AB stain wild-type adults. Morpholinos were microinjected into 1-cell stage embryos and grown until scored for each experiment (Morpholino sequences: Table 2.1). Morpholino knockdown was validated by RT-PCR confirming the presence of the maintained intron in morphant samples (data not shown).

Sorting of Primordial Germ Cells. Embryos from the transgenic line Askopos-GFP (*kop*-GFP) well collected and allowed to develop to 22-24 hours post fertilization. Embryos were then processed for FACs sorting according to the Lawson Lab protocol (http://lawsonlab.umassmed.edu/pdfs/dissociationforfacs.pdf). Dechorionated and deyolked embryos were enzymatically disaggregated. The resulting cell suspension was washed and subjected to cell sorting. GFP+ primordial germ cells were sorted, and a negative sort was also collected as a control. RNA was isolated from the sorted cells using Trizol.

o-dianisidine staining. Embryos at 48hpf were dechorionated and stained as previously described (Detrich, Kieran et al. 1995).

Immunohistochemistry and Acridine Orange Staining. Performed as in protocol (Verduzco and Amatruda 2011).

Results

Identification of Zebrafish Pumilio Homologs

To identify *pumilio* family members in zebrafish, the zebrafish genome was searched for sequences similar to the *Drosophila* PUF repeat RNA-binding domain (NP_731314.1), used as a reference for the ancestral *pumilio*. Three putative *pumilio* homologs were identified in the zebrafish: *pum1* (XP_002665632.2), *pum2* (NP_001096040.1) (Wang, Xu et al. 2012), and *pum3* (XP_002662124.1). Sequence alignment to the *Drosophila* protein shows an overall sequence homology of 87%, 80%, and 52%, respectively, for each of the 3 novel PUF domain-containing homologs (Figure 2.1 A). Each protein carries the characteristic PUF domain-containing RNA-binding repeats, however, whereas there are 8 repeats in *Drosophila*, the zebrafish proteins contain only 6 (*pum1* and *pum2*) and 7 (*pum3*) of the predicted binding repeats. Phylogenetic analysis of the novel proteins show that *pum1* and *pum2* cluster more closely with their respective homologs in other bony fishes and mammals than with each other, suggesting a duplication event in vertebrates (Figure 2.1 B). *pum3* is more distantly related to *pum1* and *pum2*, however it does maintain the PUF repeats, suggesting that it may function similarly as a translational repressor.

Pumilio is Maternally Contributed

Many genes required in early development are maternally contributed and supplied to the early embryo as packaged RNA (Abrams and Mullins 2009). Maternal genes are the sole source of transcripts until zygotic transcription is initiated at the mid-blastula transition (MBT). To determine if the identified *pumilio* homologs are maternally contributed, wild-type embryos at varying stages of development were sacrificed, RNA was isolated and used for RT-PCR analysis with homolog-specific primers (Figure 2.2 A). Transcripts of each homolog can be detected as early as the one- or two-cell stage, indicated that the homologs are maternally contributed.

Expression Patterns of Pumilio Orthologs are Restricted as Embryogenesis Progresses

To determine gene expression patterns during development, gene-specific antisense riboprobes were designed and synthesized. Wild-type embryos were fixed at varying stages of embryonic development and subjected to analysis by *in situ* hybridization. Expression of *pum1* and *pum2* are nearly identical, with global expression seen in 8-somite embryos, and expression becoming restricted to the head and anterior structures as embryos approach 24hpf (hours post fertilization) (Figure 2.2 B). The expression pattern of *pum3* is more distinct; in earlier embryos expression is global, with slight enrichment of transcripts in the dorsal brain and eye. This enrichment can be seen more clearly at 24hpf, when expression appears to be restricted to these structures.

Primordial Germ Cells Express Pumilio

pumilio has been identified and characterized for its role in germ cells, gonadogenesis, and germline maintenance (Forbes and Lehmann 1998; Parisi and Lin 1999; Jaruzelska, Kotecki et al. 2003; Moore, Jaruzelska et al. 2003). In zebrafish, a putative *pumilio* domain-containing protein, *puf-A*, has been implicated as having a role in primordial germ cell (PGC) migration (Kuo, Wang et al. 2009). To investigate if zebrafish PGCs expressed these novel *pumilio* homologs, we took advantage of the *kop*-GFP transgenic line, which expresses GFP specifically in the germ cells (Blaser, Eisenbeiss et al. 2005). Fluorescence-activated cell sorting (FACS) sorting was used to isolate cell populations enriched for germ cells (GFP+) and devoid of germ cells (GFP-). Subsequent RT-PCR analysis of the two cell populations showed that *pum1*, *pum2*, and *pum3* are each expressed in the germ cells (Figure 2.2 C). Expression of these transcripts is not enriched in the germ cells, as the GFP- sorted population shows significantly higher *pumilio* levels. RT-PCR for marker genes are included to illustrate purity of the sort, as *vasa* is specifically expressed in the germ cells, and *sdf1a* is a gene expressed in the somatic tissue of the gonad, and not expressed in germ cells.

Adult Tissues Express Pumilio and is Enriched in Immature Oocytes

To understand the expression of *pumilio* in adult zebrafish, RNA was isolated from tissues prepared from male and female mature wild-type zebrafish. By semi-quantitative RT-PCR analysis, it was determined that *pum1* and *pum3* are expressed at low basal levels in a

range of tissues. These transcripts notably were enriched in the ovary. *pum2* expression is higher globally, with enrichment in the ovary (Figure 2.3 A).

The adult zebrafish ovary is composed of various cell types, including germ cells at various stages of oogenesis as well as somatic cells. To determine the cell type that expresses these transcripts, whole ovaries were isolated and subjected them to *in situ* hybridization analysis (Figure 2.3 B). Staining reveals the smaller, immature oocytes express *pumilio* transcripts at the highest levels, diminishing as oocytes mature into eggs. This can be seen more clearly by *in situ* performed on a paraffin section of an adult ovary, in which different oocyte populations are more clearly illustrated (Figure 2.3 C).

Early Vertebrate Development Requires Pumilio

pumilio has been shown in invertebrate systems to be critical for embryonic patterning and development (Murata and Wharton 1995; Gamberi, Peterson et al. 2002; Cho, Gamberi et al. 2006; Schmitt-Engel, Cerny et al. 2012). Morpholino-mediated gene knockdown was utilized to investigate a conserved role of *pumilio* in development. Two morpholinos targeted to each of the three *pumilio* homologs were designed to target splice sites of pre-mRNA. Morpholino binding to RNA inhibits proper splicing, leading to premature stop codons and non-functional protein.

Each of the six morpholinos was injected independently at the one-cell stage of wild-type embryos. Embryos were then grown to 3 dpf and scored into classes based on the developmental defects observed (Figure 2.4 A). Embryos with loss of each of the homologs exhibited similar defects. Normal embryos at 3dpf are illustrated. As compared, the “mild”

class of embryos exhibits microcephaly, small eyes, slight body curvature, shortened body axis, and slight pericardial edema. The "severe" class of embryos exhibits more severe pericardial edema, significant body curvature, especially in the tail, and in some cases poor body axis formation. *pumilio* knockdown resulted in nearly 60% of embryos exhibiting developmental defects (Figure 2.4 B) (Uninjected: n=714; Control MO: n=408; *pum1* MO: n=265; *pum2* MO: n=200; *pum3* MO: n=500). Results from the second targeting morpholino were similar.

To confirm that the observed results were due to specific gene loss, and not off-target effects of the morpholino or toxicity, full-length, *pumilio* transcripts modified to be resistant to the morpholino were subcloned, and mRNA was synthesized, and co-injected with morpholino to rescue the developmental defects. Knockdown phenotypes were rescued to near normal levels, showing specificity of the observed phenotypes to loss of *pumilio* protein (Figure 2.4 C) (*pum1*: n=102; *pum2*: n=28; *pum3*: n=82).

The high degree of sequence conservation between species suggests that some functions of these proteins may be conserved. To test this, morpholinos were co-injected with full-length mouse *Pum1* or *Pum2* rescuing mRNA. The mouse homologs rescued the developmental phenotypes to near control levels as well, indicating a degree of functional conservation between organisms (Figure 2.4 D) (*pum1*: n=75; *pum2*: n=30).

The identified homologs have a high degree of sequence homology (72% between *pum1* and *pum2*), suggesting they may act redundantly in the zebrafish. To investigate, a cross-rescue experiment was designed in which morpholinos targeting one of the homologs was co-injected with rescuing mRNA from the second homolog. There was no rescue of the

observed phenotypes, suggesting the homologs do not act in a redundant manner, as they cannot compensate for each other (Figure 2.4 E) (*pum1* MO + *pum2* RNA: n=18; *pum2* MO + *pum1* RNA: n=26). This result may explain why knockdown of individual zebrafish *pumilio* orthologs causes severe defects in development.

p53 May Cooperate with Pumilio in Early Development

Toxicity due to morpholino injection results in abnormal embryos with specific phenotypes (Bedell, Westcot et al. 2011), and has been shown to be a result of p53 activation and apoptosis (Robu, Larson et al. 2007; Storer and Zon 2010). Morpholinos were injected into $p53^{-/-}$ embryos to confirm the observed phenotypes were not due to toxicity. If injection results in embryos developmentally normal, this would indicate the observations were due to toxicity and not gene loss. However, if the phenotypes are observed in the $p53^{-/-}$ embryos, this indicates the defects are specific to loss of *pumilio*. Loss of *pumilio* in a p53-null background resulted in a greater degree of abnormal embryos (Figure 2.5 A) (Uninjected: n=143; Control MO: n= 51; *pum1* MO: n=47; *pum2* MO: n=35; *pum3*: n=97). This further validates that the observed developmental phenotypes are due specifically to loss of *pumilio*, and also suggests that *pumilio* and p53 may cooperate in some manner during the course of embryogenesis. *pumilio* proteins have already been shown to interact with p53 during spermatogenesis (Chen, Zheng et al. 2012), and to be important during genotoxic stress events (Chang, Fan et al. 2011), however an interaction over the course of development has yet to be observed.

The Δ113p53 isoform is specifically induced by morpholino toxicity, and detection of this isoform can be used as an indicator of morpholino toxicity (Chen, Ng et al. 2009). Following protocols set forth in this publication, the effect of *pumilio* morpholinos on the presence of the Δ113p53 isoform was investigated. As anticipated, the morpholinos designed for experimentation in these studies do not induce the transcription of the Δ113p53 isoform, thus indicating morpholino toxicity is not prevalent in these embryos (Figure 2.5 B).

To further probe a possible link between *pumilio* and the p53 pathway, we investigated the incidence of DNA damage and apoptosis in knockdown embryos. Preliminary results of Acridine Orange, γH2Ax, and pH3 staining suggest *pumilio* morphants have increased apoptotic cells and increased DNA damage. Acridine orange staining shows an increase in apoptotic cells in *pumilio* morphants (Figure 2.6 A). This can be seen the most clearly in the tail of 24hpf embryos. Immunohistochemical staining using an antibody to the phosphorylated form of histone H2Ax, or γH2Ax, is used as an indicator of DNA damage. At 24hpf, morphant embryos exhibit an increased number of γH2Ax foci indicating an increase in DNA damage (Figure 2.6 B). However, this increase in apoptosis and DNA damage does not result in a cell cycle arrest. Immunohistochemisty with an antibody to the phosphorylated form of histone H3, pH3, shows mitotic cells. *pumilio* morphants do not show a mitotic arrest and have near the same number of cells dividing as in control samples (Figure 2.6 C).

Hematopoiesis is Affected in Pumilio Morphants

Observations of decreased amount of circulating blood in *pumilio* morphants was noted when scoring developmental phenotypes. To further investigate, morphant embryos

were fixed at 2dpf and processed for o-dianisidine staining to visualize red blood cells. This revealed embryos exhibiting two main defects: a loss of blood in the cardinal veins or pooling of blood as with hemorrhaging (Figure 2.7 A-B). These results are distinct from embryos exhibiting developmental defects due to *pumilio* loss (Control MO: n=38; *pum1* MO: n=38; *pum2* MO: n=25; *pum3* MO: n=22).

To investigate if the apparent hemorrhaging was due to disrupted blood vessel formation, *pumilio* morpholinos were injected into the *fli-GFP* zebrafish line, in which blood vessels can be visualized during development (Figure 2.7 C). Blood vessels at 24hpf can be seen between the boundaries of the developing somites. However, in *pumilio* morphant embryos, the branching of blood vessels does not appear to be complete and is damaged in some morphants.

Pumilio Morphants Do Not Display Ectopic PGCs

Previous studies have implicated *puf-A* in germ cell migration, an event during development in which the primordial germ cells (PGCs) specified at random, distant location from the presumptive gonad must undergo guided migration to their final locations (Kuo, Wang et al. 2009). Additionally, *pumilio* proteins are important in Drosophila gonadogenesis and germline maintenance (Forbes and Lehmann 1998; Parisi and Lin 1999).

To determine if the novel identified homologs play similar roles in primordial germ cell migration, morphants were analyzed by *in situ* hybridization with the antisense riboprobe to *vasa*, a germ cell-specific gene. Morphants embryos, as compared to controls, did not display significant ectopic germ cells (p=1.0) (Figure 2.8 A-B) (Control MO: n=28; *pum1*

MO: n=74). Injections were completed into both a wild-type background, as well as a $p53^{-/-}$ background, which yielded similar results. This indicates, at least by these studies, that *pum1* is not required for early germ cell migration.

Adult Zebrafish with Embryonic Loss of Pumilio Show Gonadal Defects

The effect of protein loss by morpholino injection only persists for 4-5 days of development. After this time period morpholinos are no longer effective in knocking down protein levels. However, with the understanding that *pumilio* is involved in gonadogenesis events that can occur early during development, it was postulated that gene loss during early development may lead to abnormal gonads in adults, long after morpholino effects have worn off.

Embryos injected with morpholino that survive to adulthood were sacrificed and processed for histological analysis. Some morphant adults display a *mini-fin* phenotype (Figure 2.9 A) (*pum1* MO: n=7/80). *mini-fin* is the designation of a mutant allele in zebrafish *tolloid,* an enzyme that modulates TGFβ/BMP signaling (Connors, Trout et al. 1999). These adults show loss of tail fin tissues and curling of the tail. Isolated adult gonads, both ovarian and testis tissue, was paraffin embedded and H&E stained, both males and females displayed abnormalities. Morphant ovarian tissues exhibited areas of oocyte breakdown, as illustrated by the disorganized, heavily pink stained areas (Figure 2.9 B) (*pum1* MO: n=6/32), as compared to controls in which oocytes of various stages of oogenesis can be observed, with no necrotic tissue areas. Morphant testes display a distinct phenotype (*pum1* MO: n=4/24). Scattered amongst properly organized spermatogenic lobules are immature oocytes. These

oocytes most likely have escaped programmed cell death during the period of zebrafish sexual determination, in which the default female gonadal tissue must apoptose and testis structures are formed.

Discussion

pumilio homologs have been identified in a variety of model organisms. This work has focused on uncovering the molecular function of *pumilio* in various cell types and settings, and is aimed at understanding how *pumilio* modulates translation of its target genes. Whether it is through attenuation of translational elongation (Friend, Campbell et al. 2012), deadenylation, or facilitating miRNA binding (Quenault, Lithgow et al. 2011), this RNA binding protein is critical in a variety of settings ranging from embryonic patterning of *Drosophila* (Gamberi, Peterson et al. 2002) to stem cell maintenance (Moore, Jaruzelska et al. 2003). However, little is known about the specific roles of *pumilio* proteins in early vertebrate development, which motivated these zebrafish studies. Here, three putative *pumilio* homologs were identified by their characteristic PUF binding domains. Through morpholino-mediated gene knockdown, these genes were found to have critical roles in early zebrafish development. While structurally, and by amino acid sequence, the functional PUF domains of *pum1* and *pum2* appear similar, they exhibit novel functions, as they cannot compensate for each other. This suggests, in this setting, these proteins do not exhibit functional redundancy and may act through regulation of different targets. While the scope of this work has focused mainly on *pum1*, similar investigations into *pum2* would be vital in

understanding the distinct and overlapping functions of these repressors. Additionally, simultaneous loss of function experiments of both *pum1* and *pum2* may uncover more dramatic roles of PUF proteins in vertebrate development.

Extensive investigation into *pum1* morphants showed not only gross developmental defects, but also more specific abnormalities, including DNA damage, hematopoietic defects, and abnormal gonadogenesis. Knockdown of *pum1* in a p53 deficient background illustrated more significant developmental defects than in wild-type embryos. These data, in conjunction with acridine orange staining and γH2AX immunostaining, which marks sites of DNA damage, suggests *pum1* may play a role in facilitating the apoptotic pathway. This has been suggested recently by work showing *pum1* acts to negatively regulate key components of the p53 pathway (Chen, Zheng et al. 2012). Other work shows *pumilio* proteins directly bind several members of the p53 pathway (Galgano, Forrer et al. 2008). This response of *pumilio* to genotoxic stress is not exclusive to the *pumilio* proteins, but extends to other PUF domain-containing proteins, as the ortholog *puf-A* is able to modulate PARP-1 (Chang, Fan et al. 2011). This regulation of the p53 pathway directly affects spermatogenesis in mice, while the data presented here illustrates a role for the *pumilio*-p53 pathway interaction in vertebrate development. Additional work focused on *pumilio* binding targets that directly regulate p53 pathway members will elucidate further molecular functions of *pumilio* proteins during development. This DNA damage, which results from *pumilio* loss and this overactivation of the p53 pathway, occurs without mitotic cell cycle arrest, suggesting that *pumilio* may also control cell cycle genes in vertebrates, similarly to its control of cyclin B in

Drosophila (Kadyrova, Habara et al. 2007). Alternatively, the DNA-damaging effects of *pumilio* knockdown may not be sufficiently severe to cause global cell-cycle arrest.

Work outlined above also highlights a role for *pumilio* in hematopoiesis. *pumilio* morphants show less red blood mass and also apparent hemorrhaging, and blood pooling by o-dianisidine staining. Hematopoiesis in zebrafish occurs in two waves, the primitive wave, occurring first from 12-32hpf, and the definitive wave, occurring from 32hpf on. Two portions of the mesoderm, the anterior lateral mesoderm (ALM) and the posterior lateral mesoderm (PLM), both express transcription factors necessary for blood and endothelial formation during somitogenesis (Hsia and Zon 2005). The defects observed in *pum1* morphants are evident between 24-28hpf, suggesting pumilio proteins may regulate the primitive wave of hematopoiesis. Utilizing markers of the ALM and PLM, such as *gata2*, *runx1*, and *c-myb*, coordinated with their expression patterns by *in situ* hybridization, it would be possible to narrow in on the affected cell type in *pumilio* morphants. These experiments would give a better understanding of whether hematopoiesis is controlled by *pumilio*, or if *pumilio* is necessary for regulating the hematopoietic stem cell. *pumilio* is critical in maintaining stem cell populations, such as the germline stem cell, in multiple organisms (Jaruzelska, Kotecki et al. 2003), however a role in the hematopoietic stem cell (HSC) has not been investigated. *pumilio* enrichment has been shown in adult murine hematopoietic stem cells, and differential expression of PUM1 and PUM2 was observed during blood cell development (Spassov and Jurecic 2003). This suggests *pumilio* proteins may not only play a role in vertebrate HSC maintenance, but possibly in regulating asymmetric cell divisions and stem cell maintenance in multiple cell types.

While the data presented here suggest *pum1* is expressed in primordial germ cells (PGCs), but not critical to PGC migration and maintenance, other PUF domain containing proteins have been shown to play a role in the migration of these cells (Kuo, Wang et al. 2009). This possibly suggests that *pum1* and *pum2* may display some level of functional redundancy in this cell type. To investigate this further, it would be necessary to simultaneously knockdown both orthologs and observe migration defects. While *pum1* does not appear to be crucial in PGC migration, it does appear to play a role in gonadogenesis. *pumilio* morphants that survive to adulthood display abnormal testis and ovarian tissues. While the gene knockdown provided by morpholinos wears off after 5 days post injection, the persisting effects of *pumilio* loss can be seen past 6 months of age. Female morphants show loss of integrity of mature oocytes as compared to controls. This suggests *pumilio* may control germline stem cell maintenance, such that when it is lost, stem cells prematurely differentiate such that there is not a continual supply of immature oocytes from asymmetric stem cell divisions to repopulate the ovary. In morphant males, a different phenotype is observed: mature testis display persisting immature oocytes. During sexual differentiation in zebrafish, which occurs between 21-30 dpf, the bipotential ovary must undergo apoptosis and upregulate genes, such as *amh,* and allow for testis formation (Masuyama, Yamada et al. 2012). In *pumilio* morphant males it is likely that this p53-dependant apoptosis is incomplete, thus a small number of immature ooctyes, or even possibly ovarian stem cells, persist in a mature testis.

However, as the effects of morpholinos are transient and wear off long before sexual differentiation and the observation of these phenotypes, it is difficult to directly study a role

of *pumilio* in gonadogenesis utilizing these gene knockdown techniques. Thus, the implication of *pumilio* knockouts would be crucial in investigating these phenotypes further. TALENs have been designed to *pum1* and could be used to create these knockout models. TALENs are transcription activator-like effector nucleases which are designed to target specific gene sequences and are capable of inducing double-stranded breaks in DNA, which are then repaired by mechanisms that can be exploited to create sequence anomalies and effective gene knockout (Joung and Sander 2013). Thus a stable knockout can be created to more accurately study the effects of *pumilio* loss not only on gonadogenesis, but many other biological processes and functions. If ubiquitous *pumilio* loss proves toxic, it would be possible to engineer a transgenic construct to specifically drive short-hairpin sequences targeting *pumilio* knockdown in the gonad specifically using the *ziwi* promoter (Leu and Draper 2010).

This work, which identified and characterized the expression of zebrafish *pumilio* homologs, suggests *pumilio* proteins are necessary components of early vertebrate development. These RBPs also likely contribute to regulation of the p53 pathway and hematopoiesis. The wide variety of observed phenotypes resulting from *pumilio* knockdown may be due to translational repression of a single gene, but it is likely that these effects stem from the wide variety of *pumilio* binding targets. Thus, it is likely that *pumilio* proteins function as master regulator of multiple pathways through direct translational control of their components. *pumilio* proteins may have different targets, or different binding partners, in different cell types this conferring the multiple observed phenotypes. Further focused work on each phenotype will enhance this work and lead to a greater understanding of the

molecular functioning of *pumilio* proteins and how they are able to pattern developmental processes.

Gene	Forward Primer Sequence	Reverse Primer Sequence
Pum1 cDNA full	atgagcgtagcgtgtgtgtt	ctggcaaacttatgctggct
Pum2 cDNA full	atgagcattccatgcagcat	tcacacacttctccacaaca
Pum3 cDNA full	catcacgctgcgtcggctac	ttaatccttcagtaattcgctg
Pum1 RT and ISH	TAGCGTGTGTGTTGAAGAGG	CATGATTGGCCGAGACACTG
Pum2 RT and ISH	AGACACAATGTGGAACGCTG	GCACCATAATGGGTTGAGAGAG
Pum3 RT and ISH	ACAGATCCAGAGGAGAAAGC	TCGTACTGCTCGACTCAAAC
RPL13a RT and qPCR	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG
Vasa RT	GACCTGTGTACGACCTGTTG	GCAGCTGGTCCCTCTTTGAG
SDF1a RT	CACCAGTGCGGATCTCTTCTTC	CCTTGTCATCTGGCAGACAT
snail ISH	Francisco Pelegri Lab	
lhx1a ISH	Lab Stock	
sox17 ISH	Francisco Pelegri Lab	
gsc ISH	Lab Stock	
chordin ISH	GGAGGCGGAGCAACTGCAC	GGCGATGTTGATGAACAGCTCG
bmp2b ISH	Thermo Scientific Clone 7993759	
ACVR1b ISH	TCTAGCCGAGCCTGGCAGACG	TCCATGGAGGGCGGTTGTGCT
DUSP6 ISH	Tsang Lab	
ACVR1b 3'UTR clone	TGAAACGCACGGGATGCTGTAG	GAAAAAAATGATTTATTACTGAATAGC
nephrin ISH	gtgccaagtgggtcggtcc	cgttacctcagctggttc
Zf ID1 qPCR	GCATCTCTGTGGAGAACGG	CGATGCTTCGATGATCTGTT
Zf TGIF1 qPCR	GACCCATCTATCCACAC	CCTTGCCATCTTTCCGG
Zf serpine1 qPCR	Qiagen Catalog # QT02180962	
Zf e-cadherin qPCR	Qiagen Catalog # QT02128063	
Zf n-cadherin qPCR	Qiagen Catalog # QT02114511	
Zf vimentin qPCR	Qiagen Catalog #QT02105908	

Zf Pumilio 1 qPCR	GAATGGACTTGATGTGGATG	CATCTCTGGTCCTCCCAAC
Zf Pumilio 2 qPCR	CATTGGCTCTGGAGTC	CACCCACAAGCATTGC
Zf ACVR1b qPCR	Qiagen Catalog # QT02084677	
Zf DUSP6 qPCR	Qiagen Catalog #QT02236423	
Zf NCOA3 qPCR	Qiagen Catalog #QT02150694	
Hs GAPDH qPCR	SA Biosciences Catalog #PPH00150E-200	
Hs PUM1 qPCR	SA Biosciences Catalog # PPH02668A-200	
Hs PUM2 qPCR	SA Biosciences Catalog # PPH02719A-200	
Hs WT1 qPCR	SA Biosciences Catalog # PPH00254A-200	
Hs ACVR1b qPCR	Qiagen Catalog #QT00053235	
Hs DUSP6 qPCR	Qiagen Catalog #QT00209986	
Hs NCOA3 qPCR	Qiagen Catalog #QT00053956	
PRE1 mutagenesis	CCACTTCAGAACACAC ACAatataatgaCTGATGAT ACATCATTC	GAATGATGTATCATCA GtcattatatTGTGTGTGTTC TGAAGTGG
PRE2 mutagenesis	CAACGGTCGTGTATTAT GgtataacatTTTTAAAAAA CGATTGCAG	CTGCAATCGTTTTTTAA AAatgttatacCATAATACA CGACCGTTG
PRE3 mutagenesis	GTGGGTTTGTTAATATA TgtatatacAATGCTATTCA GTAATAAATC	GATTTATTACTGAATAG CATTgtatatacATATATTA ACAAACCCAC
miR-200a mutagenesis	GTGTTATACCATGGTGC TCTACATattgtgcTCTCAA CCACGTTCCTTGAGG	CCTCAAGGAACGTGGT TGAGAgcacaatATGTAGA GCACCATGGTATAACA C
	Morpholino Sequences	
Pumilio 1	GCACCTTTTGTGGCACA TACCTAAA	
Pumilio 2	GGAACTGGACACTGCA AATGTAAAA	

Pumilio 3	GACTGACAGAGGACAC ATAACAGGA
Control (Non-targeting)	CCTCTTACCTCAGTTAC AATTTATA
Wt1a	Perner et. al.

Table 2.1: Primers used for experiments
Listed are the primer and morpholino sequences used for this set of experiments.

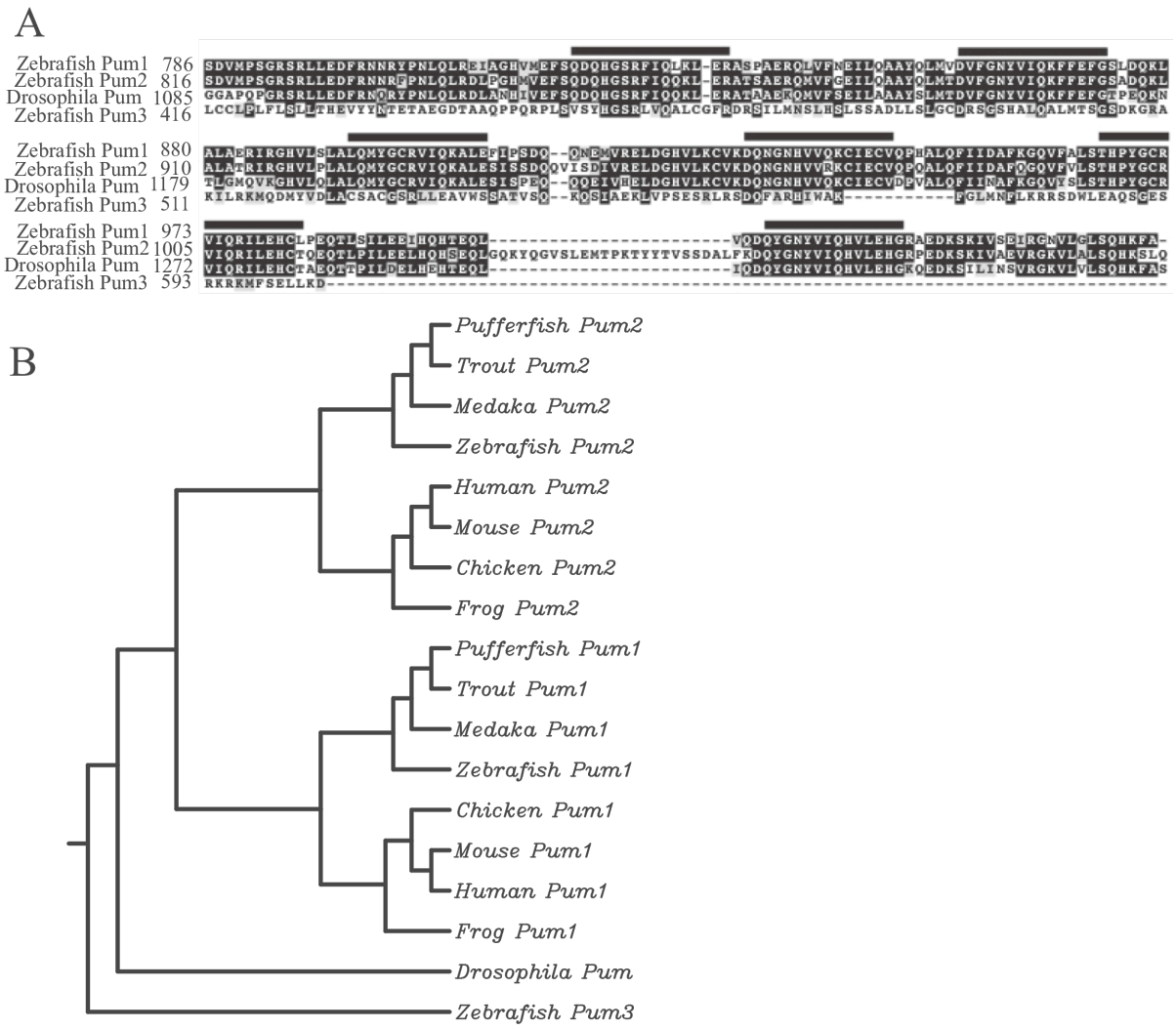

Figure 2.1: Identification of three novel zebrafish *pumilio* homologs
A: Clustal W sequence alignment of the C-terminus of the three identified zebrafish *pumilio* homologs compared to *pumilio* in *Drosophila*. Black bars highlight the 6 PUF binding repeats; amino acid residues are numbered.
B: Phylogenetic analysis illustrates the zebrafish *pum1*, *pum2*, and *pum3* proteins. Zebrafish homologs cluster near other bony fish. *pum1* and *pum2* of different organisms cluster with each other suggesting they arose from a duplication event. *pum3* appears more distantly-related to *pum1* and *pum2* along with *pumilio* of *Drosophila*.

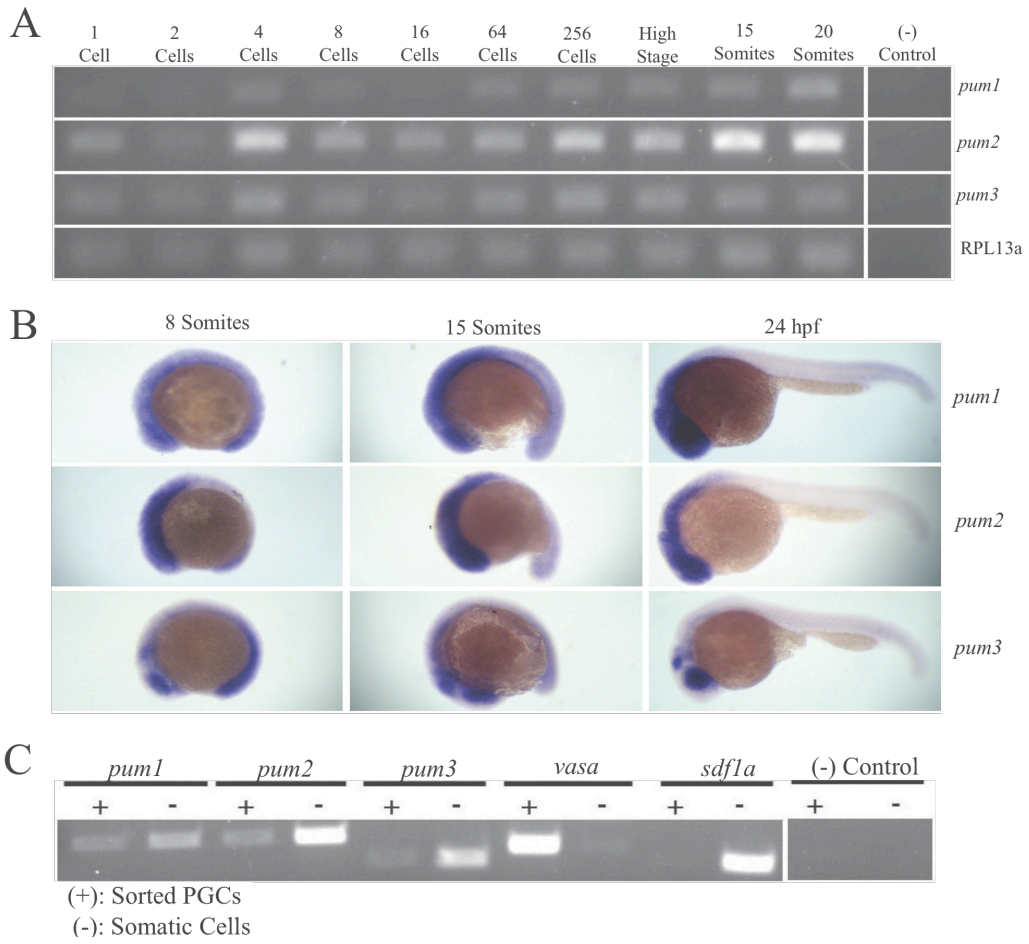

Figure 2.2: *pumilio* is expressed in early zebrafish embryos and in the primordial germ cells

A: *pumilio* can be detected in early embryos by RT-PCR as early as the 1-cell stage. Zygotic transcription is initiated between the 256-cell stage and High stage in this time course experiment. Detection of transcripts prior to Mid Blastula Transition (MBT) (approximately High stage) indicates they are maternally contributed. RPL13a is used as a loading control.
B: *in situ* hybridization with antisense riboprobes to the individual *pumilio* homologs shows similar expression patterns of *pum1* and *pum2* during development. Expression becomes restricted as embryos develop towards 24hpf from the 8-somite stage. *pum3* shows more restricted expression during development and is restricted to the eye and dorsal brain by 24hpf.
C: Isolated primordial germ cells ((+) sorted PGCs) from *kop*-GFP embryos are compared to GFP (-) surrounding somatic cells. RT-PCR shows expression of each *pumilio* homolog in the primordial germ cells, as well as in the surrounding soma. *vasa,* a PGC specific gene, indicates purity of the sort. *sdf1a,* a somatic cell marker of the presumptive gonad, also indicates purity of the sort. RPL13a shows equal loading.

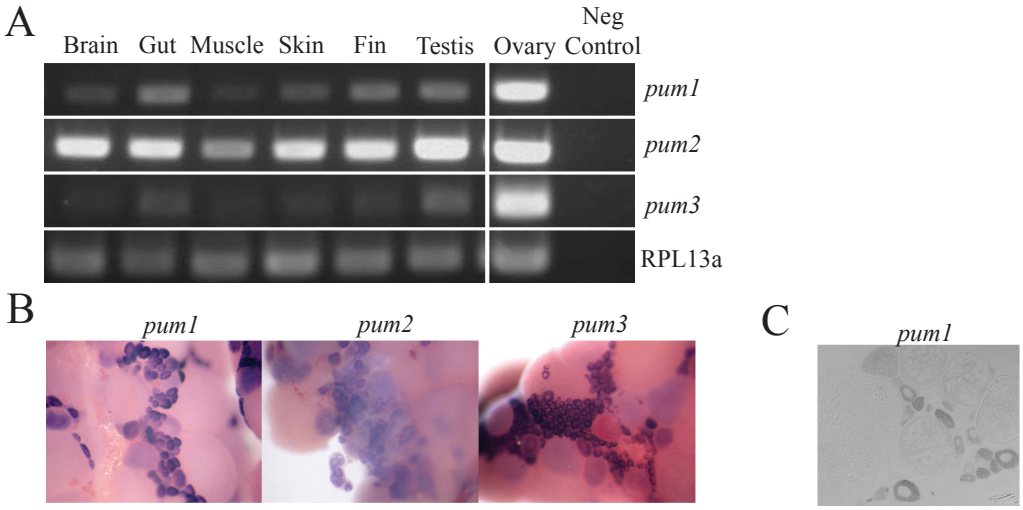

Figure 2.3: *pumilio* is expressed in adult tissues and enriched in the immature oocytes of the ovary

A: *pumilio* can be detected in multiple isolated adult zebrafish organs. *pum1* and *pum3* show low basal level of expression in most organs, and are enriched in the ovary. *pum2* shows higher detectable expression in the organs tested under the conditions of this experiment. RPL13a is used as a loading control.

B: *in situ* hybridization performed on isolated adult ovaries shows dense staining in the small, immature oocytes. Staining falls off as the oocytes enlarge and mature through oogenesis and appears undetectable in the largest, mature eggs. Staining for *pum1, pum2,* and *pum3* appear similar in the ovary.

C: *in situ* hybridization with the *pum1* antisense riboprobe performed on paraffin sections of the adult ovary illustrate the detection of transcripts more clearly in small, immature oocytes. Again, this staining is lost in enlarging oocytes with expanding yolk.

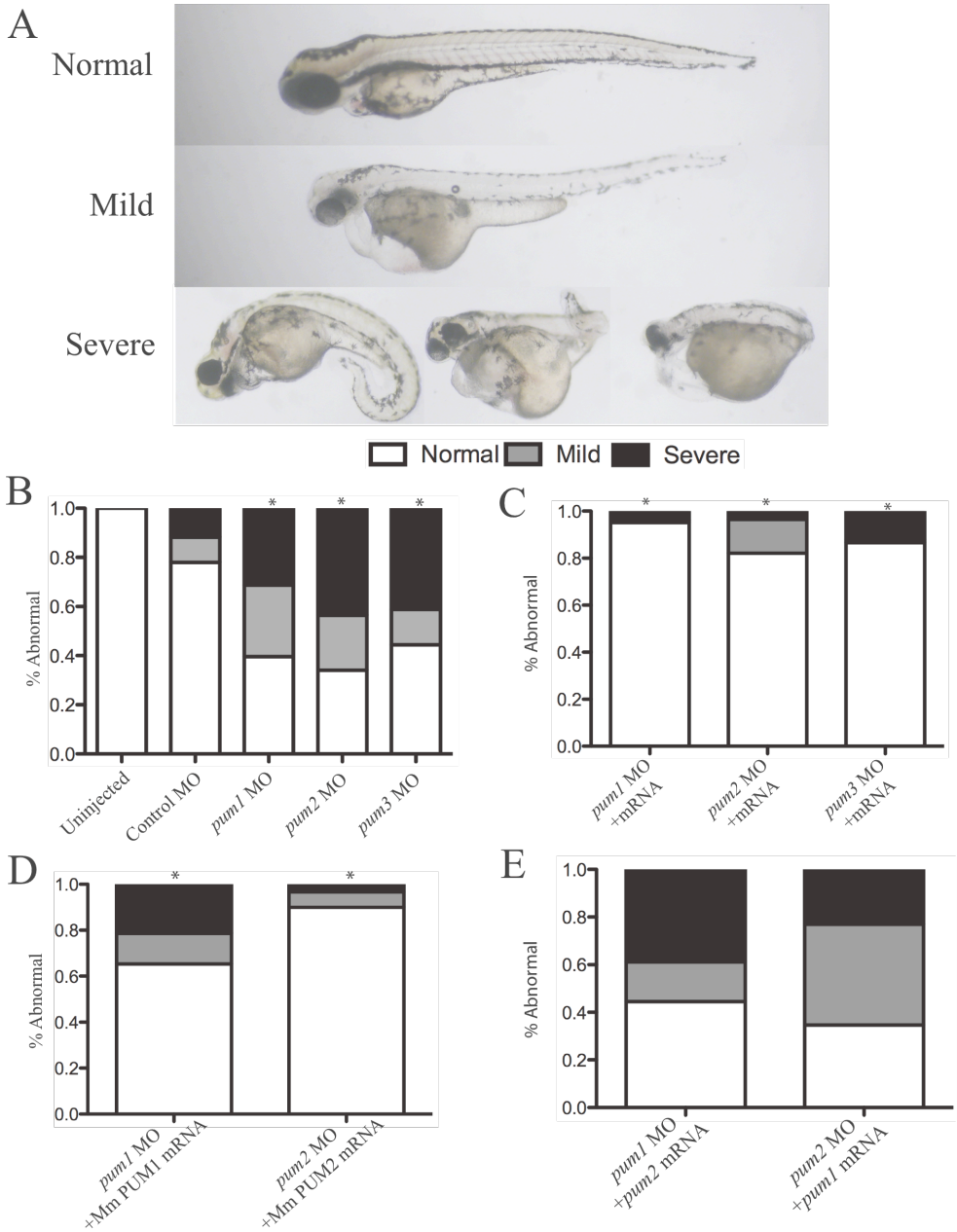

Figure 2.4: Vertebrate development requires *pumilio*
A: Morpholino injection into 1-cell stage wild-type embryos causes developmental defects as scored into three classes, normal, mild, and severe, at 3dpf. Normal embryos exhibit wild-type phenotypes at 3dpf. Mild embryos show microcephaly, smaller eyes, and pericardial edema. Severe embryos exhibit severe pericardial edema, truncated body axis and overall poor development.
B: Quantitation of the morphant phenotypes shows significant developmental defects caused by injection of morpholinos targeting each of the individual *pumilio* homologs. *p< 0.0001.

C: Co-injection of targeting morpholino with a corresponding morpholino-resistant mRNA rescues the knockdown phenotype significantly as compared to morpholino injection alone. This illustrates specificity of the morpholinos to their respective target genes. *$p < 0.0001$.
D: Co-injection of the targeting morpholino with corresponding mouse mRNA for the *pumilio* homologs is also able to rescue the knockdown phenotypes. *$p < 0.0001$.
E: Co-injection of a targeting morpholino with overexpression of the opposing homolog cannot rescue the knockdown phenotype. Thus the zebrafish *pumilio* homologs cannot cross-rescue or compensate for each other.

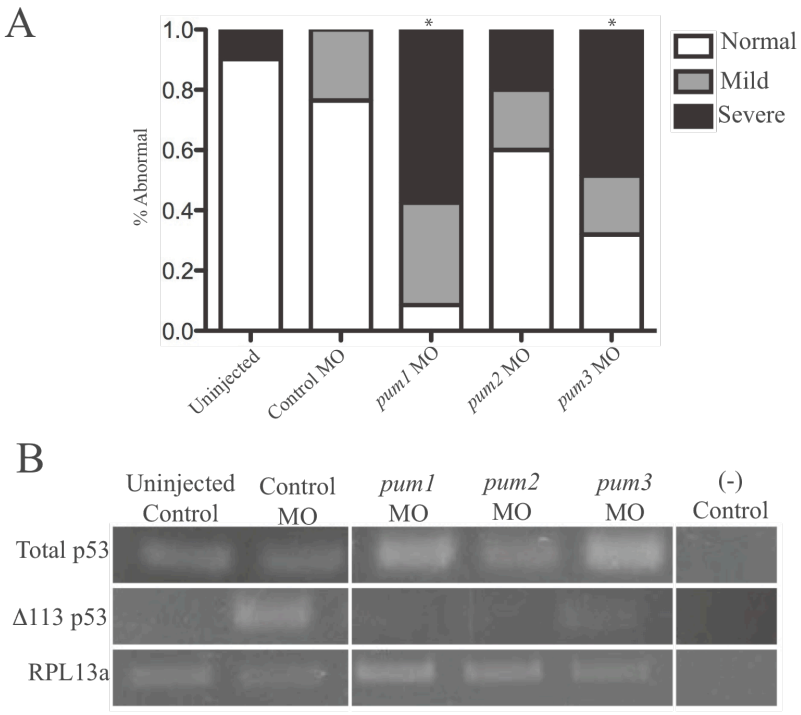

Figure 2.5: Observed phenotypes are independent of morpholino toxicity
A: Morpholino injection into 1-cell stage $p53^{-/-}$ embryos shows similar developmental defects. Quantitation of these defects shows, as compared to injection into wild-type embryos, more significant defects, illustrating that *pumilio* may cooperate with p53 in early embryos. This also shows that the developmental defects caused by morpholino injection are not due to toxicity. *$p<0.0001$.
B: RNA isolated from morpholino injected wild-type embryos was subjected to RT-PCR analysis. Detection of the Δ113 isoform of p53 indicates morpholino toxicity. This isoform is only detected slightly in the Control MO sample, again indicating that the *pumilio* morpholinos are not causing general toxicity. RPL13a is used as a loading control.

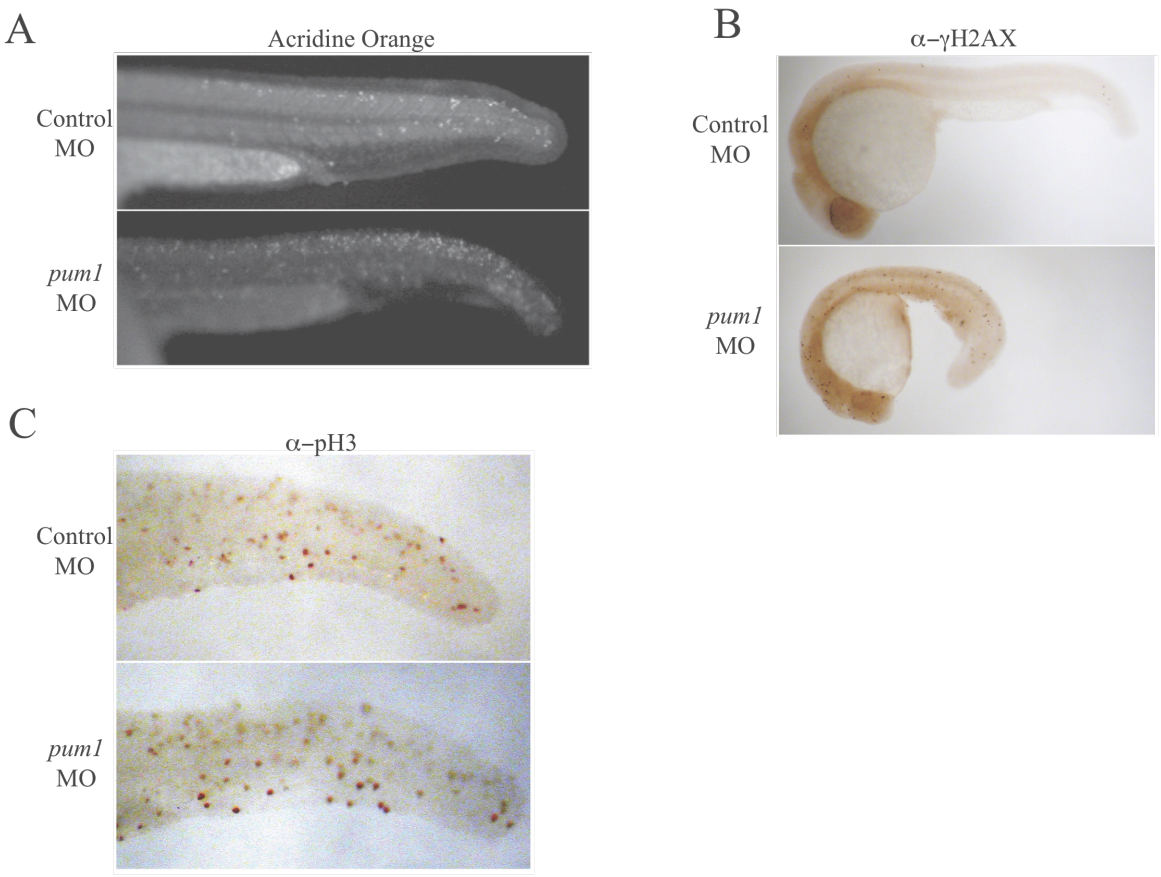

Figure 2.6: *pumilio* morphants have increased DNA damage and apoptosis
A: Acridine orange staining of *pumilio* morphants shows an increased amount of apoptotic cells in the tails of 24hpf embryos as compared to controls.
B: Immunohistochemistry shows an increased number of γH2Ax foci in *pumilio* morphants at 24hpf as compared to controls, indicating an increase in DNA damage.
C: Immunohistochemistry shows a similar number of pH3 foci in the tails of *pumilio* morphants and control embryos at 24hpf, showing that the increase in DNA damage and apoptotic cells does not lead to a cell cycle arrest.

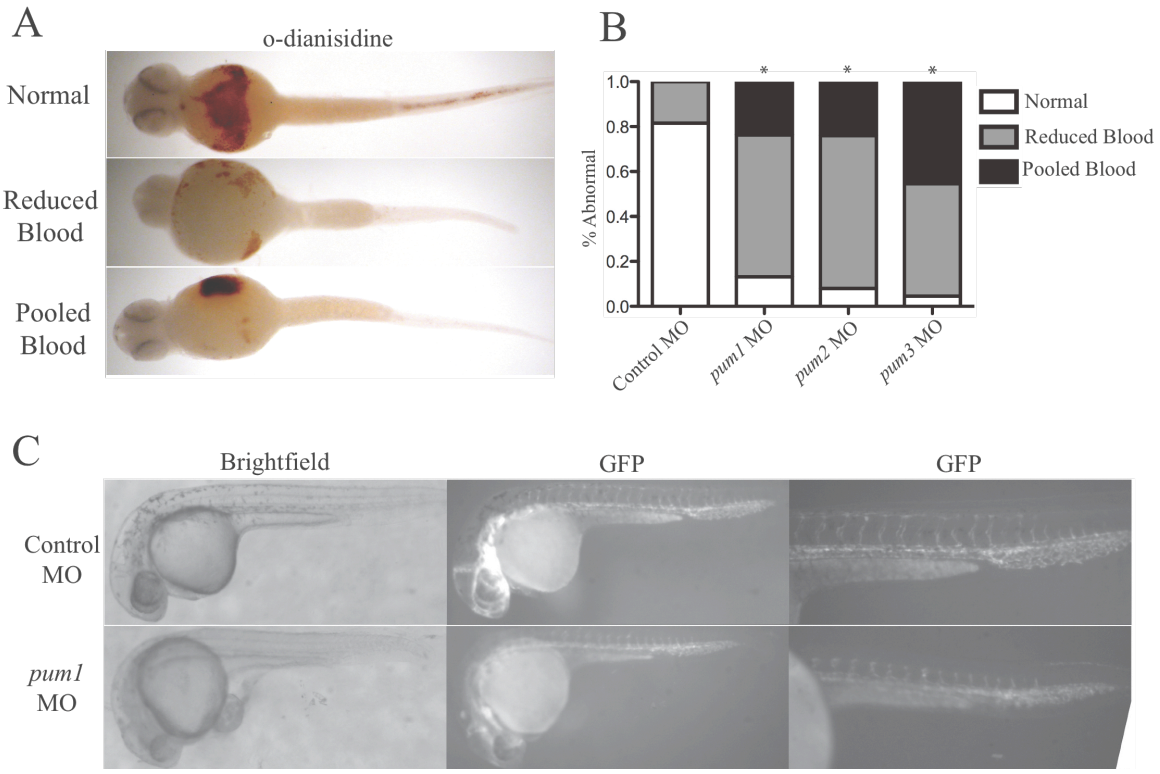

Figure 2.7: Hematopoiesis and vasculature formation is disrupted in *pumilio* morphants
A: *pumilio* morphants show a range of hematopoietic defects as detected by o-dianisidine staining at 48hpf. Normal embryos show a wild-type distribution of blood. Some embryos also show a reduced amount of blood by o-dianisidine staining. Embryos also show pooling of blood on the side of the yolk as well as within the body in some cases.
B: Quantification of the hematopoietic defects. Morphants have a significant increase in the fraction of embryos exhibiting reduced or decreased blood as compared to controls. *$p<0.0001$.
C: The *fli*-GFP zebrafish line is used to visualize vasculature in developing embryos at 28hpf. Bright field images on the left are paired with fluorescent images in the middle. Images to the extreme right are enlargements of the posterior yolk tube extension and tail of the center images. *pumilio* morphants as compared to controls exhibit reduced vasculature sprouting and growth through the somites.

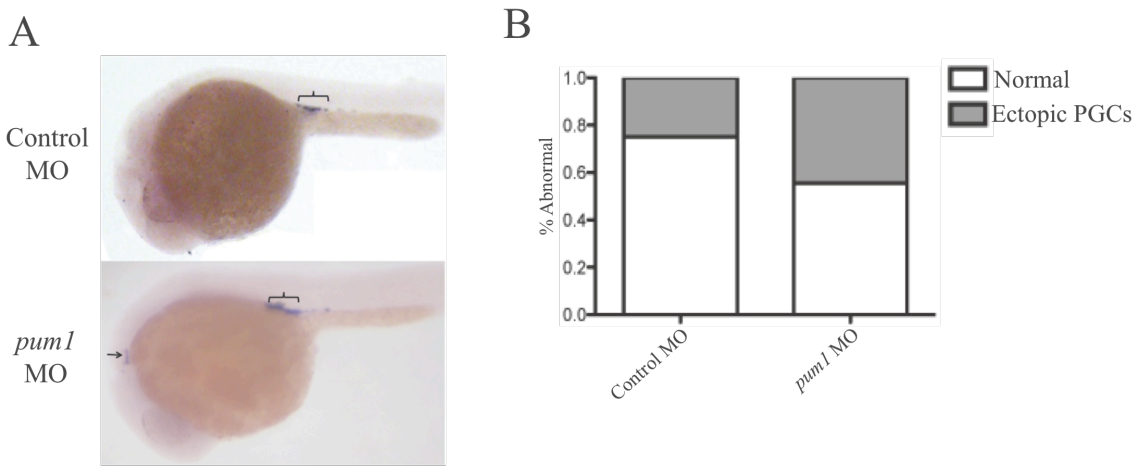

Figure 2.8: *pumilio* loss does not affect primordial germ cell migration
A: *in situ* hybridization with an antisense riboprobe to *vasa* is used to visualize PGCs at 24hpf. In control embryos, PGCs are correctly located at the site of the presumptive gonad (brackets) at the anterior end of the yolk tube extension. In some cases, PGCs migrate to ectopic locations, such as in the head (arrow).
B: Scoring of *vasa in situs* does not show a significant difference in the number of embryos displaying ectopic PGCs between control and *pumilio* morphant embryos. p=1.0.

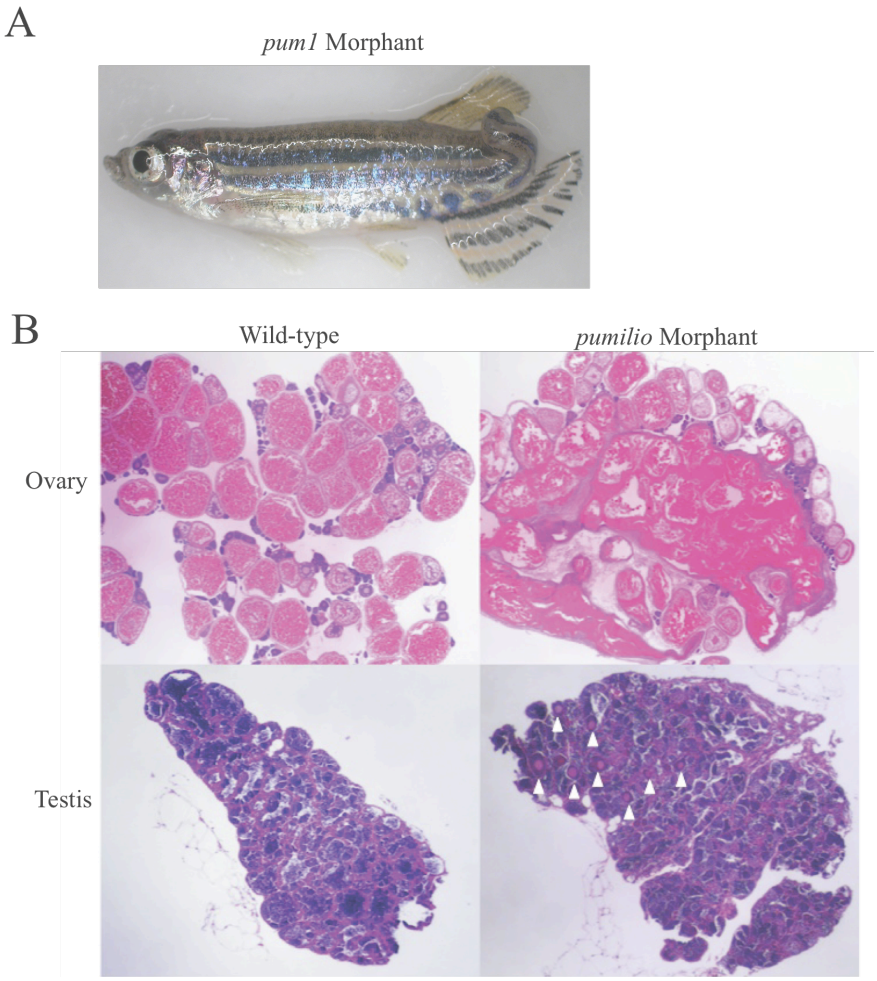

Figure 2.9: *pumilio* morphant embryos grow to adults with a *mini-fin* phenotype and gonadal defects

A: Some *pum1* morphants raised to adulthood show a *mini-fin* phenotype as scored at 7 months of age. These embryos show a protruding lower jaw as well as loss of tail fin tissues and curling of the tail.

B: *pumilio* morphant adults display gonadal defects. In control, wild-type ovaries, oocytes in varying states of oogenesis can be detected by H&E staining. Smaller immature ooctyes stain dense purple, and larger mature eggs stain pink. In *pumilio* morphants, however, there are large areas of degenerating ooplasms (pink at the center) without organization into mature eggs. In wild-type testis, lobules of cells in various stages of spermatogenesis surround a center of mature sperm. However, in morphant testis, persisting immature oocytes (white arrow heads) can be detected, and lobule formation is poor.

CHAPTER THREE:

Identification of Functional Targets of *pumilio* Required for Gastrulation and EMT

Introduction

pumilio, as illustrated above, has been shown to have a critical role in zebrafish development, especially in the early process of gastrulation and formation of the shield structure. Gastrulation commences with the first cell movements of the embryo. The blastoderm cells, sitting atop the yolk cell, undergo rearrangements in the deep cells and begin an involution at the blastoderm margin (Warga and Kimmel 1990). As the blastoderm cells move across the yolk cell, the cells thicken and form the germ ring, comprised now of two distinct layers of cells, the epiblast and the hypoblast. Once these layers are formed, cells from both the epiblast and hyboblast combine at the presumptive dorsal side of the embryos, thickening further to form the embryonic shield. Hypoblast and epiblast cells from the embryonic shield extend anteriorly and begin the formation of the germ layers (Warga and Kimmel 1990). Several zebrafish mutants have been identified by impairment and defects in gastrulation. The *one-eyed-pinhead* and *spadetail* mutants display defective cell movements and convergence ultimately affecting cell fates (Heisenberg and Tada 2002). Additionally, the *silberblick*, *trilobite*, and *pipetail* mutants display gastrulation defects predominantly affecting morphogenesis (Heisenberg and Tada 2002).

In addition, the shield is also central in the establishment of the dorsal-ventral axis. The embryonic shield acts as the dorsal organizer and is the site at which dorsal signals

originate. These dorsal signals oppose BMP ligands produced in ventral tissues. A delicate balance exists between dorsal and ventral signals to properly form the early dorsal-ventral axis, such that mutants can display both dorsalized and ventralized phenotypes. The zebrafish *chordino* mutant is deficient in chordin, a BMP antagonist and component of the dorsal organizer. These embryos exhibit severely ventralized phenotypes (Schulte-Merker, Lee et al. 1997), whereas *bmp2b* mutants conversely exhibit severe dorsalization (Schmid, Furthauer et al. 2000).

The dorsal-ventral axis is patterned by both maternal and zygotic factors. Maternal β-catenin, acting as a transcription factor for genes in the Wnt signaling pathway, accumulates at low levels in cells of the dorsal margin and is required for dorsal axis formation (Pelegri and Maischein 1998). As zygotic transcription is initiated, at the mid-blastula transition, β-catenin activates genes such as *bozozok*, *chordin*, *dickkopf1, squint*, and FGF signals which act to further establish the dorsal-ventral axis (Schier and Talbot 2005).

Zygotic transcription accounts for the continual fine-tuning of the dorsal-ventral axis, initiated by maternal determination of the dorsal pole. Pivotal work (Hemmati-Brivanlou and Melton 1997) describes how the shield, or Spemann-Mangold organizer, induces dorsal fates by inhibiting the action of ventralizing signals, such as BMPs. In this manner, development of the dorsal fate is the "default" state, such that dorsalizing factors act to block the influence of ventralizing signals rather than to actively trigger pathways that specify dorsal fates (Schier and Talbot 2005). These ventral signals are members of the BMP family, secreted ligands which bind heterodimers of transmembrane receptors to activate, by phosphorylation, intracellular SMAD family transcription factors allowing for regulating of gene expression of

pathway target genes. The knowledge of the importance of these pathway components arises from studies of mutants, and the identification of their affected genes, including *swirl/bmp2b*, *snailhouse/bmp7a*, *somitabun/smad5*, *lost-a-fin/alk5*, and *minifin/tolloid* (Langdon and Mullins 2011). Expression of bmp2b ligands immediately after MBT is restricted to the ventral tissues, such that a BMP gradient is formed. Evidence suggests that the initial wave of zygotic BMP expression is derived from maternal BMP signaling through SMAD5 (Hild, Dick et al. 1999).This gradient is attenuated by anti-BMP signals originating from the dorsal organizer. First, *bozozok*/*dharma* is induced in the organizer upon initiation of the shield formation by maternal β-catenin and inhibits the most dorsal blastomeres from expressing *bmp2* (Fekany, Yamanaka et al. 1999). This is followed by expression of further antagonists chordin, noggin, and follistatin (Dal-Pra, Furthauer et al. 2006), which act to solidify the BMP gradient across the animal pole and restrict the strongest expression of BMPs to the most ventral tissues. Although its role in dorsoventral signaling patterning is incompletely known, FGF signaling may also act to oppose BMP signaling. Evidence shows FGF expressed in the dorsal margin of early embryos and repress the initial expression of *bmp2b* and *bmp7* in a manner independent to chordin (Furthauer, Van Celst et al. 2004).

Maternal β-catenin first initiates the dorsal organizer and thus establishes the dorsoventral axis as gastrulation is initiated. Following this, zygotic Wnt signaling is required for the formation of ventral fates. *Wnt8* mutants lack ventral and posterior structures, while mutants of Wnt pathways components, *masterblind/axin* and *headless/TCF3,* lack proper eye and brain formation (Schier and Talbot 2005). Ventral Wnt, together with BMP, is essential

for maintaining transcription of the translational repressors *vox/vent/ved*, which act to restrict the dorsal promoting gene *bozozok* (Ramel, Buckles et al. 2005).

Following the initiation of the dorsoventral axis, through the interplay of the BMP, Wnt, and FGF pathways and their components, other factors, such as the secreted TGFβ family, nodal-related *cyclops* and *squint* are expressed in the dorsal organizer and are critical for the induction of endoderm and mesoderm (Poulain, Furthauer et al. 2006). Continual signaling through the BMP, TGFβ, Wnt, and FGF pathways allows for the progression through gastrulation, into epiboly, and the formation of mature germ layers.

As the dorsal organizer is the site of the initiation of cellular movements and the involution of blastomere cells to form germ layers, it also is the center for cells undergoing an epithelial-to-mesenchymal transition (EMT). EMT is a process in which a cuboidal, polarized, epithelial cell undergoes multiple biochemical changes which enables it to adopt a mesenchymal state, in which it is able to gain cell motility, migratory capacity, and invasiveness (Kalluri and Weinberg 2009). This not only enables cells during embryogenesis and organ development to become motile during gastrulation and germ layer formation, but also can adversely allow for cancer progression through metastatic events.

EMT can be tracked through the molecular changes a cell must undergo in order to transition and become motile. High levels of *E-cadherin*, which helps to maintain cell adhesion, can define the epithelial phenotype. As cells adopt a motile, mesenchymal phenotype, their gene expression signature changes, cell adhesion is lost, and cells gain expression of *N-cadherin*, *vimentin*, and *snail* (Kalluri and Weinberg 2009). This transition is under tight transcriptional and post-transcriptional control. One such regulatory network

involves the transcription factor *snail*. Embryos lacking *snail* fail to gastrulate and accumulating epithelial cells are unable to migrate (Acloque, Adams et al. 2009). Accumulation of *snail* can be through TGFβ transcriptionally inducing *snail* expression, and also through FGF signaling suppressing the activity of GSK-3β which targets *snail* for degradation (Wu, Evers et al. 2009). *Snail* acts to inhibit *E-cadherin* transcription, leading to a disruption in the adherens junctions (Acloque, Adams et al. 2009). Other transcription factors, the ZEB proteins, can also act as repressors of *E-cadherin*. As cells lose polarity, they transition, altering their molecular signature and becoming mesenchymal cells.

Recent work has begun to elucidate roles of microRNAs (miRNAs) in the regulation of epithelial phenotype by controlling EMT inducers (Thiery, Acloque et al. 2009). These small RNAs, about 22 nucleotides in length, provide post-transcriptional control of gene expression in conjunction with the Risc complex by binding 7-8 base pair seed sequences within target mRNAs, usually within the 3' untranslated region (UTR). The miR-200 family of miRNAs acts to repress expression of the ZEB transcription factors, and thus inhibit EMT by allowing for accumulation of *E-cadherin* (Xiong, Jiang et al. 2012). Thus *snail* and the miR-200 family act in opposition to regulate EMT. Maintenance of miR-200 expression prevents differentiation in embryonic stem cells. In these same cells, inhibition of Activin signaling decreases miR-200 and allows for progression of EMT and commitment to a neuroectoderm fate through differentiation (Gill, Langer et al. 2011).

Zebrafish genetic mutants, as well as the use of morpholinos, have been integral *in vivo* models in understanding vertebrate gastrulation, dorsoventral axis formation, and EMT. Here zebrafish *pumilio* protein is shown to play a role in controlling these developmental

events. *pumilio* morphants display impaired gastrulation and shield formation, with consequent misformation of the dorsal organizer, as well as the gene expression signatures necessary for proper formation of the dorsoventral axis. In addition, morphants accumulate *E-cadherin* and miR-200, and loss of *pumilio* inhibits the ability of cells to undergo EMT. It is shown that the Activin receptor *acvr1b* is a target of translational repression by *pumilio*. This suggests *pumilio*, through direct translational repression of *acvr1b*, is able to restrict expression of miR-200 and allow for EMT to progress during gastrulation and early development.

Materials and Methods

Immunofluorescence. Whole-mount immunofluorescence was done according to the Zon Lab's protocol for immunohistochemistry with some modifications. Hand-dechorionated and fixed embryos were permeabilized in cold acetone and washed thoroughly. Embryos were pre-blocked in 2% blocking reagent (Roche), 5% lamb serum, and 1% DMSO for 1 hour. Embryos were then hybridized in primary antibody at 4°C overnight. Embryos were further washed and incubated for 2 hours at room temperature in secondary antibody. Embryos were washed again and nuclear counter-stained with 0.2mg/mL DAPI in PBST. Whole embryos were visualized by confocal microscopy. pSMAD1/5/8 antibody (Cell Signaling) was used at 1:100 with the AlexaFluor 594 conjugated secondary antibody at 1:300.

Subcloning of *acvr1b* 3' UTR. The 3' UTR of *acvr1b* was RT-PCR amplified as previously described, then TA cloned into the pCDNA3.1/NT-GFP TOPO vector according to Invitrogen's protocols. The T7 mMessage machine kit (Ambion) was then used to synthesize RNA for injection, according to protocols.

Site-directed mutagenesis. Plasmids were subjected to QuikChange Lightning Multi-site directed mutagenesis as per the manufacturers protocols (Stratagene). Primers used are listed in Table 2.1.

siRNA transfection. HEK cells were transfected using the Lipofectamine reagent according to manufacturer's protocols (Invitrogen). At 24 hours or 72 hours post-transfection, cell lysates were prepared for either RNA or protein analysis.

qPCR. Isolated RNA was used to make cDNA for real-time quantitative PCR analysis with the Qiagen RT^2 First Strand Kit (Catalog #330401) according to manufacturer's protocols. Synthesized cDNA was then subjected to qPCR analysis using the Qiagen RT^2 SYBR Green Rox qPCR Mastermix (Catalog #330520) with the program: 95°C/ 10'; (95°C/15", 60°C/ 1') x 40; 95C°/15"; 60C°/1'; 95C°/15"; 60C°/15") on the Applied Biosystems 7500 Real-Time PCR System.

miRNA LNA ISH *in situ* hybridization for miRNA was performed as previous described (He, Yan et al. 2011). Digoxigenin-labeled probes were obtained from Exiqon.

TaqMan miRNA qPCR Manufacturers protocols (Applied Biosystems) were followed for Reverse Transcription and PCR set-up. Samples were run with the program: 95°C/ 10'; (95°C/15", 60°C/ 1') x 40; 95C°/15"; 60C°/1'; 95C°/15"; 60C°/15") on the Applied Biosystems 7500 Real-Time PCR System. miR-18a is used as a control small RNA in zebrafish (Lin, Kuo et al. 2008).

Results

Gastrulation is Impaired in pum1 Morphants

The pleiotropic developmental defects of *pumilio* morphants at 3dpf are interesting but do not reveal which specific processes are affected during the course of embryogenesis. Thus, morphant embryos were observed during earlier stages of morphogenesis. Animal pole cells in control embryos at 50% epiboly begin to involute during the beginning phases of gastrulation and form the germ layers. This involution occurs at a structure known as the shield, in the presumptive dorsal side of the embryo. In *pum1* morphants, this shield structure is abnormally formed, indicating that the process of gastrulation is impaired (Figure 3.1 A). Addition, in later bud-stage embryos, the body axis of *pum1* morphants is shortened as compared to controls, consistent with *pum1* depletion causing defective gastrulation (Figure 3.1 B).

Signaling Patterns of Early Axis Specification Genes are Impaired in pum1 Morphants

Earlier RT-PCR experiments showed that *pum1* transcripts can be detected as early as the 1-cell stage, however the spatial expression early in development is unknown. At 50% epiboly, when the shield structure is beginning to form and gastrulation is initiated, *pum1* transcripts are ubiquitously expressed (Figure 3.2 A). While the RNA is global, immunohistochemical staining with *pum1* antibody shows that the protein is specifically expressed in the dorsal pole of the embryo (Figure 3.2 B). These data suggest that while the mRNA is not spatially restricted, *pum1* protein is present specifically within the dorsal organizer, which may account for the apparent role of *pumilio* in gastrulation.

The zebrafish shield structure contains dorsal organizer and axis-inducing activities (Saude, Woolley et al. 2000) that form the beginning of the germ layers and body axis. Precise spatial and temporal gene expression and regulation forms the dorsal organizer, or Spemann organizer, allowing for the establishment of gastrulation and patterning of the body axis (Langdon and Mullins 2011). To examine if expression of dorsal organizer genes is affected by *pum1* loss, *in situ* hybridization was performed for a variety of genes known to be associated with the dorsal organizer.

The expression of the dorsal organizer genes *chordin* (*chd*), *lhx1a, sox 17,* and *goosecoid* (*gsc*) at 50% epiboly was investigated by *in situ* hybridization (Figure 3.2 C-J). Consistent with the gross morphological finding that the shield structure was misformed, gene expression within the dorsal organizer is disrupted in *pum1* morphants as compared to controls. In control embryos, *chd* and *gsc* are specifically expressed within the dorsal organizer, while *lhx1a* and *sox17* are transcribed along the blastula margin, where the animal

pole meets the vegetal pole, as well as enriched within the dorsal organizer. In morphants, this enrichment within the dorsal organizer is lost, and a marked reduction in expression along the blastula margin is evident. Expression of *chd*, however, is unaffected by *pum1* loss.

TGFβ/BMP Pathway Activity is Increased in pum1 morphants

The TGFβ/BMP signaling pathway is central to establishment of the dorsal-ventral axis and gastrulation events during early embryogenesis, and is enriched at the presumptive ventral side of the embryo (Langdon and Mullins 2011). With reduction of the dorsal signals, it was hypothesized that *pum1* morphants would display an expansion of ventral *bmp2b* gene expression. *In situ* hybridization confirms expanded ventral BMP signals in morphants as compared to controls (Figure 3.3 A). To determine if overexpression of *pum1* would have the opposite effect on the dorsal-ventral signaling gradients, embryos injected with *pum1* RNA were analyzed by *in situ* as well. Overexpression of *pum1* leads to a near complete loss of *bmp2b* expression. Thus, *pum1* morphants show reduced dorsal organizer gene expression, while *pum1* overexpression causes expansion of the dorsal organizer and accompanying reduction of ventral signals.

BMP pathway activation can be analyzed by assessing increasing nuclear levels of the phosphorylated form SMAD1/5/8 (Figure 3.3 B). In control embryos, detection of pSMAD1/5/8 is highest in the presumptive ventral side of the embryo, and this phosphorylation tails off in a gradient fashion as presumptive dorsal tissues are approached, consistent with the expression of *bmp2b* as observed by *in situ* hybridization. In *pum1* morphants, pSMAD1/5/8 can be detected well within dorsal embryonic tissues. This is

consistent with the expansion of the *bmp2b* expression domain as seen by *in situ* and indicates that the domain of BMP pathway is expanded dorsally in morphants. To confirm these results, expression of downstream BMP pathway targets were investigated by qPCR analysis (Figure 3.3 C). Targets of the pathway, *ID1*, *TGIF1*, and *serpine1*, are increased in *pum1* morphants, again confirming ectopic activation of the TGFβ/BMP pathway and target gene expression.

The epithelial-to-mesenchymal transition is inhibited in pum1 morphants

Formation of the shield, involution of cells, and the creation of germ layers during gastrulation is dependent on cells undergoing an epithelial-to-mesenchymal transition (EMT) (Montero, Carvalho et al. 2005; Solnica-Krezel 2005; Nakaya and Sheng 2008), requiring cells to change their cellular morphology, lose adherence to each other, and become more motile. To investigate if EMT is impaired in *pum1* morphants, expression of *snail*, an indicator of EMT, was investigated. In control samples at 50% epiboly, *snail* expression can be detected along the blastula margin and is enriched within the dorsal organizer (Figure 3.4 A). In *pum1* morphants, the expression of *snail* is reduced both along the blastula margin, as well as within the dorsal organizer. Conversely, in samples injected with *pum1* RNA, *snail* expression is expanded.

To confirm that the changes in *snail* expression indicated a block in EMT in morphants, qPCR was used to analyze the expression profiles of known markers of EMT (Figure 3.4 B). *E-cadherin* is highly expressed in epithelial cells, and as cells transition through EMT, *E-cadherin* is down regulated. In the mesenchymal state, cells more highly

express *N-cadherin* and *vimentin*. qPCR analysis reveals *pum1* morphant embryos are enriched for *E-cadherin* as compared to controls, and additionally have decreased expression of *N-cadherin* and *vimentin*. Thus, *pum1* morphant cells are unable to properly undergo EMT and cells remain in the epithelial state.

pumilio Targets Genes Expressed During Early Gastrulation

The identified function of *pumilio* as a translational repressor suggests it binds target mRNA sequences during gastrulation and alters gene expression, thus leading to the observed phenotypes. Functional targets of *pumilio*, however, are largely unknown in vertebrates and fewer have been validated *in vivo*. To investigate putative targets of translational repression by *pumilio*, genes expressed from the 1-cell stage to gastrulation (50% epiboly) were extracted from the expression database on www.zfin.org, and genes were annotated to the human genome. This list of genes was then compared to the published RIP-seq data set of mRNA sequences associated with PUM1 and PUM2 in HeLa S3 cells (Galgano, Forrer et al. 2008) (Figure 3.5 A). This revealed a list of putative targets of *pumilio* during gastrulation (Figure 3.5 B). Further analysis uncovered *pumilio* Binding Elements (PBEs) in the 3' UTRs of several candidate target genes. Of the 6 genes identified, Activin A Receptor, type 1b (*acvr1b*), dual specificity phosphatase 6 (*dusp6*), and nuclear receptor coactivator 3 (*ncoa3*) stand out due to their involvement in the TGFβ/BMP pathway and for roles in the epithelial-to-mesenchymal transition (EMT), a processes imperative for gastrulation and organogenesis.

acvr1b is a Target of Translational Repression by pum1

To validate the identified genes as *in vivo* targets of *pum1*, both *in situ* hybridization and qPCR methods were utilized. The overall change in RNA levels of the putative targets when *pum1* was lost by morpholino injection, or when *pum1* was overexpressed by RNA injection, was compared. Each identified target, *acvr1b*, *dusp6* and *ncoa3*, was significantly increased when repression by *pum1* was lost with morpholino injection (Figure 3.6 A). However, only levels of *acvr1b* are significantly decreased when *pum1* was overexpressed by RNA injection, suggesting it may be a more tightly regulated target of *pum1*.

The local expression of *acvr1b* during gastrulation, at 50% epiboly, is along the circumference of the blastula margin, as well as enriched within the dorsal organizer (Renucci, Lemarchandel et al. 1996). The expression of *dusp6* at the same stage is enriched in the presumptive dorsal tissues and is decreased approaching the ventral tissues in a gradient pattern (Molina, Watkins et al. 2007). The expression of *ncoa3* is also around the blastula margin in early zebrafish embryos. An antisense riboprobe to *acvr1b* was designed, and the Tsang lab graciously provided a probe to dusp6. Expression of *ncoa3* was not directly tested by *in situ*. As compared to controls, and consistent with qPCR observations, the expression of *acvr1b* was greatly increased in *pum1* morphants (Figure 3.6 B-D). Additionally, in overexpressed embryos, *acvr1b* was significantly decreased such that there is inconsistent staining along the blastula margin. The expression of *dusp6*, however, was inconsistently stained during multiple experiments such that no significant changes were observed between control embryos and *pum1* morphants. During the time period examined (gastrulation), only *acvr1b* appears as an *in vivo* target of *pum1* in zebrafish by these

experiments, as its levels change when the level of *pumilio* protein is modulated. Within the sensitivity of these assays, consistent effect of *pum1* knockdown on *dusp6* or *ncoa3* was not detected. Therefore, further analysis was focused on the regulation of *acvr1b* by *pum1* and its subsequent effects on gastrulation.

pum1 acts directly on the 3' UTR of acvr1b through its Binding Elements

pumilio, with its alpha-helical repeats, is able to recognize consensus sequences within the 3' UTR of target transcripts to exhibit translational control (Wickens, Bernstein et al. 2002). To investigate a direct relationship between *pum1* and *acvr1b*, the zebrafish 3' UTR of zebrafish *acvr1b* was subcloned into a GFP reporter construct. In this manner, protein levels of GFP will act as a reporter for *pum1* regulation of the *acvr1b* 3' UTR. The lack of available antibodies for zebrafish proteins made an investigation on the endogenous *acvr1b* difficult. *In vitro* transcribed RNA from the subsequent vector was injected into zebrafish embryos, along with *pum1* morpholino or *pum1* RNA and protein was isolated and subjected to western blotting. As predicted, *pum1* negatively regulates the 3' UTR of *acvr1b*, as co-injection of the vector with *pum1* morpholino increases levels of GFP as compared to injection of the vector alone (Figure 3.7 A-B). This indicates the loss of translational repression by *pum1* on the transgenic RNA construct. In contrast, when the vector is co-injected with *pum1* RNA, GFP levels are significantly decreased. Injection of the transgenic vector with control morpholino or negative control RNA (mCherry RNA) did not lead to similar changes in GFP levels.

Within the *acvr1b* 3' UTR, three putative *pumilio* Binding Elements (PBEs) were identified based on the conserved PBE sequence (UGUAN(N)AGA). To validate these as the location of *pum1* binding on the target, site-directed mutagenesis was utilized to scramble the PBEs within the created transgenic vector. RNA from the resulting vector was then similarly injected into embryos and immunoblotting showed an increased level of GFP from the mutated vector as compared to the original, non-mutated vector. Additionally, this increase of GFP when the PBEs are mutated is similar in magnitude to the increase of GFP when *pum1* is lost by morpholino injection (approximately 2.5-fold increase), showing *pum1* actively binds at the PBEs in the 3' UTR of *acvr1b* to translationally repress this target.

miR-200a acts on acvr1b and inhibits EMT

Previous studies have implicated *pumilio* as a cooperating partner in microRNA (miRNA) mediated gene regulation. Post-transcriptional control of p27 requires *pumilio* binding to the 3' UTR, which induces a conformational change in the mRNA secondary structure, to expose the miR-221 and miR-222 target sites allowing for efficient suppression of p27 expression (Kedde, van Kouwenhove et al. 2010). Similarly, PUM1 acts cooperatively with miR-410 to allow a second level of regulation to targets (Leibovich, Mandel-Gutfreund et al. 2010). Computer modeling of the 3' UTR of *acvr1b*, by RNAFold, reveals a highly conserved miR-200a binding site near a PBE (Figure 3.8). miR-200a acts to inhibit EMT and promote the epithelial state by targeting the *E-cadherin* repressors ZEB1 and ZEB2 (Park, Gaur et al. 2008). The control that miR-200a exhibits on EMT is also extended to a signaling network including TGFβ allowing for maintenance of EMT critical for embryonic

development (Gregory, Bracken et al. 2011). Additionally, miR-200a, a target of Activin signaling, acts in opposition of *snail* to regulate EMT and germ layer specification in embryonic stem cells.

This work thus far has shown that many of the key miR-200a targets are also subjected to regulation by *pumilio*. This led to the hypothesis that *pum1* may cooperate with miR-200a to regulate *acvr1b* expression and EMT during gastrulation. Expression of miR-200a early in development is ubiquitous, as detected by *in situ* hybridization utilizing a DIG-labeled LNA probe (Figure 3.9 A). To determine if miR-200a targets *acvr1b in vivo*, as predicted *in silico*, a miR-200a mimic (Qiagen) was injected and embryos were subjected to *in situ* hybridization with an antisense riboprobe to *acvr1b* (Figure 3.9 B-C). Resulting staining reveals that with ectopic miR-200a, *acvr1b* is nearly absent from the blastula margin, showing miR-200a acts to inhibit *acvr1b* expression in zebrafish embryos. To investigate if miR-200a acts in embryos to inhibit EMT, embryos injected with the miR-200a mimic were stained by *in situ* hybridization with a probe against *snail* (Figure 3.9 D-E). Decreasing levels of *snail* with ectopic miR-200a is consistent with a defect in EMT.

As miR-200a expression affects EMT and *acvr1b*, it was questioned whether *pum1* levels have an effect on the level of miR-200a. qPCR analysis of RNA isolated from embryos injected with either *pum1* morpholino or RNA revealed that altering *pum1* expression alters levels of miR-200a (Figure 3.9 F). This induction of miR-200a expression with *pum1* loss may be a consequence of enhanced *acvr1b* expression, as it has been previously described that Activins induce miR-200a expression (Gill, Langer et al. 2011). To confirm this, *acvr1b* RNA was *in vitro* transcribed and injected into embryos. Subsequent qPCR analysis revealed

that increasing levels of *acvr1b in vivo* induces miR-200a expression (Figure 3.9 G). Thus, in early embryos, *pum1* acts along with miR-200a to fine-tune *acvr1b* expression at the dorsal organizer allowing for EMT and gastrulation.

Human Cells Respond to Modulation of pumilio Similarly

Work investigating targets of *pumilio* have uncovered roles in regulating cancer-related genes such as p27 (Kedde, van Kouwenhove et al. 2010) and the E2F3 oncogene (Miles, Tschop et al. 2012). Together with the finding that *pumilio* is overexpressed in a variety of cancer cell types (Spassov and Jurecic 2002), these data suggest that understanding *pumilio* function may have therapeutic implications.

To examine if the functions outlined for *pum1* in zebrafish are conserved in humans, I utilized transient transfections in human embryonic kidney (HEK) cells. Upon transfection of siRNA-PUM1, RNA levels of ACVR1B, as observed by qPCR, were significantly increased (Figure 3.10 A). Additionally, western blot analysis confirmed that the loss of ACVR1B RNA leads to a protein loss (Figure 3.10 B-C). With help from Dr. Abhay Shukla, I also transfected a luciferase reporter vector incorporating the human ACVR1B 3' UTR along with siRNA to PUM1. PUM1 knockdown significantly increases the detected levels of luciferase as compared to controls (Figure 3.10 D). These data from human cells are consistent with the observed control regulation of *acvr1b* by *pum1* in zebrafish.

pum1 regulated EMT in zebrafish embryos. To test whether human PUM1 or PUM2 exhibits similar control, mammary epithelial ductal carcinoma cells (578T cells) were transfected with siRNA-PUM1 or siRNA-PUM2. In negative control, no treatment samples,

cells in the epithelial state are able to properly undergo EMT, change cell morphology as indicated by phalloidin staining actin at the cell membrane, and enter the mesenchymal cell state (Figure 3.10 E). A positive control, siRNA-CDC42, causes loss of a gene required for EMT, and cells remain in the epithelial state, as evident from their cuboidal morphology. Upon siRNA-PUM2, cells are unable to undergo EMT and remain in the epithelial state, similar to *pum1* zebrafish morphants.

Pumilio is a Transcriptional Target of wt1a

While few targets of *pumilio* have been validated *in vivo*, and the function of *pumilio* in various cell types remains somewhat a mystery, regulators of *pumilio* expression are even less well-understood. The developmental defects of *pumilio* morphants are reminiscent of the phenotypes caused by knockdown of *wt1a*, a zebrafish ortholog of the Wilms Tumor suppressor gene (Perner, Englert et al. 2007). Another study showed impaired expression of PUM1 and PUM2 in the urogenital ridges of WT1 null mice (Klattig, Sierig et al. 2007). WT1 is a transcription factor with both tumor suppressor and oncogenic properties, and is also a driver of EMT (Chau and Hastie 2012). To validate *pumilio* as a transcriptional target of *wt1a*, the functional homolog in zebrafish (Perner, Englert et al. 2007), *wt1a* morpholino was injected into embryos and RNA was isolated for qPCR (Figure 3.11 A). Subsequent analysis reveals that in *wt1a* morphants, *pum1* and *pum2* are expressed at lower levels as compared to controls, implicating them as transcriptional targets of *wt1a*.

WT1 is necessary for formation of the kidney and gonad. To investigate if this function of WT1 depends on *pumilio*, the ability of *pumilio* morphants to form kidneys was

assayed. *nephrin* is expressed in the embryonic kidney by 28hpf. *wt1a* morphants, as assessed by *nephrin* staining, lack kidney formation (Perner, Englert et al. 2007). However, *pum1* morphants show bilateral kidney structures formed at 2dpf (Figure 3.11 B). Thus the kidney defects seen in *wt1a* morphants are independent of *pum1*.

Pumilio and WT1 Correlate in Human Tumor Samples with Corresponding Targets Repressed

Occurrence of Wilms tumor is associated with a germline mutation in the WT1 gene in only a low percentage of cases (Li, Breslow et al. 1996) and WT1 mutations have been found some cases of acute myeloid leukemia (AML) (King-Underwood, Renshaw et al. 1996). While WT1 acts as a tumor suppressor in several studies, it also may function as an oncogene in different settings (Hohenstein and Hastie 2006).

As *pumilio* is over expressed in a variety of cancers and hematopoietic stem cells (Spassov and Jurecic 2003), the relationship between WT1 and *pumilio* may have implications in understanding and treating cancers. To analyze whether this relationship is conserved in samples of human cancers, pediatric Wilms tumors and matched normal kidney samples were obtained from Children's Medical Center. qPCR analysis reveals that in each of the 4 samples, WT1, PUM1, and PUM2 were correlated (Figure 3.12). Whereas 3 samples show increased transcription of these genes, only one showed reduction in WT1 and Pumilio levels. It is also notable that in each of the samples, levels of PUM1 and PUM2 are inversely correlated with their target ACVR1B, suggesting that the relationship between WT1, PUM1, and ACVR1B may have implications in cancer maintenance or progression.

Discussion

Few targets of *pumilio* in vertebrates are known, and those targets that have been well characterized in invertebrates, have not been validated in other systems. This work focused on identifying vertebrate *in vivo* binding targets of *pum1* that cause the various developmental defects seen previously. The gross morphological defects seen at 3dpf are consistent with an improper gastrulation event, resulting from *pum1* loss. Additionally, these morphant embryos also exhibit cell cycle abnormalities, poor vasculature formation, and abnormal gonadogenesis. *acvr1b* was characterized as a binding partner of *pum1* and shown to be important in regulating the expression of miR-200a in early development, and thus the EMT and cell involution events of gastrulation.

pum1 protein is localized to the dorsal organizer, where it restricts *acvr1b* expression. As a consequence, dorsal organizing genes such as *gsc*, *sox17* and *lhx1a* have decreased expression in morphants. These genes in themselves facilitate EMT events, as *gsc* is able to repress *E-cadherin* and is elevated in breast cancer cells to promote tumor metastasis (Hartwell, Muir et al. 2006). As a consequence of diminished dorsal signals, ventral *bmp2b* expression is expanded into dorsal tissues activating ectopic pSMAD1/5/8 and downstream BMP pathway target genes. While TGFβ induces EMT in mammary gland epithelial cells (Xie, Law et al. 2004), BMP2 is able to attenuate *snail* expression and reverse EMT (Yang, Ju et al. 2011). Thus, expanded *bmp2b* expression may further attenuate EMT in *pum1* morphants.

The ability of BMP to inhibit or reverse EMT may also be a result of driving miR-200 family transcription (Samavarchi-Tehrani, Golipour et al. 2010). This miRNA, which may act as a tumor suppressor by inhibiting EMT, is downregulated in a variety of human cancers facilitating tumor cell invasion and metastasis, and is associated with an aggressive cancer cell phenotype (Mongroo and Rustgi 2010). Additionally, p53, which acts to safeguard the epithelial phenotype, has been shown to activate miR-200 transcription (Chang, Chao et al. 2011). In the absence of p53, its isoforms p63 and p73 directly regulate this miRNA and are themselves overexpressed (Hill, Browne et al. 2013). This relationship may help in understanding the connection between *pum1* and p53, as the combined loss of both proteins leads to more severe developmental defects in early experiments shown here, possibly by the combination of two distinct pathways both contributing to overexpression of miR-200.

The role of miRNAs in zebrafish development is an emerging field; previous work has uncovered role of let-7 function in early development (Kloosterman, Wienholds et al. 2004). As more work illustrates the expression of miRNA families early in development, the coordination of this information with seed sequences located in targets also expressed in early gastrulation will uncover novel mechanisms for both spatial and temporal gene regulation during development. Highlighted in the work presented here are the synchronization of a ubiquitously expressed miRNA with a locally expressed protein, both with a common target. In other systems, miRNAs are able to restrict organizer size by directly targeting another Activin receptor, *acvr2a* (Martello, Zacchigna et al. 2007). Further investigation on the relationship between *pum1* and miR-200a will show if they are either necessary or sufficient to regulate *acvr1b*.

Data presented here illustrates that the *pumilio* binding elements in the 3' UTR of target genes are necessary for *pumilio*'s regulatory effects. Preliminary work suggests that the miR-200 seed sequence is also necessary for inhibition of the transcript (data not shown). Also, understanding if the two repressors play a combined role (ie. *pum1* allows access of miR-200a to its seed sequence), or an additive role (ie. both *pum1* and miR-200a independently repress *acvr1b*) will help to illuminate further mechanisms of *pumilio* action.

The finding that, in human cells, PUM1 similarly regulates ACVR1B is critical. It not only shows that this relationship is evolutionarily conserved, but also that the zebrafish model can accurately recapitulate human molecular pathways in an *in vivo* setting. Early gastrulation, cell movements, and germ layer formation combine various cell types, intra-and extra-cellular signaling, and interactions. As tumor cells moving through the body undergo similar processes, the zebrafish offer a simple model with which to dissect complicated human disease processes. As the interaction between Activins, miR-200, and EMT has been shown in embryonic stem cells (Gill, Langer et al. 2011), it is plausible that this may be a developmental program that neoplasms reactivate. Indeed, in renal childhood cancers, miR-200 family members are downregulated and consequently their target ACVR2B is strongly expressed (Senanayake, Das et al. 2012). A similar finding can be hypothesized for ACVR1B. Since *pumilio* is overexpressed in many cancer cells, further studies may find these cells drive EMT and metastasis through the agency of PUMILIO, via its effects on ACVR1B and the miR-200 family.

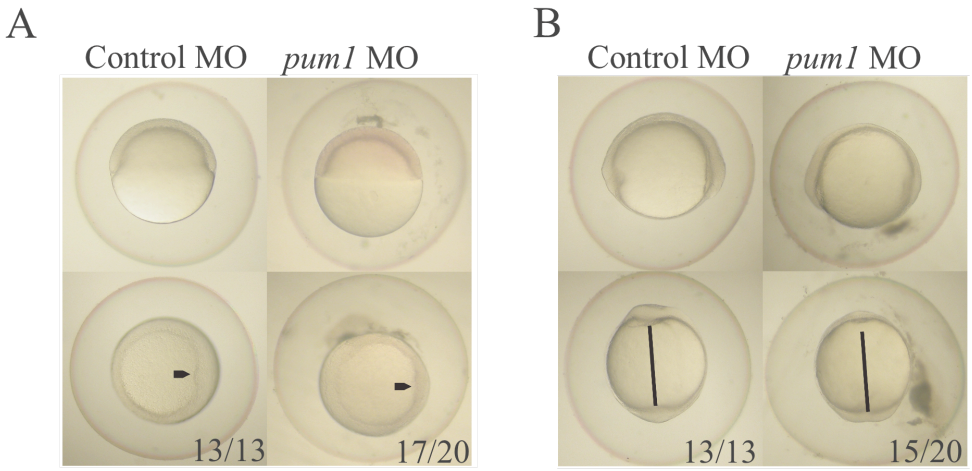

Figure 3.1: *pum1* morphants display impaired gastrulation
A: 1-cell stage wild type embryos were injected with *pum1* MO and analyzed over the course of development. At 50% epiboly, formation of the shield (arrow head) can be detected in control embryos. In morphants, however, formation of the shield is impaired. Top row is viewed animal pole to the top, vegetal pole to the bottom, dorsal to the right, and ventral to the left. Bottom row is viewed from the animal pole, dorsal to the right and ventral to the left. B: Later in development, at Bud Stage, *pum1* morphant embryos display shortened axis as compared to controls. A black bar in control embryos marks the distance between the early forming head and tail across the yolk. In morphant embryos, this distance is enlarged owing to failure of the embryo to extend fully over the dorsal surface of the yolk. Top row is viewed animal pole to the top, vegetal pole to the bottom, dorsal to the right, and ventral to the left. Bottom row is viewed from the vegetal pole (left side) or the ventral surface (right side).

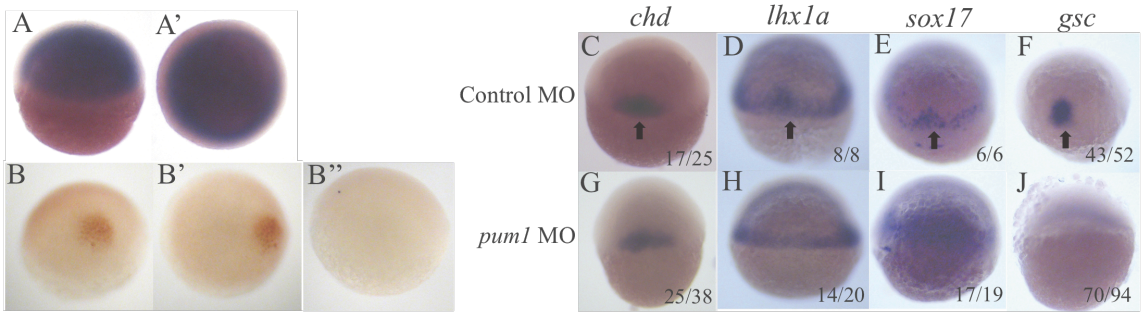

Figure 3.2: Gene expression within the dorsal organizer is altered in *pum1* morphants
A, A': *in situ* hybridization with an antisense riboprobe to *pum1* in 50% epiboly embryos shows RNA wide-spread throughout the animal pole of the embryo. A is viewed with the animal pole to the top, vegetal pole to the bottom. A' is viewed from the animal pole.
B, B', and B'': Immunohistochemical staining with an antibody targeting *pum1* highlights protein localization in the dorsal organizer in 50% epiboly embryos. B is visualized from the dorsal pole, animal pole to the top, and vegetal pole to the bottom. B' is visualized from the animal pole, dorsal to the right and ventral to the left. B'' is a negative control.
C-J: Patterning of the early embryo is controlled by dynamic signaling gradients and patterns. In control embryos, *chordin* (C) *lhx1a* (D), *sox17* (E), and *gsc* (F) are expressed in the dorsal organizer (arrow). Additionally, *lhx1a* and *sox17* are expressed along the blastula margin. In *pum1* morphants, the expression of most genes is disrupted. The expression of *chordin* (G) is unaffected in *pum1* morphants. Expression of *lhx1a* (H), *sox17* (I), and *gsc* (J) is lost in the dorsal organizer and decreased along the blastula margin in *pum1* morphants. Embryos are visualized from the dorsal pole, animal pole to the top, and vegetal pole to the bottom.

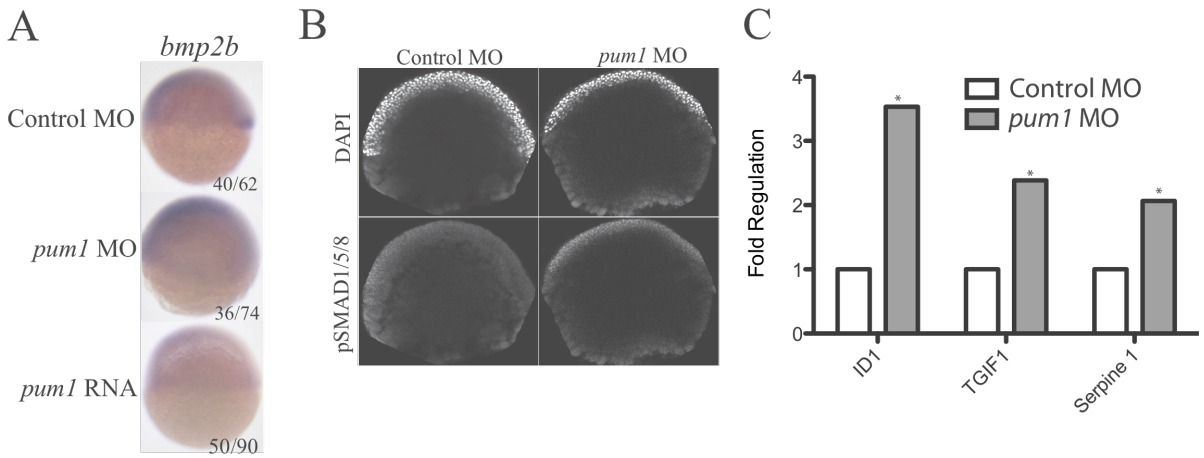

Figure 3.3: *pum1* morphants exhibit increased BMP pathway activity
A: *bmp2b* is highly expressed in ventral tissues, as seen in the control embryos, and this expression falls off in presumptive dorsal tissues in 50% epiboly embryos. In *pum1* morphants, *bmp2b* expression is expanded into the presumptive dorsal tissues. Conversely, *pum1* over expression causes near complete loss of *bmp2b* expression. Embryos are visualized animal pole to the top, dorsal to the right and ventral to the left.
B: Immunofluorescence illustrates an expanded domain of pSMAD1/5/8. Nuclear pSMAD1/5/8 can be detected in the presumptive ventral pole of embryos at 50% epiboly in control samples. In *pum1* morphants, detection of pSMAD1/5/8 is expanded well into the presumptive dorsal tissues. DAPI shows cell nuclei of the animal pole. Embryos are visualized animal pole to the top, dorsal pole to the right, and ventral pole to the left.
C: qPCR analysis of TGFβ/BMP pathway target genes shows that *pum1* morphants exhibit ectopic activation of downstream target genes. As compared to controls, *pum1* morphants have significantly increased levels of *id1*, *tgif1*, and *serpine1*. *$p < 0.005$.

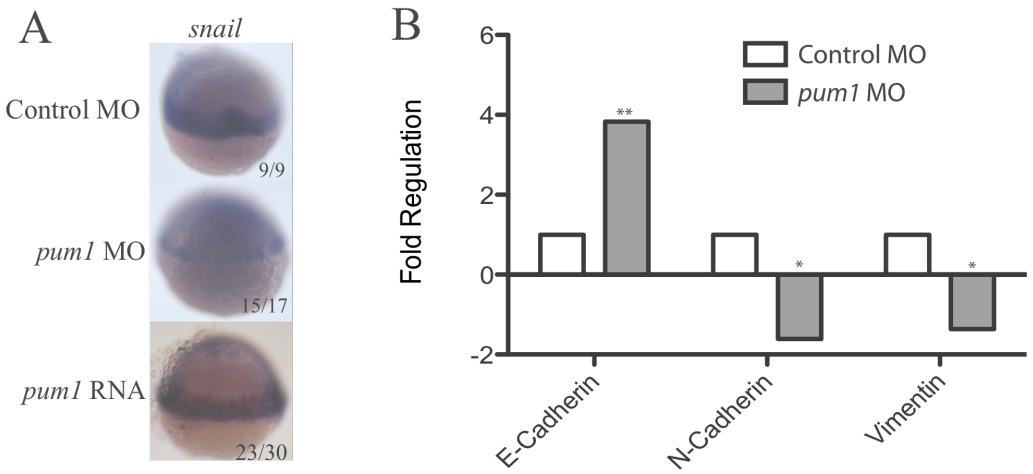

Figure 3.4: *pum1* allows for progression through EMT
A: *snail* expression illustrates EMT in 50% epiboly embryos, In control embryos, *snail* expression is along the blastula margin and enriched within the dorsal organizer, the site of active EMT during gastrulation. In *pum1* morphants, *snail* expression is markedly reduced. When *pum1* is over expressed by mRNA injection, *snail* expression is increased both within the dorsal organizer and along the blastula margin. Embryos are visualized from the dorsal pole, with animal pole to the top.
B: qPCR analysis of *pum1* morphants reveals a block in EMT. *E-cadherin* acts as a marker for epithelial cells, whereas *N-cadherin* and *vimentin* act as markers for mesenchymal cells. In *pum1* MO samples, there is an increase in *E-cadherin* levels and a corresponding decrease in *N-cadherin* and *vimentin* signals as compared to controls. This indicates *pum1* morphants are unable to properly transition through EMT. *p< 0.05, **p< 0.001.

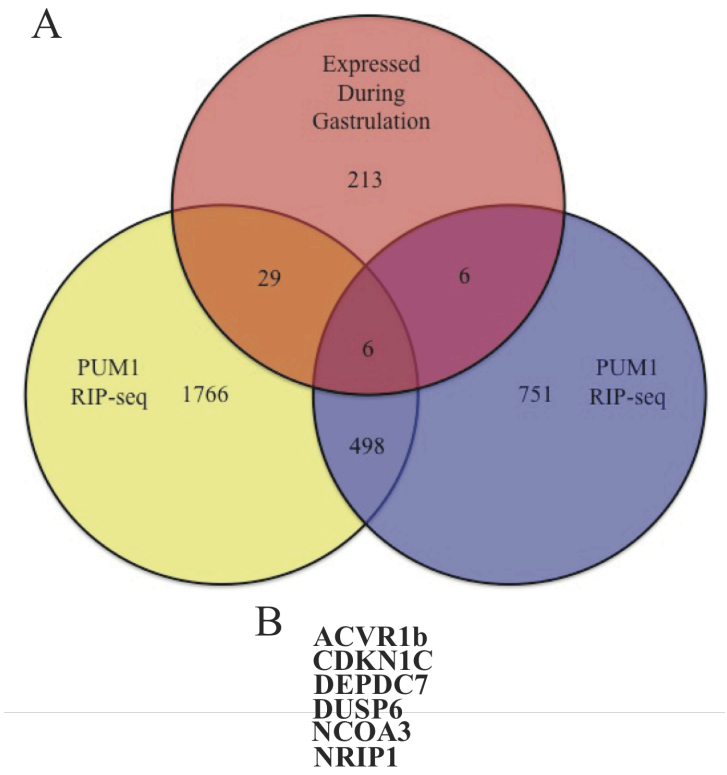

Figure 3.5: A subset of genes are expressed during gastrulation and a target of *pumilio*
A: Venn diagram shows genes both expressed during gastrulation and predicted targets of PUM1 and PUM2. Red circle contains genes expressed during zebrafish gastrulation. Yellow circle contains genes predicted to be binding targets of PUM1 in HeLa cells. Blue circle contains genes predicted to be binding targets of PUM2 in HeLa cells. 6 genes are common between the groups.
B: Listing of the 6 genes that are predicted targets of both PUM1 and PUM2, as well as expressed during gastrulation in zebrafish.

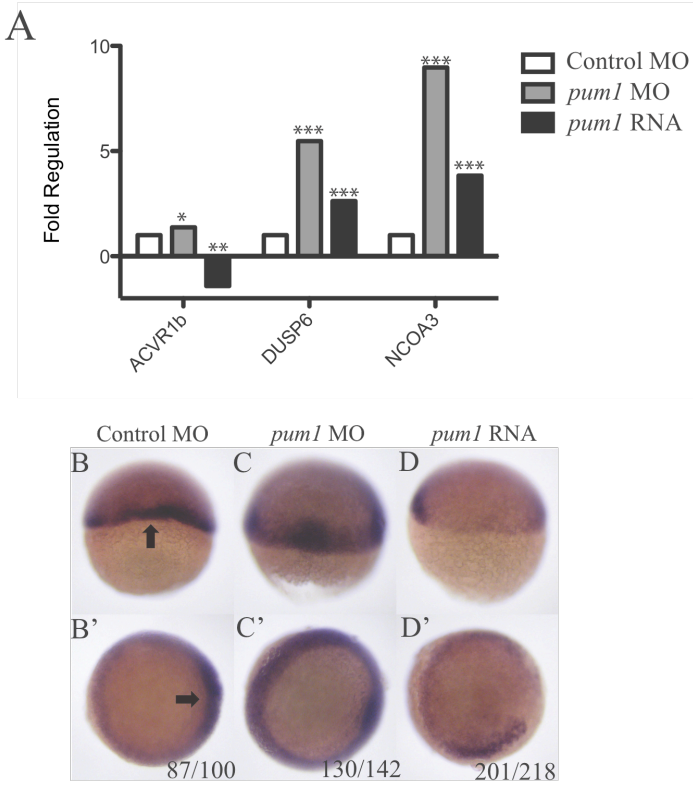

Figure 3.6: *acvr1b* is a target of *pum1* during early zebrafish development
A: qPCR analysis of three predicted target genes of *pumilio*. *pum1* MO samples have elevated levels of *acvr1b*, *dusp6*, and *ncoa3* as compared to controls. However, overexpression of *pum1* by RNA injection only decreases levels of *acvr1b*. *p< 0.05, **p<0.01, ***p<0.005.
B-D: *in situ* hybridization with an antisense riboprobe to *acvr1b* shows modulating levels of *pum1* alters *acvr1b* expression at 50% epiboly. Control embryos show *acvr1b* along the blastula margin and enriched within the dorsal organizer (arrow) (B, B'). *pum1* MO embryos show expansion of *acvr1b* transcription along the blastula margin and within the dorsal organizer (C, C'). When *pum1* RNA is injected, *acvr1b* levels are decreased and there is incomplete expression along the blastula margin (D, D'). B, C, and D are viewed from the dorsal side, animal pole to the top. B', C', and D' are viewed from the animal pole, dorsal to the right.

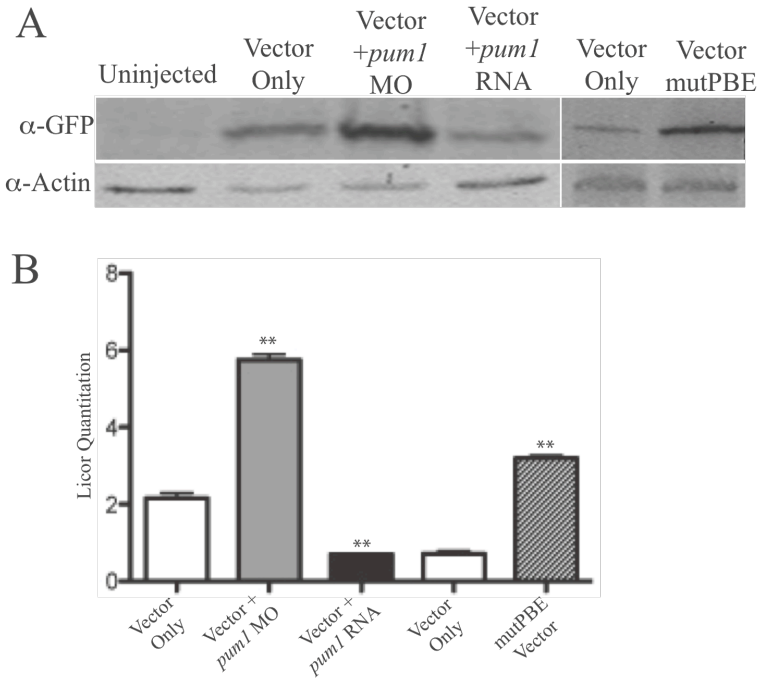

Figure 3.7: *pum1* acts through the *pumilio* Binding Elements within the 3' UTR of *acvr1b* to post-transcriptionally repress *acvr1b* expression

A: Protein isolated from injected embryos was subjected to Western Blot analysis. Injection of the transgenic vector RNA only produces a basal level of detectable GFP. When co-injected with *pum1* MO, GFP detection is increased. Co-injection with *pum1* RNA decreases GFP. If the PBEs are mutated in the vector, GFP is increased as compared to unmutated vector, showing *pum1* acts on the 3' UTR through its binding elements to repression translation of the target.

B: Quantification of the above Western Blot. **$p<0.005$.

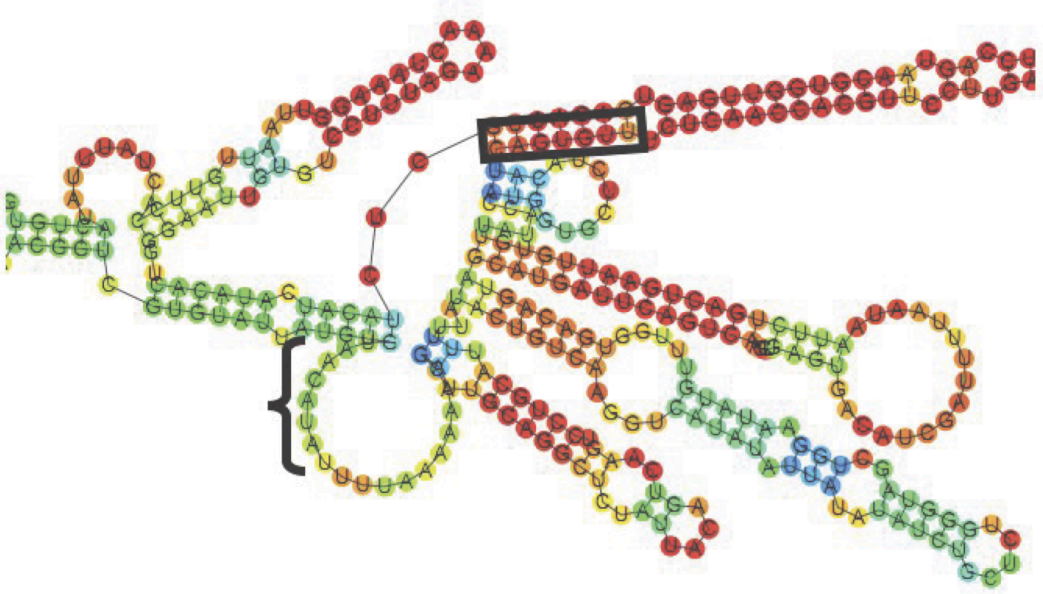

Figure 3.8: *pumilio* binds its target near a miR-200a seed sequence
Analysis of the secondary structure of the 3' UTR of *acvr1b* reveals a putative *pumilio* Binding Element (PBE) (bracket, sequence: UGUAACAUA) near a miR-200a binding site (box, sequence: CAGUGUU), suggesting *pumilio* and miR-200a may cooperate to regulate *acvr1b*.

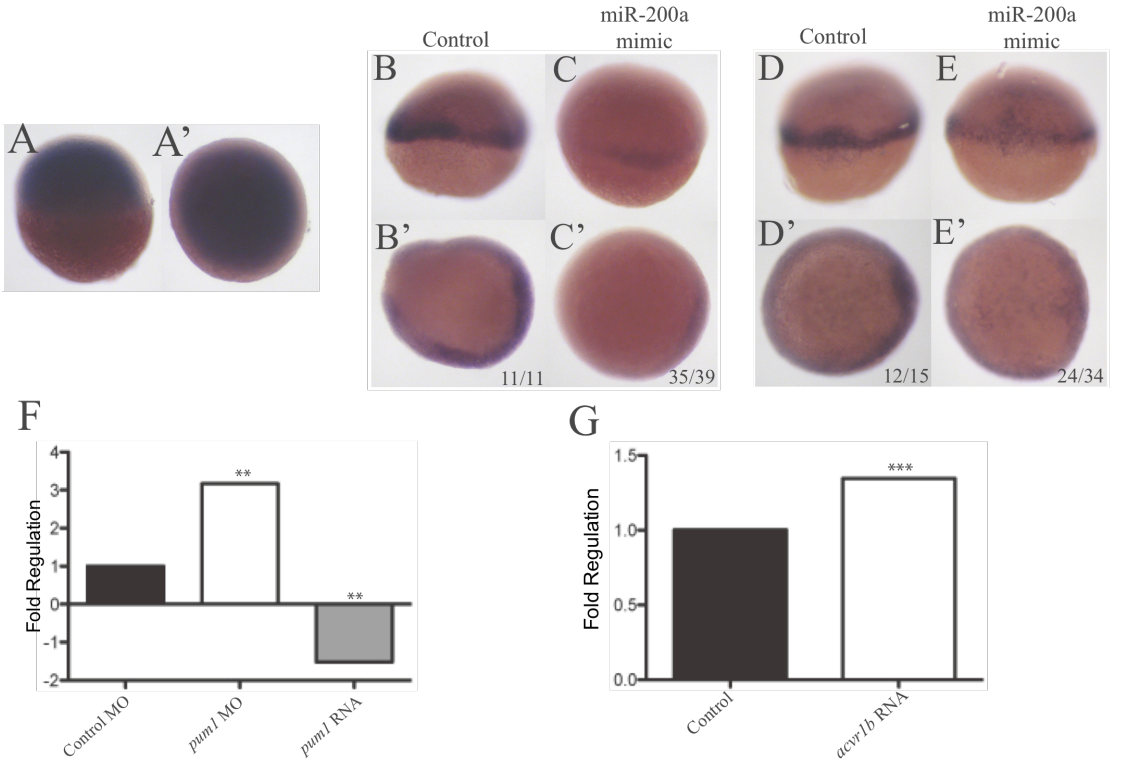

Figure 3.9: miR-200a targets *acvr1b* and inhibits EMT
A, A': *in situ* hybridization with a DIG-labeled LNA probe to miR-200a shows expression is ubiquitous at 50% epiboly. A is visualized animal pole to the top and vegetal pole to the bottom. A' is visualized from the animal pole.
B-C: *in situ* hybridization with an antisense riboprobe to *acvr1b* shows injection of a miR-200a mimic reduces levels of *acvr1b* as compared to controls at 50% epiboly. B and C are visualized from the dorsal side, animal pole to the top. B' and C' are visualized from the animal pole, dorsal to the right.
D-E: *in situ* hybridization with an antisense riboprobe to *snail* shows injection of a miR-200a mimic reduces levels of *snail* as compared to controls. *snail* is used as a marker of EMT. Reduced *snail* indicates a block in EMT when excess miR-200a is present. D and E are visualized from the dorsal side, animal pole to the top. D' and E' are visualized from the animal pole, dorsal to the right.
F: qPCR analysis shows that modulation of *pum1* levels alters expression of miR-200a. As compared to controls, *pum1* MO samples show an increase in miR-200a levels, while overexpression of *pum1* by mRNA injection reduces miR-200a levels. **$p<0.005$.
G: qPCR analysis shows injection of human *acvr1b* mRNA increases the levels of miR-200a, consistent with the model that the control of miR-200a by *pum1* is through *acvr1b*. ***$p<0.0005$.

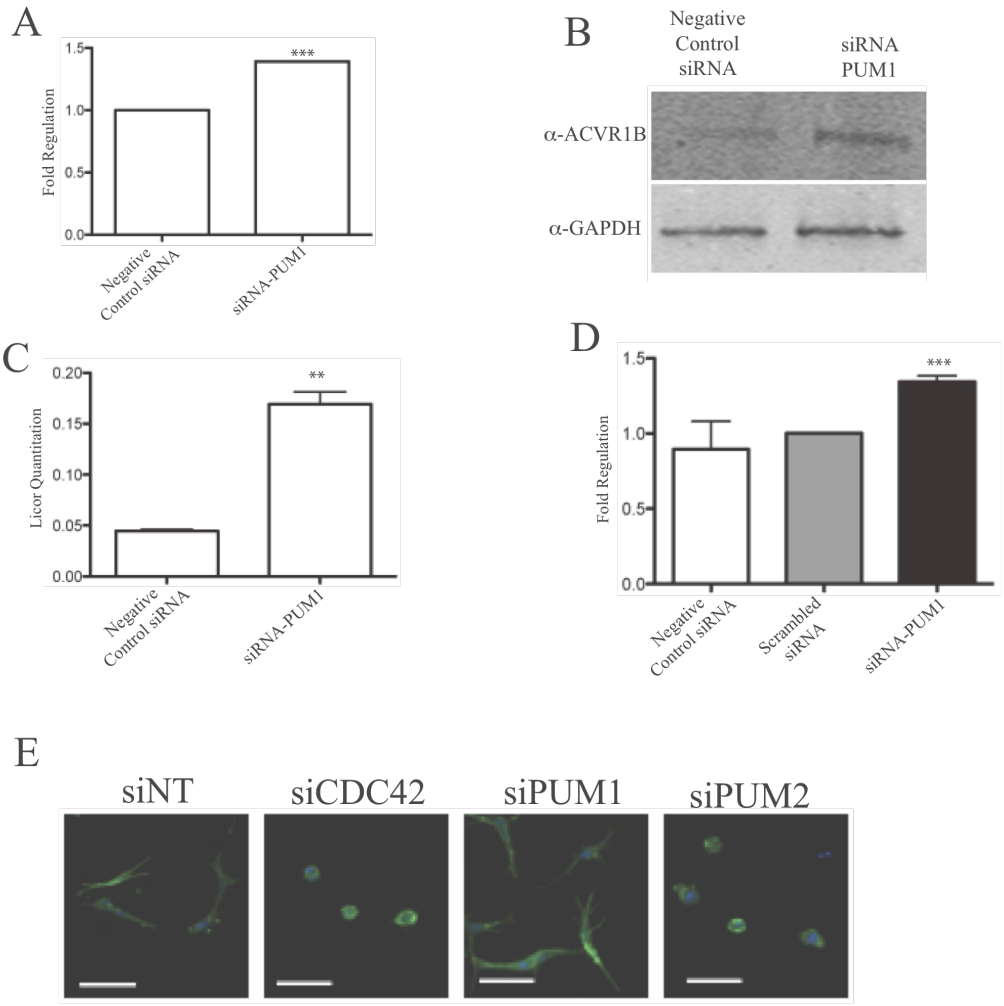

Figure 3.10: PUM1 acts similarly in human cells to target ACVR1B and inhibit EMT
A: qPCR analysis of cells transfected with siRNA-PUM1 shows increased levels of ACVR1B total RNA. ***$p<0.0005$.
B: siRNA-PUM1 causes increased levels of ACVR1B protein by immunoblot as compared to control. GAPDH shows equal loading of samples.
C: Quantification of the immunoblot in B, showing the increase in ACVR1B protein under siRNA-PUM1 conditions as compared to controls. **$p<0.001$.
D: Luciferase reporter construct with subcloned 3'UTR of human ACVR1B was transiently transfected into HEK cells. Subsequent luciferase assay showed that with PUM1 loss, by siRNA transfection, levels of ACVR1B were increased as compared to controls, consistent with previous zebrafish data. ***$p<0.0005$.
E: 578T cells were transfected with the indicated siRNAs for 48 hours then plated in organotypic culture. After 24 hours, cultures were fixed, stained with phalloidin (actin, green), and imaged. siNT (Non-targeting) and siCdc42 were purchased from Dharmacon and act as negative and positive controls respectively. Scale bar: 50μM.

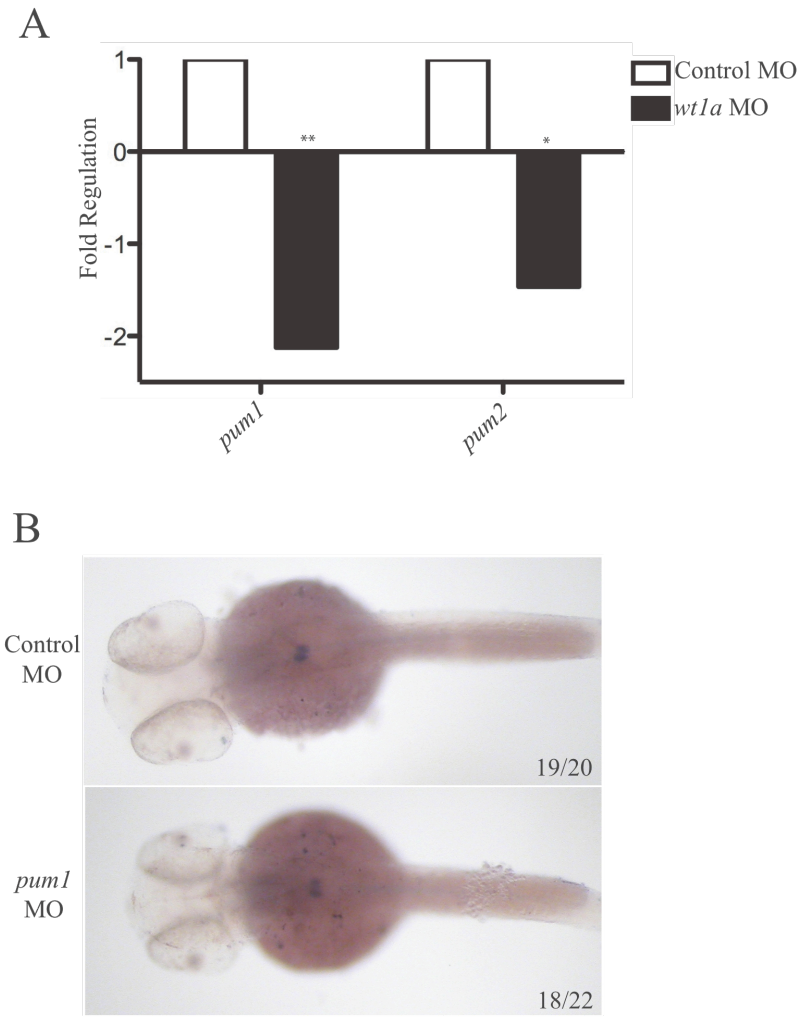

Figure: 3.11: *pumilio* is a transcriptional target of *wt1a*
A: qPCR analysis of embryos injected with morpholino to *wt1a* shows a reduction in levels of both *pum1* and *pum2*. *p< 0.05, **p< 0.005.
B: *in situ* hybridization with an antisense riboprobe to *nephrin* highlights the developing bilateral kidneys at 28hpf. *pum1* morphants display properly formed kidneys, showing *pum1* is not responsible for kidney formation under control of *wt1a*.

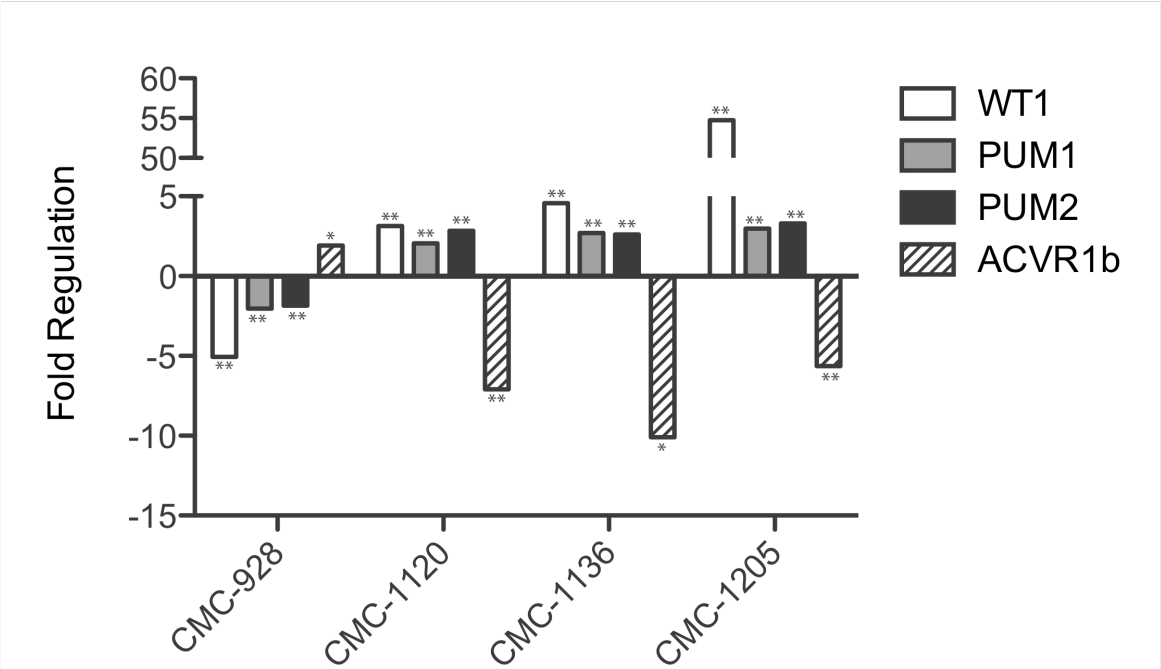

Figure 3.12: WT1, PUM1, and PUM2 are correlated in human tumor samples and inversely correlated with ACVR1B levels.
qPCR analysis of matched normal kidney tissue and Wilms Tumor samples, as scored by histology. In each of the 4 samples, WT1, PUM1, and PUM2 are inversely correlated with the putative target ACVR1B. *p<0.01, **p,<0.005.

CHAPTER FOUR:

Conclusions and Future Directions

Here, *pum1* is shown to play a critical role in patterning the embryonic shield structure, the origin of cell involution, and allowing for cells to lose adhesion and gain motility as they progress through EMT. This control is mediated by repression of the TGFβ pathway receptor, *acvr1b*, which in turn is responsible for inducing the expression of a potent EMT inhibitor, miR-200a (Figure 4.1). A negative feedback loop between *acvr1b* and miR-200a allows for dynamic control of EMT induction, while the spatial control exhibited by *pum1* confers added regulation to this molecular pathway.

Control of embryonic patterning events by *pumilio* has been shown in *Drosophila*, however not, as of yet, in vertebrates (Murata and Wharton 1995). While mouse knockout models of PUM1 and PUM2 are viable, they are smaller in size and display gonadal defects and decreased fertility (Xu, Chang et al. 2007; Chen, Zheng et al. 2012). Roles for *pumilio* in gonadal formation and germ line stem cell maintenance have been well characterized in *Drosophila* (Lin and Spradling 1997), suggesting some but not all functions of *pumilio* may be functionally conserved between vertebrates, as murine PUM1 and PUM2 are able to rescue zebrafish *pum1* and *pum2* knockdown respectively. While the role of other PUF-domain containing orthologs was not extensively studied, the data presented here strongly suggest that each ortholog may maintain distinct targets and functions, as they cannot functionally complement one another in assays of early development. While the PUF domain repeats, at the C-terminus of the protein, are highly conserved between the orthologs and between species, the N-terminus is more variable. While the N-terminus does not contain any

characterized domains, it is likely that it may confer some specificity to other protein binding partners. In *Drosophila*, *pumilio* and *nanos* cooperate to translationally repress *hunchback* mRNA and exhibit control over embryonic polarity and germline stem cell function (Wreden, Verrotti et al. 1997; Forbes and Lehmann 1998). Additionally, while the transcripts of *pum1* are relatively ubiquitously expressed throughout the animal pole early during development, protein expression is localized to the dorsal organizer. This suggests *pum1* itself is regulated post-transcriptionally by unknown factors, which likely utilize non-conserved regions that differ between orthologs to confer translational control.

These studies subsequently focused solely on the interaction between *pum1* and *acvr1b* as they related to gastrulation and EMT. The identification of other functional targets *in vivo* of *pumilio* proteins was not further investigated. Future studies utilizing RNA Immunoprecipitation and sequencing (RIP-seq) or cross-linking immunoprecipitation-high-throughput sequencing (CLIP-seq) would assist greatly in identifying other targets of *pumilio* regulation. As a few of the other identified targets of *pum1* which were not investigated, *ephrin-a* and *dusp6*, have been shown to play important roles in EMT as well, further validation of them as targets of *pum1* may illuminate how *pumilio* proteins are able to control cell movements (Frieden, Townsend et al. 2010; Wong, Chen et al. 2012). In *Drosophila*, *pumilio* controls anterior function by regulating *bicoid* and is able to recruit different partners to specifically regulate distinct RNA targets. Thus, validating targets of *pumilio* binding in combination with protein binding partners of *pumilio* may offer extensive knowledge on how translational repression can be strictly regulated in different cell settings (Gamberi, Peterson et al. 2002).

As morpholinos only transiently knockdown protein levels, these studies are restricted mostly to the period of embryogenesis, in which gene knockdown can be achieved. The fertility defects, body size reduction, and gonadal defects observed in mice thus cannot be accurately investigated using this line of experimentation (Xu, Chang et al. 2007). For these studies, creation of transgenic animals, such as through the use of TALENs, would be necessary. Additional work has implicated *pumilio* in dendrite morphogenesis and functioning of synapses in the nervous system of both *Drosophila* and mice (Ye, Petritsch et al. 2004; Chen, Li et al. 2008; Siemen, Colas et al. 2011). In this study, *pum1* expression is observed in developing brain structures of 24hpf embryos and morphants display smaller heads, suggesting *pum1* may also play a similar role in neuronal control and development in vertebrates.

pum1 and *pum2* were unable to cross-rescue for each other to recover the developmental defects scored at 3dpf. Additional work questioned the redundancy between the orthologs and attempted simultaneous knockdown; preliminary results suggest some phenotypes are exacerbated, as *bmp2b* expression can be detected throughout the animal pole of the embryos, however the severity of the developmental defects limited further analysis.

These studies highlight the role of *pum1* in the organization of the shield structure, the initiation of gastrulation, and formation of the dorsal-ventral axis. As *pum1* protein is localized to the dorsal pole, it is likely that its functions are to directly regulate translation of dorsal organizer components here. As dorsal signals are los in *pum1* morphants, ventral signals expand into dorsal tissues. This ventral expansion, as illustrated by expanding *bmp2b* expression, is likely the cause of elevated TGFβ/BMP signaling, as detected by increase

pSMAD1/5/8 and TGFβ/BMP target gene expression. As further analysis uncovers *acvr1b*, a TGFβ/BMP pathway component, as an *in vivo* functional target of *pum1*, it is likely that elevated signaling through this receptor in *pum1* morphants may be the causative factor of elevated signaling through this pathway.

pum1 targets *acvr1b* through the *pumilio* binding elements (PBEs) present within the 3' UTR. This sequence is necessary for translational repression of the transcript, however whether this repression is directly by *pum1* or in coordination with a microRNA is uncertain. As in the regulation of p27, *pumilio* cooperates with miR-221/222 to induce a structural change that allows for access of the miRNA to degrade the target (Kedde, van Kouwenhove et al. 2010). However, *pumilio* may also deadenylate targets and thus confer repression in miRNA-independent manners (Wickens, Bernstein et al. 2002). The 3' UTR of *acvr1b* contains several miRNA seed sequences conserved between species. Predictive *in silico* analysis of secondary structure of the RNA folding suggests the miR-200a binding site is near to a PBE. While it is clear that miR-200a suppresses expression of *acvr1b*, it remains unclear if this regulation is *pumilio* dependent. Further investigation and coordination modulation of both *pum1* and miR-200a, along with mutated binding sites and seed sequences will further the understanding of the regulation of this specific transcript.

The consequence of *pum1* regulation of *acvr1b* is to alter levels of miR-200a in zebrafish, which thus affects early patterning events and gastrulation. It has been shown here that this molecular pathway is conserved in human cells, as modulation of PUM1 by siRNA increases ACVR1b levels. Evidence also shows that *pum1* controls EMT processes, likely though mediation of miR-200a, a canonical EMT inhibitor. This control is present not only in

a cell culture based system, but also *in vivo* in developing embryos, as gastrulation is affected in *pum1* morphants, as well as *snail* presentation. This illustrates zebrafish as a crucial tool with which to study EMT events and metastasis. Through new knowledge implicating *pum1* as a driver of EMT events, more specifically as a repressor of *acvr1b* and thus miR-200a, it is possible to begin to outline various methods with which to investigate a role for *pum1* in tumor progression and metastatic events. Various cancer cell lines, such as leukemia, Burkitt's lymphoma, colorectal adenocarcinoma, and lung cancer, express *pumilio* proteins (Spassov and Jurecic 2002). Additionally, *pumilio* has been characterized as a maintenance factor in a variety of stem cell populations, including germline stem cells, human embryonic stem cells, neural progenitors/stem cells, and adult hematopoietic stem cells (Lin and Spradling 1997; Moore, Jaruzelska et al. 2003; Spassov and Jurecic 2003; Siemen, Colas et al. 2011). Taken together, a better understanding of the molecular functions of *pumilio* in an *in vivo* vertebrate setting, and how it is able to fine tune signaling pathways, has implications for the understanding of metastatic events, cell motility, EMT, and asymmetric cell divisions.

This study not only offers implications for a *pum1*-controlled cancer disease pathways regulation, but also offers possible insight into novel mechanisms of other diseases. Activin signaling has been implicated in hereditary hemorrhagic telangiectasia, a rare genetic disorder that leads to abnormal blood vessel formation and spontaneous bleeding (Gu, Jin et al. 2006). Cases of spontaneous intracerebral hemorrhage have also been reported in infant patients with Wilms tumor (van Toorn, Wessels et al. 2007). As morphants observed in this study display abnormal blood vessel formation and blood pooling, it is likely that a pathways involving *wt1a*, *pum1*, and *acvr1b* may be involved in vasculogenesis and angiogenesis

present in a variety of disease settings (van den Driesche, Mummery et al. 2003). Further exploration of the roles of *pumilio* proteins in the regulation of gene expression is likely to shed light on novel mechanisms of normal development and a variety of diseases.

Figure 4.1: Mechanism of *pum1* function
pum1 translationally represses *acvr1b* through its target sequences within the 3' UTR. *acvr1b* in turn is responsible for inducing the expression of the potent EMT inhibitor, miR-200a. This relationship exists in a negative feedback manner, as miR-200a suppresses *acvr1b*, allowing for dynamic control of EMT induction, while spatial control exhibited by *pum1* confers added regulation to this pathway.

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