Investigations in Early Polarity in the Sea Urchin Embryo

Author: Kathleen Moorhouse

Boston College

The Graduate School of Arts and Sciences

Department of Biology

INVESTIGATIONS IN EARLY POLARITY IN THE SEA URCHIN EMBRYO

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KATHLEEN S. MOORHOUSE

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Kathleen S. Moorhouse

Thesis Advisor: Dr. David R. Burgess

Abstract

Establishment and maintenance of cell polarity has become an increasingly interesting biological question in a diversity of cell types and has been found to play a role in a variety of biological functions. Previously, it was thought that the echinoderm embryo remained relatively unpolarized until the first asymmetric division at the 16cell stage of development. However, there is mounting evidence to suggest that polarity is established much earlier. I analyzed roles of the cell polarity regulators, the PAR complex proteins, and how their disruption in early development affects later developmental milestones such as blastula formation. I found that PAR6 along with aPKC and CDC42 localize to the apical cortex (free surface) as early as the 2cell stage of development and this localization is retained through the gastrula stage. Interestingly, PAR1 also colocalizes with these apical markers through the gastrula stage, despite the formation of a polarized epithelium and a series of asymmetric divisions. Additionally, PAR1 was found to be in complex with aPKC, but not PAR6, during these developmental stages. PAR6, aPKC, and CDC42 are anchored in the cortex by assembled myosin; however, a clear role for myosin assembly in PAR1 localization could not be determined. Furthermore, myosin assembly was found to be necessary to maintain proper PAR6 localization through subsequent cleavage divisions. Interference with myosin assembly prevented the embryos from reaching the blastula stage, while transient disruptions of either actin or microtubules did not have this effect. Similarly, inhibition of aPKC

activity during early cleavage stages impeded blastula formation; however, aPKC is not involved in the regulation of the first asymmetric division at the 16cell stage in sea urchin embryos. These observations suggest that disruptions of the polarity complex in the early embryo can have a significant impact on the ability of the embryo to reach later critical stages in development.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ABC- ATP-binding cassette

aPKC – Atypical protein kinase C

aPKCBC – aPKC binding domain

ASW – Artificial seawater

ATP – Adenosine triphosphate

AV – Animal-vegetal

Brat – Brain tumor

CAB - Centrosome attracting body

CaFSW- Calcium free seawater

CDC42 - Cell division control protein 42 homolog

CLASP - Cytoplasmic linker associated protein

CPC – Chromosomal passenger complex

CR1 – Conserved region domain 1

Dlg – Discs large

DMSO – dimethyl sulfoxide

DN – Dominant negative

DNA - Deoxyribonucleic acid

FSW – Filtered seawater

GEF – Guanine exchange factor

GFP – Green fluorescent protein

GTP – Guanosine triphosphate

HRP - Horseradish peroxidase

KA – Kinase associated domain

LGL – Lethal giant larvae

LGN - Leucine-Glycine-Asparagine

MAP – Microtubule associated protein

MARK – Microtubule associated regulatory kinase

MLCK – myosin light chain kinase

mRNA – Messenger RNA

Mud – Mushroom body defect

NuMA -Nuclear mitotic apparatus protein

PABA - Para-aminobenzoic acid

PAR – Partitioning defective

PB1- Phox and bem1 domain

PBS - Phosphate buffered saline

PBT – Phosphate buffered saline with 0.1% Triton

PCP – Planar cell polarity

PDZ – Post-synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (Dlg1), and Zonula occludens-1 protein (zo-1)

PEM - Posterior end mark

PI3 kinase - Phosphatidylinositol-4,5-bisphosphate 3-kinase

Pros- Prospero

RHO -Ras homolog gene family

RING – Really interesting new gene

RNA – Ribonucleic acid

ROCK – Rho kinase

ROI – Region of interest

RT-PCR – Reverse transcriptase polymerase chain reaction

Scrib – Scribble

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBS – Tris buffered saline

TBS-T – Tris buffered saline with 0.1% Tween

TE – Tris, EDTA

UBA – Ubiquitin associated domain

Zn – Zinc finger domain

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Chapter 1. Introduction: Mechanisms of Polarity

1.1. Overview of Polarity

1.1.1. Establishment and Maintenance of Polarity

The establishment and maintenance of polarity is now a well-studied biological phenomenon because it is required for such a vast array of biological processes. Biological polarity is defined by segregation of molecules into discrete domains and it is this segregation that produces distinct functionality in different regions. Some examples of biological polarity include migrating cells, maintenance of neuronal asymmetries, and asymmetric distributions of developmental determinants during embryogenesis. These examples highlight the necessity of a differential distribution of cellular components in order to carry out specialized functions, such as the release of a neurotransmitter or the development of filopodia. The signaling components required for these polarity events are well conserved across a wide array of organisms and biological activities.

The establishment of polarity can be broken into four primary components: breaking symmetry, establishing cortical landmarks, polarizing the cytoskeleton, and amplifying and maintaining the polarized state (McCaffrey and Macara, 2009). Maintenance of the polarized state or its disassembly then becomes essential for carrying out various functions. While embryos may only need to maintain a signal for endomesoderm specification for a short duration in development, neurons need to be able to maintain the signal for their dendritic and axonal processes throughout the lifetime of the cell. The maintenance of polarity is carried out by a variety of cellular factors such as the cytoskeleton and endocytic machinery. These cellular components are then utilized for a range of processes including translocation, anchoring, active exclusion, and positive

feedback loops (McCaffrey and Macara, 2012). The asymmetric distribution of cellular components can then ensure that cellular events only occur in the particular regions in which all the necessary factors are properly localized.

1.1.2. Types of Polarity: Cell, Embryonic, and Planar Cell

There are three main types of biological polarity: embryonic, cell, and planar cell polarity (PCP). All of these types of polarity exhibit asymmetric distribution of RNAs, lipids, and proteins that allow for the development of a specialized function in one region over another. Neurons are a common example of a highly polarized cell; their axons function to transmit signals and dendrites are found on the opposing end to receive those signals (Nishimura et al., 2005). In addition to neurons, epithelial cells are also frequently studied models of polarity. Epithelial cells are widely present throughout the body and polarize to form epithelial sheets that are responsible for lining organs and ensuring that transcellular transport occurs in the correct direction (McCaffrey and Macara, 2012;St Johnston and Ahringer, 2010). Embryonic polarity, on the other hand, refers to the asymmetric distribution of regulators of different developmental fates. These developmental determinants must reach the right location at the right time during development; any errors in this precision could be extremely detrimental to the developing embryo. Dishevelled is a common example of embryonic polarity as its accumulation in the vegetal pole of sea urchins, among other species, is known to regulate endomesoderm formation (Weitzel et al., 2004;Wikramanayake et al., 1998). Planar cell polarity, on the other hand, is used to describe the orientation of cells along an axis within the plane of an epithelium (Vladar et al., 2009). A typical model of planar cell polarity is the convergent extension movement that occurs during gastrulation, as

blastomeres from the blastula stage alter their phenotype and begin their migration into the blastocoel in order to form the three primary germ layers. An example of this can be seen during zebrafish gastrulation when planar cell polarity is required for cell elongation and mediolateral alignment (Dohn et al., 2013). While these three different types of polarity polarize an organism in distinct ways, there is a core group of proteins that are often utilized by all three to regulate polarity.

1.2. The Discovery of the PAR Proteins

1.2.1. Initial Findings in C. elegans Embryos

The PAR proteins are a core group of signaling proteins that are major regulators of cell polarity. They were first discovered by Kempues and colleagues in a screen of maternal embryonic lethal genes in *Caenorhabditis elegans* (Kemphues, 2000;Kemphues et al., 1988). The first division following fertilization is asymmetric in *C. elegans* embryos and the PAR proteins were found based on their involvement in the regulation of this first division. Since their initial discovery each of the PAR proteins has been further characterized (Figure 1.2.1.). PAR1 and PAR4 have both been identified as serine-threonine kinases (Guo and Kemphues, 1995;McCaffrey and Macara, 2009;Watts et al., 2000). PAR2 is a nematode specific PAR protein; it is a RING (Really Interesting New Gene) finger domain protein that functions as an E3 ubiquitin ligase (Boyd et al., 1996;Levitan, et al., 1994). PAR3 and PAR6 are both PDZ (post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)) domain proteins that act as scaffolding proteins for the other PAR proteins as well as for other polarity regulators, such as aPKC (atypical protein kinase C) (Etemad-

Moghadam et al., 1995;Hung and Kemphues, 1999;Tabuse et al., 1998;Watts et al., 1996). Lastly, PAR5 is a 14-3-3 protein that is recruited to phosphorylated serine and threonine residues (Morton et al., 2002). While PAR1 and PAR2 become enriched at the posterior cortex, PAR3 and PAR6 are enriched in the anterior cortex in C. *elegans* embryos (Kemphues, 2000). PAR4 and PAR5 are then symmetrically localized in both the cortex and the cytoplasm (Goldstein and Macara, 2007). Localization of the posterior proteins is necessary for the exclusion of the anterior proteins and vice versa (Kemphues, 2000). These mutual exclusion events ensure proper segregation of the PAR proteins and help to maintain the polarized domains found in the *C. elegans* embryo (Figure 1.2.1.). Asymmetry is not established until after fertilization in *C. elegans* embryos when the PAR proteins help to coordinate the localization of the mitotic spindle and cause asymmetric division (Ahringer, 2003).

1.2.2. The Role of the PAR Complex

In addition to the PAR proteins, the kinase aPKC and the GTPase CDC42 (Cell division control protein 42 homolog) have been found to play a significant role and function along with the PAR proteins in the establishment and maintenance of polarity (Figure 1.2.1.). PAR3, PAR6, and aPKC are known to interact in what is referred to as the PAR complex (Joberty et al., 2000;Lin et al., 2000;McCaffrey and Macara, 2009;Suzuki et al., 2001;Welchman et al., 2007). This PAR complex is one of the major regulators of polarity across a diversity of cell types and organisms (Figure 1.2.2.). The PAR complex is utilized to regulate polarity in anterior or apical domains, depending on the organism (McCaffrey and Macara, 2012). CDC42 was later found to act upstream of the PAR complex through its interaction with PAR6 (Joberty et al., 2000;Lin et al.,

2000a;McCaffrey and Macara, 2009). aPKC is known to inhibit PAR6 activity, but through its association with CDC42, this repression is partially relieved (Goldstein and Macara, 2007). CDC42 has also been shown to be necessary for the proper localization of the PAR complex in the cell cortex (Goldstein and Macara, 2007). CDC42 can be used to recruit the PAR complex to regions of the cortex where CDC42 is activated (McCaffrey and Macara, 2012). Before the discovery of its interaction with the PAR complex, CDC42 was already a known polarity protein as this small GTPase is a major regulator of the actin cytoskeleton (Iden and Collard, 2008). aPKC activity maintains the anterior domain through phosphorylation events that exclude other proteins from the anterior domain. aPKC phosphorylates PAR1, which both inhibits the kinase activity of PAR1 and causes its disassociation from the membrane (Hurov et al., 2004). The self-oligomerization of PAR3 is additionally necessary for the formation of higher-order complexes and maintenance of the polarized state (Dawes and Munro, 2011;Feng et al., 2007). The PAR complex, however, is not a constitutive complex; the interaction of PAR3, PAR6, and aPKC is regulated by numerous GTPases, kinases, and other binding partners (McCaffrey and Macara, 2012). The sometimes transient nature of the interactions of the PAR complex allow for an additional level of regulation of polarity.

1.2.3. The Role of the Posterior PAR Proteins

PAR1 and PAR2 function to regulate polarity in the posterior end of the *C. elegans* embryo (Figure 1.2.1). PAR1 is a well-conserved kinase that was independently discovered as microtubule-associated-regulatory-kinase (MARK), which is known to phosphorylate microtubule-associated-proteins (MAPs) such as Tau and MAP4 (Hurov and Piwnica-Worms, 2007). Depending on the organism, these phosphorylation events

serve to either stabilize or destabilize microtubules (Doerflinger et al., 2003). Similar to aPKC in the anterior cortex, PAR1 functions to maintain polarity in posterior cortex through phosphorylation events. PAR3 is a known target of PAR1 and its phosphorylation causes the dissociation of PAR3 from the cortex. Interestingly, *par-1* mutants in *C. elegans* embryos do not show severe defects in polarity (Boyd et al., 1996;Nance and Zallen, 2011).

PAR2 has to date only been identified in the *C. elegans* embryo. In these embryos it is not required for the establishment of polarity, but is needed to maintain the polarized state of the zygote. PAR2 functions to antagonize PAR3 recruitment of myosin, which allows for cortical flow towards the anterior (Zonies et al., 2010). Although PAR2 and PAR1 both localize the posterior cortex, it is not known if they regulate each other (Nance and Zallen, 2011). Furthermore, both of these proteins independently bind to the cortex (Boyd et al., 1996). While PAR2 expression has only been verified in the *C. elegans* embryo, another protein lethal giant larvae (LGL) is believed to function redundantly with PAR2 (Beatty et al., 2010;Hoege et al., 2010). While it not yet known how LGL regulates polarity, LGL is known to bind to myosin and to be involved in vesicle trafficking (Nance and Zallen, 2011;Strand et al., 1994).

1.2.4. The Role of the Cytoplasmic PAR Proteins

While the PAR complex proteins and the posterior PAR proteins have been extensively studied, there is significantly less research on the roles of PAR4 and PAR5. Unlike the other PAR proteins, PAR4 and PAR5 are symmetrically localized following fertilization in the *C. elegans* embryo (Figure 1.2.1.) (Morton et al., 1992;Morton et al., 2002;Watts et al., 2000). The 14-3-3 protein PAR5 is known to shuttle the other PAR

proteins to their respective domains, following phosphorylation events (McCaffrey and Macara, 2012). PAR5 recognizes and binds proteins with certain phosphorylated serine residues (Macara, 2004). In addition, binding of PAR3 with PAR5 prevents oligomerization of PAR3, which is required for its localization (Benton and Johnston, 2003). In the absence of PAR5, the anterior and posterior PAR proteins are not properly segregated and become intermixed (Morton et al., 2002). PAR5 is also involved in the regulation of cell cycle timing and in WNT signaling during early development in the *C. elegans* embryo (Aristizábal-Corrales et al., 2013). The targets of the kinase PAR4, however, to date remain relatively unknown (Nance and Zallen, 2011). PAR4 is required for polarization during oogenesis in *C. elegans*, which occurs prior to the establishment of the anterior-posterior axis. This suggests that PAR4 functions to prepare the oocyte for polarization (Morton et al., 1992). Further work has shown that PAR4 mutations reduce nonmuscle myosin contractility through its regulation of annilin (Chartier et al., 2011). Additional data in *Drosophila* have shown that PAR4 regulates apical-basal polarity under normal conditions (Martin and St Johnston, 2003). Future work will more clearly elucidate the functions of these proteins in the establishment and maintenance of polarity.

1.2.5. Cytoskeletal Regulation of the PAR Proteins Localization and Function

The cytoskeletal regulation of the PAR proteins has been an intensive area of study. In the *C. elegans* embryo the PAR proteins have been found to segregate to their anterior and posterior domains following fertilization due to changes in the actomyosin cortex. Polarization of the PAR proteins can be blocked by the knockdown of myosin and the inhibition of actin (Cowan and Hyman, 2007;Gonczy and Rose, 2005).

Following fertilization the sperm centrosome moves to the pole closest to the site of sperm entry and specifies this region as the posterior. Non-muscle myosin II and actin are initially distributed throughout the cortex and contract with no net directionality. Once the embryo begins polarization, however, contractile cortical ruffles become limited to the anterior. This leaves the posterior cortex smooth and the PAR proteins localize to distinct anterior and posterior domains (Nance and Zallen, 2011). Actomyosin flow is directed towards the anterior and induces the translocation of PAR3, PAR6, and aPKC (Munro et al., 2004). Advective flow causes the anterior PAR proteins to be passively transported to the anterior domain and clear from the posterior domain (Goehring et al., 2011). Myosin first begins to clear in the posterior adjacent to the sperm centrosome; centrosome ablation experiments have shown that the anterior PAR domain will not form in the absence of the sperm centrosome (Cowan and Hyman, 2004b;Munro et al., 2004;Nance and Zallen, 2011). The sperm centrosome can initiate polarity from any position within the embryo; however, increased distance from the cortex increases the time needed to initiate polarity (Bienkowska and Cowan, 2012). It is not yet known if a polarity cue emanates from the centrosome and the involvement of microtubules in the polarization of the PAR proteins remains controversial (Nance and Zallen, 2011). Rho GTPases such as CDC42 and RHO-1/RhoA also play important roles in the maintenance of polarity (Aceto et al., 2006;Schonegg and Hyman, 2006). Their interactions with actin, myosin, and the PAR proteins help to generate positive feedback loops that are required for maintenance of the polarized state (McCaffrey and Macara, 2012). This dependence on the actomyosin cortex for the polarization of the PAR proteins is

conserved in other model systems such as *Drosophila*, and mice (David et al., 2010;Leibfried et al., 2013;Simões et al., 2010;Solinet et al., 2011).

1.2.6. The Role of the PAR Proteins in Cells, Cancer, and Embryos

Since their initial discovery in the *C. elegans* embryo, the PAR proteins have been found to be a well conserved group of proteins that regulate polarity in wide variety of multicellular eukaryotes (Figure 1.2.2.). The PAR proteins are involved in many different biological processes at multiple times in both development and disease. The manner in which the PAR proteins polarize and control polarity in all of the various biological processes, however, is well maintained. Here their roles in the polarity of cells, cancer, and embryos are examined.

The PAR proteins regulate polarity in variety of mature cell types, including epithelial and neuronal cells. Neurons are a classic example of a polarized cell. They must be able to form and maintain both dendritic and axonal processes at opposing ends of the cell so that they can receive and then transmit neurological signals from one synapse to the next. The PAR proteins are essential for maintaining neuronal asymmetries. PAR3 and PAR6 are involved in axon formation in hippocampal neurons, while PAR1 function is needed for dendrite formation (Shi et al., 2003;Terabayashi et al., 2007). PAR3 has subsequently been found to regulate microtubule stability, which is necessary for axon specification whereas PAR1 is needed for microtubule growth (Chen et al., 2012;Hayashi et al., 2011a).

Epithelial cells must also be able maintain apical-basal polarity in order to carry out their barrier and transport functions. The apical domain faces the outer surface of the surrounding tissue, while the basolateral domain is defined as regions of cell contact and

the site of adherens junctions. The PAR proteins have been found to have important roles in the establishment and maintenance of polarity in the epithelial tissue as well as in spindle orientation during their divisions. In the epithelium of *Drosophila* PAR6 and aPKC regulate polarity in the apical marginal zone, while PAR3 regulates polarity at the adherens junctions (Doerflinger et al., 2010;Morais-de-Sá et al., 2010). Experiments in epithelial cell tissue culture demonstrated the involvement of PAR3, PAR6 and aPKC in the orientation of the mitotic spindle (Durgan et al., 2011;Hao et al., 2010). In *C. elegans,* the intestinal epithelial cells require PAR3 to recruit adherens junction proteins like E-cadherin to the apical surface during apical junction maturation (Achilleos et al., 2010). The posterior PAR protein, PAR1, also plays a significant role in the regulation of epithelial cell polarity. For example, PAR1 is involved epithelial lumen polarity and spindle orientation through its regulation of microtubules, myosin-II, E-cadherin, and LGN (Leucine-Glycine-Asparagine)-NuMA (Nuclear mitotic apparatus protein 1)(Cohen et al., 2004;Cohen et al., 2007;Lázaro-Diéguez et al., 2013).

Many cancers are derived from epithelial tissue and there is mounting evidence to suggest that a loss of polarity is a hallmark feature of cancerous tissues (McCaffrey and Macara, 2012). As the PAR proteins are known to regulate both polarity and spindle orientation, particularly in epithelial tissue, their involvement in cancer development and progression has begun to be studied. PAR6 and aPKC have been found to upregulated in certain cancers (Eder et al., 2005;Nolan et al., 2008). aPKC has also been found to be mislocalized from the apical membrane in both breast and ovarian cancers (Kojima et al., 2008). PAR6 activity is required for proper tight junction formation and aPKC localization, which becomes misregulated in breast cancer (Cunliffe et al., 2012). PAR3,

on the other hand, has been shown to have both tumor-suppressive and tumor-promoting properties depending on the type of tumor in skin cancer, while loss of PAR3 promotes tumor progression and metastasis in breast cancer (Iden et al., 2012;McCaffrey et al., 2012). Mutations in PAR4 are also known to cause the heritable Peutz-Jegers syndrome, which elevates risks of multiple types of cancer including pancreatic and liver cancer (Wodarz and Näthke, 2007). Collectively, these results highlight that, depending on the mutations in particular cancers, the PAR proteins can either contribute to tumor suppression or progression. A better understanding of how the PAR proteins function in cancer development is required before these proteins can be utilized as possible drug targets and therapeutic interventions.

The PAR proteins have been found to play a conserved role in polarity during development across a multitude of species including *Drosophila, Xenopus*, mice, and humans (McCaffrey and Macara, 2009) (Figure 1.2.2.). PAR6 along with aPKC and CDC42 have been shown to regulate neuroblast polarity in *Drosophila* (Atwood et al., 2007). *Drosophila* also requires the presence of aPKC in order to properly form adherens junctions around the apical domain during gastrulation (Harris and Peifer, 2007). In mouse embryos PAR3 and aPKC become polarized at the 8 cell stage of development and help to regulate cell divisions (Plusa et al., 2005;Vinot et al., 2005). PARD6B also plays a role in trophoectoderm formation in the mouse embryo and aPKC is involved in endoderm maturation (Alarcon, 2010;Saiz et al., 2013). Additionally, in *Xenopus* PAR1 along with aPKC and 14-3-3 (PAR5) are critical for gastrulation (Hyodo-Miura et al., 2006;Kusakabe and Nishida, 2004). Other studies have also shown that aPKC is involved in polarized cell divisions during the blastula stage of development (Chalmers et

al., 2003;Chalmers et al., 2005;Saiz et al., 2013). Further work has shown that the apical localization of the PAR complex proteins is required for the asymmetric division at the 8 cell stage in ascidian embryos (Patalano et al., 2006). While the PAR proteins have been extensively studied for their roles in early polarity in protostome embryos, less is known about their role during the early development of the deuterostomes.

1.2.7. Conclusions

There has been an explosion in research on the PAR proteins since their initial discovery in the *C. elegans* embryo. This has been in large part because of their involvement in so many different processes that are well conserved across a variety of multicellular eukaryotes. The manner in which they polarize to anterior/posterior domains or apical/basolateral domains seems to be both well conserved and necessary for their regulation of polarity. The localization of the PAR proteins is dependent on a number of factors including the active exclusion of opposing PAR proteins and the activity of the actomyosin cortex. The PAR proteins are required for a number of different cellular processes. They are key regulators of spindle orientation in both symmetric and asymmetric divisions. They are also needed to maintain cellular asymmetries in mature cell types such as epithelial cells and neurons. Their misregulation is now known to contribute to the development and progression of several different types of cancer. The role of the PAR proteins during the development and embryogenesis of many model organisms is well established. However, while the PAR proteins involvement throughout embryogenesis and oocyte maturation in protostomes is well known, research on deuterostome development has largely focused on later developmental events such as gastrulation and apical constriction.

1.3. Sea Urchin Embryos as a Model of Deuterostome Development

1.3.1. Historical Perspective on Sea Urchins as a Model System

The sea urchin embryo has long been used as model system of deuterostome development (Ernst, 2011). The sea urchin embryo has been a classic model organism for the study of cellular and developmental biology since the 1800s because of the numerous advantages this system has in the laboratory. Their gametes are easy to obtain and some species have large, optically clear embryos that are perfectly suited for live cell imaging. A single adult urchin also produces copious amounts of gametes that readily allow for biochemical analysis and the generation of large data sets. Additionally, although they are not a genetic model system like *Drosophila* and *C. elegans*, they are a genomic system and thus molecular components can be identified (Cameron et al., 2009;Sodergren et al., 2006). Recent work has also developed a pantropic retrovirus transduction tool in the sea urchin embryo, which can be utilized to study molecular components in later development (Core et al., 2012). Their development following fertilization is synchronous, which additionally allows for greater analysis of specifically timed events, either during the cell cycle or during embryogenesis. Sea urchin embryos also have external development and develop very similar to higher order vertebrates and mammals through gastrulation. The combination of all of these factors has allowed for significant analysis of biological processes in the sea urchin embryo.

There have been numerous seminal contributions to the fields of cellular and developmental biology through the observation of sea urchin embryos. Oskar Hertwig first utilized echinoderm and frog eggs to assess the role of the mitotic spindle in

positioning the cleavage plane. He created what would become known as "Hertwig's rules" (Hertwig, 1884). He pioneered the idea that cells divided perpendicular to their longest axis, which worked well to explain the division plane of symmetrically-dividing, cleavage stage echinoderm embryos. Hertwig also was the first to observe the fusion of the sperm and egg nuclei following fertilization in sea urchin embryos (Ernst, 2011). Later work by Ray Rappaport further expanded the experiments of Hertwig and the prediction of the division plane. Rappaport performed experiments that changed the shape of the normally spherical echinoderm embryo into various shapes including cigars, ice cream cones or dumbbells. Through these experiments he found that that although most divisions followed Hertwig's rules, there are some instances of unusual sites of furrow formation. In these cases, Rappaport observed that a normal furrow that bisected the spindle formed, but a second additional furrow ingressed at one pole where an aster intersected (Rappaport, 1996). These would later become known as Rappaport furrows. Rappaport also classically demonstrated that there existed a positive cue that emanated from the astral microtubules, which stimulated contraction during cytokinesis (Rappaport, 1996).

Theordor Boveri famously found that chromosomes located in the nucleus were the determinants for development (Boveri, 1902). Later work by Jean Brachet helped to establish what was considered the central dogma of molecular biology that is that DNA (deoxyribonucleic acid) codes for RNA (ribonucleic acid), which codes for protein. Brachet discovered that chromosomes were made of DNA, RNA was found in the cytoplasm, and that RNA regulated protein synthesis (Brachet, 1950;Brachet et al., 1963;Ernst, 2011). Other contributions to the field from work in sea urchin embryos

include the discovery of long-lived maternal RNAs, polyadenylation of mRNAs (messenger RNA), and the cloning of the first eukaryotic gene (Gross and Cousineau, 1963;Kedes et al., 1975;Slater et al., 1973;Wilt, 1973). It was not until 2002, however, that a Nobel Prize was won for work using sea urchin embryos. Tim Hunt shared the Nobel Prize for the discovery of cyclins, which are critical for the regulation of the cell cycle (Evans et al., 1983). These early experiments greatly contributed to our knowledge of cellular and molecular biology.

There have additionally been significant discoveries in developmental biology using sea urchin embryos. Driesh showed that the blastomeres separated up to the 4 cell stage were able to make normal plueteus larvae, demonstrating the potency of an individual blastomere. He also found that causing the normally equatorial third division (Figure 1.3.1.) to become meridional forced nuclei that would have normally produced dorsal structures to be in ventral cells. These experiments found that the fate of the nucleus depended on its location in the embryo. The combination of these data revealed that sea urchin embryos undergo both regulative development and conditional specification (Driesch, 1892;Gilbert, 2006). Further work by Sven Hörstadius examined the developmental potential of different regions of the embryo. His data showed that if the animal half of the embryo was isolated at the 60cell stage of development, the embryo developed into a ciliated ball of ectoderm. However, if the animal half was also combined with micromeres a normal larva formed. A secondary gut could additionally be formed if the micromeres were transplanted to the animal half of the embryo (Hörstadius, 1939). The ability to form a secondary axis has been an area of intensive investigation since Hörstatius' initial experiments (McClay et al., 2000;Ransick and

Davidson, 1993;Ransick and Davidson, 1995). Recent work by Eric Davidson has pioneered the examination of the gene regulatory networks in the sea urchin embryo and his work on the specification of the endomesoderm network has made the sea urchin embryo the most well annotated gene regulatory network (Davidson et al., 2002a;Davidson et al., 2002b;Ernst, 2011). This collective work has demonstrated the impact the study of sea urchin embryos has had on our current understanding of cellular and developmental biology.

1.3.2. Previous Views and Findings of Early Polarity in the Sea Urchin Embryo

The sea urchin embryo was previously thought to remain relatively unpolarized until at least the 16 cell stage of development, the first time that they undergo asymmetric division (Schroeder, 1987) (Figure 1.3.1.). At this stage β catenin becomes nuclear only in the micromeres of the vegetal pole, which is critical for endomesoderm specification during gastrulation (Weitzel et al., 2004;Wikramanayake et al.; 2004;Wikramanayake et al., 1998). These micromeres have also recently been found to be germ line precursors (Juliano et al., 2006;Yajima and Wessel, 2012). Classic experiments by Driesch had already revealed that the blastomeres of these embryos can be separated up until the 4 cell stage and produce viable adults, demonstrating that developmental determinants are still symmetrically segregating during the first two divisions (Driesch, 1892). Examples of later polarity in the sea urchin embryo have been well established. At the blastula stage the sea urchin embryo forms a polarized epithelium. Here the embryo has hatched from its fertilization envelope and has become free swimming, using the cilia that now line the free cell surface (Lepage et al., 1992)(Figure 1.3.1.). Significant work has also investigated the 60 cell stage of development in these embryos, which has generated a

developmental fate map for these embryos. At this stage each blastomere has been reversibly committed to a particular cell fate. The animal half of the embryo will give rise to ectoderm, while the vegetal tiers will give rise to the endoderm and mesoderm (Logan and McClay, 1997) (Figure 1.3.1.). Even later in development during gastrulation, the sea urchin embryo exhibits the classic planar cell polarity movements of convergent extension in order to form the archenteron or primitive gut (Martins et al., 1998). Thus, while there are examples of polarity later in development in the sea urchin embryo, polarity during the early cleavage stages has remained largely ignored.

However, there is now mounting evidence to suggest that these embryos polarize earlier than originally thought (Figure 1.3.2.). For example, lectin receptors have been shown to have an apical surface localization (McCaig and Robinson, 1982). Schroeder also demonstrated that the apical surface is enriched in microvilli, whereas the basolateral surface is relatively free of these actin protrusions even upon dissociation (Schroeder, 1988). Additionally, cadherin localization polarized to new sites of cell-cell contact (Miller and McClay, 1997). More recently Burke and colleagues have shown that βC integrins are found only on the outer surface that is exposed to the extracellular matrix as early as the 2 cell stage of development and that these integrins associate with focal adhesion kinase, which is necessary for proper cortex formation (Burke et al., 2004;Chan et al., 2013). Lipid rafts are additionally polarized to the free cell surface following the first cleavage in sea urchin embryos (Alford et al., 2009;Ng et al., 2005). Developmental determinants such as dishevelled have also been found to have polarized localization in the vegetal cortex in the unfertilized egg (Leonard and Ettensohn, 2007;Weitzel et al.,

2004). These examples highlight that there is an early establishment of polarity in the sea urchin embryo, despite symmetric cleavages.

1.3.3. The PAR Proteins in the Sea Urchin Embryo

Although the PAR proteins have been examined across a wide variety of species, it was not until recently that they were explored in sea urchins (Alford et al., 2009;Pruliere et al., 2011;Shiomi and Yamaguchi, 2008;Shiomi et al., 2012). The PAR proteins were utilized to show that the sea urchin embryo exhibits polarity following the first cleavage (Figure 1.3.2.). PAR6, aPKC, and CDC42 all localize to the apical cortex as early as the 2 cell stage of development in these embryos and this apical polarity was found to be functional, as endocytosis was only observed on the apical surface even upon dissociation of the blastomeres (Alford et al., 2009). PAR6, aPKC, and PAR1 were also cloned in *Hemicentrotus pulcherimmus* and their expression pattern was examined through *in situ* hybridizations and RT-PCR. PAR6, aPKC, and PAR1 were uniformly distributed along the animal-vegetal (AV) axis throughout early cleavage stages. PAR1 becomes transiently vegetal following hatching, while aPKC was restricted to the primary mesenchyme cells and the vegetal plate at the blastula stage (Shiomi and Yamaguchi, 2008). Additional work has shown through morpholino knockdown of PAR6 expression, PAR6 is required for skeletogenesis and gut differentiation at the larval stage of development. The authors suggest that PAR6 plays a role in the deposition of biominerals in the syncytial cable and are necessary to stabilize the skeletal rods (Shiomi et al., 2012). Other work in the *Paracentrotus lividus* species of sea urchin embryos has found that aPKC activity is required for both ciliogensis and swimming starting at the blastula stage of development (Pruliere et al., 2011). These initial findings in the sea

urchin embryo suggest that the PAR proteins could play an important role in early development and that their activity during the early cleavage stages could be required for the later developmental processes that are already known to involve the PAR proteins.

1.3.4. Conclusions

The sea urchin embryo has long stood as a model for deuterostome development. Scientists have utilized these embryos since the 1800s and they have been upheld as a classic model organism to present day. The numerous advantages of the sea urchin embryo have allowed them to remain relevant to current science, despite the fact that they are not a genetic system. While once thought to remain unpolarized until the first asymmetric division at the 16 cell stage of development, there is now growing evidence to suggest that these embryos polarize during earlier cleavage stages. Additionally, although the PAR proteins have been extensively studied for their roles in the development of protostomes such as *C. elegans* and *Drosophila*, their role during the early development of deuterostome embryos has not been thoroughly reviewed. The PAR proteins are now known to be imperative for later developmental events in the sea urchin embryo, but their role in early cleavage stages has not been well characterized (Pruliere et al., 2011;Shiomi et al., 2012). Here I will examine the role and polarization of the PAR proteins during early development and how disruptions in these proteins during early cleavage stages affect later developmental events.

1.4. Specific Aims

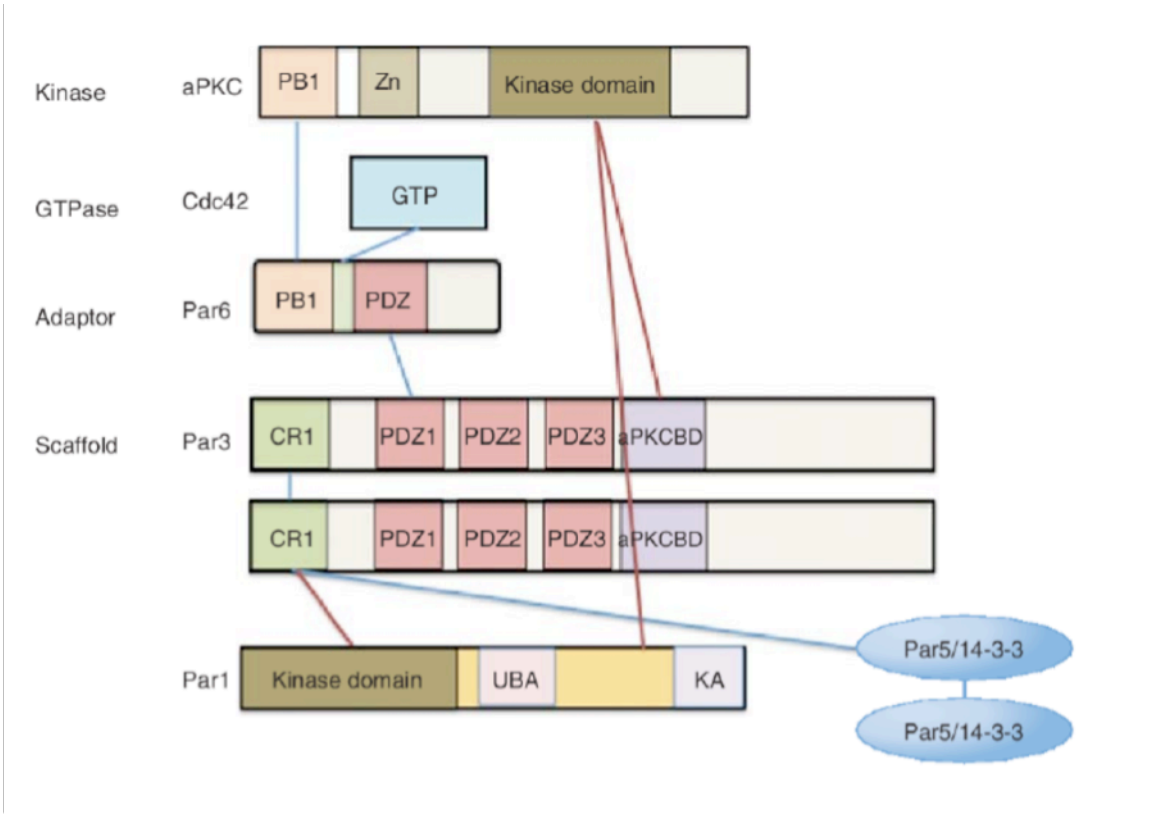
1.4.1. Studies on the Impact of Disruptions in Early Polarity on Later Development

Previous studies from our lab have shown that PAR6, aPKC, and CDC42 localize to the apical cortex as early as the 2 cell stage of development in the sea urchin embryo, while other groups have examined the role of these proteins during later development (Alford et al., 2009;Pruliere et al., 2011;Shiomi and Yamaguchi, 2008;Shiomi et al., 2012). The goal of this project was to determine what role the establishment of early polarity plays in later development. Specifically, I examined how disruptions in the localization of PAR6, aPKC, and CDC42 affect later polarity dependent events in development, such as the formation of a polarized epithelium at the blastula stage. My hypothesis was that these embryos required the early polarized localization of the PAR complex in order for development to proceed normally. I found that PAR6, aPKC, and CDC42 remain localized to the apical cortex through the gastrula stage of development and that these proteins are anchored in the cortex specifically by myosin assembly as disruption of myosin light chain kinase (MLCK) activity led to the cytoplasmic pooling of these proteins. Cell-cell contact was additionally needed to maintain the apical, but not cortical, localization of PAR6, aPKC, and CDC42. While prior work in our lab has found that aPKC activity is necessary for proper spindle orientation during early cleavages, here I found that aPKC is not involved in the first asymmetric division in the sea urchin embryo at the 16 cell stage. However, aPKC activity during early cleavage stages is essential for blastula formation. Similarly, perturbations of MLCK activity severely impeded the ability of the embryos to reach the blastula stage. These data demonstrate the importance of early polarity in deuterostome embryos and the significant functional impact it has on later developmental events.

1.4.2. A Closer Examination of the role of PAR1 in Sea Urchin Development

The second aim of this project was to determine what role the traditionally posterior PAR protein, PAR1, plays in the establishment of polarity in the sea urchin embryo. Prior to this work, the mRNA transcript expression pattern of PAR1 was examined in sea urchins and was found to be expressed throughout embryogenesis, only becoming more vegetal in localization following the hatching of the embryo from the fertilization envelope (Shiomi and Yamaguchi, 2008). Here I found that the PAR1 protein colocalizes with the apical markers, PAR6, aPKC, and CDC42 in the cortex of cleavage stage embryos. The colocalization of these proteins is maintained through the first asymmetric division at the 16 cell stage and is further retained at the blastula stage after the formation of the polarized epithelium. Furthermore, PAR1 remains colocalized with the PAR complex proteins at the gastrula stage, well after hatching from the fertilization envelope, and after the embryo has undergone the PCP movements of convergent extension during gastrulation. Through co-immunoprecipitation assays, PAR1 was also found to be in complex with aPKC during these developmental stages. While PAR1 was found to be dependent on cell-cell contact to maintain its apical localization similar to the PAR complex proteins, which cytoskeletal components anchor PAR1 in the cortex remains to be determined. Additionally, I generated a DNPAR1 (dominant negative) construct to be used for microinjection into the sea urchin embryos in order to assess what role PAR1 may play in spindle orientation during early development. These insights into PAR1 function in the sea urchin embryo highlight a unique localization pattern that may be required for proper development.

A.


Kinase
aPKC
PB1
Zn
Kinase domain
GTPase
Cdc42
GTP
Adaptor
Par6
PB1
PDZ
Scaffold
Par3
CR1
PDZ1
PDZ2
PDZ3
aPKCBD
CR1
PDZ1
PDZ2
PDZ3
aPKCBD
Par5/14-3-3
Par1
Kinase domain
UBA
KA
Par5/14-3-3

B.

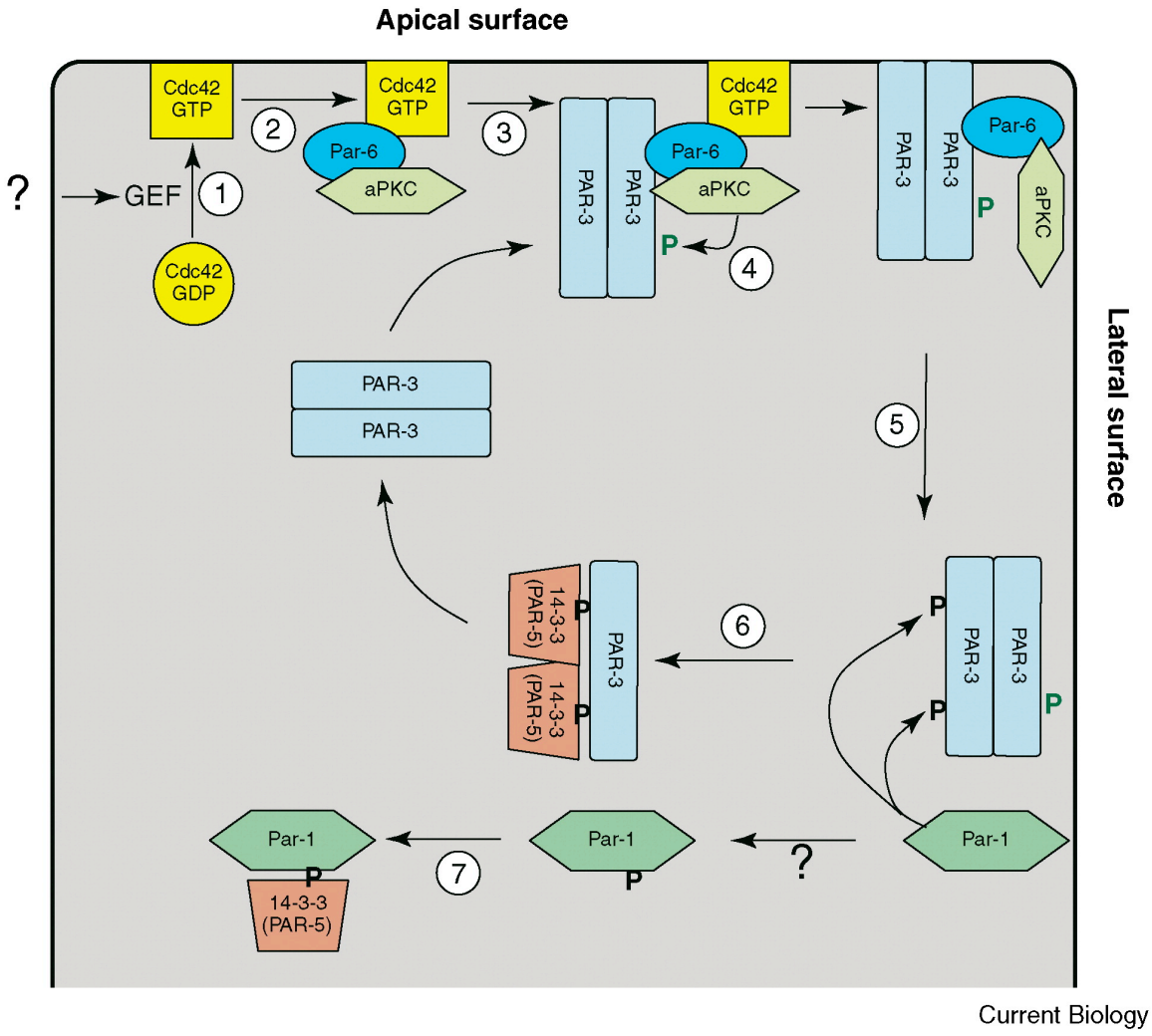
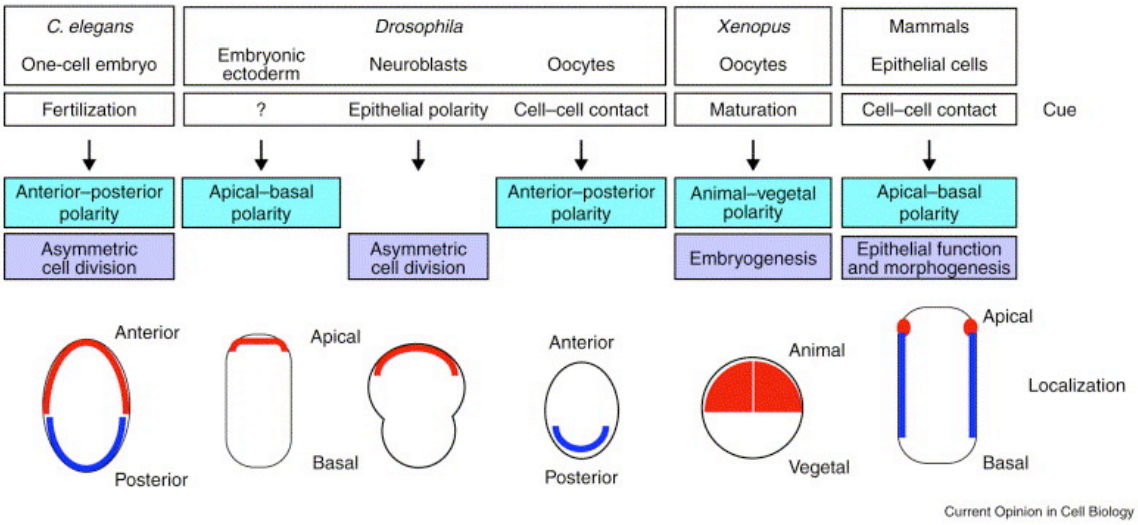


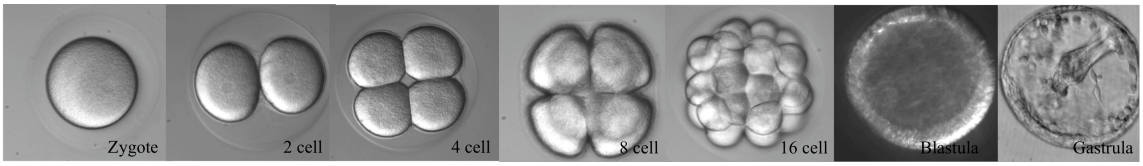
Figure 1.2.1. The signaling pathway of the PAR proteins. (A) Schematic diagram of the protein domains and interactions of each PAR protein (McCaffrey and Macara, 2012). The Phox and Bem1 domain (PB1) is found in both aPKC and PAR6 and forms both homodimers and heterodimers. aPKC additionally has a zinc finger domain (Zn) and a kinase domain. PAR6 and PAR3 act as scaffolding proteins through their PSD95, Dlg1, ZO-1 domains (PDZ). PAR3 also has atypical protein kinase C binding domain (aPKCBD), which is utilized in PAR complex formation, and a conserved region domain

(CR1), which is required for self-oligomerization. PAR1 proteins contain a kinase domain, ubiquitin-associated domain (UBA), and kinase-associated domain (KA). (B) Schematic diagram of the signaling pathway of the PAR proteins (Macara, 2004). After receiving an unknown polarization cue, CDC42 becomes activated by a guanine nucleotide exchange factor (GEF). Activated CDC42 recruits PAR6 and aPKC to the cell cortex, which is needed for PAR3 recruitment and phosphorylation. PAR3 can also be phosphorylated by PAR1 in the lateral domain, which promotes its association with PAR5. PAR5 then shuttles PAR3 back to the apical surface where it associates with the PAR complex.

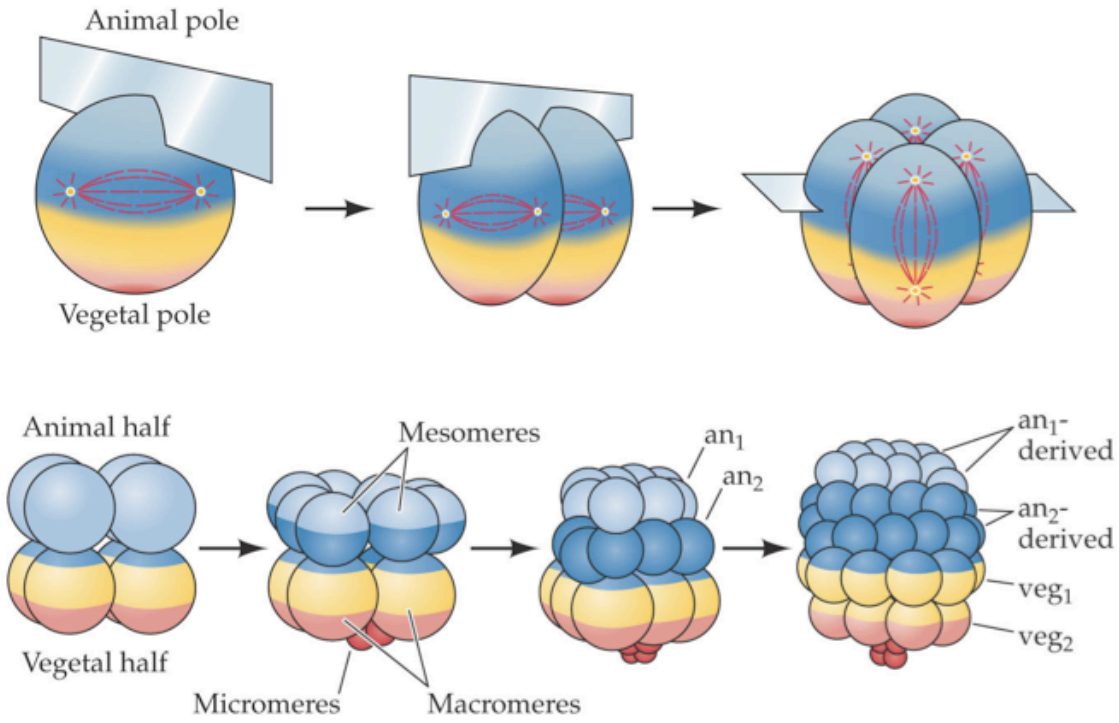


1.2.2. The localization pattern of the PAR proteins during development. Schematic representation of the polarization of the PAR proteins in several different model organisms (Ohno, 2001). Anterior or apical PAR proteins (PAR3, PAR6, and aPKC) are outlined in red. Posterior PAR proteins (PAR1) are outlined blue. The asymmetric and polarized distribution of the anterior and posterior PAR proteins has been conserved in a variety of multicellular eukaryotes and for a variety of biological processes.

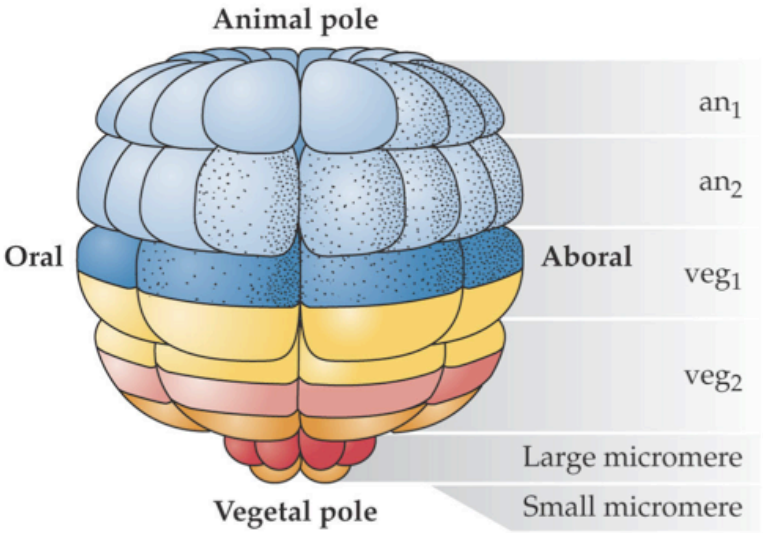
A.

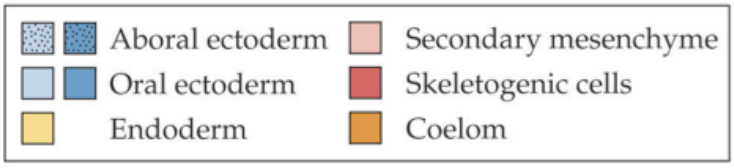

Zygote
2 cell
4 cell
8 cell
16 cell
Blastula
Gastrula

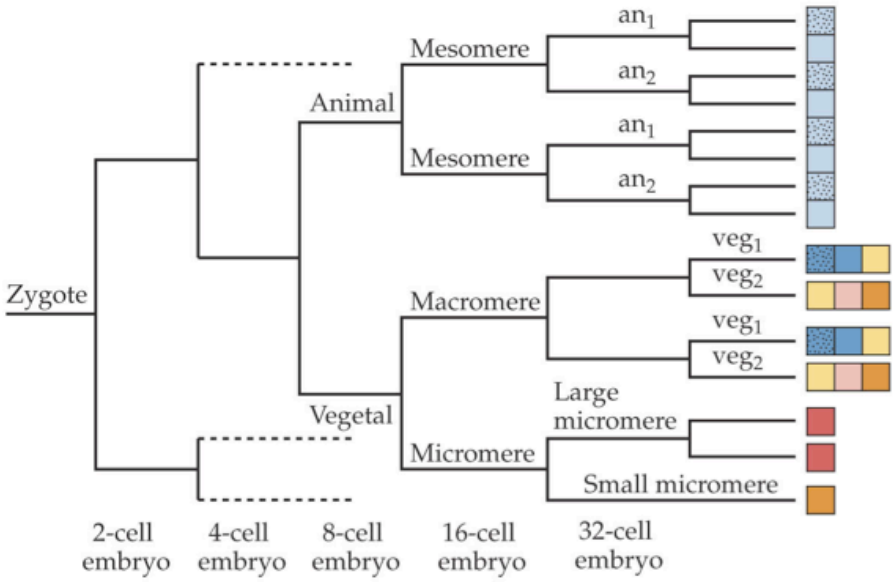
B.


Animal pole
Vegetal pole
Animal half
Vegetal half
Mesomeres
Micromeres
Macromeres
an1
an2
an1-derived
an2-derived
veg1
veg2

C.


Animal pole
Oral
Aboral
Vegetal pole
an1
an2
veg1
veg2
Large micromere
Small micromere


Aboral ectoderm
Oral ectoderm
Endoderm
Secondary mesenchyme
Skeletogenic cells
Coelom


Zygote
Animal
Vegetal
Mesomere
Mesomere
Macromere
Micromere
an1
an2
an1
an2
veg1
veg2
veg1
veg2
Large micromere
Small micromere
2-cell embryo
4-cell embryo
8-cell embryo
16-cell embryo
32-cell embryo

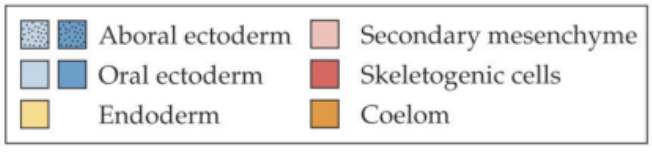

Aboral ectoderm
Oral ectoderm
Endoderm
Secondary mesenchyme
Skeletogenic cells
Coelom

Figure 1.3.1. Normal development of the sea urchin embryo. (A) *Lytechinus pictus* embryos undergo stereotypic symmetric cleavages until the 8 cell stage of development. The first asymmetric division does not occur until the 16 cell stage. At the end of the cleavage stage of development the blastula is formed, which consists of the first polarized epithelium surrounding the blastocoel. It is not until gastrulation that sea urchin embryos exhibit planar cell polarity movements like convergent extension that allow for the migration of cells at the vegetal pole into the blastocoel and the formation of the archenteron. (B) Schematic diagram of the first 6 divisions of the sea urchin embryo (Gilbert, 2006). Direction of the cleavage plane and the formation of tiers are outlined. Future ectoderm is designated in blue, endoderm in yellow, and mesoderm in red. (C) Schematic diagram of the fate map and cell lineage of the sea urchin embryo at the 60 cell stage of development (Gilbert, 2006). Future ectoderm, endoderm, and mesoderm derived cells are outlined. Each of these examples highlights the polarity dependent processes that were known to occur later in development in the sea urchin embryo.

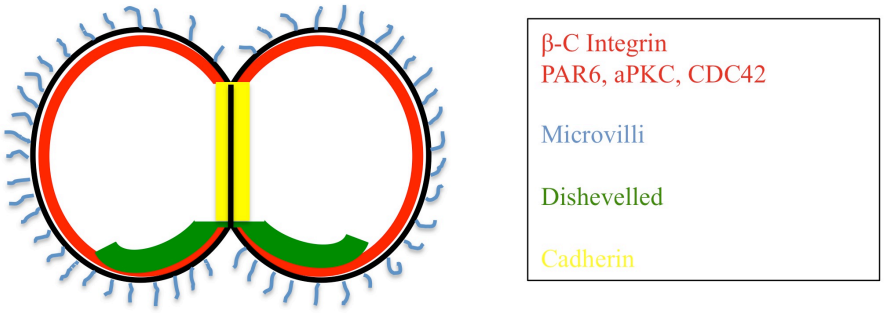


Figure 1.3.2. Examples of early polarity in the sea urchin embryo. There are now several examples of early polarity in the sea urchin embryo. β-C integrins, PAR6, aPKC, and CDC42 all localize to the apical cortex at the 2 cell stage of development (red). At this stage microvilli are only found at the free cell surface (blue), cadherins localize to sites of cell-cell contact (yellow), and the developmental determinant, dishevelled, is partitioned to the vegetal cortex (green).

Chapter 2. Early Disruptions in Polarity Impede Later Development

2.1. Introduction

Polarization of a developing embryo is critical to ensure proper segregation of developmental determinants throughout embryogenesis. Since their initial discovery in the *C. elegans* embryo the PAR proteins have been found to play a vital role in polarization during the embryogenesis of a variety of different model organisms (Kemphues et al., 1988;McCaffrey and Macara, 2009). They regulate a variety of processes during development ranging from spindle orientation, junction formation, gastrulation, and tissue development (Alarcon, 2010;Chalmers et al., 2003;Etemad-Moghadam et al., 1995;Hao et al., 2010;Harris and Peifer, 2007;Kemphues et al., 1988;Niessen et al., 2013;Watts et al., 1996). The PAR proteins consist of a core group of signaling proteins that cooperatively function to control polarity in embryos. The scaffolding proteins PAR3 and PAR6 along with the kinase aPKC form the PAR complex and are regulated upstream by the GTPase CDC42 (McCaffrey and Macara, 2009). The collective action of these proteins controls polarity in the anterior or apical domain of embryos. The kinase PAR1, along with either the E3 ubiquitin ligase PAR2 or the tumor suppressor LGL, is required for polarity in either the posterior or basolateral domains (Nance and Zallen, 2011).

The role of the PAR proteins in the development of the sea urchin embryo has only recently begun to be explored (Alford et al., 2009;Pruliere et al., 2011;Shiomi and Yamaguchi, 2008;Shiomi et al., 2012). PAR6, aPKC, and CDC42 have all been annotated in the genome of *Strongylocentrotus purpuratus* through both GLEAN predictions and RNAseq data (Cameron et al., 2009). Each of these annotations contains

the predicted conserved domains of each of these proteins. PAR6 is predicted to contain the classical PB1 and PDZ domains. aPKC includes a kinase domain, and CDC42 is predicted to have the P-loop containing nucleoside triphosphate hydrolases superfamily domain, which is typically found in G proteins (Cameron et al., 2009). In addition, immunofluoresence assays show that PAR6, aPKC, and CDC42 all localize to the apical cell cortex at the 2 cell stage of development in these embryos. Further analysis demonstrated the functionality of this apical polarity as endocytosis was only observed on the apical surface even upon dissociation of the blastomeres (Alford et al., 2009). Other studies have focused on the roles of PAR6 and aPKC during later development. aPKC activity was required for ciliogenesis at the blastula stage, while PAR6 was found to be needed for skeletogenesis at the larval stage (Pruliere et al., 2011;Shiomi et al., 2012). These observations on the roles of the PAR proteins during the blastula and larval stages of development highlight the utilization of these proteins for polarization during the embryogenesis of the sea urchin embryo. However, similar to other deuterostome embryos, a clear role for the PAR proteins during early development has not been elucidated.

In this study I further examine the role of the PAR proteins during the early development of the sea urchin embryo. In particular, I examined how the polarization of the PAR proteins during early cleavage stages is required for later developmental processes. Following first cleavage, the apical localization of the PAR complex proteins is retained through the blastula stage. I find that these proteins are anchored in the apical cortex specifically by assembled myosin and that perturbations in their localization during early cleavage stages severely impeded later development. While aPKC activity

was not found to be required for the first asymmetric division at the 16 cell stage, its activity during early cleavage stages was necessary for blastula formation. These data demonstrate the importance of early polarity establishment in deuterostome embryos and the significant functional impact it has on later developmental events.

2.2. Results

2.2.1. The PAR Complex Proteins Localize to the Apical Cortex Through the Blastula Stage

Previous studies have shown that PAR6, aPKC, and CDC42 localize to the apical cortex as early as the 2 cell stage of development in the sea urchin embryo (Alford et al., 2009). These observations suggest that the early polarization of these proteins may be important for the proper development of the sea urchin embryo, similar to their role in other model systems (Nance and Zallen, 2011). In order to investigate further, I first examined the localization pattern of these proteins through the early cleavage stages of *Lytechinus pictus* embryos using immunofluoresence assays. I found that PAR6, aPKC, and CDC42 remain localized to the apical cortex through the 16 cell stage of development, the first asymmetric division in sea urchins (Figure 2.2.1.). As aPKC was recently found to be involved in ciliogenesis in the sea urchin embryo (Pruliere et al., 2011), I also determined the localization of PAR6, aPKC, and CDC42 at the blastula stage of development. At the blastula stage, in addition to the presence of cilia, embryos have formed an epithelium and junctions are present. I found that these polarity proteins remain localized to the apical or free cell surface at the blastula stage (Figure 2.2.1.).

Additionally, under high magnification these proteins can be observed in the cilia, similar to the aPKC localization that was seen previously (Pruliere et al., 2011) (Figure 2.2.1.).

2.2.2. aPKC Does Not Regulate the First Asymmetric Division, but is Necessary for Blastula Formation

The PAR proteins were initially discovered based on their involvement in the regulation of the first asymmetric division in the *C. elegans* embryo and have since been found to regulate the divisions of many other organisms at various stages of development (Chalmers et al., 2003;Etemad-Moghadam et al., 1995;Hao et al., 2010;Kemphues et al., 1988;Niessen et al., 2013;Watts et al., 1996). Previous results have shown that treatment with a specific peptide inhibitor of PKCζ resulted in the formation of multipolar spindles, short asters, and improper spindle rotation during early cleavages (Alford et al., 2009). This peptide inhibitor is able to interfere with aPKC activity because it is a pseudosubstrate sequence modeled from the human PKCζ (amino acids 113-125:N-SIYRRGARRWRKL-C) that has been completely conserved in rabbit, rat, mouse and *Xenopus*. The N-terminus has also been myristoylated to allow for cell permeability (Enzo Life Sciences). This peptide sequence is mostly conserved in the sole aPKC predicted in the *S. purpuratus* genome with a single amino acid substitution of serine to asparagine at the N-terminus (amino acids 38-50) (Cameron et al., 2009;Samanta et al., 2006). Here I investigated if aPKC is involved in the first asymmetric division in the sea urchin embryo, at the 16 cell stage of development. I found that, in both intact and dissociated blastomeres, treatment with the specific peptide inhibitor of PKCζ at the 8 cell stage did not affect the formation of mesomeres, macromeres, or micromeres at the 16 cell stage (Figure 2.2.2.A, B.).

Because the inhibition of aPKC during early cleavage stages resulted in misplaced spindle alignment and asymmetric divisions, I next investigated if aPKC activity during early cleavage stages was required for later development. In these assays, embryos were treated the specific peptide inhibitor of PKCζ at the fertilized egg, 2 cell, 4 cell, 8 cell, and 16 cell stages of development. The embryos were raised until controls had reached the blastula stage of development (24 hours post fertilization). Embryos that were treated with the specific peptide inhibitor of PKC at the 16 cell stage had the greatest percentage of embryos reach the blastula stage (>60%), while those that were treated at the fertilized egg stage had the smallest percentage (<10%) (Figure 2.2.2.C.). In addition, the embryos treated at the 16 cell stage that were able to reach the blastula stage had an altered phenotype. They appeared to be highly compacted compared to controls (Figure 2.2.2.C. inset). These data suggest that aPKC activity is critical during early cleavage stages in sea urchin embryos as the earlier the treatment with the specific peptide inhibitor of PKCζ the less likely the embryos reached the blastula stage. This highlights the importance of early polarity for later developmental events.

2.2.3. Myosin Assembly and Cell-Cell Contact are Required for the Localization of the PAR Complex to the Apical Cortex

In order to assess what anchors the PAR complex proteins in the apical cortex, I tested a series of small molecule inhibitors of the cytoskeleton. I first assessed inhibitors of the acto-myosin cortex as its involvement in the localization of the PAR complex has been well documented (Cowan and Hyman, 2004a;Munro et al., 2004;Munro, 2006). Additionally, prior research has found that polarized plasma membrane rafts in sea urchin embryos, marked by the ganglioside GM1, are dependent on myosin filament assembly (Alford et al., 2009;Gudejko et al., 2012;Ng et al., 2005). Embryos were treated with the

various inhibitors at the 2 cell stage of development, after the PAR complex proteins had polarized to the apical cortex. I used specific small molecule inhibitors in order to dissect the different functions of myosin. Phosphorylation of serine19 on myosin light chain results in both bipolar filament assembly and an enhancement of the actin activated ATPase activity (Bresnick, 1999). The effects of each activity can be determined through the use of specific inhibitors.

First, 2 cell stage embryos were treated with either ML-7 or ML-9, which are inhibitors of myosin light chain kinase (MLCK) and thus bipolar filament assembly (Saitoh et al., 1987). ML-7 and ML-9 act as ATP (adenosine triphosphate) competitors in order to inhibit MLCK activity. Blocking MLCK activity then blocks a variety of cellular activities including the formation of the contractile ring during cytokinesis (Uehara et al., 2008). Treatment with either ML-7 or ML-9 resulted in the cytoplasmic pooling of PAR6, aPKC, and CDC42 (Figure 2.2.3.A.). To test the specificity of the role of MLCK, I also treated the embryos with H1152, a Rho kinase (ROCK) inhibitor, as ROCK is also indirectly involved in the phosphorylation of myosin light chain by the phosphorylation and thus the inhibition of myosin phosphatase (Kosako et al., 2000). H1152 acts as an ATP competitor to block ROCK activity (Sasaki et al., 2002). Treatment with H1152 had no effect on the localization of PAR6, aPKC, or CDC42 (Figure 2.2.3.A.). I further dissected the role of myosin in the localization of the PAR complex, by treating with blebbistatin, an inhibitor of myosin motor function. Blebbistatin preferentially binds to the ATPase intermediate of myosin with ADP and phosphate bound at the active site. It then slows down the release of the phosphate. Blebbistatin interferes with neither the binding of myosin to actin nor with the ATP-

induced actomyosin dissociation, instead, it blocks the myosin heads in a conformation, which has low actin-affinity (Kovacs et al., 2004). Unlike treatment with ML-7, ML-9 or H1152, which block the formation of the contractile ring, treatment with blebbistatin inhibits constriction of the contractile ring during cytokinesis (Miyoshi et al., 2006). Here, treatment with blebbistatin did not perturb the apical or cortical localization of the PAR complex proteins (Figure 2.2.3.A.). Because blebbistatin was not found to have an effect on the PAR complex, these results demonstrate that it is bipolar filament assembly and not the ATPase activity of myosin that is necessary for the PAR complex localization.

Myosin motors along with actin filaments are the dominant structural proteins in the cellular cortex. Recent data demonstrate that assembled myosin forms clusters that act as network nucleators to organize and remodel the cortex (Ideses et al., 2013). Additional evidence suggests that inhibition of myosin can affect actin turnover because myosin motors are able to dissemble actin filaments (Haviv et al., 2008;Murthy and Wadsworth, 2005;Salbreux et al., 2012). As the other dominant structural protein in the cortex, and because of its regulation by myosin, actin involvement was also analyzed by the inhibition of actin polymerization with latrunculin B. Latrunculin B is a structurally unique marine toxin (isolated from the red sea sponge) that is 10- to 100-fold more potent than cytochalasins. Whereas cytochalasin D induces dissolution of F-actin and stress fiber contraction in fibroblasts in culture, latrunculin B causes a shortening and thickening of stress fibers (Schatten et al., 1986;Spector et al., 1983;Wakatsuki et al., 2001). Cytochalasin D was also tested (data not shown) to determine if a different mechanism of actin inhibition affected the PAR proteins; however, because it was not reversible in sea

urchin embryos, I did not continue experiments with this drug. I found that treatment with neither latrunculin B nor cytochalasin D affected the localization pattern of the PAR6, aPKC, or CDC42 (Figure 2.2.3.A.).

In another series of experiments, I also inhibited microtubules with either urethane or nocodazole to assess what role another major cytoskeletal component may play in anchoring the PAR complex within the apical cortex (Strickland et al., 2005). Astral microtubules are known to be involved in the maintenance of cortical PAR domains in *C. elegans*, although the role of microtubules in PAR polarization remains somewhat controversial (Ai et al., 2011;Nance and Zallen, 2011). Treatment with urethane shortens and destabilizes astral microtubules and promotes astral microtubule catastrophe. This disrupts the ability of the microtubules to contact the cortex and furrow formation is not initiated (Strickland et al., 2005). Nocodazole, on the other hand, is a microtubule depolymerizer and antimitotic agent that disrupts microtubules by binding to β tubulin and preventing the formation of one of the two interchain disulfide linkages, thus inhibiting microtubule dynamics, causing disruption of mitotic spindle function and fragmentation of the Golgi complex (Jordan et al., 1992;Luduena and Roach, 1991;Storrie and Yang, 1998;Vasquez et al., 1997). Treatment with nocodazole arrests cells at the G_2/M phase in the cell cycle. Similar to cytochalasin D, further experiments with nocodazole were not continued because its effects were not reversible in sea urchin embryos (data not shown). Treatment either urethane (Figure 2.2.3.A.) or nocodazole (data not shown) did not affect the localization of PAR6, aPKC, or CDC42, confirming a specific role for assembled myosin in PAR complex localization.

Once I determined that myosin bipolar filament assembly was involved in anchoring the PAR complex in the apical cortex, I next analyzed the stability of these disruptions in PAR complex localization by MLCK inhibition. I treated embryos at the fertilized egg stage of development with a 15 minute pulse of ML-7 and allowed the embryos to divide to the 2 cell stage before fixation and staining for PAR6, which I used as a marker of the PAR complex (Figure 2.2.3.B.). I found that treatment with ML-7 at the fertilized egg stage resulted in the same cytoplasmic pooling of PAR6 as it does at the 2 cell stage (Figure 2.2.3.B.ii. and iv.). Additionally, I find that the pulse treatment with ML-7 at the fertilized egg stage causes PAR6 to remain cytoplasmic through the next cell division to the 2 cell stage (Figure 2.2.3.B.v.). This suggests that the initial disruption is maintained because after only a short disruption in myosin assembly during the fertilized egg stage PAR6 did not return to the cortex at the 2 cell stage.

Previous studies had shown that PAR6 localization is dependent on calcium mediated cell adhesion (Alford et al., 2009). Here embryos were dissociated with gentle pipetting in CaFSW (calcium free sea water) and PAR6 along with aPKC, and CDC42 were examined in intact and dissociated 2 cell stage embryos. Like PAR6, aPKC and CDC42 become evenly distributed throughout the cortex upon dissociation (Figure 2.2.4.).

2.2.4. Myosin Light Chain Kinase Activity During Early Cleavage Stages is required for Blastula Formation

After determining that the PAR complex proteins were anchored in the cortex by assembled myosin and that these disruptions in localization could be maintained through the next division, I analyzed the effects of disruptions in PAR complex localization on later development. I pulse treated *L. pictus* embryos with the same cytoskeletal inhibitors

(ML-7, ML-9, H1152, latrunculin B, urethane, and blebbistatin) for 15 minutes at the fertilized egg, 2 cell, 4 cell, 8 cell, and 16 cell stages of development and then monitored the progression of the embryos. Pulse treatments were done instead of continuous treatments because each of these cytoskeletal inhibitors causes cells to arrest either during mitosis and cytokinesis. Therefore, experiments with continuous treatments of these inhibitors would have assessed cell cycle defects, rather than developmental deficiencies. All inhibitors used in these experiments were found to be reversible in sea urchin embryos; they could be washed out and the embryos would continue through the cell cycle normally (data not shown). A 15 minute pulse of ML-7 or ML-9 was determined to be sufficient to disrupt the localization of PAR6, aPKC, and CDC42 (Figure 2.2.3.A.). Embryos that were pulse treated with either ML-7 or ML-9 continued to cleave normally, but failed to reach the blastula stage when pulse treated at these early cleavage stages. Embryos underwent apoptosis prior to forming the polarized epithelium that is characteristic of the blastula stage (Figure 2.2.5.). This effect appears to be specific to assembled myosin, as cleavage stage embryos pulse treated with the other cytoskeletal inhibitors were able to reach the gastrula stage successfully (Figure 2.2.6.). Embryos pulse treated with latrunculin B, urethane, or blebbistatin underwent normal gastrulation, similar to controls (Figure 2.2.6.). Embryos pulse treated with H1152 still reached the gastrula stage, although there was slight variation in morphology as compared to controls (Figure 2.2.6.A, B.).

2.3. Discussion

The development of an embryo is a tightly regulated process by which an entire organism is generated from a single fertilized egg. While the sea urchin embryo has long been used as a model to study this process, little was known until recently about its polarization during early cleavage stages. The PAR proteins have been extensively studied for their roles in polarity; however, a specific role for these proteins in the early development of deuterostome embryos had not been fully illuminated. Here I find that the polarization of the PAR proteins during early cleavage stages also is critical for proper development of the sea urchin embryo.

The PAR complex proteins, PAR6 and aPKC, as well as its upstream regulator, CDC42, colocalize to the apical cortex as early as the 2 cell stage of development in sea urchin embryos (Alford et al., 2009). I now find that these proteins retain their apical localization in the cortex through the blastula stage of development. While others have shown that aPKC is excluded from the vegetal pole at the 16 cell stage of development in the *Hemicentrotus pulcherrimus* species of sea urchin, I find that the PAR complex retain their apical localization in both the micromere and macromeres of the vegetal pole (Pruliere et al., 2011). The colocalization of PAR6, aPKC, and CDC42 mirrors the results seen in other model organisms (McCaffrey and Macara, 2012;Nance and Zallen, 2011).

The cleavage to the 16 cell stage is an imperative step in the development of the sea urchin embryo. It is the time of the first asymmetric division and it lays down some important foundational work for later developmental events. At this stage β-catenin

becomes nuclear only in the micromeres of the vegetal pole, which is critical later during gastrulation for endomesoderm specification (Weitzel et al., 2004;Wikramanayake et al., 2004;Wikramanayake et al., 1998). These micromeres have also recently been found to be germ line precursors (Juliano et al., 2006;Yajima and Wessel, 2012). aPKC has been found to regulate a number of other asymmetric divisions and I was interested in determining its involvement in the sea urchin embryo (Chalmers et al., 2003;Durgan et al., 2011;Niessen et al., 2013;Suzuki et al., 2002). While prior research has shown that inhibition of aPKC at early cleavages stages resulted in several spindle defects (Alford et al., 2009), I find here that it does not appear to regulate the first asymmetric division in sea urchin embryos. Given these data it may be that other polarity proteins besides aPKC are involved in this particular asymmetric division. Candidates include LGL, Scribble, Pins, Crumbs, and CDC42 (McCaffrey and Macara, 2012).

While aPKC activity may be not necessary for proper micromere formation at the 16 cell stage, it was already known to be required for normal symmetric cleavages prior to the 16 cell stage (Alford et al., 2009). The data here also suggest that aPKC activity is additionally required for blastula formation; demonstrating a clear role for early polarity. At the blastula stage, sea urchin embryos have formed a polarized epithelium that surrounds a fluid-filled cavity, the blastocoel. In other models, aPKC activity is required at the blastula stage for junction formation and polarized cell divisions (Chalmers et al., 2003;Harris and Peifer, 2007). These data suggest that the polarity that is established by aPKC during early cleavage stages may be the beginning of the polarization of the sea urchin embryo that is required later for junction formation at the blastula stage.

Previously, MLCK activity was shown to be important in maintaining the stability of polarized plasma membrane domains in sea urchin embryos (Alford et al., 2009;Gudejko et al., 2012;Ng et al., 2005). Here I have demonstrated the importance of MLCK activity in maintaining the localization of the PAR proteins at the apical cortex, as treatment with ML-7 or ML-9 resulted in the cytoplasmic pooling of these proteins. This disruption in localization by inhibition of myosin assembly is sustained through the next division. Additionally, assembled myosin was found to be required during these early cleavage stages in the embryo in order to reach the blastula stage. Pulse treatments with ML-7 or ML-9 resulted in apoptosis of the embryos prior to becoming blastula. In contrast, treatment with other small molecule inhibitors of the actin or microtubule cytoskeleton did not perturb the localization of the PAR proteins nor did it alter the embryos' ability to reach the gastrula stage of development. These data demonstrate the specificity of the MLCK activity and its necessity during early cleavage stages for normal development.

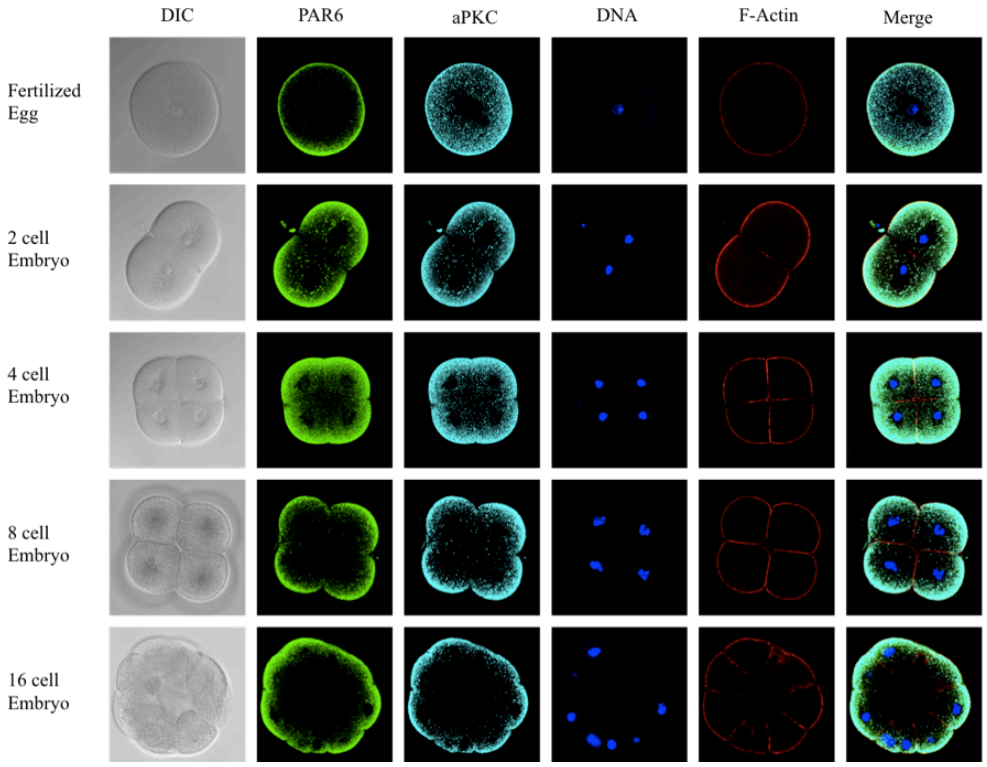
The reliance of calcium dependent adhesion to maintain the apical localization of PAR6, aPKC, and CDC42 highlights the importance for these proteins to remain cortical, but not necessarily apical for proper development. Driesch showed in his classic experiments that the early blastomeres of sea urchin embryos could develop into normal, albeit smaller, adults (Driesch, 1892). However, here I have shown that the cortical reorganization of the PAR proteins after myosin filament disruption results in apoptosis prior to blastula formation. These results also mirror my data that established a specific role for aPKC during early development as early cleavage stage embryos treated with a

specific peptide inhibitor of aPKC were also unable to reach the blastula stage (Alford et al., 2009).

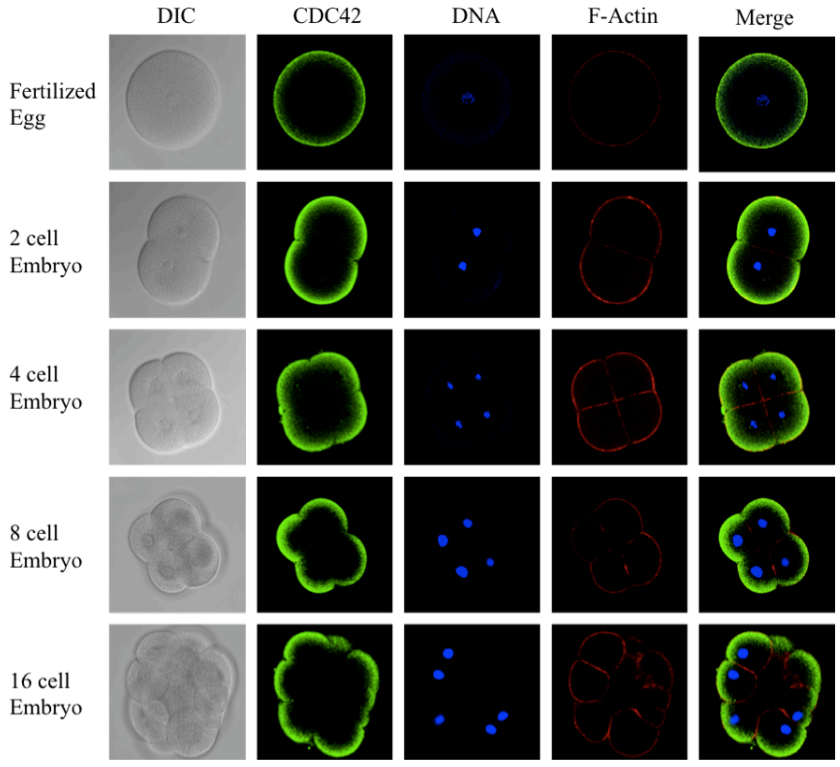
Interestingly, recent data has found that perturbations in MLCK activity during early cleavage stages in sea urchin embryos do not disrupt myosin localization, but rather actin localization in the cortex (Gudejko, 2013). These results support the idea that assembled myosin acts a molecular scaffold that can regulate cortical dynamics (Ideses et al., 2013;Murthy and Wadsworth, 2005;Salbreux et al., 2012). The formation of Arp 2/3 nucleated actin comets by treatment with ML-7 or ML-9 can be seen in both live and fixed embryos (Gudejko, 2013). These changes in the structure of the cortex may be responsible for the cytoplasmic pooling of the PAR proteins that are observed with the same drug treatments.

These studies on the sea urchin embryo have explored how and when polarity is established and the impact of disturbing this polarity during stages of critical developmental decisions. From these data there is a clear role for the PAR proteins during the early development of the sea urchin embryo. The polarization of PAR6, aPKC, and CDC42 to the apical cortex as well as aPKC activity during early cleavage stages is essential for blastula formation. These insights into the generation and maintenance of polarity are crucial to our understanding of how a developing embryo properly partitions components to ensure that the right function occurs in the correct location.

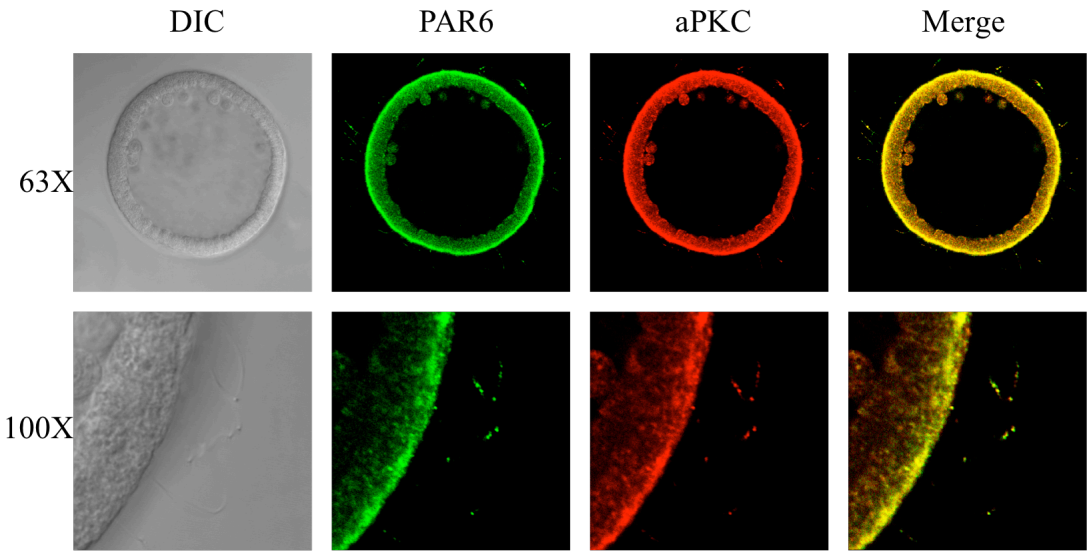
A.


DIC
PAR6
aPKC
DNA
F-Actin
Merge
Fertilized Egg
2 cell Embryo
4 cell Embryo
8 cell Embryo
16 cell Embryo

B.


DIC
CDC42
DNA
F-Actin
Merge
Fertilized Egg
2 cell Embryo
4 cell Embryo
8 cell Embryo
16 cell Embryo

C.



D.

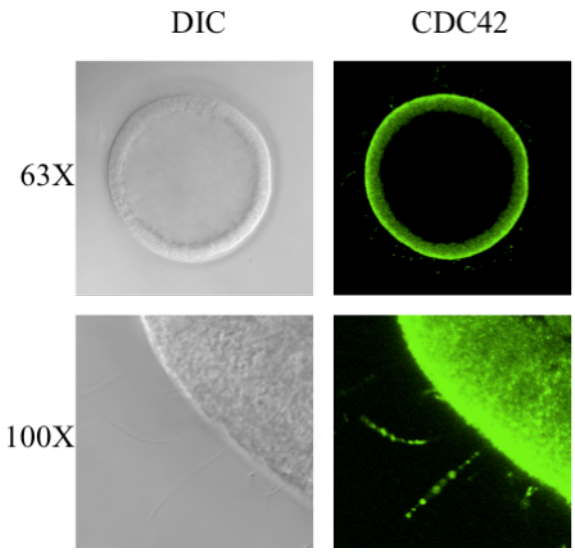
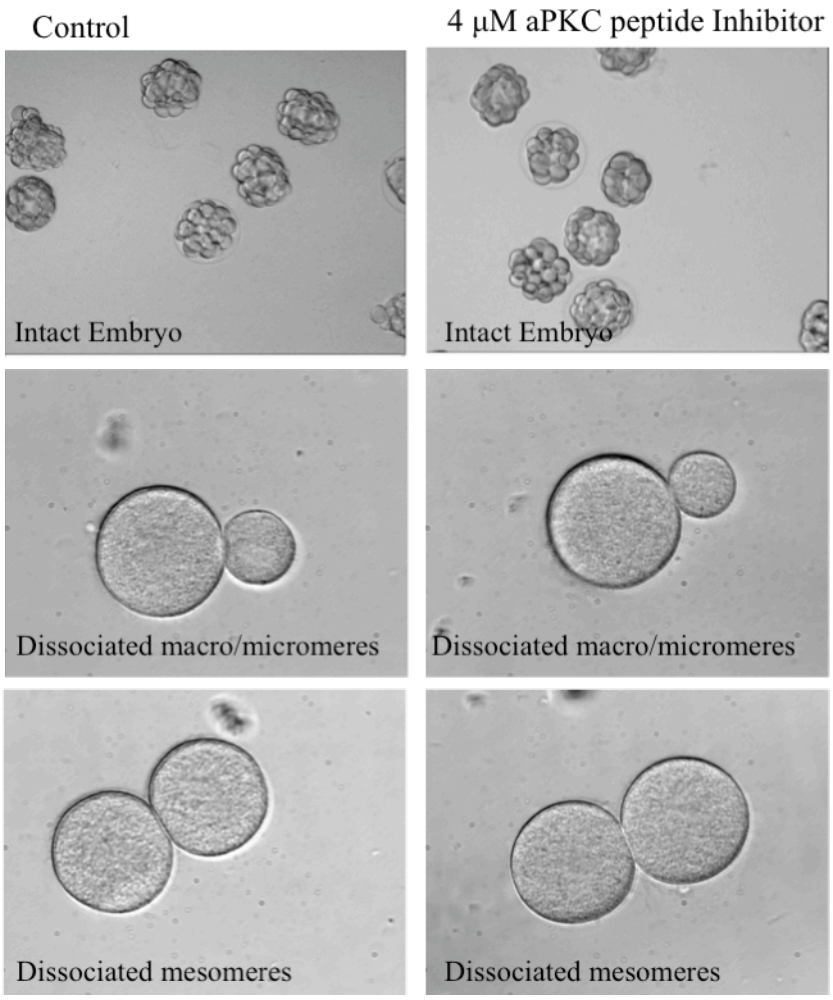
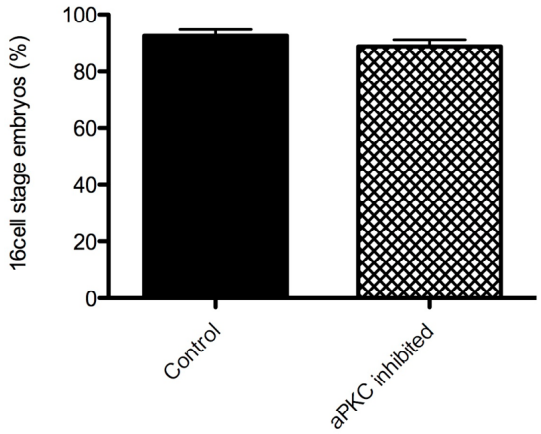


Figure 2.2.1. PAR6, aPKC, and CDC42 colocalize at the apical cortex through the blastula stage. *L. pictus* embryos were fixed and then stained for PAR6 and aPKC (A and C) or CDC42 (B and D). Cleavage stage embryos (A and B) were additionally stained with phalloidin to label F-actin and Hoescht to label DNA. PAR6, aPKC, and CDC42 maintain the apical localization pattern initially seen at the 2 cell stage through the 16 cell stage (A and B). This colocalization at the apical surface is further retained at the blastula stage after an epithelium has formed and junctions are present (C and D). Expression of PAR6, aPKC, and CDC42 is additionally found in the cilia at the blastula stage of development under high magnification and zoom (C and D). For scaling reference *L. pictus* embryos are approximately 120 μm in diameter.

A.


Control
4 μM aPKC peptide Inhibitor
Intact Embryo
Intact Embryo
Dissociated macro/micromeres
Dissociated macro/micromeres
Dissociated mesomeres
Dissociated mesomeres

B.


16cell stage embryos (%)
100
80
60
40
20
0
Control
aPKC inhibited

C.

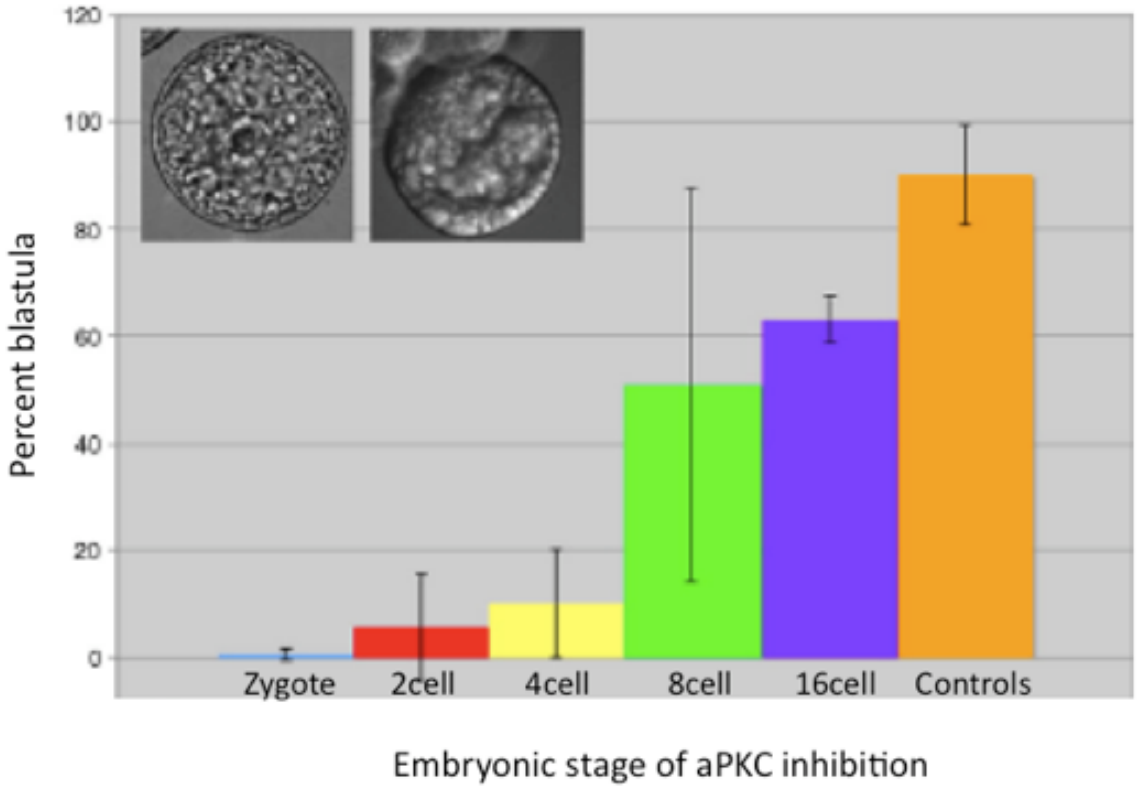
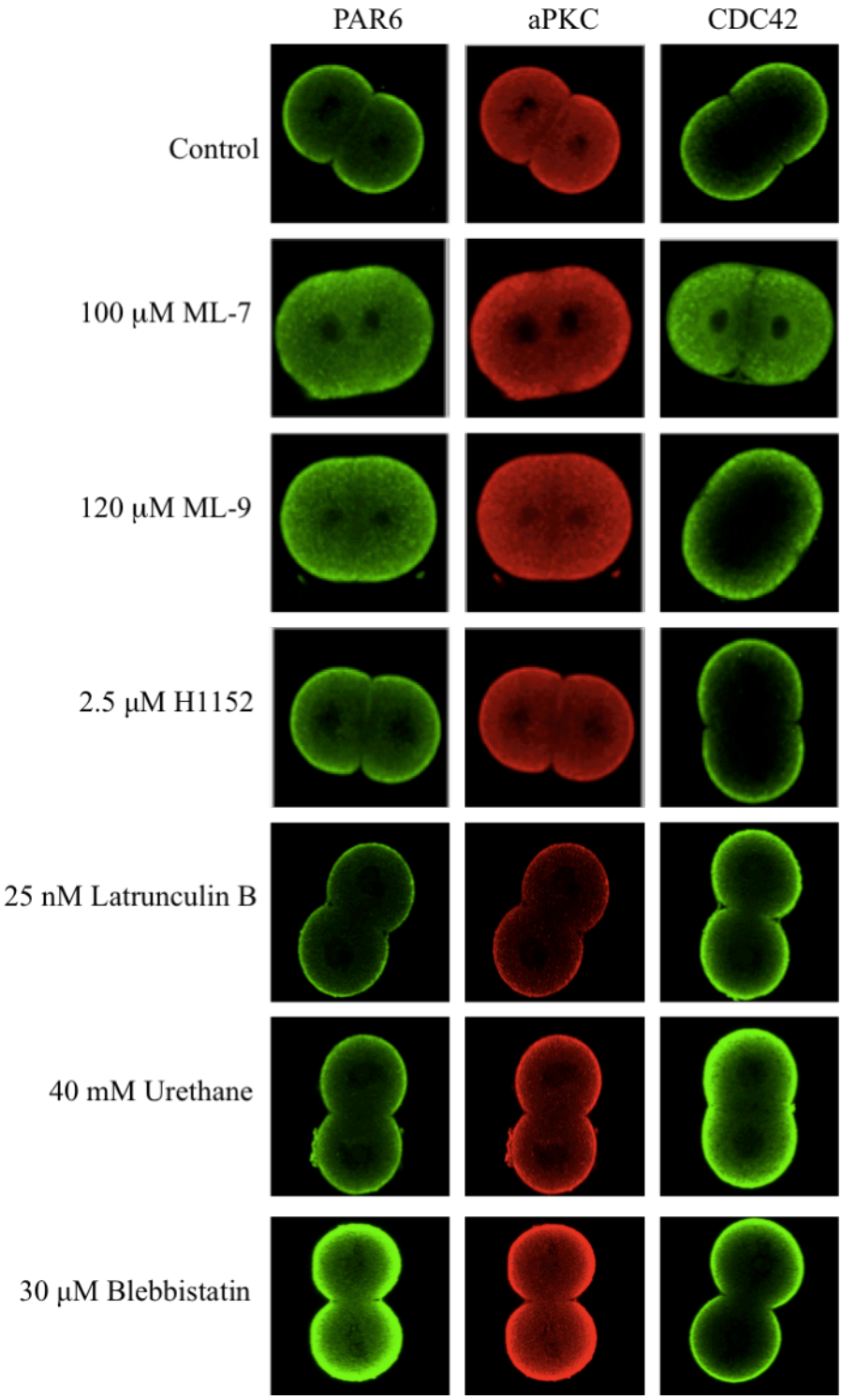


Figure 2.2.2. aPKC does not regulate the first asymmetric division in the sea urchin embryo, but is required for blastula formation. 4 μM PKCζ peptide inhibitor was added at the 8 cell stage of development in intact and dissociated embryos through the subsequent division to the 16 cell stage (A). No effect was observed on the formation of micromeres, macromeres, or mesomeres compared to controls in trials of 100 embryos each (n=6). Normal 16 cell embryo formation is quantified (B) in controls (92.5±5.4%) and treated groups (88.8±5.7 %) (mean ± SD). 4 μM PKCζ peptide inhibitor was added at the fertilized egg, 2 cell, 4 cell, 8 cell, and 16 cell stages of development (C). Embryos were raised until control embryos had reached the blastula stage of development (Alford et al., 2009). The earlier the PKCζ peptide inhibitor was added the less likely the

embryos reached the blastula stage. Treatment at the 16 cell stage additionally resulted in compaction at the blastula stage (inset). For scaling reference *L. pictus* embryos are approximately 120 μm in diameter.

A.


PAR6
aPKC
CDC42
Control
100 µM ML-7
120 µM ML-9
2.5 µM H1152
25 nM Latrunculin B
40 mM Urethane
30 µM Blebbistatin

B.

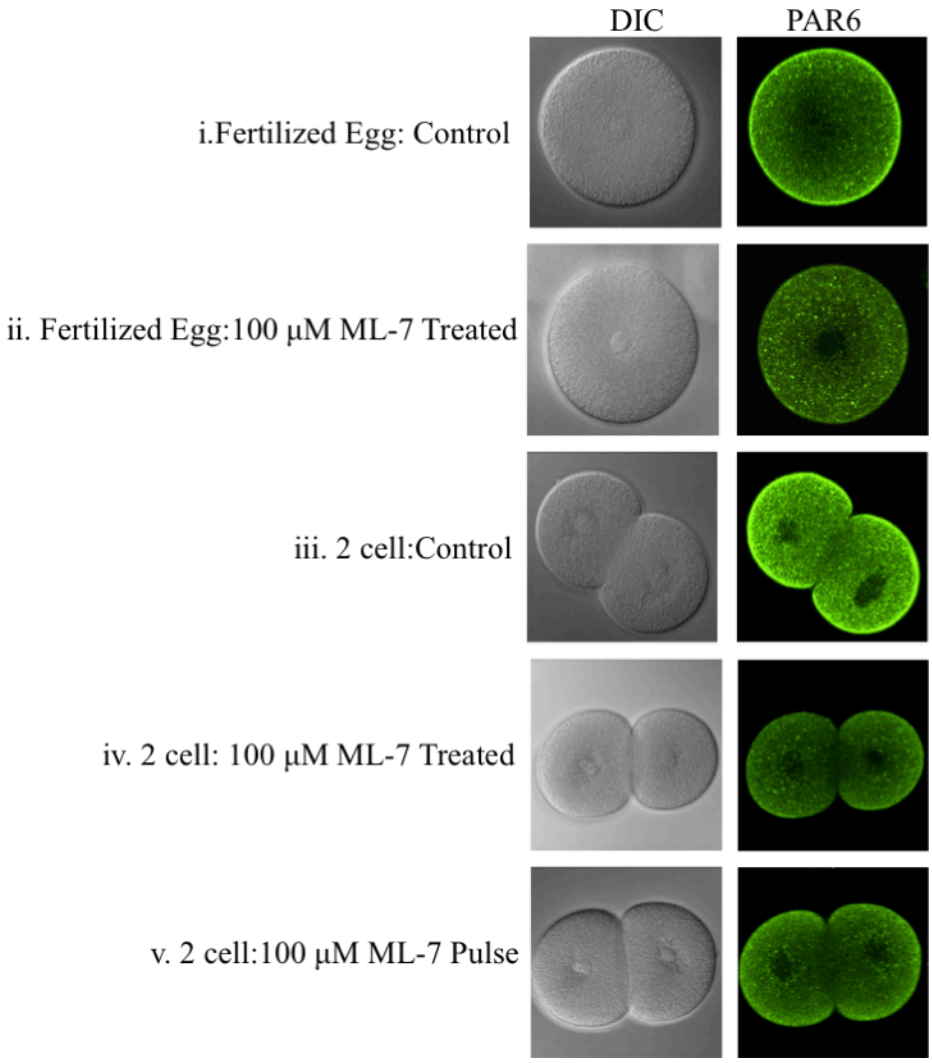
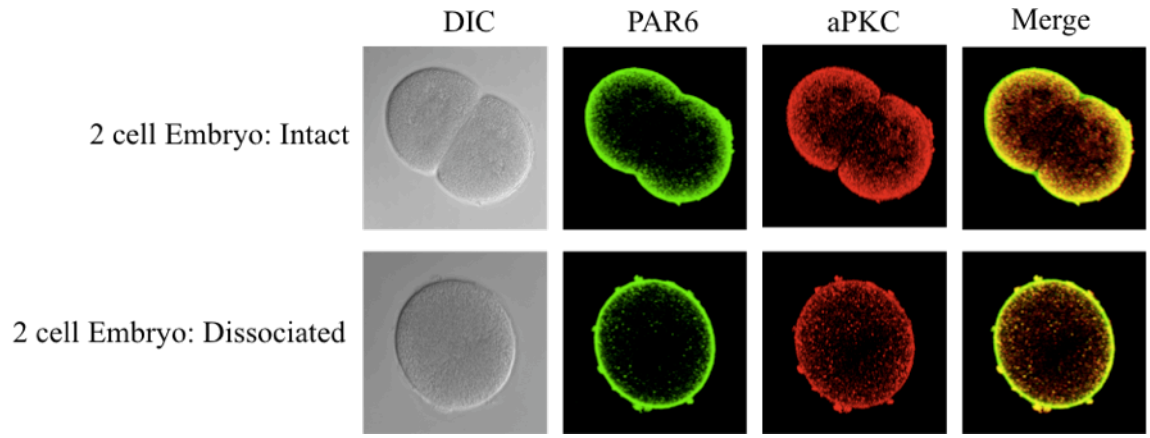


Figure 2.2.3. Myosin assembly is required for maintaining the cortical localization of the PAR complex proteins. *L. pictus* embryos were fixed at the 2 cell stage after treatment with various inhibitors for 15 minutes (A). Fixed embryos were stained for PAR6, aPKC, and CDC42. PAR6, aPKC, and CDC42 are apically localized in the cortex of the sea urchin embryo at the 2 cell stage of development. Inhibition of myosin light chain kinase (MLCK) by ML-7 resulted in cytoplasmic pooling of these cell polarity regulators.

To a lesser extent this same effect was observed with ML-9, a less specific MLCK inhibitor. Treatment with a general Rho kinase inhibitor, H1152, an actin polymerization inhibitor, latrunculin B, an astral microtubule inhibitor, urethane, or a myosin ATPase inhibitor, blebbistatin did not effect the cortical localization of these proteins. *L. pictus* embryos were fixed and then stained for PAR6 (B). PAR6 localizes to the cortex and apical cortex at the fertilized egg and 2 cell stages (i and iii). Treatment with ML-7 at both the fertilized egg and 2 cell stages for 15 minutes resulted in cytoplasmic pooling of PAR6 (ii and iv). Pulse treatment with ML-7 for 15 minutes at the fertilized egg and analysis of PAR6 localization at the next cleavage (2 cell) reveals that PAR6 remains cytoplasmic (v). For scaling reference *L. pictus* embryos are approximately 120 μm in diameter.

A.



B.

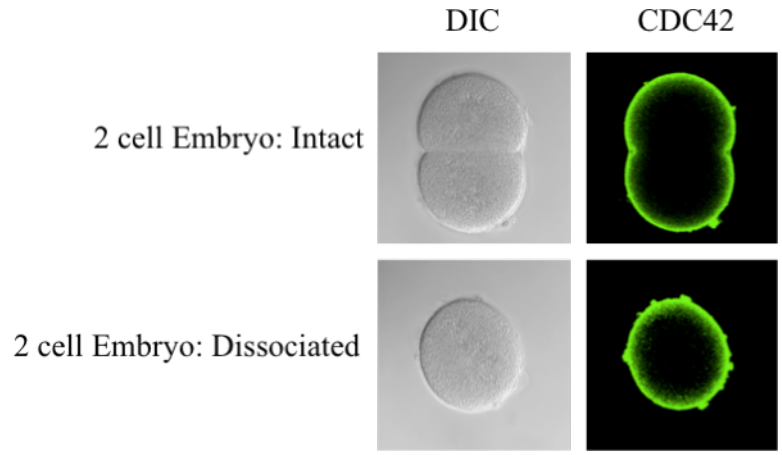
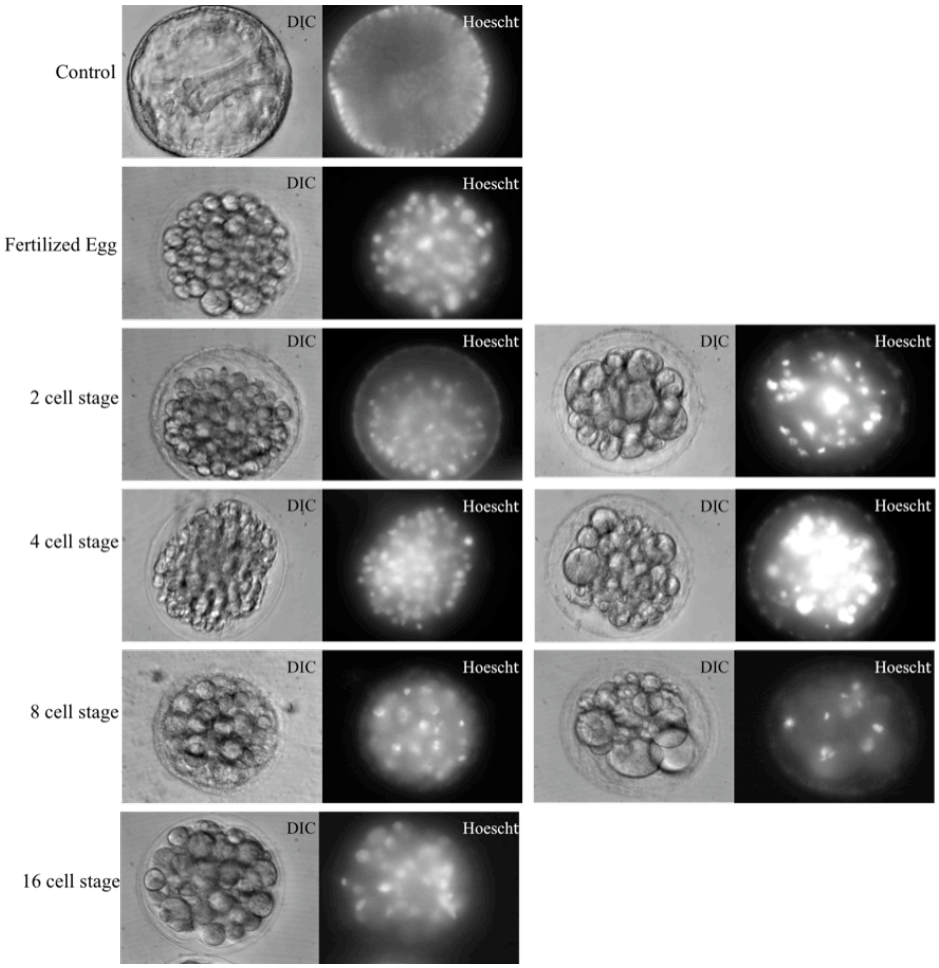
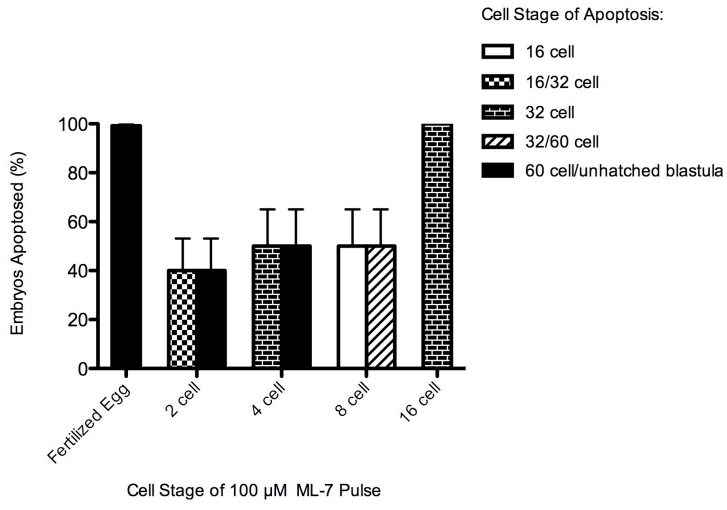


Figure 2.2.4. PAR6, aPKC, and CDC42 are reliant upon calcium dependent cell adhesion for apical localization. *L. pictus* embryos were fixed and stained for PAR6 and aPKC (A) or CDC42 (B) in both intact and dissociated 2 cell embryos. All proteins were found to have a uniform distribution in the cortex upon dissociation. For scaling reference *L. pictus* embryos are approximately 120 μm in diameter.

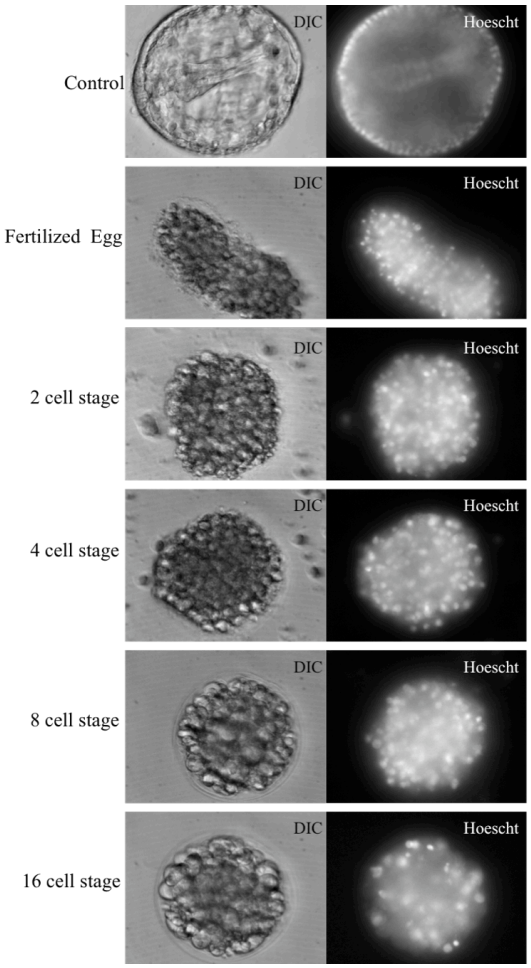
A.


Control
Fertilized Egg
2 cell stage
4 cell stage
8 cell stage
16 cell stage
DIC
Hoescht

B.


Cell Stage of Apoptosis:
16 cell
16/32 cell
32 cell
32/60 cell
60 cell/unhatched blastula
Embryos Apoptosed (%)
100
80
60
40
20
0
Fertilized Egg
2 cell
4 cell
8 cell
16 cell
Cell Stage of 100 µM ML-7 Pulse

C.


Control
Fertilized Egg
2 cell stage
4 cell stage
8 cell stage
16 cell stage
DIC
Hoescht

D.

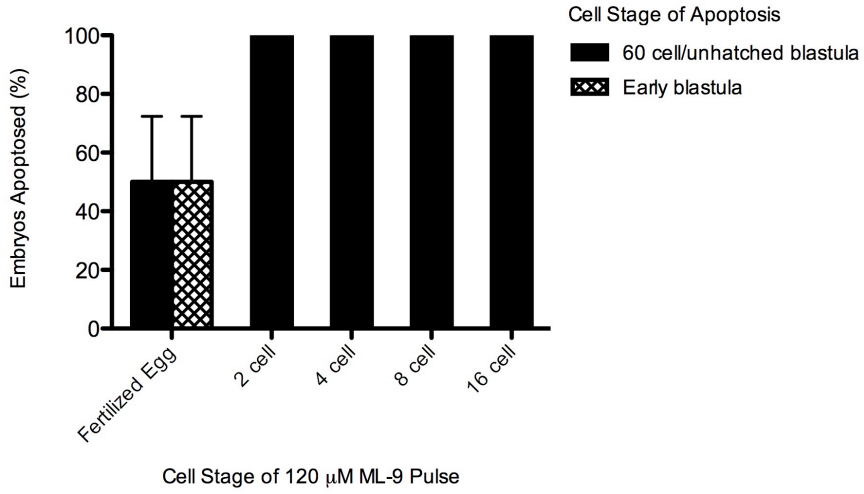
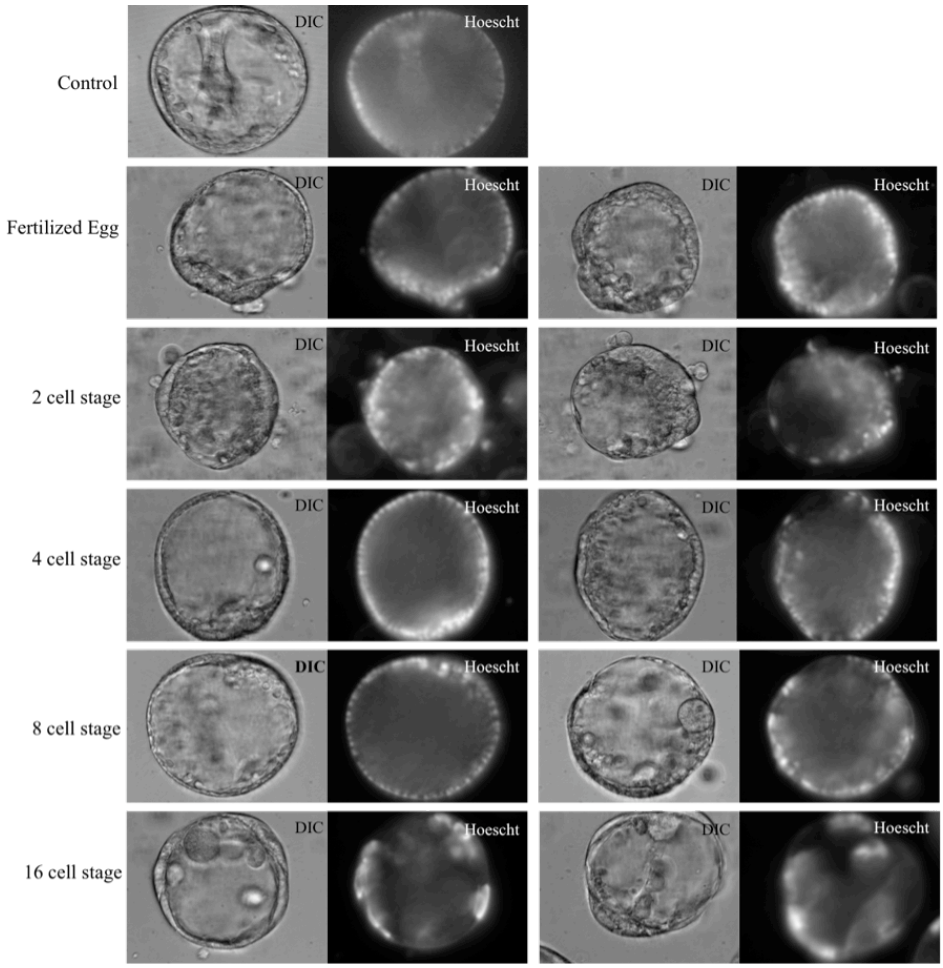
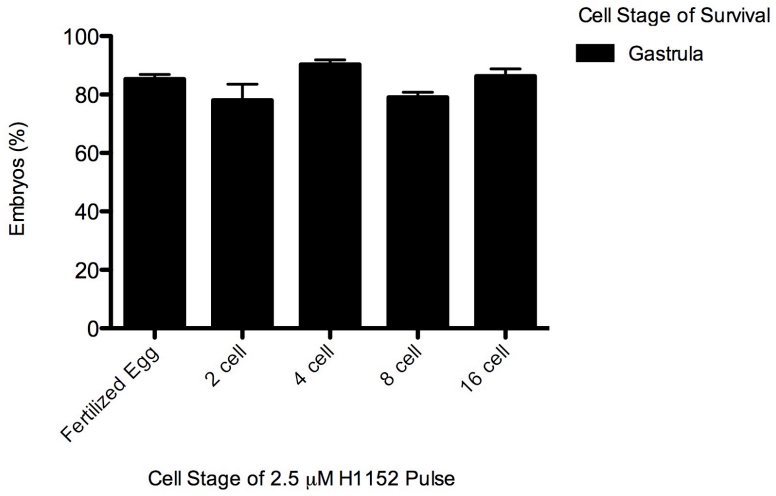

Cell Stage of Apoptosis
60 cell/unhatched blastula
Early blastula
Embryos Apoptosed (%)
100
80
60
40
20
0
Fertilized Egg
2 cell
4 cell
8 cell
16 cell
Cell Stage of 120 μM ML-9 Pulse

Figure 2.2.5. Assembled myosin is required during early cleavage stages for blastula formation. 100 embryos were treated with a 100 μM ML-7 (A) (n=12), or 120 μM ML-9 (C) (n=6) pulse for 15 minutes at various cell stages as indicated and allowed to develop until the control embryos had reached the gastrula stage (≥90% of controls reached gastrulation). Results are quantified (B and D) (mean±SD). Embryos treated with the MLCK inhibitors, ML7 and ML-9, failed to reach the hatched blastula stage. For scaling reference *L. pictus* embryos are approximately 120 μm in diameter.

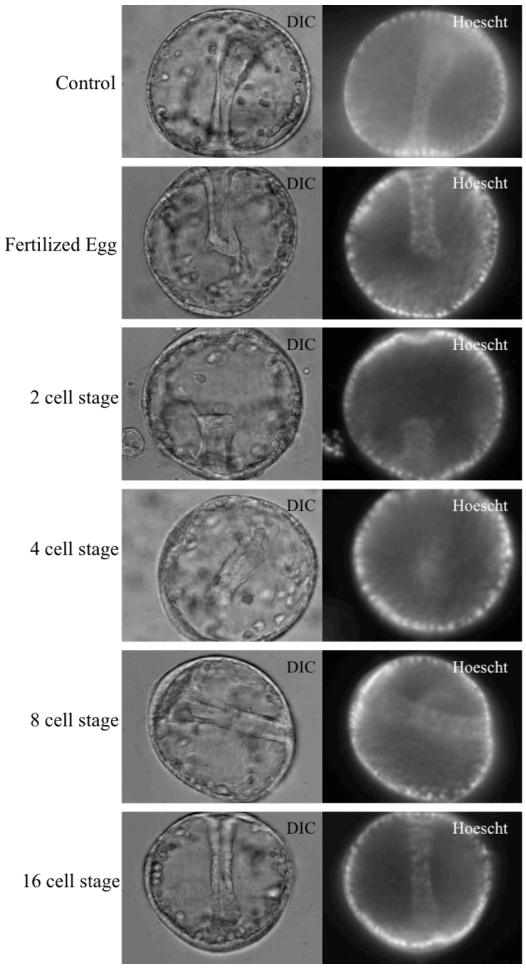
A.


Control
Fertilized Egg
2 cell stage
4 cell stage
8 cell stage
16 cell stage
DIC
Hoescht

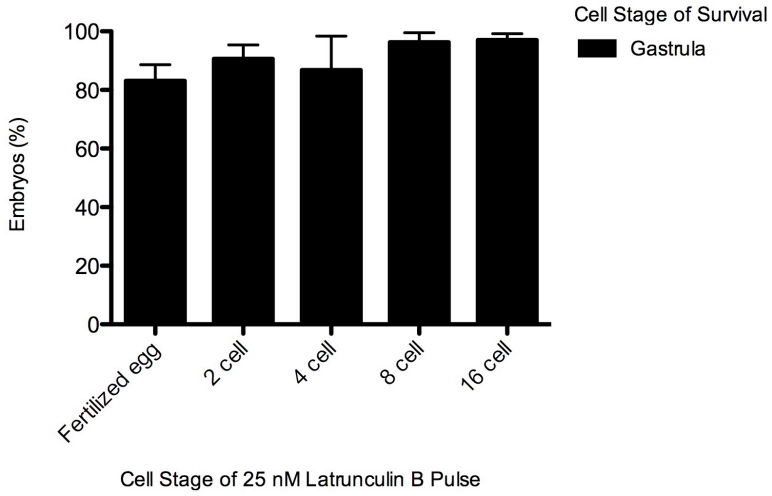
B.


Cell Stage of Survival
Gastrula
Embryos (%)
100
80
60
40
20
0
Fertilized Egg
2 cell
4 cell
8 cell
16 cell
Cell Stage of 2.5 μM H1152 Pulse

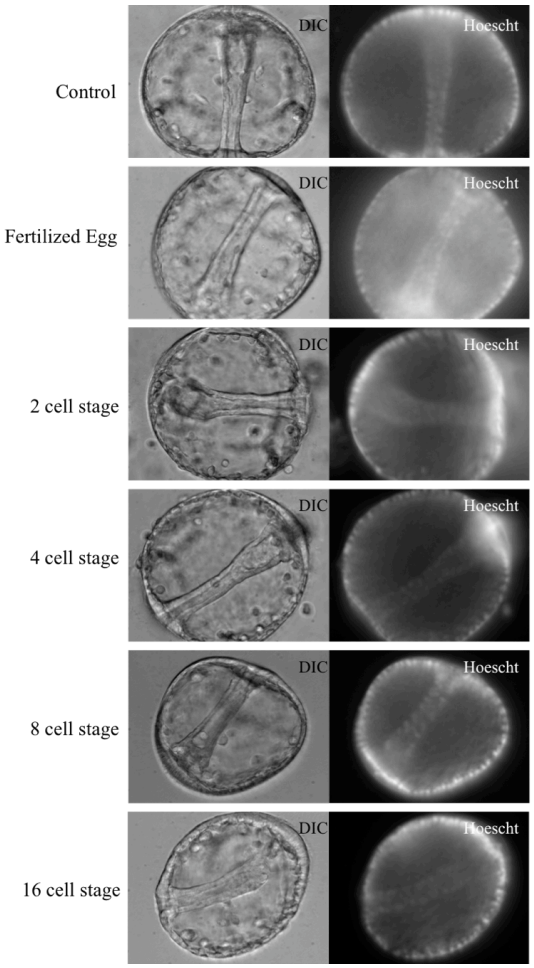
C.


Control
Fertilized Egg
2 cell stage
4 cell stage
8 cell stage
16 cell stage
DIC
Hoescht

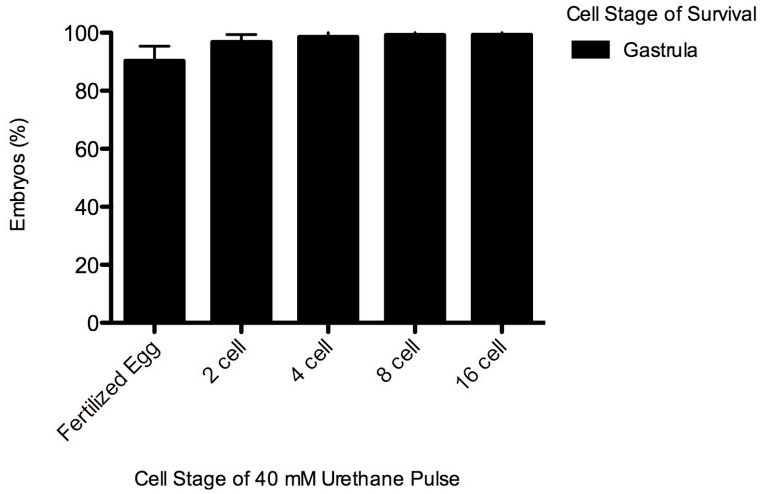
D.


Cell Stage of Survival
Gastrula
Embryos (%)
100
80
60
40
20
0
Fertilized egg
2 cell
4 cell
8 cell
16 cell
Cell Stage of 25 nM Latrunculin B Pulse

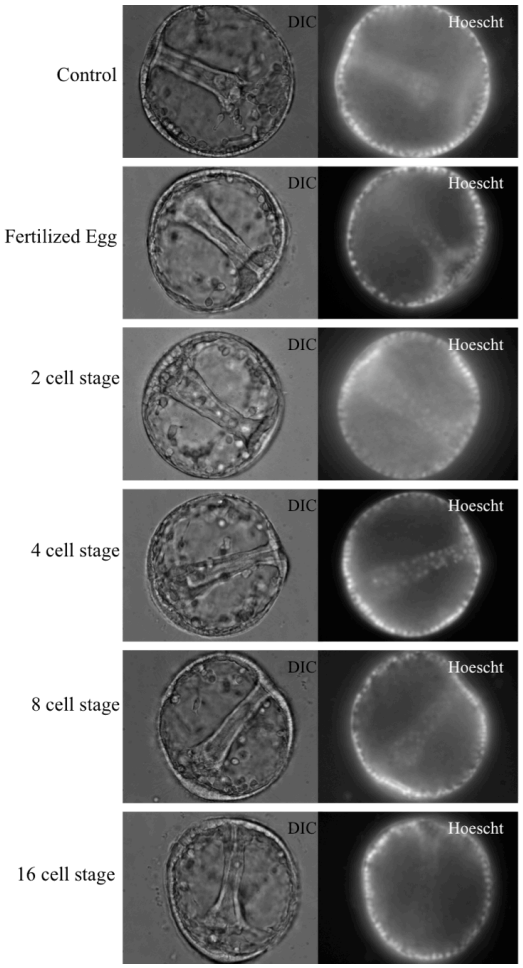
E.


Control
Fertilized Egg
2 cell stage
4 cell stage
8 cell stage
16 cell stage
DIC
Hoescht

F.


Cell Stage of Survival
Gastrula
Embryos (%)
100
80
60
40
20
0
Fertilized Egg
2 cell
4 cell
8 cell
16 cell
Cell Stage of 40 mM Urethane Pulse

G.


Control
Fertilized Egg
2 cell stage
4 cell stage
8 cell stage
16 cell stage
DIC
Hoescht

H.

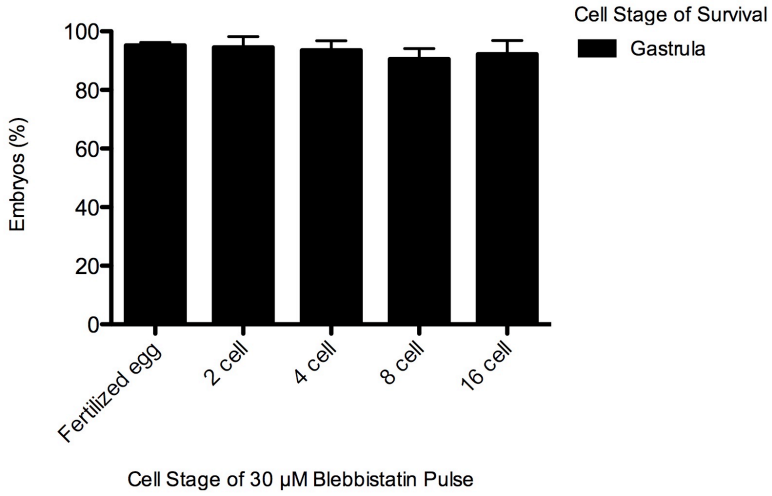

Cell Stage of Survival
Gastrula
Embryos (%)
100
80
60
40
20
0
Fertilized egg
2 cell
4 cell
8 cell
16 cell
Cell Stage of 30 µM Blebbistatin Pulse

Figure 2.2.6. Rho kinase, actin, microtubules, and myosin ATPase activity during early cleavage stages are not required for blastula formation. 100 embryos were treated with a 2.5 μM H1152(A) (n=6), 25 nM latrunculin B(C) (n=6), 40 mM urethane(E) (n=6), or 30 μM blebbistatin(G) (n=6) pulse for 15 minutes at various cell stages as indicated and allowed to develop until the control embryos had reached the gastrula stage (≥90% of controls reached gastrulation). Results are quantified (B, D, F, and H) (mean±SD). Embryos treated with either the Rho kinase inhibitor (H1152) reached the gastrula with an abnormal morphology. Embryos treated with the actin inhibitor (latrunculin B), the astral microtubule inhibitor (urethane), or the myosin ATPase inhibitor (blebbistatin) reached the gastrula stage and had similar morphology to controls. For scaling reference *L. pictus* embryos are approximately 120 μm in diameter.

Chapter 3. PAR1 Polarizes in a Distinct Pattern in the Sea Urchin Embryo

3.1. Introduction

PAR1 is a serine/threonine kinase that was discovered because of its involvement in the first asymmetric division in the *C. elegans* embryo (Guo and Kemphues, 1995). Unlike the PAR complex proteins, PAR1 localizes to posterior end of the *C. elegans* embryo following fertilization; the asymmetry between these proteins is well conserved (Benton and Johnston, 2003;Kemphues, 2000;Suzuki et al., 2004). Since its initial discovery, PAR1 has been found to regulate polarity and a variety of other cellular processes in many other model systems. PAR1 was also independently discovered as microtubule associated regulatory kinase (MARK), which phosphorylates microtubule associated proteins (MAPs) (Drewes et al., 1995). PAR1 was identified in these experiments because it phosphorylates the MAP, Tau, which is an important component of the neurofibrillary tangles in Alzheimer's disease (Drewes et al., 1995). Phosphorylation of MAPs can result in either the stabilization (in *Drosophila*) or destabilization (in mammals) of microtubules (Doerflinger et al., 2003). PAR1 specifically phosphorylates KXGS repeats in the microtubule binding domains of MAPs, which causes MAPs to dissociate from microtubules (Drewes et al., 1997). PAR1 plays a critical role in the organization of the microtubule cytoskeleton in oocytes, developing embryos, neurons, and epithelial cells (Cohen et al., 2004;Cox et al., 2001;Doerflinger et al., 2003;Terabayashi et al., 2007).

In neurons, PAR1 is involved in the formation of both axonal and dendritic processes as well as in neuronal migration. In mouse hippocampal neurons, PAR1 is necessary for proper dendritic spine morphology (Hayashi et al., 2011a) and positively

regulates microtubule dynamics (Hayashi et al., 2011b). Other studies have found that PAR1 is also needed for axon formation in hippocampal neurons (Chen et al., 2006). PAR1 is tightly regulated as elevated activity of this protein results in reduced axon and dendritic growth, while downregulation of PAR1 results in the formation of multiple axons (Matenia and Mandelkow, 2009). PAR1 is also required for neuroblast migration to the olfactory bulb and the maintenance of microtubule dynamics in the migrating neurons of the cerebral cortex (Mejia-Gervacio et al., 2011;Sapir et al., 2008).

Additionally, PAR1 is essential for polarity in epithelial cells. Here, PAR1 regulates morphogenetic branching decisions by organizing the radial array of microtubules in nonpolarized cells into the horizontal array of polarized hepatocytes and the vertical arrays in columnar epithelia (Cohen et al., 2004). Further studies suggest that PAR1 promotes cell-cell adhesion, regulates myosin light chain phosphorylation, and is involved in mitotic spindle orientation (Cohen et al., 2007;Elbert et al., 2006;Lázaro-Diéguez et al., 2013;Slim et al., 2013).

There has also been significant analysis of the role of PAR1 during development. In addition to regulating the first asymmetric division in the *C. elegans* embryo (Guo and Kemphues, 1995), PAR1 is required later in development for vulva formation in the *C. elegans* embryo (Hurd and Kemphues, 2003). Other studies have shown that PAR1 is also involved in the asymmetric divisions at the blastula and gastrula stages of development in *Xenopus* (Ossipova et al., 2007). In *Xenopus* PAR1 additionally promotes ciliated cell differentiation, stimulates the generation of deep cell progeny from the superficial epithelium of the neural plate, controls gastrulation, and stimulates neuronal differentiation (Kusakabe and Nishida, 2004;Ossipova et al., 2007;Ossipova et

al., 2009;Ossipova and Sokol, 2011;Tabler et al., 2010). In *Drosophila* PAR1 activity is required for axis formation in the oocyte, the selection of one cell in the germline cyst to become an oocyte, and cell polarity in the blastoderm and ectoderm (Bayraktar et al., 2006;Cox et al., 2001;Huynh et al., 2001). Similar to studies on the PAR complex proteins, the role of PAR1 has not been well reviewed in the early development of deuterostome embryos.

Here, I generated a specific antibody generated against the *S. purpuratus* PAR1 protein and used the antibody to examine the localization pattern of PAR1 in the sea urchin embryo. Unlike in other model organisms, PAR1 colocalizes with the PAR complex proteins in the apical cortex at the 2 cell stage of development. This colocalization is retained through the gastrula stage, after a series of asymmetric divisions, the formation of an epithelium and the convergent extension movements of gastrulation. Co-immunoprecipitation assays further revealed that PAR1 is in complex with aPKC during early development in the sea urchin embryo. Collectively, these data highlight a unique role for PAR1 in the polarization and development of the sea urchin embryo.

3.2. Results

3.2.1. Anti-PAR1 Antibody Design and Verification

Having examined the localization of the PAR complex proteins and their upstream regulator, CDC42, the localization pattern of a traditionally posterior polarity regulator, PAR1, was studied in order to determine how it polarized in the sea urchin embryo. Using the predicted PAR1 sequence, a specific antibody against the *S.*

purpuratus PAR1 protein was created (Cameron et al., 2009). Based on its annotation in the *S, purpuratus* genome, the predicted PAR1 protein contains the three conserved domains of PAR1 proteins: the kinase domain, the kinase associated domain, and the ubiquitin associated domain (Cameron et al., 2009). The antigenic sequence used for antibody production was specifically chosen to be outside of the three conserved domains of the PAR1 protein (Figure 3.2.1.A.). Previously screens of commercial antibodies against mouse and human MARK proteins that were designed in conserved regions (usually the kinase domain) were found to be nonspecific (data not shown). Two rabbits were immunized with the antigenic peptide generated by Covance. Bleeds from days 0, 28, 56, and 70/72 were tested against the purified antigen. Bleeds from the first rabbit on day 70/72 were found to have the strongest reactivity against both the target peptide and protein extracts of 2 cell and 4 cell stage *L. pictus* embryos and were subsequently chosen for purification against the initial target peptide (Figure 3.2.1.B. and C.). Purified PAR1 antibody was tested against 2 cell and 4 cell stage *L. pictus* embryos and was found to be specific and a Western blot shows a single band at the correct molecular weight of 81.5 kD and additionally identifies PAR1 dimers at 163 kD (Figure 3.2.1.D.).

3.2.2. PAR1 Colocalizes with the PAR complex Proteins in the Apical Cortex Through the Gastrula Stage

Immunofluoresence assays revealed that during early cleavage stages, PAR1 colocalizes with the PAR complex proteins in the apical cortex through the 8 cell stage (Figure 3.2.2.A.). Furthermore, PAR1 retains this colocalization with the apical PAR complex at the 16 cell stage, after the first asymmetric division, and at the blastula stage, after junctions have formed within the epithelium (Figure 3.2.2.A. and B.).

In order to determine if PAR1 polarizes to a distinctive region later in development in the sea urchin embryo, its localization pattern at the gastrula stage was examined. mRNA transcripts of PAR1 are known to be expressed throughout embryogenesis, but appear to become more vegetal in localization following hatching from the fertilization envelope in the sea urchin species *Hemicentrotus pulcherrimus* (Shiomi and Yamaguchi, 2008). During the gastrula stage, the embryo is laying the foundation for the three primary germ layers, the endoderm, mesoderm, and ectoderm, by undergoing massive cellular rearrangements. PAR6, aPKC, CDC42, and PAR1 retain their localization to the apical or free cell surface at this stage (Figure 3.2.3.A.). However, there is a significant downregulation of the expression of all of these proteins within the epithelium of the archenteron compared to both the apical tuft and the epithelium of the ectoderm (Figure 3.2.3.B.). There does appear to be a slight enrichment of these proteins in the apical tuft compared to the epithelium of the ectoderm, but this was not found to be statistically significant (Figure 3.2.3.B.).

Because of this unique PAR1 localization, co-immunoprecipitation assays were performed in order to determine if PAR1 was interacting with the PAR complex proteins PAR6 and aPKC (Figure 3.2.4.). Previous results demonstrated that PAR6 and aPKC, as in other model systems, were associated with each other (Alford et al., 2009;Joberty et al., 2000;Li et al., 2010;Lin et al., 2000;McCaffrey and Macara, 2009). Co-immunoprecipitation assays at the 2 cell (first appearance of polarization to the apcial cortex), 16 cell (first asymmetric division), and blastula (formation of an epithelium) stages were carried out in order to study the relationship of these proteins. Across all studied stages of development that aPKC was found to be in complex with both PAR6

and PAR1; however, PAR6 and PAR1 were not in complex with each other (Figure 3.2.4.B., C., and D.).

Several control co-immunoprecipitation assays were performed to assess the specificity of the Dynabeads kit (Life Technologies) and confirm the interaction of aPKC with PAR1 and PAR6. First positive controls tested if antibody coupled beads pulled down the same protein the antibody was generated against. For example, PAR6 coupled beads were used for the co-immunoprecipitation assay and then probed for the presence of PAR6 in the Western blot. Positive controls were confirmed for PAR6, aPKC, and PAR1 (Figure 3.2.4.A.). An additional control tested and confirmed that the magnetic beads of the Dynabeads kit (Life Technologies) did not non-specifically interact with either the PAR6, aPKC, or PAR1 proteins (Figure 3.2.4.E.). The specificity of the Dynabeads was further confirmed with a control that utilized buffer instead of lysed embryos as the starting sample for co-immunoprecipitation assay and there were no non-specific interactions with aPKC (Figure 3.2.4.F.).

3.2.3. Regulation of PAR1 Localization

In order to evaluate the cytoskeletal regulation of PAR1 localization, the same series of small molecule inhibitors that were used previously to examine PAR6, aPKC, and CDC42 localization patterns were again utilized. The acto-myosin cortex is involved in the localization of the PAR complex proteins in other model systems (Cowan and Hyman, 2004a;Munro et al., 2004;Munro, 2006). Myosin assembly is known to regulate polarized plasma membrane rafts in the sea urchin embryo (Alford et al., 2009;Gudejko et al., 2012;Ng et al., 2005) and had already been confirmed to be specifically required for the cortical localization of the PAR complex in the sea urchin embryo (Figure 2.2.3.).

The MLCK inhibitors, ML-7 and ML-9, were tested to determine the role of assembled myosin in PAR1 localization. 2 cell stage embryos were treated for 15 minutes with ML-7 or ML-9 immediately prior to fixation. PAR1 localization was unpertubed in the apical cortex by treatment with either ML-7 or ML-9. However, unlike previous experiments, CDC42 localization was also not affected by treatment with either ML-7 or ML-9 (Figure 3.2.5.A.). In order to investigate further, the efficacy of ML-7 and ML-9 were tested. A series of dose-response assays in three different species of sea urchins (*S. purpuratus, L. pictus, and Lytechinus variegatus*) in multiple batches across several different seasons found that these embryos were no longer responding to ML-7 or ML-9. In addition to no longer causing the cytoplasmic pooling of PAR6, aPKC, or CDC42, they also no longer became binucleate when the embryos were treated prior to entering mitosis and they no longer formed the F-actin comets that had been observed previously (Gudejko, 2013) (data not shown).

Other cytoskeletal inhibitors were still tested in order to determine if there was a role for ROCK, myosin ATPase, actin, or microtubules in the localization of PAR1 to the apical cortex. Unlike, ML-7 and ML-9, *L. pictus* embryos still responded appropriately to each of the other cytoskeletal inhibitors. 2 cell stage embryos were treated for 15 minutes with either H1152 (ROCK inhibitor), blebbistatin (myosin ATPase inhibitor), latrunculin B (actin inhibitor), or urethane (astral microtubule inhibitor). Similar to the PAR complex proteins, PAR1 remained localized to the apical cortex when embryos were treated with the listed inhibitors (Figure 3.2.5.A.). The cytoskeletal regulation of PAR1 localization thus far remains unclear. However, a role for cell-cell contact was clearly elucidated for PAR1 localization. Similar to PAR6, aPKC, and CDC42 (Figure

2.2.4.), PAR1 became evenly distributed throughout the cortex upon dissociation (Figure 3.2.5.B.).

3.2.4. DNPAR1 and PAR1 Construct Development

In order to evaluate both PAR1 function and PAR1 dynamics in live embryos, a DNPAR1 (dominant negative) and PAR1 construct were developed. Because there are currently no commercial small molecule inhibitors of PAR1, a dominant negative construct is an alternative to inhibit PAR1. The dominant negative construct consists of the full-length PAR1 protein without the N-terminal kinase domain. In this manner microinjection of DNPAR1 in live embryos can outcompete the native PAR1 and inhibit PAR1 function. The full-length PAR1 construct was then created in order to assess PAR1 dynamics in live embryos by tagging it with green fluorescent protein (GFP).

RNA was isolated from several different batches of *S. purpuratus* zygotes, 30 minutes post-fertilization. cDNA sequences were amplified using the ProtoScript M-MuLV *Taq* RT-PCR (reverse transcriptase polymerase chain reaction) kit (NEB) and primers designed against either the GLEAN prediction PAR1 sequence or the RNAseq prediction sequence (Table 6.6.1., Figure 3.2.6.) (Cameron et al., 2009). Both constructs were successfully cloned into the bacterial expression vector pEXP5-CT (Life Technologies). However, only the DNPAR1 construct was successfully expressed and purified from bacterial cultures (Figure 3.2.6.C). As a control, *L. pictus* and *L. variegatus* were microinjected with rhodamine dextran diluted into injection buffer (10mM HEPES, 150mM aspartic acid, pH 7.2) during interphase. Control embryos failed to enter mitosis and subsequently apoptosed (data not shown). For these reasons the efficacy of the DNPAR1 construct could not be determined.

3.3. Discussion

The PAR proteins have been well characterized in a number of systems and are strictly regulated in order to carry out diverse polarity dependent processes such as spindle positioning, axon formation, and gastrulation (Kusakabe and Nishida, 2004;Lázaro-Diéguez et al., 2013;Yoshimura et al., 2010). The manner in which they polarize has been well conserved among multicellular eukaryotes. While PAR6, aPKC, and CDC42 often colocalize to a single region, PAR1 is typically found in the opposing domain. Here the data show a unique localization pattern of the PAR proteins in the sea urchin embryo, which despite its nontraditional pattern, may be critical for proper development of the embryo.

PAR6, aPKC, and CDC42 localize to the apical cortex as early as the 2 cell stage of development and retain their apical localization through the blastula stage; these data reflect a conserved localization pattern for these proteins (McCaffrey and Macara, 2012;Nance and Zallen, 2011). However, the colocalization of PAR1 with these proteins in the apical cortex appears to be unique to the sea urchin embryo. Despite having an asymmetric cell division at the 16 cell stage, forming tight junctions in an epithelium at the blastula stage, and undergoing convergent extension movements during gastrulation PAR1 remains localized to the apical surface along with the other PAR proteins. Given the distinctive localization pattern of PAR1 in the sea urchin embryo, determining the localization pattern of other traditionally posterior polarity regulators will be imperative to explore. Lethal giant larvae (LGL) is known to regulate polarity in *C. elegans,* function redundantly with PAR2 in *Drosophila*, and is a predicted protein based on the sequenced sea urchin genome (Beatty et al., 2010;Beatty et al., 2013;Cameron et al.,

2009;Hoege et al., 2010;Prehoda and Bowerman, 2010). In sea urchin embryos, it may be that other posterior polarity regulators, such as LGL, function to regulate polarity in areas of cell-cell contact.

The data suggest that although the PAR proteins are polarized, the manner in which they polarize is unique to the sea urchin embryo. Furthermore, the downregulation of the expression of these proteins in the archenteron may reflect a functional significance, as they may be more important in the formation and maintenance of the epithelium that is formed at the blastula stage. Recent data, however, has found that PAR6 regulates skeletogenesis and gut differentation in the *Hemicentrotus pulcherrimus* species of sea urchin (Shiomi et al., 2012). Inhibition of aPKC did not appear to affect archenteron ingression or spicule formation in the *Paracentrotus lividus* species of sea urchin (Pruliere et al., 2011). Future experiments could determine if there is a species specific or protein specific requirement for the PAR proteins during and post gastrulation.

Here I find that PAR1 is actually in complex with aPKC at the 2 cell, 16 cell, and blastula stages of development, which is surprising given the traditionally antagonistic behavior of these proteins (Hurov et al., 2004). Because PAR1 is a substrate of aPKC, it is perhaps less surprising that they are associated with one another as opposed to PAR6 and PAR1, which are not known to interact in other systems. In other model organisms aPKC phosphorylates PAR1 and PAR1 phosphorylates PAR3 as a means to exclude each other from their respective domains (Benton and Johnston, 2003;Hurov and Piwnica-Worms, 2007). These typically transient phosphorylation events may be an indicator of why PAR1 and aPKC are found in the same complex in the sea urchin embryo.

While previous results had indicated that PAR6, aPKC, and CDC42 were anchored in the apical cortex specifically by assembled myosin, it remains to be determined what cytoskeletal components affect PAR1 localization. Similar to PAR6, aPKC, and CDC42 PAR1 does not appear to be anchored in the apical cortex by ROCK activity, myosin ATPase activity, actin or microtubule polymerization. PAR1 localization to the apical cortex is also similarly reliant upon cell-cell contact to maintain apical localization. However, a role for myosin assembly in PAR1 localization has not yet been elucidated.

In numerous previous seasons of both *L. pictus* and *S. purpuratus* ML-7 and ML-9 worked effectively to inhibit the activity of MLCK in these species of sea urchin embryos. It was through the use of these inhibitors that MLCK activity was found to be required for the stability of lipid rafts in the membrane of sea urchin embryos (Alford et al., 2009;Gudejko et al., 2012;Ng et al., 2005). One of the major phenotypes associated with MLCK inhibition is the formation of binucleate cells because while mitosis can proceed normally, the contractile ring is unable to form without myosin assembly and without a contractile ring cells do not undergo cytokinesis (Mabuchi and Takano-Ohmuro, 1990). In sea urchin embryos it has been shown that mono-phosphorylated regulatory light chain is required for cleavage furrow formation as well as for contraction of the furrow (Uehara et al., 2008). Additional studies have found that myosin II is involved in coordination of the global activation of the cortex prior to the onset of cytokinesis and the maintenance of contractility in the furrow only during division (Miyoshi et al., 2006). These studies all utilized either ML-7 or ML-9 in order to assess the function of the MLCK in sea urchin embryos. However, for the past few seasons of *L.*

pictus, S. purpuratus and *L. variegatus* both ML-7 and ML-9 have been ineffective. In addition to not causing cytoplasmic localization of PAR6, aPKC, or CDC42 and not resulting in the formation of actin comets (Gudejko, 2013), embryos treated with either ML-7 or ML-9 did not become binucleate. Either embryos undergo cytokinesis normally, or they do not enter mitosis and apoptose, mostly likely to due to solvent, dimethyl sulfoxide (DMSO), toxicity (Mabuchi and Takano-Ohmuro, 1990).

This switch in the efficacy of ML-7 and ML-9 may be due to several changes in sea urchin embryos. All of the sea urchins used for scientific studies are wild-caught and are therefore not a genetically uniform system. They are also subject to any changes in their environment, which cannot be precisely controlled, as they would be in a laboratory setting. Sea urchins are well documented to express ATP-binding cassette (ABC) transporters (Campanale and Hamdoun, 2012;Shipp and Hamdoun, 2012;Whalen et al., 2012). There are over 100 predicted ABC transporters in the *S. purpuratus* genome and 40 annotated transporters that are multidrug efflux transporters (Cameron et al., 2009;Shipp and Hamdoun, 2012). These multidrug efflux transporters are well-known to cause drug resistance in variety of diseases, including cancer (Borst and Elferink, 2002). While the relative expression levels of the ABC transporters in sea urchin embryos is unknown, it is possible that there has been a recent increase in their expression which may be causing certain drugs like ML-7 and ML-9 to be pumped out of the embryos and rendering them ineffective. ML-7 and ML-9 are not the only inhibitors that have been ineffective recently. Sea urchin embryos have also not been responding to both the peptide inhibitor of PKCζ (data not shown) and inhibitors of Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3 kianse) (Sluter, personal communications). These data

suggest that there may be an increased expression of the ABC transporters in the wild caught sea urchin population in response to a possible increase in environmental toxins that must be pumped out for survival.

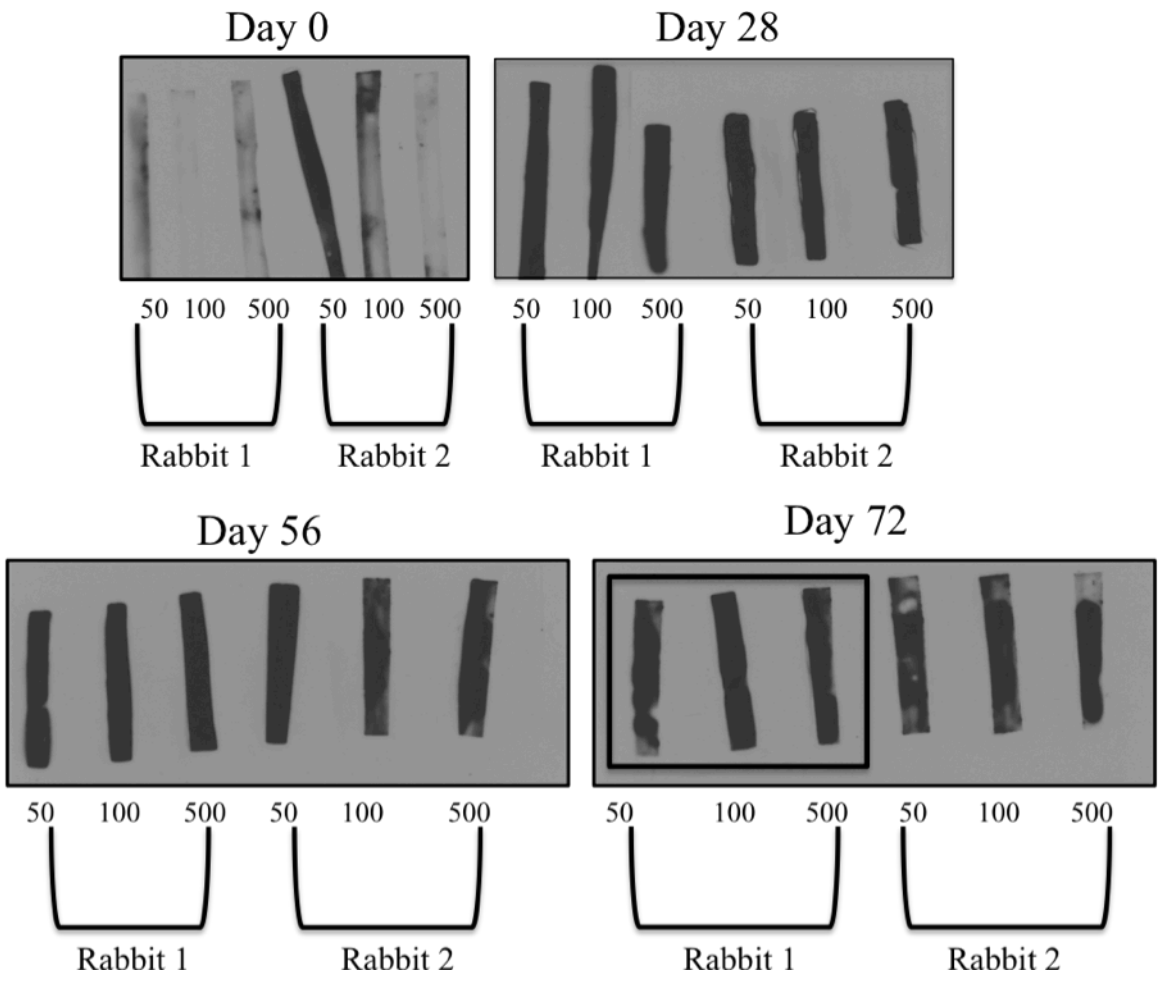
There may be other environmental changes that are causing the resistance to drugs like ML-7 and ML-9 and may also explain why the embryos in the control microinjection experiments failed to divide. Global warming is now contributing to rising ocean temperatures and carbon dioxide levels. Because sea urchin embryos are a well-studied model of cellular and developmental biology, the impact of these changes in temperature and ocean acidification have been explored using this model system. Increases in temperature and carbon dioxide levels have been shown to cause widespread changes in gene expression in sea urchin embryos (Pespeni et al., 2013;Runcie et al., 2012). Further work has demonstrated that specific genes involved in spiculogensis and biomineralization vary in response to changes carbon dioxide levels (Hammond and Hofmann, 2012). Other studies have found that although sea urchins embryos are able to compensate for the acidification of the seawater, it comes at increased costs, which impact growth and mortality (Stumpp et al., 2012). The long-term impact of rising temperatures and ocean acidification are not yet known. While it cannot be definitively determined, these changes in their environment may be affecting the sea urchins used for my experiments. The adult sea urchins have been noticeably less stable during their shipment from coastal California and in some batches the embryos have seemed less healthy. It remains to be determined if the drug resistance and failed microinjection experiments were due to a seasonal variation in sea urchins or were an indicator of more long-term effects in response to environmental changes.

The data here demonstrate a unique localization pattern for the PAR1 protein in sea urchin embryos. Unlike in other model organisms, PAR1 colocalizes with the PAR complex proteins in the apical cortex through the gastrula stage of development. Further analysis revealed that PAR1 is in complex with aPKC at the 2 cell, 16 cell, and blastula stages of development. Future studies will determine the cytoskeletal regulation of PAR1 localization as well as the function of PAR1 in cleavage stage embryos. The polarization of PAR1 to the apical cortex may be an indicator of novel regulations of polarity in the sea urchin embryo.

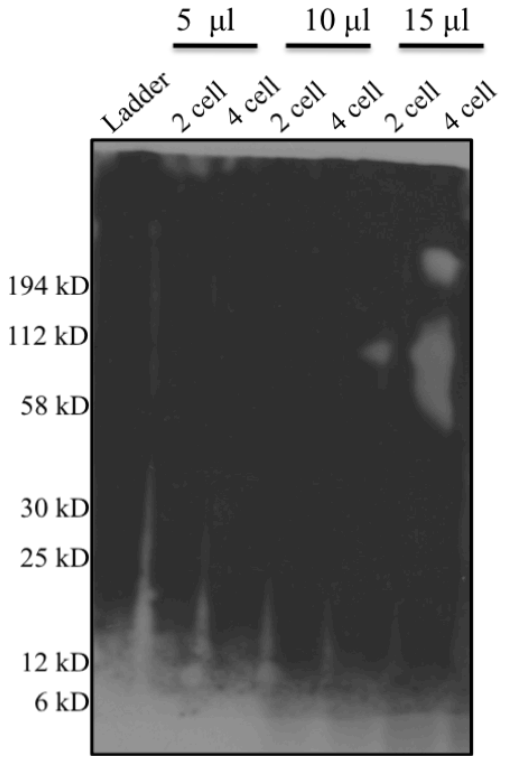
A.

MDLHDDSTNSGSSRSRGRTGDDQPHVGKYRLIKTIGKGNFAKVKLAKHIPTGKE
VAIKIIDKTQLNPSSLQKVYREVKIMKLLDHPNIVKLFEVIETDKTLYLAMEYASG
GEVFDYLVAHGRMKEKEARAKFRQIVSAVQYCHQKRVVHRDLKAENLLLDKD
LNIKIADFGFSNEFTIGCKLDTFCGSPPYAAPELFQGKKYDGPEVDVWSLGVILYT
LVSGSLPFDGQNLKELRERVLRGKYRIPFYMSTDCENLLKRFLMLNPAKRAMLE
TIMKDKWMNAGFEEHELKPHQDNQEDFYDERRIETMTGMGFKRKEIEDSLRNH
KYDEHYATYLLLGRRHSDQAEDADSTSGSCLSLPQRTVSDLSSSITQSPSSQGKK
QRSIPSNQKERRFSHGGENYGQHSYKRHMDSSLKENMGNPPHRDRGRSSHGPIG
SKDSSSQSNTSNNPNQSDIPDRKAKSTPKSASKLPTKTPPGMSRRNTYVAGERDF
PGSSRTNGSSERSTRHQKSSSTSSHPVKGALLPPIDDPSNRPSTAPNKRHQIGGSPS
VRGPPVQGSNLHRGTVNRATIHGAPGRRPMFNGPASAQGSSQDTSTRNMGGPH
NISLFSKFAS**KFSRRSLVMAEPPSEYVKPR**SLRFTFSMKTTSSKEPDSIIGEIRRVL
ESNGVDFEQRERYLLFCVHGDGRGDNLIQWEMEDSNLHQLTEDSQDSTVLLFLF
ESSEGRNMKQTPKHSTFKSSVKSPDSAHVLPE

B.



C.


5 µl
10 µl
15 µl
Ladder
2 cell
4 cell
2 cell
4 cell
2 cell
4 cell
194 kD
112 kD
58 kD
30 kD
25 kD
12 kD
6 kD

D.

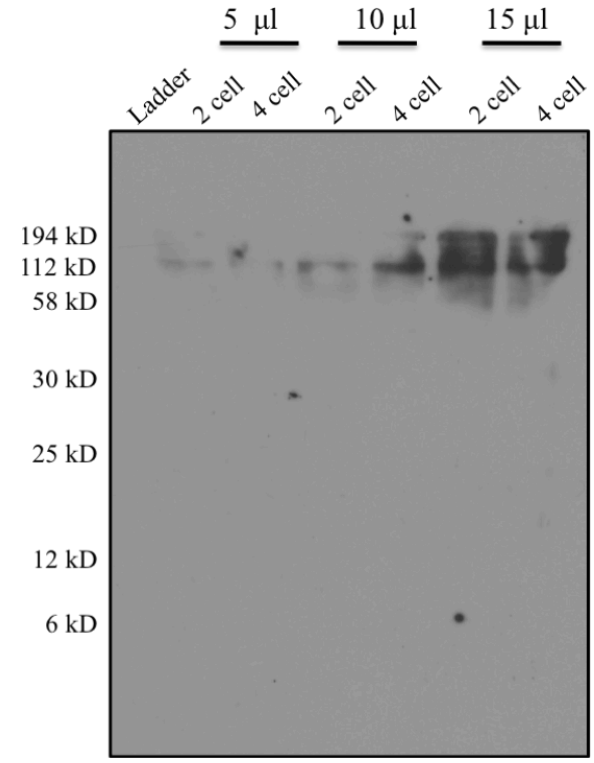
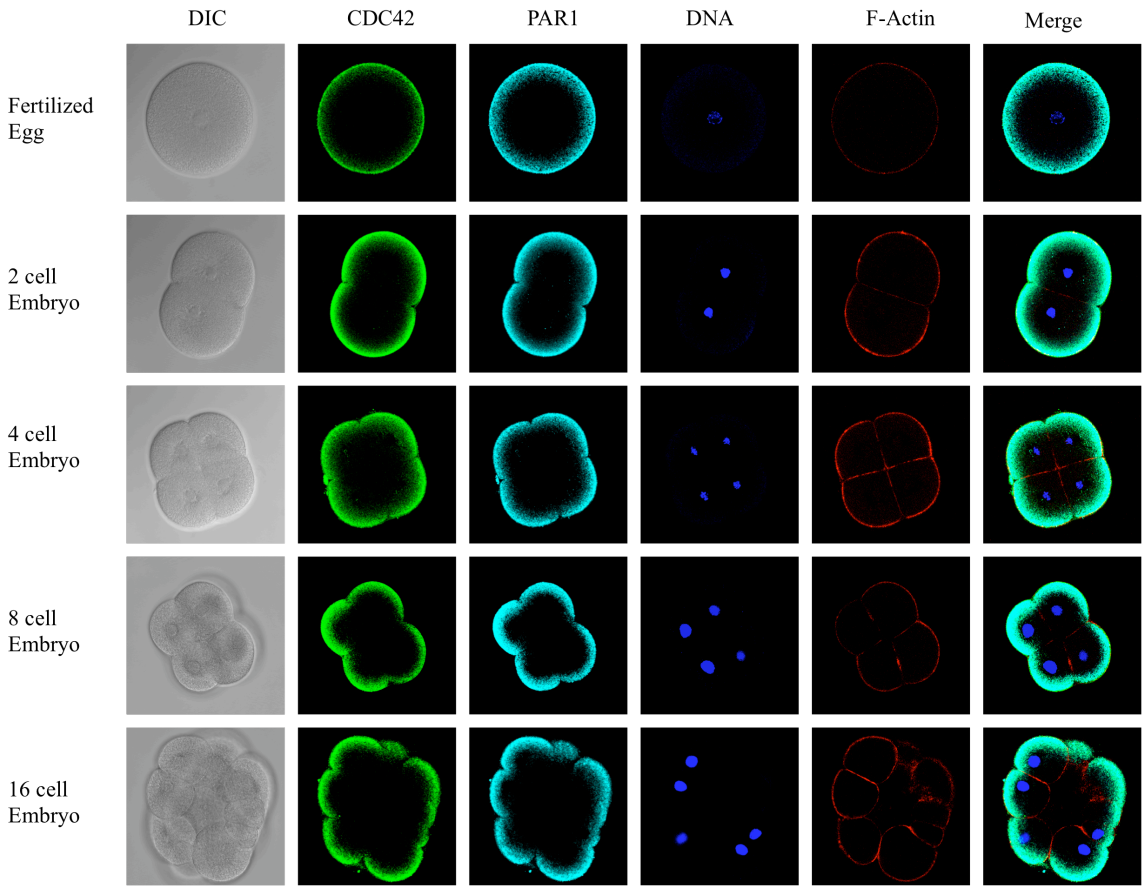

5 µl
10 µl
15 µl
Ladder
2 cell
4 cell
2 cell
4 cell
2 cell
4 cell
194 kD
112 kD
58 kD
30 kD
25 kD
12 kD
6 kD

Figure 3.2.1. PAR1 antibody design and verification. (A) The GLEAN prediction Sp-Mark3 (PAR1) amino acid sequence of *S. purpuratus.* The antigen sequence is highlighted. (B)Slot blot analysis of the bleeds of two rabbits from days 0, 28, 56, and 70/72 post inoculation with the target peptide used for the PAR antibody generation. The target peptide was spotted onto nitrocellulose membrane and probed with the different bleeds. Bleeds were tested at three different dilutions: 1:50, 1:100, and 1:500. The bleed from rabbit 1 on day 70/72 was found to have the strongest reactivity and were chosen for antibody purification (boxed). (C) Crude serum analysis of the bleed from rabbit 1 on day 70/72. Cell extracts isolated from 2 cell and 4 cell stage *L. pictus* embryos were run on a 12% SDS-PAGE gel and probed with a 1:1000 dilution of the crude serum of the bleed from rabbit 1 on day 70/72. Strong cross-reactivity was observed at 81.5kD, the predicted molecular weight of the PAR1 protein. (D)Western blot of *L. pictus* extracts with the purified polycloncal rabbit anti-*S. purpuratus* PAR1 antibody. Cell extracts isolated from 2 cell and 4 cell stage *L. pictus* embryos were run on a 12% SDS-PAGE gel and probed with a 1:10,000 dilution of the purified polycloncal rabbit anti-*S. purpuratus* PAR1 antibody. Western blot shows a single band at the correct molecular weight of 81.5 kD and additionally identifies PAR1 dimers at 163 kD.

A.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CDC42
PAR1
DNA
F-Actin
Merge
Fertilized Egg
2 cell Embryo
4 cell Embryo
8 cell Embryo
16 cell Embryo

B.

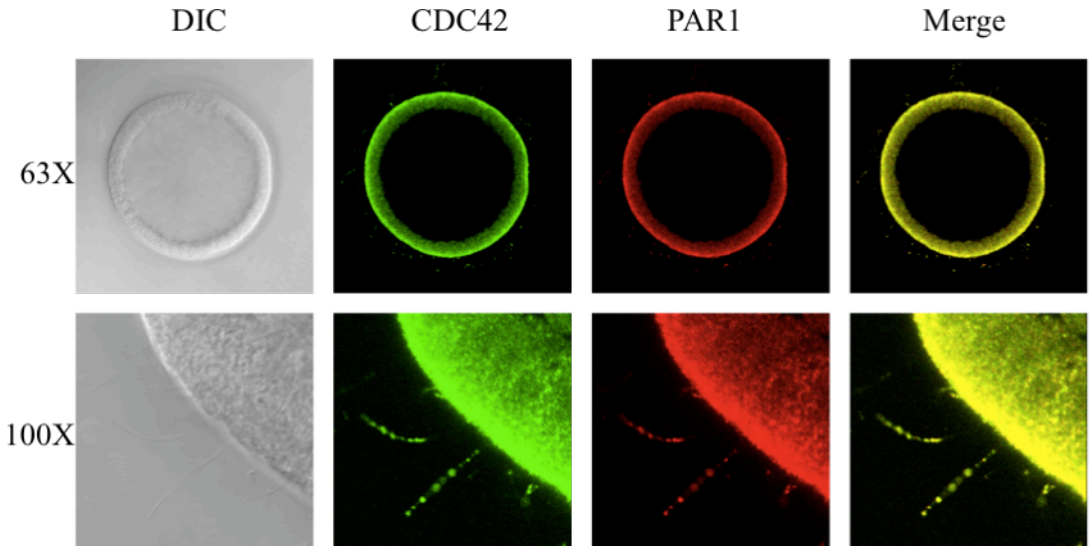
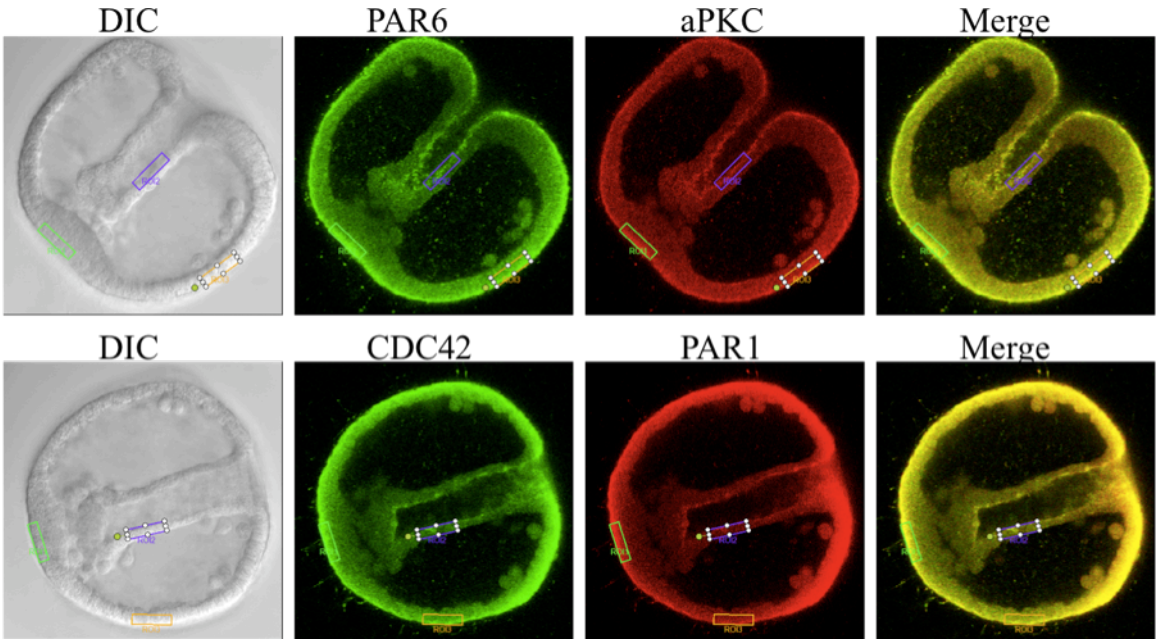
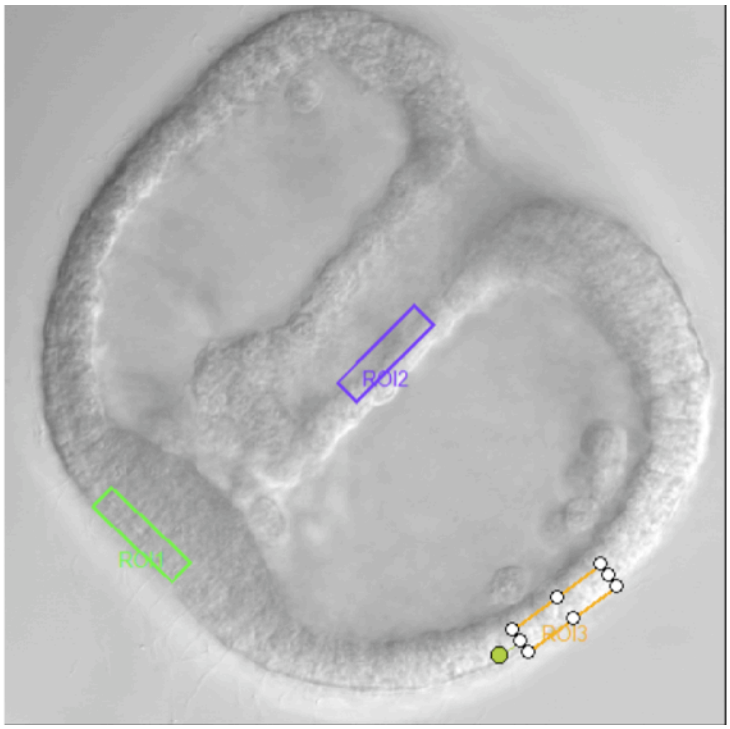

DIC
CDC42
PAR1
Merge
63X
100X

Figure 3.2.2. PAR1 colocalizes with the PAR complex marker CDC42 at the apical cortex through the blastula stage. *L. pictus* embryos were fixed and then stained for CDC42 and PAR1. Cleavage stage embryos (A) were additionally stained with phalloidin to label F-actin and Hoescht to label DNA. PAR6, aPKC, and CDC42 maintain the apical localization pattern initially seen at the 2 cell stage through the 16 cell stage (Figure 2.2.1.). At these early cleavage divisions PAR1 also colocalizes with these known apical markers(A). This unique colocalization at the apical surface is further retained at the blastula stage after an epithelium has formed and junctions are present (B). Expression of PAR1 is additionally found in the cilia at the blastula stage, similar to PAR6, aPKC, and CDC42 (B). For scaling reference *L. pictus* embryos are approximately 120 μm in diameter.

A.



B.



ROI 1: Apical Tuft
ROI 2: Archenteron
ROI 3: Epithelium

C.

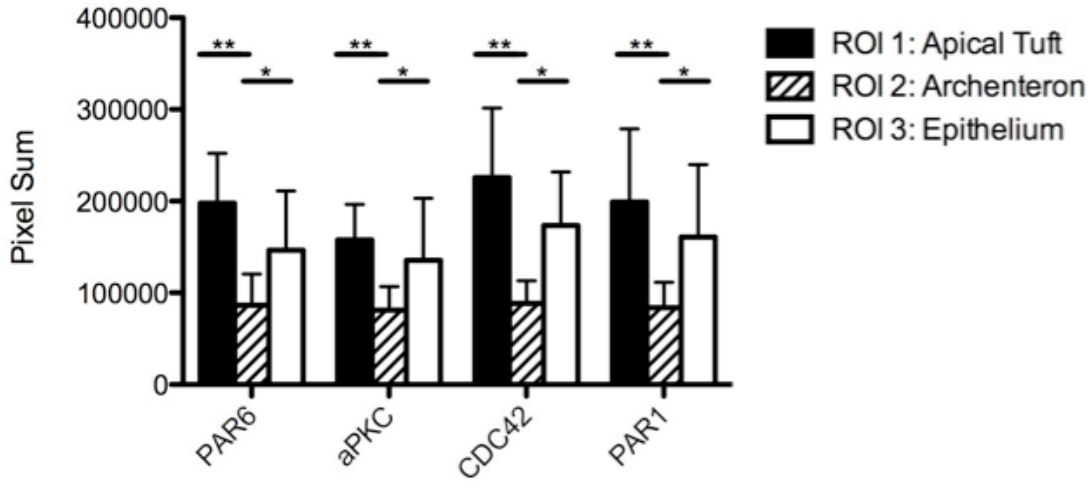
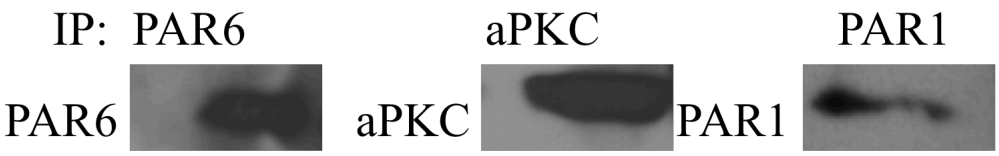


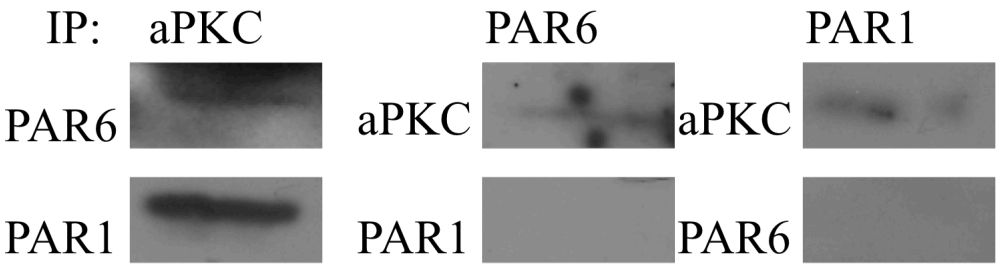
Figure 3.2.3. The PAR complex along with PAR1 remain enriched in the apical epithelium of the ectoderm and are downregulated in the archenteron at the gastrula stage. *L. pictus* embryos were raised to the gastrula stage and then fixed and stained for PAR6, aPKC, CDC42, and PAR1 (A). Fluorescence intensity of each protein was determined in three regions: the apical tuft, the archenteron, and the surrounding epithelium (B). These proteins were all found to have a significant decrease in expression in the archenteron (ROI 2) as compared to both the apical tuft (ROI 1) (**$p \leq 0.001$) and the surrounding epithelium (ROI 3) (*$p \leq 0.05$) (C). The fluorescence intensity (pixel sum in arbitrary units (AU)) of PAR6 was 197360.4±54422.1 in the apical tuft, 86450.5±34162.4 in the archenteron, and 146407.6±64839.5 in the epithelium (n=10 embryos, mean±SD). The fluorescence intensity of aPKC was 157470.9±38619.6 in the apical tuft, 81117.3±25727.6 in the archenteron, and 135381.9±67839.2 in the epithelium (n=10 embryos, mean±SD). The fluorescence intensity of CDC42 was

225433.3±75871.2 in the apical tuft, 88369.8±24944.3 in the archenteron, and 173483.6±58318.1 in the epithelium (n=10 embryos, mean±SD). The fluorescence intensity of PAR1 was 198961.5±79431.1 in the apical tuft, 83908.9±27518.6 in the archenteron, and 160788.5±78914.1 in the epithelium (n=10 embryos, mean±SD). For scaling reference *L. pictus* embryos are approximately 120 μm in diameter.

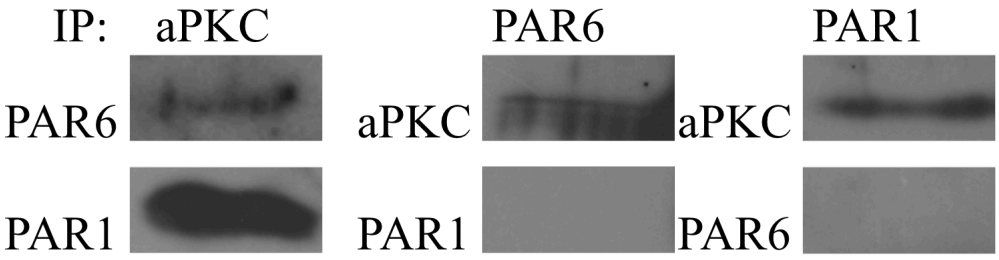
A. Controls



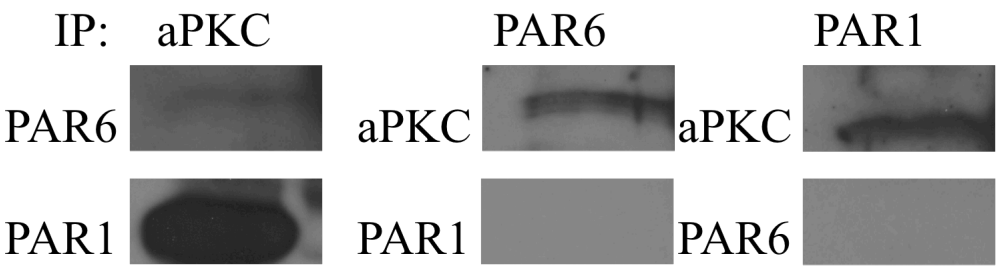
B. 2 Cell Stage Embryos



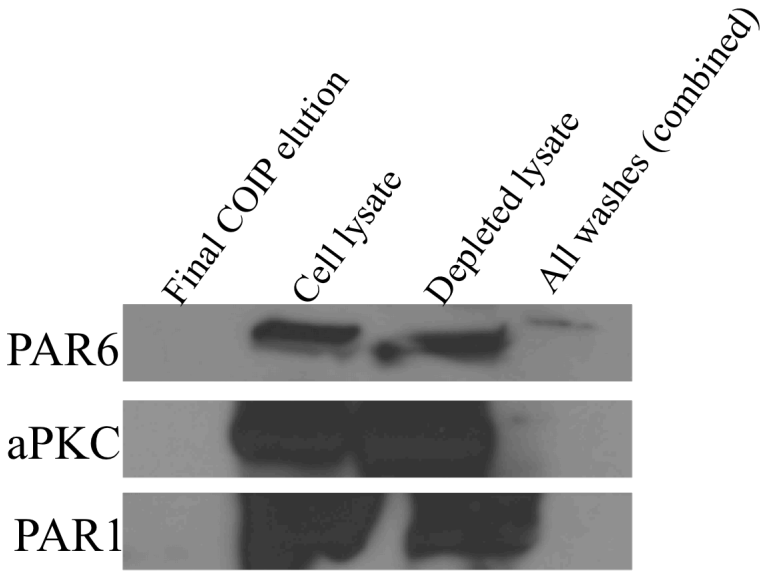
C. 16 Cell Stage Embryos



D. Blastula Stage Embryos



E.



F.

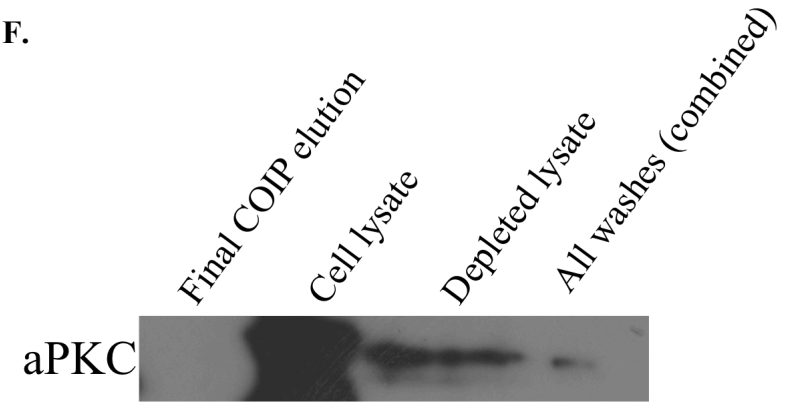
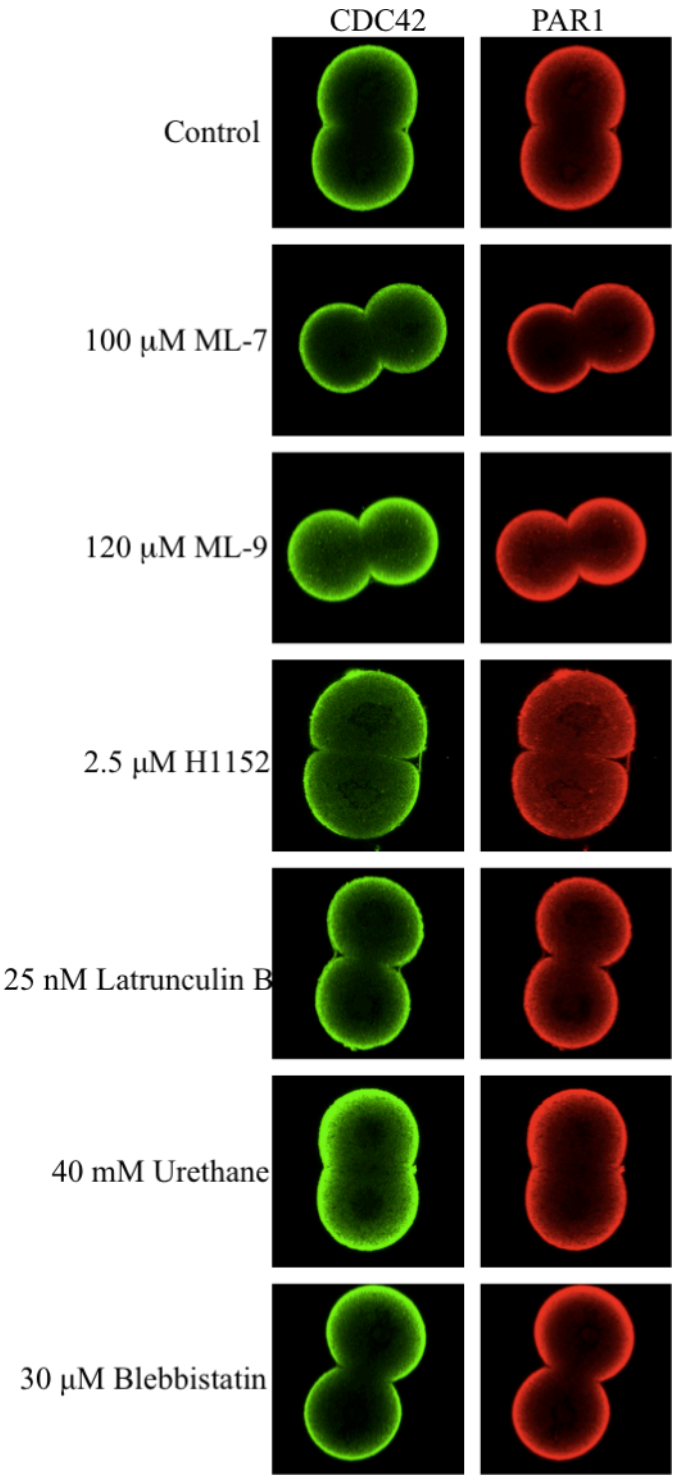


Figure 3.2.4. aPKC is in complex with PAR6 and PAR1 at the 2 cell, 16 cell and blastula stages of development. *S. purpuratus* embryos were grown to the stages indicated: 2 cell (B), 16 cell(C), and Blastula (D). Embryos were then lysed and a co-immunoprecipitation assay was performed using the Dynabeads kit (Life Technologies) with Dynabeads that had been coupled to either PAR6, aPKC, or PAR1 antibodies as specified (IP). Western blots were run and samples were probed for PAR6 (1:200), aPKC (1:200), and PAR1 (1:10,000) as specified. PAR6, aPKC, and PAR1 were found

at the correct molecular weights of 43 kD, 80 kD, and 81.5 kD, respectively. aPKC was found to be in complex with both PAR6 and PAR1 at the 2 cell, 16 cell, and blastula stages of development. However, PAR6 and PAR1 did not associate with each other at any of the examined stages. (E) *S. purpuratus* embryos were grown to the 2 cell stage and then lysed. A co-immunoprecipitation assay was performed using the Dynabeads kit with Dynabeads beads that had not been coupled to an antibody. Final co-immunoprecipitation elution, 2 cell stage *S. purpuratus* cell lysate, depleted 2 cell stage *S. purpuratus* cell lysate (after incubation with Dynabeads), and co-immunoprecipitation assay washes were run on a 12% SDS-PAGE gel. Western blots were run and samples were probed for PAR6 (1:200), aPKC (1:200), and PAR1 (1:10,000) as specified. The Dynabeads were found to not interact with PAR6, aPKC, or PAR1. (F) A co-immunoprecipitation assay was performed using the Dynabeads kit that utilized buffer only instead of lysed embryos as the starting sample. Final co-immunoprecipitation elution, 2 cell stage *S. purpuratus* cell lysate, depleted 2 cell stage *S. purpuratus* cell lysate (after incubation with Dynabeads), and co-immunoprecipitation assay washes were run on a 12% SDS-PAGE gel. Western blots were run and samples were probed for aPKC (1:200). Non-specific interactions were not observed.

A.


CDC42
PAR1
Control
100 µM ML-7
120 µM ML-9
2.5 µM H1152
25 nM Latrunculin B
40 mM Urethane
30 µM Blebbistatin

B.

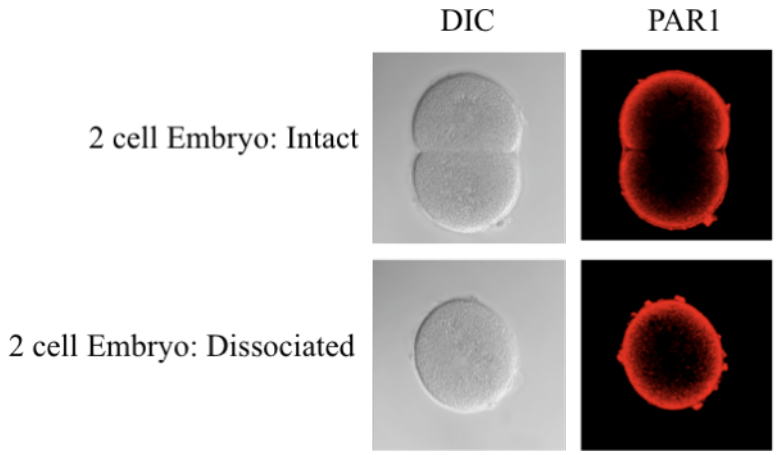
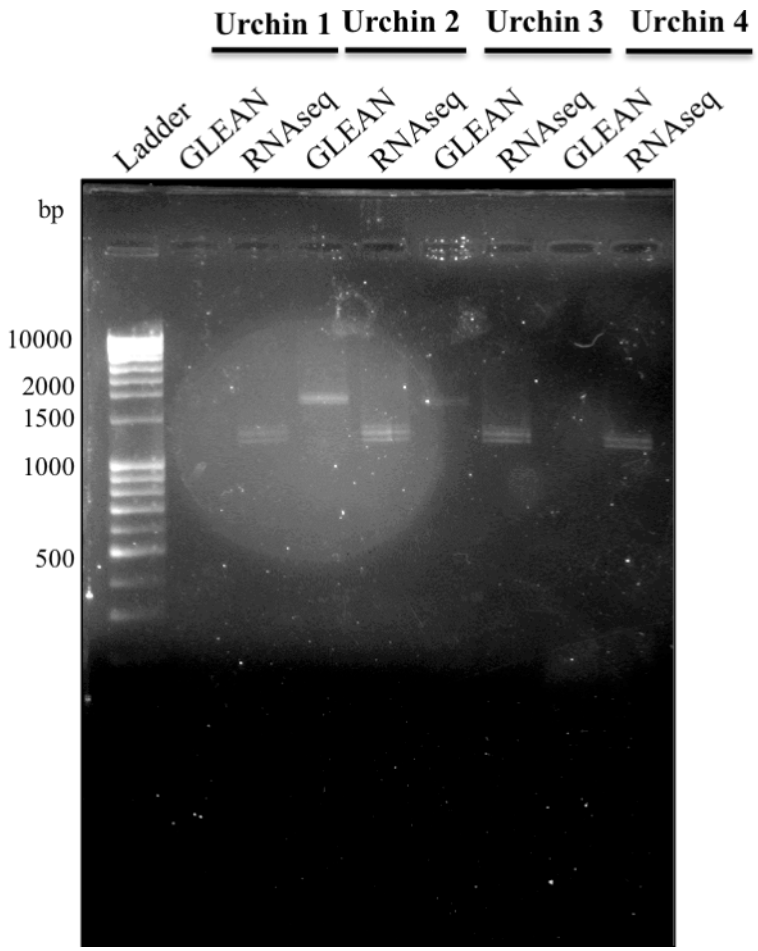
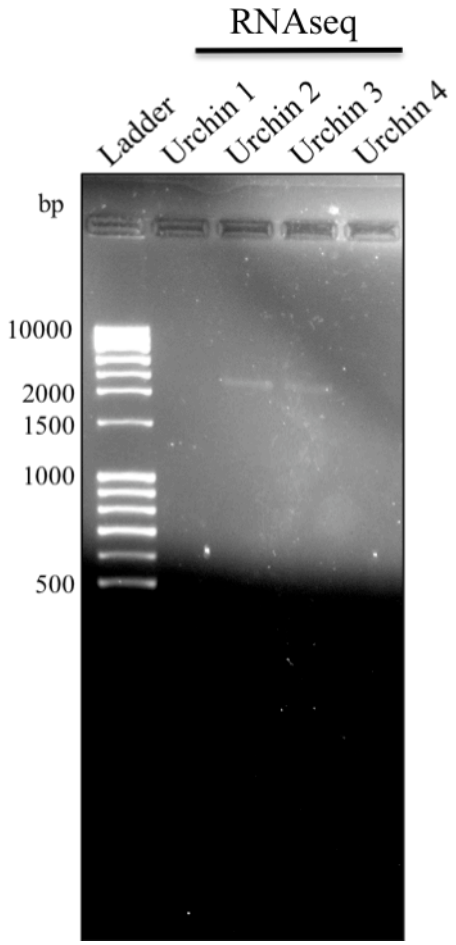


Figure 3.2.5. Calcium mediated cell adhesion is required for apical localization of PAR1, but the cytoskeletal regulation of PAR1 localization is unclear. (A) *L. pictus* embryos were fixed at the 2 cell stage after treatment with the various inhibitors for 15 minutes. Fixed embryos were stained for CDC42 and PAR1. CDC42 and PAR1 are apically localized in the cortex of the sea urchin embryo at the 2 cell stage of development. Previously, inhibition of MLCK by ML-7 or ML-9 resulted in cytoplasmic pooling of CDC42. However, *L. pictus* embryos appear to no longer respond to these inhibitors and CDC42 along with PAR1 remain cortical. Treatment with a general Rho kinase inhibitor, H1152, an actin polymerization inhibitor, latrunculin B, an astral microtubule inhibitor, urethane, or a myosin ATPase inhibitor, blebbistatin still did not effect the cortical localization of CDC42 or PAR1. These inhibitors were effective in *L. pictus* embryos. (B) *L. pictus* embryos were fixed and stained for PAR1 in both intact and dissociated 2 cell embryos. PAR1 had a uniform distribution in the cortex upon dissociation. For scaling reference *L. pictus* embryos are approximately 120 μm in diameter.

A.


Urchin 1
Urchin 2
Urchin 3
Urchin 4
Ladder
GLEAN
RNAseq
GLEAN
RNAseq
GLEAN
RNAseq
GLEAN
RNAseq
bp
10000
2000
1500
1000
500

B.


RNAseq
Ladder
Urchin 1
Urchin 2
Urchin 3
Urchin 4
bp
10000
2000
1500
1000
500

C.

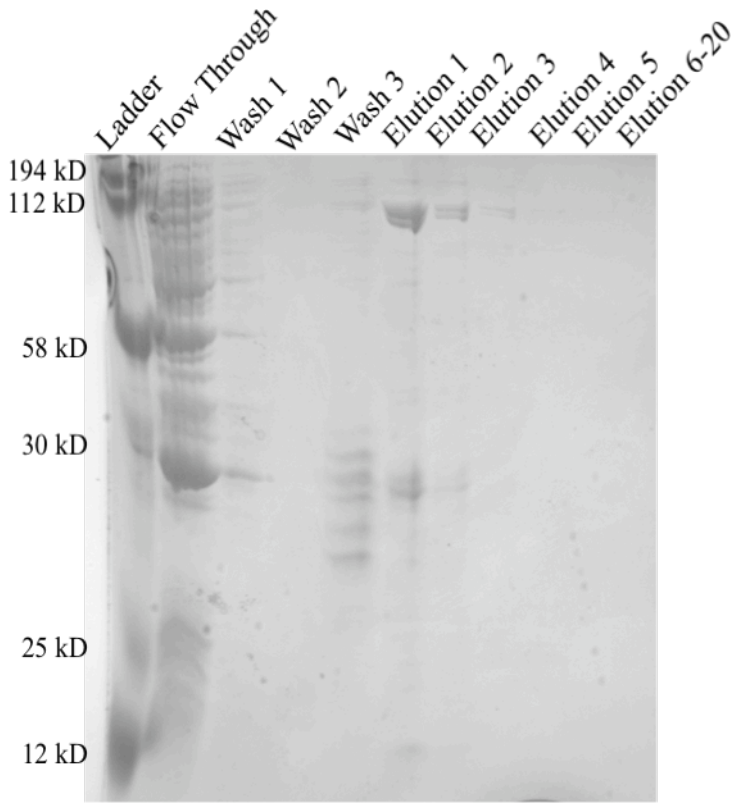


Figure 3.2.6. DNPAR1 and PAR1 construct development. Total RNA was isolated from four different batches of *S. purpuratus* zygotes and cDNA sequences were amplified using the ProtoScript M-MuLV *Taq* RT-PCR kit (NEB). Primers were designed based on the GLEAN prediction of *S. purpuratus* PAR1 or RNAseq data for the amplification of DNPAR1 (A) or the RNAseq data alone for the amplification of PAR1 (B) (Cameron et al., 2009). Samples were run on a 2% gel for 1 hour. Positive PCR reactions were observed around 1266 bp for DNPAR1 using RNA isolated from each of the four different batches of *S. purpuratus* embryos and the RNAseq generated primers(A). Positive PCR reactions were observed around 2190 bp for PAR1 using the RNA isolated from two of the different batches of *S. purpuratus* embryos and the RNAseq generated primers(B). (C) DNPAR1 was then cloned into the bacterial expression vector pEXP5-

CT (Life Technologies). The BL21-D3pLys strain of *Escherichia coli* cells were then transformed with the pEXP5-CT-DNPAR1 plasmid and expression was induced with 1mM IPTG overnight at 30°C. Samples were run through a NiNT3 columns and then run on a 12% SDS-PAGE gel and stained with Coomasie. Isolated DNPAR1 dimers are observed in the first three elutions.

Chapter 4. How to be at the Right Place at the Right Time: The Importance of Spindle Positioning in Embryos, a Review

4.1. Introduction

The mitotic spindle is an essential cellular apparatus that is required for cleavage plane determination and faithful separation of the chromosomes during mitosis. It is a microtubule-based structure that must be properly positioned within a cell for successful embryogenesis and survival of the daughter cells. While spindle positioning has been extensively studied in adult tissues, oocyte formation and unicellular organisms, it also plays a vital role during cell morphogenesis and the development of embryos. During the development of multi-cellular organisms the coordination of the mitotic spindle and subsequent cleavage plane is imperative for differing developmental outcomes. Asymmetric and symmetric divisions are well known regulators of morphogenesis. Localization of a spindle to one end of developing embryo can result in the segregation of differing developmental determinants within the cortex and the cytoplasm of a cell following division. Without precise positioning of the spindle, the formation of different cell and tissue types within an embryo would not be possible.

Spindle positioning is a fundamental cellular process that has intrigued scientists for well over 100 years. Oskar Hertwig first utilized echinoderm and frog eggs to assess the role of the mitotic spindle in positioning the cleavage plane. He created what would become known as "Hertwig's rules" (Hertwig, 1884). His rules, as concluded by Wilson, stated that "1) The typical position of the nucleus (and hence the mitotic figure) tends towards the center of the its sphere of influence, i.e., of the protoplasmic mass in which it lies. 2) The axis of the spindle typically lies in the longest axis of the protoplasmic mass,

and division therefore tends to cut this axis transversely." (Wilson, 1924) This idea that cells divided perpendicular to their longest axis seemingly worked well to explain the division plane of symmetrically dividing cleavage stage echinoderm embryos. Experiments in the early 1900s further demonstrated that there was a clear correlation between the division plane and cell fate specification in ascidian embryos (Conklin, 1905). Later work by Ray Rappaport further expanded the experiments of Hertwig (Rappaport, 1996). Rapport performed experiments that changed the shape of the normally spherical echinoderm embryo into various shapes including cigars, ice cream cones or dumbbells. Through these experiments he found that that although most divisions followed Hertwig's rules, there are some instances of unusual sites of furrow formation. In his famous Torus experiments, Rapport created a donut-shaped embryo by pressing in a glass ball and observed that a normal furrow that bisected the spindle formed, but a second additional furrow ingressed at one pole where an aster intersected. These would later become known as Rappaport furrows. Rappaport also classically demonstrated that there existed a positive cue that emanated from the astral microtubules which stimulated contractile ring formation for cytokinesis (Rappaport, 1996). Astral microtubules are now known to be key players in the determination of spindle positioning as they interact with the cell cortex and generate pulling forces against the cortex and the cytoplasm (For review see McNally, 2013). Additionally, the minus end directed motor protein, dynein, is thought to exert the pulling forces necessary for spindle movement. However, there remains some debate about which forces, pushing or pulling, are required for spindle movement (For review see McNally, 2013).

These early experiments examined and helped to explain a very complex cellular process. Since their discoveries, cellular and developmental biologists have now built upon these findings to explore the molecular machines that are responsible for these processes. In this review I highlight the most current work on the important role that spindle positioning plays in developing embryos. Specifically I will examine the molecular mechanisms of both polarized and nonpolarized divisions in the diverse array of model organisms in which spindle positioning has been studied, including: *Caenorhabditis elegans, Drosophila melanogaster,* sea urchin embryos, ascidians embryos, and *Xenopus laevis*. Each model system presents its own advantages that have allowed for biologists to dissect the many pieces that must be coordinated to position the spindle at the right place and at the right time. Finally I will discuss the future directions of this field and the emerging importance of computer-aided mathematical modeling to our understanding of this intricate process.

4.2. The *C. elegans* Embryo: A model for polarity dependent spindle positioning

One of the most exploited model systems utilized to study spindle positioning is the *C. elegans* embryo. With the many genetic tools that are available for this organism, the *C. elegans* embryo is an ideal system that has allowed the identification of the molecular components necessary for proper spindle positioning. Furthermore, the optical clarity of these embryos has readily allowed for the imaging of intricate cellular processes like spindle positioning. Additionally, as this embryo is a well-established model of polarized cell divisions, research into the *C. elegans* embryo has determined how cell polarity factors influence spindle positioning.

The first division following fertilization in the *C. elegans* embryo is asymmetric. The spindle initially assembles in the center of the embryo, moving towards the posterior end of the embryo during metaphase and early anaphase. Genetic screens have revealed that a ternary complex consisting of two Gα subunits (GOA-1 and GPA-16), two TPR/GoLoco-domain proteins (GPR-1 and GPR-2), and the large coiled-coil protein LIN-5 plays a substantial role in spindle positioning in this embryo (Figure 4.2.1.) (Lorson et al., 2000;Gotta and Ahringer, 2001;Srinivasan et al., 2003; for review see Kotak and Gönczy, 2013). Depletion studies of each of these proteins demonstrated that they are involved in pulling forces of the astral microtubules and without their function the spindle remains centered and the first cell division becomes symmetric (Lorson et al., 2000; Gotta and Ahringer, 2001; Colombo et al., 2003). These proteins are also known to regulate dynein and become concentrated at the posterior end during metaphase and early anaphase; it is the higher posterior concentration of the ternary complex that pulls the spindle to the posterior end (Grill et al., 2001; Grill et al., 2003; Park and Rose, 2008). Dynein activity has been proposed to be most critical during early prophase for proper spindle positioning (Figure 4.2.1.) (Gusnowski and Srayko, 2011). GPR-1 is also known to sense and respond to the mechanical properties of the cortex, which may be important for its role in spindle positioning (Bringmann, 2012). Laser severing experiments have additionally shown that the pulling forces in the posterior end of the cell are stronger than those found in the anterior and this results in the spindle displacement (Grill et al., 2001; for review see Gillies and Cabernard, 2011). Other studies demonstrated the importance of the CLASP proteins, which are microtubule-associated proteins that are necessary for the astral microtubules to be able to reach the

cell cortex at the right time during spindle positioning (Espiritu et al., 2012). Thus data from the *C. elegans* embryo overwhelmingly favors a role for pulling forces in generating the asymmetric spindle position during their first division.

In addition to the ternary complex, the PAR proteins play a well-known role in the establishment of the anterior-posterior axis and subsequent asymmetric division in the *C. elegans* embryo. The PAR proteins were initially discovered in these embryos based on a genetic screen that identified these proteins based on their involvement in the regulation of the first division (Kemphues et al., 1988; Morton et al., 1992; Levitan, 1994; Guo and Kemphues, 1995; Etemad-Moghadam et al., 1995; Watts et al., 1996; Tabuse et al., 1998; for review see McCaffrey and Macara, 2009; McCaffrey and Macara, 2012). Following fertilization PAR-3 and PAR-6 become enriched at the anterior cortex, while PAR-1 and PAR-2 localize to the posterior cortex (Figure 4.2.1.) (Kemphues, 2000). Mutual exclusion of proteins from each domain ensures proper segregation of the PAR proteins and is used to maintain the polarized distribution of these proteins (Cuenca et al., 2003; Boyd et al., 1996; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Tabuse et al., 1998; Watts et al., 1996). Once the asymmetry of these proteins has been established, the PAR proteins help to coordinate the localization of the mitotic spindle and subsequent asymmetric division (Ahringer, 2003; Galli et al., 2011; Hao et al., 2010). The atypical protein kinase C, PKC-3, associates with the scaffolding proteins PAR-6 and PAR-3 in the PAR complex and is now known to negatively regulate LIN-5 by phosphorylation (Galli et al., 2011; Joberty et al., 2000; Lin et al., 2000). Additionally, the PAR proteins regulate the cortical localization of the GPR-1/2 to the posterior of the zygote during anaphase through their interactions with casein kinase I

and PI(4)P5-kinase (Panbianco et al., 2008). The asymmetry that is established by the PAR proteins ensures that developmental determinants are properly positioned following the first cleavage in this embryo. The importance of the segregation of developmental determinants is demonstrated by the zinc-finger proteins MEX-5/MEX-6, which are partitioned to the anterior cell. The segregation of MEX-5/MEX-6 is needed for germline development (Schubert et al., 2000). Since their initial discovery in the *C. elegans* embryo the PAR proteins have been shown to be involved in the regulation of many other divisions in higher order eukaryotes (Hao et al., 2010; Lázaro-Diéguez et al., 2013; Slim et al., 2013; Dormoy et al., 2012; Durgan et al., 2011).

C. elegans embryos have thus proven to be a powerful tool for analyzing the proteins that control the localization of the spindle and the subsequent site of division. Work on these embryos also highlights the fact that cell polarity proteins can play a central role in spindle positioning that overcomes the simple geometrical ideas originally put forth by Hertwig.

4.3. The *Drosophila melanogaster* Embryo: A model for stem cell renewal

The *Drosophila* embryo has been an excellent model for studying spindle positioning in specific cell types. Like the *C. elegans* embryo, powerful genetic tools are available for use in *Drosophila* as a model. *Drosophila* embryos are an excellent model for the study of the mechanisms of spindle positioning in stem cells, especially in the study of neuroblast formation, as well as in early embryogenesis (Baena-López et al., 2005; da Silva and Vincent, 2007; for review see Gillies and Cabernard, 2011). Here, however, we will focus on the asymmetric divisions found in neuroblast cells.

Through asymmetric cell divisions neuroblasts are able to maintain one self-renewing, undifferentiated cell and one cell that will begin differentiating into a neuron based on the developmental determinants that are inherited following cell division. Numerous molecular components are required for this asymmetric division and this work has supplemented our understanding of stem cell generation in vertebrates (For review see Williams and Fuchs, 2013). *Drosophila* neuroblasts orient their spindles along an internal polarity axis, which sets up the division plane so that cell fate determinants such as Miranda, Numb, Brain tumor (Brat), and Prospero (Pros) are only segregated to the developing neuron and not into the self-renewing stem cell (Figure 4.2.1.) (Gillies and Cabernard, 2011; Doe, 2008; Cabernard and Doe, 2009). The spindle rotates 90 degrees prior to division only during the first neuroblast cell cycle; subsequent cycles rely on the attachment of the centrosome to the apical cortex in order to determine spindle orientation (Kaltschmidt et al., 1999; Rebollo et al., 2009; Rebollo et al., 2007; Rusan and Peifer, 2007). Recent data also suggests that there is an asymmetry between mother and daughter centrioles in interphase *Drosophila* neuroblasts and that the daughter centriole retains the pericentriolar material and organizes the aster that is required for asymmetric division (Januschke et al., 2013). Furthermore, these neuroblasts must also remain associated with neuroepithelial cells in order to maintain their division axis as dissociation resulted in random division (Siegrist and Doe, 2006). Thus, in neuroblasts there are cellular cues (centrosomes or centriole) that are the primary control of spindle positioning that override simple cell geometry as the mechanism of controlling spindle positioning and thus the cell division plane.

The molecular machinery that is required for these divisions has been investigated using various mutants. Mushroom body defect (*mud*), an ortholog of the vertebrate nuclear mitotic apparatus (NuMa), is necessary for the asymmetric division of neuroblasts; *mud* mutants divide symmetrically as a result of a misaligned spindle to produce two neuroblast cells instead of a neural progenitor and a differentiating neuron (Cabernard and Doe, 2009). Mud colocalizes with Pins (LGN ortholog) in the apical cortex through metaphase, and becomes symmetrically distributed to both ends at anaphase (Figure 4.2.1.) (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). It has been proposed that the Pins/Mud/GαI complex interacts with dynein; however, a physical interaction between dynein and Mud has yet to be demonstrated (Figure 4.2.1.) (Gillies and Cabernard, 2011). Mud interaction with molecular motors may then generate pulling forces on the mitotic spindle; however, a direct role for these pulling forces has yet to be established. Genetic examination of two dynein associated proteins, Lis1 and Glued, have resulted in similar spindle defect phenotypes as *mud* mutants (Siller and Doe, 2008). Another microtubule motor, kinesin Khc73, has been shown to connect Dlg (Discs large), a PDZ protein, with Pins and act a linker between the astral microtubules and Pins (Siegrist and Doe, 2005; Johnston et al., 2009). In this Mud independent pathway, Pins recruits Dlg to the cortex and Dlg then anchors microtubules in the cortex via Khc73 in order to position the spindle (Gillies and Cabernard, 2011). While Pins/Mud/GαI appears to play a role in microtubule pulling, this Pins/Dlg/Khc-73 pathway is more actively involved in microtubule anchoring (Morin and Bellaīche, 2011). These differing microtubule-based forces must both contribute to the asymmetric

spindle positioning that generates the progenitor and daughter cells and may suggest that there are multiple forces that actively position the spindle.

Similar to the *C. elegans* embryo, *Drosophila* neuroblasts also require the PAR complex to regulate their asymmetric division (For review see Nance and Zallen, 2011). In these cells Par3 (Bazooka), Par6, and aPKC localize to the apical cortex from late interphase onward. Par3 recruits Insc (Inscuteable) to the apical cortex, which then interacts with Pins/GαI. Analysis of Pins and GαI mutants have shown that these interactions are necessary for mitotic spindle orientation and for the apical localization of Par3, aPKC, and Incs (Figure 4.2.1.) (For review see Morin and Bellaīche, 2011). The association of the PAR proteins with stem cell divisions has been evolutionarily conserved as recent data suggests aPKC is involved in the division orientation of the self-renewing epithelium in mice (Niessen et al., 2013).

The *Drosophila* neuroblasts have thus proven to be an excellent model for studying the process of stem cell regeneration and differentiation. It is from this model that that we have learned how spindle components, molecular motors, and polarity factors work together to accurately orient the mitotic spindle and following division produce two distinct cell types: a progenitor cell and a differentiating cell. Further work will establish whether pulling forces and the location of these forces, play a key role in spindle orientation in this system.

4.4. The Sea Urchin Embryo: A model for studying cell shape

The sea urchin embryo has long been utilized as a model for cellular and developmental biology (For review see Ernst, 2011). Their gametes are easy to obtain

and some species have large, optically clear embryos that are perfectly suited for live cell imaging. Additionally, although they are not a genetic model system like *Drosophila* and *C. elegans*, the genome of *Stronglyocentrotus purpuratus* has been sequenced and thus molecular components involved in spindle positioning can be identified (Cameron et al., 2009; Sodergren et al., 2006). Following fertilization, the first few divisions of the sea urchin embryo are symmetric; the first asymmetric division does not occur until the 16 cell stage (Schroeder, 1987). While cell polarity has now been shown to emerge following the first cleavage in the sea urchin embryo, the first division remains unpolarized and thus can be studied to determine how spindles become oriented without the influence of polarity cues (Figure 4.4.1.) (Ng et al., 2005; Alford et al., 2009). Classic experiments by Driesch revealed that the blastomeres of these embryo can be separated up until the 4 cell stage and produce viable adults; demonstrating that developmental determinants are still symmetrically distributed during the first two divisions (Driesch, 1892).

The symmetric division of the first cleavage in the sea urchin embryo is a well-suited model for the study of the influence of cell geometry on spindle positioning. The role of mitotic shape and an apparent requirement for cell rounding prior to division has been garnering attention as mechanism in which to properly construct the spindle and ensure proper segregation of both the chromosomes and cellular components (For review see Cadart et al., 2014). Pioneered as an experimental system by Hertwig and later by Rappaport, sea urchin embryos can be easily manipulated and forced into different shapes in order examine how spatial cues influence the mitotic spindle (Hertwig, 1884; Rappaport, 1996). Further work, modeled after Rappaport's experiments, has shown that

both membrane curvature and fluid membrane movements also play a role in the positioning of the cleavage furrow (Yoshigaki, 2001; Yoshigaki, 2002; Yoshigaki, 2003). Recent experiments using microfabricated molds have further explored the original findings of Hertwig (Figure 4.4.1.) (Minc et al., 2011). The ability to replicate these experiments and produce large data sets using embryos fit into molds allowed for critical analysis of Hertwig's rules. Although the site of division in the sea urchin embryos followed Hertwig's rules in most shapes examined, there were a few that "broke" his rules. An example can be seen in embryos that were placed in the rectangular molds and subsequently divided along instead of perpendicular to the longest axis of symmetry. This suggests that there may be more complexity to determining the site of furrowing than simply placing the division site perpendicular to the longest axis. The embryos appeared to set their division axis in early prophase before nuclear envelope breakdown when long microtubules emanated from the duplicated centrosomes that exert balanced pulling forces on the nucleus (Minc et al., 2011). Modeling was then used to determine that these microtubules sense the geometric space and exert pulling forces that are scaled to the cube of their length. Shape sensing was proposed to be due to the longest microtubules generating the greatest pulling forces. These pulling forces were likely generated by dynein that is found on particles dispersed in the cytoplasm rather than on the cell cortex (Minc et al., 2012). Thus multiple molecular motors in different locations may provide the force that is required to pull on the spindle. However, these ideas have yet to be formally tested. These studies did provide new insight into how a spindle is positioned in a non-adherent embryonic cell that does not have traditional polarity cues.

Other work has investigated the molecular components involved in spindle positioning in the sea urchin embryo. From Rappaport's famous Torus experiments, we know that sea urchin zygotes can be induced to form an artificial secondary "Rappaport" furrow (Rappaport, 1996). From these types of experiments, cues emanating from the spindle can be separated from other microtubule populations, such as astral microtubules, in order to determine which are required for furrow formation and positioning (Rappaport, 1996). Centralspindlin and the chromosomal passenger complex (CPC) organize the central spindle and are both involved in furrow positioning; recently their roles in Rappaport furrows were examined (Argiros et al., 2012). While in mononucleate zygotes mitotic-kinesin-like-protein1 (MKLP1), a member of the Centralspindlin complex, and Survivin, a member of the CPC, were localized to the central spindle upon anaphase onset and subsequently spread towards the equatorial cortex, neither protein was detected on the secondary Rappaport furrows in binucleate cells unless chromosomes were abnormally localized between opposing astral arrays (Argiros et al., 2012). These data show that in mononucleate zygotes components of the central spindle are localized to the tips of astral microtubules concurrent with furrow initiation and are thus are the right place at the right time to be part of the signaling pathway that specifies furrow position. Despite the fact that MKLP1 and Survivin were not detected on secondary furrows, inhibition of another component of the CPC, aurora kinase, by the inhibitor VX-680 revealed that CPC signaling was required for Rappaport furrows to form (Argiros et al., 2012). Collectively, these data demonstrate that components of the central spindle are required for furrow induction and bridge the gap between our understanding of the

central spindle furrow specification model and Rappaport's classic cleavage plane specification model.

The use of the sea urchin embryo has made important contributions to the field of spindle positioning. It is from these embryos that the influence of cell shape and the ability to predict division orientations has been established. Work on these embryos has also help to resolve the complex relationship between the spindle and the subsequent cleavage furrow that forms to divide and separate the daughter blastomeres.

4.5. Ascidian Embryos: A model for chordate spindle positioning

Because of their evolutionary position between invertebrate deuterostomes, like sea urchins, and vertebrates, like *Xenopus*, and the fact that their cleavage divisions are stereotypically invariant, ascidians are a useful developmental model for studying the conserved processes between these different lineages. Like *C. elegans* and sea urchin embryos, some ascidian embryos are transparent, which makes them optimal for imaging. Furthermore, they undergo rapid embryogenesis, have well-documented cell lineages, have invariant cleavages, and have a relatively simple body plan. The genome of *Ciona intestinalis* was also sequenced in 2002, which has allowed for whole-genome analysis (Dehal et al., 2002; Satoh et al., 2003).

While most of the work on spindle positioning in embryos has focused on more established model organisms, analysis of this process in basal chordates has only begun to be explored. The first asymmetric division in ascidians occurs at the 8 cell stage in the posterior blastomeres. This division begins to separate the muscle cell fate and germline precursors (Nishida, 2002; Nishida, 2005). A cortical structure called the centrosome

attracting body (CAB) is found in the posterior blastomeres at the 8 cell stage and regulates this asymmetric division (Hibino et al., 1998; Nishikata et al., 1999). Recent data suggest that the cortical accumulation of the PAR complex in the CAB is required for the asymmetric division at the 8 cell stage (Patalano et al., 2006). These researchers find that aPKC localization to the CAB is actin-dependent and astral microtubules make contact with the aPKC cortical domain (Patalano et al., 2006). Other studies found that another protein, Posterior End Mark (PEM), also localizes to the CAB in the posterior blastomeres and is required for correct orientation of the cleavage plane. PEM appears to play a role in the anchoring of microtubules between the centrosome and the cortex (Negishi et al., 2007). A live 4D confocal study of microtubule dynamics during the asymmetric cell division at the 16 cell stage in *Phallusia mammillata* further demonstrated that one spindle pole of the mitotic spindle moves toward the CAB during prometaphase and it was this spindle placement that drove asymmetric division (Prodon et al., 2010). Collectively, these data highlight the utility of ascidian embryos to study the process of spindle positioning and a clear justification for their use as a model as the evolution of conserved processes between invertebrate and vertebrate lineages can be established using this model.

4.6. *Xenopus laevis* Embryos: A model for size scaling in spindle positioning and vertebrate development

Xenopus laevis embryos serve as an influential model of spindle positioning for higher vertebrates. Their eggs are easily obtained in the laboratory and can be microinjected for analysis of the roles of various molecular components. Additionally,

fixed embryos can be used for immunofluoresence assays, and cell-free extracts can be made form *Xenopus* eggs, which can be used for biochemical and physical manipulations. These eggs are also large in size (1200μm), cleave completely and have a fast cell cycle (20-30 minutes following the first division) (Mitchison et al., 2012).

Because of the large size of *Xenopus* eggs, they have been utilized to assess the role of size scaling and the spindle (Figure 4.6.1.). The regulation of aster size during the cell cycle has important implications for the spatial organization of the embryo (Mitchison et al., 2012). The spindle in early blastomeres of *Xenopus* embryos is centered, yet because of the large size of the blastomeres astral microtubules are unable to reach the cortex (Wühr et al., 2009; Wühr et al., 2010). In smaller embryos, the astral microtubules have contact with the cell cortex at prophase and metaphase and can thus position the spindle as the chromosomes align at the metaphase plate; however, it is not until anaphase onset that the asters are able to reach the cell cortex in *Xenopus* embryos (Figure 4.6.1.) (Wühr et al., 2010). In *Xenopus* embryos it was found that interphase asters preposition the centrosomes using dynein motors and the spindle is then positioned between these centrosomes. Dynein motors, most likely anchored to organelles, must then pull on sites in the bulk cytoplasm, rather than the cortex, to orient the centrosomes (Wühr et al., 2010). These data highlight that microtubule pulling forces can still be generated in embryos that are too large to reach cortical sites.

Additional studies have investigated the role of size scaling in *Xenopus* embryos by examining how spindle length and cytoplasmic volume are related with spindle length increasing with larger cytoplasmic volumes to a limit (Wühr et al., 2008; Hazel et al., 2013). During embryogenesis, *Xenopus* embryos are rapidly dividing without any

accompanying growth, decreasing the diameter of individual blastomeres 100-fold. The cellular components in these individual blastomeres must then adapt to these increasingly smaller volumes. While it was initially thought that spindle size would scale with cell size, experiments using *Xenopus* embryos at multiple stages of development and thus at multiple cell sizes, have shown that there is an upper limit of 60μm for the mitotic spindle. This was largest size the mitotic spindle would reach to in these embryos, which suggests that spindle length is both independent of cell length and determined by mechanisms intrinsic to the spindle (Wühr et al., 2008). Recent work then explored if it was a developmental program or cell size and shape that determined the spindle size during embryogenesis. Interestingly, by placing *Xenopus* extracts in engineered cell-like compartments of defined sizes, researchers were able to find that cytoplasmic volume regulated the size of the spindle (Good et al., 2013; Hazel et al., 2013). This scaling trend demonstrated that in a cell free system spindle size scaled with compartment volume rather than shape or developmental cues. Similar observations of size scaling and spindle alignment in large embryos have been made in zebrafish embryos, further confirming an evolutionary conserved mechanism of spindle positioning during vertebrate development (Wühr et al., 2008; Wühr et al., 2009; Wühr et al., 2010).

Spindle alignment is important in later development in *Xenopus* embryos. Polarized blastomeres of *Xenopus* embryos at the blastula stage have spindles that orient themselves based on the shape of the cells (Strauss et al., 2006). These polarized blastomeres are utilized to generate the inner and outer cells with different fates in the blastula. While cell shape was previously thought to be the default mechanism, isolated blastomeres from these embryos oriented randomly unless introduced into an

experimentally long axis early in the cell cycle. These experiments demonstrated the importance of cell shape in the generation of cell fate diversity (Strauss et al. 2006). The polarity of these blastomeres was regulated by aPKC, Crumbs3, and Lgl2 as has been shown in invertebrate embryos (Chalmers et al., 2005). Other studies have also shown that geometric constraints result in perpendicularly oriented divisions that are necessary for the formation of superficial, epithelial cells and non-epithelial deep cells (Chalmers et al., 2003).

Xenopus embryos have also been utilized to demonstrate that the symmetric cell divisions during epiboly are controlled by an apically directed cortical flow of F-actin and myosin-2 and a basally directed force that is generated by microtubules and myosin-10 (Woolner and Papalopulu, 2012). Myosin-10 and F-actin had already been shown to associate with the spindle and were additionally necessary for the regulation and maintenance of spindle length (Woolner et al., 2008). Similarly F-actin was shown to be necessary to maintain the central localization of the spindle in mouse zygotes (Chew et al., 2012) and has been garnering attention as another manner in which spindle positioning is regulated in a number of cell types (Bezanilla and Wadsworth, 2009). These studies highlight the roles of actin and myosin in spindle regulation as opposed to just the regulation of microtubule-based forces and continue the debate over pushing vs. pulling mechanisms of spindle movement.

Our knowledge of spindle positioning during both early and later embryogenesis has been greatly supplemented by experiments in *Xenopus* embryos and extracts. These embryos have demonstrated that the importance of regulating spindle positioning applies

to many stages of development that require different molecular components at different times of development.

4.7. The Mouse Oocyte: A unique mechanism for asymmetric spindle positioning

The mouse oocyte offers an unusual case of spindle positioning that is accomplished in a seemingly unique manner as it is independent of both polarity and geometric cues. Studies utilizing the mouse oocyte has illuminated the roles of F-actin and myosin-II in generating the forces required for asymmetric spindle positioning in a system that lacks both astral microtubules and centrosomes. The formation of mammalian oocytes is an imperative process to understand as it begins the segregation of developmental determinants prior to fertilization. There is much known on the positioning of the meiotic spindle in mouse oocytes (For review see Chaigne et al., 2012; Almonacid et al., 2014). Some have proposed that F-actin along with a myosin II motor may be pulling on the spindle towards the cortex (Shchuh and Ellenberg, 2008), while others have postulated that the F-actin cloud surrounding the chromosomes, may actually push the spindle towards the cortex (Li et al., 2008). Additional studies have shown that the asymmetric division in mouse oocytes that results in polar body formation also requires a soft cortex and a dynamic actin network (Schuh and Ellenberg, 2008; Azoury et al. 2008; Chaigne et al., 2013). Other work using live cell imaging has demonstrated that the actin network becomes dense during prophase I before undergoing remodeling and destabilization during meiosis. These dynamics appear to be the cue for symmetry breaking in the mouse oocyte (Azoury et al., 2011). The actin filament nucleator, Formin-2, organizes the actin network, while myosin activity regulates spindle movement

(Schuh and Ellenberg, 2008; Azoury et al., 2008). These data highlight the utilization of other cytoskeletal components, apart from microtubules, that can orient the meiotic spindle and ensure the proper segregation of chromosomes along with maternal storage RNAs and proteins into the oocyte.

4.8. Conclusions and Future Directions:

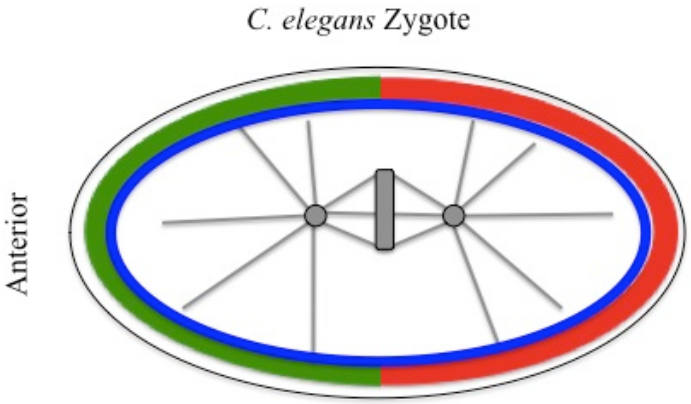
The work reviewed here has examined the importance of spindle positioning in a number of different developmental model organisms and how that positioning is accomplished. Genetic model organisms such as *C. elegans* and *Drosophila* embryos have been utilized to assess the roles of varying molecular components that influence spindle positioning. Studies in these organisms have identified roles for proteins in the ternary complex and the PAR complex, as well as molecular motors like kinesin and dynein and how they override simple cell geometry and the long axis rule to position spindles. Further experiments have found how the polarity inducing factors influence the placement of the spindle and the developmental impact that they have on processes such as embryogenesis and stem cell renewal. Experiments in ascidian embryos have further supplemented our knowledge of the conserved role the PAR proteins play in asymmetric divisions in development in deuterostomes. On the other hand, the sea urchin embryo has been a historical model system for the study of the influence of cell shape on spindle positioning and remains the model system with which to further analyze the long axis rule and the molecular basis and location of pulling forces that position the future spindle. *Xenopus* embryos have been employed in the study of size scaling and examining how such a large embryo is able to correctly orient a spindle with astral microtubules that do

not reach the cortex until anaphase onset (Wühr et al., 2010). Further work in the mouse oocyte has demonstrated the roles of F-actin and myosin-II as an alternative force generator in asymmetric spindle positioning. All of the models discussed here have greatly supplemented our knowledge of spindle positioning and demonstrated the importance of correct spindle orientation for proper development.

An emerging trend in the field of spindle positioning is the use of mathematical modeling. The combination of computational and experimental approaches allows for predictions to be both produced and tested in a quantitative manner. By generating computational models developed with experimentally tested parameters scientists will obtain more quantitative data leading to a greater understanding of this biological process. It will also allow for the examination of more complicated models of spindle positioning such as the coordination of multiple spindles during tissue morphogenesis (Minc and Piel, 2012). Modeling may also allow for further analysis of the influence individual cytoskeletal elements have on the position of the spindle. The regulation of pushing vs. pulling forces and microtubule vs. actin-based mechanisms may be more clearly elucidated in models that can tease apart each individual component, providing a framework for future experimental design. Although there remains debate about which forces are most imperative for positioning the spindle, reality may be that in fact both pushing and pulling mechanisms must work simultaneously through multiple signaling pathways and cytoskeletal elements in order to properly orient the spindle. Which of these forces is most dominant may then vary in different organisms or at different times during development. This complexity may be required to ensure that the spindle is at the right place at the right time.

The process of spindle positioning has long fascinated scientists and continues to be a significant field of study. It plays an important role in embryos as the determination of the placement of the spindle has a significant impact on subsequent development. In the future it will be imperative to be able to establish how all of the different factors – cell shape, molecular motors, the cytoskeleton and polarity proteins – work together to establish the position of the spindle.

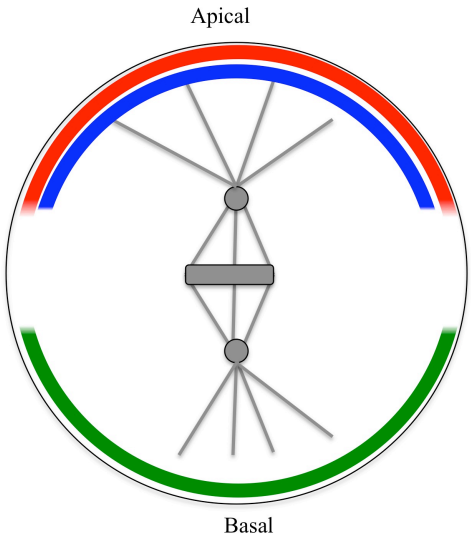
A.



Par-3/Par-6/PKC-3
Par-2/Par-1
GPR-1/2

B.

Drosophila Neuroblast



Baz-aPKC-Par6
Insc
Pins/Gαi
Brat/Pros/Numb/Miranda
Mud

C.

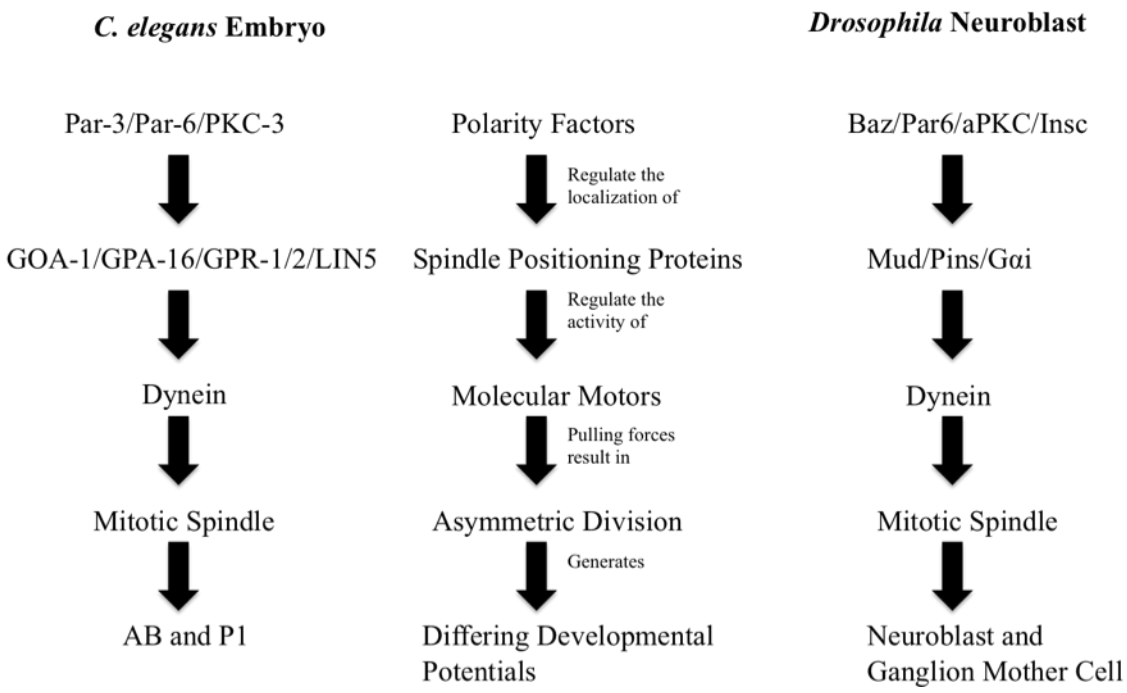


Figure 4.2.1. Asymmetric divisions in the *C. elegans* zygote and the *Drosophila* neuroblast. (A) The cortical organization of the *C. elegans* zygote during the first round of mitosis following fertilization influences the asymmetric placement of the spindle (Morin and Bellaïche, 2011). The PAR complex proteins (Par-3/Par-6/PKC-3) regulate polarity at the anterior end of the zygote, while Par-1/Par-2 regulate polarity in the posterior end. The ternary complex member GPR-1/2 is symmetrically distributed throughout both anterior and posterior ends. (B) Similar to the *C. elegans* zygote the *Drosophila* neuroblast has an asymmetric distribution of the cellular components that regulate spindle positioning (Morin and Bellaïche, 2011). The PAR complex proteins (Baz/aPKC/Par6) localize to the apical end of the neuroblast, while the cell fate determinants (Brat/Pros/Numb/Miranda) segregate to the basal end. Mud is enriched at

the apical end through metaphase and becomes symmetrically distributed in both the apical and basal ends at anaphase. (C) The pathways for both the *C. elegans* zygote and the *Drosophila* neuroblast are outlined. Both utilize a combination of polarity factors, spindle positioning proteins, and molecular motors to cause an asymmetric division that results in two daughter cells with different developmental fates.

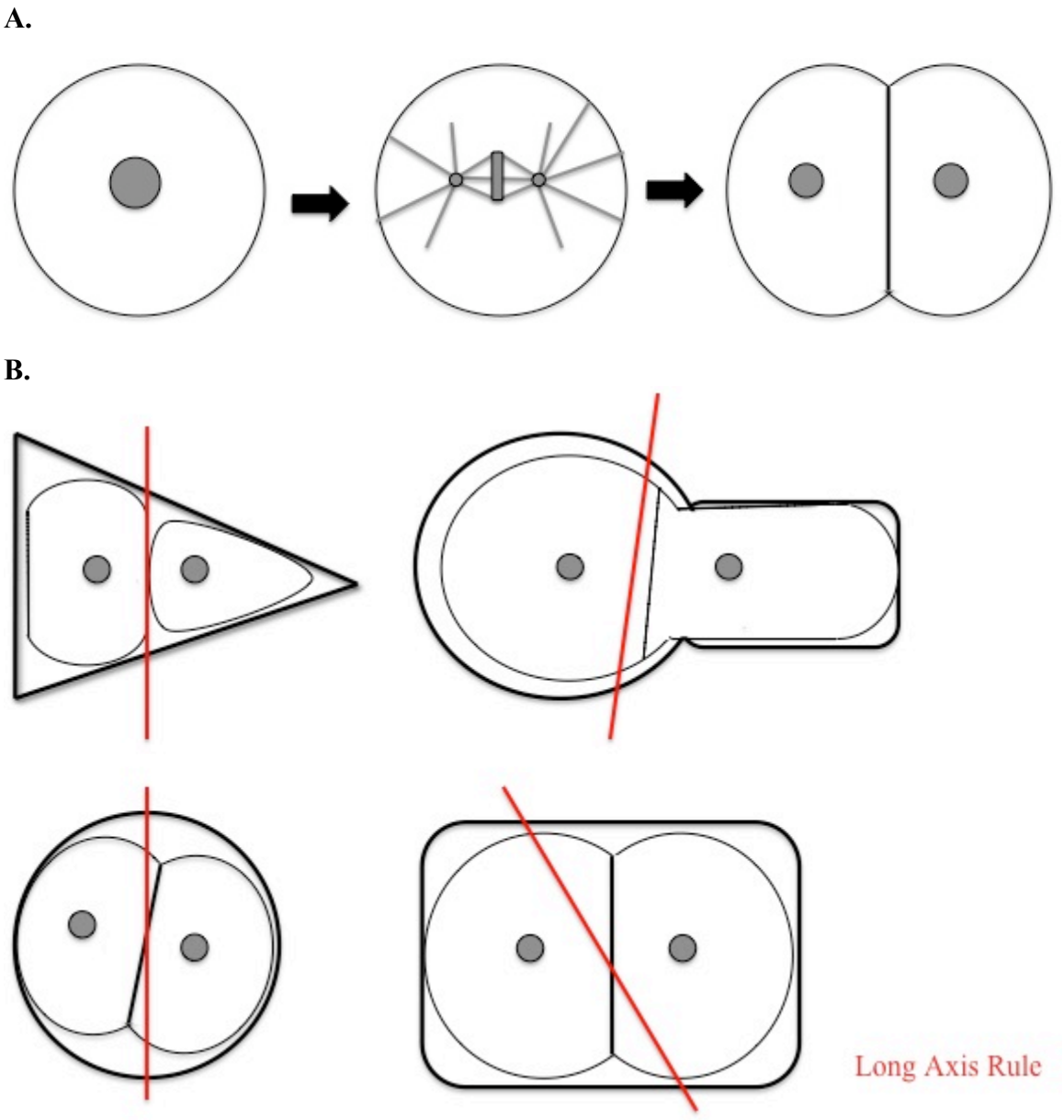


Figure 4.4.1. Spindle positioning in the sea urchin zygote. (A) Normal spindle positioning and cell division in a sea urchin embryo outside of a microfabricated mold. (B) Division patterns observed in sea urchin embryos placed in different shape microfabricated molds (Minc et al., 2011). The embryos in the top row followed Hertwig's rules, while the embryos in the bottom row had a division plane that violated Hertwig's rules. Red lines are used to indicate the predicted division plane based on Hertwig's rules.

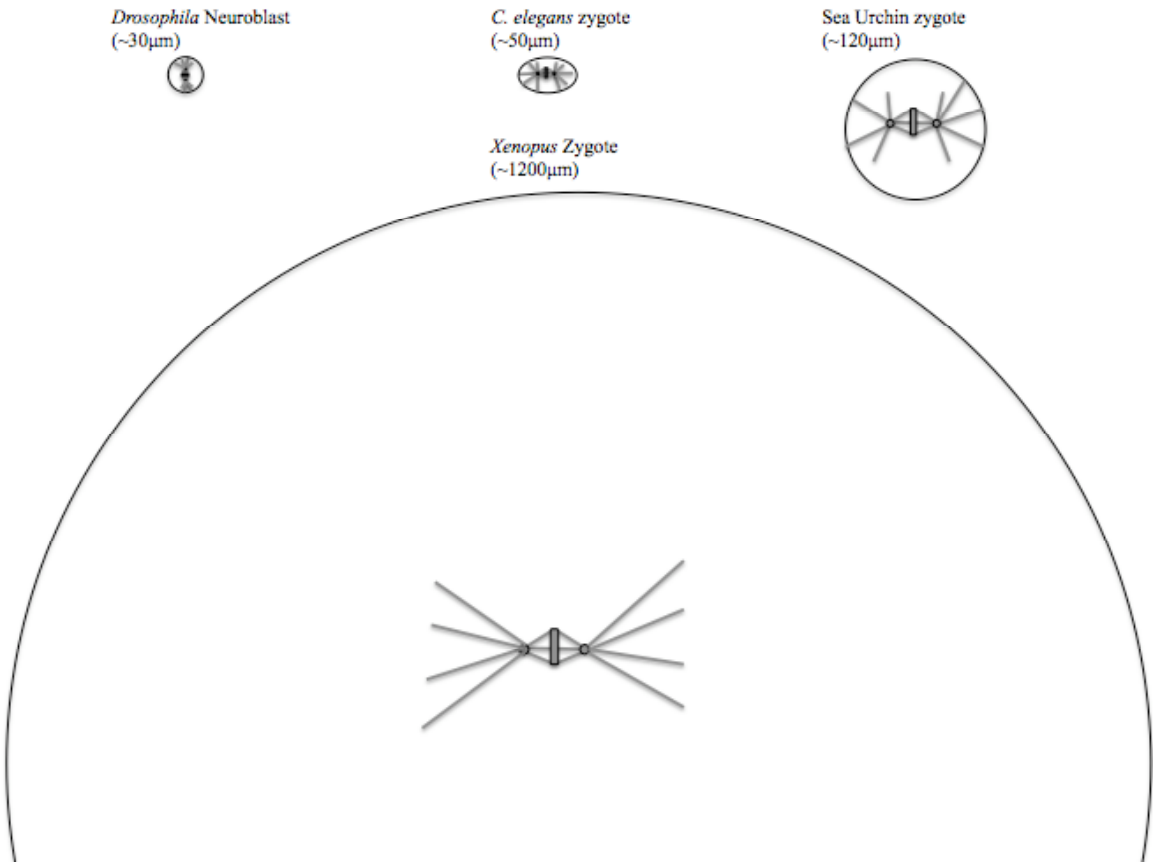


Figure 4.6.1. Size scaling in *Xenopus* embryos. *Drosophila* neuroblast, *C. elegans* zygote, sea urchin zygote, and *Xenopus* zygote are drawn to scale. *Xenopus* embryos are utilized as a model in order to address questions of size scaling. Because of their large size *Xenopus* embryos must center their spindles without astral microtubule contact with the cortex. During interphase astral microtubules pull on dynein in the cytoplasm to orient the centrosomes. The spindle is then centered between the centrosomes during metaphase. It is not until anaphase onset that the astral microtubules make contact with the cortex.

Chapter 5. Conclusions and Future Directions

The sea urchin embryo is a classic model organism for the study of the cellular and developmental biology. Because of their numerous experimental advantages and a developmental pattern that is similar to vertebrates through gastrulation, sea urchin embryos serve as useful models of early deuterostome development. Here sea urchins embryos were utilized to study the role of the PAR proteins in the establishment of polarity in an early deuterostome embryo. While the PAR proteins were initially discovered in the *C. elegans* embryo and have been subsequently studied for their roles in the polarity of cells and embryos, there was little data on the role of the PAR proteins in the early development of deuterostome embryos (McCaffrey and Macara, 2009;McCaffrey and Macara, 2012;Nance and Zallen, 2011). The data here find both the anterior polarity proteins PAR6, aPKC, and CDC42 along with the posterior polarity protein, PAR1 localize to the apical cortex at the 2 cell stage of development and that this colocalization is retained through the gastrula stage. While this polarization pattern is unique to the sea urchin embryo, it does appear to be required for proper development. PAR6, aPKC, and CDC42 are anchored in the cortex by myosin assembly and further analysis found that there was a clear role for assembled myosin during early cleavage stages. These data demonstrate that deuterostomes, like protostomes, polarize early in development and that this polarity is required for normal development.

Future studies will be necessary to more fully understand the role of early polarity in the sea urchin embryo. Here the data suggest that myosin assembly anchors the PAR complex proteins and is additionally needed for blastula formation. While inhibition of MLCK was actually found to disrupt actin and not myosin localization (Gudejko, 2013),

it remains to be determined if the resulting actin comets actually cause the cytoplasmic pooling of the PAR proteins. The cortical reorganization caused by inhibition of myosin assembly would best be studied in live embryos in order to assess the effects in real time. Previous experiments have successfully microinjected sea urchin embryos with the F-actin-binding probe, Lifeact-GFP (Gudejko, 2013;Riedl et al., 2008). Future experiments could co-inject Life-act-GFP and differentially tagged PAR proteins into ML-7 or ML-9 treated embryos and then examine if the PAR proteins become cytoplasmic as a result of the F-actin restructuring in the cortex. Although an initial disruption in PAR6 localization was maintained through a single division, future experiments are required to determine how long these disruptions are maintained. Embryos could again be pulse treated with MLCK inhibitors in order to resolve the number of divisions that the PAR proteins remain cytoplasmic and then how the subsequent cytoplasmic localization impacts development.

The first true asymmetric division in the sea urchin embryo occurs at the 16 cell of development. This is a key event in sea urchin development as this begins endomesoderm specification as well as the generation of germline precursors (Juliano et al., 2006;Weitzel et al., 2004;Wikramanayake et al., 2004;Wikramanayake et al., 1998;Yajima and Wessel, 2012). aPKC activity was not directly involved in the asymmetric division at the 16 cell stage, but inhibition of aPKC has been shown to cause the normally symmetric cleavage from the 2 cell to the 4 cell stage of development to become asymmetric (Alford et al., 2009). While the cytoplasmic pooling of the PAR complex proteins caused by MLCK inhibition may have an impact on spindle positioning, initial observations suggest that the cytoplasmic pooling of these proteins

does not impact the asymmetric division at the 16 cell stage, furthering support that a different protein most likely regulates micromere formation. However, aPKC activity and myosin assembly during early cleavage stages are both required for blastula formation, suggesting a link between proper cortical organization and polarity in the early development of a deuterostome embryo.

The specific effects of aPKC activity need to be more fully explored in the sea urchin embryo. While aPKC activity in early cleavage stage embryos appears to be essential for blastula formation, further analysis of its role in later development is needed. aPKC is required for ciliogenesis (Pruliere et al., 2011), but aPKC involvement in gastrulation and the planar cell polarity movements that occur during convergent extension and archenteron formation remains to be determined. Embryos could be treated with the peptide inhibitor of PKCζ at the blastula stage of development and observed to see if gastrulation proceeds normally. It would also be interesting to examine how aPKC effects actin and myosin localization during early cleavage stages since aPKC regulates these cytoskeletal proteins in other systems (David et al., 2010;McCaffrey and Macara, 2012;Röper, 2012). aPKC activity could be required for the cortical integrity during early cleavages as a mechanism for both the establishment and maintenance of polarity. Again embryos could be treated with the peptide inhibitor of PKCζ and any changes in actin and myosin localization patterns could be observed by immunofluoresence assays and live cell probes. Given the traditionally antagonistic relationship between PAR1 and aPKC it would also be imperative to examine how aPKC activity influences PAR1 localization. In most other model systems studied thus far aPKC and PAR1 polarize to distinct domains and it is in fact the segregation of these

proteins that controls polarity (McCaffrey and Macara, 2012). Conventionally, aPKC phosphorylates PAR1, which causes PAR1 dissociation from aPKC and subsequent binding to PAR5, which then shuttles PAR1 to an opposing domain (Benton and Johnston, 2003;Hurov and Piwnica-Worms, 2007). However, in the sea urchin embryo PAR1 not only colocalizes with aPKC, but is also found within the same complex. Embryos could be treated with the peptide inhibitor of PKCζ and the localization of the PAR1 could be determined in immunofluoresence assays. Further analysis could explore whether or not each of these proteins are phosphorylated during early cleavage stages and if they are phosphorylated by each other. These experiments would address the activity level of aPKC during early cleavage stages and what influence aPKC has on PAR1 in the sea urchin embryo.

In addition to aPKC, the specific function of PAR1 activity should be explored in the sea urchin embryo. Given its unique localization pattern, PAR1 may have a very distinct function in these embryos. Although microinjections have thus far been unsuccessful, future seasons of *L. pictus* and *L. variegatus* may be better suited for this type of experiment and allow for normal division of control-injected embryos. Microinjections of either the DNPAR1 protein or the antibody generated against the *S. purpuratus* PAR1 protein may be utilized to inhibit the native PAR1 since there are currently no commercially available small molecule inhibitors of PAR1. These experiments could then examine in real time the effects of PAR1 inhibition. PAR1 may regulate cellular events such as microtubules dynamics, myosin organization, or spindle orientation and site of cell division or PAR1 may regulate developmental events such as gastrulation, axis formation, or cell fate specification (Cohen et al., 2007;Cox et al.,

2001;Kusakabe and Nishida, 2004;Lázaro-Diéguez et al., 2013;Ossipova et al., 2007;Slim et al., 2013). Additional microinjection experiments with a GFP tagged PAR1 could then observe PAR1 dynamics in live embryos. These studies would thus expound the function of PAR1 in the sea urchin embryo.

Because of the unique localization pattern of PAR1 in the sea urchin embryo, future studies could examine if other proteins regulate polarity in the basolateral domain (areas of cell-cell contact) in the sea urchin embryo. Cadherins have already been found in regions of cell-cell contact during early cleavage stages in sea urchin embryos and are required for the epithelial to mesenchymal transition at the gastrula stage of development (Miller and McClay, 1997). However, their role in the polarization in the early embryo has yet to be clearly elucidated. In mouse embryos, E-cadherin is needed for epithelial integrity in pre-implantation embryos; however, E-cadherin does not appear to be involved in the initial polarization (Stephenson et al., 2010). In the *C. elegans* embryo PAR1 and PAR2 function to regulate polarity in the posterior domain; however, PAR2 is a nematode specific protein and thus far has not been identified in any other model system (Levitan, et al., 1994;McCaffrey and Macara, 2009). Another protein, LGL, however, has been found to function redundantly with PAR2 and is a predicted protein based on the sea urchin genome (Beatty et al., 2010;Beatty et al., 2013;Cameron et al., 2009;Hoege et al., 2010;Prehoda and Bowerman, 2010). LGL is often associated with two other proteins, discs large (Dlg) and Scribble (Scrib) in basolateral domains and both Dlg and Scrib are predicted proteins in the sea urchin genome (Cameron et al., 2009;Elsum et al., 2012). Originally identified in *Drosophila* because of their involvement in epithelial organization and the proliferation of both the imaginal discs and

larvae, LGL, Dlg, and Scrib have now been implicated in junction formation and cell polarity in other model systems such as *C. elegans,* zebrafish and mammalian tissue culture (Elsum et al., 2012). Pins is another possible candidate for the regulation of polarity in the basolateral domains in the sea urchin embryo and is also annotated as a predicted protein in the sea urchin genome (Cameron et al., 2009). Pins helps to control spindle orientation in both epithelial tissues and *Drosophila* neuroblasts by attaching astral microtubules to the cortex (McCaffrey and Macara, 2012). Similar to PAR1, both Pins and LGL are actively excluded from the apical cortex by aPKC activity (McCaffrey and Macara, 2012;Yamanaka et al., 2003;Yamanaka et al., 2006). There are clearly a number of candidates that may regulate the polarity in the regions of cell-cell contact in the early sea urchin embryo and further work will be required to determine which, if any, of these proteins are involved in this process.

How the PAR proteins, critical regulators of cell polarity, interact and influence embryonic regulators of polarity, such as dishevelled, remains an important question to address. Dishevelled has been documented to interact with PAR proteins in *Xenopus, C. elegans,* and in cultured cells (Dollar et al., 2005;Ossipova et al., 2005;Schlessinger et al., 2007;Sun et al., 2001;Terabayashi et al., 2008;Wharton, 2003). In sea urchins, dishevelled has been extensively studied because of its vital importance for β-catenin signaling and endomesoderm specification. In canonical signaling, dishevelled first becomes active at the 16 cell stage of development, which is when β-catenin becomes nuclear in the micromeres of the vegetal pole (Kumburegama and Wikramanayake, 2007). The crosstalk between dishevelled and the PAR proteins may occur in early development in order to establish axis specification before individual blastomeres have

committed to their respective cell fates. Others have examined the role of dishevelled in early cleavages stages in sea urchin embryos (Peng and Wikramanayake, 2013). Their results suggest that the actin cytoskeleton anchors dishevelled in the vegetal cortex. However, in their studies embryos were treated with cytochalasin for a very extended time period, far beyond normal treatments for actin disruption, which may have caused additional effects. I had wanted to determine if perturbations in the myosin scaffold in early cleavage stages additionally affected developmental determinants like dishevelled. However, in our hands I had very inconsistent immunostaining and reactivity in a Western blot with their sea urchin specific dishevelled antibody and could not resolve the role myosin assembly played in dishevelled localization or determine if dishevelled interacted with any of the PAR proteins through co-immunoprecipitation assays and Western blot analysis. Insights into the communication between these pathways will broaden our knowledge of the mechanisms that lead to the generation of a polarized state.

Embryogenesis is a complex, well-organized, and precisely timed process that must ensure that every sequence of events in development occurs on time and in the correct location. Subtle changes can have significant impacts on developing embryo. The sea urchin embryo has long served as useful model for the study of embryogenesis. While originally thought to remain relatively unpolarized until at least the 16 cell stage of development, the evidence presented here adds to a growing list of polarized factors in early cleavage stage sea urchin embryos. PAR6, aPKC, CDC42, and PAR1 have all been found to colocalize in the apical cortex from the 2 cell stage of development through gastrulation. Additionally, aPKC activity was found to be required for symmetric cleavages and for proper blastula formation. Similarly, assembled myosin anchored the

PAR proteins in the apical cortex and was needed during early cleavage stages for blastula formation. Collectively, these data highlight the importance of polarity during the early development of a model deuterostome embryo and demonstrate that disruptions in this polarity can have a significant impact on later development. Future work will delve deeper in the specific roles of the PAR proteins throughout the development of the sea urchin embryo as well as illuminate the roles of other polarity factors in the establishment and maintenance of polarity during early cleavage stages. The findings presented here shed light on the role of the PAR proteins and assembled myosin in early polarity in the development of deuterostome embryos.

Chapter 6. Materials and Methods

6.1. Sea Urchin Embryo Culture

Lytechinus pictus and *Stronglyocentrotus pupuratus* (Marinus Scientific, Long Beach, CA) gametes were obtained by intracoelomic injection of 0.5M KCl. Eggs were shed into artificial seawater (ASW) and sperm was dry collected. Eggs were swirled twice to expand the jelly coat. Sperm was diluted 1:1000 in ASW prior to use and added to a culture of eggs in ASW for fertilization. Fertilization was monitored by the formation of the fertilization envelope. In order to remove the fertilization envelope, *L. pictus* eggs were cultured in 4mM para-aminobenzoic acid (PABA) and run through a 118 μm nytex. Fertilized embryos were then cultured in either ASW, filtered seawater (FSW) or calcium-free seawater (CaFSW) as indicated at 15°C. Embryos were treated with 100 μM ML-7 (Sigma-Aldrich), 120 μM ML-9 (Tocris Bioscience), 2.5 μM H1152 (Alexis- Biochemicals), 4 μM myristolated protein kinase c zeta peptide (PKCζ) inhibitor (Enzo Life Sciences), 25 nM latrunculin B (Sigma-Aldrich), 40 mM urethane (Sigma-Aldrich), or 30 μM blebbistatin (Sigma-Aldrich) for 15 minutes at various cell stages as indicated. Embryos were stained with Hoescht (1:10,000; Life Technologies) as indicated and then imaged using either a Nikon TE 200 inverted microscope or a Nikon TE2000 inverted microscope with a Yokogawa spinning disk head, both controlled by Metamorph software.

6.2. PAR1 Antibody Generation

6.2.1. Antibody Design

The GLEAN prediction Sp-Mark3 amino acid sequence of *S. purpuratus* was utilized to generate a specific antibody against the PAR1 protein (Cameron et al., 2009). KFSRRSLVMAEPPSEYVKPR was used as the antigenic sequence for antibody production. Antibody generation in rabbits and purification using the target antigen was performed by Covance and then verified through both Western blot and immunofluoresence analysis.

6.2.2. Slot Blot Analysis of Crude Serum Against Target Peptide for PAR1 Antibody

Lyophilized target peptide was resuspended in a resuspension buffer (50mM Tris HCl, 150mM NaCl, pH 7.5) to final concentration of 1 mg/mL. The resuspended peptide solution was spotted onto nitrocellulose membrane and blocked in 5% milk in TBS-T for 1 hour at room temperature in the slot blot. Day 0, Day 28, Day 56, and Day 70/72 crude serums of 2 rabbits (designated 1 and 2) were added at 3 different concentrations: 1:50, 1:100, and 1:500 and incubated overnight at 4°C. Blots were washed 3 times with Tris buffered saline with 0.1% Tween (TBS-T) and then incubated in a 1:10,000 dilution of horseradish peroxidase(HRP) conjugated donkey-anti-rabbit (Amersham Bioscience) in 5% milk in TBS-T for 1 hour at room temperature. Blots were washed with TBS-T three times before the addition of the HRP substrate.

6.2.3. Crude Serum Analysis in PAR1 Antibody Extracts of 2 cell and 4 cell Stage L. pictus Embryos

L. pictus were raised in ASW until the 2 cell or 4 cell stage of development and then lysed in Laemmli's SDS-Sample Buffer (250 mM Tris-HCl, pH 6.8, 8% SDS, 40%

glycerol, 8% βME, and 0.02% Bromophenol Blue) . Lysed samples were then spun down for 2 minutes at 13,000 x g and run on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred to an Immobilon-P membrane (Millipore) for Western blot analysis. Blots were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBS-T) for 1 hour at room temperature. Blots were then incubated with the crude serum from Day 70/72 of rabbit 1 in 5% milk in TBS-T overnight at 4°C. Blots were washed 3 times with TBS-T and then incubated in a 1:10,000 dilution of HRP conjugated donkey-anti-rabbit (Amersham Bioscience) in 5% milk in TBS-T for 1 hour at room temperature. Blots were washed with TBS-T three times before the addition of the HRP substrate.

6.3. Fixation and Immunofluorescence

L. pictus embryos were treated with inhibitors as indicated and then incubated in fixation buffer (3.2% formaldehyde, 0.125% glutaraldehyde, 0.2 M $NaH_2PO_4H_2O$, 0.136 M NaCl) for 45 minutes. Following fixation, embryos were permeabilized in fixation buffer with 0.1% NP-40 for an additional 20 minutes and treated with 50 mM glycine for 15 minutes. Embryos were washed three times with phosphate buffered saline (PBS). Primary antibody, polyclonal goat anti-PARD6A(1:100; Santa Cruz, sc-14405), polycloncal goat anti-Cdc42 (1:50; Santa Cruz, sc-87), polyclonal rabbit anti-PKCζ (1:100; Santa Cruz, sc-216), or polyclonal rabbit anti- *S.purpuratus* PAR1 (1:100) antibody were added in PBS+0.1% Triton (PBT) overnight at 4°C(Alford et al., 2009;Gudejko et al., 2012). Fixed embryos were then washed three times with PBT for 20 min. Secondary antibody, Alexa 488-conjugated donkey anti-goat (1:1000) (for anti-

PAR6 and Cdc42 primary antibodies) or Alexa 647 or 555-conjugated donkey anti-rabbit (1:1000) (for anti-PKCζ and PAR1 primary antibodies) were added in PBT and incubated for 3 hours at room temperature (Life Technologies). For some fixations, Alexa 546 conjugated phalloidin (1:500) and Hoescht (1:10,000) were added simultaneously with the secondary antibodies (Life Technologies). Fixed embryos were again washed three times with PBT for 20 minutes, resuspended in mounting media (50:50 glycerol:PBS) and imaged on a Leica DM I 6000 inverted microscope equipped with the Leica TCSSP5 confocal system.

For immunofluoresence assays of gastrula stage embryos, a region of interest (ROI) of 6.488 x 26.199 μm^2 was utilized to analyze the pixel intensity of three regions using the Leica LAS AF software: the apical tuft, the archenteron, and the epithelium. These data were analyzed in Microsoft Excel and GraphPad Prism. Statistical significance between each ROI was then determined using an unpaired *t*-test and a *P*-value of ≤ 0.05 was considered significant.

6.4. Co-immunoprecipitation Assays

Co-immunoprecipitation assays were performed using the Dynabeads Co-immunoprecipitation Kit (Life Technologies). Polyclonal rabbit anti-*S. purpuratus* PAR1, polyclonal goat anti-PARD6A, and polyclonal rabbit anti-PKCζ antibodies were coupled to the Dynabeads per the manufacturer's instructions. Each antibody was coupled to the Dynabeads at a final concentration of 10 μg/ml. *S. purpuratus* embryos were raised to the desired developmental stages as indicated, pelleted, washed once with PBS, and then lysed in extraction buffer B (100 mM $MgCl_2$, 1X IP buffer, 150 mM NaCl,

1 mM DTT, and 500 μM PMSF) for 15minutes on ice. The embryo lysis suspension was centrifuged at 2600 x g for 5 minutes at 4°C. 1.5 mg of antibody-coupled beads were washed with extraction buffer B, resuspended in the embryo lysate, and rotated for 30 minutes at 4°C. Beads were then washed 3 times with extraction buffer B and once with the last wash buffer for 5 minutes (1xLWB, 0.02% Tween 20) at room temperature. Lastly, the beads were incubated with the elution buffer for 5 minutes at room temperature. The eluted supernatant was analyzed by SDS-PAGE and Western blot.

Several control coimmunoprecipitation assays were performed in order to determine if the Dynabeads themselves interact with any of the antibodies or proteins examined here. First an antibody diluent solution (0.1% sodium azide, 0.1% gelatin) was used as the coupling agent to Dynabeads per manufacturer's instructions. The co-immunoprecpitation assay was performed as described above with cell lysates from 2 cell stage *S. purpuratus* embryos and the eluted supernantant was analyzed by SDS-PAGE and Western blot. An additional control was performed that utilized Dynabeads that had been coupled with polyclonal rabbit anti-*S. purpuratus* PAR1 to a final concentration of 10μg/ml; however, instead of using a cell lysate made from embryos, the co-immunoprecipitation assay was performed using just extraction buffer B as described above. The eluted supernatant was again analyzed by SDS-PAGE and Western blot.

6.5.Western Blot Analysis of Purified PAR1 Antibody and Coimmunoprecipitation Samples

L. pictus embryos were lysed in Laemmli's SDS-Sample Buffer for polycloncal rabbit anti-PAR1 verification Western blots. These lysates or the co-immunoprecipitation samples were run on a 12% SDS-PAGE and then transferred to an

Immobilon-P membrane (Millipore) for Western blot analysis. Blots were blocked in 5% nonfat dry milk in TBS-T for 1 hour at room temperature. Blots were then incubated with polyclonal rabbit anti-*S. purpuratus* PAR1 (1:10,000), polyclonal goat anti-PARD6A (1:200), or polycloncal rabbit anti-PKCζ(1:200) in 5% milk in TBS-T overnight at 4°C. Blots were washed 3 times with TBS-T and then incubated in a 1:10,000 dilution of HRP conjugated donkey-anti-rabbit or donkey-anti-goat secondary antibody (Amersham Bioscience) in 5% milk in TBS-T for 1 hour at room temperature. Secondary antibodies were preincubated with a cold acetone extraction of *S. purpuratus* eggs prior to use in order to minimize cross-reactivity. Blots were washed with TBS-T three times before the addition of the HRP substrate.

6.6. DNPAR1 and PAR1 Construct Development

6.6.1. RNA Isolation

RNA was isolated as previously described (Ettensohn et al., 2004). Fertilized eggs of *S. purpuratus* were raised for 30 minutes post fertilization, hand centrifuged, and resuspended in a 10x volume of Tri-reagent. Following a 5 minute incubation at room temperature, samples were spun down at 12,000 x g at 4°C for 10 minutes. The supernatant was collected and 0.2 mL of cholorform/mLstarting volume of Tri-reagent was added to each sample and shaken vigorously. Samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 x g for 15 minutes at 4°C to separate the phases. The aqueous phase was transferred and 0.25 mL isopropanol and 0.25 mL of RNA precipitation solution (1.2 M NaCl, 0.8 M disodium citrate) was added for each 1 mL of initial Tri-reagent used. The samples were again shaken vigorously,

incubated at room temperature for 10 minutes, and centrifuged at 13,000rpm for 10 minutes at 4°C. To the pellet, 1 mL of 75% ethanol/mL of Tri reagent was added. This wash was repeated and the ethanol was allowed to evaporate. Pellets were resuspended in TE buffer (10 mM Tris, 1 mM EDTA) and purity was determined using the A_{260}/A_{280} ratio.

6.6.2. RT-PCR of DNPAR1 and PAR1

DNPAR1 and PAR1 cDNA sequences were amplified from RNA isolated from *S. purpuratus* using the ProtoScript M-MuLV *Taq* RT-PCR kit (NEB) according to the manufacture's instructions. Primer sequences were designed using the different predicted PAR1 sequences from the *S. purpuratus* genome based on either the GLEAN predictions or RNAseq data (Table 6.6.1) (Cameron et al., 2009).

DNPAR1 and PAR1 were then TOPO cloned into the bacterial expression vector pEXP5-CT (Life Technologies). Proper insertion of each sequence was determined using restriction digestion and sequence analysis. The BL21-D3pLys strain of *Escherichia coli* cells were then transformed with the pEXP5-CT-DNPAR1 plasmid and expression was induced with 1 mM IPTG overnight at 30°C. His-tagged DNPAR1 was purified using NiNT3 columns. Samples were run a 12% SDS-PAGE and stained with Coomasie. Protein concentrations were determined suing a Bradford Assay (Pierce) and purified DNPAR1 was then dialyzed to 1 mg/ml in injection buffer (10 mM HEPES, 150 mM aspartic acid, pH 7.2).

	Forward Primer (5'→3')	Reverse Primer (5'→3')
DNPAR1; GLEAN Prediction Sequence	ATGGCTGGGTTTGAGGAGCAT	CTCAGGTAAGACATGAGCAGAAT
DNPAR1; RNAseq data	ATGGCTGGGTTTGAGGAGCAT	CAAGCACAGTTCATTGGAAATCT
PAR1; RNAseq data	GAATTCATGTCCAGGATGCCCAAC	AAGCTTCAAGCACAGTTCATTGGAAATCT

Table 6.6.1. Primers for DNPAR1 and PAR1 constructs. DNPAR1 primers were designed based off both the GLEAN prediction sequences and RNAseq data sequences of *S. purpuratus* PAR1 and were designed to amplify the full-length PAR1 sequence without the N-terminal kinase domain. PAR1 primers were designed based on the RNAseq data sequence of the full-length *S. purpuratus* PAR1 (Cameron et al., 2009). An EcoRI site was added to the 5' end of the forward primer and a HindIII site was added to the reverse primer.

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