THE ROLE OF THE SMALL GTPase ARF6 DURING MORPHOGENESIS IN SEA URCHIN EARLY DEVELOPMENT

by

Priscilla Adowa Konadu Kobi

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Masters of Science in Biological Sciences

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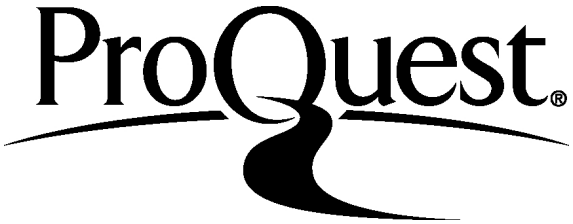

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ABSTRACT

ADP ribosylation factor 6 (Arf6) is a small GTPase that acts as a molecular switch, which regulates endocytosis by mediating membrane trafficking between the plasma membrane and endosomal compartments, as well as cell motility by remodeling the actin cytoskeleton. This protein is evolutionarily highly conserved and has been identified in organisms ranging from yeast to human. Although *in vitro* studies have revealed the cellular functions of Arf6, relatively little is known about its physiological role in development. In this study we examined the function of Arf6 in early development using the purple sea urchin as a model organism. We found that perturbation of Arf6 with loss-of-function morpholino antisense oligonucleotide (MASO), the constitutively active GTPase defective Arf6 mutant Arf6 Q67L, and the dominant negative Arf6 mutant Arf6 T27N led to embryonic defects in gut development, skeletal structures, PMC patterning, and pigment cell motility and pseudopodial projections. The cells types that we focus on are those that undergo cellular morphogenesis during gastrulation: 1) endodermal cells which undergo morphogenesis in order to form the larval gut, 2) primary mesenchyme cells (PMCs) which undergo migration and give rise to the larval skeleton, and 3) pigment cells which are immune cells that are highly motile. Results indicate that Arf6 perturbed embryos led to a range of dose-dependent developmental defects, including a delay in

development, exogastrulation, defective larval gut, aberrant PMC development, and pigment cell morphological changes. These results support the hypothesis that Arf6 is essential for proper morphogenesis and cell movements during early development; hence, this study provides insights into the functional role of Arf6 during early development.

Chapter 1

INTRODUCTION

1.1 ADP Ribosylation Factor (Arf)

ADP ribosylation factor (Arf) family is a small G protein that belongs to the Ras superfamily and is composed of small guanine-nucleotide-binding proteins. Arfs are N-terminally myristoylated proteins with an N terminal amphipathic helix, these modifications have been shown to contributes to their various functions in membrane trafficking (D'Souza-Schorey and Chavrier, 2006). Arfs are categorized into three different classes in mammals based on sequence similarity; Class I (Arf1, Arf2 and Arf3), Class II (Arf4 and Arf5) and Class III (Arf6) (Gillingham and Munro, 2007). Similar to other small GTPases of the Ras family, Arfs acts as a molecular switch, cycling between an active GTP-bound and inactive GDP-bound conformation (D'Souza-Schorey and Chavrier, 2006). Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) regulate nucleotide exchange, which allow for proper activation and inactivation, respectively. The organization of the secretory and endocytic pathways is regulated by Arfs. Retrograde vesicle transport from the Golgi to the ER, such as the COPI coat protein and clathrin adaptor protein 1, and the activation of lipid-modifying enzyme is regulated by Class I (Arf1, Arf2 and Arf3) (Gillingham and Munro, 2007). Hydrolysis of Arf1-GTP is required for integration of

vesicle recycling between the Golgi and the ER compartments. The role of class II family of Arfs is currently undefined (Chun et al., 2008). However, *in vitro* studies have suggested that Arf5 (class II family of Arfs) may play a role in early Golgi transport and in the recruitment coat complexes at the *trans*-Golgi membrane. These functions are similar to that of class I Arfs. Arf6 is the only member of class III Arfs and it mediates plasma membrane to endosomal trafficking and actin remodeling (Donaldson, 2003; Sabe, 2003) It has been hypothesized that these different Arfs most likely optimize particular functions at the Golgi complex (Gillingham and Munro, 2007). Arf proteins are ubiquitously expressed and highly conserved in all eukaryotes, from yeast to humans (Donaldson and Honda, 2005; Donaldson and Jackson, 2011; Johnson et al., 2011)

1.2 ADP Ribosylation Factor 6 (Arf6) Function

Arf6 is the only Arf trafficking proteins between the plasma membrane in its active GTP bound form and endosomal compartments in its GDP bound form. (D'Souza-Schorey et al., 1995; Donaldson, 2002; Donaldson and Radhakrishna, 2001). Arf6 regulates an assortment of cellular processes, including endocytic membrane trafficking, clathrin-dependent and independent exocytosis, phosphoinositide metabolism and actin remodeling (Donaldson, 2003; Sabe, 2003). Arf6 has also been shown to mediate phagocytosis, cell motility, disassembly of adhesion junctions, regulated secretion, cell signaling and endosomal membrane traffic (Brown et al., 2001; D'Souza-Schorey et al., 1995; Palacios et al., 2001; Radhakrishna et al., 1999; Santy et al., 2001; Zhang et al., 1998) (Fig. 1).

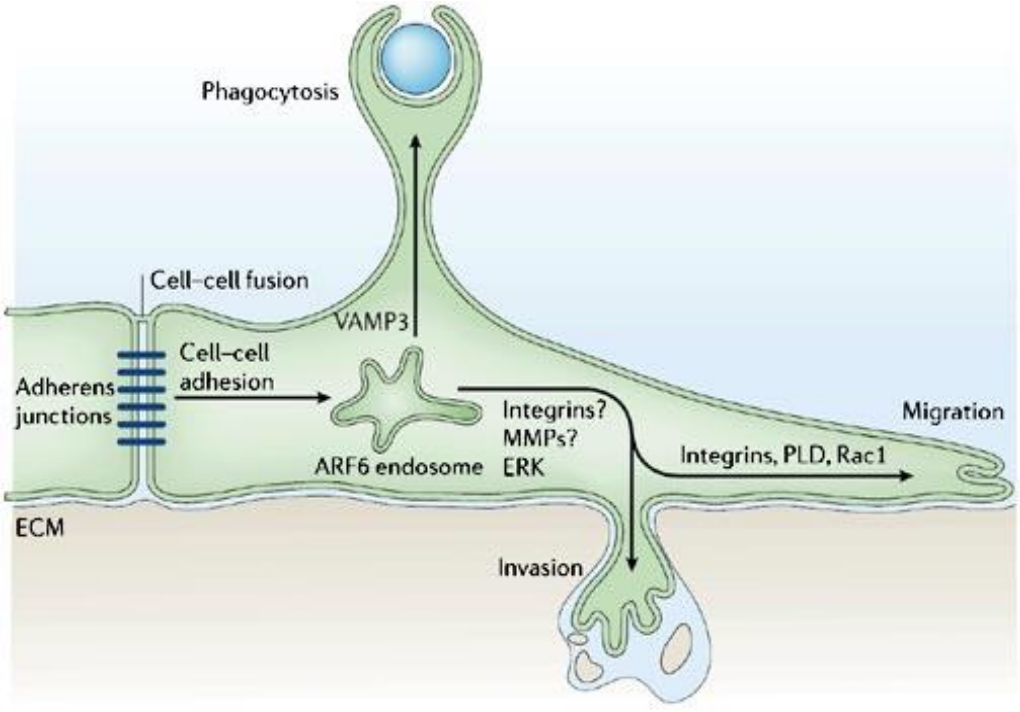

Figure 1.The cellular function of Arf6. Arf6 has been shown to regulate phagocytosis, cell motility, disassembly of adhesion junctions, secretion, cell signaling, and endosomal membrane trafficking (Brown et al., 2001; D'Souza-Schorey et al., 1995; Palacios et al., 2001; Radhakrishna et al., 1999; Santy et al., 2001; Zhang et al., 1998)

1.2.1 Arf6 Impacts Signaling Pathways

In vitro studies revealed multiple functions of Arf6, including regulating membrane traffic, membrane recycling, endocytosis and organizing actin cytoskeleton (Al-Awar et al., 2000; Brown et al., 1993; Massenburg et al., 1994). Cellular polarity is essential for proper intrinsic function such as protein secretion and fate determination during cell division. *In vitro* studies in yeast and animal cell lines have shown the importance of vesicular trafficking for protein secretion and asymmetric

cellular division (Mostov et al., 2003). Arf6 mediates the recycling of cell surface proteins that impact distinct signaling pathways, such as activation of the phospholipase D (PLD) (Cockcroft et al., 1994; Massenburg et al., 1994), EGFR induced MEK-ERK pathway (Tague et al., 2004), the G-protein coupled receptors induced G protein signaling pathway (Claing, 2011; Cotton and Claing, 2009), β-integrin induced tyrosine receptor-kinase signaling pathway (Allaire et al., 2013; Chen et al., 2014; Powelka et al., 2004) (Li et al., 2005), and cadherin recycling which affects β-catenin abundance downstream of the Wnt signaling pathway (Grossmann et al., 2013; Onishi et al., 2013; Pellon-Cardenas et al., 2013). Hence, Arf6 plays a critical role in signal transduction pathways.

1.2.2 Arf6 Mediates Cell Motility

During epithelial-to-mesenchymal transition (EMT), cell-to-ECM and cell-to-cell adhesions must be disassembled in order for the cell to migrate. Adherens junctions are anchorage sites, which form connections between cell to cell via the transmembrane protein cadherin. In the cytoplasm, cadherins bind to α-catenins, which bind to the actin filaments in the cytoskeleton, providing a stable anchorage site (Yap et al., 1997). It has been demonstrated that Arf6 regulates E-cadherin recycling between the plasma membrane and early endosomes in polarized epithelial cells (Schweitzer et al., 2011). Integrins are the major transmembrane proteins found in focal adhesions, which connect the cell with the ECM (Wozniak et al., 2004). The turnover rate of focal adhesions is important during cell motility. A cell must be able

to attach to the ECM with its integrins but also inhibit the maturation of focal complexes into a focal adhesion in order to continue migration (Wozniak et al., 2004).

Arf6 was identified to plays a role in actin cytoskeleton rearrangements, via its interaction with Rac1 GTPase (Radhakrishna et al., 1999). *In vitro* studies have identified that Arf6 regulates the recycling of the small GTPase Cdc42 to the migrating end of the cell (Osmani et al., 2010). Cdc42 is a member of Rho GTPase family of small GTPases has been established to play a role in formation of the actin-based filopodia (Kozma et al., 1995; Nobes and Hall, 1995). Filopodia, which are thin, actin-based protrusions, are found at the leading edge of the migrating cells and act as a probe providing sensory information to the cell about the microenvironment (Mattila and Lappalainen, 2008). Filopodia are able to achieve this because they contain receptors for different signaling molecules, thus becoming a location for signal transduction (Mattila and Lappalainen, 2008). Cell adhesion molecules, such as integrin and cadherin, are also found in filopodial protrusions (Mattila and Lappalainen, 2008). Localization of adhesion molecules allows the filopodia to promote cell migration and cell adhesion. Arf6 is essential for actin based filopodia formation and rearrangement.

1.2.3 Physiological Function of Arf6

In vivo studies in mammalian systems ranging from yeast to mice have identified that Arf6 is important for proper development. In yeast Arf6 has been shown to be important for bud formation (Fujita, 2008; Huang et al., 2003;

Smaczynska-de Rooij et al., 2008). In filamentous fungi, Arf6 is important in polarity establishment and endocytosis (Lee et al., 2008). In the fly, Arf6 is critical for spermatogenesis (Dyer et al., 2007) and pupal eye patterning (Johnson et al., 2011). In zebrafish, Arf6 regulates epiboly progression and gastrulation by regulating the recycling of syndecan, which is a transmembrane protein that acts as a co-receptor for adhesion molecules and growth factors, such as the morphogens FGFs and Wnt (Lambaerts et al., 2012a). In mammalian systems, homozygous deletion of Arf6 in mice resulted in defective liver development due to apoptosis, although the exact molecular mechanism is unknown (Suzuki et al., 2006).

Studies in mammalian hippocampal neurons have identified that Arf6 and its regulatory proteins are essential for dendritic spine formation and axonogenesis (Hall and Lalli, 2010; Hernandez-Deviez et al., 2004; Kobayashi and Fukuda, 2012). These dendritic spines are actin rich filopodia structures, which are essential for presynaptic inputs in the central nervous systems (Kim et al., 2015; Rochefort and Konnerth, 2012). Dendritic spines maturation occurs when filopodia undergo spine conversion. Arf6 and its GEF, EFA6A, have been shown to regulate spine conversion and stabilization of the early spine which promotes mature dendritic spines (Choi et al., 2006). *In vivo* overexpression studies of Arf6 T157A lead to early spine formation; conversely, the knockdown using siRNA against Arf6 suppressed spine formation (Choi et al., 2006a). Formation of neurons depends on actin cytoskeleton rearrangements and sensing, similar to cellular activities during gastrulation (Hall and Lalli, 2010). It has been shown Arf6 induced PIP5K signaling pathway is essential in

synaptic vesicle recycling (Krauss et al., 2003), regulation of exocytosis (Aikawa and Martin, 2003) and limiting axonal elongation and branching (van Rossum and Hanisch, 1999). Arf6 is a key activator of phospholipase D (PLD), an enzyme known for its function in catalyzing the hydrolysis of phosphatidylcholine in order to create phosphatidic acid (PA), leading to actin cytoskeleton remodeling, cell migration, and cell signa choline al., 1993; Cockcroft et al., 1994) (Brown et al., 1998; Du et al., 2003) (Du et al., 2004; O'Luanaigh et al., 2002; Santy and Casanova, 2001) (Fig. 2).

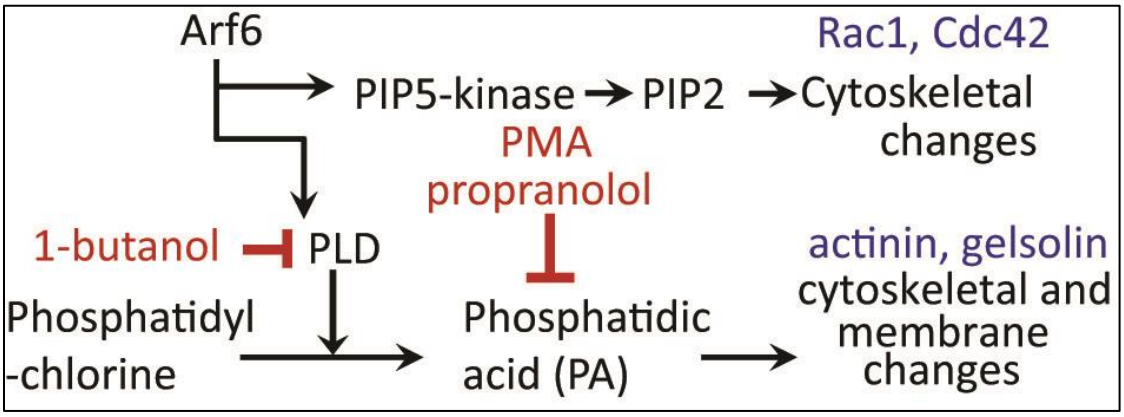

Figure 2.Actin remodeling pathway mediated by Arf6. Arf6 mediates actin remodeling via at least two pathways: 1) via the Rac1, Cdc42 pathway and 2) via PLD pathway (Honda et al., 1999).

1.3 Sea Urchin Development

Sea urchin development begins with external fertilization where gametes are shed directly into the sea water.

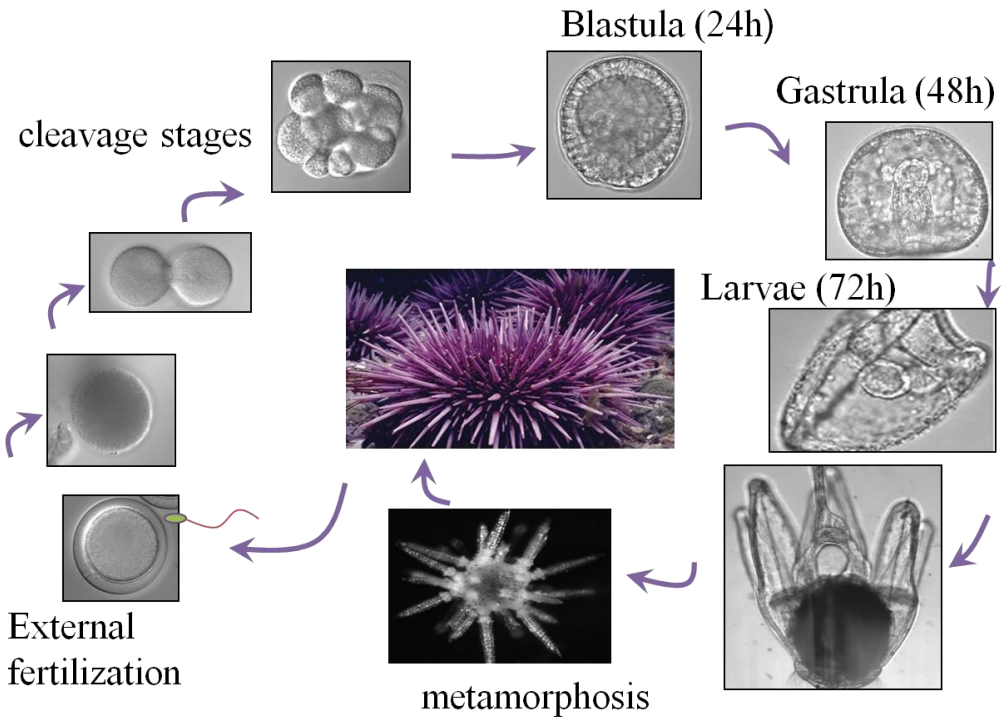

Figure 3.Sea urchin life cycle. The gametes undergo external fertilization. By 24 hours post fertilization (hpf), the embryos reach the blastula stage. By 48 hpf, all three germ layers are specified. At 72 hpf, the larvae begin to feed. The embryo is an indirect developer that undergoes metamorphosis to form a juvenile adult (Song, unpublished).

The blastula forms a hollow sphere around the central body cavity known as the blastocoel. The cells around the vegetal pole began to thicken and the mesenchymal cells ingress into the blastocoel, which will give rise to the mesodermally derived structures. Specification of the endoderm, mesoderm, ectoderm and gut formation marks the gastrulation stages at 48 hpf, during which the mesoendodermal cells from the vegetal pole begin to invaginate into the blastocoel and give rise to the gut (Logan and McClay, 1997). The ectodermal cells give rise to the larval skin and the nervous

system. The mesoderm gives rise to the skeleton spicule, muscle, blastocoelar cells, and pigment cells. The primary mesenchyme cells (PMCs) give rise exclusively to the larval skeleton, and the secondary mesenchyme cells (SMCs) give rise to the rest of the mesodermal cell types (Fig. 4).

Gastrulation is divided into four stages: 1) The PMC are derived from the micromeres specifically the mesoderm, undergo epithelial to mesenchymal transition (EMT) migrating and transitioning which begins to ingress into the blastocoel between 9-10 hpf in response to cues from the ectoderm (Logan and McClay, 1997). This ingression leads to the formation of the spicules, which are the mineralized rods that constitute the skeleton of the embryo, important for the shape, support, feeding and swimming of the larva (Hart and Strathmann, 1994; Pennington and Strathmann, 1990), 2) the vegetal plate bends inward to forms the blastopore which becomes the anus of the embryo at a later stage of development (Kimberly and Hardin, 1998; Lane et al., 1993; Nakajima and Burke, 1996), 3) embryonic gut elongation occurs to forms the tubular structure of the gut, (Ettensohn and Ingersoll, 1992; Hardin, 1989; Logan and McClay, 1997), and 4) the SMCs migrate to the animal pole and is important for gut elongation. The SMCs also give rise to the blastocoelar, coelomic pouch, pigment, muscle and spicule tip cells (Ruffins and Ettensohn, 1993; Shoguchi et al., 2002) (Fig. 4). Cell signaling and cell movements are crucial for the cell differentiation during gastrulation. Transmembrane proteins such as cadherins and integrins are important during EMT and their regulation is essential for proper cellular morphogenesis (Kalluri and Weinberg, 2009).

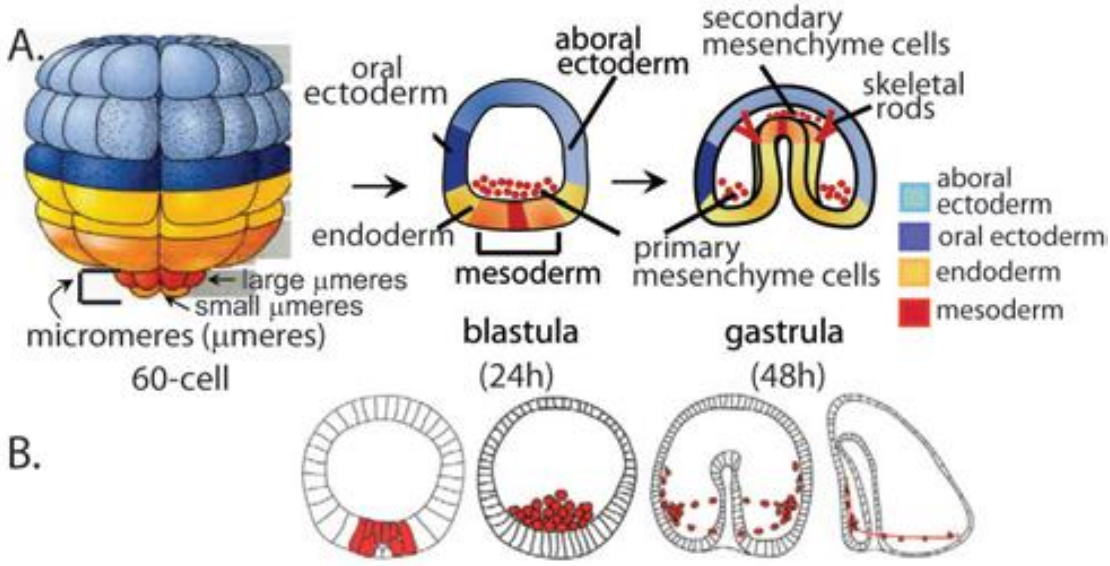

Figure 4.Schematic of embryo cell specification. A) During the blastula and gastrula stages, endomesodermal specification occurs.
(B) Migration pattern of skeletogenic PMCs are shown in red (Gilbert, 2000).

Chapter 2

SPECIFIC AIMS

Central Hypothesis:

We hypothesize that Arf6 directs cell movement and cell specification of mesenchymal cells during gastrulation.

2.1 Specific Aim 1- Characterization of the Spatial and Temporal Expression of Arf6

To examine the spatial and temporal expression of Arf6 mRNA, we collected embryos at various time points. Previously, real time, quantitative PCR (qPCR) was used to detect Arf6 expression. I used RNA *in situ* hybridization to detect the spatial distribution of Arf6 mRNA in the early developmental stages. I immunostained sea urchin embryos using Arf6 monoclonal antibody made against human Arf6 in order to identify the spatial and temporal expression of the Arf6 protein.

2.2 Specific Aim 2- Test the Function of Arf6 in Early Development

To investigate the physiological function of Arf6, embryos were injected with an Arf6 morpholino antisense oligonucleotide (MASO) to knockdown endogenous Arf6 in the developing embryo. Arf6 dominant negative (T27N) and constitutively active (Q67L) mRNA constructs previously generated were also used to further

elucidate the function of Arf6. This study was initiated by a former undergraduate researcher, Megan Dumas. I completed the final analysis of these Arf6 perturbation experiments.

2.3 Specific Aim 3- Identify the Molecular Mechanism of Arf6-Mediated Cell Movement

To investigate the molecular mechanism responsible for developmental defects induced by Arf6 MASO and Arf6 Q67L mRNA, we generated additional Arf6 mutants that were defective in actin remodeling, membrane trafficking, and PLD activation to dissect the multiple functions of Arf6. I contributed to the manufacturing of these mutant constructs along with *in vitro* transcription for microinjections.

Chapter 3

MATERIALS AND METHODS

3.1 Animals

Adult Strongylocentrotus purpuratus were obtained from Point Loma Marine Company in California. Adult male and female sea urchins were given 0.5M KCl intracoelomic injections to induce gamete shedding. Embryos were cultured in filtered sea water that was collected from Indian River Inlet; University of Delaware Lewes campus at 15 °C.

3.2 Microinjection

Microinjections (by Dr. Jia L. Song) were performed as previously described (Song et al., 2012). Using a Femto Jet injection system (Eppendorf; Hamberg, Germany), newly fertilized embryos were injected with Arf6 MASO 5' TCTTTGATAGTACCTTCCCCATCGT 3', negative control MASO (Genetools, OR), constitutively active Q67L, and dominant negative T27N Arf6 mRNAs.

3.3 Immunolabeling

In order to examine the expression and localization of Arf6 (Santa Cruz Biotechnology, Inc. Dallas, TX) or PMCs (McClay et al., 1983), embryos were fixed in 4% paraformaldehyde for 10 min at a specific time point, then washed 4 times in 1X Phosphate Buffered Saline (BioRad, Hercules, CA) in addition with 0.05% Tween (PBST). Embryos were blocked in 4% sheep serum in PBST (Sigma, St. Louis, MO) for 1 hour at room temperature and then incubated in primary antibody (1:200 for Endo1, 1:50 for 1D5, and 1:50 or 1:100 for Arf6) in blocking buffer (4% sheep serum in PBST) overnight at 4°C. Next, embryos were washed 4 times with PBST, subsequently incubated with goat anti-rabbit Alexa Fluor 488 conjugated antibody at 1:300 (Invitrogen, Carlsbald, product code A10520) in blocking buffer for 45 min to 1 hour at room temperature. Embryos were then washed 4 times for 10 mins with PBST and then imaged on an Axio Observer Z.1 epifluorescence microscope or on LSM 710 or LSM 780 confocal microscopes (Carl Zeiss, Inc.; Thornwood, NY).

3.4 Endocytosis

Arf6 has been shown to play a major role in endocytosis. I used the Tetramethylrhodamine dextran and Cholera toxin subunit B (CTB) (Invitrogen, Carlsbald, CA) to label endosomal compartments (Whalley et al., 1995) (Alford et al., 2009). Dejellied eggs were fertilized in the presence of 1 mM 3-amino-triazol (3AT, Sigma, St. Louis, MO) in order to soften the fertilization envelope. Fertilization

envelopes were removed by passing the zygotes through a 65 μm nytex mesh 2 to 3times. Fertilized eggs were cultured in filtered sea water (FSW) on ice until the embryos reach the 4-8 cell stage. CTB (final concentration at 5 μg/ml, Invitrogen) conjugated with Alexa 488 was added to live embryos 2 hours post-fertilization and incubated for 20 mins. This was followed with 5 rinses of ice-cold FSW and hyaline extraction medium for 5 mins. Afterwards the embryos were fixed on ice with 0.3% glutaraldehyde, 3% paraformaldehyde, 70% FSW on ice for 2 hours (Campanale and Hamdoun, 2012). Alternatively, 20 μl/ml of tetramethylrhodamine dextran 10,000 MW (10 mg/ml) was added to embryos at 4 cell stage in 1 ml of sea water and incubated for 10 mins at room temperature. Embryos were rinsed thoroughly with FSW 5 times and fixed with 4% formaldehyde in ASW for 1 hour at 4° C. Prior to imaging, embryos were washed with PBS and imaged on an Axio Observer Z.1 epifluorescence microscope (Carl Zeiss, Inc.; Thornwood, NY) or on LSM 780 confocal microscopes.

3.5 *In vitro* Transcription

Previously, the sea urchin Arf6 mRNA was cloned into a plasmid containing Xenopus β-globin 5' and 3'UTRs (a functional plasmid in the sea urchin embryo) to ensure proper translation of Arf6 (Gustafson et al., 2011). I linearized and in vitro transcribed Arf6 mRNA constructs by using the mMessage machine kit (Ambion). SP6 RNA polymerase was used to generate all mRNA constructs according to manufacturer's instructions. However, the DNase treatment step was carried for 40

mins instead of the recommended 15 mins. mRNA was purified using Qiagen microRNeasy kit according to manufacturer's specifications (Qiagen Inc., Valencia, CA) and purified mRNAs were loaded onto the Millipore spin columns in order to further clean the mRNAs prior to microinjections (Millipore ULTRAFREE-MC filter units, catalog #UFC30GVOS) (Fig. 5).

Table 1: *In vitro* transcribed constructs for microinjections.

Constructs	**Linearize**
Sea urchin wild type Arf6	EcoRV
Sea urchin Q67L	SalI
Membrane mCherry	EcoRI
LifeAct mCherry	NotI
Farnesyl-GFP	BglII
PH-GFP	NotI
F-actin	NotI

3.6 Alkaline Phosphatase Treatment

Larvae (72 hpf) embryos were fixed in MOPS-paraformaldehyde based fixative (4% paraformaldehyde, 100 mM MOPS pH 7, 2 mM MgSO4, 1 mM EGTA, and 0.8 M NaCl) for 10 mins at room temperature as previously described (Stepicheva et al., 2015). Embryos were then washed with alkaline phosphatase buffer 3 times (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl2, 0.1% Tween-20), followed by staining until the desired color was developed with the staining solution which was approximately 7 mins (0.1 M Tris pH 9.5, 50 mM MgCl2, 0.1 M NaCl, 1 mM Levamisole, 10% Dimethylformamide, 45 µl of 75 mg/mL NBT and 35 µl of 50 mg/mL BCIP per 10 ml of solution). Staining was stopped with multiple washes of MOPS buffer (0.1 M MOPS pH 7, 0.5M NaCl, and 0.1% Tween-20). Images were acquired using Nikon D90 digital camera connected to a Zeiss Axio Observer Z.1 microscope.

3.7 Lipopolysaccharide (LPS) Treatment/Time Lapse Video

To assay the immune response and morphology of the pigment cells in wild type and Arf6 perturbed larvae, these embryos were incubated with a final concentration of 50 µg/mL LPS from Escherichia coli 0111:B4 (Sigma Aldrich, St. Louis, MO) in filtered sea water for 30 mins at 15°C. The treated embryos were placed in a 12-well imaging plate coated with 1% protamine sulfate and imaged using the Nikon D90 on Zeiss Axio Observer Z.1 epifluorescence microscope at 400X

magnification (Carl Zeiss, Inc.; Thornwood, NY) and the Camera Control Pro 2 software. Videos can be seen at http://youtu.be/KeMczbxGNRM.

3.8 Whole Mount RNA In situ Hybridization (WMISH)\

Arf6 coding sequence was cloned into a PCRII vector and used as a RNA in situ hybridization probe to detect the spatial and temporal distribution of Arf6 (Stepicheva et al., 2015). All embryos were fixed with Fixative II (4% paraformaldehyde, 32.5% artificial sea water, 32.5 mM MOPS pH 7, 162.5 mM NaCl). Embryo were washed in MOPS buffer (0.1 M MOPS pH 7, 0.5 M NaCl, 0.1% Tween-20) and incubated with the Arf6 riboprobe for one week in hybridization buffer (70% formamide, 0.1 M MOPS pH 7, 0.5 M NaCl, 0.1% Tween-20, 1mg/ml BSA). After the incubation period, embryos were washed with blocking solution I (0.1 M MOPS pH 7, 0.5 M NaCl, 10 mg/ml BSA, 0.1% Tween-20) and blocking solution II (0.1 M MOPS pH 7, 0.5 M NaCl, 10 mg/ml BSA, 0.1% Tween-20, 10% goat serum). This was followed by incubation with alkaline phosphatase conjugated antibody overnight in blocking solution II. Lastly, embryos were washed in MOPS buffer, alkaline phosphatase buffer (10 mM Tris Base, 100 mM NaCl, 5 mM MgCl2) and staining solution. Staining was then monitored and reaction was stopped using several washes of MOPS buffer.

3.9 Site-Directed Mutagenesis

The coding sequence of S. purpuratus Arf6 was cloned, sequenced, and used as a DNA template for the mutagenesis reaction to generate Arf6 N48I (impaired activation of PLD), Arf6 Q37E:S38I (Arf6 impaired Arf6 actin functions) (Al-Awar et al., 2000; Jovanovic et al., 2006). Primers for mutagenesis were designed using a primer design program provided by Stratagene (www.agilent.com/genomics/qcpd) (Table 2). Arf6 mutant coding sequences were mutated using the QuikChange Lightning mutagenesis kit according to manufacturer's specifications (Stratagene, La Jolla, CA). The fidelity of the mutagenesis positive clones were sequenced for validation (Genewiz Inc., South Plainfield, NJ).

Table 2: Primer sequences

	FOR (5' to 3')	**REV (5' to 3')**
UBQ qPCR	CACAGGCAAGACCATCACAC	GAGAGAGTGCGACCATCCTC
Arf6 qPCR	AACACAGGGGTTGATCTTCG	ATTGGCGAAGATGAGGATGA
Arf6 *in situ*	CATGGATCCATGGGGAAGGT ACTATCAAA	ACAGTCTCGAGTCAGGGTTTATTA TTAGATGTTA
Q67L	ATGGGATGTTGGTGGTCTGG ATAAAATTCGGCCTC	GAGGCCGAATTTTATCCAGACCA CCAACATCCCAT
T27N	GGGCTTGGATGCAGCCGGCA AGAACACGGTGTTATAT	ATATAACACCGTGTTCTTGCCGGC TGCATCCAAGCCC
T44N	CAGTCACAACCATCCCAAAC GTGGGCTTCAACGTAGAAAC AG	CTGTTTCTACGTTGAAGCCCACGT TTGGGATGGTTGTGACTG
Q37E:S38I	TGTTATATAAACTGAAGCTG GGCGAGATAGTCACAACCAT CCCAACGGTGG	CCACCGTTGGGATGGTTGTGACTA TCTCGCCCAGCTTCAGTTTATATA ACA
Arf6N48I	TGACTGTTTCTACGATGAAG CCCACCGTTGGG	CCCAACGGTGGGCTTCATCGTAG AAACAGTCA
The underlined base pairs are used to create the corresponding amino acid mutations.		

Chapter 4

RESULTS

4.1 Spatial and Temporal Expression of Arf6

Previously we examined the endogenous mRNA expression of Arf6 during the early developmental stages of the sea urchin using qPCR analysis to determine mRNA levels of Arf6 at different developmental time points. We determined that the mRNA expression of Arf6 peaked at the 32-cell stage after which it continued to decrease from early blastula stage until the gastrula stage (Fig. 5) (Dumas et al, 2013). I examined the spatial expression of Arf6 using RNA *in situ* hybridization. We observed that Arf6 RNA is ubiquitously expressed throughout the different developmental time points (Fig. 5).

4.2 Arf6 Localizes to Distinct Punctate Structures

The human and sea urchin Arf6 protein shares 91.4% identity (Fig. 6). Based on this high level of protein identity, we used the antibody against human Arf6 to immunolabel sea urchin Arf6 proteins. We found that Arf6 accumulates in punctate structures ubiquitously, as well as on the skeletal spicules (Figs. 6, 7). These Arf6 positive punctate structures are likely to be endosomal compartments (Peters et al., 1995a, b). To test if Arf6 antibody labels the endosomal compartment, my plan was to test if the Arf6 positive stained compartments could co-localize with endosomal

markers. First I incubated embryos with CTB, and planned to follow with immunolabeling of Arf6 antibody to determine if CTB co-localizes with Arf6 in endosomal compartment. My preliminary data suggest that CTB is endocytosed by the embryo at 4 hpf (Fig. 8); however, we cannot conclude at this time if CTB and Arf6 co-localize in the same endosomal compartments and future studies will be conducted.

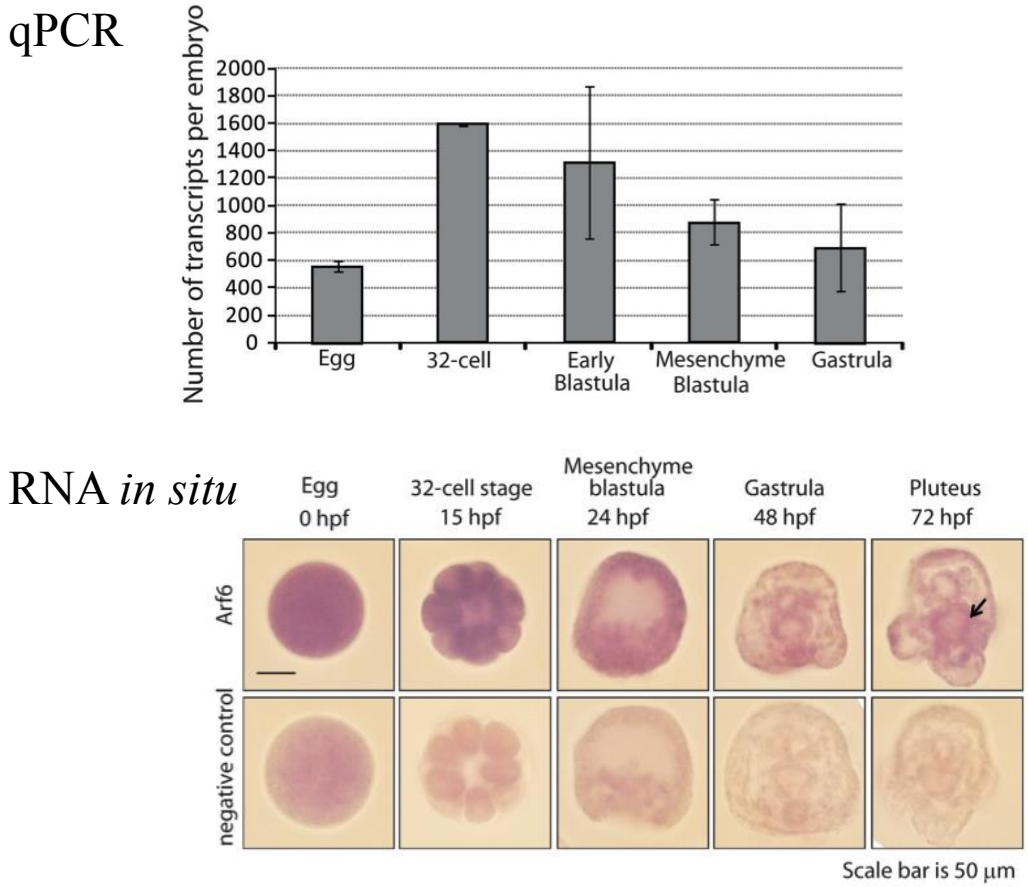

Figure 5.Temporal and spatial expression of Arf6. Levels of Arf6 are measured at various developmental time points. Arf6 transcripts are calculated from 3 biological replicates based on the number of internal control ubiquitin mRNAs (Materna and Davidson, 2012; Materna et al., 2010). (B) Whole mount *in situ* hybridization

indicates ubiquitously expression of Arf6 throughout the different developmental time points, increased expressing on gut at 72 hpf (indicated by arrow).

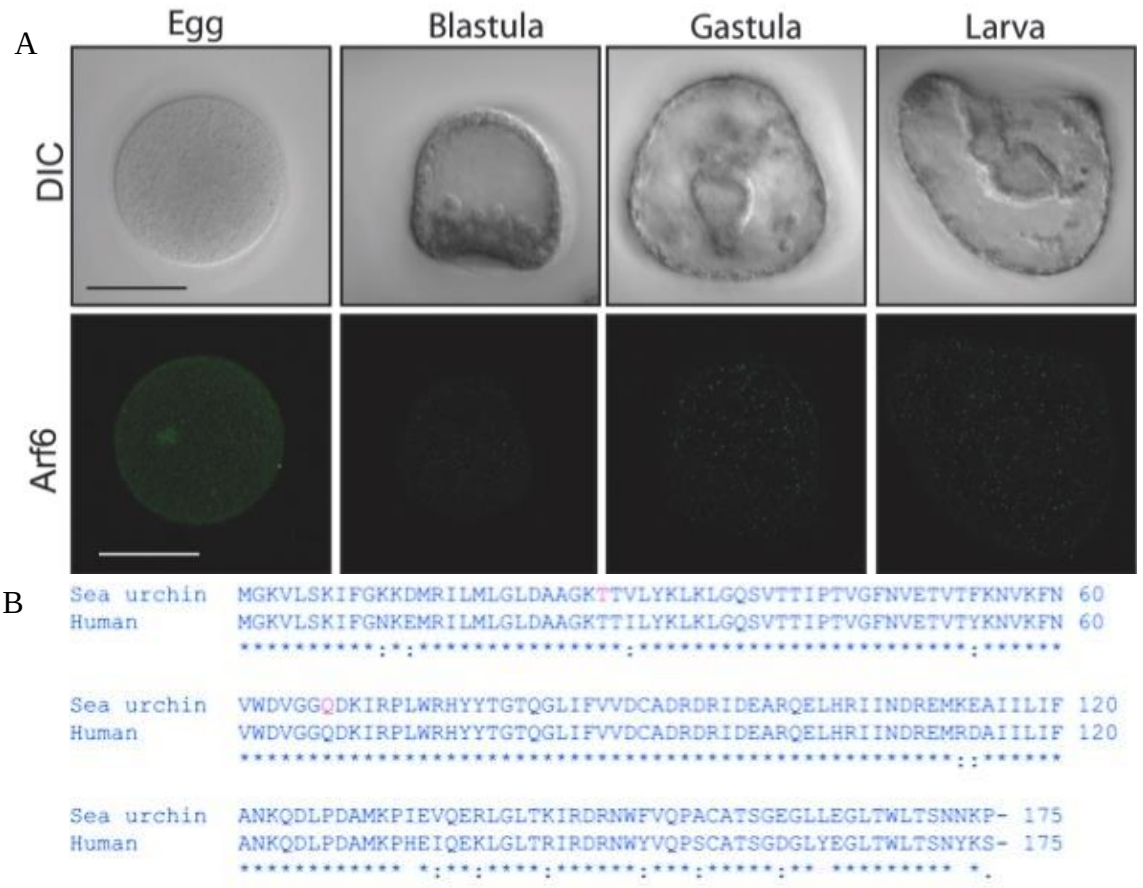

Figure 6.Arf6 localizes to punctate structures. (A) Immunostaining of embryos at various stages with monoclonal Arf6 antibody indicated that Arf6 accumulates in distinct punctate structures throughout the embryo. (B) Protein alignment indicates that Arf6 is highly conserved between humans and sea urchins, with a conservation of 91.4% identity. Image was taken with epifluorescence microscope (Zeiss Axio Observer Z.1). Scale bar 50μm.

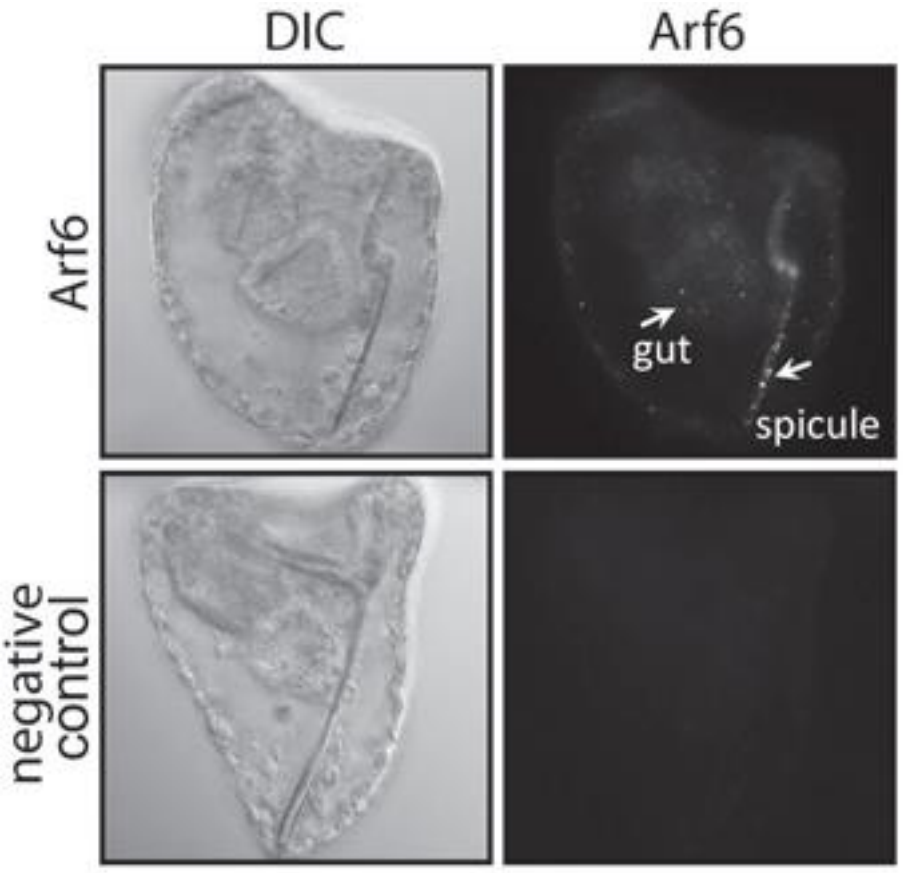

Figure 7.Arf6 is expressed in larvae skeleton spicules. Immunolabeled sea urchin larvae (72 hpf) with Arf6 antibody against the human Arf6, indicates that Arf6 proteins localize in the skeletal spicules (arrow). The negative control sample contains only the secondary antibody labeling. Image taken with epifluorescence microscope (Zeiss Axio Observer Z.1).

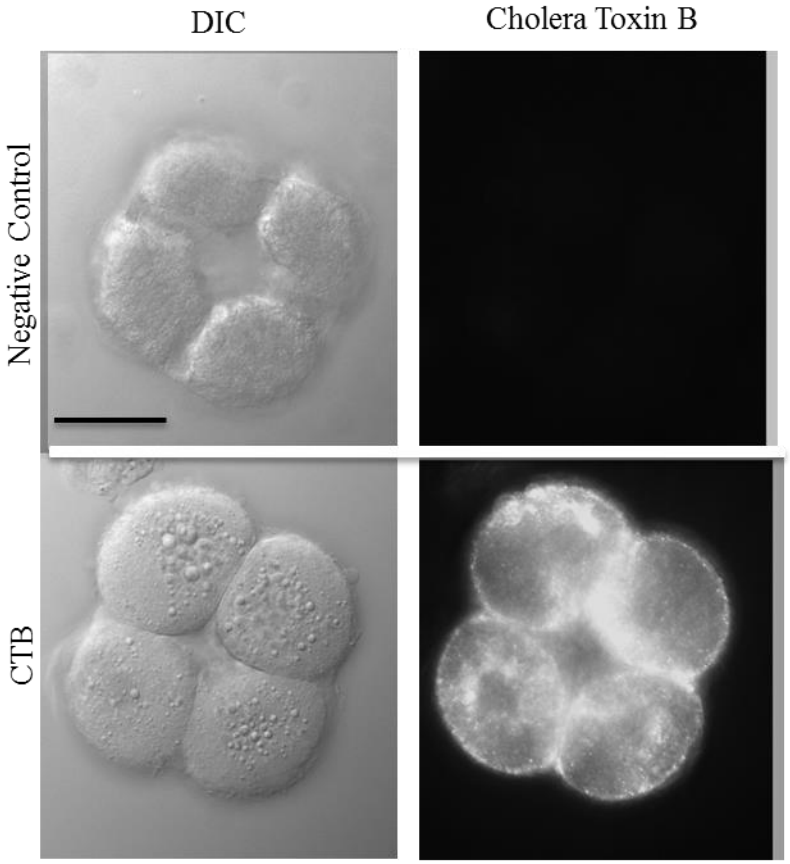

Figure 8.Embryos treated with cholera toxin subunit B show active endocytosis during development. 4 cell stage embryos were treated with CTB conjugated to Alexa 488 at 4 hpf and imaged with epifluorescence microscope (Zeiss Axio Observer Z.1). Treated embryos actively endocytosed CTB.

4.3 Arf6 is Essential for Early Development.

To test the function of Arf6, embryos were injected with Arf6 MASO to inhibit newly synthesized Arf6. We tested a range of Arf6 MASO and observed a dose-dependent severity of defects (conducted by Dr. Jia L. Song). Phenotypes of Arf6 knockdown embryos included thickened and finer filopodial structures, lack of PMC migration, PMC clumping, cell clumping, and shorten skeleton spicule (Fig. 9). We

tested the specificity of the Arf6 MASO by co-injecting the Arf6 MASO embryo with the sea urchin Arf6 mRNA and the human Arf6 mRNA (gift from Dr. Julie Donaldson, NIH). Embryos were injected with Texas Red dextran, Arf6 MASO, Arf6 MASO + sea urchin Arf6 mRNA and Arf6 MASO + human Arf6 mRNA. Gastrulae were immunolabeled with 1D5 antibody which recognizes PMCs, filopodial structures, and skeletal spicules (McClay et al., 1983). Mock treated embryo expressed normal PMCs

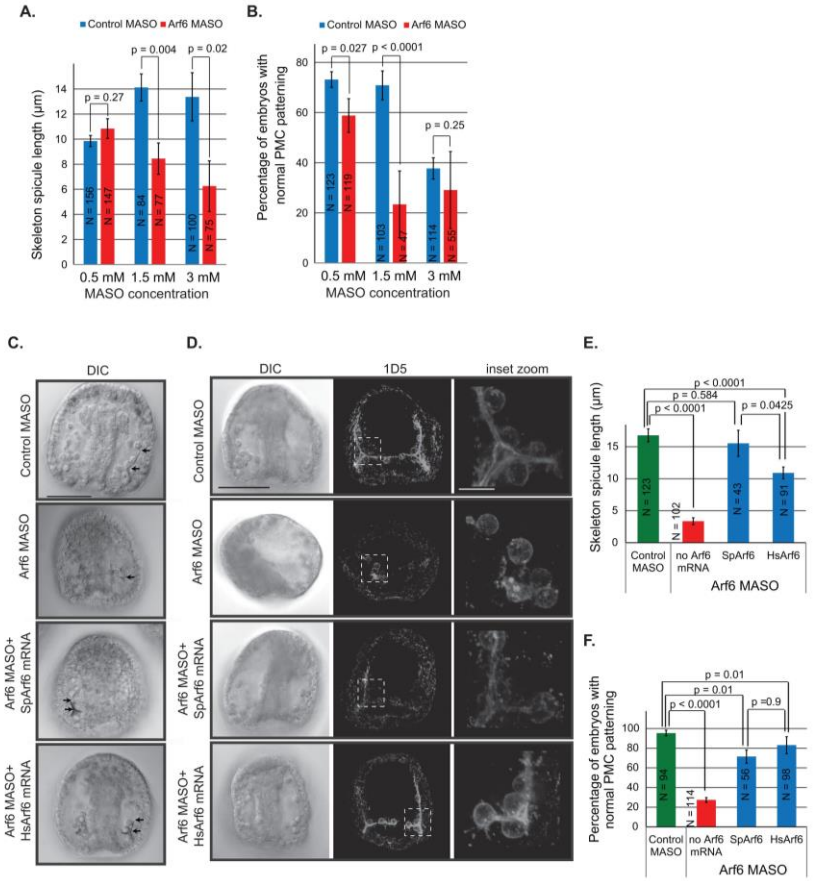

Figure 9.Arf6 perturbation results in defective PMC migration and patterning, which can be rescued with Arf6 mRNA. Newly fertilized embryos were perturbed with Arf6 MASO, and immunolabled with ID5 PMC antibody. Arf6 MASO exhibited cell clumps with no visible PMC migration in the gastrula embryos. Arf6 MASO-induced PMC defects were rescued with sea urchin or human Arf6 mRNA.

structure and migration. Arf6 MASO treated embryos exhibited PMC clusters at the ventral region and lack proper migration to the anterior pole. We found that Arf6 MASO induced phenotypes were rescued with either the sea urchin or human Arf6 mRNA co-injection, and that the percent of normal gastrula in embryos co-injected with Arf6 MASO and Arf6 mRNA were similar to the percent of normal gastrula in the control MASO injected embryos (Fig. 9).

4.4 Arf6 Regulates Gut Morphology

During gastrulation, endodermal cells undergo extensive morphological movement where they invaginate into the blastocoel to form the larval gut. We examined the effect of Arf6 perturbation on gut development by injecting newly fertilized eggs with the constitutively active Arf6 (Q67L) mRNA, the dominant negative Arf6 (T27N) mRNA or Arf6 MASO. At gastrula stage, these embryos were assayed with endodermal immunolabeling, using the Endo1 antibody, which recognizes an antigen expressed by the endodermal cells in the midgut and hindgut of the embryo (Wessel and McClay, 1985). Constitutively active Arf6 (Q67L) mRNA injected embryos exhibited disorganized endodermal gut epithelial cells that seem to be detached from the tight epithelial layer of the gut, whereas dominant negative Arf6 (T27N) mRNA injected embryos expressed a reduction in endodermal staining with Endo1 antibody (Fig. 10). Arf6 MASO (knockdown) embryos in severe cases

exhibited exogastrulation (protruding gut) (Fig. 10), and in mild cases, displayed disorganized epithelial cells lining of the larval gut (data not shown). Less than 10 percent of the Arf6 MASO injected embryos had a visible gut (Fig. 11). To assess the specificity of gut defects induced by Arf6 MASO-injected embryos, we tested if these gut defects could be rescued with co-injection with sea urchin or human Arf6 mRNA. Normal gastrula is defined as having a visible gut structure. These results indicate that Arf6 MASO–induced gut phenotypes is a result of the loss of Arf6, since these defects were rescued with supplementing the embryo with exogenous sea urchin or human Arf6 mRNA (Fig. 11). These results also strongly indicate that the Arf6 function is highly conserved, since the human Arf6 is able to rescue Arf6 KD in the sea urchin embryo. Further, blastopore size was also affected in perturbed embryos. Arf6 Q67L mRNA and Arf6 MASO-injected embryos exhibited a significantly larger blastopore when compared to control, which suggest that Arf6 could play a role in blastopore formation and the gut elongation process (Fig. 12).

Gut structure and function was also assessed in Arf6 perturbed embryos using alkaline phosphatase, which is used as a marker for differentiated endoderm known to be only expressed in the gut epithelium of sea urchin larvae (Annunziata et al., 2013; Drawbridge, 2003; Hinman and Degnan, 1998; Kumano and Nishida, 1998; Nishida and Kumano, 1997; Whittaker, 1990). Embryos injected with constitutively active Arf6 (Q67L) mRNA expressed significant developmental delay and less endogenous alkaline phosphatase, while embryos injected with dominant negative Arf6 (T27N) mRNA appear to have a slight developmental delay compared to control (Fig. 13). The

Arf6 MASO (knockdown) embryos exhibited a significant delay in development (similar to embryos injected with Arf6 Q67L mRNA) and aberrant larval gut structures (Fig. 13). Arf6 MASO gut defects were rescued with co-injection of sea urchin or human Arf6 mRNA respectively (Fig. 14). We can conclude that Arf6 is highly conserved and may regulate various aspects of gut development, including proper gut morphology and endodermal differentiation in early development.

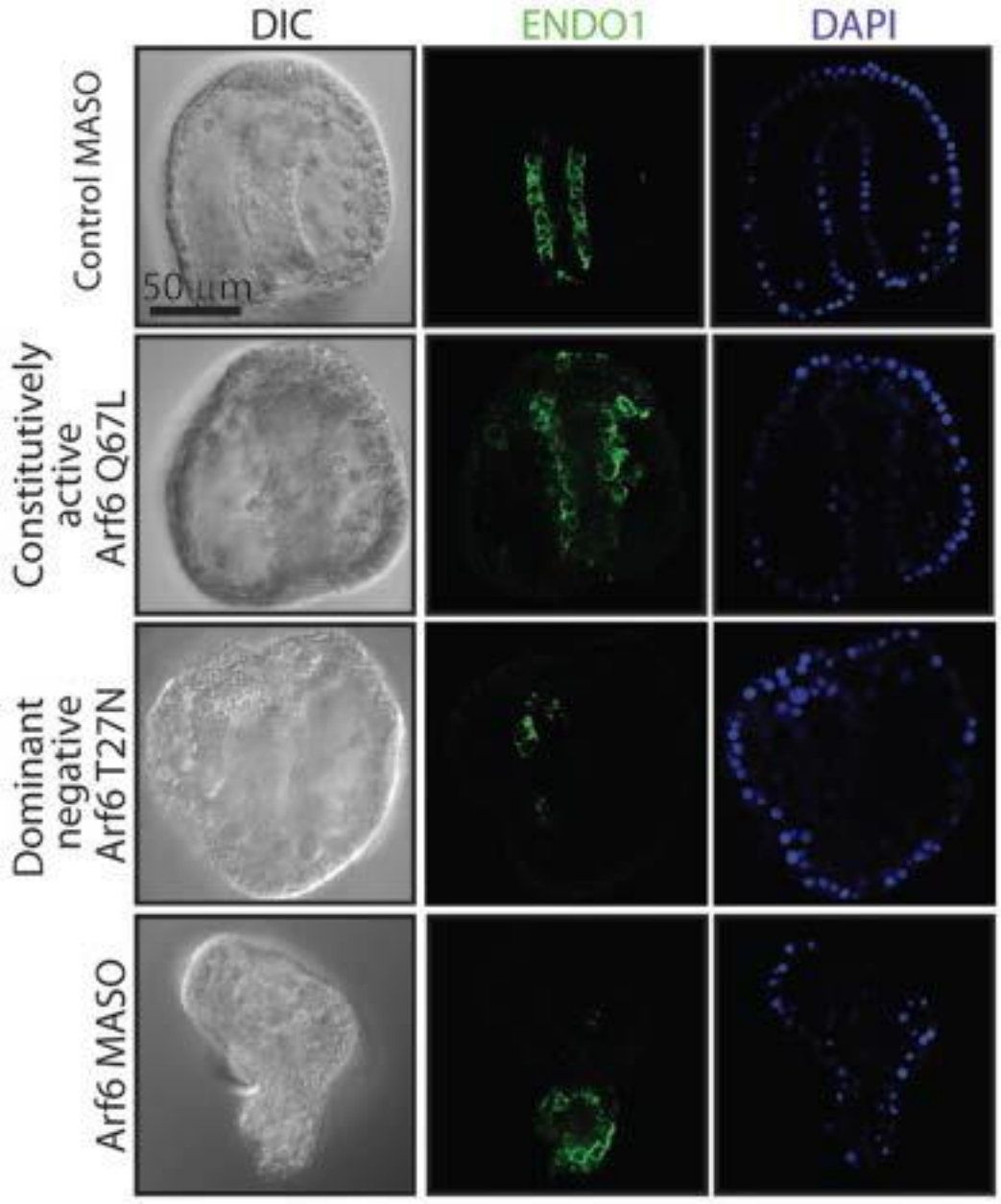

Figure 10.Arf6 perturbation results in endodermal defects. Embryos injected with the constitutively active Arf6 mRNA leads to disorganized endodermal gut cells, whereas embryos injected with the dominant negative Arf6 T27N mRNA have decreased endodermal staining with the Endo1 antibody, which recognizes the mid- and hindgut. Arf6 knockdown embryos exhibited exogastrulation (protruding gut) in severe cases.

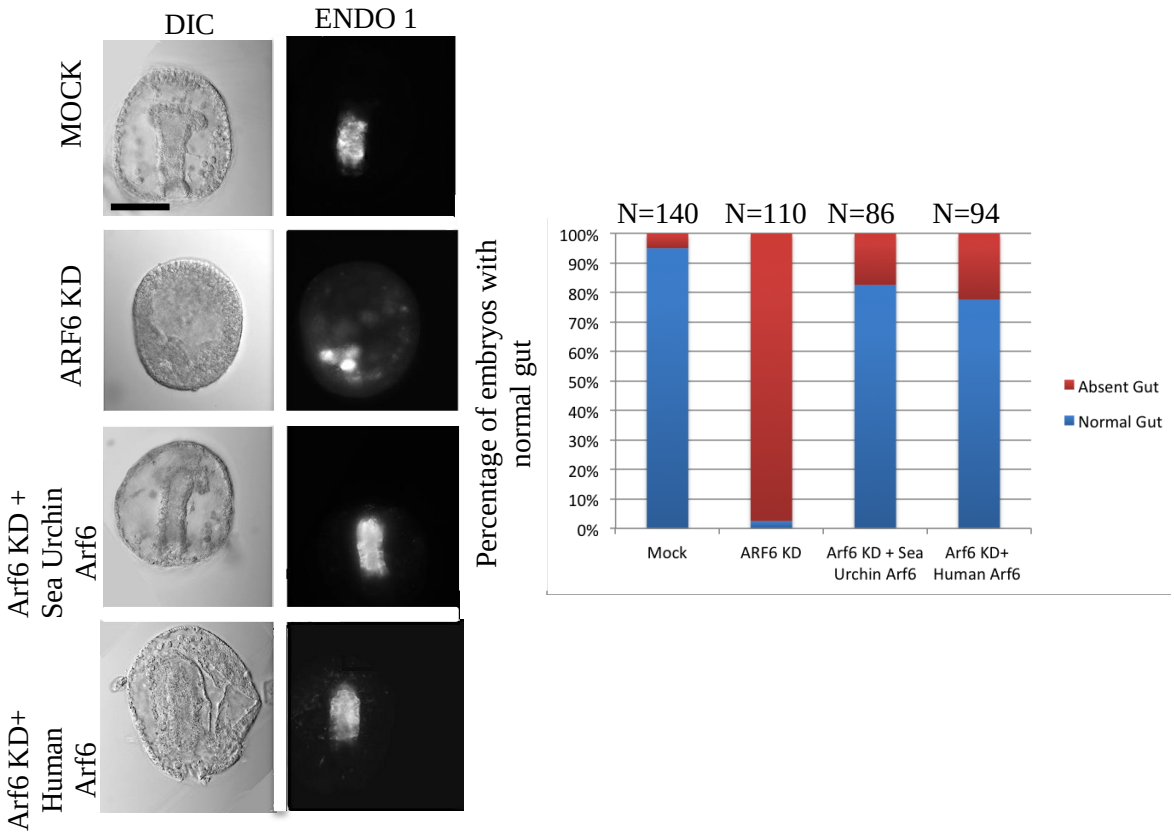

Figure 11.Endodermal defects are rescued with Arf6 mRNA. Embryos injected with Arf6 MASO led to disorganized endodermal gut cells. Gastrulae were immunolabeled with Endo1 antibody against the midgut and hindgut. Arf6 knockdown (KD) exhibited cell clumps with no visible gut. Arf6 MASO-induced gut defects were rescued with sea urchin or human Arf6 mRNA (2 biological replicates).

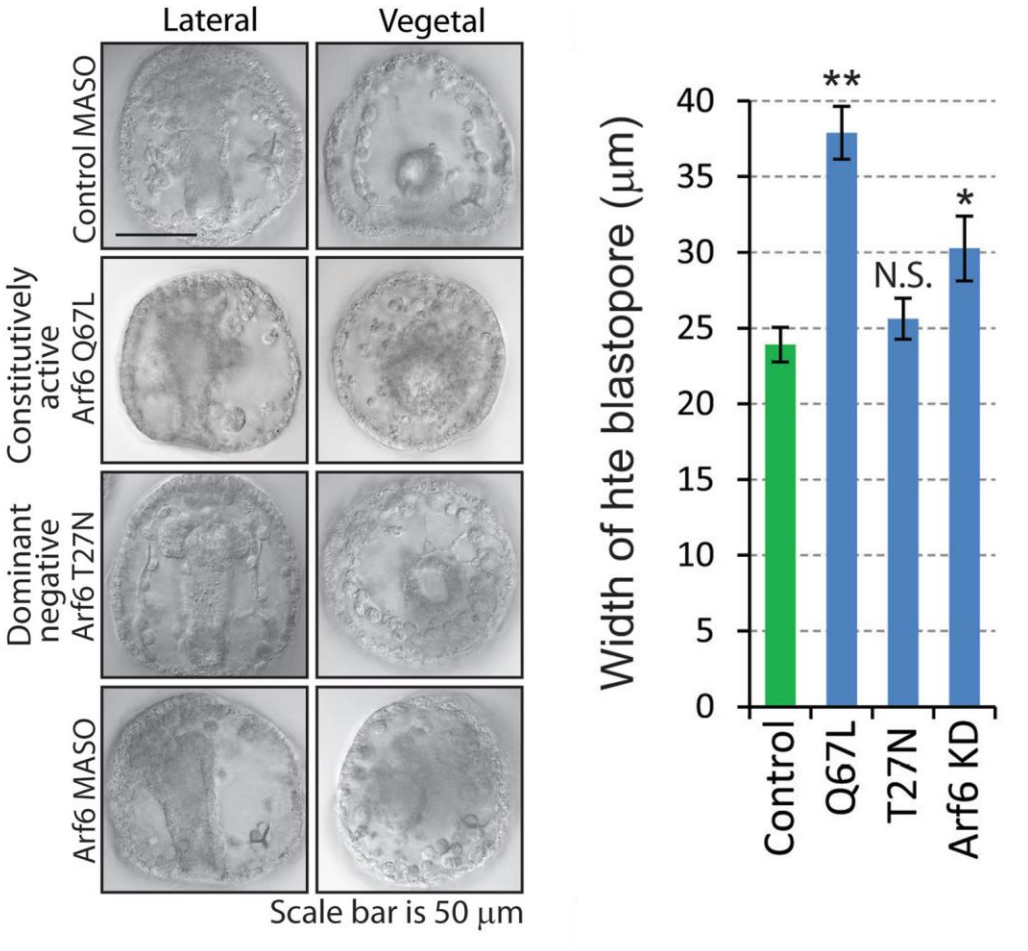

Figure 12.Arf6 perturbation results in gut developmental defects. The size of the blastopore of the embryos injected with the constitutively active Arf6 Q67L mRNA and Arf6 MASO is significantly larger than the control, suggesting that Arf6 may be involved in blastopore formation and gut elongation process.

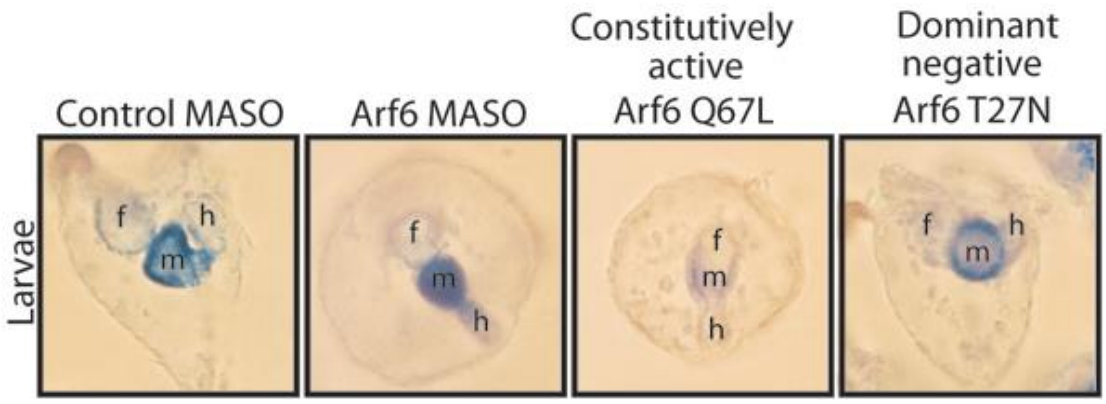

Figure 13.Arf6 perturbation results in endodermal differentiation defects. Embryos injected with active Arf6 Q67L mRNA have significant developmental delay as well as reduced endogenous alkaline phosphatase. Embryos injected with the inactive form of Arf6 T27N mRNA appear to have a slight developmental delay with a similar level of alkaline phosphatase and normal gut morphology compared to the control. Although the Arf6 knockdown embryos stained for alkaline phosphatase, these embryos are significantly delayed in development. f=foregut, m=midgut, and h=hindgut.

4.5 Arf6 Regulates PMC Patterning

To test the effect of Arf6 perturbation on skeletogenic cell specification and patterning, we microinjected newly fertilized eggs with Arf6 MASO, constitutively active Arf6 (Q67L) mRNA and dominant negative Arf6 (T27N) mRNA. To assay for PMC specification and patterning, we used RNA *in situ* probe against SM-50, which is specifically expressed in PMCs (Fig. 14) (Benson et al., 1991) and a 1D5 PMC specific antibody (McClay et al., 1983). We found that all Arf6 perturbed embryos expressed defects in PMC patterning and/or spicule formation (Fig. 15). Embryos injected with either the constitutively active Arf6 (Q67L) or dominant negative Arf6

(T27N) mRNAs resulted in a lower percentage of embryos with equatorial ring pattern in comparison to the control embryos (Fig. 15). The majority of the Arf6 KD embryos exhibited PMCs equatorial ring formation; however, the PMCs are clearly clustered bilaterally at the border ectoderm and dorsal ventral margin intersection next to the embryonic gut (Fig. 15), indicating PMC patterning defect.

Arf6 Q67L mRNA injected embryos resulted in seemingly random PMC localization that fail to pattern appropriately with extensive filopodial projections (Fig. 14). Embryos injected with T27N Arf6 mRNA appear to have more extensive, finer, membranous connections among the PMCs and longer spicules (Figs. 14). Arf6 KD embryos failed to migrate anteriorly (Fig. 14). The spicule length of embryos injected with Arf6 Q67L mRNA and Arf6 KD embryos also have significantly shorter spicules as compared to control. We performed a rescues experiment on Arf6 KD embryos by co-injecting with sea urchin Arf6 mRNA or human Arf6 mRNA (Fig. 15). Results indicated that PMC migration and spicule formation defects were rescued, indicating that the Arf6 MASO-induced PMC defects were specific to the loss of Arf6 and that this protein is evolutionarily conserved in function.

4.6 Arf6 Regulates Pigment Cell Morphology

We previously noticed that Arf6 perturbation resulted in defects in pigment cell morphology and movement (initial observation by Kelsie Landis, undergraduate

researcher). Pigment cells have significantly shorter pseudopodial projections in Arf6 KD embryos, and significantly longer pseudopodial projections in Arf6 Q67L or T27N perturbed embryos (Landis, unpublished). This observation leads me to the hypothesis that Arf6 may play a regulatory role in pseudopodial projections formation in pigment cells.

Previous study has shown adult sea urchin phagocytes mount an immune response in the presence of LPS (Majeske et al., 2013). To elucidate the effects of Arf6 perturbation on the ability of pigment cells to elicit an immune response, we treated Arf6 perturbed embryos in the presence or absence of LPS. Embryos were imaged prior to and after LPS exposure in Arf6 perturbed embryos and we video recorded how pigment cells react in the presence of LPS. In the absence of LPS, the pigment cells undergo dynamic extension and contractions. However, in the Arf6 MASO-treated embryos, their pigment cells are rounded in morphology. In the presence of LPS, the pigment cells changed morphology in control MASO- injected embryos from long dendritic projections to rounded appearance (Fig. 15). This change in pigment cell morphology was used as a marker for an immune response mounted by the pigment cells when expose to LPS. We found that upon exposure to LPS, all Arf6 Q67L and Arf6 T27N mRNAs perturbed embryos pigment cells underwent a morphological change from having pseudopodial projections to having minimal projections (Fig. 15), which suggest that the pigment cells are able to mount an immune response. However, Arf6 knockdown embryos had round pigment cells prior to LPS exposure, so we could not assess their ability to mount an immune response.

Based on these findings, we propose that Arf6 perturbation affects the dendritic morphology but does not seem to affect the ability of pigment cells to mount an immune response to LPS in constitutively active Arf6 Q67L and dominant negative Arf6 T27N mRNA-injected embryos (Fig. 15).

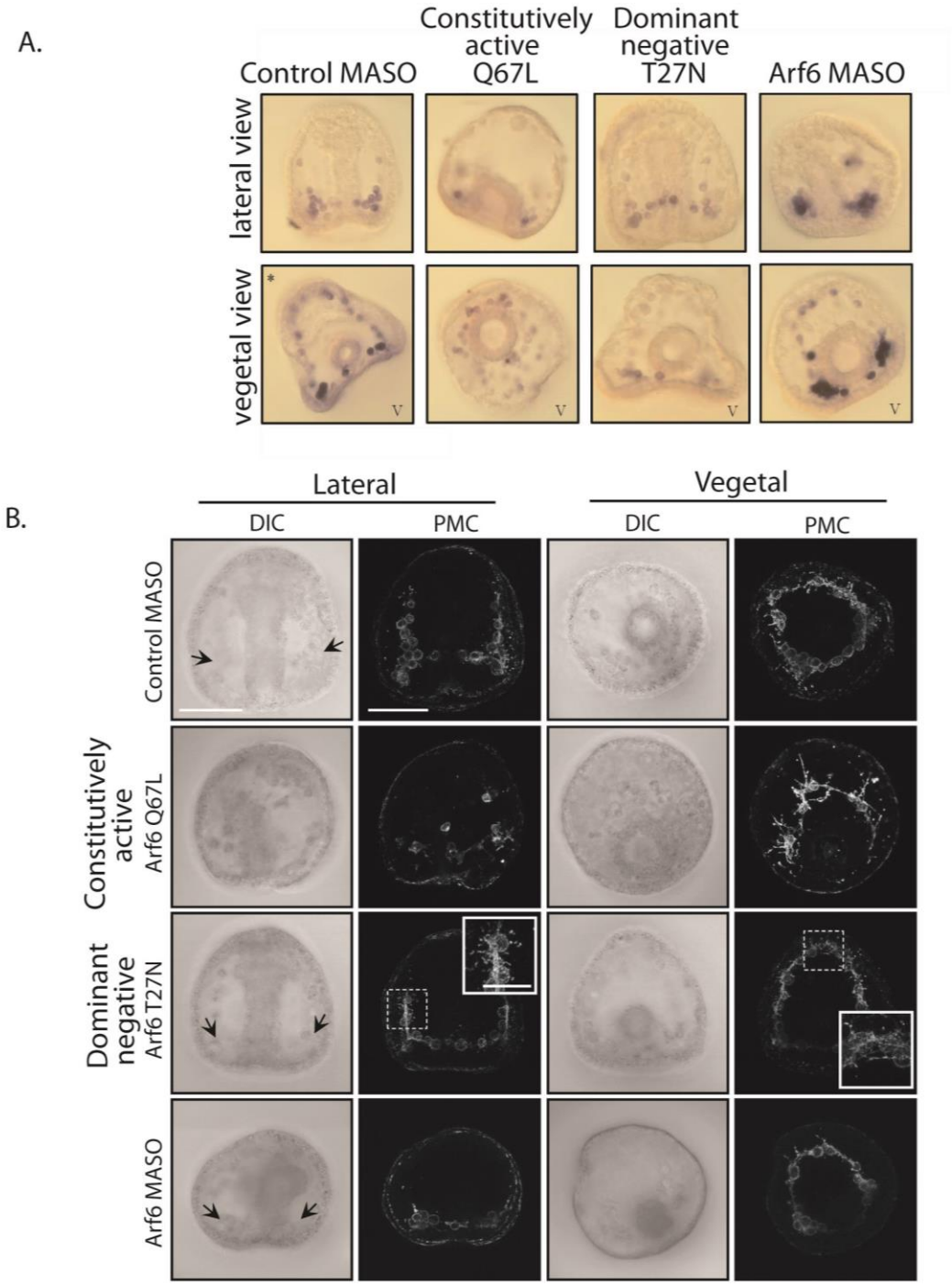

Figure 14.Arf6 regulates PMC patterning. (A) Arf6 perturbed embryos stained SM-50 *in situ* probe showed PMC arrangement in embryos injected with constitutively active Arf6 Q67L mRNA is not in an orderly ring structure around the blastopore from the vegetal view (V). Arf6 knockdown embryos have PMCs that fail to migrate anteriorly. Representative images of the mutant and knockdown embryos are shown

for each construct. One biological replicate was conducted. (B) Arf6 Q67L or Arf6 T27N mRNAs have lower percentage of embryos with highly predictable equatorial ring pattern in comparison to the control embryos. The majority of the Arf6 KD embryos have PMCs formed in a ring in the ventral part of the embryo, but the PMCs failed to migrate anteriorly. Q67L Arf6-injected embryos have thick filopodia and T26N Arf6-injected embryos have finer filopodia.

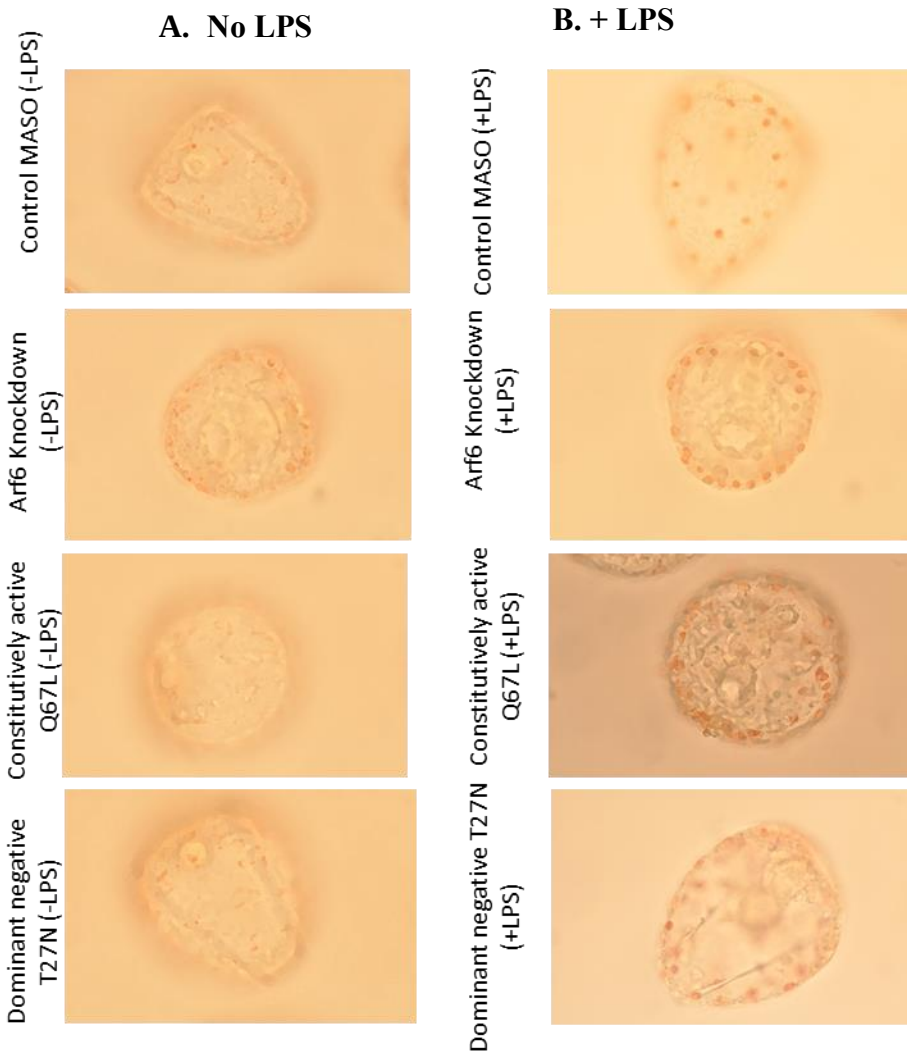

Figure 15.LPS response of pigment cells in Arf6 perturbed larvae (72 hpf). (A) Untreated embryos were imaged prior to LPS exposure. Pigment cells in control embryos have numerous pseudopodial projections undergoing dynamic contraction and extension. Embryos injected with constitutive active Arf6 Q67L or dominant negative T27N Arf6 mRNA have pigment cells with similarly long, extensive pseudopodial projections. Arf6 knockdown (MASO) larvae had pigment cells that lacked pseudopodial projections. (B) Upon LPS exposure (50 µg/mL) for 30 min, all embryos have darker pigment cells with a rounded morphology.

Chapter 5

DISCUSSION

As the sole member of class III Arf family, Arf6 is a major regulator of cytokinesis, recycling of adherens junctions, clathrin-dependent and independent receptor mediated endocytosis, and actin rearrangements (Donaldson, 2003; Sabe, 2003). Despite the fact that cellular functions of Arf6 have been well characterized, their physiological function in development is moderately obscure. In this study we examined the physiological function of Arf6, using a loss-of-function reagent and various Arf6 mutants that have defective membrane trafficking as characterized in mammalian cells.

Perturbation of sea urchin embryos with Arf6 MASO resulted in dose-dependent developmental defects in PMC patterning, gut morphogenesis, and pigment cell morphology. Homozygous deletion of Arf6 in mice models (*arf6* -/-) resulted in embryonic lethality starting at midgestation stage (Suzuki et al., 2006). In contrast, the sea urchin embryos have Arf6-induced defects earlier than the *arf6* -/- mouse embryo. This lethality in *arf6* -/- mice is due to liver hepatic cord formation defects; however, the exact molecular mechanism of Arf6 is unclear. The ability of the mouse embryo to survive into the midgestation stage suggest that there may be redundant mechanisms of protein and membrane trafficking which may allow for proper cell migration and actin cytoskeleton reorganization. Internalization of factors such as E-

cadherin by Arf6 is essential for epithelial cell motility; therefore, perturbation of Arf6 can lead to a defect in E-cadherin recycling which may explain the defect in hepatic cord formation. Arf6 MASO perturbed zebrafish embryos expressed mild effects on epiboly; however, the dominant negative T27N mRNA-treated embryos exhibited delay in epiboly progression, which could be as a result of the loss of Arf6-dependent recycling of syndecan (Lambaerts et al., 2012b; Zimmermann et al., 2005). The delay development in the dominant negative T27N mRNA-treated zebrafish embryos is similar to the phenotypes in the sea urchin T27N perturbed embryos. Hence, Arf6 is crucial for early embryonic development.

Embryos microinjected with the constitutively active Arf6 Q67L mRNA resulted in disorganized endodermal epithelium (Figs. 10-14). During gastrulation, the endodermal cells maintain cadherin connections as they invaginate into the blastocoel as a sheet of cells (Miller and McClay, 1997). During this morphogenetic process, the localization of cadherin and its abundance in these cells were found to be unchanged, suggesting that endothelial cells reconfigure their cell adhesive properties via the mediation of cadherin and cadherin associated proteins (such as β-catenin) interaction, which allows it to maintain its association with the actin cytoskeleton (Miller and McClay, 1997). Embryos injected with Arf6 Q67L seem to have gut epithelial cells that are detaching from the main gut epithelium, indicating that constitutively active Arf6 may potentially disrupt the adherens junction homeostasis either through cadherin trafficking or actin remodeling.

Microinjections of embryos with the constitutively active Arf6 Q67L mRNA resulted in widened blastopore and gut when compared to control (Fig. 12). One possible explanation for this observation is the role of Arf6 in actin rearrangements in the cells that form the lip of the blastopore. It has been shown that in the sea urchin species *L. pictus*, blastopore formation is controlled by cells that constrict apically followed by the width of the blastopore decreasing as elongation of the gut progresses (Hardin, 1989; Kimberly and Hardin, 1998). It has been proposed that these morphological changes in these cells generate the driving force required for invagination to occur (Nakajima and Burke, 1996). The decrease in blastopore width leads to increasing archenteron (or primitive gut) length during gastrulation; therefore, the gut elongation which involves non-skeletogenic mesenchyme (NSM) cells pulls the archenteron in order for the gut to reach its final length (Hardin, 1988). Studies using laser ablation of the filopodia connections of NSM cells at the animal pole results in shortening of gut elongation to two-thirds of its final length, demonstrating that the filopodia connections of the NSM are critical for proper gut elongation (Hardin, 1988; Hardin, 1987). Hence, Arf6 perturbed NSMs may have a disruption in the filopodial structures that prevented their filopodia from extending and elongating the gut, resulting in an alteration of the width of the blastopore (Miller et al., 1995).

Arf6 perturbed embryos resulted in defective PMC development and function. PMCs undergo directional migration from ventrolateral position toward the anterior end of the embryo during differentiation (Peterson and McClay, 2003). During the

process they undergo a sequence of patterning in order to form the skeleton spicules (Peterson and McClay, 2003). In contrast with the endodermal cells which migrate as a sheet of cells to form the gut, PMCs ingress into the blastocoel as individual cells. Embryos injected with the constitutively active Arf6 Q67L mRNAs resulted in PMCs with thicken, and extensive filopodial connected especially prominent once observed from the vegetal view (Fig. 14). The embryos injected with dominant negative Arf6 T27N mRNA, however, exhibit finer filopodial connections among the PMCs (Fig. 14). These finding are reminiscent of what has been shown in the rat nervous system, where Arf6 was shown to regulate dendritic spine and axons formation in hippocampal neurons (Choi et al., 2006; Hernandez-Deviez et al., 2004). Active Arf6 Q67L promotes thicker dendritic spines formation and the dominant negative Arf6 T27N promotes thinner dendritic spines formation and filopodial structures in rat hippocampal cells (Choi et al., 2006; Hernandez-Deviez et al., 2004). Results demonstrate that Arf6 KD and Arf6 Q67L mRNA-injected embryos have defects in PMC patterning and spicule formation (Fig. 14). PMCs undergo directed migration in response to FGF and VEGF signaling (Adomako-Ankomah and Ettensohn, 2013; Duloquin et al., 2007; Röttinger et al., 2008). Arf6 may play regulate the recycling of growth factor receptors that are critical for PMC patterning. Hence, Arf6 likely regulates PMC patterning through its actin remodeling ability in forming filopodial structures, as well as its ability to mediate recycling of growth factor receptors.

Pigment cells are exceptionally motile and are thought to be the immune cells of the sea urchin larvae (Ruffins and Ettensohn, 1993; Shoguchi et al., 2002). We found that Arf6 knockdown embryos have altered pigment cell morphology with significantly shorter pseudopodial extensions. Embryos injected with Arf6 Q67L and T27N mRNAs have longer pseudopodial extensions compared to the control embryos (Fig. 15). Interestingly, sea urchin pigment cells and the dendritic cells in the vertebrate immune system behave in a similar manner. Dendritic cells are antigen-presenting cells that function in the immune system of vertebrates. They have numerous pseudopodal projections, similar to those of sea urchin pigment cells (Leon et al., 2014). When vertebrate dendritic cells mount an immune response, they exhibit a rounded shape by utilizing amoeboid-like migration via actin network and actomysin contractions (Heuze et al., 2013). Activated dendritic cells utilize pseudopods and podosomes which are mediated by small GTPases Arf6 and Cdc42 (Heuze et al., 2013). Since the pseudopodial extensions may be indicative of the pigment cell's ability to move and potentially its ability to mount an immune response, we examined their behavior in the presence of LPS. In normal sea urchin pigment cells, upon exposure to LPS, the pigment cells adopt a spherical morphology, which is similar to rounded morphology of dendritic cells in response to an immune challenge (Fig. 15) (Heuze et al., 2013). Pigment cells injected with Arf6 MASO did not change in their morphology upon LPS, suggesting that the pigment cells in these embryos may fail to mount an immune response or that they are always in an activated state (Fig. 15). Pigment cells in embryos injected with either the Arf6 Q67L or the T27N mRNAs

underwent a change to a rounded morphology, indicating that they are able to mount an immune response (Fig. 15). Thus, Arf6 may regulate dendritic projections of pigment cells, but not affecting their ability to respond to LPS.

Overall, we demonstrated that Arf6 is essential for proper early development and plays a critical role in the regulation of endodermal gut morphology, PMC development, and pigment cell morphology. The regulatory mechanism of Arf6 may involve (1) mediate plasma membrane to endosomal membrane trafficking of cell surface receptors and proteins including signaling molecules, adhesion molecules, and growth factor receptors, and (2) remodeling actin based structures in order to regulate cell movement and the formation of filopodial structures. This study shed light on the key functions of Arf6 in sea urchin early development, a point that has not been investigated. In future studies, we plan to explore the molecular mechanism of Arf6 in regulating endodermally and mesodermally-derived cell types.

Chapter 6

FUTURE WORK

6.1.1 Examining the Molecular Mechanism of Arf6 in Mediating Cell Movement

We demonstrated that during the gastrula stage of early development, perturbation of Arf6 results in a wide, stunted gut. Adhesion molecules such as integrin and cadherins have been shown to associate with the Arp2/3 complex in order to mediate actin assembly and adhesive contacts (Kovacs et al., 2002). We propose using integrin and cadherin reporter constructs to examining expression of these adhesion factors in Arf6-perturbed embryos. We expect to observe internalization of cadherin and integrin, which may explain some of the defects that we observed.

6.1.2 PMC Migration in Response to Growth Factors.

Upon format ion of the sub-equatorial ring surround the gut, a subset of PMCs migrate in response to VEGF signaling from the posterior end of the embryo toward the anterior end of the embryo (Adomako-Ankomah and Ettensohn, 2013; Duloquin et al., 2007). We will test the hypothesis that the defective PMC patterning in Arf6 perturbed embryos have defects in trafficking of VEGF receptors expressed on the cell surface of PMCs. We plan to perform in vitro experiments where the PMCs will be extracted from wild type, Arf6 KD, constitutively active and dominant negative

embryos, and culture them in the presence of VEGF ligand. We will measure their migration direction and velocity in the presence or absence of the VEGF ligand. This will further elucidate the molecular mechanism underlying the PMC patterning defects in Arf6-perturbed embryos.

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