THE ROLE FOR ECT-2 AND RHO-1 IN POLARIZATION OF THE ACTOMYOSIN NETWORK OF THE C. ELEGANS ONE-CELL EMBRYO

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by
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Asymmetric cell division plays a critical role in embryogenesis and organogenesis of multicellular organisms. In *C. elegans*, asymmetric divisions take place during the earliest stages of embryogenesis, generating 6 founder cells, each of which is responsible for development of a unique subset of tissues. During the first cell cycle after fertilization, the contractile actomyosin network on the cortex is locally down-regulated on the future posterior pole by an unknown cue associated with the centrosomes containing paternally provided centrioles. Due to the internal tension, the actomyosin network retracts toward the anterior, generating cortical and cytoplasmic flows that serve as the driving force for initial polarization of the zygote. I studied RHO-1, a Rho GTPase, and ECT-2, a RhoGEF protein that activates Rho GTPases, for their possible roles in polarization of the zygote. My data suggest that RHO-1 and ECT-2 are required for activation of the contractile actomyosin network, and local down-regulation of RHO-1 and ECT-2 on the future posterior pole in response to the polarizing signal is responsible for local down-regulation of the actomyosin network. I also determined that the C-terminal region of ECT-2 containing a membrane-targeting domain and a C-terminal tail, but not the catalytic domain with guanine-nucleotide exchanging activity, is necessary and sufficient for both cortical accumulation and transient local reduction of ECT-2. These data suggest that the guanine-nucleotide exchanging activity of ECT-2 is required for the full extent of
posterior reduction, but not for cortical accumulation and initial reduction of ECT-2 in response to the polarizing signal. I also tested the physiological importance of the interactions between the anterior PAR proteins, PAR-3, PAR-6, and PKC-3, in collaboration with other members in the Kemphues lab. My data suggest that the interaction of PAR-6 and PKC-3 is required for proper distribution of PAR-6 in larvae and adults, and that PDZ2 domain of PAR-3 is partially required for accumulation of PAR-3 on the cortex, but fully required for cortical accumulation of PAR-6 and PKC-3.
Heon Sang Kim was born in Seoul, Korea, in 1977, to Ki-Taek Kim and Myung-Ok Oh. Although Seoul, the capital city of Korea, is one of the most crowded cities in the world, his home was located near the edge of the city, in a clean and quiet neighborhood, which may account for his affinity for peaceful small towns. Early in his childhood, he started to show interest in science and was selected to attend various extracurricular science activities. He decided to become a scientist while he was still in elementary school. During his middle school and high school years, he became fascinated by the mystery of life, which made him decide to study biological science in college. He entered Seoul National University in 1996, which is considered to be one of the best universities in Korea. He received several competitive scholarships during his time in college. In late 2000, he and his parents came to Seattle, three years after his brave younger brother left his family to go to high school in the United States. To pursue his career as a scientist, Heon Sang Kim came to Cornell University in 2002 and joined Dr. Kenneth J. Kemphues' lab, where he carried out his research on asymmetric division of the *C. elegans* one-cell embryo, described in this thesis.
To my parents and my brother.
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# TABLE OF CONTENTS

BIOGRAPHICAL SKETCH........................................................................................................... iii
DEDICATION................................................................................................................................ iv
ACKNOWLEDGMENTS.................................................................................................................. v
TABLE OF CONTENTS................................................................................................................ v
LIST OF FIGURES..................................................................................................................... x
LIST OF TABLES........................................................................................................................ xii

CHAPTER ONE. Introduction..................................................................................................... 1
  Part I: Overview of morphological changes during the first cell cycle of the *C. elegans* embryo................................. 1
    Fertilization, egg activation, and the first division...................................................................... 1
    Polarization of the actomyosin network.................................................................................... 4
    Tension within the contractile actomyosin drives cortical and cytoplasmic flows..................... 6
  Part II: PAR proteins: Key mediators of the zygote polarization............................................ 7
    PAR proteins are required for polarization of the zygote...................................................... 7
    Anterior and posterior PAR proteins..................................................................................... 9
    Localization of PAR-3, PAR-6, and PKC-3 requires the cortical flow..................................... 10
    Anterior and posterior PAR proteins antagonize each other during establishment and maintenance phases of zygote polarization.................................................................................. 12
    PAR-1 is the link between cortical PAR polarization and cytoplasmic polarization................. 14
  Part III: Centrosomes: Source of the polarizing cue............................................................. 17
  Part IV: Rho GTPases: Key regulators of actomyosin cytoskeleton......................................... 19
    Brief overview of actin cytoskeleton and its regulation.......................................................... 19
    Brief overview of non-muscle myosin II structure, function, and regulation............................ 21
    Rho-family small GTPases...................................................................................................... 22
    Rho, CDC42, and Rac facilitate actin polymerization............................................................. 23
    Non-muscle myosin II can be activated by Rho...................................................................... 26
    The cortical actomyosin network of *C. elegans* zygote is similar to stress fibers and contractile ring.................................................................................................................... 27
    Function of Rho GTPases in *C. elegans*............................................................................... 28
    Ect2/Pebble/ECT-2 are Dbl-family RhoGEF proteins that activate Rho GTPases...................... 29
    Functional domains of Ect2/Pebble/ECT-2........................................................................... 30
    Thesis organization.............................................................................................................. 34
CHAPTER TWO. RHO-1 and ECT-2 play critical roles in cortical polarization of the *C. elegans* one-cell embryo.................................................36

Introduction ........................................................................................................36
Materials and methods .......................................................................................37
Results and discussion .......................................................................................42
  RHO-1 is required for establishing cortical polarity ........................................42
  GFP::RHO-1 accumulates at the cell periphery and becomes reduced at the posterior pole........................................................................49

ECT-2 is required for establishing cortical polarity ............................................53
GFP::ECT-2 accumulates at the cell periphery and becomes reduced at the posterior pole.......................................................................56

Reduction of RHO-1 and ECT-2 at the posterior pole requires the polarizing signal...........................................................60
ECT-2 is required for RHO-1 posterior reduction, but neither protein depends on the other for accumulation at the cell periphery.....................................................60

Posterior reduction of ECT-2 does not require PAR-6........................................64
The C-terminal region of ECT-2 containing the Pleckstrin homology domain and a C-terminal tail is necessary and sufficient for the accumulation and local reduction of ECT-2 at the cell periphery........................................................64
Yeast two-hybrid screen for identifying factors required for localization of ECT-2(PH-C)........................................................................69
Analysis of the PH domain of ECT-2 for its possible interaction with Phosphatidylinositol phosphates and its role in ECT-2 localization...........................................................73

CHAPTER THREE. Physiological importance of the interactions between the anterior PAR proteins..............................................................84

Introduction .......................................................................................................84
Materials and methods .....................................................................................86
Results and discussion ......................................................................................87
  Interaction of PAR-6 with PKC-3 is required for proper PAR-6 localization during late stages of *C. elegans* development .........................................................87
  Interaction of the highly conserved PDZ domain of PAR-6 and its conventional target proteins is not required during late stages of *C. elegans* development ........................................................................87
  Disruption of the direct binding between PAR-3 and PAR-6 does not affect their co-localization dynamics in the *C. elegans* one-cell embryo.................................91
  PAR-3 PDZ2 is partially necessary for PAR-3 accumulation on the cortex, but absolutely necessary for recruitment of PAR-6 and PKC-3 on the cortex in the *C. elegans* early embryo.................................97
LIST OF FIGURES

Figure 1.1. Founder cells are generated during early embryogenesis of C. elegans .......................................................................................................................2

Figure 1.2. Actomyosin network becomes polarized during the early phase of the first division .................................................................................................5

Figure 1.3. Par (partitioning defective) phenotypes .................................................................................................................................8

Figure 1.4. Interaction between the anterior PAR proteins ..............................................................................................................................11

Figure 1.5. Sequential suppression model ...............................................................................................................................16

Figure 1.6. Rho-family GTPases function as molecular switches ......................................................................................................................24

Figure 1.7. Functional domains of Ect2/Pebble/ECT-2 ..........................................................................................................................32

Figure 1.8. Three-dimensional fold of the PH domain ..............................................................................................................................32

Figure 2.1. RHO-1 and ECT-2 are required for cortical ruffling, cortical polarization, and cytokinesis .................................................................................46

Figure 2.2. RHO-1 and ECT-2 are required for foci formation and polarization of NMY-2 ......................................................................................................48

Figure 2.3. RHO-1 and ECT-2 are required for PAR-6 polarization .......................................................................................................................50

Figure 2.4. RHO-1 and ECT-2 accumulate at the cell periphery and become reduced at the posterior pole ...........................................................................51

Figure 2.5. PAR-2 polarization is abnormal when ECT-2 is depleted ...............................................................................................................55

Figure 2.6. Reduction of RHO-1 and ECT-2 at the posterior pole requires the polarizing signal ........................................................................................61

Figure 2.7. ECT-2 is required for RHO-1 posterior reduction, but neither protein depends on the other for accumulation at the cell periphery ...............62

Figure 2.8. Posterior reduction of ECT-2 does not require PAR-6 .........................................................................................................................65
Figure 2.9. Summary of mutational analysis of ECT-2

Figure 2.10. The C-terminal region of ECT-2 containing the PH domain and a C-terminal tail is necessary and sufficient for the accumulation and local reduction of ECT-2 at the cell periphery.

Figure 2.11. Identification of the PH domain in ECT-2 by comparing secondary structures, and identification of putative PIP-binding residues.

Figure 2.12. The PH domain of ECT-2 shows marginal affinity for PI(4)P and PI(3,4,5)P₃ in vitro.

Figure 2.13. PI(3,4,5)P₃ enrichment on the membrane in the gonads is gradually downregulated during oocyte development due to DAF-18/PTEN activity.

Figure 3.1. par-6 gene model.

Figure 3.2. Interaction of PAR-6 with PKC-3, but not with the conventional target proteins of the PDZ domain, is required for proper PAR-6 localization during late stages of C. elegans development.

Figure 3.3. Disruption of the direct binding between PAR-3 and PAR-6 does not affect their co-localization dynamics in the C. elegans one-cell embryo.

Figure 3.4. PAR-6 and PKC-3 do not accumulate on the cortex in par-3ΔPDZ2::gfp; par-3(it71) one-cell and two-cell embryos.

Figure 4.1. Short patches of homologous regions in the C-terminal tail of ECT-2 and its orthologues.
LIST OF TABLES

Table 2.1. Injection RNAi of *ect-2* or *rho-1* results in sterility and embryonic lethality. ........................................................................................................................................43

Table 2.2. *gfp::ect-2 (cDNA)* rescues 3’-UTR RNAi of endogenous *ect-2*...........58

Table 2.3. Genetic analysis of the candidates identified from yeast two-hybrid screen. ........................................................................................................................................72

Table 2.4. *gfp::ect-2 (-4)* rescues 3’-UTR RNAi of endogenous *ect-2*.................74
CHAPTER ONE

INTRODUCTION

PART I

OVERVIEW OF MORPHOLOGICAL CHANGES DURING THE FIRST CELL CYCLE OF THE C. ELEGANS EMBRYO

Fertilization, egg activation, and the first division

Asymmetric cell division plays a critical role in embryogenesis and organogenesis of multicellular organisms. During the early development of the C. elegans embryo, 6 founder cells called AB, MS, E, C, D, and P4 are generated by a consecutive series of asymmetric cell divisions, and each founder cell is responsible for development of a unique subset of tissues (Sulston et al. 1983) (Figure 1.1).

In C. elegans, the oocytes are arrested at the prophase of meiosis I until fertilization. Oocytes show no sign of asymmetry except for the position of the meiotic spindle. During ovulation, mature oocytes are fertilized while passing through the spermatheca where sperm is stored; the position of sperm entry determines the posterior pole (Hirsh et al. 1976; Goldstein and Hird 1996). The position of the meiotic spindle is not directly relevant to the polarization of the embryo, but the meiotic spindle typically marks the anterior because the opposite side enters the spermatheca first and is more likely to be the site of sperm entry. During the first 30 minutes after fertilization, the newly fertilized embryo, or zygote, undergoes dramatic morphological changes. The zygote resumes Meiosis I and produces two polar bodies by the end of meiosis II (Hirsh et al. 1976). The fertilized egg produces an egg shell
Figure 1.1. Founder cells are generated during early embryogenesis of *C. elegans* (Kemphues 2000) (with permission)
to protect the embryo from physical stresses including osmotic shock (Hirsh et al. 1976). The cortex starts to ruffle, which is characterized as a series of dynamic small invaginations throughout the cortex. The symmetry-breaking event takes place about 30 minutes after fertilization, when ruffling ceases on a small region over the decondensing paternal pronucleus. The contractile cortex moves away from the site of ruffling cessation, resulting in cortical flow toward the anterior and cytoplasmic flow toward the posterior (Hird and White 1993). Cortical and cytoplasmic flow continues until the non-contractile smooth posterior domain of the cortex expands up to approximately 50% egg-length. At the boundary of the contractile and non-contractile cortex, a deep invagination called pseudocleavage forms, then retracts while the maternal pronucleus migrates toward the paternal pronucleus, which is referred to as pronuclear migration (Hirsh et al. 1976; Albertson 1984). The two pronuclei meet near the posterior pole then migrate together until they reach the center of the zygote, with the juxtaposed centrosomes aligning transverse to the antero-posterior (A-P) axis of the embryo (pronuclear meeting and centration) (Hirsh et al. 1976; Albertson 1984; Hyman and White 1987). At the center of the embryo, the pronuclei and associated centrosomes rotate 90-degrees while nuclear envelope breaks down and the spindle elongates, so that the elongating spindle lies along the A-P axis (Albertson 1984; Hyman and White 1987). During spindle elongation, the spindle is displaced toward the posterior and the posterior centrosome temporarily swings around, or rocks, perpendicular to the A-P axis (spindle displacement and rocking) (Albertson 1984; Hyman and White 1987). A cytokinesis furrow forms transverse to the A-P axis over the center of the spindle, resulting in a larger anterior cell called AB and a smaller posterior cell called P1 (Hirsh et al. 1976; Albertson 1984). The zygote is called P0. The P1 cell undergoes three more successive rounds of asymmetric divisions to produce 5 additional founder cells (Sulston et al. 1983). During asymmetric divisions
in the early embryo, germ-line-specific cytoplasmic granules rich in RNA and RNA-binding proteins called P granules and germ-line fate regulator PIE-1 segregate into the P-cell lineage, which eventually forms the germ-line (Strome and Wood 1982; Mello et al. 1996; Tenenhaus et al. 1998).

**Polarization of the actomyosin network**

At the time of completion of meiosis II, small invaginations form throughout the whole cortex. These invaginations are quite dynamic. They are in constant motion and the duration of each invagination varies. Some of them form and disappear, while others eventually merge, forming deeper invaginations. The spatial and temporal dynamics of these invaginations coincides with that of the inter-connected dense foci of non-muscle myosin NMY-2, as visualized by time-lapse confocal micrographs of cortical sections of zygotes expressing NMY-2::GFP, suggesting that the cortical ruffling is correlated with the contractility of the actomyosin network (Munro et al. 2004) (Figure 1.2). Symmetrically distributed NMY-2 foci start to disappear on a small region on the future posterior side, and this clearing co-localizes with the decondensing paternal pronucleus. This clearing of NMY-2 foci also coincides with disappearance of the invaginations that results in smooth-looking cortex (Munro et al. 2004). This cessation of ruffling is then propagated, expanding the smooth domain of the cortex until it reaches approximately 50% egg-length, where pseudocleavage forms. This symmetry-breaking event is often referred to as posterior smoothing. During posterior smoothing, the movement of the contractile cortex results in cortical flow and opposing cytoplasmic flow, initially identified as a streaming of granules on the cortex toward the anterior and in the cytoplasm toward the posterior, is one of the most distinctive morphological changes during polarization of the zygote (Hird and White 1993).
**Figure 1.2. Actomyosin network becomes polarized during the early phase of the first division.** Confocal micrographs of cortical sections of zygotes expressing NMY-2::GFP. Blue asterisk represents the position of the paternal pronucleus. Arrowheads indicate the position of deep invaginations. Anterior is to the left and posterior is to the right. (Munro et al. 2004) (with permission)
Tension within the contractile actomyosin network drives cortical and cytoplasmic flows.

Several lines of evidence, in addition to the NMY-2::GFP imaging experiments by Munro et al., suggest that the tension within the actomyosin network serves as the driving force for the cortical and cytoplasmic flow, and that the cortical and cytoplasmic flow, but not pseudocleavage, is required for asymmetric division of the zygote. Compromising the integrity of the actomyosin network by depletion of its components including non-muscle myosin NMY-2 (Guo and Kemphues 1996), myosin regulatory light chain MLC-4 (Shelton et al. 1999; Munro et al. 2004), or profilin PFN-1 (Severson et al. 2002), as well as inhibition of actin polymerization with Cytochalasin D treatment (Strome and Wood 1983; Hill and Strome 1988; Hill and Strome 1990; Hird and White 1993) effectively blocks cortical contractility. When cortical contractility is inhibited by any of these treatments, cortical and cytoplasmic flow does not take place, pseudocleavage does not occur, and polarity markers such as P granules are mis-localized. Notably, loss of pseudocleavage seems to reflect loss of cortical contractility, but pseudocleavage itself is not required for polarization (Rose et al. 1995). On the contrary, the cortical and cytoplasmic flow, pseudocleavage formation, and P-granule segregation appear to be independent of microtubules, since treating zygotes with microtubule polymerization inhibitors including Nocodazole and Colcemid does not affect those processes (Strome and Wood 1983; Hird and White 1993).
PART II
PAR PROTEINS: KEY MEDIATORS OF THE ZYGOTE POLARIZATION

PAR proteins are required for polarization of the zygote.

A group of genes called par (partitioning defective) relays the morphological asymmetry of the cortex into cytoplasmic asymmetry. Six par genes, par-1, par-2, par-3, par-4, par-5, and par-6, were identified by Kemphues and colleagues in screens designed to identify factors required for polarization of the early embryo (Kemphues et al. 1988; Watts et al. 1996; Morton et al. 2002). par mutant embryos show a variety of polarity defects during early embryogenesis (Figure 1.3). Mutations or RNAi-mediated depletion of the par genes result in polarity phenotypes including absence of spindle displacement and symmetric first division, synchronous second division, abnormal spindle orientation during the second division, and abnormal segregation of P granules and germ-line specific fate regulator PIE-1, with exception of asymmetric first division in par-4 and normal spindle orientation during the second division in par-1 and par-4 (Kemphues et al. 1988; Morton et al. 1992; Cheng et al. 1995; Etemad-Moghadam et al. 1995; Guo and Kemphues 1995; Boyd et al. 1996; Watts et al. 1996; Tenenhaus et al. 1998; Morton et al. 2002). These phenotypes are often referred to as Par phenotypes. A worm homolog of atypical Protein Kinase C (aPKC), designated as PKC-3, was identified by its sequence homology to human PKCζ, and was characterized to have similar phenotypes with par-3 when depleted and to show localization pattern similar to PAR-3 (Tabuse et al. 1998; Wu et al. 1998). Depletion of CDC-42, a Rho-family small GTPase, also leads to symmetric first division, more synchronous second division, and spindle orientation defects in the second division (Gotta et al. 2001; Kay and Hunter 2001).
Figure 1.3. Par (partitioning defective) phenotypes. The first column shows DIC images of two-cell embryos. The size of the two cells are same in some par mutants due to symmetric first division. The second column shows spindle orientation of two-cell embryos. The third column shows distribution of P granules in four-cell embryos. (The Kemphues lab web page; http://mbg.cornell.edu/cals/mbg/research/kemphues-lab/research-details.cfm)
*par-1, par-4, and pck-3* encode serine/threonine protein kinases (Guo and Kemphues 1995; Tabuse et al. 1998; Wu et al. 1998; Watts et al. 2000). *par-2* encodes a protein unique to nematodes, containing a ring-finger domain which is found in E3 ubiquitin ligase subunits (Levitan et al. 1994). *par-3* and *par-6* encode proteins with PDZ (PSD-95/ Discs Large/ ZO-1) domains which mainly function to bind other factors (Izumi et al. 1998; Hung and Kemphues 1999). *par-5* encodes a member of 14-3-3-family proteins which bind to numerous proteins implicated in signaling pathways (Morton et al. 2002).

**Anterior and posterior PAR proteins**

Among the PAR proteins, PAR-3, PAR-6, and PKC-3 accumulate on the anterior cortex, while PAR-1 and PAR-2 accumulate on the posterior cortex (Etemad-Moghadam et al. 1995; Guo and Kemphues 1995; Boyd et al. 1996; Watts et al. 1996; Tabuse et al. 1998; Hung and Kemphues 1999; Cuenca et al. 2003). During the first meiotic division, PAR-3, PAR-6, and PAR-2 accumulate to a low level throughout the whole cortex suggesting that they overlap at this stage. After onset of cortical ruffling but before posterior smoothing, PAR-3 and PAR-6 accumulate throughout the whole cortex to a higher level, while cortical PAR-2 is barely detectable except on a small region where meiosis II is taking place. During posterior smoothing, PAR-3, PAR-6, and PKC-3 are restricted to the ruffling anterior cortex, while PAR-1 and PAR-2 accumulate on the expanding smooth domain of the posterior cortex. PAR-2 accumulation around the second polar body extrusion site on the anterior often persists until early stage of posterior smoothing, but it eventually disappears during posterior smoothing. Due to their polarized localization, PAR-3, PAR-6, and PKC-3 are called the anterior PARs, and PAR-1 and PAR-2 are called the posterior PARs. PAR-4 and
PAR-5 accumulate without detectable asymmetry on the cortex and in the cytoplasm (Watts et al. 2000; Morton et al. 2002).

The anterior PAR proteins interact with each other (Figure 1.4). PAR-6 and PKC-3 interact through their PB1 (Phox and Bem1p) domains (Li et al. 2010). A region of PAR-3, named CR3 (conserved region 3), interacts with the kinase domain of PKC-3 (Izumi et al. 1998; Tabuse et al. 1998). CR3 includes PDZ3 and a conserved domain C-terminal to PDZ3 that contains a likely PKC phosphorylation site. PAR-3 can oligomerize through its CR1 domain at the N-terminus (Bingsi Li et al., in press). PAR-3, through its PDZ1 domain, binds the PDZ domain of PAR-6 (Li et al. 2010).

Localization of PAR-3, PAR-6, and PKC-3 requires cortical flow.

Cortical flow is important for localization of PAR-3, PAR-6, and PKC-3, or the anterior PAR proteins. Initial observations were that compromising the contractility of actomyosin network by depleting NMY-2 or MLC-4, leading to absence of cortical flow, results in uniform distribution of the anterior PARs (Guo and Kemphues 1996; Shelton et al. 1999). More recent work showed that cortical puncta of GFP::PAR-6 and NMY-2::GFP move at the same speed with nearby yolk granules during cortical flow, and that depletion of MLC-4 results in attenuated flow of all three groups of puncta, suggesting that they move within a common flow (Munro et al. 2004). These observations suggest that the cortical flow is the driving force that localizes the anterior PAR proteins. However, the anterior PARs do not seem to be simply riding along on the cortical flow, since they are also required for full-extent of cortical and cytoplasmic flow, suggesting that they form a positive feedback loop upon the actomyosin network (Cheeks et al. 2004; Munro et al. 2004).
Figure 1.4. Interaction between the anterior PAR proteins
Anterior and posterior PAR proteins antagonize each other during establishment and maintenance phases of zygote polarization.

One important role of anterior PAR proteins appear to be antagonizing PAR-2. When any of the anterior PAR proteins is knocked down, PAR-2 accumulates on the entire cortex throughout all the stages of the first cell cycle, and when cortical flow is blocked by disruption of the actomyosin network leading to uniform accumulation of the anterior PARs, PAR-2 does not associate with the cortex, while knocking down the anterior PARs in this condition allows PAR-2 to accumulate uniformly on the cortex, suggesting that the anterior PARs antagonize accumulation of PAR-2, and that absence of the anterior PARs on the posterior cortex allows PAR-2 accumulation (Etemad-Moghadam et al. 1995; Guo and Kemphues 1996; Watts et al. 1996; Tabuse et al. 1998; Hung and Kemphues 1999; Shelton et al. 1999; Cuenca et al. 2003). PKC-3 appears to be important for regulation of PAR-2 distribution. Human aPKC can phosphorylate PAR-2 in vitro, and mutations on PAR-2 that block phosphorylation by PKC-3 result in PAR-2 accumulation on the entire cortex, while phosphorylation-mimicking mutations on PAR-2 block cortical accumulation of PAR-2, suggesting that phosphorylation of PAR-2 by PKC-3 is the key mechanism for restricting PAR-2 on the posterior cortex (Hao et al. 2006).

The role for the posterior proteins appears to be slightly different. When PAR-2 is knocked down, the anterior PARs localize to the ruffling anterior cortex as in wild-type until the end of posterior smoothing, then retract toward the posterior to re-occupy the whole cortex, suggesting that PAR-2 is not the reason the anterior PARs clear from the posterior during the early stages of the first cell cycle, but is required during later stages to keep the anterior PARs on the anterior (Cuenca et al. 2003). The polarization period when cortical flow drives the anterior PARs toward anterior is called establishment phase, and the later period when PAR-2 keeps the anterior PARs
on the anterior is called maintenance phase (Cuenca et al. 2003).

Interaction between PAR-6 and CDC-42, a member of Rho-family small GTPases, is also required during maintenance phase. Depletion of CDC-42 results in Par phenotypes, but the initial polarization during establishment phase appears normal (Gotta et al. 2001; Kay and Hunter 2001). In cdc-42(RNAi) embryos, GFP::PAR-6 accumulates weakly but asymmetrically on the cortex during establishment phase, but disappears from the cortex during maintenance phase. Mutations in the semi-CRIB (Cdc42/Rac-interactive binding) domain of PAR-6 (CM1 and CM2) have been shown to disrupt interaction with CDC-42 in yeast two-hybrid assays, and GFP::PAR-6(CM2) proteins show similar localization with GFP::PAR-6 in cdc-42(RNAi) embryos (Aceto et al. 2006). Another study has shown, by yeast two-hybrid assays, that both the semi-CRIB domain and the PDZ domain of PAR-6 are required for interaction with PKC-3 (Gotta et al. 2001). These data suggest that PAR-6 interacts with CDC-42 through its semi-CRIB and PDZ domains and that this interaction is required for cortical accumulation of PAR-6 during maintenance phase. A constitutively active mutant of CDC-42 expressed in the presence of wild type CDC-42 co-localizes with PAR-6, while a dominant negative mutant of CDC-42 expressed under the same conditions accumulates evenly on the cortex. Depletion of PAR-6 by RNAi results in even accumulation of the constitutively active mutant of CDC-42 on the cortex, suggesting that activation of CDC-42 enables its association with PAR-6, leading to its anterior localization (Aceto et al. 2006).

Posterior PAR-2 accumulation is required for posterior PAR-1 accumulation. When PAR-2 alone or both PAR-2 and PAR-3 are knocked down, PAR-1 fails to accumulate on the cortex, while PAR-3 knock-down allows PAR-1 to accumulate on the entire cortex, suggesting that the presence of PAR-2 but not the absence of the anterior PARs on the posterior cortex is the requirement for the posterior accumulation

13
of PAR-1 (Boyd et al. 1996). In contrast, knocking down PAR-1 does not have pronounced effect on localization of PAR-3, PAR-6, or PAR-2, except that it results in a slightly larger PAR-2 domain, suggesting that PAR-1 does not play a critical role in polarizing the anterior and posterior PARs (Etemad-Moghadam et al. 1995; Boyd et al. 1996; Hung and Kemphues 1999; Cuenca et al. 2003).

**PAR-1 is the link between cortical PAR polarization and cytoplasmic polarization.**

Rather than regulating localization of other PARs, PAR-1 appears to function to regulate distribution of cytoplasmic factors. Posterior localization of PAR-1 is required for anterior accumulation of cytoplasmic factors MEX-5 and MEX-6 which are then required for posterior accumulation of germ-line fate regulator PIE-1 (Schubert et al. 2000; Cuenca et al. 2003). In par-1 mutant zygotes, an anterior cytoplasmic factor MEX-5 accumulates evenly in the cytoplasm, while mex-5; mex-6 double mutant zygotes show normal PAR-1 distribution (Schubert et al. 2000). Double mutation of mex-5 and mex-6 causes even accumulation of P granules (Schubert et al. 2000). A recent study showed that phosphorylation of a serine residue on MEX-5 is required for anterior localization, and that among 57 serine/threonine kinases that function and/or express in oocytes or embryos, only depletion of PAR-1 or PAR-4 blocks the accumulation of the phosphorylated form of MEX-5 in the cytoplasm, suggesting that kinase activity of PAR-1 and PAR-4 might be responsible for anterior accumulation of MEX-5 and MEX-6 (Tenlen et al. 2008).

When PAR-1, MEX-5, and MEX-6 are simultaneously knocked down, the expansion of PAR-2 domain observed in PAR-1 knock-down is suppressed, suggesting that ectopic accumulation of MEX-5 and MEX-6 is the cause of expansion of PAR-2 domain in par-1 zygotes. Additionally, when MEX-5 and MEX-6 are knocked down,
expansion of PAR-2 domain is delayed and occasionally PAR-2 does not accumulate at all, suggesting that MEX-5 and MEX-6 may form a feed back loop to insure full extent of polarization of the anterior and posterior PAR domains (Cuenca et al. 2003). In a separate study, MEX-5 and MEX-6 were shown to be required for cytoplasmic flow, which is consistent with their possible role in polarization of the anterior and posterior PAR domains (Cheeks et al. 2004).

Sequential repression model

Taken together, these results suggest that PAR proteins relay the morphological asymmetry of the actomyosin network into cytoplasmic asymmetry by a series of antagonizing interactions, which is summarized in a sequential repression model (Kemphues 2000; Cuenca et al. 2003) (Figure 1.5). These results also suggest that there are two phases during cortical polarization of the zygote. During the establishment phase the cortical flow drives the anterior PARs toward the anterior, while the anterior PARs and cytoplasmic MEX-5 and MEX-6 insure the full extent of cortical flow. During the maintenance phase, the posterior PAR-2 domain prevents the anterior PARs from leaking back to the posterior. PAR-1 accumulates where PAR-2 is present, and executes polarization of the cytoplasm that results in posterior accumulation of germ-line specific fate regulators.

PAR-4 and PAR-5

Although PAR-4 and PAR-5 have been shown to be required for the polarization of the early embryo, their exact roles are not yet clear. PAR-4 and PAR-5 are exceptional among the PAR proteins in that they accumulate both in the cytoplasm and on the cortex and that they do not distribute asymmetrically (Kemphues et al. 1988; Morton et al. 1992; Watts et al. 2000; Morton et al. 2002). Loss of PAR-4 does
Figure 1.5. Sequential suppression model.
(Adopted from Kemphues 2000 and Cuenca et al.2003) (with permission)
not produce prominent polarity defects in the zygote, although polarity defects in later cell divisions are detected, including synchronous second division and dispersed P granules in the four-cell stage embryo (Kemphues et al. 1988; Morton et al. 1992). PAR-5 appears to be required for proper segregation of the anterior and posterior PAR domains, as knocking down par-5 by mutation or RNAi results in uniform and overlapping distribution of PAR-3 and PAR-2, or PAR-6 and PAR-2 (Morton et al. 2002; Cuenca et al. 2003). In these embryos, cortical activities are somewhat abnormal with deeper and more posteriorly positioned pseudocleavage that often forms only on one side (Morton et al. 2002; Cuenca et al. 2003). However, the posterior smoothing is seen in these zygotes, albeit to a lesser extent than in wild-type, raising the possibility that PAR-5 might be required for the anterior and posterior PARs to respond to the local changes in cortical contractility.

PART III
CENTROSOMES: SOURCE OF THE POLARIZING CUE

Since it was shown that the position of the sperm entry dictates which side of the zygote becomes the posterior, one of the key questions has been what is the nature of the polarizing cue brought in by the sperm. When the sperm fuses with the oocyte, it contributes a small amount of plasma membrane and cytoplasm along with haploid genome and a pair of centrioles. Among these, the centrioles appear to be the key contribution of the sperm for constructing the polarizing cue, suggested by work of several groups. Cessation of ruffling and clearance of NMY-2 spatially and temporarily coincide with the decondensing paternal pronucleus and the accompanying centrosomes assembled around the sperm-provided centrioles (Munro et al. 2004). Depletion of proteins required for centrosome maturation and/or centriole
duplication, SPD-2, SPD-5, or AIR-1 causes polarity phenotypes including absence of the cortical and cytoplasmic flow and mis-localization of polarity markers, without affecting completion of meiotic divisions or cortical ruffling (Schumacher et al. 1998; O’Connell et al. 2000; Hamill et al. 2002; Kemp et al. 2004; Pelletier et al. 2004).

When anucleate sperm derived from mutants with meiotic chromosome segregation defects fertilizes the oocyte, the resulting zygote successfully undergoes two meiotic divisions, producing two polar bodies, initiates the cortical and cytoplasmic flow, and segregates the polarity markers normally (Sadler and Shakes 2000). Taken together, these observations suggest that the centrosomes containing sperm-provided centrioles are the source of the polarizing cue, and that the sperm-provided DNA and the resulting paternal pronucleus are not required for fertilization, egg activation, and more importantly, polarization of the zygote. The nature of the polarizing cue is currently unknown.

The main function of the centrosomes is to serve as the microtubule organizing centers, so it is only natural for microtubules to be among the most attractive candidates for the polarizing cue, except that initial observations suggested that microtubules are not required for polarization of the zygote (Strome and Wood 1983; Hird and White 1993). Despite these observations, whether microtubules are absolutely dispensable for the polarizing cue is somewhat controversial. Evidence for a role for microtubules comes from analysis of mutants with compromised anaphase promoting complex (APC) (Wallenfang and Seydoux 2000). In these mutants, egg activation is blocked and the zygote is arrested at metaphase of meiosis I, and the centrosomes fail to nucleate microtubules to form asters. In this condition, it was observed that the presumptive anterior near the meiotic spindle became posteriorly polarized, and that the reverse-polarization in these mutant zygotes was dependent on microtubules, suggesting that asymmetrically localized microtubules can function as
the polarizing cue. More recently, Tsai and Ahringer have reported similar results. They showed that \textit{spd-5(RNAi)} zygotes often exhibit reversed polarity of PAR-2 and NMY-2, suggesting that when centrosome maturation is compromised, the meiotic spindle can mark the posterior (Tsai and Ahringer 2007). They also showed that expression of GFP::PAR-2 enhanced the reversal of PAR-2 polarity, suggesting that PAR-2 is involved in the establishment of the reversed polarity when the centrosome maturation is compromised by \textit{spd-5} depletion.

On the other hand, it has been shown that depletion of \(\alpha\)-/\(\beta\)-tubulin, nocodazole treatment, or combination of both does not block polarization of the zygote that went through normal egg activation (Cowan and Hyman 2004). In this work, the level of GFP::\(\beta\)-tubulin was quantified so that the researchers could monitor the extent of depletion of microtubules in \(\alpha\)-/\(\beta\)-tubulin(RNAi) zygotes, and the results indicated that microtubules were not detectable in their RNAi conditions, suggesting that microtubules are not required for normal polarization of the zygote.

One possible explanation for these seemingly contradictory observations is that microtubules are indeed capable of posteriorly polarizing the cortex but only when egg activation is blocked, and that the centrosomes serve as the primary source of polarizing cue which polarizes the zygote through microtubule-independent mechanisms when the egg has gone through normal activation.

\textbf{PART IV}

\textbf{RHO GTPASES: KEY REGULATORS OF ACTOMYOSIN CYTOSKELETON}

\textbf{Brief overview of actin cytoskeleton and its regulation}

Actin filaments function as structural backbones that interact with other factors to provide mechanical supports and generate forces for a variety of cellular functions,
including migration, cytokinesis, and morphogenesis. Actin filaments also function as tracks along which motor proteins transport their cargoes. Actin filaments are composed of globular actin (G-actin) monomers. When G-actin monomers are in ATP-bound form, they undergo nucleation and elongation to form a filament. The two ends of an actin filament are different in shape and dynamics. The barbed end is the site of ATP-bound G-actin monomer addition, so the filament grows toward the barbed end. Over time, ATP is hydrolyzed to ADP, and the ADP-bound G-actin monomers dissociate from the filament at the pointed end. This process is often called aging. Assembling the first three ATP-bound G-actin monomers, which is called nucleation, is an unfavorable and rate limiting step, but once nucleation is done, monomers can spontaneously polymerize on a pre-existing barbed end (Pollard and Cooper 2009) (for review).

The nucleation process can be aided by capping factors including Arp2/3 (actin-related protein 2/3) and formin. Arp2/3 complex facilitates nucleation of a new branch on the side of a newly polymerized actin filament, generating branched actin filaments, then remains capping the pointed end of the nascent filament (Machesky et al. 1994; Welch et al. 1997b; Mullins et al. 1998; Ichetovkin et al. 2002), (Goley and Welch 2006) (for review). In contrast, formin binds the barbed end and facilitates both nucleation and elongation of unbranched actin filaments (Pruyne et al. 2002; Sagot et al. 2002; Romero et al. 2004), (Chesarone et al. 2010) (for review). Networks of Arp2/3-dependent branched actin filaments generate protrusive forces and are found in protrusive membrane ruffles called lamellipodia at the leading edge of fibroblasts, as well as trafficking vesicles (Machesky et al. 1997; Welch et al. 1997a; Mullins et al. 1998; Svitkina and Borisy 1999). Networks of unbranched actin filaments are found in stress fibers throughout the cell cortex (Tominaga et al. 2000), and in spike-like membrane protrusions called filopodia at the leading edge (Schirenbeck et al. 2005).
Nucleation of these unbranched actin filaments are dependent on formins, rather than Arp2/3. Formin also facilitates elongation at the barbed-ends of the branched filaments nucleated by Arp2/3 complex in lamellipodia (Yang et al. 2007). A formin binding protein profilin also binds to G-actin monomers and accelerates formin-dependent elongation in a dose-dependent manner, by bringing G-actin monomers to formin (Perelroizen et al. 1994; Kaiser et al. 1999; Pruyne et al. 2002; Sagot et al. 2002; Paul and Pollard 2008).

**Brief overview of non-muscle myosin II structure, function, and regulation**

Non-muscle myosin (NM II) complex is present as a dimer consisting of two heavy chains, two regulatory light chains, and two essential light chains. The heavy chain has an N-terminal globular domain called head and a C-terminal coiled-coil domain called tail. The region between the head and the tail domains is called neck region. The head domain contains a catalytic site with Mg\(^{2+}\)-dependent ATPase activity that provides energy for generating contractile forces. The tail domain is required for dimerization with another non-muscle myosin heavy chain. Two pairs of non-muscle myosin complexes can form a short bipolar filament, which then multimerizes to form longer filaments. Activation of the non-muscle myosin head domains of the bipolar filaments results in contractile forces within the actomyosin network (Clarke and Spudich 1977) (for review). The power-generating cycle begins when one molecule of ATP binds at the catalytic site of the head domain and the head domain detaches from the actin filament. Hydrolysis of ATP causes a conformational change from low-energy to high-energy conformational state at the neck region. With ADP and P\(_i\) still attached, the head domain binds to the actin filaments. Subsequent release of P\(_i\) is followed by a conformational change of the head domain, back to low-energy state, executing the power stroke that slides the myosin and the actin to the
opposite direction in a way that generates contraction. The detachment of the head domain from the actin filament upon ADP-to-ATP exchange at the beginning of the next cycle allows the head group to bind next available site on the actin filament upon ATP hydrolysis and generate productive strokes (Spudich 2001) (for review). The essential light chain binds to the neck region of the heavy chain and is required for stabilization of the heavy chain. The regulatory light chain, which also binds to the neck region, regulates NM II activity. Phosphorylation of the Ser19 on the regulatory light chain leads to conformational changes in the NM II heavy chain releasing the auto-inhibiting interaction between the head and the tail domains, and increases the ATPase activity of the head domain (Vicente-Manzanares et al. 2009) (for review). Several kinases, including MLCK, MRCK, ROCK, ZIPK, and Citron kinase, have been reported to phosphorylate the regulatory light chain on Thr18, Ser19 or both. Ser19 serves as the primary phosphorylation site for activation of NMII ATPase activity, and phosphorylation of Thr18 further increases the ATPase activity. On the other hand, phosphorylation of Ser1, Ser2, and Thr9 by PKC decreases NMII activity by preventing phosphorylation by MLCK (Vicente-Manzanares et al. 2009) (for review). The regulatory light chain is not the only point of regulation. Although less well studied, the heavy chain has been shown to have a role in NMII regulation. For example, TRPM7, PKC and Casein kinase II can phosphorylate different subset of Serine and Threonine residues at the C-terminal tail of the heavy chain, and decrease stability of the NMII complex (Vicente-Manzanares et al. 2009) (for review).

**Rho-family small GTPases**

Rho (Ras homology) -family members are well known as cytoskeleton modulators among the Ras-superfamily small GTPases. Three Rho-family members, Rho, Rac, and Cdc42 are most extensively studied for their roles in modulating the

Like other small GTPases, Rho-family GTPases function as molecular switches that cycle between active GTP-bound and inactive GDP-bound states (Figure 1.6). Balance between these two states can be regulated by other factors. Guanine nucleotide exchange factors (GEFs) facilitate GDP-to-GTP exchange to tip the balance toward the active state (Schmidt and Hall 2002) (for review), while GTPase-activating proteins (GAPs) stimulate the intrinsic GTP-hydrolyzing activity of small GTPases to push the balance toward the inactive state (Bernards 2003) (for review). Guanine nucleotide dissociation inhibitors (GDIs) sequester GDP-bound GTPases in the cytoplasm, which is probably important to prevent spontaneous activation of GTPases (Olofsson 1999) (for review). In their activated state, GTPases act on target proteins, often referred to as effectors, which leads to cellular changes specific to each effector protein. Rho GTPase effectors have been implicated in a broad spectrum of pathways. Among the Rho GTPases, the most extensively studied are Rho, Cdc42, and Rac, for their roles in actin cytoskeleton regulation. These Rho GTPases positively regulate actin polymerization and non-muscle myosin activation.

**Rho, CDC42, and Rac facilitate actin polymerization.**

Cdc42 and Rac play major roles in actin polymerization through activation of Arp2/3 complex. To become active, Arp2/3 complex requires nucleation promoting factors (NPFs), including N-WASP (neural Wiskott-Aldrich syndrome protein) and WAVE (WASP-family verprolin-homologous protein) (Machesky and Insall 1998; Machesky et al. 1999), (Campellone and Welch 2010) (for review). In addition to
Figure 1.6. Rho-family GTPases function as molecular switches.

(Etienne-Manneville and Hall 2002) (with permission)
their binding to Arp2/3, NPFs can also bind to G-actin monomers, suggesting that they activate Arp2/3 complex by bringing G-actin monomers to the complex (Marchand et al. 2001; Kelly et al. 2006). NPFs can also induce conformational changes in Arp2/3 complex that bring Arp2 and Arp3 closer, which activates Arp2/3 complex (Goley et al. 2004). Cdc42 stimulates N-WASP by directly binding to its CRIB (Cdc42 and Rac interactive binding) domain and releasing auto-inhibition of N-WASP (Rohatgi et al. 1999). Cdc42 can also act through its effector TOCA1 (transducer of Cdc42-dependent actin assembly 1) that binds to N-WASP to release its auto-inhibition (Ho et al. 2004). Rac activates WAVE indirectly by binding to another factor SRA1 (specifically Rac-associated 1), a member of WAVE regulatory complex (WRC), and disrupting trans-inhibitory interaction between WAVE and WRC complex (Steffen et al. 2004; Ismail et al. 2009). Arp2/3 activation through Rac/WAVE is mainly required for lamellipodia formation, while Cdc42/N-WASP are required for vesicle trafficking and filopodia formation, in addition to lamellipodia formation (Campellone and Welch 2010) (for review).

Rho appears to be more important for actin polymerization through activation of formin which is required in stress fiber and filopodia formation. There are 7 subfamilies of formins in mammals and 4 of them achieve auto-inhibition by intramolecular interaction of an N-terminal domain DID (Dia inhibitory domain) and a C-terminal domain DAD (Dia autoregulatory domain) (Li and Higgs 2003), (Chesarone et al. 2010) (for review). Activated Rho can release auto-inhibition of a formin mDia1 (mouse Diaphanous 1) by directly binding to RBD domain which is directly N-terminal to the DID domain (Rose et al. 2005; Nezami et al. 2006). In addition, Rho can activate a formin FHOD1 (FH1/FH2 domain-containing protein 1) indirectly through ROCK (Rho-associated coiled-coil-containing protein kinase) by phosphorylating the DAD domain (Takeya et al. 2008), (Chesarone et al. 2010) (for
Release of auto-inhibition is required for proper localization and activation of formin proteins. Rho1 activates a yeast formin homolog Bni1 which is required for its localization at the bud neck and bud cortex (Qi and Elion 2005; Yoshida et al. 2006), and RHOA activates mDia1 which is required for its localization at adherens junctions in mammalian epithelial cells (Li and Higgs 2003; Carramusa et al. 2007). Activation of FHOD1 by ROCK stimulates stress fiber contractions in HeLa cells (Hannemann et al. 2008; Takeya et al. 2008), (Chesarone et al. 2010) (for review).

Non-muscle myosin II can be activated by Rho

Activation of non-muscle myosin II (NM II) is achieved either directly by activation of the regulatory light chains through phosphorylation or indirectly by inhibition of myosin phosphatase through phosphorylation of myosin phosphatase targeting subunit (Vicente-Manzanares et al. 2009) (for review). In animal cells, Rho and its effector ROCK play central roles in formation and function of contractile ring at the cytokinesis furrow and stress fibers in fibroblasts, through activation of NM II (Kishi et al. 1993; Ridley and Hall 1994; Ishizaki et al. 1996; Leung et al. 1996; Matsui et al. 1996). In its GTP-bound form, Rho binds to its effector ROCK and it is believed that the binding disrupts the inhibitory intramolecular interactions of ROCK (Amano et al. 1999; Chen et al. 2002). When activated, ROCK directly activates the myosin regulatory light chains by phosphorylation or indirectly by inhibition of myosin phosphatase by phosphorylation (Amano et al. 1996; Kimura et al. 1996; Totsukawa et al. 2000). Another Rho effector, citron kinase, can also phosphorylate the regulatory light chains to activate NM II in the contractile ring (Madaule et al. 1998). Phosphorylation of the regulatory light chain can also be achieved, independently of Rho GTPases, through MLCK (myosin light chain kinase) which is activated by Ca$^{2+}$/calmodulin during cytokinesis (Totsukawa et al. 2000).
The cortical actomyosin network of *C. elegans* zygote is similar to stress fibers and contractile ring.

In summary of what has been reviewed in this part, activation of the contractile force-generating unbranched actin network and protrusive force-generating branched actin network appear to be quite different. Stress fibers and contractile ring are constructed by Arp2/3-independent mechanisms and are associated with NM II, while actin filaments in lamellipodia are Arp2/3-dependent and are not associated with NM II. In fact, it has been shown that Arp2/3 complex cannot facilitate branching on tropomyosin-associated contractile actin filaments (Blanchoin et al. 2001). Tropomyosin is a long coiled-coil protein that polymerizes along actin filaments and regulates binding of NM II head domain to the actin (Fanning et al. 1994). Formation and function of stress fibers and contractile ring are mainly activated by Rho, while formation and function of lamellipodia is mainly activated by Rac.

The cortical actin network of the *C. elegans* zygote appears to be similar to the one in stress fibers and contractile ring, rather than the one in lamellipodia, for several reasons. First, the cortex of *C. elegans* zygote generates contractile forces rather than protrusive forces (Hird and White 1993; Munro et al. 2004). Second, the cortex of the zygote contains non-muscle myosin II heavy chain and regulatory light chain (NMY-2 and MLC-4, respectively), and both NMY-2 and MLC-4 are required for cortical contractility (Guo and Kemphues 1996; Shelton et al. 1999). Last, CYK-1 and PFN-1, *C. elegans* homologs of formin and profilin, respectively, but not Arp2/3 complex, are required for cortical accumulation of both actin and NMY-2, and for cortical contractions (Severson et al. 2002).
Function of Rho GTPases in *C. elegans*

*C. elegans* genome encodes a RhoA homolog *rho-1*, a Cdc42 homolog *cdc-42*, and three Rac homologs *ced-10, rac-2*, and *mig-2* (Lundquist 2006) (for review). Studies suggest that RHO-1, ECT-2 (RhoGEF homolog), and LET-502 (ROCK homolog) are required for cytokinesis of the zygote and epidermal P-cells (LET-502 does not show cytokinesis defects in epidermal P-cells), morphogenesis during late embryogenesis (ECT-2 has not been studied in embryo morphogenesis), and epidermal P-cell migration during larval development (Wissmann et al. 1997; Jantsch-Plunger et al. 2000; Piekny et al. 2000; Spencer et al. 2001; Piekny and Mains 2002; Canevascini et al. 2005; Morita et al. 2005). CYK-4 (RhoGAP homolog) has been shown to be required for central spindle formation and cytokinesis of the zygote (Jantsch-Plunger et al. 2000). Two additional RhoGAP homologs, RGA-3 and RGA-4, have been shown to have a role in NMY-2 regulation in the zygote. In one study, depletion of RGA-3 and RGA-4 by RNAi caused hyper contractions and smaller NMY-2 domain (Schonegg et al. 2007). In another study, depletion of RGA-3 and RGA-4 by RNAi increased cortical NMY-2 level without causing defects in polarity (Schmutz et al. 2007). Interestingly, Schmutz et al. also reported that when RGA-3, RGA-4, and CYK-4 were simultaneously depleted, NMY-2 domain expanded and PAR-2 domain became smaller. Although the two reports present somewhat different results, the bottom line is that RGA-3 and RGA-4 function to downregulate NMY-2 in the zygote. CDC-42 has been shown to be required during maintenance phase of the zygote polarization through interaction with PAR-6 (Aceto et al. 2006). A recent study has shown that a RhoGEF protein, CGEF-1, and a RhoGAP protein, CHIN-1, regulate CDC-42 activity in the zygote (Kumfer et al. 2010). CED-10 and MIG-2 have been shown to be required for phagocytosis and gonadal tip cell migration, while *ced-10; mig-2* double mutants show defects in axon pathfinding and migration of CAN neuron
Depletion of RAC-2 does not result in any phenotype, but depletion of RAC-2 in *ced-10; mig-2* double mutants causes embryonic and early larval lethality with severe morphological defects, suggesting that *C. elegans* RAC homologs might function redundantly during development (Lundquist et al. 2001). 

**Ect2/Pebble/ECT-2 are Dbl-family RhoGEF proteins that activate Rho GTPases**

Ect2/Pebble/ECT-2 are members of the Dbl (diffuse B-cell lymphoma)-family of RhoGEF proteins. Ect2 (epithelial cell transforming gene 2) was first identified as an oncogene in a screen of a mouse keratinocyte cDNA library, looking for factors capable of inducing transformation of 3T3 fibroblast cells (Miki et al. 1993).

Studies of mammalian Ect2 has been concentrated on its role in cytokinesis through RhoA activation (Tatsumoto et al. 1999). However, it has been suggested that the morphological changes in 3T3 cells induced by constitutively active Ect2 may also require Rac1 and Cdc42 (Solski et al. 2004), and Ect2 has recently been implicated in tumorigenesis of lung cancer through Rac1 activation, independent of cytokinesis regulation (Justilien and Fields 2009). In addition, it has been reported that Ect2 shows GTP-exchange activity on RhoA, Cdc42, and Rac1 *in vitro* and that stimulation of SRF-regulated transcription by expressing constitutively active Ect2 can be suppressed by co-expression of dominant negative RhoA, Cdc42, and Rac1 in an assay in COS cell (Tatsumoto et al. 1999; Saito et al. 2004). These data suggest that Ect2 can activate any of the three Rho GTPases, depending on the cell type and the pathway being regulated. Studies on Pebble, the Drosophila homolog of Ect2, also showed similar results. Studies of Pebble *in vivo* indicate that it acts through Rho1 in cytokinesis, but not Cdc42 or Rac1, and yeast two-hybrid studies revealed positive interaction only to Rho1 (Prokopenko et al. 1999). However, recent studies showed
that Pebble catalyzes GTP exchange on Rho1, Rac1, and Rac2 in vitro, and revealed that Pebble genetically interacts with Rac1 and Rho1 in eye development, and is required for mesoderm migration during gastrulation through Rac1, rather than Rho1 (Prokopenko et al. 1999; van Impel et al. 2009). In *C. elegans*, although less extensively studied, ECT-2 has been shown to be required for cytokinesis of the zygote, migration and cytokinesis of epidermal P-cell, and vulval cell fate specification through RHO-1 (Dechant and Glotzer 2003; Canevascini et al. 2005; Morita et al. 2005). My work on ECT-2 has shown that ECT-2, through RHO-1, functions to regulate contractility of the actomyosin cortex and is required for establishment of polarity in the *C. elegans* zygote (This thesis, Chapter 2). In parallel, other groups have reported similar results (Jenkins et al. 2006; Motegi and Sugimoto 2006; Schonegg and Hyman 2006).

**Functional domains of Ect2/Pebble/ECT-2**

All of the ECT-2 orthologues have a tandem repeat of BRCT domains on the N-terminal half, and a DH (Dbl homology) domain and a PH (pleckstrin homology) domain on the C-terminal half (Figure 1.7). The BRCT repeats, which are mainly found in DNA damage-responsive cell cycle checkpoint proteins (Bork et al. 1997), is required for auto-inhibition through intramolecular interaction with the DH domain (Saito et al. 2003; Saito et al. 2004; Canevascini et al. 2005). The BRCT repeats of Ect2/Pebble/ECT-2 interact with MgcRacGAP/RacGAP50C/CYK-4 and this interaction activates ECT-2 orthologues during cytokinesis (Somers and Saint 2003; Oceguera-Yanez et al. 2005; Yuce et al. 2005). The DH domain, found in all Dbl-family RhoGEFs, catalyzes guanine-nucleotide exchange, and the PH domain functions as a membrane-targeting domain in numerous proteins (Rossman et al. 2005) (for review).
In almost all Dbl-family RhoGEFs, the DH domain is immediately followed by the PH (pleckstrin homology) domain, which is often referred to as DH-PH cassette (Rossman et al. 2005) (for review). PH domains were first identified as possible signaling modules present in the N-terminus and the C-terminus of pleckstrin, a major protein kinase C substrate in platelets (Tyers et al. 1988). Subsequent sequence comparison identified similar modules of about 100 amino acids in length in several signaling factors, and the modules were named Pleckstrin Homology domains (Haslam et al. 1993; Mayer et al. 1993). PH domains have a conserved structure of β barrel composed of seven β-strands and a C-terminal α-helix (Figure 1.8) (DiNitto and Lambright 2006) (for review). Despite their strict conservation in secondary structures and the 3-dimensional fold, PH domains are known to have low homology in amino-acid sequences (DiNitto and Lambright 2006). In fact, the PH domain of human Ect2 was among the first to be identified to have a hidden PH domain that cannot be detected by sequence homology search alone, and the identification of such hidden PH domains requires comparison of the secondary structures and, if available, 3-dimensional folds (Habets et al. 1994).

Results from several studies suggest that PH domains function as membrane targeting domains, either by binding to other factors or phosphatidylinositol phosphates (PIPs). Purified PH domains from several proteins have been shown to bind directly to bovine brain G-protein βγ-subunit (Koch et al. 1993; Touhara et al. 1994). PH domains from several proteins, including PLCδ Akt, Btk, and Grp1 have been shown to bind to PIPs with high affinity and specificity (Garcia et al. 1995; Lemmon et al. 1995; Fukuda et al. 1996; Salim et al. 1996; Klarlund et al. 1997), while PH domains from several other proteins showed low affinity and/or specificity (Kavran et al. 1998).
Figure 1.7. Functional domains of Ect2/Pebble/ECT-2

Figure 1.8. Three-dimensional fold of the PH domain. Left panel shows the PH domain of Grp1. Right panel shows superposition of PH domains from Grp1, Btk, Akt, and PLDδ1. Conserved core is colored in gray. Variable loops are colored in brown, green, and purple. The head group of PI(3,4,5)P3 is colored in yellow and red. (DiNitto & Lambright 2006) (with permission)
PH domains of Dbl-family RhoGEFs have also been shown to be important for their membrane targeting. PH domains of Dbl, Dbs, Lfc, Lsc, and Tiam, are required for their transforming activity, which requires their proper localization, and replacing the PH domains of Dbs, Lfc, and Tiam with membrane targeting domains can restore the transforming activity (Whitehead et al. 1995a; Whitehead et al. 1995b; Whitehead et al. 1996; Zheng et al. 1996; Michiels et al. 1997). The PH domain of Dbl-family RhoGEF proteins have been shown to bind other proteins. Dbl binds ezrin, which connects the plasma membrane to the actin cytoskeleton and Trio binds actin-binding proteins, filamin and Tara. PH domains from Sos and Tiam have been shown to bind PI(4,5)P₂ and PI(3)P, respectively, with high affinity and specificity, but their membrane targeting has been shown to be independent of PIP-binding (Chen et al. 1997; Rameh et al. 1997; Baumeister et al. 2003). In addition, PH domains of several Dbl-family RhoGEFs, have been reported to have low affinity and/or specificity to PIPs, suggesting that interaction of PH domain with PIPs may not be the main mechanism of membrane targeting of Dbl-family RhoGEF, and other factors may be needed (Rossman et al. 2005) (for review). Some Dbl-family RhoGEFs might employ different membrane-targeting mechanisms. For example, about 40% of human Dbl-family RhoGEFs have PDZ-binding motifs at their C-termini, and some of them, including Syx1, β-PIX, and Tiam1 have been shown to be recruited to membrane by PDZ-domain containing proteins, Scribble, Synectin, and Par3, respectively (Garcia-Mata and Burridge 2007) (for review).

The C-terminal tail of Ect2/Pebble/ECT-2 does not contain any known functional domain, but it appears that the C-terminal tail is important for the function or membrane association of the protein. It has been shown that the mammalian Ect2 requires the C-terminal tail for its transforming activity and accompanying morphological changes in NIH 3T3 cells, although the C-terminal tail is not required
for the membrane association of Ect2 in fractionation assays (Westwick et al. 1998; Solski et al. 2004). Solski et al. also showed that expression of the constitutively active form of Ect2 with the C-terminal tail induces lamellipodia formation in 50% of the cells and stress fiber formation, concentrated near the cell periphery, in 25% of the cells, while expression of the constitutively active Ect2 without the C-terminal tail only causes mild increase in stress fibers along the body and does not result in morphological changes. The data suggest that the C-terminal tail is required for activation of Rac, but not Rho, although in vitro assays showed interaction with Rho with or without the C-terminal tail (Solski et al. 2004). In a recent study of Pebble during mesoderm migration of the Drosophila embryo, genetic analysis using various constructs of Pebble provided another line of evidence for a role for the C-terminal tail in GEF specificity regulation, although it appears to regulate the specificity in the opposite direction to that observed in mammalian cells. Data from van Impel et al. suggest that the C-terminal tail enables Pebble to activate Rho1, but not Rac1, and that the C-terminal tail needs to be antagonized for Rac1 activation during mesoderm migration, although the mechanism is not clear (van Impel et al. 2009). The same group also observed that the C-terminal tail is important, but not fully required, for cortical accumulation of Pebble in mesodermal cells (van Impel et al. 2009). Understanding the exact roles for the C-terminal tail requires further investigation.

THESS ORGANIZATION

Chapter 2 of this thesis presents evidence for a role for the small GTPase RHO-1 and the RhoGEF protein ECT-2 in polarizing the actomyosin network of the C. elegans zygote in response to the polarizing signal. It also presents results suggesting that the C-terminal region of ECT-2 is important for its cortical accumulation and initial posterior reduction. In addition, it describes my efforts to
understand the mechanism of the posterior reduction of ECT-2. Chapter 3 describes my collaboration with former members of our lab, Jin Li and Bingsi Li, on studying the physiological importance of interactions between the anterior PAR proteins, PAR-3, PAR-6, and PKC-3. It presents evidence that association of PAR-6 and PKC-3 through their PB1 domains is important for proper distribution of PAR-6 in larvae and adult worms, and that PDZ2 domain of PAR-3 is partially required for cortical accumulation of PAR-3, but fully required for cortical accumulation of PAR-6 and PKC-3. This work on the anterior PAR proteins have been published as two separate journal articles (Li et al. 2010) (Li et al. 2010b, in press).
CHAPTER TWO

RHO-1 AND ECT-2 PLAY CRITICAL ROLES IN CORTICAL POLARIZATION OF THE C. ELEGANS ONE-CELL EMBRYO

INTRODUCTION

During the earliest stages of C. elegans embryogenesis, 6 founder cells are produced by a consecutive series of asymmetric cell division, starting from the first division, and each founder cell is responsible for development of a unique subset of tissues.

Actomyosin contractions provide the driving force for polarization of the one-cell embryo. At the end of meiosis II, the actomyosin network is activated, and the resulting contractile forces generate small invaginations, called ruffling, throughout the cortex. The centrosomes send out an unknown polarizing signal that locally downregulates actomyosin network on the future posterior cortex. The tension imbalance causes the actomyosin network to shrink toward the anterior, generating cortical flow, which serves as the driving force for polarization of the zygote. When the actomyosin network is compromised, cortical flow does not occur and polarity markers such as P granules are mis-localized.

Rho-family of small GTPases are well known as the key regulators of actomyosin cytoskeleton. RhoA and its orthologues have been extensively studied for their role in formation and function of stress fibers and the contractile ring, which are contractile force-generating structures containing actin filaments and non-muscle myosin II complex. Like other small GTPases, Rho-family GTPases function as molecular switches that cycle between active GTP-bound and inactive GDP-bound
states. Balance between these two states can be regulated by other factors. Guanine nucleotide exchange factors (GEFs) facilitate GDP-to-GTP exchange to tip the balance toward the active state, while GTPase-activating proteins (GAPs) stimulate the intrinsic GTP-hydrolyzing activity of small GTPases to push the balance toward the inactive state.

In search for factors that regulate actomyosin contractions in the C. elegans zygote, a former lab member, Donato Aceto, found that RHO-1, a homolog of RhoA is required for cortical ruffling and proper localization of PAR-6 and PAR-2, and that a Dbl-family RhoGEF protein, ECT-2, is also required for cortical ruffling. In this chapter, I describe my work on RHO-1 and ECT-2, inspired by the initial observations by Donato Aceto. I show that RHO-1 and ECT-2 are required for polarization of the actomyosin network and cortical PAR proteins. I show that RHO-1 and ECT-2 accumulate at the cell periphery, and that local down-regulation of RHO-1 and ECT-2 on the future posterior pole, in response to the polarizing signal, is responsible for local downregulation of the actomyosin network. I also show that ECT-2 functions upstream of RHO-1. In parallel to my work, other groups have reported similar results (Jenkins et al. 2006; Motegi and Sugimoto 2006; Schonegg and Hyman 2006). In addition, I also show that the C-terminal region of ECT-2, containing a PH domain and a tail, but not the catalytic domain with GEF activity, is necessary and sufficient for both cortical accumulation and transient local reduction of ECT-2.

MATERIALS AND METHODS

Nematode strains

Nematodes were cultured under standard conditions (Brenner 1974). The Bristol N2 strain was used as wild type. Strains used for this analysis were KK0818,
par-6(zu222) unc-101(m1)/hIn1 [unc-54(h1040)]I; VC114, ect-2/let-21(gk44)/mIn1 [mIs14 dpy-10(e128)]II; JJ1473, unc-119(ed3) III; zuIs45[Pnmy-2::NMY-2::GFP + unc-119(+)] V (Nance et al. 2003); KK0881, itIs160 [Ppie-1::GFP::PAR-6 unc-119(+)]; unc-119(ed3); KK866, itIs153[pMW1.03 GFP:PAR-2; pRF4]; WH401, unc-119(ed3) III; Ppie-1::GFP::RHO-1 unc-119(+).

Construction of transgenes and production of transgenic lines

A gfp::ect-2(cDNA) translational fusion was constructed by amplifying t19e10.1b, one of the two isoforms of ect-2, from a cDNA library, and inserting it into pAJS100 (pJunc) (Beers and Kemphues 2006), which expresses unc-119 and has a cassette for insertion of cDNAs of interest under the control of the pie-1 promoter and 3’ UTR, and a translational fusion to gfp at the 5’end of the cDNA. Constructs for expression of various forms of GFP::ECT-2 were generated in a similar manner. Genomic gfp::ect-2 translational fusion was constructed by amplifying genomic DNA encoding ect-2, including 3kb upstream of the translation start and 3kb downstream of the translation stop and inserting it into pBluescript vector, along with genomic unc-119 with its own promoter and 3’-UTR. Translational fusions for expressing GFP::PH Akt1 and GFP::PH-PH Akt1 were constructed by inserting the PH domain of mouse Akt1 (cDNA) into the pAJS100 vector. All constructs were transformed into unc-119(ed4) strain using biolistic particle bombardment (Praitis et al. 2001).

RNA interference

For injection RNAi, double stranded RNAs were prepared using the RiboMAX™ Large Scale RNA Production System. Final dsRNA product was diluted 1:2 or 1:3, heated at 95°C for about 5 minutes and cooled at room temperature to ensure formation of double stranded RNA, and injected into the gut cells of young
adults. Injected worms were incubated at 25°C until dissected for time-lapse microscopy. For feeding RNAi, HT115(DE3) bacteria were transformed with constructs that contain the genes of interest in L4440 vector (Timmons et al. 2001). Double stranded RNA production was induced with IPTG. After induction, bacteria were seeded onto non-nutrient agar plates upon which worms were placed to feed.

**Microscopy**

Worms were dissected in diH2O, and early embryos were transferred to 2% agar pads on a glass slides, which were then covered with cover slips and sealed with Vaseline. A Leica compound microscope (DMRA2) was used to make movies of embryos from N2, *nmy-2::gfp, gfp::par-2*, and *gfp::par-6* strains. For imaging of GFP signals, neutral density filters were used to attenuate the excitation to ¼ and one image of a single focal plane was taken every 30 seconds at 500ms exposure time, focused at the median section, with a 63X oil immersion lens. Confocal images of GFP fluorescence in embryos from *gfp::ect-2* and *gfp::rho-1* strains were collected on a Leica TCS SP2 system with a Leica DMRE-7 microscope with a 63X oil immersion lens. GFP was excited with an Argon laser, at about 40% of its full power. Scanning was performed at 200Hz, and two simultaneous scans were accumulated to construct one single focused median plane image, and one image was acquired every 30 seconds. Images were processed using the Leica Confocal SP2 software program and Adobe Photoshop.

**Quantification**

For quantification, I traced arcs of approximately 20% embryo length along the cortex in the anterior and posterior, and measured the mean pixel intensity along these arcs. For each time point, I repeated each cortical measurement three times and
averaged them. I divided each cortical signal by the cytoplasmic signal to obtain a normalized measure of the cortical GFP. Cytoplasmic GFP level was determined by averaging the pixel intensity along a line in the center of the embryo that avoided cortex and nuclear areas. The first movement of the male pronucleus away from the cortex served as a reference point (minute 0) to synchronize the movies for quantification.

**Yeast two-hybrid screen**

An ECT-2 fragment containing the PH domain and the C-terminal tail (aa. 567-932), which served as bait, was cloned into pGBDU, containing a LEU2 gene, and transformed into PJ69-4a yeast strain [ade2-, his3-, trp1-, ura3-, leu2-] (James et al. 1996), then ProQuest mixed stage full-length cDNA library in pPC86, containing a TRP1 gene (Invitrogen), was sequentially transformed. Transformation was performed using Frozen-EZ yeast transformation II kit (Zymo Research). Transformed yeast cells were plated on selective medium [CSM-His-Trp-Ura with 1mM 3-amino 1,2,4 triazole (3-AT)]. Yeast colonies identified in this selective medium were then streaked on different selective medium [CSM-Ade-Trp-Ura], where white color of colonies indicates positive bait-prey interaction and pink color indicates no interaction. To test if the white color is truly induced by positive interaction, selected yeast cells were streaked on CSM-Ade-Trp medium containing 0.2% 5-Fluoroorotic acid (5-FOA) to selectively grow yeast cells that have lost the ECT-2(PH-C)-pGBDU plasmid. From the candidates that passed all three testes, the “prey” plasmids were recovered and sequenced, using primer sets specific to the pPC86 plasmid, to identify the factors interacting with the “bait”. Each identified gene was then reintroduced to the PJ69-4a with ECT-2(PH-C)-pGBDU for retests on the same selective media used for initial screening.
**PIP-binding assay**

PI(4)P, PI(4,5)P2, and PI(3,4,5)P3 (0.1mg in glass vials; Echelon inc.) were resuspended to 0.5mM by adding Chloroform directly into the glass vial. I mixed 4µl of each PIP stock solution with 11µl spot buffer (mixture of Chloroform, MeOH, 50mM HCL, and Ponceau S in 250:600:200:5 ratio) to make 200µM solution, then took 2µl of the 200µM solution and mixed it with 11µl spot buffer to make 40µM solution. I used excess amount of spot buffer to compensate for its rapid evaporation. I spotted 1µl of each diluted PIP solution on Whatman OPTITRAN BA-S 85 nitrocellulose membrane, to make one set of spots containing a total of 200pmole of each PIP and another set of spots containing 40pmole of each PIP. The spotted membrane was dried at room temperature in a dark place for 1hr, then at 4°C overnight in a dark container. I blocked the membrane with 5% non-fat dry milk in TBS with 0.1% Tween 20, at RT for 1hr. I drained the blocking solution, then incubated the membrane with GST-fusion protein in 0.5% fatty-acid free BSA in TBS with 0.1% Tween20, at 4°C for 10–12 hrs. 5µg/ml of fusion proteins were used in assays with GST, GST-PLCd1(PH-PH), GST-PLCd1(PH), and GST-Akt1(PH), each of which revealed only one major band on SDS-PAGE gel, stained with GelCode Blue (Coomassie G-250-based reagent; Pierce). Unlike these proteins, Coomassie staining of SDS-PAGE and Western blot of GST-ECT-2 fusion proteins, either with anti-GST or anti-ECT-2 antibodies, showed multiple bands, possibly due to breakdown. To ensure that 5µg/ml of the intact fusion proteins were used, I estimated the amount of intact GST-ECT-2 fusion proteins by comparing the band intensity with GST standards on SDS-PAGE gel, stained with GelCode Blue. I washed the membrane twice with TBS + 0.1% Tween20 quickly, then three times with 10 min incubation. I incubated the membrane with Goat anti-GST antibody (1:7,500, Amersham), at 4°C overnight. I
washed the membrane as described above, then incubated the membrane with Donkey anti-Goat antibody with HRP (1:10,000), at room temperature for 1hr. After washing the membrane as described above, I incubated the membrane in Amersham ECL Western blotting reagents, following its protocol. Chemiluminescence on the membrane was visualized by exposure on films.

RESULTS AND DISCUSSION

RHO-1 is required for establishing cortical polarity

To test whether RHO-1 plays a role in establishing cortical polarity in *C. elegans* early embryos, I depleted RHO-1 by RNAi via injection of double stranded RNA and examined the morphology of the embryos using differential interference contrast optics. N2 (wild type) worms injected with *rho-1* dsRNA showed 100% embryonic lethality among embryos laid from 15 to 28hrs post injection (n=12; table 2.1A) and the embryos died as multinucleated cells due to cytokinesis defects. *rho-1(RNAi)* embryos that were dissected out at 24-30hrs post-injection showed reduced cortical contractility and cytokinesis failure, while wild-type embryos show extensive ruffling that flows toward the anterior pole (Figure 2.1). The worms stopped producing embryos at about 30hrs after injection, suggesting that cytokinesis fails during oogenesis. Thus, the embryos I was able to recover likely retained varying amounts of active RHO-1 protein. I presumed that failure in forming the cleavage furrows in the one-cell embryos represents the strongest depletion phenotype I can achieve and I restricted my subsequent analysis to such embryos. In wild-type embryos, the spindle moves toward the posterior during spindle elongation. However, among 17 embryos that failed to form cleavage furrows, 10 embryos showed absence of spindle displacement. Eight embryos caught early enough to observe pronuclear
Table 2.1. Injection RNAi of *ect-2* or *rho-1* results in sterility and embryonic lethality. Depletion of ECT-2 or RHO-1 caused sterility in the injected worms. Injected worms were collected from 15 to 28hrs after injection, and scored at 48hrs after injection. *rho-1* RNAi (A) or *ect-2* RNAi (B) results in near 100% embryonic lethality, similar to the *par-3* RNAi (C; positive control), while *dh11.5* RNAi (D; negative control) causes near 0% embryonic lethality.
A. rho-1(RNAi)

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B. ect-2(RNAi)

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Table 2.1. (continued)

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D. *dh11.5(RNAi)* – negative control

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Figure 2.1. RHO-1 and ECT-2 are required for cortical ruffling, cortical polarization, and cytokinesis. Selected images from wide-field time-lapse DIC micrographs, focused on the midsection, show cortical morphology during the first cell cycle in uninjected, *rho-1(RNAi)*, and *ect-2(RNAi)* embryos. Note that *rho-1(RNAi)* and *ect-2(RNAi)* result in smaller or larger embryos, which may be caused by mild defects in cytokinesis during oogenesis. Anterior is to the left and posterior is to the right. (All images of embryos are presented in the same orientation in this thesis.)
meeting showed pronuclear meeting at or near the center of the embryos (i.e. 40% embryo length or more apart from either pole, compared to about 25% embryo length from the posterior pole in wild-type). All of these phenotypes are consistent with an essential role for RHO-1 in cortical actomyosin activity in the one-cell embryo.

I next asked how depletion of RHO-1 affects asymmetry of the cortical actomyosin network by injecting \textit{rho-1} dsRNA into worms expressing GFP-tagged myosin heavy chain of the class II non-muscle myosin, NMY-2, and then monitoring myosin behavior during the first cell cycle via time-lapse microscopy (Figure 2.2). In wild-type embryos, NMY-2::GFP forms dense foci that colocalize with ingressions throughout the contractile cortex and move toward the anterior during polarity establishment. Near the end of pronuclear decondensation and the onset of pronuclear migration, which is the earliest stage of cortical polarization, dense foci of NMY-2::GFP disappear from a small region over the paternal pronucleus where posterior smoothing of the cortex has just begun. At pronuclear meeting, clearance of NMY-GFP reaches about 50% of the embryo length. At metaphase, NMY-2::GFP transiently decreases to a low level, then quickly accumulates again asymmetrically at early anaphase. RHO-1 depletion eventually blocks oogenesis; embryos collected before sterility onset show a range of phenotypes. To ensure that I was examining the strongest possible embryonic phenotypes, I restricted data analysis to 22 embryos that failed the first cytokinesis. In these embryos, NMY-2::GFP was found at a low level without dense foci throughout the whole cortex, near the end of pronuclear decondensation and near the onset of pronuclear migration, which is the earliest stage of cortical polarization (n=10; Figure 2.2). NMY-2::GFP gradually accumulated on the cortex in \textit{rho-1(RNAi)} embryos until prometaphase, which is right after nuclear envelop breakdown. During this period, NMY-2::GFP formed foci, but they were smaller and more transient than those in wild-type. After reaching its maximum at
uninjected \textit{nmy-2::gfp} embryo

\textit{rho-1(RNAi) nmy-2::gfp} embryo

\textit{ect-2(RNAi) nmy-2::gfp} embryo

\textbf{Figure 2.2.} \textbf{RHO-1 and ECT-2 are required for foci formation and polarization of NMY-2.} Selected images from wide-field time-lapse micrographs, focused on the midsection, show NMY-2::GFP distribution during the first cell cycle in uninjected, \textit{rho-1(RNAi)}, and \textit{ect-2(RNAi)} embryos.
prometaphase, cortical NMY-2::GFP level decreased quickly to a low level by the end of metaphase, and stayed low until the end of the first cell cycle. In all 22 *rho-1(RNAi)* embryos, NMY-2::GFP remained symmetric until pronuclear meeting, then in 16 embryos, NMY-2::GFP suddenly became reduced on one side of the cortex before NMY-2::GFP level decreased at anaphase, while in 6 embryos, NMY-2::GFP remained unpolarized. These data suggest that RHO-1 is required for normal activation of actomyosin network at the beginning of polarization, and for proper polarization of the cortex.

To determine the requirement of RHO-1 for PAR protein asymmetry, I depleted RHO-1 by RNAi in *gfp::par-6* worms. In embryos that failed to cleave at the first division, GFP::PAR-6 remained symmetric during the first cell cycle, in contrast to the anterior localization of PAR-6 in wild-type (n=12 of 13; Figure 2.3). In one embryo, GFP::PAR-6 was reduced on the posterior cortex but the reduction was delayed relative to wild type. These results indicate that RHO-1 is required for proper segregation of cortical PAR proteins.

**GFP::RHO-1 accumulates at the cell periphery and becomes reduced at the posterior pole.**

One possible way for the polarizing signal to regulate cortical contractility is by reducing the amount or activity of cortical RHO-1 in the posterior. To test this hypothesis, I obtained *gfp::rho-1(cDNA)* transgenic lines from Kraig Kumfer and John White at the University of Wisconsin-Madison. I examined GFP::RHO-1 distribution by time-lapse fluorescence confocal microscopy. During the cortical ruffling period prior to symmetry breaking, GFP::RHO-1 accumulated uniformly at the cell periphery (n=7; quantified n=5; Figure 2.4). After the onset of posterior smoothing, GFP::RHO-1 became reduced in a graded fashion at the posterior pole near the paternal...
Figure 2.3. RHO-1 and ECT-2 are required for PAR-6 polarization. Selected images from wide-field time-lapse micrographs, focused on the midsection, show GFP::PAR-6 distribution during the first cell cycle in uninjected, rho-1(RNAi), and ect-2(RNAi) embryos.
Figure 2.4. **RHO-1 and ECT-2 accumulate at the cell periphery and become reduced at the posterior pole.** Upper two panels show selected images from confocal time-lapse micrographs of GFP::RHO-1 and GFP::ECT-2 in the midsection, during the establishment phase of one-cell embryo polarization. Middle panel describes cortical GFP mean intensity acquisition methods. Lower two panels show quantification data. Horizontal black bars at the bottom of each graph indicate length of each time-lapse micrograph used for quantification.
$gfp::rho-1$ embryo

$gfp::ect-2$ embryo

Cortical GFP mean intensity acquisition methods

Normalization:

GFP on the cortex (A or P) divided by GFP in the cytoplasm (C)

Quantification of GFP::RHO-1

Quantification of GFP::ECT-2
pronucleus (n=12). The domain of reduced GFP::RHO-1 expanded along with the expanding smooth domain of the cortex. GFP::RHO-1 was also found at a high level at the cleavage furrow during the first division of the embryo, which is consistent with observations in *C. elegans* embryos and other model systems that reveal a role for RHO-1 in cytokinesis. These data are consistent with my hypothesis that the polarizing signal generates asymmetric cortical flow by down-regulating RHO-1 at the posterior pole.

**ECT-2 is required for establishing cortical polarity**

ECT-2 is a *C. elegans* homolog of the Dbl-family RhoGEF Ect2/Pebble which is required for cytokinesis (Miki et al. 1993; Prokopenko et al. 1999; Tatsumoto et al. 1999). Reducing activity of *C. elegans* ECT-2 by mutation or RNAi results in sterility (Morita et al. 2005). The cytokinesis defects and the Sterile phenotype are reminiscent of *rho-1(RNAi)* phenotypes that I have observed, so I hypothesized that ECT-2 functions upstream of RHO-1 as its RhoGEF during one-cell embryo polarization. To test this hypothesis, first I tested if ECT-2 is required for polarization of the zygote. I depleted ECT-2 by RNAi and examined the resulting embryos with differential interference contrast optics. Embryos were collected from 15 to 28hrs after injection. N2 worms injected with *ect-2* dsRNA showed 100% embryonic lethality (n=18; table 2.1B). The other three worms showed 67% to 94% embryonic lethality. I also noticed that injected worms stopped producing embryos about 20hrs after injection. The morphology of N2 embryos after ECT-2 depletion was similar to that seen after RHO-1 depletion. Among 13 embryos that failed to form cytokinesis furrows, 9 embryos showed absence of spindle displacement. Among 9 embryos that were captured before pronuclear meeting, 8 embryos showed pronuclear meeting at or near the center of the embryo (Figure 2.1). Distribution of NMY-2::GFP and GFP::PAR-6 in *ect-2(RNAi)*
embryos were also similar to those of rho-1(RNAi) embryos. All 7 ect-2(RNAi) embryos with failed cytokinesis showed no asymmetry in NMY-2::GFP until pronuclear meeting, then in 5 embryos, NMY-2::GFP suddenly became reduced on one side of the cortex before NMY-2::GFP level decreased at anaphase, while in 2 embryos, NMY-2::GFP remained unpolarized (Figure 2.2). GFP::PAR-6 persisted at the posterior cortex in all 10 ect-2(RNAi) embryos with failed cytokinesis, similar to what I observed in rho-1(RNAi) embryos (Figure 2.3). In ect-2(RNAi) embryos, GFP::PAR-2 failed to accumulate at the cortex throughout the first cell cycle (n=4 of 13) or was delayed in its appearance (n=8 of 13; Figure 2.5). Those 8 embryos with delayed accumulation showed various onset of GFP::PAR-2 accumulation, ranging from about a minute before pronuclear meeting to prometaphase, which is more than 7 minutes late compared to the wild-type. One of these 8 embryos only showed a transient patch of marginally detectable GFP::PAR-2. In 2 of these 3 embryos, GFP::PAR-2 started accumulating laterally, then slid to one pole. Surprisingly, in 1 of the 8 embryos, GFP::PAR-2 accumulated on the anterior pole, judging by the polar body. In one other embryo, GFP::PAR-2 accumulated on both poles. These data show that ECT-2 is required for proper polarization of the one-cell embryo. A recent report suggested that PAR-2 can drive late polarization on the posterior pole when ECT-2 activity is decreased, and it can be suppressed by over-expression of PAR-6 (Zonies et al. 2010). This is consistent with my results in that I observed the late symmetry-breaking in the majority of NMY-2::GFP and GFP::PAR-2 embryos with rho-1(RNAi) or ect-2(RNAi), but it was rare among GFP::PAR-6 embryos. My results suggest that the late symmetry-breaking event requires ECT-2 activity and the resulting cortical tension, because some ect-2(RNAi) embryos completely lacked polarity; this result differs from that reported by Zonies et al. (2010). My data also showed that a few ect-2(RNAi) embryos expressing GFP::PAR-2 were reverse-polarized or bipolar,
Figure 2.5. PAR-2 polarization is abnormal when ECT-2 is depleted. Selected images from wide-field time-lapse micrographs, focused on the midsection, show GFP::PAR-2 distribution during the first cell cycle in uninjected and *ect-2(RNAi)* embryos.
suggesting that the late symmetry-breaking event caused by PAR-2 is not under strict control of the polarizing signal from the centrosomes. It had been reported that the meiotic spindle can posteriorly polarize the presumptive anterior pole when the oocytes are arrested at metaphase of meiosis I shortly after fertilization, in embryos with defective anaphase-promoting complex (APC) (Wallenfang and Seydoux 2000). One possible explanation for my observation is that when cortical flow is significantly reduced due to severe ECT-2 depletion, the weak polarizing signal from the meiotic spindle can win over the normal polarizing signal from the centrosomes, which had also been suggested by Zonies et al. 2010. This is consistent with previous observations in wild-type embryos that PAR-2 can transiently accumulate on the anterior cortex (Boyd et al. 1996). When cortical flow is significantly weakened, this transient anterior PAR-2 might be able to persist and polarize the cortex. Taken together, these data suggest that ECT-2 is required for proper polarization of the actomyosin network and proper segregation of anterior and posterior PAR proteins. These data also show that ect-2(RNAi) phenocopies rho-1(RNAi), suggesting that ECT-2 and RHO-1 function in the same pathway and that ECT-2 probably functions as a RhoGEF for RHO-1.

**GFP::ECT-2 accumulates at the cell periphery and becomes reduced at the posterior pole.**

If ECT-2 acts as a GEF for RHO-1, it should colocalize with RHO-1. To test this hypothesis, I generated transgenic lines bearing gfp::ect-2(cDNA) or gfp::ect-2(genomic DNA) by biolistic transformation. Several lines of gfp::ect-2(cDNA) and gfp::ect-2(genomic DNA) showed embryonic expression of GFP::ECT-2. To confirm that the GFP::ECT-2(cDNA) transgene product functions as wild-type ECT-2, I tested if the transgene product can rescue ect-2(RNAi) embryos. To selectively deplete
endogenous ECT-2, I injected dsRNA designed against 3’-UTR of the endogenous
ect-2. Degradation of the 3’-UTR will remove Poly-A tail from the mRNA and cause
instability of the message. Since the transgene expression is controlled by the pie-1
promoter and pie-1 3’-UTR, GFP::ECT-2(cDNA) should be resistant to such RNAi.

After RNAi by injection of the same dsRNA preparation into control and experimental
worms, I found that embryonic lethality ranged from 0 to 40% in the experimental
animals (n=29 injected worms) but from 64 to 100% in the controls (n=25 injected
worms) (Table 2.2). These data suggest that GFP::ECT-2(cDNA) fusion protein is
functional in the early embryo. I also tested whether the GFP::ECT-2(genomic) can
function as wild-type. To do so, I crossed the gfp::ect-2(genomic) lines with ect-
2[gk44]/mIn1 line. My results suggested that GFP::ECT-2(genomic) rescues the
sterile phenotype of ect-2[gk44] (Data not shown).

GFP::ECT-2 distribution was examined by time-lapse fluorescence confocal
microscopy. GFP::ECT-2(cDNA) showed distribution patterns that were nearly
identical to that of GFP::RHO-1 (n=6; Figure 2.4). Prior to posterior smoothing,
GFP::ECT-2 accumulated throughout the whole cell periphery (n=2). GFP::ECT-2
became reduced at the posterior cell periphery near the paternal pronucleus after the
onset of posterior smoothing, and the reduction expanded along with the smooth
domain of the cortex (n=6). Quantification also showed the reduction (n=5; Figure
2.4). GFP::ECT-2(genomic) showed distribution patterns indistinguishable from that
of GFP::ECT-2(cDNA) (data not shown). Because GFP::ECT-2(cDNA) was slightly
brighter, I decided to use GFP::ECT-2(cDNA) for all further assays.
Table 2.2. *gfp::ect-2 (cDNA) rescues 3’-UTR RNAi of endogenous ect-2.* To test if the transgene product can function as wild-type ECT-2, I selectively depleted the endogenous ECT-2 without depleting the transgene, by injecting worms with dsRNA targeting 500bp of the 3’-UTR of the endogenous *ect-2*, which is absent in the *gfp::ect-2 (cDNA) transgene*. Embryos were collected between 12.5hrs and 19hrs post injection and scored at 45hrs post injection.
**Experiment**

`gfp::ect-2` worms were injected with 500bp dsRNA targeting 3'-UTR of genomic `ect-2`, with 100bp gap from the stop codon to suppress transitive RNAi.

**Control**

`par-3S863A::gfp` worms (from Bingsi Li), which carry a mutation with no obvious phenotype, were injected with the dsRNA used in the experiment.

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Reduction of RHO-1 and ECT-2 at the posterior pole requires the polarizing signal.

To test whether the polarizing signal from the centrosomes is a requirement for the reduction of RHO-1 and ECT-2 at the posterior pole, I depleted SPD-2 or SPD-5 by RNAi and examined the distribution of GFP::RHO-1 and GFP::ECT-2. SPD-2 and SPD-5 are centrosomal proteins that are required for centriole duplication and centrosome maturation, and for the polarizing signal in *C. elegans* one-cell embryo (O'Connell et al. 2000; Hamill et al. 2002; Kemp et al. 2004; Pelletier et al. 2004). I found that, after RNAi-mediated depletion of SPD-2, GFP::ECT-2 remains symmetric during the first cell cycle (n=8 of 8; quantified n=4; Figure 2.6). GFP::ECT-2 in *spd-5(RNAi)* embryos showed similar distribution (n=5 of 5; quantified n=3). Similarly, GFP::RHO-1 did not become reduced at the posterior pole during the first cell cycle in *spd-2(RNAi)* (n=1 of 1; quantified n=1; data not shown) or *spd-5(RNAi)* (n=3 of 4; Figure 2.6; quantified n=2; data not shown) embryos. These data suggest that RHO-1 and ECT-2 become reduced at the posterior pole in response to the polarizing signal.

ECT-2 is required for RHO-1 posterior reduction, but neither protein depends on the other for accumulation at the cell periphery.

To understand better the functional relationship between RHO-1 and ECT-2, I tested the effects of depleting RHO-1 on the distribution of GFP::ECT-2 and of depleting ECT-2 on GFP::RHO-1. I found that GFP::ECT-2 and GFP::RHO-1 were still found at the cell periphery when RHO-1 and ECT-2 were depleted by RNAi, respectively, suggesting that neither protein requires the other to accumulate at the cell periphery (n=7 for each experiment; Figure 2.7). In *ect-2(RNAi)* embryos, GFP::RHO-1 level at the posterior pole remained high throughout the cell cycle (n=7 of 7; quantified n=4; Figure 2.7), suggesting that posterior RHO-1 reduction requires
Figure 2.6. Reduction of RHO-1 and ECT-2 at the posterior pole requires the polarizing signal. Upper two panels show selected images from confocal time-lapse micrographs of GFP::ECT-2 and GFP::RHO-1 in the midsection, during the first cell cycle of spd-2(RNAi) and spd-5(RNAi) embryos. Lower panel shows quantification of GFP::ECT-2 in spd-2(RNAi) embryos.
Figure 2.7. ECT-2 is required for RHO-1 posterior reduction, but neither protein depends on the other for accumulation at the cell periphery. Upper two panels show selected images from confocal time-lapse micrographs of GFP::RHO-1 and GFP::ECT-2 in the midsection of *ect-2(RNAi)* and *rho-1(RNAi)* one-cell embryos, during the establishment phase. Lower two panels show quantification data.
GFP::RHO-1 in \textit{ect-2(RNAi)} embryo

GFP::ECT-2 in \textit{rho-1(RNAi)} embryo

Quantification of GFP::RHO-1 in \textit{ect-2(RNAi)} embryos

Quantification of GFP::ECT-2 in \textit{rho-1(RNAi)} embryos
ECT-2. However, in *rho-1(RNAi)* embryos, GFP::ECT-2 became reduced on a small region at the cell periphery over the paternal pronucleus prior to pronuclear meeting but became symmetric again after the paternal pronucleus started migrating toward the center of the embryo (n=7 of 7; quantified n=5; Figure 2.7). These data suggest that the initial reduction of ECT-2 does not require RHO-1 activity, but propagation of the reduction does. Taken together, these data suggest that ECT-2 functions upstream of RHO-1 to respond to the polarizing signal.

**Posterior reduction of ECT-2 does not require PAR-6.**

Although the anterior PAR proteins are not required for the initiation of the cortical flow, they are required for the full-extent cortical flow (Munro et al. 2004). To test if the anterior PAR proteins play any role on reduction of ECT-2 level at the posterior pole, I examined the behavior of GFP::ECT-2 in *par-6(zu222)* embryos. I introduced *gfp::ect-2* transgene into a *par-6(zu222)* strain by genetic crosses. Although not as obvious as in control, GFP::ECT-2 was reduced at the posterior pole (n=11 of 13; quantified n=6; Figure 2.8), suggesting that anterior PAR activity is not required for ECT-2 reduction.

**The C-terminal region of ECT-2 containing the Pleckstrin homology domain and a C-terminal tail is necessary and sufficient for the accumulation and local reduction of ECT-2 at the cell periphery.**

To identify the regions that are necessary for cortical accumulation of ECT-2, I constructed various truncated versions of GFP::ECT-2 and generated transgenic lines by biolistic transformation, then examined the distribution of GFP in the one-cell embryos. Structures of each fusion protein and their sub-cellular distribution are summarized in Figure 2.9. A fragment containing the Dbl Homology domain, the
Figure 2.8. Posterior reduction of ECT-2 does not require PAR-6. Upper panel shows selected images from confocal time-lapse micrographs of GFP::ECT-2 in the midsection of $par-6(zu222)$ one-cell embryos, during the establishment phase. Lower panel shows quantification data.
Figure 2.9. Summary of mutational analysis of ECT-2. Upper panel summarizes the mutational analysis of ECT-2. Numbers under each diagram indicates amino-acids at the end points. Lower panels show examples of cortical and cytoplasmic accumulation. ECT-2(-4) is missing four amino-acids of the putative PDZ-binding motif at the C-terminus. This mutation was tested because PDZ domain-containing proteins are identified from yeast two-hybrid screen.
Summary of ECT-2 structure-function analysis

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GFP::ECT-2(PH-C)  GFP::ECT-2(BRCT-DH)  GFP::ECT-2(-4)
Pleckstrin Homology domain and the rest of the C-terminus, named GFP::ECT-2(DH-PH-C), and another fragment containing the Pleckstrin Homology domain and the rest of the C-terminus, named GFP::ECT-2(PH-C), were able to accumulate at the cell periphery in wild-type one cell embryos (Figure 2.9; GFP::PH-C image shown in lower panel). All the other fragments tested accumulated in the cytoplasm (Figure 2.9; GFP::BRCT-DH image shown in lower panel). Numbers of observed embryos are n=49 for PH-C, n=51 for BRCT-DH, n=42 for DH-PH-C, n=22 for PH-only, n=21 for C-only, n=8 for PH-C\textsuperscript{1/2}, and n=7 for PH\textsuperscript{1/2}-C. Interestingly, I noticed that the majority of the adult worms expressing GFP::ECT-2(DH-PH-C) became sterile and some embryos from non-sterile worms failed to hatch (data not shown). This observation is consistent with the findings in C. elegans and human that the BRCT repeats function as an auto-inhibitory domain of the protein (Tatsumoto et al. 1999; Kimura et al. 2000; Canevascini et al. 2005; Kim et al. 2005).

To test if the accumulation of GFP::ECT-2(PH-C) at the cell periphery depends on endogenous ECT-2, I injected dsRNA against the 3'-UTR of the endogenous ect-2 in the worms expressing GFP::ECT-2(PH-C). When I examined the one cell embryos, I detected only a marginal level of GFP::ECT-2(PH-C). I reasoned that this occurred because not only the endogenous ECT-2 but also the transgene was depleted significantly, due to transitive RNAi. Transitive RNAi is a phenomenon of mRNA depletion that spreads over the boundary of the targeted region, in a 3' to 5' direction, caused by anti-sense RNA polymerization by RNA-dependent RNA polymerase, involved in the gene silencing amplification process (Alder et al. 2003; Fischer 2010).

To avoid depletion of the transgene by transitive RNAi, I decided to change the RNAi target to the N-terminal half of ect-2, which is missing in GFP::ECT-2(PH-C). This time, the level of GFP::ECT-2(PH-C) was not decreased after RNAi. GFP::ECT-2(PH-C) accumulated at the cell periphery when the endogenous ECT-2 was depleted.
(n=16; Figure 2.10), suggesting that PH-C fragment is able to accumulate at the cell periphery without association with the endogenous ECT-2. These embryos did not show any detectable cortical contractility or cortical flows, but GFP::ECT-2(PH-C) became transiently reduced on a small region over the paternal pronucleus, indicating, as expected, that the guanine-nucleotide exchange activity is required for activating the actomyosin network, that the PH-C fragment is depleted at the posterior pole in response to the polarizing signal from the centrosomes, and that the guanine-nucleotide exchange activity is not required for this partial reduction of PH-C (n=16; quantified n=7; Figure 2.10). Taken together, these results suggest that the C-terminal fragment of ECT-2 that contains the PH domain and the rest of the C-terminus is the smallest region that is sufficient and necessary for cortical accumulation of ECT-2. All the fragments that accumulated in the cytoplasm did so even when there was endogenous ECT-2, and the PH-C fragment accumulated at the cell periphery with or without endogenous ECT-2, suggesting that there is no significant dimerization through any part of the protein. The fact that neither the PH domain nor the C-terminal tail is sufficient for localization indicates that elements in both domains are required.

**Yeast two-hybrid screen for identifying factors required for localization of ECT-2(PH-C)**

Based on my findings, I hypothesized that the sub-cellular localization of ECT-2 is modulated by factors that associate with the PH-C region. In hope of identifying such factors, I carried out a Yeast 2-Hybrid screen using the PH-C fragment as bait. To compensate for possible variation in target gene representation (i.e. copy numbers) in the cDNA library, I designed my Yeast 2-Hybrid to cover approximately $10^7$ transformants, which is roughly 10-times the number of transformants needed for
Figure 2.10. The C-terminal region of ECT-2 containing the PH domain and a C-terminal tail is necessary and sufficient for the accumulation and local reduction of ECT-2 at the cell periphery. To examine the distribution of ECT-2(PH-C) in the absence of wild-type ECT-2, the endogenous ECT-2 was selectively depleted by RNAi, targeting the 3'-UTR of ECT-2 (as described in Table 2.2). Upper panels show confocal micrographs of GFP::ECT-2 in the midsection of par-6(zu222) one-cell embryos, during the establishment phase. Lower panel shows quantification data.
saturation assuming that each gene in the cDNA library is presented equally. I identified over 90 transformants as positives, and 16 of them retested as positives, representing 5 genes. The 5 genes are *mig-5*, *magi-1*, *dlg-1*, *eps-8*, and *zk849.2*. *zk849.2* was of special interest among these candidates, because its function is unknown and it has a closely related homologue, *zk849.1*, immediately adjacent to it. The two proteins are 23% identical and 44% homologous overall, raising the possibility of functional redundancy. These two genes are immediately adjacent to each other so that it is impossible to generate double knock-out using alleles currently available, yet their DNA sequences are diverged enough that RNAi of one gene would not deplete the other. To test if association of ECT-2 with the candidate proteins is of physiological significance, I depleted these genes either singly or in combination, but none of these RNAi experiments revealed polarity phenotype in the one-cell embryo (table 2.3).

Interestingly, four of these candidate genes have PDZ domains. I examined the sequence of ECT-2 and found out that the last four amino-acids at the C-terminus of ECT-2 falls into a rare category of PDZ-binding motifs. In fact, about 40% of human RhoGEFs have a putative PDZ-binding motif at the C-terminus (Garcia-Mata and Burridge 2007) (for review). To test if binding to these PDZ-containing proteins is important for ECT-2 localization and function, I generated transgenic lines expressing GFP-tagged ECT-2 lacking the last four amino-acids, named GFP::ECT-2(-4). Surprisingly, GFP::ECT-2(-4) distribution in the one-cell embryos was not distinguishable from that of GFP::ECT-2 (n=8; Figure 2.9). I tried to examine the distribution of GFP::ECT-2(-4) after depletion of endogenous ECT-2 by RNAi targeting 3'-UTR, but the RNAi caused significant depletion of the transgene as well as the endogenous ECT-2, because of transitive RNAi which also occurred in my previous RNAi experiments with GFP::ECT-2(PH-C). Although I was not able to
Table 2.3. Genetic analysis of the candidates identified from yeast two-hybrid screen. ect-2(gk44)/+ was used as a sensitized genetic background. DLG-1, EPS-8, and MIG-5 are required during late embryogenesis, so I examined if depletion of these genes in ect-2(gk44)/+ or zk849.2(tm1466) causes polarity defects in early embryos, instead of scoring embryonic lethality. Depletion of MAGI-1, ZK849.1, and ZK849.2 does not cause any lethality on their own, so I scored embryonic lethality for genetic analysis of these genes in ect-2(gk44)/+ or zk849.2(tm1466).

<table>
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<tr>
<th>genotype</th>
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<tr>
<td>dlg-1(RNAi); ect-2(gk44)/+</td>
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</tr>
<tr>
<td>eps-8(RNAi); ect-2(gk44)/+</td>
<td>no polarity defects</td>
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<td>zk849.1(RNAi); zk849.2(RNAi); ect-2(gk44)/+</td>
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<td>magi-1(RNAi); ect-2(gk44)/+</td>
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</tr>
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<td>mig-5(RNAi); zk849.2(tm1466)</td>
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</table>
examine GFP::ECT-2(-4) distribution in ECT-2 knock-down background, it is highly unlikely that accumulation of GFP::ECT-2(-4) at the cell periphery is dependent on endogenous ECT-2, because ECT-2 is not likely to dimerize, based on my findings from previous structure-function analysis. To test if GFP::ECT-2(-4) functions as wild-type ECT-2, I depleted 3'-UTR of the endogenous ECT-2, and scored embryonic lethality. The results suggest that GFP::ECT-2(-4) resces (table 2.4). Taken together, these data suggest that the 6 genes that I have identified do not play any role in early embryonic development and the last four amino-acids of ECT-2, which appears to be a possible PDZ binding motif, is not required for its proper localization and function in the one-cell embryo.

Analysis of the PH domain of ECT-2 for its possible interaction with Phosphatidylinositol phosphates and its role in ECT-2 localization

In almost all Dbl-family RhoGEFs, the DH domain is immediately followed by the PH domain, which is often referred to DH-PH cassette (Rossman et al. 2005) (for review). However, sequence homology-based domain search of ECT-2 did not reveal the expected PH domain. PH domains are known to have relatively low homology in amino-acids sequences while the secondary structures and the 3-dimensional fold of the secondary structures are highly conserved. PH domains have a β barrel structure, composed of seven β-strands and a C-terminal α-helix (Figure 2.11) (DiNitto and Lambright 2006) (for review). To predict the secondary structures of ECT-2, I used a protein structure prediction server, PSIPRED (McGuffin et al. 2000) (http://bioinf4.cs.ucl.ac.uk:3000/psipred/). The prediction suggests that ECT-2 has a region that shares homology with the conserved structure of seven β-strands and a C-terminal α-helix, and it is immediately C-terminal to the DH domain, suggesting that ECT-2 has a PH domain (Figure 2.11). Because ECT-2 has a PH domain and I have
Table 2.4. *gfp::ect-2* (-4) rescues 3’-UTR RNAi of endogenous *ect-2*. To test if removing the putative PDZ-binding motif from the C-terminus of ECT-2 affects its function during early embryogenesis, I selectively depleted the endogenous ECT-2 without depleting the transgene, as described in Table 2.2.

<table>
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74
Figure 2.11. Identification of the PH domain in ECT-2 by comparing secondary structures, and identification of putative PIP-binding residues. Upper panel shows sequences of PH domains that bind PIPs (Ceccarelli et al. 2007) (adopted from). Blue boxes highlight residues that interact with the phosphate groups of PIPs. Secondary structure representation is shown above the sequences, colored in light green. Note that their amino-acid sequences do not reveal high level of conservation. Middle panel shows secondary structure prediction of ECT-2. Lower panel shows sequence comparison of ECT-2 and its orthologues. Acidic and basic residues are colored in blue and red, and Threonine and Serine residues are colored in light green. Putative PIP-binding acidic residues, K615 and R667, are highlighted with red asterisks.
Sequence comparison of PH-domains known to bind PIPs

Secondary structure prediction of ECT-2

Sequence comparison of PH-domain of ECT-2 and its homologs
observed that it is required for proper sub-cellular localization of ECT-2, I hypothesized that the PH domain of ECT-2 binds PIPs and it recruits ECT-2 at the cell periphery. To test if the PH domain of ECT-2 binds PIPs, I carried out in vitro PIP-binding assays (Kavran et al. 1998). In this dot-blot assay, purified PIPs are immobilized on a nitrocellulose membrane and the membrane is incubated with purified ECT-2 proteins tagged with GST. Proteins bound to PIPs are detected by primary antibodies against GST and secondary antibodies conjugated with Horseradish peroxidase (HRP), and Amersham ECL™ Western Blotting Reagents. The chemiluminescence resulting from HRP activity on the membrane, which reflects presence of the ECT-2 proteins, is visualized by exposure on a film. I tagged three different fragments of ECT-2, which are PH-C, PH-only, and C-only, with GST, and expressed them in an E. coli strain. I have tested PI(4)P, PI(4,5)P₂, and PI(3,4,5)P₃ because they are known to accumulate on the plasma membrane (Di Paolo and De Camilli 2006) (for review). I used GST-PLCδ1(PH-PH) and GST-PLCδ1(PH) proteins that bind PI(4,5)P₂ as positive controls. They showed binding to PI(4,5)P₂, as expected, but with extremely high background in my assays for unknown reasons. I included GST-Akt1(PH) protein that binds PI(3,4,5)P₃ in my later assays as an additional positive control, which showed strong binding to PI(3,4,5)P₃, as expected, without high background. GST-ECT-2(PH-C) and GST-ECT-2(PH-only) showed very weak interaction with PI(4)P and PI(3,4,5)P₃, marginally above detection limit (Figure 2.12). To test whether this weak binding is due to the interaction between the PH domain and the PIPs, I targeted two conserved residues for mutagenesis, one in the β2 strand and the other in the β7 strand. Structural studies have shown that conserved positively charged residues in β1 and β2 strands, and around the N-terminal region of β7 strand make direct contact with PIPs and are thought to be important for PIP
Figure 2.12. The PH domain of ECT-2 shows marginal affinity for PI(4)P and PI(3,4,5)P<sub>3</sub> in vitro. Upper panel shows in vitro PIP-binding assays with ECT-2(PH-C), ECT-2(PH-only), and ECT-2(C-only). Lower panel shows assays with ECT-2(PH-C), ECT-2(PH-C<sup>K615A</sup>), and ECT-2(PH-C<sup>R667A</sup>). PLCδ1(PH-PH) and PLCδ1(PH) proteins, used as positive controls, show high background in my assays for unknown reasons. Akt1(PH) was added to the assays as another positive control. In each PIP strip, dots containing 200pmole PIPs are on the left and dots containing 40pmole PIPs are on the right.
Spot buffer only
PI(4)P
PI(4,5)P₂
PI(3,4,5)P₃

200 pmole
40 pmole
binding, and ECT-2 has two of the conserved residues in the \( \beta_2 \) strand and one in the \( \beta_7 \) strand (Figure 2.11) (Ferguson et al. 1995; Isakoff et al. 1998; Baraldi et al. 1999; Dowler et al. 2000; Ferguson et al. 2000; Lietzke et al. 2000; Lemmon and Ferguson 2001; Thomas et al. 2002; Cronin et al. 2004; Komander et al. 2004). I mutated a Lysine in the \( \beta_2 \) strand to an Alanine (K615A), and an Arginine in the \( \beta_5 \) strand to an Alanine (R667A), separately. When I repeated the PIP-binding assay to test these mutant ECT-2(PH-C), I noticed that GST-ECT-2(PH-C) showed even weaker binding to PI(4)P and PI(3,4,5)P\(_3\) while two positive control proteins showed strong binding, and binding pattern of GST-ECT-2(PH-C\(^{K615A}\)) and GST-ECT-2(PH-C\(^{R667A}\)) was indistinguishable from GST-ECT-2(PH-C) (Figure 2.12). Suggesting that the PH domain does not bind to the tested PIP species with high specificity and affinity under these conditions.

PI(4,5)P\(_2\) and PI(3,4,5)P\(_3\) have been implicated in epithelial cell polarization (Leslie et al. 2008) (for review). The apico-basal asymmetry of epithelial cells shares similarities with the antero-posterior asymmetry of the \textit{C. elegans} one-cell embryo. Orthologues of the anterior PAR proteins, PAR-3, PAR-6, PKC-3, and CDC-42, are found at the cell-cell junctions on the apical side of the epithelial cells, and orthologues of the posterior PAR protein, PAR-1, are found on the basolateral surface (Goldstein and Macara 2007) (for review). PI(4,5)P\(_2\) is found at the apical surface and PI(3,4,5)P\(_3\) is found at the basolateral surface, and PI(4,5)P\(_2\) works with the PAR proteins to polarize the epithelial cells (Leslie et al. 2008) (for review). Bazooka, the Drosophila homolog of PAR-3, has been shown to recruit PTEN (phosphatase and tensin homolog), which converts PI(3,4,5)P\(_3\) to PI(4,5)P\(_2\) at the cell-cell junctions (Pinal et al. 2006). In mammals, PTEN, through its product PI(4,5)P\(_2\), recruits Cdc42 at the cell-cell junctions, which in turn recruits Par6 and aPKC (Martin-Belmonte et al. 2007).
PI(4,5)P₂ distribution in the *C. elegans* one-cell embryo has been examined by two other groups, by imaging GFP::PH₇₅₆ which serves as a PI(4,5)P₂ probe, but the results are controversial. One lab showed that GFP::PH₇₅₆ accumulates on the whole membrane without any asymmetry, by mid-section microscopy (Motegi and Sugimoto, 2006). Another lab showed that GFP::PH₇₅₆ becomes anteriorly enriched in PAR-6 dependent manner, by cortical-section microscopy, but their mid-section images do not reveal asymmetry (Nakayama et al. 2009). Yet another lab showed that PPK-1, a homolog of Phosphatidylinositol-4-phosphate 5-Kinase which is an enzyme that converts PI(4)P to PI(4,5)P₂, is posteriorly enriched, which might suggest posterior enrichment of PI(4,5)P₂ (Panbianco et al. 2008). Due to these confusing results on PI(4,5)P₂ from multiple groups, I decided to examine how PI(3,4,5)P₃ distributes by expressing GFP::PH₄₃₆, a PI(3,4,5)P₃ reporter, instead of examining PI(4,5)P₂ reporters. GFP::PH₄₃₆ was highly enriched on the membrane of the gonad but the level decreased gradually as the oocytes mature so that GFP::PH₄₃₆ was not detectable on the membrane in one-cell embryos. Instead, GFP::PH₄₃₆ accumulated in the cytoplasm (Figure 2.13). I also tried GFP::PH-PH₄₃₆ which has two PH domains, because others had reported that the probes with tandem PH repeats can recognize its target PIPs better than the ones with single PH domain, *in vivo* (Stefan et al. 2002). The distribution of the reporter was similar to GFP::PH₄₃₆, except that the reporter formed small patch-like structures near the cell periphery (Figure 2.13). To test if the reporter reflects true PI(3,4,5)P₃ distribution, I depleted daf-18, a homolog of PTEN which converts PI(3,4,5)P₃ to PI(4,5)P₂ (Maehama and Dixon 1998; Ogg and Ruvkun 1998). Blocking the enzyme would be expected to increase the amount of PI(3,4,5)P₃. In *daf-18(RNAi)* embryos, GFP::PH-PH₄₃₆ was highly enriched even in the developing oocytes and the one-cell embryos but did not become polarized during the first cell cycle. The patch-like structures were not detectable in these embryos (Figure 2.13).
Figure 2.13. PI(3,4,5)P3 enrichment on the membrane in the gonads is gradually downregulated during oocyte development due to DAF-18/PTEN activity. Wide-field micrographs of worms and embryos expressing GFP::PH\(^{Akt1}\) and GFP::PH-PH\(^{Akt1}\) are shown.
These data suggest that in wild-type worms, PI(3,4,5)P$_3$ enrichment on the membrane is gradually down-regulated during oocyte development due to DAF-18/PTEN activity. However, unnaturally high enrichment of PI(3,4,5)P$_3$ does not seem to interfere with any aspect of the one-cell embryo polarization and further development, since *daf-18(RNAi)* embryos were viable even when fed with dsRNA for two generations (data not shown). Thus, I found no evidence that suggests a role for PIPs during one-cell embryo polarization.

In summary, my data indicate that the RhoGEF protein ECT-2, through the Rho GTPase RHO-1, serves as an important point of regulation for the polarization of the cortical actomyosin network in the *C. elegans* one-cell embryo. I have provided evidence that the unknown polarizing signal from the centrosomes reduces cortical accumulation of ECT-2 at the posterior pole and that the reduction of ECT-2 results in the reduction of RHO-1. My data suggest that ECT-2 and RHO-1 are required for activation of the actomyosin network, and the posterior reduction of ECT-2 and RHO-1 causes local downregulation of the actomyosin network. The resulting cortical flow has been previously shown to serve as the driving force that brings the anterior PAR proteins, PAR-3, PAR-6, and PKC-3, to the anterior cortex. My mutational analyses of ECT-2 revealed that the C-terminal region, containing a PH domain and a tail, is necessary and sufficient for its cortical accumulation, and sufficient for its local reduction. These data suggest that the PH domain and the C-terminal tail are important for ECT-2 regulation. To understand the exact mechanisms by which the polarizing signal regulates ECT-2 localization through the C-terminal region requires further research.
CHAPTER THREE

PHYSIOLOGICAL IMPORTANCE OF THE INTERACTIONS BETWEEN THE ANTERIOR PAR PROTEINS

INTRODUCTION

Work of the former members of the Kemphues lab have provided insights into the physiological importance of the interactions between the anterior PAR proteins, PAR-3, PAR-6, and PKC-3, in the *C. elegans* early embryo, using maternal-effect lethal alleles. In *C. elegans*, mRNAs and proteins are produced by the mother and loaded into the oocytes. These maternally provided materials support the embryo during early embryogenesis, until transcription of the embryonic genome takes place. A maternal-effect mutation blocks production of maternally provided materials. As a result, embryos homozygous for a maternal-effect mutation develop as wild type during early embryogenesis, due to the maternal contribution from its heterozygous mother. However, the embryos from a homozygous mother fail to develop normally, due to lack of the maternal contribution. Maternal-effect alleles are useful in studying function of genes during early embryogenesis. On the other hand, studying gene function during later development requires alleles that block production of zygotically provided mRNAs and proteins. The genes *par-3* and *par-6* are unusual in that both genes can mutate to specifically eliminate the maternal contribution without affecting the embryonically produced gene product. Thus, complete loss-of-function mutations are zygotic lethal: homozygous animals die in early larval stages. In contrast, animals homozygous for maternal-specific mutations live to be egg-laying adults but produce nothing but inviable embryos.
Donato Aceto revealed that the interaction of PAR-6 with PKC-3 through the PB1 domain is required for early embryogenesis, as maternally expressed PAR-6Δ15-28::GFP, which cannot bind to PKC-3, does not rescue a maternal-effect lethal allele of par-6, zu222. PAR-6Δ15-28::GFP also accumulated in the cytoplasm, which is different from cortical accumulation of PAR-6WT::GFP. Jin Li, in her work on PAR-6, made a surprising discovery, confirmed by studies of the PAR-3 protein by another lab member, Bingsi Li, that direct binding between PAR-3 PDZ-1 and PAR-6 PDZ appears not to be required during early embryogenesis. She found that PAR-6F192Y, D198P::GFP, a mutant that blocks PAR-6 PDZ binding to PAR-3 PDZ1 in vitro, can rescue the maternal effect mutant par-6(zu222). Surprisingly, data from Jin Li suggested that the interaction of PAR-6 with PDZ-binding proteins through the highly conserved hydrophobic binding pocket of the PDZ domain does not play an essential role during early embryogenesis, as par-6(zu222) embryos were rescued by maternally expressed PAR-6R166A, P167A::GFP and PAR-6P167G::GFP that contain mutations that disrupt the interaction of the hydrophobic binding pocket with its conventional target Pals1. Bingsi Li’s work on PAR-3 suggested that PDZ2, but not PDZ1 or PDZ3, is required for the localization and function of PAR-3. PAR-3 protein lacking PDZ2 accumulated on the cortex in sparse and large puncta early in the cell cycle but disappeared at later stages.

This chapter describes my work, in collaboration with other lab members, that extends the studies of the anterior PAR proteins. In collaboration with Jin Li, I present evidence consistent with Jin Li’s results that interaction between PAR-6 and PKC-3 is required for proper PAR-6 localization during late stages of C. elegans development, and that interaction of the highly conserved PDZ domain of PAR-6 and its conventional target proteins is not required during late stages of C. elegans development. I also show that disruption of the direct binding between PAR-3 and
PAR-6 does not affect their co-localization dynamics in the *C. elegans* one-cell embryo. This work was recently published as a co-first authored paper (Li et al. 2010). In collaboration with Bingsi Li, I show that PAR-3 PDZ2 is partially necessary for PAR-3 accumulation on the cortex, but absolutely necessary for recruitment of PAR-6 and PKC-3 on the cortex in the *C. elegans* early embryo. This work has been accepted for publication and is in press.

**MATERIALS AND METHODS**

**Construction of transgenes and production of transgenic lines**

To test the effect of PAR-6 mutants on the zygotic requirement for PAR-6, I used biolistic bombardment to generate transgenic lines expressing GFP-tagged wild-type or mutated genomic *par-6* under the control of its own promoter. Mutations were generated by fusion PCR using pJN284 as template (Nance et al. 2003). I bombarded *par-6::gfp* transgenes directly into KK1002, *par-6(tm1425)/hIn1[unc-54(h1040)]:unc-119(ed3)*. Strains expressing PAR-6<sup>F192Y, D198P</sup>::GFP in *par-6(zu222)* background (Jin Li's thesis), and expressing PAR-3<sup>ΔPDZ2</sup>::GFP in *par-3(it71)* (Bingsi Li's thesis) background were generated in a similar manner by Jin Li and Bingsi Li.

**Analysis of rescue by transgene**

Progeny from *par-6(tm1425)/hIn1[unc-54]* heterozygotes segregate three genotypes: *par-6(tm1425)/par-6(tm1425)*, which arrest as L2 larvae, *par-6(tm1425)/hIn1[unc-54]*, which are phenotypically wild-type, and *hIn1[unc-54]/hIn1[unc-54]*, which are paralyzed Unc. Rescue was scored as the ability to recover individual wild-type-looking fertile worms that failed to segregate Unc-54 progeny. To verify the presumed genotype of the rescued lines, I collected fertile
wild-type appearing adults from one line per each transgene, and genotyped them by single-worm PCR using primer sets that could detect the *tm1425* deletion, the rescuing *par-6::gfp* transgene, and the *par-6(+) gene in the balancer.

**Immunostaining**

Embryos were fixed in methanol following previously published procedures (Guo and Kemphues 1995). The following primary antibodies and dilutions were used: anti-PAR-3 mouse monoclonal (Nance et al. 2003) at 1:20; anti-PAR-6 rabbit polyclonal (Hung and Kemphues 1999) at 1:20. Incubation times and temperatures were as described by Nance et al. (2003). Primary antibodies were detected by Cyc3 labeled goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc.) at 1:200 and Alexa Fluor 488 labeled goat anti-Rabbit (Invitrogen) at 1:200. Confocal images were collected on a Leica TCS SP2 system with a Leica DMRE-7 microscope and an HCX PL APO 63× oil immersion lens. Images were processed using the Leica Confocal SP2 software program and Adobe PhotoShop. To quantify degree of co-localization of PAR-3 and PAR-6, three to four embryos were analyzed for each genotype. Six cortical sections 0.25μm apart were projected to obtain each image for analysis. For each embryo, two independent regions were analyzed. For each image, background, as defined by the cytoplasmic signal at the posterior cortex, was removed; remaining cortical puncta were analyzed for overlap.

**RESULTS AND DISCUSSION**

**Interaction of PAR-6 with PKC-3 is required for proper PAR-6 localization during late stages of *C. elegans* development.**

To test whether interaction of PAR-6 with PKC-3 is important during later
development of *C. elegans*, I tested whether expression of PAR-6^{Δ15-28}::GFP from the *par-6* endogenous promoter could rescue the putative *par-6* zygotic null allele, *tm1425* (Totong et al. 2007). The *par-6(tm1425)* allele is a 853-bp deletion that spans from the first exon to the second intron of T26E3.3a, which is the longer of the two isoforms of PAR-6, confirmed by cDNA (Figure 3.1). These two exons are absent in the shorter isoform. *tm1425* homozygotes can proceed through embryogenesis due to the maternal load of PAR-6, but arrest as young larvae. Out of 29 lines transformed with *par-6^{WT}::gfp* from a biolistic transformation experiment, I recovered 9 integrated lines, 3 of which expressed PAR-6^{WT}::GFP driven by the endogenous promoter. The *par-6^{WT}::gfp* transgene fully rescued homozygous *tm1425* worms and exhibited tissue and subcellular distributions consistent with previous analyses (Totong et al. 2007).

For *par-6^{Δ15-28}::gfp*, I recovered 34 lines in two independent biolistic transformation experiments; only 2 lines integrated the transgene into the genome and neither expressed GFP. I examined 10 non-integrated lines, and recovered 7 lines that expressed some GFP. When I examined expression in these lines, I noted that GFP accumulated in the cytoplasm of various tissues including hypodermis, vulva, and cells near the pharynx. Surprisingly, very few worms showed accumulation of PAR-6^{Δ15-28}::GFP in either pharynx or intestine, where PAR-6^{WT}::GFP is readily detected. In those few worms, expression was mosaic with only one or two of the intestinal cells or the pharyngeal cells expressing GFP. In those cells the mutant protein accumulated in the cytoplasm, but not the apical cortex (Figure 3.2), consistent with Jin Li’s results from expression of this same mutated transgene in the early embryo. I tested 2 lines with rare mosaic expression for rescue of the larval lethality in *par-6(tm1425)* and as expected for such mosaic expression, saw no rescue. Because the few lines I recovered did not integrate the transgene and showed only rare and mosaic GFP expression in the tissues where PAR-6 is normally expressed, it is possible that
Figure 3.1. *par-6* gene model. Exons are shown as boxes and introns are shown as lines connecting the boxes. The exons that are translated to form cDNA are shown in pink. Untranslated exons are shown in grey. *tm1425* allele is indicated as a red box. (Adopted from Wormbase; http://www.wormbase.org)
Figure 3.2. Interaction of PAR-6 with PKC-3, but not with the conventional target proteins of the PDZ domain, is required for proper PAR-6 localization during late stages of *C. elegans* development. Wide-field micrographs show distribution of PAR-6<sup>WT</sup>::GFP, PAR-6<sup>R166A,P167A</sup>::GFP, for PAR-6<sup>P167G</sup>::GFP, and PAR-6<sup>Δ15–28</sup>::GFP in larvae and late embryos. PAR-6<sup>Δ15–28</sup>::GFP shows mosaic expression and cytoplasmic accumulation.
expressing PAR-6Δ15-28::GFP in late stage embryos or larvae is toxic. Alternatively, coding sequences deleted in creating the transgene may be essential for proper expression. Although these experiments are inconclusive, they are consistent with the hypothesis that the association of PAR-6 and PKC-3 is required during late embryogenesis.

**Interaction of the highly conserved PDZ domain of PAR-6 and its conventional target proteins is not required during late stages of C. elegans development.**

To test whether a functional hydrophobic binding pocket is essential for PAR-6 PDZ domain function during later development of *C. elegans*, and to verify the surprising results with the maternally expressed protein from Jin Li's work, I generated transgenic lines that express wild-type or mutant PAR-6::GFP driven by the endogenous *par-6* promoter in a *par-6(tm1425)/hIn1(unc-54)* background. I recovered integrated and expressing lines for PAR-6WT::GFP (3 lines), PAR-6R166A,P167A::GFP (4 lines), and for PAR-6P167G::GFP (5 lines). For 2 lines from each genotype, I determined the pattern of GFP accumulation in homozygous *par-6(tm1425)* worms by whole-mount fluorescence imaging and tested the ability of the lines to rescue the lethality. In transgenic lines expressing PAR-6WT::GFP, GFP accumulated as previously reported for this construct (Totong et al. 2007), including on the apical surface of the pharynx and apical junctions of intestine cells in late embryos from comma stage to about 2-fold stage. In larvae and adults, GFP accumulated on the apical surface of the pharynx, intestine, anus, spermatheca, uterus, and vulva. Both mutant proteins showed a distribution indistinguishable from wild-type (Figure 3.2). Consistent with this, all of these mutant transgenic lines rescued *tm1425* homozygotes as well as PAR-6WT::GFP. Indeed, for both mutant transgenes, I was able to establish stable lines homozygous for *tm1425* and carrying the transgene as the only source of
full-length PAR-6 as verified by PCR genotyping. These data suggest that the highly conserved PDZ domain of PAR-6 and its conventional hydrophobic binding pocket does not play a critical role in later development of *C. elegans*.

**Disruption of the direct binding between PAR-3 and PAR-6 does not affect their co-localization dynamics in the *C. elegans* one-cell embryo.**

To test the effects of disruption of the direct binding between PAR-3 and PAR-6 on their co-localization dynamics *in vivo*, I examined the distribution of PAR-3 and PAR-6^{F192Y, D198P}::GFP in *par-6(zu222)* homozygotes and quantified their co-localization in the cortical puncta in the one-cell embryo. In wild-type embryos, PAR-3 and PAR-6 co-localization is dynamic such that only about 40% of the cortical puncta recognized by the two proteins contain detectable levels of both proteins (Hung and Kemphues 1999). If the direct interaction between the two proteins plays a significant role in complex formation, I expected that I might see fewer cortical puncta that contained both PAR-3 and PAR-6^{F192Y, D198P}::GFP relative to PAR-6^{WT}::GFP. I found no significant difference between the extent of co-localization of the two proteins; 41±4% of the puncta contained both PAR-3 and PAR-6::GFP (n=3 embryos; puncta were counted in two different areas of each embryo) and 44±1% of the puncta contained both PAR-3 and PAR-6^{F192Y, D198P}::GFP (n=3 embryos) (Figure 3.3). As a control for possible effects of transgene over-expression, I also examined untransformed wild-type embryos (N2 strain) and noted that although the overall level of PAR-6 protein appeared lower, the extent of co-localization was similar; 35%±4 (n=4 embryos). These data suggest that disrupting the potential for direct binding between PAR-3 and PAR-6 through their PDZ domains does not change their co-localization dynamics.
Figure 3.3. Disruption of the direct binding between PAR-3 and PAR-6 does not affect their co-localization dynamics in the *C. elegans* one-cell embryo. Confocal micrographs of the cortical section of one-cell embryos stained with anti-PAR-3 (shown in red) and anti-PAR-6 (shown in green) antibodies are shown. Yellow dots indicate colocalization. The three smaller panels at the bottom of each panel show the puncta in the boxed regions (anti-PAR-6, anti-PAR-3, and merge) after image processing to remove cytoplasmic background.
Figure 3.4. PAR-6 and PKC-3 do not accumulate at the cortex in par-3^{ΔPDZ2}::gfp; par-3(it71) one-cell and two-cell embryos. (A) Confocal images of midsections of one-cell and two-cell embryos. Images of par-3^{ΔPDZ2}::gfp; par-3(it71) are projections of six adjacent sections. All the other images are single sections. Cytoplasmic puncta in anti-PAR-6 stained embryos are P granules. (B) Confocal images of cortical sections of one-cell embryos.
Figure 3.4. (continued)

B

one-cell embryos

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PAR-3 PDZ2 is partially necessary for PAR-3 accumulation on the cortex, but absolutely necessary for recruitment of PAR-6 and PKC-3 on the cortex in the *C. elegans* early embryo.

In collaboration with Bingsi Li, I tested whether PDZ2 deletion affected the ability of PAR-3 to associate with PAR-6 and PKC-3 in these puncta. In *par-3::gfp; par-3(it71)* embryos, PAR-3 puncta co-localized extensively with PAR-6 and PKC-3. However, the large puncta containing PAR-3\(^{ΔPDZ2}\)::GFP failed to co-localize with PAR-6 (n=15 embryos) or PKC-3 (n=15 embryos). Indeed, PAR-6 and PKC-3 were not enriched on the cortex at all in these embryos (Figure 3.4). The two-cell embryos in Figure 3.3 represent the most striking examples; PAR-3 is enriched at the boundary between two cells whereas PAR-6 and PKC-3 are not detectable. These data suggest that PDZ2 is partially necessary for PAR-3 accumulation on the cortex, but absolutely necessary for recruitment of PAR-6 and PKC-3 on the cortex.

In summary, our data suggest that the interaction of PAR-6 and PKC-3 is required for proper localization and function of PAR-6 throughout the development of *C. elegans*. Surprisingly, our data also suggest that the direct interaction between PAR-6 and PAR-3, and interaction of PAR-6 PDZ domain and its putative binding partners are dispensible throughout the development of the animal. Interestingly, we have shown that PAR-3 PDZ2 domain plays an important role in cortical accumulation of PAR-3, PAR-6, and PKC-3. Identifying factors interacting with PAR-3 PDZ2 will help us understand its exact role in localization of the anterior PAR proteins.
CHAPTER FOUR

SUMMARY AND FUTURE DIRECTIONS

After fertilization, activation of the cortical actomyosin network of the *C. elegans* one-cell embryo causes the cortex to ruffle. This cortical contractility is locally down-regulated on the future posterior pole, near the centrosomes containing paternally provided centrioles. Local down-regulation of the contractility and imbalance of the tension within the actomyosin network result in cortical and cytoplasmic flows which serve as the driving forces for the polarization of the zygote. The nature of the polarizing cue is not known yet, but the centrosomes have been identified as the source of the cue.

In this thesis, I have presented evidence that Dbl-family RhoGEF protein ECT-2 and Rho GTPase protein RHO-1 serve as one of the links between the polarizing cue and polarization of the actomyosin network. My data suggest that ECT-2 acts through RHO-1 to activate the contractile actomyosin network on the cortex of the zygote, and ECT-2 responds to the polarizing signal and its cortical accumulation becomes locally reduced on the future posterior pole near the centrosomes. Full extent of ECT-2 reduction appears to be dependent on the cortical flow, but its initial reduction can still occur even when cortical flow is blocked by depletion of RHO-1, suggesting that its initial response to the polarizing signal is accomplished by cortical flow-independent mechanism. I determined that the C-terminal region of ECT-2, containing a PH domain and a C-terminal tail, is necessary and sufficient for both its accumulation at the cell periphery and posterior reduction. I have carried out yeast two-hybrid screens to identify factors interacting with the C-terminal region of ECT-2, in hope of elucidating mechanisms by which the polarizing signal reduces cortical accumulation.
of ECT-2. I have identified several candidates, and interestingly, most of them have PDZ domains. However, results from my further analysis suggest that the PDZ-binding motif at the tail of ECT-2 is not required for its localization and function, and that none of the candidate factors are required during early embryogenesis. I have also carried out in vitro PIP-binding assays, using the C-terminal region of ECT-2, to test if the interactions the PH domain with PIPs could be important for cortical accumulation and posterior reduction of ECT-2. My data suggest that the PH domain of ECT-2 does not show high affinity to PIPs in vitro. I have analyzed the distribution of PI(3,4,5)P3 by visualizing two probes, GFP::PHAkt1 and GFP::PH-PH Akt1. The probes accumulated on the cortex in the early oocytes, but the cortical accumulation decreased during oogenesis so that the zygote showed no enrichment on the cortex. Blocking the conversion of PI(3,4,5)P3 to PI(4,5)P2 by depletion of DAF-18, a homolog of PTEN, resulted in enrichment of GFP::PH-PH Akt1 in early embryos, suggesting that PI(3,4,5)P3 is down-regulated during oogenesis and early embryogenesis in wild-type, due to the DAF-18 activity. However, down-regulation of PI(3,4,5)P3 does not appear to be required for embryogenesis, because the daf-18(RNAi) embryos are viable.

The fact that neither the PH domain nor the C-terminal tail is sufficient for localization of ECT-2 indicates that elements in both domains are required. The PH domains from many proteins have been implicated in membrane-targeting through binding to either PIPs or other proteins. On the other hand, the C-terminal tails of ECT-2 and its orthologues do not possess any known functional domains, and their binding partners have not yet been identified. Studies in mammals and Drosophila suggest that the C-terminal tail is involved in regulating specificity of Ect2/Pebble between Rho and Rac. However, it is unlikely that asymmetric localization of ECT-2 is achieved through regulation of its specificity to RHO-1, because my data indicate that ECT-2 accumulates at the cell periphery and becomes partially reduced at the
posterior pole independent of RHO-1.

The C-terminal tail of Pebble, in Drosophila, has also been shown to be important for its cortical localization. There are several short stretches with homology among Ect2, Pebble, and ECT-2, despite low overall homology in the C-terminal tail (Figure 4.1). It is a possibility that these homologous regions are important for their localization. Determining the requirements of these regions will require additional mutational analysis. If we are able to determine which region is important, we can use that knowledge to identify factors that associate with it, for example, by pull-down assays and mass spectrometry. Further investigation of these homologous regions may provide insights into the mechanism by which the polarizing signal reduces ECT-2 accumulation at the posterior pole. It may also provide insights into how the localization of its orthologues is regulated in mammals and Drosophila.

I also studied the physiological importance of the interactions between the anterior PAR proteins, in collaboration with former lab members. My work on PAR-6 suggests that interaction of PAR-6 with PKC-3 is required for proper PAR-6 localization during late stages of *C. elegans* development, and that interaction of the highly conserved PDZ domain of PAR-6 and its conventional target proteins is not required during late stages of *C. elegans* development. My data also indicate that disruption of the direct binding between PAR-3 and PAR-6 does not affect their colocalization dynamics in the *C. elegans* one-cell embryo. My work on PAR-3 suggests that PAR-3 PDZ2 is partially necessary for PAR-3 accumulation on the cortex, but absolutely necessary for recruitment of PAR-6 and PKC-3 on the cortex in the *C. elegans* early embryo.

To understand more precisely how PAR-3 localization is regulated, it is important to identify binding partners of PDZ2. In mammalian epithelial cells, Par3 PDZ2 is recruited to the tight junctions by binding to PI(4,5)P$_2$ (Wu et al. 2007). To
Sequence comparison of the C-terminal tail of ECT-2 and its homologs

**Figure 4.1. Short patches of homologous regions in the C-terminal tail of ECT-2 and its orthologues**
determine if PAR-3 PDZ2 also binds PI(4,5)P₂ and if the binding is sufficient for PAR-3 localization, experiments including in vitro PIP-binding assays and substitution of PDZ2 with the PH domain of PLCδ1 have been proposed (Bingsi Li's thesis).
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