DIFFERENT DOMAINS OF C. ELEGANS PAR-3 ARE REQUIRED AT

DIFFERENT TIMES IN DEVELOPMENT

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DIFFERENT TIMES IN DEVELOPMENT

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Polarity is a fundamental cellular feature that is critical for generating cell diversity and maintaining organ functions during development. In *C. elegans*, the one-cell embryo is polarized via asymmetric localization of the PAR proteins, which in turn are required to establish the future anterior-posterior axis of the embryo. PAR-3, a conserved PDZ domain-containing protein, acts with PAR-6 and PKC-3 (atypical protein kinase; aPKC) to regulate cell polarity and junction formation in a variety of cell types. To understand how PAR-3 localizes and functions during *C. elegans* development, we have produced targeted mutations and deletions of conserved domains of PAR-3 and examined the localization and function of the GFP-tagged proteins in *C. elegans* embryos and larvae. We find that neither PDZ1 not nor PDZ3 are essential for localization or function. PDZ2, however, is required for PAR-3 to accumulate stably at the cell periphery in early embryos and at the apical surface in pharyngeal and intestinal epithelial cells. CR1, the PAR-3 self-oligomerization domain, is required for PAR-3 cortical distribution and function only during early embryogenesis. We also show that phosphorylation at S863 by PKC-3 is not essential in early embryogenesis, but is important in later development. Our results indicate that the different domains and phosphorylation forms of PAR-3 can have different roles during *C. elegans* development.

BIOGRAPHICAL SKETCH

Bingsi Li was born in November, 1979 in Beijing, China. At the early stage of her life she showed interest and talent in reading and writing. When she was six, she won a nation-wide writing contest. She also served as a student reporter for local journals during her early teen years. For a long time her family, which include several linguists and historians, thought there would be another writer in the family. However things changed unexpectedly. Bingsi's hidden enthusiasm for science was revealed when she studied in Beijing No.8 middle school, where she met several great science teachers and inspirational friends. She spent almost every Saturday in the Beijing Olympic School in Biology for two years in high school. Her favorite memories are the times she spent with her classmates arguing about unusually designed experiments and sharing knowledge about peculiar animals. After she graduated, she decided to be a medical doctor to explore the mystery of life and to help others. In 1996 she entered Peking University Health Science Center, one of the leading medical schools in China, and was ranked as one of the top students in her class. In 2001, she obtained her Bachelor of Medicine degree (B.S.) and worked with Dr. Bo Zhang in Peking University on transcriptional regulation of telomerase for one year. In 2002, she came to Cornell University and then joined Dr. Ken Kemphues' lab, where she performed the work described in her thesis.

To my mother Yan and my husband Haizhi.

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AJ	adherence junction
A-P	anterior-posterior
CRIB	Cdc42/Rac interaction binding
GFP	green fluorescence protein
GAP	GTPase activation protein
GEF	guanine nucleotide exchange factor
GMC	ganglion mother cell
MDCK	Madin-Darby canine kidney
MTOC	microtubule organization center
PATJ	PALS1 associated TJ proteins
PB1	Phox and Bem1p domain
PCR	polymerase chain reaction
PDZ	PSD95, Dlg, ZO-1 domain
RNAi	RNA interference
SAR	subapical region
TJ	tight junction
ZA	zonula adherens
SJ	septate junctions

CHAPTER ONE

INTRODUCTION

Polarity is a fundamental feature of cells, from yeast positioning their budding site to neurons forming axons and dendrites. The development of multicellular organisms requires choreographed cell polarization to generate diverse tissue structures and maintain distinct organ functions. Some cells become polarized to segregate cell fate determinants unequally. For example, in *Drosophila*, most neural precursor cells divide asymmetrically to generate neurons and their support cells (Betschinger and Knoblich, 2004). Some cells become polarized to execute specialized functions. For example, the distinct apical and basolateral domains in epithelial cells provide barriers that regulate ionic homeostasis; while in nervous system the specification of extended axons are required for signal transduction over long distances (Macara, 2004b).

During the past several decades, the mechanisms that drive cell polarization have been widely explored in many model systems. The *C. elegans* embryo provides many advantages for studying the mechanisms controlling polarized cell division: First, asymmetric divisions are a prominent feature during early embryogenesis (Sulston et al., 1983). Second, the large, transparent early blastomeres are favorable for microscopy and physical manipulations. Third, powerful forward genetics and reverse genetics tools are well established in *C. elegans*, which makes studies on gene function rapid and thorough (Brenner, 1974; Chalfie et al., 1981; Cuppen et al., 2007; Kamath et al., 2003; Timmons and Fire, 1998). Finally, the development of transgenic animal production techniques allowed us to study some germ-line expressed genes that had been impossible by traditional microinjections (Praitis et al., 2001).

This thesis describes the work I have done to gain insight into how PAR-3, a conserved scaffold protein, controls embryonic and epithelial polarity in *C. elegans*. It

focuses on the structure and function analysis of PAR-3 in early worm embryos and developing larvae. In this introduction I will first discuss the events and the underlying mechanisms in the early worm embryo. Then I will review epithelial polarity development in flies, worms, and mammals. Finally I will discuss the structure and function of each PAR-3 domain in other organisms.

I. ASYMMETRIC DIVISIONS IN THE EARLY C. ELEGANS EMBRYO
1.1. The events of the first asymmetric cleavage

A wild-type *Caenorhabditis elegans* hermaphrodite can produce about 300 self-progeny at 20°C. Oocytes are produced in the two arms of the ovary. During larval development, sperm are made in each arm before the germline permanently switches to produce oocytes. During the first ovulation and fertilization event, mature sperm are pushed into the spermathecae, where they are stored and used to fertilize oocytes as they pass through (Hall et al., 1999).

Before fertilization, oocytes are arrested at meiotic prophase for days and have no inherent polarity (Goldstein and Hird, 1996; McCarter et al., 1997). The symmetry is broken upon sperm entry, which promotes the completion of meiosis. The position of the sperm at the end of meiosis determines the future posterior pole of the embryo (Albertson, 1984; Browning and Strome, 1996; Goldstein and Hird, 1996). After meiosis, the surface of the newly fertilized egg begins to ruffle, seen as a number of small and transient invaginations of the cortex. As a sign of active cortical contractility, ruffling can last up to several minutes (Cowan and Hyman, 2004a; Hird and White, 1993; Munro et al., 2004). Initially, the contractions are present all over the surface, but shortly after the paternal chromosomes decondense, a local cessation of ruffling occurs at the cortex adjacent to the sperm centrosome (Cowan and Hyman, 2004a; Hird and White, 1993; Munro et al., 2004). The smooth region rapidly expands

toward the opposite pole and ends at a deep invagination of the membrane, called the pseudocleavage furrow (Cowan and Hyman, 2004a; Hird and White, 1993; Munro et al., 2004). The sudden weakening of the tensioned cytoskeleton network at the cortex over the sperm centrosome promotes a dramatic movement of the cell contents: over a period of 10 to 15 minutes, cortical cytoplasm streams away from, and central cytoplasm streams toward, the sperm centrosome in a fountainhead-like fashion (Hird and White, 1993; Munro et al., 2004; Strome, 1986). The flow is believed to be responsible for the initial partitioning of molecules that are involved in zygotic polarity establishment (Cheeks et al., 2004; Munro et al., 2004) Meanwhile, the decondensing maternal pronucleus migrates toward the paternal pronucleus. After meeting in the posterior, they move back as a unit to the center of the embryo, and execute a 90° rotation to align the centrosomes along the A-P axis (Albertson, 1984; Hyman and White, 1987)

In metaphase and anaphase, the mitotic spindle shifts to the posterior, leading to an asymmetric division, which generates two daughter cells different in size and fate. The larger anterior AB cell gives rise to neurons, anterior pharynx, skin and other tissues; and the smaller posterior P1 cell produces germ line, intestine, posterior pharynx, muscle and other tissues (Kemphues, 2000). Moreover, the AB cell and P1 cell divide at different times and in different orientations. In the AB cell, which divides first, its spindle is perpendicular to the A-P axis. However, in the P1 cell, which divides second, an additional centrosome-nuclear rotation aligns the mitotic apparatus parallel to the A-P axis (Albertson, 1984; Cowan and Hyman, 2004a)

1.2. The sperm sends the initial polarity cue

Fertilization presumably brings the sperm contents—paternal DNA, centrosome, cytoplasm, and plasma membrane--into the egg (Siomos et al., 2001).

Unlike in *Drosophila*, there is no evidence to indicate that asymmetries before fertilization in *C. elegans* oocytes are relevant to embryonic polarity (Pellettieri and Seydoux, 2002; Wodarz, 2002). Investigations from several labs suggest that the sperm centrosome, which organizes astral microtubules, specifies the posterior pole. First, despite the entry position relative to oocyte polarity, the pole occupied by the sperm centrosome always becomes the posterior (Goldstein and Hird, 1996). Second, embryos mutant for genes that are required for paternal centrosome maturation fail to establish AP axis (Hamill et al., 2002; O'Connell et al., 2000). Third, anucleated sperm with a pair of centrioles can fertilize eggs and establish A-P polarity (Sadler and Shakes, 2000).

Although the paternal centrosome is believed to account for sending the initial polarity cue, the role of microtubules in this process is still controversial. In mutant embryos that arrest in meiosis with immature sperm asters, the persistent maternal meiotic spindle can specify some aspects of the posterior pole (Wallenfang and Seydoux, 2000). Since the meiotic spindle lacks centrioles and the reversed A-P polarity can be abolished by microtubule elimination, it suggests an important role of microtubule in at least this abnormal polarity establishment (Wallenfang and Seydoux, 2000). However, this polarity establishment is transient and partial, as P granules, a posterior marker, are dispersed throughout the cytoplasm instead of anteriorly concentrated (Wallenfang and Seydoux, 2000). In addition, in separate experiments, depletion of visible α-/β-tubulin by RNA interference (RNAi) fails to prevent polarization of the zygote, raising the possibility that centrosomal components may signal directly to the cortex (Cowan and Hyman, 2004b).

1. 3. The PAR proteins

1.3.1 The identification of the PARs

The key players for setting up embryonic polarity were identified in genetic screens aimed for mutants affecting the first asymmetric division (Kemphues et al., 1988; Morton et al., 2002; Watts et al., 1996). Six genes were identified in these screens and were named as *par* (*par-1* to *par-6*), standing for <u>par</u>titioning defective. Mutations in these genes perturb the early cleavage patterns without reducing the total cell number (Kemphues et al., 1988). The changes in the developmental potential of the blastomeres correlated with the absence of intestine and germline, and excess of pharyngeal and body-wall muscle to various extents (Bowerman et al., 1997; Crittenden et al., 1997; Draper et al., 1996). A seventh gene, *pkc-3* (atypical kinase C), can be classified as a *par* gene because depletion of it in the early embryos leads to phenotypes indistinguishable from those of *par-3* and *par-6* (Tabuse et al., 1998).

Mutants in each *par* gene exhibit most or all of a common set of polarity defects during the first few cell cycles, defined as the Par phenotypes: equal first cleavage, synchronous second cleavage, misorientated spindles at second cleavage, and mislocalization of cell fate determinants and P granules (Bowerman et al., 1993; Boyd et al., 1996; Etemad-Moghadam et al., 1995; Grill et al., 2001; Guo and Kemphues, 1995a; Hung and Kemphues, 1999; Kemphues et al., 1988; Tabuse et al., 1998). In addition, cytoplasmic flow is attenuated, and the pronuclei meet more medially during the first cell cycle (Etemad-Moghadam et al., 1995; Kemphues et al., 1988; Kirby et al., 1990). The phenotypes of some of the *par* mutants are displayed in Figure 1.1. The fact that most known cell fate regulators depend upon PAR activities for their asymmetric distribution implies that the PAR proteins are among the earliest, key molecules setting up embryonic polarity (Rose and Kemphues, 1998; Schneider and Bowerman, 2003).
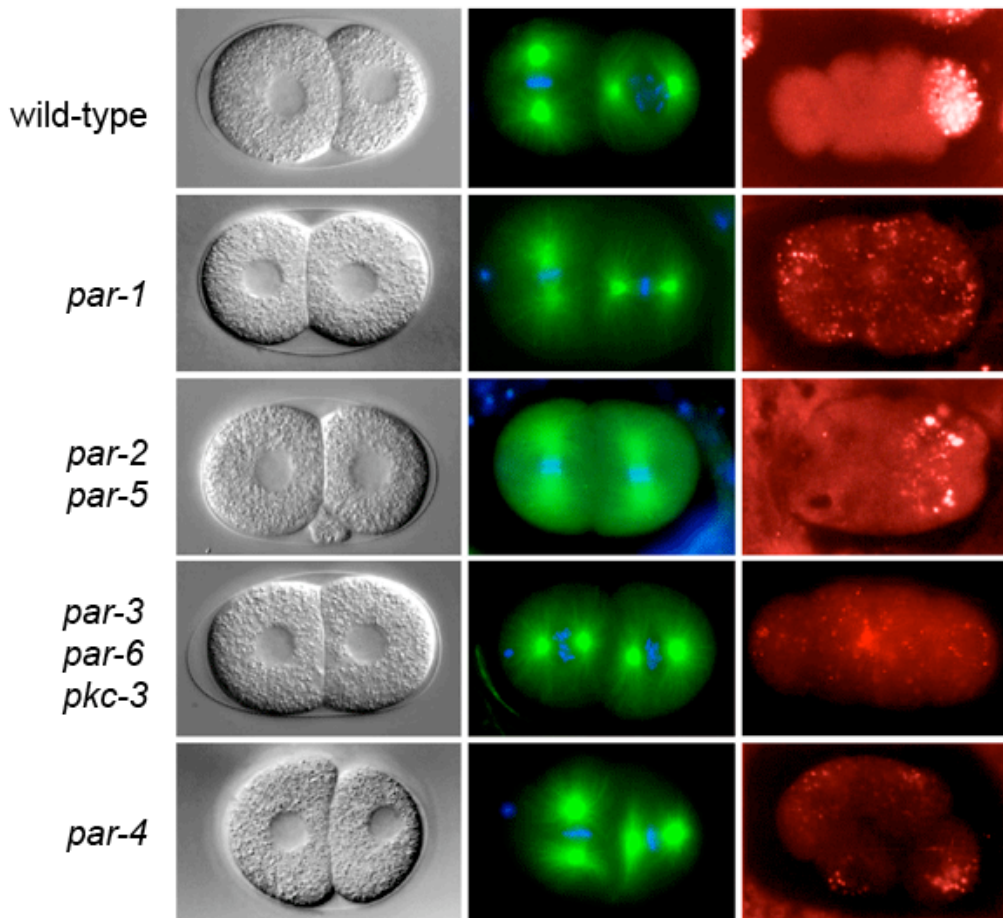
Figure 1.1 Par phenotype Each *par* mutant exhibits most or all of a common set of polarity defects during the first few cell cycles, defined as the Par phenotypes: equal first cleavage (right column), synchronous second cleavage and misoriented spindles at second cleavage (middle column; microtubules in green, chromosomes in blue), and mislocalization of cell fate determinants and P granules (left column; embryos are at four cell stage). Modified from K. Kemphues.

1.3.2 PAR Protein structures

Figure 1.2 is a summary of the PAR proteins' primary structures and identified motifs.
An evident feature of them is that the motifs they contain are often involved in
intracellular signal transduction (Rose and Kemphues, 1998). PAR-1, PAR-4, and
PKC-3 are serine/threonine protein kinases (Guo and Kemphues, 1995a; Tabuse et al.,
1998; Watts et al., 1996). PAR-3 and PAR-6 contain PDZ domains, which are well
known to mediate protein-protein interactions and scaffold signaling complexes
(Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Watts et al., 1996).
PAR-5 is a member of the 14-3-3 protein family, which has many roles in stimulating
protein-protein interactions and regulating protein activity and localization (Morton et
al., 2002). PAR-2 contains a ring-finger domain, which has been found in E3 ubiquitin
ligase subunits (Boyd et al., 1996; Levitan et al., 1994). Recent studies also added
CDC42, a member of the Rho GTPases, to this group since its removal in early
embryos causes phenotypes similar to those of the *par* mutants (Aceto et al., 2006;
Gotta et al., 2001; Kay and Hunter, 2001).

1.3.3 PAR Protein localization

The first clue to interpret how the PAR proteins control the polarization of the
zygote came from their distinct asymmetric localization. Studies on fixed embryos
revealed that four of the PAR proteins localize to the reciprocal cortical regions of the
one-cell embryo. PAR-3 and PAR-6 colocalize to the anterior cortex, and PAR-2 and
PAR-1 define the posterior cortical domain (Boyd et al., 1996; Etemad-Moghadam et
al., 1995; Guo and Kemphues, 1995b; Hung and Kemphues, 1999; Levitan et al.,
1994; Tabuse et al., 1998; Watts et al., 1996). PAR-4 and PAR-5 are uniformly
distributed in the cytoplasm as well as at the cortex (Morton et al., 2002; Watts et al.,
2000). Later work also showed that PKC-3 and active CDC42 also localize to the
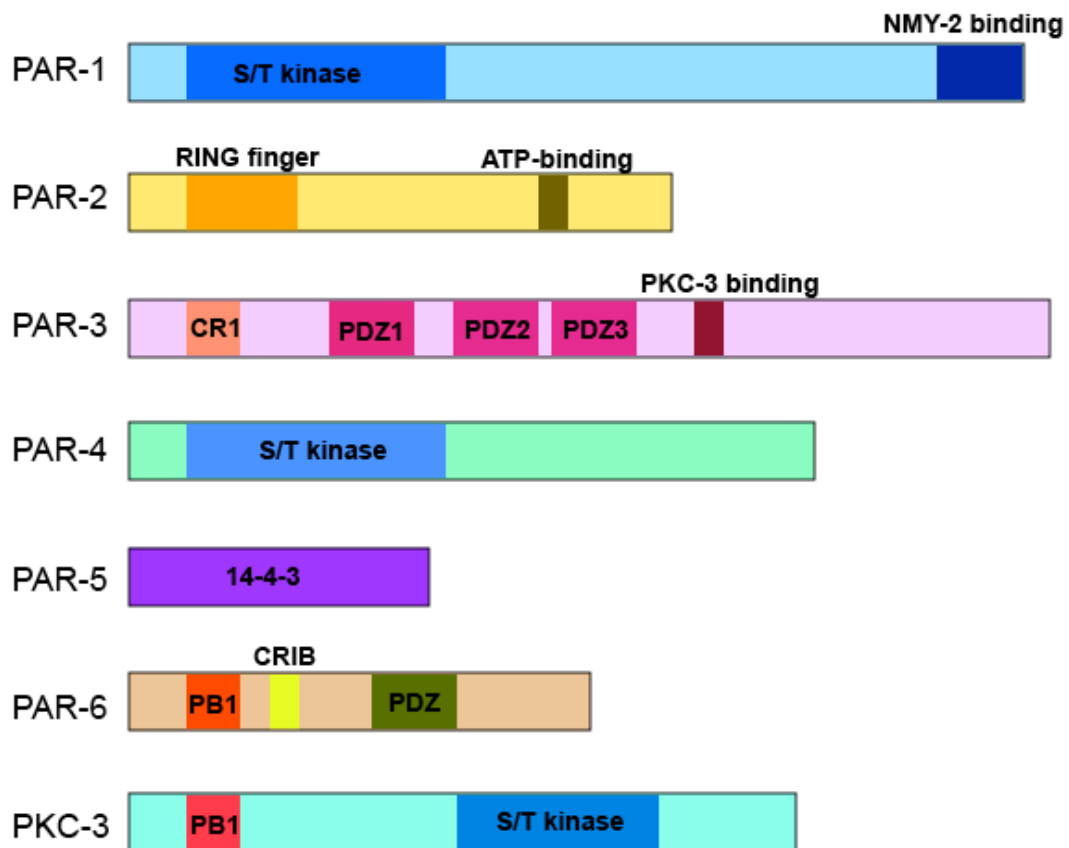
Figure 1.2 The structure of the PAR proteins. PAR-1, PAR-4, and PKC-3 are serine/threonine protein kinases; PAR-3 and PAR-6 contain PDZ domains, which are well known to mediate protein-protein interactions and scaffold signaling complexes; PAR-5 is one of the two 14-3-3 proteins in *C.elegans*; PAR-2 contains a ring-finger domain, which has been found in E3 ubiquitin ligase subunits.

anterior cortex of the zygote (Gotta et al., 2001; Kay and Hunter, 2001; Motegi and Sugimoto, 2006). The anterior PARs and the posterior PARs act antagonistically to maintain the A-P asymmetry. How they achieve their asymmetric localization and execute their functions is discussed below.

1.3.4 The interplay between actomyosin and PAR-3/PAR-6/PKC-3 during polarity establishment

The polarization of the worm zygote can be categorized into two phases: the initial polarity establishment phase (fertilization to pronuclear meeting) and the later maintenance phase (pronuclear meeting to telophase) (Cuenca et al., 2003). These two phases differ not only in time, but also in the proteins controlling them. A series of molecules have been found acting antagonistically to translate the initial polarity signal to a persistent asymmetry in the one-cell embryo. The complex interactions are summed up as a sequential repression model, seen in Figure 1.3.

PAR-3, PAR-6 and PKC-3 are the key regulators in the first phase. They interact with each other both *in vitro* and *in vivo*, forming a ternary complex and acting co-dependently (Hung and Kemphues, 1999; Tabuse et al., 1998; Watts et al., 1996) (Li et al., unpublished). Depletion of any of them results in the same phenotypes. In *par-3, par-6, and PKC-3(RNAi)* embryos, the polarity completely fail to establish, as cortical and cytoplasmic flow is abolished, P granules are partitioned incompletely or not at all, and the mitotic spindle is centrally positioned, resulting in an equal first cleavage (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Tabuse et al., 1998; Watts et al., 1996). At the two-cell stage, both AB and P1 are the same size and divide synchronously. The centrosome-nuclear rotation fails to occur in the P1 cell, resulting in the spindle forming parallel to the long axis of the embryo
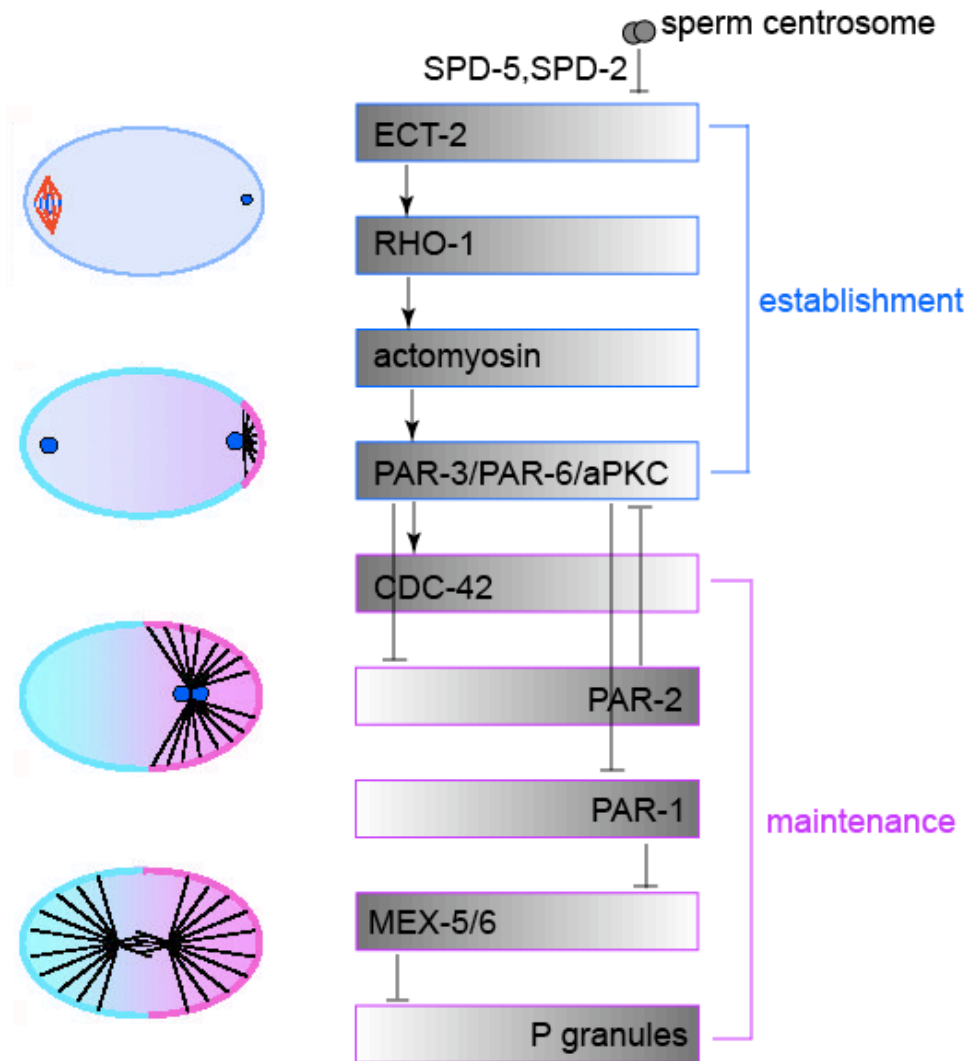
Figure 1.3 The sequential repression model for polarization of the *C.elegans* zygote Proteins that localize to the anterior are in blue (PAR-3, PAR-6, PKC-3 on the cortex, MEX-5 and MEX-6 in the cytoplasm). Proteins that localize to the posterior are in purple (PAR-2 and PAR-1 on the cortex, and P granules in the cytoplasm). Lines with bars depict antagonistic interactions, whereas lines with arrows depict positive interactions. Modified from Cuenca et al., 2003 and Motegi and Sugimoto, 2006.

(Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Tabuse et al., 1998; Watts et al., 1996).

PAR-3, PAR-6, and PKC-3 are the earliest PAR proteins that respond to the polarity signal and redistribute asymmetrically (Cuenca et al., 2003; Munro et al., 2004). During meiosis II, PAR-3, PAR-6 and PKC-3 accumulate at a high level around the entire cortex, while PAR-2 is uniformly distributed in the cytoplasm (Boyd et al., 1996; Cuenca et al., 2003; Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Tabuse et al., 1998). About 30 minutes after fertilization, PAR-3, PAR-6 and PKC-3 start to clear from the posterior pole in response to the polarity signal, allowing PAR-2 and PAR-1 to fill the cortical region left by the retraction of the anterior PARs (Cuenca et al., 2003; Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Munro et al., 2004; Tabuse et al., 1998). In the absence of PAR-2, the anterior PARs can still clear from the posterior pole and establish the anterior domain until pronuclear meeting, after which they spread to the posterior cortex gradually (Cuenca et al., 2003). In any of the anterior PAR mutant embryos, PAR-1, PAR-2 occupy the entire cortex (Boyd et al., 1996; Guo and Kemphues, 1995b). This evidence indicates that the anterior PARs react actively to the polarity cue, and PAR-2 functions to restrict the anterior PARs from leaking back to the posterior domain (Cuenca et al., 2003).

How the PAR proteins associate with the cortex is still not clear, but their cortical localization requires an intact microfilament network. Microfilament fibers and foci are present around the cell periphery and then are enriched in the anterior half in the early one-cell embryo (Hill and Strome, 1988). Chemical disruption of microfilaments with latrunculin A in early one-cell embryos blocks the cortical and cytoplasmic flows, and greatly reduces, if not eliminates, all cortical accumulation of PAR-3, PAR-6 and PKC-3 (Severson and Bowerman, 2003).

The mechanisms by which the anterior PARs establish their anterior domain appear to depend on the asymmetric contraction of the actomyosin network (Munro et al., 2004). Upon fertilization, the sperm-provided polarity signal causes a local weakening of the tensioned actomyosin network, resulting in an anterior-directed cortical flow (Munro et al., 2004). During the cortical flow, microfilaments, myosin, PAR-6, and yolk granules move toward the anterior pole at the same velocity, indicating that all they are components of the same profound cytoskeleton rearrangement (Munro et al., 2004). Depletion of non-muscle myosin II (NMY-2), myosin regulatory light chain (MLC-4), profilin (PFN-1), or the Formin Homology protein (CYK-1) abolishes the cortical contractility as well as the cytoplasmic and cortical flows, resulting in the failure of the clearance of PAR-3/PAR-6/PKC-3 from the posterior pole (Guo and Kemphues, 1996a; Munro et al., 2004; Severson and Bowerman, 2003; Shelton et al., 1999). Taken together, all these findings indicate that cortical transport is a dominant mechanism for the establishment of the PAR polarity (Munro et al., 2004).

The PAR proteins are not simply passive cargo carried by the flow. They also modulate cortical actomyosin dynamics actively to promote a sustainable movement (Munro et al., 2004). In *par-3* mutants, NMY-2 transiently clears away from the cortex next to the sperm centrosome, but fails to produce a directional flow (Munro et al., 2004). In summary, the cortical flows driven by asymmetric contraction of the actomyosin carry the anterior PARs as well as other cytoplasmic contents to the anterior side of the zygote. And the PAR proteins feed back to the loop by promoting a persistent flow.

1.3.5 RHO-1 and its regulators link the polarity cue to the PARs

Since the posterior relaxation of the actomyosin network directly links to the

PAR asymmetry, one of the main questions to be answered is what causes the local change in response to the initial polarity cue. Growing evidence implied that the response to the sperm-provided signal is mediated by RHO-1, a small GTPase that regulates actomyosin contractility in many other systems (Goldstein and Macara, 2007). GTPases are small signaling proteins that cyclebetween an active GTP-bound state and an inactive GDP-bound state (Erickson and Cerione, 2001; Etienne-Manneville and Hall, 2002).

Prior to meiosis II, RHO-1 is uniformly distributed around the periphery of the zygote as small foci and short filaments (Motegi and Sugimoto, 2006). As the sperm de-condenses, RHO-1 is excluded from the posterior cortex and forms an anterior cap in large foci (Motegi and Sugimoto, 2006). In *rho-1(RNAi)* embryos, the cortical contractility is lost, cortical and cytoplasmic flows are abolished, the PAR-3/PAR-6/PKC-3 fail to clear from the posterior pole, and PAR-2 is uniformly cytoplasmic (Jenkins et al., 2006; Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006).The initial exclusion of RHO-1 is dependent on the sperm signal, as in *spd-2* or *spd-5* mutant embryos, which fail to assemble functional centrosomes (Hamill et al., 2002; O'Connell et al., 2000), RHO-1 never clears from posterior pole (Motegi and Sugimoto, 2006; Kim et al., unpublished). However, none of the PARs and the components of actomyosin network are required for the initial clearance of RHO-1, indicating RHO-1 acts upstream of the asymmetric PAR proteins (Jenkins et al., 2006; Motegi and Sugimoto, 2006).

The activity of the Rho-family GTPases is regulated by guanine nucleotide exchange factors (GEFs) and the GTPase-activating proteins (GAPs) (Erickson and Cerione, 2001; Etienne-Manneville and Hall, 2002). GEFs promote the active state of GTPases by inducing GDP dissociation; and GAPs promote the inactive state of

GTPases by stimulating GTP hydrolysis (Erickson and Cerione, 2001; Etienne-Manneville and Hall, 2002).

Increasing evidences implies that the GEFs and GAPs are widely involved in sending/sensing the polarity signal and refining the PAR asymmetry in the worm zygote (Jenkins et al., 2006; Motegi and Sugimoto, 2006; Schonegg et al., 2007). Jenkins and colleagues presented evidence that the sperm-provided RhoGAP, CYK-4, acts as the initial polarity cue (Jenkins et al., 2006). *cyk-4(RNAi)* embryos display similar polarity defects as *rho-1(RNAi),* as the cortical flow is abolished, and PAR-3, PAR-6 are uniformly distributed at the cortex(Jenkins et al., 2006). Depletion of paternal CYK-4 causes impaired polarization in about half of the embryos produced by the wild-type mothers (Jenkins et al., 2006). CYK-4 in the wild-type sperm can rescue the polarity defects but not the impaired meiotic cytokinesis for fertilized *cyk-4(RNAi)* eggs (Jenkins et al., 2006). These are difficult experiments to carry out and several labs, including ours, have tried to repeat these results with no success.

In mammals, ECT-2, a RhoGEF protein, works with CYK-4 to regulate RHO activity during cytokinesis (Glotzer, 2001). Recent studies of the worm ECT-2 revealed its role in controlling asymmetric localization and/or activity of RHO-1, and subsequently the early polarity establishment (Jenkins et al., 2006; Motegi and Sugimoto, 2006). Despite some subtle dynamic differences, ECT-2 shows a similar localization to RHO-1, as it is uniformly distributed at the cell cortex before polarization, then clears from the posterior pole to occupy a common anterior domain shared by RHO-1, NMY-2, and the anterior PARs (Jenkins et al., 2006; Motegi and Sugimoto, 2006). Depletion of RHO-1 does not abolish the initial decrease of ECT-2 at the cortex nearest to the sperm centrosome; but RHO-1 fails to clear from the posterior pole in the absence of ECT-2 (Jenkins et al., 2006; Motegi and Sugimoto,

2006). This evidence indicates that ECT-2 acts upstream of RHO-1 to promote its asymmetric localization in response to the polarity cue.

One downstream effector of RhoA is RhoA kinase (ROCK), which activates myosin contraction by phosphorylating myosin light chain (MLC) (Riento et al., 2003). Staining of early embryos with antibodies that can specifically recognize phospho-MLC revealed co-localization of phopho-MLC to RHO-1 and ECT-2 (Jenkins et al., 2006). In *ect-2(RNAi)* or *rho-1(RNAi)* embryos, no phospho-MLC was detected at the cell periphery (Jenkins et al., 2006).

RGA-1 and RGA-4, two similar RhoGAP proteins are required to control the activity of the actomyosin network and the size of the PAR domains (Schonegg et al., 2007). *rga-3/-4 (RNAi)* embryos exhibit increased cortical contractility and an anterior shift of the boundary between the two reciprocal PAR domains (Schonegg et al., 2007). This also supports the "actymyosin relaxation" model in which upregulated cortical contractility will lead to an over-contraction of the meshwork toward the anterior, which in turn results in the expansion of PAR-2 domain and the shrinking of the PAR-3/PAR-6/PKC-3 domain (Schonegg et al., 2007).

1.3.6 CDC42 and PAR-2 in polarity maintenance

As PAR-3, PAR-6 and PKC-3 gradually clear away from the posterior, PAR-2 fills the cortical region left by the anterior PARs (Boyd et al., 1996; Cheeks et al., 2004; Cuenca et al., 2003; Levitan et al., 1994). During the maintenance phase of the cell cycle, posterior PAR-2 and anterior PAR-3/PAR-6/PKC-3 exclude each other from invading their respective territories to maintain the established AP axis (Boyd et al., 1996; Cheeks et al., 2004; Cuenca et al., 2003).

PAR-2 is the only PAR protein for which no homologs have been identified in other animals (Levitan et al., 1994). It has a myosin-like ATP binding site and a

RING finger domain that is typical for E3 ligases (Levitan et al., 1994). The relevant biochemical activity of PAR-2 has not been characterized, but it appears to maintain the polarity by preventing back-flow of the actyomyosin meshwork (Munro et al., 2004). In *par-2* mutant embryos, an ectopic posterior-directed cortical flow redistributes PAR-6 to the posterior cortex by metaphase (Munro et al., 2004). This reverse flow is not observed in wide-type embryos, suggesting that after the polarity is established, PAR-2 normally functions to prevent aberrant flows that otherwise carry the anterior PARs back to the posterior (Munro et al., 2004).

The anterior PARs also function to exclude PAR-2 from the anterior cortex (Hao et al., 2006). Human aPKC, which is very similar to PKC-3, phosphorylates PAR-2 directly *in vitro* (Hao et al., 2006). PAR-2::GFP with alanine substitutions in its PKC-3 sites spreads to the entire cortex, and a construct with glutamic acid substitutions fails to associate with the cell periphery (Hao et al., 2006). This evidence indicates that PAR-2 is a target of the PKC-3 kinase and is excluded from the anterior cortex by PKC-3-dependent phosphorylation (Hao et al., 2006). Similarly, PKC-3 may also phosphorylate PAR-1 to prevent PAR-1 extending into the anterior domain (J. Li, personal communication)(Hao et al., 2006).

CDC42, the Rho family GTPase, has been shown to participate in polarity maintenance and proper spindle orientation in the one-cell worm embryo (Gotta et al., 2001; Kay and Hunter, 2001). As a well-studied molecular switch, CDC42 controls many aspects of polarity, including yeast budding and mammalian epithelia formation (Etienne-Manneville and Hall, 2002; Hutterer et al., 2004; Johnson, 1999).

The phenotype of C. elegans embryos depleted of CDC42 by RNAi resembles the polarity phenotypes of *par-3, par-6,* and *pkc-3* mutants (Gotta et al., 2001; Kay and Hunter, 2001). Constitutively active CDC42(Q61L), but not constitutively inactive CDC42(T17N), co-localizes with PAR-6 (Aceto et al., 2006). Further analysis

showed that CDC42(Q61L) interacts with the semi-CRIB (Cdc42/Rac Interaction Binding) domain of PAR-6 (Aceto et al., 2006; Gotta and Ahringer, 2001). Disruption of CDC42(Q61L)-PAR-6 interaction results in CDC42(Q61L) and PAR-6 mislocalization during the first cell cycle(Aceto et al., 2006).

1.3.7 PAR-5 is required for the segregation of the reciprocal PAR domains

PAR-5, one of the two 14-3-3 proteins in *C. elegans*, is required for the formation of two distinct PAR domains (Cuenca et al., 2003; Morton et al., 2002). In *par-5* mutant embryos, the cortical contraction is aberrant, P granules fail to localize, the first cleavage is equal, and the second cleavage is synchronous and transverse (Morton et al., 2002), PAR-3/PAR-6/PKC-3 and PAR-2 expand into the corresponding opposite pole, resulting in the partial overlap of the anterior and the posterior domains (Cuenca et al., 2003; Morton et al., 2002). Similar to PAR-4, PAR-5 localizes uniformly in the cytoplasm as well as at the cell cortex (Morton et al., 2002). In mammalian epithelial cells, 14-3-3 binds to aPKC-phosphorylated PAR-1, inhibits its kinase activity and prevents its membrane association (Hurov et al., 2004; Suzuki et al., 2004). The mechanisms by which PAR-5 segregates the anterior and the posterior PARs in worm zygote is not known yet.

1.3.8 PAR-4 is required for polarized development

PAR-4 is present uniformly in the cytoplasm with a slight enrichment around the cell cortex (Watts et al., 2000). *par-4* mutant embryos differ in phenotype from mutants in other par genes. In *par-4* embryos, although P granules are dispersed and the second division is synchronous, cortical contractions still occur and the first cleavage is asymmetric (Morton et al., 1992; Watts et al., 2000). LKB1, the mammalian homolog of PAR-4 has been found correlated with the Peutz-Jeghers

Syndrome (PJS). PJS Patients are subjected to develop tumors originating from epithelial cells of a variety of organs (Baas et al., 2004). In mammals, LKB-1 acts as a master kinase that can phosphorylate and activate al least 14 downstream kinases, including PAR-1 (Alessi et al., 2006). The full kinase activity of LKB1 requires two cofactors: a pseudokinase, STRAD, and an armadillo-repeat-containing protein MO25 (Baas et al., 2003). Depletion of the worm STRAD can enhance the embryonic lethality of a *par-4* temperature-sensitive mutant dramatically, indicating PAR-4 may need STRAD to function in worm embryogenesis (D. Morton, personal communication).

1.3.9 PAR-1 partitions cell fate determinants

PAR-1 is a serine/threonine kinase involved in a variety of aspects of cell polarization. For example, in *Drosophila* oocyte, PAR-1 is required for the posterior localization of the germline determinant *Oscar* by stabilizing microtubules and phosphorylating *Oscar* (Shulman et al., 2000). It also regulates the anterior localization of *bicoid* mRNA by phosphorylating Exuperantia, a mediator required for *bicoid* transport (Riechmann and Ephrussi, 2004).

The worm *par-1* mutant embryos have polarity defects including an equal first cleavage, synchronous second division, misoriented spindles and a failure to segregate P granules and lineage determinants (Guo and Kemphues, 1995b). Similar to PAR-2, PAR-1 accumulates at the posterior cortical region left by the anterior PAR clearance. The observation that PAR-1 is mislocalized in all other *par* mutants but *par-1* mutants have no effects on the distribution of other PARs suggest that PAR-1 is the most downstream protein among the PARs (Boyd et al., 1996; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995b; Guo and Kemphues, 1996c; Tabuse et al., 1998; Watts et al., 1996)).

The molecules that anchor PAR-1 at the posterior cortex are still unknown. Interaction between PAR-1 C-terminal region and the tail part of NMY-2 was indentified in *in vitro* binding assays (Guo and Kemphues, 1996b). So far no direct substrate of PAR-1 has been identified in *C. elegans*, but it appears to function by preventing P granules and other germ-line destined molecules from ubiquitin-dependent degradation in the posterior of the zygote (Cheeks et al., 2004; DeRenzo et al., 2003). This function of PAR-1 requires MEX-5 and MEX-6, two highly similar CCCH finger proteins that function redundantly (Cuenca et al., 2003; Schubert et al., 2000). Depletion of MEX-5/-6 only has subtle effects on PAR localization, but causes mislocalization of most known fate determinants such as MEX-1, MEX-3, SKN-1, PIE-1, POS-1, GLP-1 and P granules (Cuenca et al., 2003; Schubert et al., 2000).

MEX-5 and MEX-6 are distributed in the anterior cytoplasm of the one-cell embryo and the asymmetry depends on PAR-3 and PAR-1 (Cuenca et al., 2003; Schubert et al., 2000). Recent studies revealed that MEX-5 asymmetry requires phosphorylation at a single serine S458, and this phosphorylation is dependent on PAR-1 and PAR-4 kinase activity, although it is not yet known whether either kinase acts directly on MEX-5 (Tenlen et al., 2008).

1.4 Spindle orientation

In the one-cell worm embryo, the spindle needs to be placed correctly to bisect across the axis of asymmetry, generating two daughter cells different in molecular composition and size. The spindle positioning results from a series of movements. The maternal pronuleus migrates to meet the sperm pronucleus in the posterior, and the joined pronuclei move back as a unit to the center, termed centration (Albertson, 1984; Hyman and White, 1987). As the apposed pronuclei approach the cell center, the nuclear-centrosome complex rotates 90° to align along the AP axis, ensuring the

future cleavage plane is perpendicular to the long axis (Albertson, 1984; Hyman and White, 1987). These processes are dependent on astral microtubules, dynein and dynactin (Gonczy et al., 1999; Skop and White, 1998).

After rotation and centration, the spindle moves toward the posterior pole as it elongates during anaphase (Albertson, 1984; Hyman and White, 1987). The posterior displacement of the spindle results in a larger AB cell and a smaller P1 cell after the first division (Albertson, 1984). Disruption of the central spindle using a laser beam causes the two centrosomes to move apart rapidly from each other, with the posterior centrosome moving farther, and faster, indicating the presence of unequal pulling forces on astral microtubules, presumably from the cortex (Grill et al., 2001).

PAR-3 and PAR-2 are required to generate asymmetric cortical pulling forces on microtubules (Grill et al., 2001). In *par-2* and *par-3* mutants, the first mitotic spindle remains centrally positioned, producing daughter cells of equal size (Kemphues et al., 1988). Severing the central spindle in *par-2* mutants results in both centrosomes moving apart with the same speed as the anterior centrosome in wild-type zygote; while in *par-3* mutants both move apart with the same speed as the posterior centrosome (Grill et al., 2001). As previously discovered by Cheng et al., 1995, the *par-3 par-2* double mutant behaves like *par-3*, indicating that PAR-3 reduces the cortical forces at the anterior pole, and, PAR-2 functions to restrict PAR-3 to the anterior cortex (Grill et al., 2001). Although how the PAR proteins modulate the cortical pulling forces is still unclear, they appear to act through two redundant heterotrimeric G protein α subunits, called GOA-1 and GPA-16 (Colombo et al., 2003; Gotta and Ahringer, 2001). GOA-1/GPA-16 are present in the cytoplasm, diffusely at centrosomes and at the cortex (Colombo et al., 2003; Gotta and Ahringer, 2001). Depletion of GOA-1/GPA-16 dramatically reduces the pulling forces applied on the spindle poles (Colombo et al., 2003; Gotta and Ahringer, 2001). GPR-1 and

GPR-2 are two nearly identical proteins working redundantly to control cortical pulling forces (Gotta et al., 2003). They are postertiorly localized regulators of Gα subunits, linking the cortex and the asymmetric pulling forces (Colombo et al., 2003; Gotta and Ahringer, 2001). Depletion of them results in phenotypes similar to those in embryos lacking GOA-1/GPA-16 (Colombo et al., 2003; Gotta and Ahringer, 2001; Gotta et al., 2003). Another regulator of spindle pulling forces is LIN-5, a coiled-coil protein. Recent studies showed that LIN-5 becomes enriched at the anterior cortex during rotation and centration, and is required for the cortical localization of GPR-1/-2 (Gotta et al., 2003; Lorson et al., 2000; Park and Rose, 2008; Srinivasan et al., 2003). LIN-5 is a distant homologue of MT-binding protein NuMA and Mud. Mammalian and fly homologs of LIN-5, GPR-1/-2 form a trimeric complex with Gα to regulate spindle positioning in flies and mammals (Bowman et al., 2006; Du and Macara, 2004; Izumi et al., 2006).

PAR-2 and PAR-3 control the spindle placement in early cleavages at least in part by determining the posterior enrichment of GPR-1/2 and the circumferential cortical localization of LET-99, a DEP domain proteins that may negatively regulate cortical forces (Colombo et al., 2003; Gotta and Ahringer, 2001; Tsou et al., 2002). The PAR proteins may also regulate the pulling forces by affecting microtubule dynamics, as PAR-3 acts, presumably indirectly, to stabilize microtubules that reach the anterior cortex (Labbe et al., 2003).

1.5 General cellular mechanisms that affect embryonic polarity

The identification of the *pod* mutants, many of which are components of anaphase promoting complex (APC), revealed that cell cycle machinery is involved in proper polarity establishment (Rappleye et al., 2002). Mutations in *pod3, pod-4, pod-5,* and *pod-6* lead to meiotic delay, osmotic sensitivity, and various polarity defects

including failed sperm apposition, mislocalization of the PAR proteins, and centrally positioned spindle (Rappleye et al., 2002). However, it is difficult to conclude if the polarity defects reflect a direct regulation by the APC pathway or a general requirement of proper progression of meiosis.

Fatty acids have also been shown to be required for correct polarity setup in the one-cell embryo (Rappleye et al., 2003). POD-2, the only acetyle-CoA carboxylase in *C. elegans*, is the rate-limiting enzyme for *de novo* fatty acid biosynthesis. EMB-8, a NADPH-cytochrome P450 reductase, is one of the key molecules in fatty acid desaturation pathway (Rappleye et al., 2003). Mutations in both genes lead to defects in PAR localization, spindle positioning and P granules restriction (Rappleye et al., 2003). Other members of fatty acid metabolism also appear to be required in the polarization process, and exogenous supplied fatty acids can rescue the polarity defects to some extent (Rappleye et al., 2003) (D. Morton, personal communication). It is possible that proper fatty acid composition of the zygote membranes is necessary for proper embryonic polarity establishment (Rappleye et al., 2003).

Recent works from several labs have revealed roles of anterior PAR proteins in endocytic traffic regulation, and phospholipid synthesis pathway in spindle positioning (Balklava et al., 2007; Panbianco et al., 2008). All these findings provide new insight into the mechanism underlying the polarity establishment and maintenance in the *C. elegans* embryo.

II: PAR-3 IN EPITHELIAL POLARIZATION IN FLIES, WORMS AND MAMMALS

As discussed above, PAR-3, PAR-6, and PKC-3 play fundamental roles in *C. elegans* embryo polarization. In addition, their homologues have been identified in yeast, flies, frogs, fish, and mammals, and control diverse aspects of cell polarization

(Goldstein and Macara, 2007). How they achieve their special cellular localization and function together or independently to polarize cells has remained unsolved in many areas. Here I will review some of the cases in which mechanisms of PAR-3/PAR-6/aPKC-dependent cell polarization have been well investigated.

2.1. Asymmetric divisions in the *D. melanogaster* neuroblasts

In the past several decades, the asymmetric division of *Drosophila* neuroblasts has been studied deeply and provided tremendous knowledge about polarity establishment and cell fate determination (Yu et al., 2006). In the fly central nervous system, neural precursors called neuroblasts (NBs) delaminate from ventral neuroectoderm and undergo several rounds of asymmetric divisions in a stem cell-like manner (Betschinger and Knoblich, 2004). Each division produces a smaller basal ganglion mother cell (GMC) and a larger apical NB. The GMC divides to generate neurons or glia, while the newly formed NB keeps the stem cell potential and continues to divide asymmetrically. The difference in fate between GMC and NB results from exclusive segregation of neural cell fate determinants such as Prospero and Numb to the GMC (Betschinger and Knoblich, 2004).

The polarity of *Drosophila* NBs is established when they become specified within the neuroectoderm (Jan and Jan, 2001). During NBs delamination, Bazooka (Baz), DmPar-6, and aPKC (the *Drosophila* homologues of *C.elegans* PAR-3, PAR-6 and PKC-3, respectively) spread from the subapical region to the entire apical cortex (Petronczki and Knoblich, 2001; Rolls et al., 2003; Wodarz et al., 2000). During this process, an adaptor protein called Inscuteable is recruited to the apical cortex by Bazooka (Schober et al., 1999; Wodarz et al., 1999). Inscuteable in turn recruits Pins (Partner of Inscuteable) and its downstream effector Gαi, the α subunit of

heterotrimeric G protein to the apical complex (Schaefer et al., 2001; Schaefer et al., 2000; Yu et al., 2000).

The correct build-up of the apical complexes ensures two key processes to occur properly. First, Gαi is important for mitotic spindle alignment during NB division, and Pins appear to be essential for Gαi activity at the apical side. *Gαi* and *pins* mutants have partial defects in spindle orientation. NBs devoid of both of them divide equally, resulting in two same-sized daughter cells (Cai et al., 2003; Fuse et al., 2003; Izumi et al., 2004; Kimple et al., 2002; Schaefer et al., 2001; Yu et al., 2003). How Pins and heterotrimeric G proteins control the NB spindle orientation is not fully understood yet, although it may involve direct binding to microtubules or dynein-associated protein as is the case for their homologues in mammals (Du et al., 2001; Merdes et al., 1996; Roychowdhury et al., 1999; Wang et al., 1990). Second, the Baz/Par-6/aPKC complex directs neural fate determinants (Prospero and Numb) and their adaptor proteins (Miranda and Pon, respectively) to segregate only into the basal GMC in a sequential repression manner (Betschinger et al., 2003). During the interphase of NB division, Miranda and Prospero accumulate apically, while Pon and Numb distribute uniformly around the cortex. When the NB enters prophase of mitosis, both complexes redistribute to the basal crescent, and then segregated into the basal GMC during cytokinesis (Betschinger and Knoblich, 2004). The redistribution of the neural fate determinants requires a restricted inactivation of Lethal giant larvae, a WD-40 domain containing proteins, on the apical cortex (Albertson and Doe, 2003). Lgl is uniformly localized around the NB periphery and is a kinase target of aPKC (Betschinger et al., 2003; Ohshiro et al., 2000). On the apical cortex, Lgl is phosphorylated and inactivated, allowing myosin II to accumulate and prevent determinants from localizing apically by an unknown mechanism (Barros et al., 2003; Betschinger et al., 2005). On the basal side, Lgl remains active and inhibits the

assembly of myosin filaments, allowing the association of the determinants with the cortex (Peng et al., 2000; Strand et al., 1994). During NB division, myosin II moves down to the cleavage furrow and appears to "push" determinants to GMC (Barros et al., 2003). In addition, myosin VI Jaguar binds to Miranda directly and probably transports Miranda to the basal cortex (Petritsch et al., 2003).

In all cases, Bazooka is the most upstream gene in the linear hierarchy. In Bazooka null germline clones, all molecules mentioned above are mislocalized, basal crescents do not form and spindles are randomly oriented (Kuchinke et al., 1998; Rolls et al., 2003; Schober et al., 1999; Wodarz et al., 1999). How Bazooka associates with the cortex and achieves its apical distribution remains unknown.

2.2 Epithelial Polarization in Worms, Flies and Mammals

Epithelial cells are present in most metazoa and the acquisition of apico-basal polarity is crucial for epithelial cells to perform their specialized functions. They confine the neighboring tissues from the environment, control the exchange of ions and molecules, and govern the shape change and directional movement during morphogenesis (Nelson, 2003). The formation of cell-cell junctions is a key step in epithelial polarization, defined as the establishment of asymmetric composition and organization of cellular components along the apicobasal axis (Macara, 2004b). For example, overexpression of E-cadherin, one of the main components of adherens junctions (AJ), can drive AJ formation and differential distribution of polarity markers in non-polarized fibroblasts (McNeill et al., 1990).

In most animals examined thus far, AJs form an adhesive belt that encircles the apex of the epithelial cell (Knust and Bossinger, 2002). In vertebrates, epithelial cells also develop tight junctions (TJ) apical to AJ, which form an impermeable barrier and tight seal between the neighboring cells. Although *Drosophila* epithelial cells do not

develop TJ, they bear a distinct region apical to the AJ, the subapical region (SAR), which is composed of proteins similar to vertebrate TJs. The *Drosophila* cells also develop septate junctions (SJ), which lie basal to the AJ, to provide a seal between the cells. In *C. elegans,* only one tripartite junction, the *C. elegans* apical junction (CeAJ) has been identified, which resembles the AJ of flies and vertebrates (Knust and Bossinger, 2002). These structures are summarized in Figure 1.4.

Several conserved groups of genes have been found to act in controlling junction formation and apico-basal polarity: the PAR-3-PAR-6-aPKC complex, the Scrib-Dlg-Lgl complex, and the Crb-Sdt-Patj complex (Betschinger et al., 2003; Bilder et al., 2003; Hurd et al., 2003b; Johnson and Wodarz, 2003; Tanentzapf and Tepass, 2003; Yamanaka et al., 2003). In the following sections I will focus on reviewing how PAR-3, PAR-6, and aPKC control epithelial polarity in flies, worms, and mammals.

2.2.1. *PAR-3 in cellular junction formation of Drosophila embryos*

In *Drosophila*, epithelial polarity is first established during cellularization. Blastoderm, the first epithelium of the fly embryo develops from a syncytium. After 13 nuclear divisions without cytokinesis, the embryo plasma membrane forms numerous invaginations simultaneously, called cleavage furrows, to segregate ~5000 nuclei into individual cells (Lecuit and Wieschaus, 2002). During this process, AJ components such as E-cadherin, α-and β-catenin, accumulate as spots along the furrow surface. After cellularization is complete, the dispersed AJ members coalesce apically and form the circumferential AJ belt in each cell. Some of these junction components also localize transiently at the furrow canal to promote persistent growth of furrows in concert with other molecules (Bhat et al., 1999; Lecuit and Wieschaus, 2002).

26
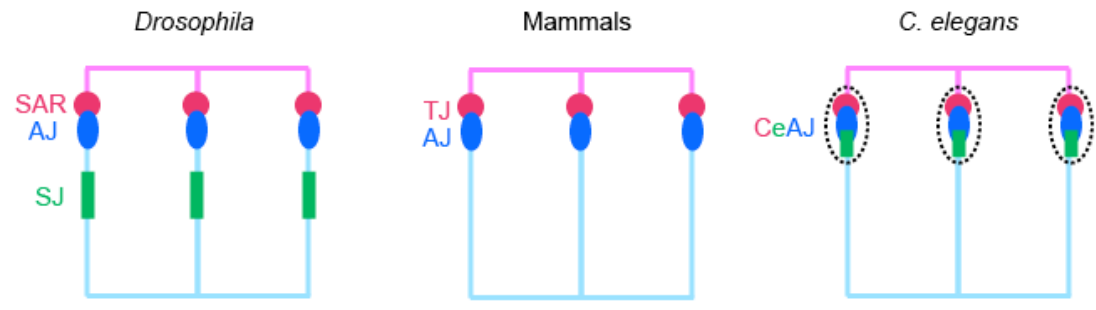
Figure 1.4 Comparison of epithelial organization in *Drosophila*, vertebrates, and *C. elegans*. In *Drosophila* and vertebrate epithelia, junctional regions are made up of common elements that are organized differently. The *C. elegans* junction presents a single structure, subdivided into three parts. (SAR, subapical region; TJ, tight junction; ZA, zonula adherens; SJ, septate junction; CeAJ, *C. elegans* apical junction). Modified from Knust and Bossinger, 2002.

The proper formation of AJ in *Drosophila* embryos depends on three well-conserved protein complexes (Muller and Bossinger, 2003). The Baz-DmPar-6-aPKC complex is targeted to the apical membrane domain and enriched at the SAR in *Drosophila* embryonic ectoderm. Depletion of any of them disrupts proper epithelial polarization (Kuchinke et al., 1998; Petronczki and Knoblich, 2001; Wodarz et al., 2000). Surprisingly, when the embryonic epithelia first polarize, each member of this complex displays distinct localization and roles (Harris and Peifer, 2005). Baz serves as the initial apical landmark for AJ assembly, and DmPar-6/aPKC is required to maintain AJ in gastrulation and later development. (Harris and Peifer, 2004; Harris and Peifer, 2005; Hutterer et al., 2004; Petronczki and Knoblich, 2001; Tepass et al., 2001; Wodarz et al., 2000). During cellularization, Baz does not co-localize with its typical binding partners DmPar-6 and aPKC, but is positioned more basally at the future AJ sites (Harris and Peifer, 2005). Even after the blastoderm is fully polarized at stage 14, Baz still remains separated from DmPar-6/aPKC (Harris and Peifer, 2005). Mutant analysis revealed that initial positioning of Baz does not require an intact AJ, or aPKC, or DmPar-6. However, the formation of AJ and aPKC localization depends upon Baz. In $baz^{m/z}$ embryos, E-cadherin and aPKC are dispersed along the entire furrow surface (Harris and Peifer, 2004; Harris and Peifer, 2005). Interestingly, at this stage DmPar-6 shows a Baz-independent widespread cytoplasmic and cortical distribution with slight enrichment above Baz (Harris and Peifer, 2005). During gastrulation DmPar-6 concentrates apically, which depends upon cdc42 but only partially requires Baz during this process (Hutterer et al., 2004). As epithelial development progresses, Baz, DmPar-6, and aPKC also exhibit a gradual dependence between each other (Hutterer et al., 2004).

The apical scaffold that anchors Baz initially is not known yet, but Baz positioning requires intact microfilaments and dynein-mediated transport (Harris and

Peifer, 2005). Chemical disruption of microfilaments with cytochalasin D causes Baz to diffuse along the lateral membrane with reduced level of accumulation. In embryos with decreased dynein pool, Baz and E-cadherin are recruited to cleavage furrows, but fail to be recruited to the apical region (Harris and Peifer, 2005).

The maintenance of the embryonic epithelial polarity also requires the other two complexes. In the SAR, the transmembrane protein Crumbs (Crb), MAGUK (membrane-associated guanylate kinase) protein Stardust (Sdt), and PDZ protein Patj (previously called Discs lost) form a complex to maintain AJ at least partially by interacting with the intracellular cytoskeleton (Bachmann et al., 2001; Grawe et al., 1996; Hong et al., 2001; Medina et al., 2002; Tepass et al., 1990). In maternal *crb* or *sdt* mutant embryos, AJ is fragmented and never forms a continuous belt (Bachmann et al., 2001; Bilder et al., 2000; Grawe et al., 1996; Tepass, 1996). Crb is also required to separate BAZ from DmPar-6 and aPKC, since depletion of Crb leads to extensive intermixing of BAZ, DmPar-6, and aPKC (Harris and Peifer, 2005).
Another protein complex is composed of the multi-PDZ protein Scribble (Scrib), the MAGUK protein Discs large (Dlg), and Lgl. The ancient Scrib-Dlg-Lgl complex is localized at the SJ and lateral domain, serving as the guardian of the basolateral identity in epithelial cell (Bilder et al., 2000; Bilder and Perrimon, 2000). The localization of each of these proteins requires the presence of the other two proteins. In the absence of any of them, functional AJ fails to form and the epitheliabecomes multilayered (Bilder et al., 2000)

In the past, a single regulatory hierarchy between the Crb-Sdt-Dlt, the BAZ-DmPar-6-aPKC, and the Scrib-Lgl-Dlg complexes has been suggested in polarizing the fly embryonic epithelia, which is summarized in Figure 1.5 (Bilder, 2004; Brumby and Richardson, 2005). According to this model, BAZ-DmPar-6-aPKC complex forms
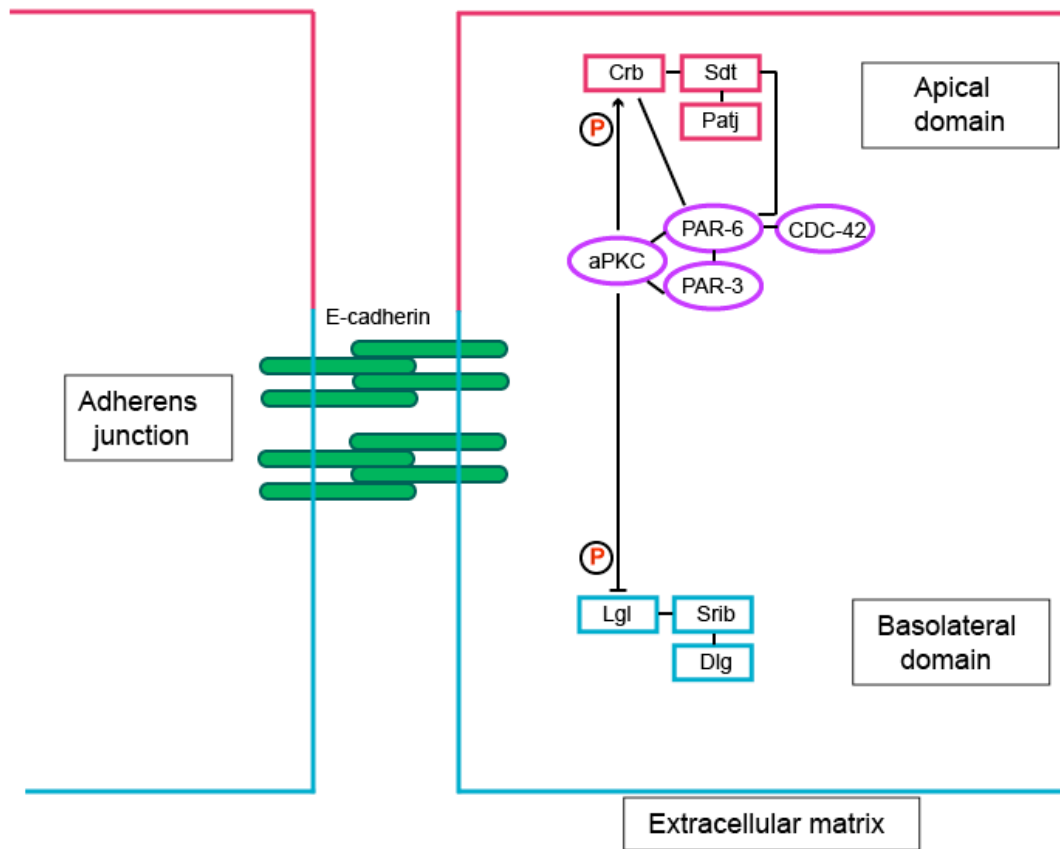
Figure 1.5 Regulation of epithelial polarity Par3–Par6–aPKC–Cdc42 and Crb–Sdt–
Patj function to promote apical membrane formation during adherence junction
assembly, whereas Scrib–Dlg–Lgl functions to antagonize Par3–Par6–aPKC–Cdc42,
and is itself antagonized by Crb–Sdt–Patj, to promote basolateral membrane identity.
Lines with bars depict antagonistic interactions, whereas lines with arrows depict
positive interactions. Circles with "P" denote phosphorylation. Modified from Brumby
and Richardson, 2005.

first and specifies the apical membrane identity. The Scrib-Lgl-Dlg complex defines the basolateral region by restricting the BAZ-DmPar-6-aPKC complex to the apical side. To fight back, BAZ-DmPar-6-aPKC complex recruits Crb-Sdt-Dlt to antagonize the activity of the Scrib-Lgl-Dlg complex. The interplay between these complexes controls the build-up and reinforcement of the AJ, ensuring precise control of epithelial organization and growth (Bilder, 2004; Brumby and Richardson, 2005).

Recent investigations uncovered more roles of the Par complex and their associated-proteins. aPKC maintains epithelial polarity by regulating apical microtubule organization (Harris and Peifer, 2007). Cdc42, the partner of DmPar-6, participates in this process by controlling transport of apical proteins, including Crb, Baz, DmPar-6 and aPKC (Harris and Tepass, 2008). The PAR proteins also appear to actively facilitate this process by regulating endocytosis (Harris and Tepass, 2008). A Genome-wide RNAi screen of epithelial morphogenesis uncovered bitesize, a synaptotagmin-like protein, as one of the downstream effectors of Baz. Bitesize displays a Baz-dependent apical localization. In *bitesize* mutant, AJ assembly and Baz localization are normal initially, but rapidly become dispersed as epithelial polarization progresses (Pilot et al., 2006). All these emerging findings imply a complex crosstalk between the PAR proteins and other molecules in controlling epithelial polarization in *Drosophila*.

2.2.2. PAR-3 in cellular junction formation of C. elegans embryos

The hypodermis, the pharynx and the intestine are the main epithelial organs in the *C. elegans* embryo (Muller and Bossinger, 2003). Only one type of intercellular junction, the CeAJ has been well investigated (Koppen et al., 2001; Leung et al., 1999; McMahon et al., 2001). Since PAR-3, PAR-6 and aPKC have not been reported in

epidermis, I will focus on their roles in the digestive tract and somatic gonad (Bossinger et al., 2004; McMahon et al., 2001).

The *C. elegans* intestine is made of only 20 polarized cells, forming a single layered tube (Sulston et al., 1983). As morphogenesis starts, the junctional components and PAR-3, PAR-6, and aPKC-3 accumulate in puncta at the apical pole (Leung et al., 1999). Later, the CeAJ components segregate more laterally, while PAR-3, PAR-6 and PKC-3 occupy the apical surface. As morphogenesis progresses, the CeAJ form a circumferential adhesion belt around the cell, closely resembling the AJ in flies and mammals (Bossinger et al., 2001; Leung et al., 1999).

However, it has been difficult to test whether and how the PAR-3/PAR-6/PKC-3 complex participates in epithelial polarization due to their early requirement in embryogenesis (Pellettieri and Seydoux, 2002). Recent experiments in which PAR-3 and PAR-6 were only provided to the early embryo but degraded in the somatic precursors provide evidence that they also play roles in zygotic development (Nance et al., 2003). In those embryos, depletion of maternal PAR-3 or PAR-6 from the 4-cell stage leads to defects in endodermal precursor ingression and cell-cell adhesion during gastrulation (Nance et al., 2003). Embryos lacking both maternal and zygotic PAR-6 arrest during morphogenesis with severely impaired epithelial adhesion and junction formation (Totong et al., 2007). Surprisingly, these epithelial cells are still polarized, indicating that PAR-6 regulates epithelial junction formation but epithelial polarization does not require PAR-6 (Totong et al., 2007). Interestingly, PAR-6 and PKC-3 always co-localize, but they only overlap with PAR-3 transiently during the early stage of polarization. In fully polarized epithelial cells, PAR-3 develops a more lateral localization (Totong et al., 2007).

During larval development, PAR-3, PAR-6 and PKC-3 are transiently localized in the somatic gonad (Aono et al., 2004). From the late-L3 stage, PAR-

3/PAR-6/PKC-3 start to accumulate in developing uterinesheath and spermathecal cells, and gradually localize apically until mid-L4 stage(Aono et al., 2004). Depletion of zygotic PAR-3 in larvae results in compromised distal spermatheca polarity and CeAJ assembly. In turn, functional CeAJ is required for signaling between the spermatheca and the germline (Aono et al., 2004).

Besides the PAR proteins, the apical members work cooperatively to regulate epithelial polarization in the intestine. For example, CRB-1, the worm homologue of Crumbs, is localized at the apical surface in the gut. Depletion of CRB-1 alone does not cause any polarity phenotype. However, when CRB-1 is knocked down together with HMP-1(α-catenin), the intestinal polarity is severely compromised (Bossinger et al., 2004; Bossinger et al., 2001). Similarly, worms devoid of DLG-1(Dlg) are still able to retain established cell-cell adhesion. When DLG-1 was reduced with other CeAJ components such as HMR-1(E-cadherin), synergistic effects were uncovered as the epithelial cells dissociate from each other and the polarity is lost (McMahon et al., 2001; Segbert et al., 2004).

The apically enriched cytoskeletal bundles are also important for the apico-basal polarity in intestine, although the mechanism is still unclear. Recently, two cytoskeleton associated proteins, kinesin ZEN-4 and RhoGAP CYK-4, have been found essential in pharyngeal polarity establishment. In *zen-4* or *cyk-4* mutant embryos, PAR-3 and PKC-3 fail to localize at the apical membrane and lumen formation is disrupted, possibly due to the compromised microtubule and actin organization (Portereiko and Mango, 2001; Portereiko et al., 2004).

The apical restriction of the junctional proteins requires the ancient basolateral identity keeper, LET-413, which is the only worm homologue of the fruit fly Scribble (Bossinger et al., 2001; Koppen et al., 2001; Legouis et al., 2000; McMahon et al., 2001). In the absence of LET-413, apical markers gradually expand to the basolateral

domain in the gut. Interestingly, LET-413 does not co-localize with its typical binding partner DLG-1 in worm epithelia. Further biochemical studies are necessary to dissect the role of LET-413 in maintaining epithelial polarity (Bossinger et al., 2001; Koppen et al., 2001; Legouis et al., 2000; McMahon et al., 2001).

In summary, the *C. elegans* apical junctions share many characteristics of the intercellular junctions in *Drosophila*. The identification of other polarity molecules and their characterization will help to elucidate the nature of the CeAJ and contribute to an understanding of junction development.

2.2.3. *PAR-3 in cellular junction formation in mammals*

In vertebrate epithelia, the TJ serves as physical barrier to separate the apical and basolateral domain as well as to provide cell-cell adhesion and vesicle targeting sites (Shin et al., 2006). Transmembrane proteins occludin, claudin, and JAM (junctional adhesion molecule) are the important components of TJ. All of them interact directly with the PDZ-containing ZO proteins to link to the beneath cytoskeleton meshwork (Shin et al., 2006).

Two conserved protein complexes, the CRB-Pals1-PATJ and the Par3-Par6-aPKC complexes, are localized at the TJ and control the epithelial polarity (Shin et al., 2006). Expression of CRB3, one of the human homologues of Drosophila Crb, in MCF10A cells drives ectopic TJ formation in those poorly polarized cells (Fogg et al., 2005). Overexpression of CRB3 in MDCK cells leads to delayed TJ formation and disrupted cell polarity (Lemmers et al., 2004; Roh et al., 2003). PALS1 (Stardust) is also required in polarity establishment, as depletion of PALS1 causes defects in TJ assembly and polarity marker recruitment (Straight et al., 2004). Another member of this complex is PATJ(Discs lost), which participates in the polarization process at least partially by stabilizing the CRB3 complex (Latorre et al., 2005; Michel et al., 2005;

Shin et al., 2005). In the absence of PATJ, the TJ is disrupted and CRB3 is redistributed into intracellular compartments, suggesting impaired vesicular transport (Michel et al., 2005; Shin et al., 2005). All these results imply that the conserved CRB-Pals1-PATJ polarity complex controls epithelial polarity in mammals as well.

Recent results also indicate that the Par3-aPKC-Par6-Cdc42 complex is involved in several aspects of mammalian epithelial polarization (Macara, 2004b). In this complex, the interaction between activated Cdc42 and Par6 induces a conformational change in Par6, resulting in the activation of the kinase activity of aPKC (Garrard et al., 2003; Yamanaka et al., 2001). The activated aPKC promotes the polarization process through the phophorylation of downstream effectors such as Lgl or Par-1. The aPKC-dependent phosphorylation causes the dissociation of Lgl or Par-1 from the apical membrane, resulting in the restricted basolateral distribution of Lgl and Par-1 (Hurov et al., 2004; Plant et al., 2003; Suzuki et al., 2004; Yamanaka et al., 2003).

Par6 signals to the CRB-Pals-PATJ complex by direct binding to the C-terminus of CRB3. Mutations in CRB3 that specifically disrupted Par6-CRB3 binding can abolish the detrimental effects of CRB3 overexpression in MDCK cells, indicating that the Pals1-Par6 interaction is important for CRB3 function (Lemmers et al., 2004). Par6 has also been shown to physically interact with Lgl. However, it is difficult to assess the significance of this interaction since the mutations that abolished Par-6-Lgl binding also disrupted Par-6-Pals1 interaction (Hurd et al., 2003b; Plant et al., 2003; Wang et al., 2004; Yamanaka et al., 2003).

.   Par3 directly interacts with the C-terminus of JAM, and this interaction is important for Par3 to localize at the TJ and functions to assemble the Par3-aPKC-Par6-Cdc42 complex (Ebnet et al., 2001; Hirose et al., 2002; Itoh et al., 2001). Moreover, Par3 may also regulate epithelial polarity by binding to 14-3-3 in a

phosphorylation-dependent manner. Disruption of interaction between Par3 and the 14-3-3 proteins leads to the failure in the lumen formation of epithelial cyst (Hurd et al., 2003a). In *Drosophila*, this phosphorylation is dependent on PAR-1 kinase, and excludes Baz-DmPar6-aPKC from the basolateral membrane in follicular cells (Benton and St Johnston, 2003b). The mammalian kinase that is responsible for this regulation is still not known.

Recently, additional roles of the PAR proteins have been reported. However, the mechanisms are still controversial. Par3 can recruit T lymphoma invasion and metastatis protein (Tiam1), a RacGEF, through its C-terminus. This Cdc42-independent interaction leads to a local activation of Rac and regulates the polarity of epidermal keratinocytes (Mertens et al., 2005). In contrast, Chen and Macara (2005) showed that Par3 binds to and inhibits Tiam1-mediated Rac activation, allowing the proper formation of TJ in MDCK cells (Chen and Macara, 2005). A third study in neuroblastoma cells suggests that Par3 mediated Rac activation requires activated Cdc42 at the front edge, which promotes the directional cell migration (Nishimura et al., 2005). Further studies are necessary to understand the basis for these contrasting results. In summary, all the evidence indicates that various cell polarity complexes are physically linked and functionally interdependent during the epithelial polarization and junction formation processes.

III: THE STRUCTURE AND FUNCTION OF PAR-3 IN OTHER ORGANISMS
3.1. The interactions between PAR-3, PAR-6, and aPKC

PAR-3, PAR-6 and PKC-3 are highly conserved proteins and their homologues have been identified in frogs, flies and mammals (Goldstein and Macara, 2007). In worms as well as in other species, PAR-3 is able to form a tripartite complex with PAR-6 and aPKC, and the interactions have been extensively investigated in many

systems (Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Petronczki and Knoblich, 2001; Suzuki et al., 2001; Wodarz et al., 2000; Yamanaka et al., 2001)(J. Li, personal communication).

The *C.elegans par-3* genomic sequence spans 12.5kb on Chromosome III. The ORF is 4140 nucleotides, encoding a 138 kD protein (Etemad-Moghadam et al., 1995). Three conserved regions (CR) have been indentified in PAR-3, named CR1, CR2 (consisting of three PDZ domains in tandem), and CR3. In *C.elegans*, PAR-3 binds to PAR-6 via its first PDZ domain (Etemad-Moghadam et al., 1995; Tabuse et al., 1998) (J. Li, personal communication). The N-terminal PB1 domain of PAR-6 interacts with that of PKC-3 (D. Aceto, personal communication). PAR-6 also interacts with the active form of CDC42 via its semi-CRIB domain (Gotta et al., 2001; Aceto et al, 2006). PKC-3 binds to and phosphorylates PAR-3 at S863 in its CR3 region (Izumi et al. M. Beers, personal communication; see Chapter 2).

Based on their physical interactions, co-localization and co-dependence, PAR-3, PAR-6, and PKC-3 are considered to function as an obligate complex in many cases (Macara, 2004a). However, growing evidence suggest that they can localize and/or function independently. In *drosophila*, Baz localizes at the apical membrane below aPKC and DmPar-6, and is positioned there by distinct mechanisms during epithelial development (Harris and Peifer, 2005). In *C. elegans* zygotes, PAR-3, PKC-3, and PAR-6 co-localize at the anterior cortex in clusters, but these different clusters only partially co-localize (Hung and Kemphues, 1999; Tabuse et al., 1998). aPKC and Par6 colocalize without Par3 at the leading edge of migrating mammalian astrocytes (Etienne-Manneville and Hall, 2001). In MDCK cells, Par3 localizes dominantly to the TJ, while aPKC and Par6 are also distributed at the apical surface and the cytoplasm (Vogelmann and Nelson, 2005). Par3 can regulate TJ assembly independently of aPKC and Par6 (Chen and Macara, 2005). In summary, the

interactions between PAR-3, aPKC, and PAR-6 are dynamic and regulated in various circumstances.

3.2 PAR-3 is an ancient scaffolding protein

Our understanding on PAR-3 structure and function heavily relies on the studies of its mammalian homologues in cultured epithelial cells. Par3 localizes at the TJs of hese extremely differentiated cells which bears distinct apical and basolateral domains (Balda and Matter, 2003; Nelson, 2003). This system is favorable for physical manipulations and biochemical treatments, allowing rapid and thorough studies of protein structure and function both *in vitro* and *in vivo*.

One of the limitations of much of the previous Par3 analysis in cultured epithelial cells is that endogenous Par3 could mask the abnormal distribution and functions of the transfected Par3 mutants by self-recruitment and/or dose compensation. To overcome this difficulty, an endogenous Par3 suppression system in cultured epithelial cells was established by Chen and colleagues in 2005 (Chen and Macara, 2005). MDCK II cells are canine epithelial cells that have been widely used for studying epithelial polarization. Depletion of Ca2+ from the cell medium causes rapid disruption of cellular junctions, while replenishing Ca2+ can reverse this process. Thus this treatment is usually referred to as "calcium switch" (Matter and Balda, 2003). Overexpression of Par3 in MDCK II cells does not cause dramatic effects on TJ formation (Hirose et al., 2002). However, reducing Par3 by RNAi leads to a profound disruption of TJ in MDCK cells (Chen and Macara, 2005). The robust Par3 knockdown is also specific, as AJ assembly is only slightly affected and little effects were observed on other polarity markers and junctions (Chen and Macara, 2005). Normally, TJs start to reassemble within 30 minutes after adding Ca2+ back to the medium. However, this process was greatly delayed when Par3 is reduced by

RNAi. In addition, the formation of TJs can be rescued by expressing human Par3, which is not degraded by the canine-Par3-specific RNAi (Chen and Macara, 2005). In summary, this system clearly showed that Par3 is required for TJ formation in MDCK cells. These newly developed tools made it possible to reduce endogenous Par3 and to express various exogenous Par3 constructs simultaneously, thus providing an excellent opportunity to study Par3 structure and function *in vivo*.

3.2.1 CR1 domain (69-152)

The N-terminally located CR1 domain of PAR-3 is highly conserved in all PAR-3 homologues, but is not found in any other known proteins (Benton and St Johnston, 2003a). By comparing the possible predicted CR1 structures with known protein sequences, Benton and colleagues uncovered the similarity of Baz CR1 and the dimerization domain of a bacterial gene Din1 (Benton and St Johnston, 2003a). They provided evidence that CR1 mediates oligomerization of Baz both *in vitro* and *in vivo*. BAZ::GFP with CR1 deleted is largely diffused in the cytoplasm of follicular cells, and exhibits poor ability to rescue *baz* mutant phenotype (Benton and St Johnston, 2003a). Similarly, in mammals Par3 CR1 is also able to self-associate, and the overexpression of CR1 in MDCK cells leads to compromised Par3 localization and delayed TJ formation (Mizuno et al., 2003).

In 2007, Feng and colleagues solved the structure of a monomeric form of Par-3 CR1 (called NTD in that study, standing for <u>N</u>-terminal <u>d</u>omain) (Feng et al., 2007). The CR1 monomer adopts a PB1-like fold and is able to self-oligomerize in a front-to-back manner, forming a helical filament(Feng et al., 2007). Although PB1 domains can form dimers in many cases, Par3 CR1 does not interact with that of Par6 or aPKC. Whether self-oligomerization is essential for Par3 cellular localization and/or function are still controversial (Chen and Macara, 2005; Feng et al., 2007).

Mutations in two conserved residues (V13D and D70K) can abolish the CR1-mediated oligomerization without significantly affecting the overall structure (Feng et al., 2007). Either CR1Δ or V13DD70K can disrupt the junctional localization of Par3. Only subtle difference were observed, as CR1Δ mutant is completely cytoplasmic while V13DD70K mutant still shows residual junctional distribution. Moreover, replacing CR1 by a GCN leucine zipper motif, which is able to form tight tetramers, fully restored the membrane localization of the chimaric Par3. Taken together, the results indicate that CR1 is absolutely required for the TJ localization of Par3 (Feng et al., 2007).

In contrast, Chen and colleagues found that the Par3 construct missing the CR1 domain is still able to localize correctly and to rescue TJ assembly efficiently after a Ca2+ switch in MDCK cells (Chen and Macara, 2005). In addition, Expression of Par3 in neuroblastoma cells can induce the formation of multiple axon-like neurites. CR1 deletion does not affect Par3's ability to promote neurite growth (Nishimura et al., 2005).

3.2.2. PDZ1 (383-463)

PDZ domains are one of the most common protein-protein interaction domains in eukaryotes (Nourry et al., 2003; Sheng and Sala, 2001). They typically function through scaffolding molecular complexes in signal pathways, thus enhancing the fidelity and rate of signal transduction within the complex (Sheng and Sala, 2001). PDZ domains are usually 80-90 amino acid-long modules, forming a barrel-like structure consisting of 5-6 β stands and 2 α helices (Nourry et al., 2003). To date, four types of interactions have been identified for PDZ domains: They can harbor the C-terminal signature sequence of transmembrane proteins (Ebnet et al., 2001; Takekuni et al., 2003), or bind to the internal peptides (Penkert et al., 2004; Wang et al., 2004),

or form dimmers with other PDZ domains (Brenman et al., 1996; Hillier et al., 1999), and even interact with phosphatidylinositol moieties (Wu et al., 2007; Yan et al., 2005; Zimmermann et al., 2002). In addition, PDZ domains also show significant sequence variation, presumably reflecting the diversity of their binding partners (Nourry et al., 2003).

So far, several proteins have been identified as PAR-3 PDZ1 partners (Chan et al., 2006; Ebnet et al., 2001; Itoh et al., 2001; Joberty et al., 2000; Schober et al., 1999; Takekuni et al., 2003; Wodarz et al., 1999). For example, in *Drosophila* neuroblast Baz recruits Inscuteable via its PDZ1 domain to the apical surface, regulating the downstream effectors that control spindle orientation (Schober et al., 1999; Wodarz et al., 1999). In vertebrate nervous systems, Par3 recruits p75 neurotrophin receptor to the axon-glial side of Schwann cells to promote myelination. Overexpression of Par3 PDZ1 domain or the Par3-binding motif of P75 significantly inhibits myelination (Chan et al., 2006). In MDCK cells, Par3 is recruited to TJs by interacting with the C-terminus of JAM directly. JAM lacking the Par3 binding motif fails to recruit endogenous Par3 in several types of cultured epithelial cells. Both PDZ1 and PDZ3 appear to be required for this binding, and overexpression of JAM C-terminal fragment disrupts Par3 localization at TJs (Ebnet et al., 2001; Itoh et al., 2001).

The interaction between PAR-3 and PAR-6 has been verified in many species, although the consequence of this binding still remains elusive (Gibson and Perrimon, 2003). In one study, overexpressed Par6 can perturb epithelial polarity, but mutations in Par6 that disrupt Par3-Par6 interaction abolished this activity (Joberty et al., 2000). Whether this effect is due to abolished Par3-Par6 interaction remains unclear since the same mutations (KPLG167-170AAAA) can also abolish the interaction between Par6 and Pals1 (Hurd et al., 2003b). In contrast, in MDCK cells Par3 lacking PDZ1 domain can localize normally and rescue the TJ formation efficiently in the absence of

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endogenous Par3, indicating that Par3-Par6 binding is not required for MDCK cell polarization (Chen and Macara, 2005).

3.2.3. PDZ2 (515-584) and PDZ3 (659-738)

Little research has been reported on PAR-3 PDZ2 and PDZ3 domains until recently, when the structure of mammalian Par3 PDZ2 and PDZ3 were solved and their roles in epithelial polarization were examined (Feng et al., 2008; Wu et al., 2007). Both PDZ2 and PDZ3 domains of Par3 adopt the canonical PDZ fold. Surprisingly, PDZ2 shows high affinity to PIP lipids (Wu et al., 2007). It contains a flat surface rich in positively charged residues, which are critical for the interaction with negatively charged inner cell membrane. It also bears a binding pocket to harbor PIP head directly as well as several residues that can insert into the lipid bilayer (Wu et al., 2007). However, whether PDZ2 is the necessary for Par-3 localization and function in epithelial polarization is still controversial (Chen and Macara, 2005; Wu et al., 2007). In one Ca2+ switch experiment, a C-terminal Par3 fragment without PDZ2 is sufficient to localize normally and rescue the TJ assembly in MDCK cells lacking endogenous Par3 (Chen and Macara, 2005). While in another study, Par3 with PDZ2 deletion is completely cytoplasmic in MDCK cells. After Ca2+ switch, the protein bearing PDZ2 deletion fails to rescue the TJ formation in the absence of endogenous Par3. Replacing Par3 PDZ2 with PLCδ PH domain that exhibits similar lipid-binding affinity to Par3 PDZ2 domain was able to rescue the polarization efficiently (Wu et al., 2007).

The role of PAR-3 PDZ3 is linked to its conserved interaction with the phosphoinositide phosphatase PTEN. In *Drosophila*, Baz co-localizes and binds to PTEN directly through a region containing PDZ2 and PDZ3 domain (von Stein et al., 2005). PTEN is required for *Drosophila* germline fate determinants localization and

mammalian epithelial lumen formation (Martin-Belmonte et al., 2007; von Stein et al., 2005). In mammals, Par3 PDZ3 binds to the very C-terminus of PTEN directly, and the interaction is important for the membrane enrichment of PTEN in MDCK cells (Feng et al., 2008). Mutations that specifically disrupt Par3-PTEN interaction (DED3A) eliminates PTEN from the cell-cell contacts. Knockdown of PTEN in MDCK cells dramatically delayed TJ formation after Ca2+ switch, which closely resembles the *par-3(RNAi)* results. RNAi-resistant human PTEN is able to rescue the TJ formation caused by endogenous Par3 reduction, while Par3-binding deficient PTEN (DED3A or ΔC) fails to rescue those repolarization defects (Feng et al., 2008).

3.2.4. CR3 (854-868)

In mammals, aPKC binds to the CR3 region of Par3 directly and phosphorylates serine 827 (Lin et al., 2000; Nagai-Tamai et al., 2002). This phosphorylation significantly reduces the affinity between Par3 and aPKC (Nagai-Tamai et al., 2002). Antibody that specifically recognizes S827-phosphorylated Par3 can identify this form in MDCK cell lysate, suggesting at least some population of Par3 is phosphorylated at S827 *in vivo*. Furthermore, the kinase activity of aPKC decreases by 40% in the presence of Par3 CR3, indicating that the interaction with Par3 may hold aPKC in an inactive conformation (Lin et al., 2000).

The physiological significance of this phosphorylation is still unclear. Overexpression of an S827A mutant in MDCK cells causes polarity defects similar to dominant-negative aPKC in MDCK cells (Nagai-Tamai et al., 2002). However, Par3 lacking aPKC binding region is still able to rescue the polarization of MDCK cells when endogenous Par3 is suppressed (Chen and Macara, 2005). In *D. rerio* embryos, PAR-3 lacking aPKC binding motif localizes the same as the wide-type PAR-3::GFP

in neuroepithelial cells, although the function is hard to dissect due to the presence of endogenous fish PAR-3 (von Trotha et al., 2006).

Recent studies also revealed Par3 as a direct kinase target of RhoA kinase (ROCK), which is a downstream effector of RhoA (Nakayama et al., 2008). ROCK directly phosphorylates Par3 at T833. This phophorylation disrupts the assembly of Par3/aPKC/Par6/Cdc42 complex, thereby locally preventing the Cdc42-induced activation of Rac. Moreover, an antibody that specifically recognize the phospho-T833 form of Par3 shows that this phosphorylation occurs *in vivo* and the T833 phosphorylated Par3 is present in the central and rear cytoplasm as well as the leading edge of cultured migrating cells, which closely resembles the distribution of active RhoA (Jaffe and Hall, 2005). Depletion of Par3 inhibits Hela cell migration and decreases lamellar length in the wound-healing assay. Compared to wide-type Par3, Par3 T833D that mimics constitutive ROCK phosphorylation exhibits much weaker ability to rescue the defects in front-to-rear polarity and migration caused by endogenous Par3 knockdown. All these results imply that the ROCK-dependent phosphorylation of Par3 is context-specific and may function in distinct pathways to regulate polarized cell migration and epithelial polarization.

3.2.5. C terminus (869-1379)

The sequence of the PAR-3 C terminal region can vary greatly between species or even within the same animal (Etemad-Moghadam et al., 1995; Lin et al., 2000; von Trotha et al., 2006). In cultured rat hippocampal neurons, Par3/Par6/aPKC complex accumulates at the tip of growing axons and plays important roles in neuron polarization (Shi et al., 2003). Ectopic expression of Par6 or Par3 leaves neurons with no axon specified (Shi et al., 2003). A direct interaction between Par3 C-terminal region and KIF3A was identified (Nishimura et al., 2004). KIF3 is a plus-end-directed

MT motor protein that participates in anterograde axonal transport in neurons (Goldstein and Yang, 2000). Par3 and KIF3A accumulate and colocalize at the tip of the axon in stage-3 neurons. The expression of dominant-negative Par3 or KIF3A fragments deficient in Par3-binding inhibits the accumulation of Par3 and aPKC at the neurite tip and impairs axon formation (Nishimura et al., 2004). These results suggest that Par3 is transported to the distal tip of the axon by KIF3A and that the proper localization of Par3 is required to establish neuronal polarity.

Par3 C-terminus also interacts with Tiam1, the RacGEF, directly. In MDCK cells, The PDZ3 plus C-terminal region of Par3 is sufficient and essential for its proper localization and TJ-rescue ability when endogenous Par3 is suppressed (Chen and Macara, 2005). When Tiam1 is reduced, the polarity defects caused by *Par3(RNAi)* can be partially suppressed. In addition, Par3 fragments deficient in Tiam1-binding fails to rescue the TJ formation in cells lacking Par3 (Chen and Macara, 2005). All these data suggest that Par3 controls TJ formation by recruiting Tiam1, providing local control to Rac activity. Similarly, in dendritic spines of hippocampal neurons, Par3 also recruits Tiam1 to locally activate Rac, thereby contributing to spine maturation (Zhang and Macara, 2006).

3.2.6. *Putative 14-3-3 binding sites (947-952)*

PAR-3 C terminus also contains a conserved putative 14-3-3 binding site. As discussed in 1.3.5, PAR-5, a member of the 14-3-3 family, is required to restrict the PAR-3/PAR-6/PKC-3 complex to the anterior cortex (Cuenca et al., 2003; Morton et al., 2002). In mammalian epithelial cells, 14-3-3 protein binds to Par3 both *in vitro* and *in vivo,* and the phosphorylation of Par3 at S144 is required for the interaction (Hurd et al., 2003a). MDCK cells stably expressing Par3 S144A mutant exhibit profound defects in cell organization and lumen formation, while cells expressing WT

Par3 can form relatively normal cysts with a central lumen (Hurd et al., 2003a).
Together with other data, they concluded that the phosphorylation-dependent binding
between 14-3-3 and Par3 regulates epithelial polarity, although the interaction is
unlikely to control the subcellular distribution of Par3 (Hurd et al., 2003a).

In *Drosophila*, Par-1 phosphorylates Baz at S151 and S1085 to generate 14-3-3
binding sites (Benton and St Johnston, 2003b). S1085 is conserved in *Drosophila*,
human and *C.elegans* (corresponding to serine 950 in *C.elegans*). The 14-3-3-Baz
interaction blocks the interaction between Baz and aPKC, thus preventing the
assembly of the Baz/DmPar-6/aPKC complex (Benton and St Johnston, 2003b).
Disruption of Baz and14-3-3 binding leads to the spread of Baz to the basolateral
surface, resulting in compromised epithelial polarity (Benton and St Johnston, 2003b).

Thesis Organization

Chapter 2 of this thesis focuses on the role of different PAR-3 domains and
phosphorylation forms in early embryonic patterening and in late epithelial
development. The appendices are a compilation of projects I initiated to understand
how PAR-3 is regulated throughout *C. elegans* development.

CHAPTER TWO

DIFFERENT DOMAINS OF C. ELEGANS PAR-3 ARE REQUIRED AT DIFFERENT TIMES IN DEVELOPMENT

INTRODUCTION

Acquisition of cell polarity is a critical process for specifying body axis and maintaining distinct organ function in metazoan development. The PAR (<u>par</u>titioning defective) proteins, which are highly conserved from worms to mammals, are part of the core machinery to control cell polarization in many different cell types (Goldstein and Macara, 2007). PAR-3, a multi-PDZ domain scaffold protein, can interact with PAR-6 and PKC-3 (atypical protein kinase C; aPKC) to control cell polarization in different developmental stages and in different tissues. For example, in the *Drosophila* central nervous system, PAR-3 (also called Bazooka), PAR-6 and aPKC co-localize at the apical surface of neuroblasts and ensure that the neural fate determinants segregate into one of the two daughter cells (Rolls et al., 2003; Schaefer et al., 2001; Schober et al., 1999; Wodarz et al., 1999). In mammalian epithelial cells, PAR-3, PAR-6 and aPKC localize to the tight junctions to control apical-basolateral polarity (Chen and Macara, 2005; Izumi et al., 1998; Yamanaka et al., 2001).

The role of PAR-3, PAR-6 and PKC-3 as regulators of polarity was first identified in *C. elegans*, where they play critical roles in the establishment of embryonic polarity and organization of epithelial cells (Aono et al., 2004; Etemad-Moghadam et al., 1995; Kemphues et al., 1988; Nance et al., 2003; Tabuse et al., 1998; Totong et al., 2007; Watts et al., 1996). Early in the first embryonic cell cycle,

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*This Chapter is written in a format compatible with submission for journal publication. Figure 2.10 A was contributed by M. Beers.

PAR-3, PAR-6, and PKC-3 are uniformly distributed at the cell periphery of the fertilized egg (Cuenca et al., 2003; Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Tabuse et al., 1998). In response to the polarity cue provided by the sperm centrosomes or microtubules emanating from them, localized reduction of actomyosin contractility at the posterior pole results in the cortical actin network flowing away from the sperm, carrying PAR-3, PAR-6 and PKC-3 toward the anterior (Cowan and Hyman, 2007). This restricted localization of the anterior PAR proteins is critical for the first asymmetric division, which generates two daughter cells different in size, fate, and spindle orientation (Boyd et al., 1996; Cheeks et al., 2004; Etemad-Moghadam et al., 1995; Hao et al., 2006; Munro et al., 2004; Tabuse et al., 1998; Watts et al., 1996). PAR-3 appears to act upstream to localize PAR-6 and PKC-3 at the cell periphery (Beers and Kemphues, 2006; Tabuse et al., 1998; Watts et al., 1996). However, little is known about how PAR-3 associates with the cortex in the one-cell stage worm embryo.

Recent studies in *C. elegans* organogenesis have revealed that PAR-3, PAR-6 and PKC-3 also play important roles in epithelial development (Aono et al., 2004; Nance et al., 2003; Totong et al., 2007). Maternal PAR-3, PAR-6 and PKC-3 gradually diminish after the 26-cell stage, and zygotic expression of PAR-3 initiates when the embryo approaches 400 cells (Leung et al., 1999; McMahon et al., 2001; Nance et al., 2003). The re-expressed PAR-3, PAR-6 and PKC-3 proteins localize at the apical surface of developing pharynx, intestine, vulva, spermatheca, uterus, and male tail rays (Aono et al., 2004; Nance et al., 2003; our unpublished results). Interestingly, PAR-3 localizes basolaterally to PAR-6 and PKC-3 in fully polarized epithelial cells, suggesting that PAR-3 may act independently from the other two proteins (Totong et al., 2007), similar to results reported in flies (Harris and Peifer, 2005). Targeted degradation of maternal PAR-3 in embryonic somatic precursor cells

leads to aberrant cell adhesion and cell ingression (Nance et al., 2003) and knockdown of zygotic PAR-3 protein in larvae causes defects in distal spermathecal junctions (Aono et al., 2004).

Like its homologues, *C. elegans* PAR-3 contains an N-terminal CR1 domain, three PDZ domains in tandem (PDZ1, PDZ2, PDZ3) followed by a CR3 region containing a conserved PKC-3 binding site (Figure 1A) (Etemad-Moghadam et al., 1995). There has been considerable progress in understanding PAR-3 function and localization in mammalian cultured cells (Goldstein and Macara, 2007). However, less is known about how the domains of PAR-3 contribute to its function in cells of living animals. To understand how PAR-3 localizes and functions during worm development, we have introduced targeted mutations and deletions into PAR-3::GFP and examined the localization and function of the mutated proteins in the genetic background of two different *par-3* alleles that allow us to assess maternal versus zygotic requirements. Our results indicate that although the role of PAR-3 in controlling cell polarity is widely conserved, the protein acts via different mechanisms in early embryos and epithelial cells.

MARTERIALS AND METHODS

Nematode strains

Caenorhabditis elegans strains were cultured under standard procedures (Brenner, 1974), except that all transgenic strains were maintained at 25°C. The Bristol N2 strain was used as wild type. The *par* mutant strains used in this study are KK653, *unc32(e189)par-3(it71)/qC1 III* (Etemad-Moghadam et al., 1995), and KK928, *par-3(tm2010)/qC1 III. par-3(tm2010),* generated by the National Bioresource Project (S. Mitani, Tokyo Women's Medical University), was outcrossed 6 times, balanced, and sequenced.

Transgene construction and transformation

All *par-3* transgenes were derived from plasmid pJN210 (gift from Dr. Jeremy Nance (Nance et al., 2003). Mutations or deletions were constructed by site-directed mutagenesis (Quickchange kit, Stratagene) or recombinant PCR. All constructs included a wild-type *unc-119* gene on the vector used for transformation marker. *unc-119(ed3)* worms were transformed by microparticle bombardment (Praitis et al., 2001). Only 5-10% of the Unc + transgenic lines stably express GFP both maternally and zygotically, and the maternal expression in most *par-3^{CT}::gfp* lines was lost within 6 weeks after the lines were generated.

Analysis of transgene rescue of *par-3(it71)* and *par-3(tm2010)*

We integrated homozygous transgenic lines that express mutated variants of PAR-3::GFP both maternally and zygotically. We tested at least two independent lines from each construct for rescue and fusion protein distribution, except for the PAR-3^{ΔPDZ3}::GFP mutant, for which we recovered only one line. The identity of the transgene in each rescue experiment was confirmed by single-worm PCR followed by DNA sequencing (Barstead et al., 1991; Williamson et al., 1991).

To assess maternal function of the mutant construct, we mated *unc32(e189)par-3(it71)/qC1 III* males to transgenic hermaphrodites. F1 outcross progeny were allowed to self individually. The recessive marker *unc-32* was used to select *par-3(it71)* homozygotes in the F2. We identified individuals expressing the PAR-3 transgene by scoring for GFP expression in the pharynx, developing vulva and embryos by fluorescence microscopy.

To assess the ability of mutated forms of PAR-3 to rescue the zygotic requirement for the gene, we mated *par-3(tm2010)/qC1 III* males to hermaphrodites

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from each homozygous integrated transgenic line. Offspring from the F1 worms that did not segregate *qC1* homozygotes were scored for embryonic and larval lethality. If the transgene fully rescues in a single copy, we expect approximately 15/16 larvae to be viable in the F2; if the transgene fails to rescue, we expect ¾ of the worms to be viable. Therefore we define full zygotic rescue as 93.75% viability, and no rescue as 75% viability. The percentage rescue was determined by the following formula: (X-75%)/(93.75%-75%), X=scored viability. Each cross was performed in parallel with crosses using the wild-type PAR-3::GFP line as positive controls and N2 as negative controls.

To determine the localization of non-rescuing PAR-3::GFP fusion proteins in homozygous *par-3(tm2010)* embryos and larvae, we constructed *par-3(tm2010)/qC1* strains expressing *par-3^{S863A}::gfp (itIs182), par-3^{CT}::gfp (itIs195), par-3^{NT}::gfp (itIs200)* and *par-3$^{\Delta PDZ2}$::gfp (itIs232)* respectively. For control experiments, we also generated *par-3(tm2010)/qC1* strains expressing wild-type *par-3::gfp* (*itIs179*) and rescuing construct *par-3^{S863E}::gfp(itIs166)*. Synchronous populations of embryos from these worms were mounted for microscopy and assayed for PAR-3::GFP distribution. One fourth of the embryos are expected to be homozygous for *tm2010*. Counts verified that ¼ of the progeny died as embryos or arrested near the L1 to L2 molt.

Microscopy

Observations of live embryos were made on a Leica DM RA2 microscope with a 63X Leica HCX PL APO oil emersion lens and Hamamatsu ORCA-ER digital camera. Digital images were captured using Openlab software (Improvision). Unless indicated otherwise, for each construct images were obtained from at least two independent lines and more than 50 embryos.

Immunostaining and Western analysis

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) 1:70, anti-GFP goat polyclonal (Rockland Immunochemicals) 1:400. Primary antibodies were detected by goat anti-mouse Cy3 at 1:250, or donkey anti-goat Cy3 1:400 (Jackson ImmunoResearch Laboratories, Inc.). Unless indicated otherwise, immunostaining observations were based on the analysis of more than 10 embryos at the appropriate stage.

For detection of proteins in embryo extracts, embryos were collected from hypochlorite-treated adult worms and boiled in SDS-sample buffer (Etemad-Moghadam et al., 1995). Gel electrophoresis and Western blots were performed by standard procedures.

***in vitro* kinase assays**

His-PKC-3 and His-PKC-3^{K266A} were expressed and purified from baculovirus-infected Sf21 cells (Fujise et al., 1994). GST-PAR-3$^{678-935}$, GST-PAR-3$^{1-152}$, GST-PAR-3$^{153-382}$, GST-PAR-3$^{759-868}$, and GST-PAR-3$^{869-1379}$ were produced in *Escherichia coli* and purified by standard procedures. His-PKC-3 and His-PKC-3^{K266A} were incubated with 10μCi[γ-$_{32}$P]ATP (ICN Biomedicals, Inc.) and GST-PAR-3 fragments in 100 μl kinase buffer (25mM Tris-HCl, pH 7.4, 25ng phosphatidylserine , 5mM MgCl$_2$, 500μM EGTA, 1mM dithiothreitol). Reactions were incubated at 30°C for 2 hours and terminated by addition of SDS sample dilution buffer. Proteins were separated by 10% SDS-PAGE, and phosphorylation was visualized by autoradiography.

RESULTS

A *par-3* deletion mutant causes larval lethality

All previously reported *par-3* alleles are strict maternal-effect-lethal mutations (Cheng et al., 1995; Kemphues et al., 1988; Kirby et al., 1990). *par-3(it71),* the strongest of these, contains a nonsense mutation in exon 3 and shows no detectable protein in early embryos (Etemad-Moghadam et al., 1995). However, PAR-3 accumulates normally in epithelial cells of the digestive tract and somatic gonad in embryos from homozygous *it71* mothers, indicating that *it71* is not a null allele (Aono et al., 2004). We obtained a *par-3* deletion allele (*tm2010*, generously provided by the National Bioresource Project, Tokyo), which contains a 409bp internal deletion (5049-5457, start codon=1) including part of intron 6 and exon 7 (Fig. 2.1A). In contrast to the previously identified *par-3* mutants, *par-3(tm2010)* homozygotes die as L1 larvae (33/47) or embryos (14/47). The mutation failed to complement *par-3(it71)* and was rescued by a *par-3::gfp* transgene (viability 95.1±3%, n=523). The larval lethality of *tm2010* indicates that zygotic expression of PAR-3 is required for viability.

The N-terminal region of PAR-3 containing amino acids 1-809 is required for its cortical recruitment

To identify the core sequence in PAR-3 important for localization and function, we tested the ability of truncated PAR-3::GFP proteins to localize and function in *par-3* mutants. We first made reciprocal constructs producing either the N-terminal (NT) or C-terminal (CT) portions of the PAR-3 fused to GFP: *par-3^{NT}::gfp* (aa 1-809) and *par-3^{CT}::gfp* (aa 809-1379), driven by its native promoter. We introduced both constructs and a control full-length *par-3::gfp* into worms by biolistic transformation of an *unc119(-); par-3(+)* strain (Praitis et al., 2001) and examined the distribution of the GFP fusion proteins in early embryos, late embryos

Figure 2.1. PAR-3^{NT}::GFP and PAR-3^{CT}::GFP in early embryos.

(A) Schematic drawing of the *par-3* gene (upper row) and PAR-3 protein structure (lower row). *par-3* exons are represented by black boxes, introns are black lines and untranslated sequences are grey boxes. Asterisk shows the location of the *it71* nonsense mutation, and bracket shows the *tm2010* deletion. Blue, red and green rectangles denote the genomic regions corresponding to CR1, PDZ1, 2, 3 and CR3 domains respectively. In the lower row, colored boxes indicate the conserved domains of PAR-3 protein; numbers denote the amino acids marking the endpoints of each conserved domain (Etemad-Moghadam et al., 1995; Izumi et al., 1998). (B-I) Fluorescence images of PAR-3::GFP, PAR-3^{NT}::GFP and PAR-3^{CT}::GFP in *par-3(+)* embryos (B-E) and in *par-3(it71)* embryos (F-H). (I) shows an embryo with no transgene under the same microscopy conditions. In this and all figures, anterior is to the left of the embryo and the scale bar is approximately 10μm.

A

par-3

it71

tm2010

PAR-3

1   69   152   383   463   515   584   659   738   854   868   1379

CR1   PDZ1   PDZ2   PDZ3   CR3

PAR-3::GFP	PAR-3^NT^::GFP	PAR-3^NT^::GFP	PAR-3^CT^::GFP; *it71*
B	**C**	**D**	**E**

PAR-3::GFP; *it71*	PAR-3^NT^::GFP; *it71*	PAR-3^NT^::GFP; *it71*	negative control
F	**G**	**H**	**I**

55
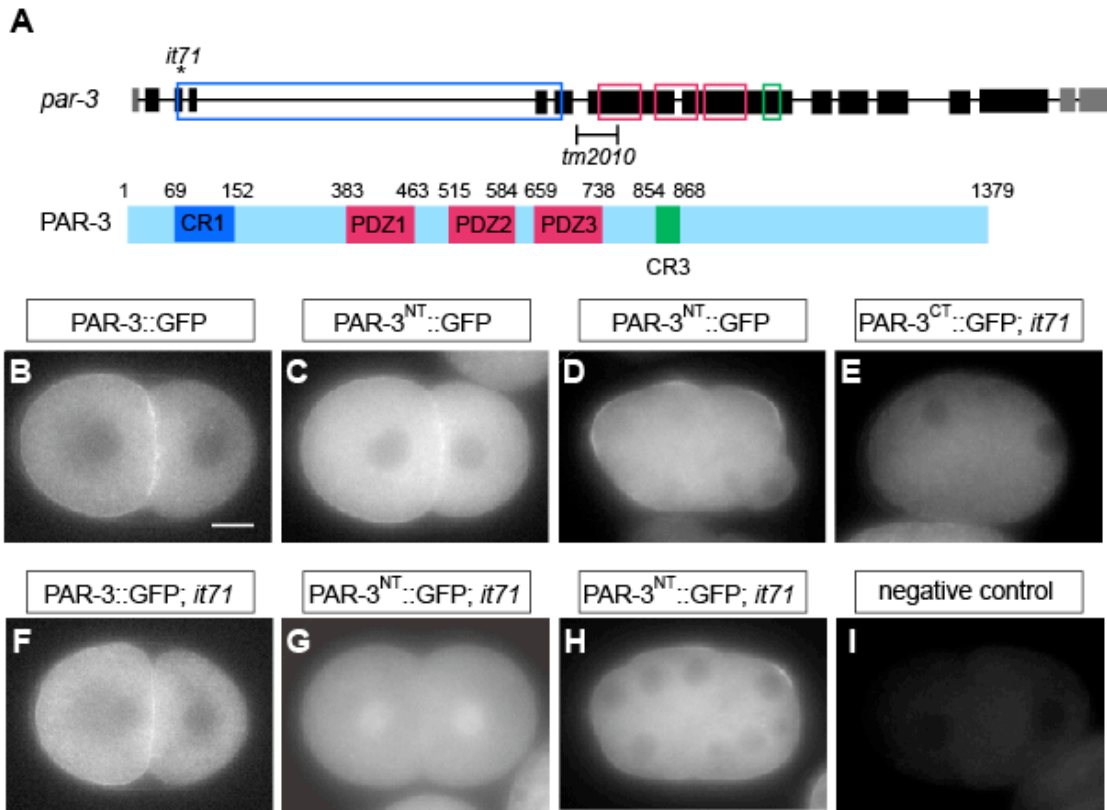
and developing larvae. Because PAR-3 can self-oligomerize via its CR1 domain in flies and mammals (Benton and St Johnston, 2003a; Feng et al., 2007; Mizuno et al., 2003), it is possible that the wild-type PAR-3 present in the worms could recruit the mutant protein via oligomer formation and thus mask any abnormal localization. Therefore we also examined GFP distribution after crosses to replace the endogenous wild-type *par-3* gene with *par-3(it71)* or *tm2010* mutations. These crosses also enabled us to test whether the truncated transgenes could provide *par-3* function in early embryogenesis (*it71*) and in late embryogenesis or post-embryonic development *(tm2010)*.

In both *par-3(+)* and *par-3(it71)* worms, full-length PAR-3::GFP displayed an identical distribution to endogenous PAR-3 protein as reported before (Aono et al., 2004; Etemad-Moghadam et al., 1995; Nance et al., 2003); PAR-3::GFP distributed uniformly at the cortex early in the cell cycle, then cleared from the posterior cortex during the first mitotic prophase. After the first mitotic division, PAR-3::GFP covered the anterior AB cell cortex as well as the anterior periphery of the posterior P1 cell (Fig. 2.1B, F). In L4 larvae, PAR-3::GFP localized to apical surfaces of pharyngeal and vulval epithelial cells (Fig. 2.2A, B). In addition, both *par-3(it71)* and *par-3(tm2010)* were rescued by PAR-3::GFP (Figure 2.3A, B), indicating that this fusion protein functions normally throughout development.

Neither PAR-3[NT]::GFP nor PAR-3[CT]::GFP functioned like wild-type PAR-3. When expressed in *par-3(+)* embryos, PAR-3[NT]::GFP localized to the anterior cortex with much of the protein present in the cytoplasm (Figure 2.1C). However, in *par-3(it71)* embryos, little PAR-3[NT]::GFP was detected at the cortex, the cytoplasmic GFP signal was higher than for the wild-type transgene in these strains, and the mutant protein showed a cell-cycle dependent nuclear accumulation (Figure 2.1G). The cortical accumulation started to increase after the 16-cell stage, in both the *par-3(+)*
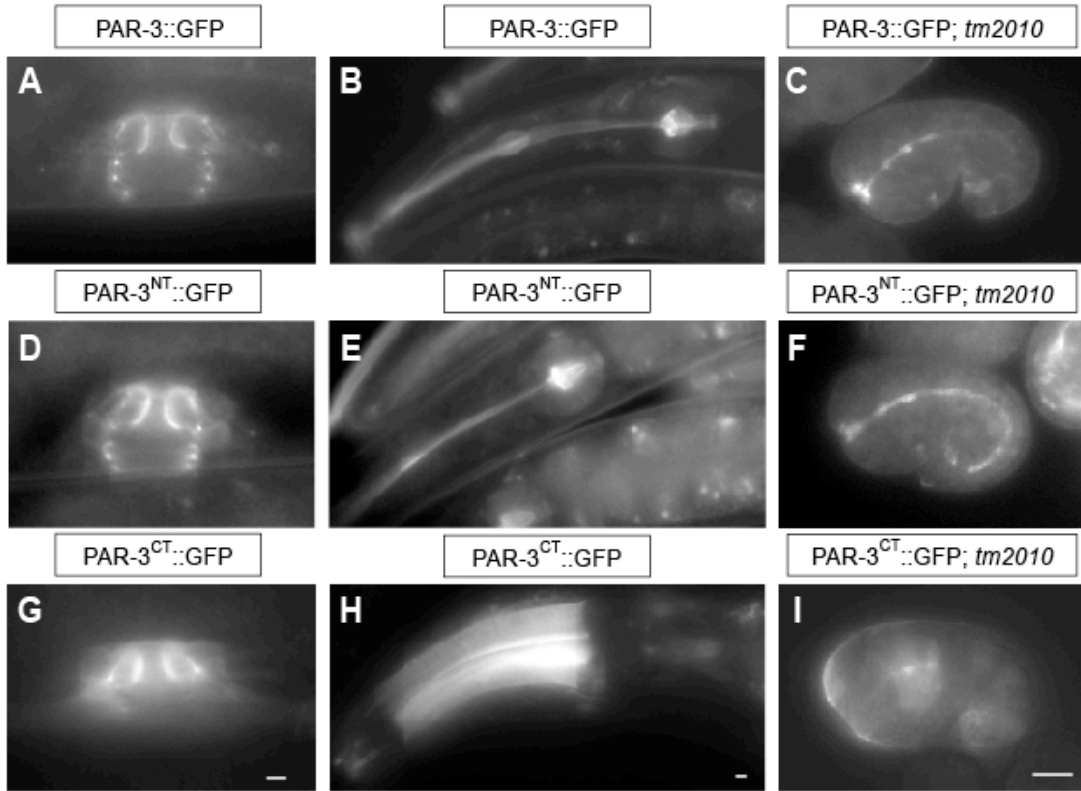
Figure 2.2. PAR-3^NT::GFP and PAR-3^CT::GFP in late *par-3(+)* embryos, developing larvae and *par-3(tm2010)* embryos. Fluorescence images of *par-3(+)* larvae (A, B, D, E, G, H) and 1.5-fold stage *par-3(tm2010)* embryos (C, F, I) expressing PAR-3::GFP (A-C), PAR-3^NT::GFP (D-F) and PAR-3^CT::GFP (G-I). (A, D, G), vulva; (B, E, H), pharynx.

and *par-3(it71)* backgrounds, indicating that PAR-3^{NT}::GFP was still capable of associating with the cortex (Fig. 2.1D, H). Since *par-3(it71)* embryos lack maternal PAR-3, and zygotic PAR-3 does not express until 300-400 cell stage (Nance et al., 2003), this late cortical localization is likely a result of gradual accumulation of PAR-3^{NT}::GFP rather than the recruitment from re-expressed wild-type PAR-3, although we cannot rule out the possibility that there could be different mechanisms to localize PAR-3^{NT}::GFP in one-cell and >16-cell stage embryos. When introduced into *par-3(it71),* PAR-3^{NT}::GFP completely failed to rescue the maternal-effect-lethality (Fig. 2.3A). Thus the C-terminal region of PAR-3 is required for the maternal function of the protein, and contains information necessary for robust accumulation at the cell cortex. Zygotic expression of PAR-3^{NT}::GFP showed the same pattern as wild-type PAR-3:GFP (Fig. 2D, E), except that the best expressing lines (n=5) showed consistently higher signal in the somatic gonad compared to wild-type PAR-3::GFP (data not shown). In spite of this normal cortical distribution, PAR-3^{NT}::GFP completely failed to rescue *par-3(tm2010)*, indicating that the C-terminal region of the protein is necessary for PAR-3 zygotic function as well (Fig. 2.3 B).

In *par-3(+)* embryos, maternally-expressed PAR-3^{CT}::GFP failed to localize to the cortex and was barely detectable in the cytoplasm. One-cell embryos of transgenic worms had consistently higher levels of cytoplasmic signal than the negative controls (Fig. 2.1E, I; 121.6±8% of background fluorescence, n=31 embryos, single-tail t-test, $P<0.005$). We verified that this weak signal was due to expression of the PAR-3^{CT}::GFP by Western blot (Fig. 2.4). Compared to wild-type PAR-3::GFP, which displayed restricted apical localization in epithelial tissues (Fig. 2.2A, B), zygotically
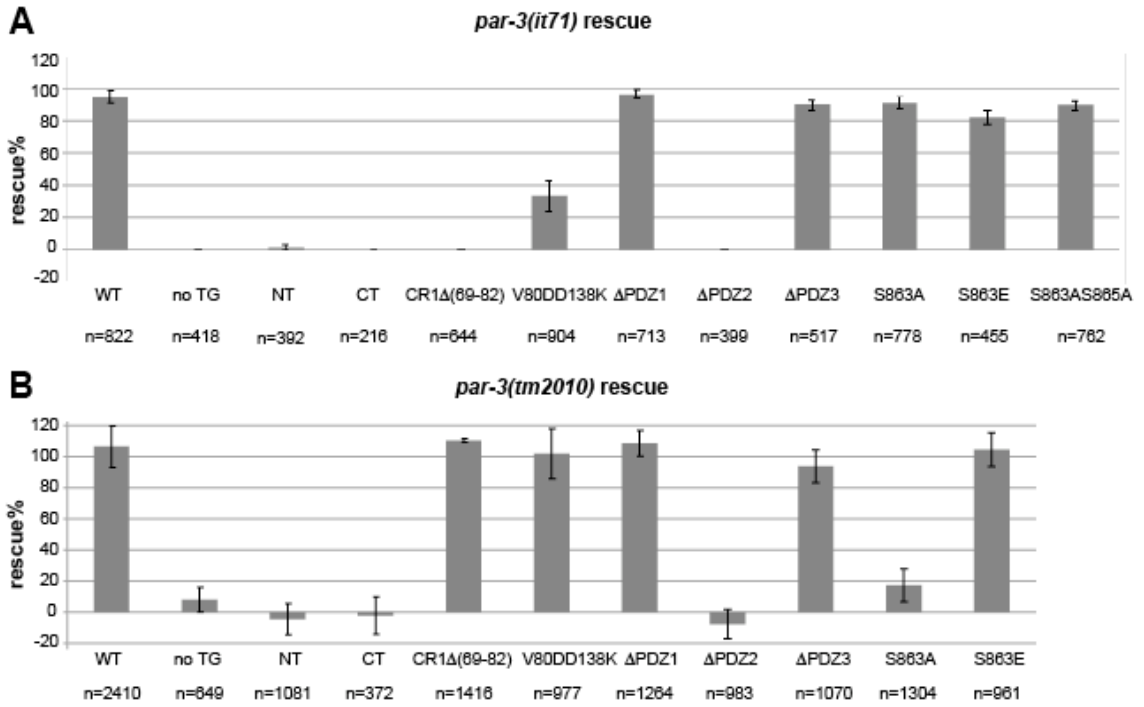
Figure 2.3. PAR-3 transgene rescue of *par-3(it71)* and *par-3(tm2010).*

(A) Percentage of viable embryos from *it71* homozygous mothers carrying the indicated transgene. WT= wild-type PAR-3 transgene; no TG = no transgene (See text for explanation of abbreviations for transgene constructs). (B) Percentage rescue of progeny viability from *par-3(tm2010)/+* mothers calculated as described in Materials and Methods. Error bars represent standard deviation of the values obtained for each experiment. n=total embryos checked for viability.
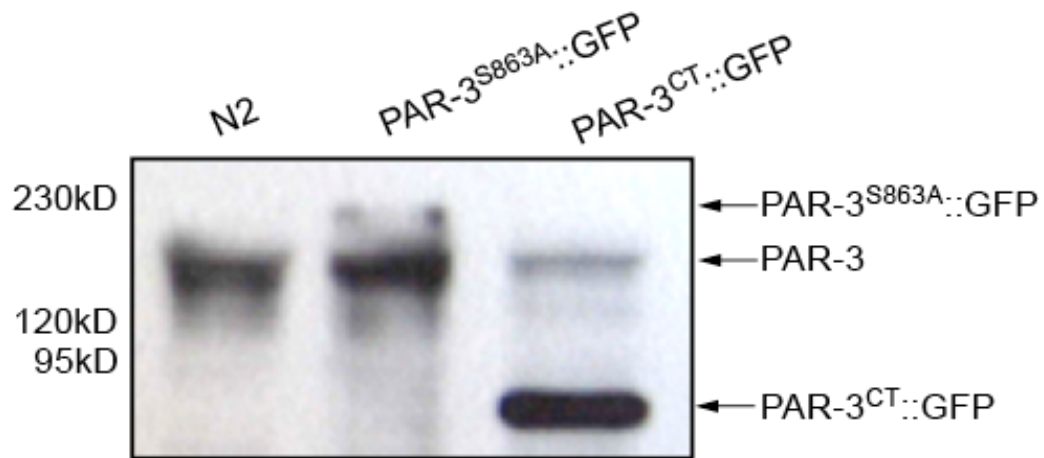
Figure 2.4. Western blot of PAR-3^{CT}::GFP protein in *par-3(+)* embryos. Embryo extracts were prepared from wild type (N2) and from lines expressing PAR-3 ^{S863A}::GFP and PAR-3^{CT}::GFP, then probed with anti-PAR-3 antibody following separation by 7% SDS-PAGE. Note that because the collected embryos were somewhat asynchronous and might include late stage embryos, we cannot rule out the possibility that some of the positive signal is due to zygotic expression.

expressed PAR-3^{CT}::GFP was diffuse in pharynx, spermatheca, vulva, uterine muscles, and anus (Fig. 2.2G, H and data not shown). Consistent with its failure to localize, PAR-3^{CT}::GFP also completely failed to rescue *par-3(it71)* (Fig. 2.3B).

To examine the localization of the NT and CT truncated proteins in the absence of endogenous PAR-3 in developing larvae, we performed crosses to obtain *par-3(tm2010)/qC1 III* hermaphrodites homozygous for *par-3^{NT}::gfp* or *par-3^{CT}::gfp*. Of the progeny from these mothers, 25% should be *par-3(tm2010)* homozygotes and express the transgene. We observed that 97/343 progeny from *par-3^{NT}::gfp; par-3(tm2010)/qC1 III* and 84/273 progeny from *par-3^{CT}::gfp; par-3(tm2010)/qC1 III* died as embryos or L1 larvae, and thus confirmed that neither transgene is capable of rescuing the *tm2010* mutation. We also examined the localization of these transgenic proteins in bean, comma, 1.5-fold, 2-fold and 3-fold stage embryos. We detected no defects in embryonic localization for PAR-3^{NT}::GFP-expressing embryos (n=87), among which a quarter are *par-3(tm2010)* homozygotes (Fig. 2.2F). The intestinal GFP signal appeared stronger than wild type, although after immunostaining the samples to enhance the signal, there was no detectable difference (data not shown). As expected, *tm2010* homozygous embryos expressing PAR-3^{CT}::GFP displayed diffuse expression, with concentrated signal in pharynx, developing digestive tract and hypodermis (Fig 2.2I; n=33).

Overall, these results indicate that the first 808 amino acids of PAR-3 (NT), contain information sufficient for cortical localization but not for proper function, and that amino acids 809 to 1379 (CT) contribute to cortical accumulation or protein stability and are required for function.

PDZ2, but not PDZ1 or PDZ3, is necessary for PAR-3 localization and function

Our attempts to identify smaller fragments sufficient for localization by sequential deletion of the N-terminal fragment failed because we were unable to recover lines expressing any fragment smaller than PAR-3^{NT}::GFP. Therefore we took an alternative approach by making targeted deletions of conserved domains (summarized in Fig. 2.5). We started our analysis by deleting each of the PDZ domains. We generated lines expressing constructs PAR-3$^{\Delta PDZ1}$::GFP (Δaa 383-463), PAR-3$^{\Delta PDZ2}$::GFP (Δaa 515-584), PAR-3$^{\Delta PDZ3}$::GFP (Δaa 659-738) and examined the distribution of the GFP-tagged transgenes. In wild-type *par-3(+)* embryos, we found that deletion of any one of the three PDZ domains had no obvious effect on the cortical localization of the corresponding fusion protein (summarized in Fig. 2.5). Occasionally we observed embryos expressing PAR-3$^{\Delta PDZ2}$::GFP that showed *par-3(it71)*-like phenotypes in the presence of the endogenous wild-type copy of PAR-3, indicating that PDZ2 deletion may cause some dominant-negative effects (data not shown). When endogenous maternal PAR-3 was absent, as in progeny from homozygous *par-3(it71)* mothers, PAR-3$^{\Delta PDZ1}$::GFP and PAR-3$^{\Delta PDZ3}$::GFP proteins showed distributions indistinguishable from PAR-3::GFP (Fig. 2.6A, B, D) and rescued the progeny from homozygous mothers to near wild-type viability (Figure 2.3A). In contrast, PAR-3$^{\Delta PDZ2}$::GFP deviated from wild type, forming cortical and cytoplasmic puncta that were sparser and larger than the puncta formed by PAR-3::GFP (n>50, Figure 2.6C). In addition, the mutant protein failed to maintain its cortical localization through the first cell cycle. In prophase of the first division, a

Embryonic localization of PAR-3::GFP protein and the mutant variants

constructs	par-3(+) cortical	par-3(+) asymmetric	par-3(it71) cortical	par-3(it71) asymmetric	par-3(tm2010) apical
full-length	+	+	+	+	+
V80D, D138K	transient	-	-	-	+
CR1Δ(69-82)	-	-	-	-	+
ΔPDZ1	+	+	+	+	+
ΔPDZ2	+	+	punctate	-	punctate
ΔPDZ3	+	+	+	+	+
NT	+	+	weak	-	+
CT	-	-	-	-	-
S863A	+	+	+	+	+
S863E	+	+	+	+	+
S863AS865A	+	+	+	+	N/A
S950A	+	+	+	+	N/A
S251AS950A	+	+	+	+	N/A

CR1 (oligomerization domain)　PDZ domains　CR3 (PKC-3 binding motif)

Figure 2.5. Embryonic localization of PAR-3::GFP protein and the mutant variants. Summary of the localization of indicated transgenic protein in early *par-3(+)* embryos, early *par-3(it71)* embryos and late *par-3(tm2010)* embryos respectively. Asterisks show positions of point mutations. "+" indicates normal localization; "-" designates failure to localize. If abnormally large and sparse GFP puncta were observed, this was noted as "punctate".
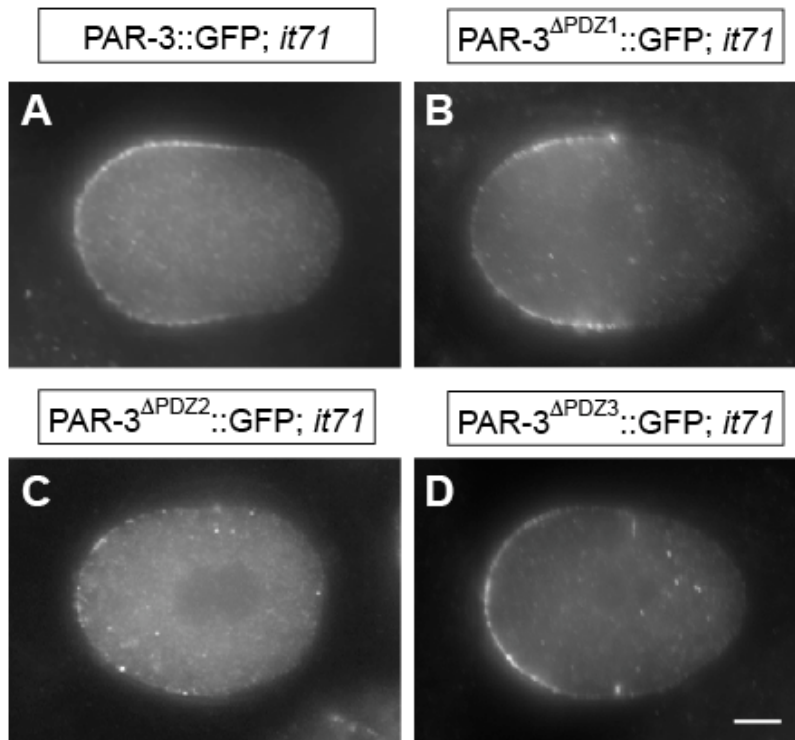
Figure 2.6. PAR-3$^{\Delta PDZ1}$::GFP, PAR-3$^{\Delta PDZ2}$::GFP and PAR-3$^{\Delta PDZ3}$::GFP in one-cell

***par-3(it71)* embryos.** Anti-PAR-3 stained one-cell *par-3(it71)* embryos that express

PAR-3::GFP (A), PAR-3$^{\Delta PDZ1}$::GFP (B), PAR-3$^{\Delta PDZ2}$::GFP (C) and PAR-3$^{\Delta PDZ3}$::GFP

(D). Note that PAR-3$^{\Delta PDZ2}$::GFP forms large and sparse puncta at the cell periphery

and in the cytoplasm (C).
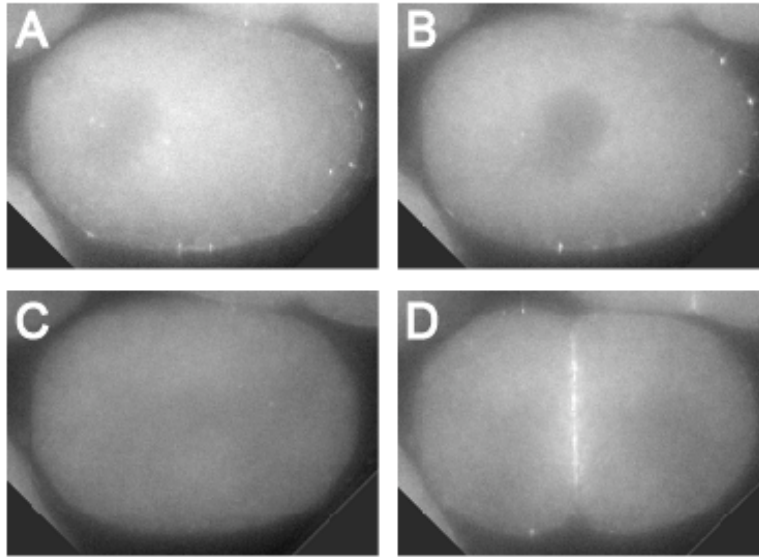
Figure 2.7. PAR-3$^{\Delta PDZ2}$::GFP in *par-3(it71)* embryos. Fluorescence images of *par-3(it71)* embryo expressing PAR-3$^{CR1\Delta(69\text{-}82)}$::GFP at pronuclear meeting (A), centration (B), metaphase (C) and cytokinesis (D).

reduced amount of PAR-3$^{\Delta PDZ2}$::GFP accumulated at the anterior cortex but then lost its restriction as the cell cycle progressed and was uniformly distributed at the time of cytokinesis (Fig.2.7). Consistent with these defects, the transgenes failed to rescue and the embryos exhibited phenotypes typical for loss of maternal PAR-3. These observations showed that PDZ2, but not PDZ1 or PDZ3, is required for PAR-3 to localize and function properly in early embryos.

We obtained similar results when we introduced these three constructs into *par-3(tm2010)*: homozygous *tm2010* embryos were rescued by PAR-3$^{\Delta PDZ1}$::GFP and, to a slightly lesser extent, by PAR-3$^{\Delta PDZ3}$::GFP, but not by PAR-3$^{\Delta PDZ2}$::GFP (Figure 2.3B). We examined the localization of the fusion protein among embryos of *tm2010/+; par-3$^{\Delta PDZ2}$::gfp* mothers, in which 25% of the offspring were expected to express PAR-3$^{\Delta PDZ2}$::GFP and lacked endogenous PAR-3. We found 21% of embryos lacked apical accumulation of GFP but showed accumulations of large GFP puncta adjacent to or in the lumen of the developing pharynx during morphogenesis (n=8/38; Fig. 2.8A, C) while the remaining embryos showed normal localization (n=30/38; Fig. 2.8A, B). Control embryos lacking the transgene showed no signal (data not shown). These results suggested that PDZ2, but not PDZ1 or PDZ3, is required for apical localization and function of PAR-3 in late embryogenesis or larval development.

CR1 is necessary for PAR-3 function in early embryos but dispensable in late-embryogenesis and post-embryonic development

CR1 (<u>c</u>onserved <u>r</u>egion 1), also called NTD (<u>N</u>-terminal <u>d</u>omain), is highly conserved among PAR-3 homologues (Benton and St. Johnston, 2003a; Feng et al., 2007; Mizuno et al., 2003). It has been shown to mediate PAR-3 oligomerization both *in vitro* and *in vivo* and is necessary for PAR-3 apical localization in *Drosophila* and
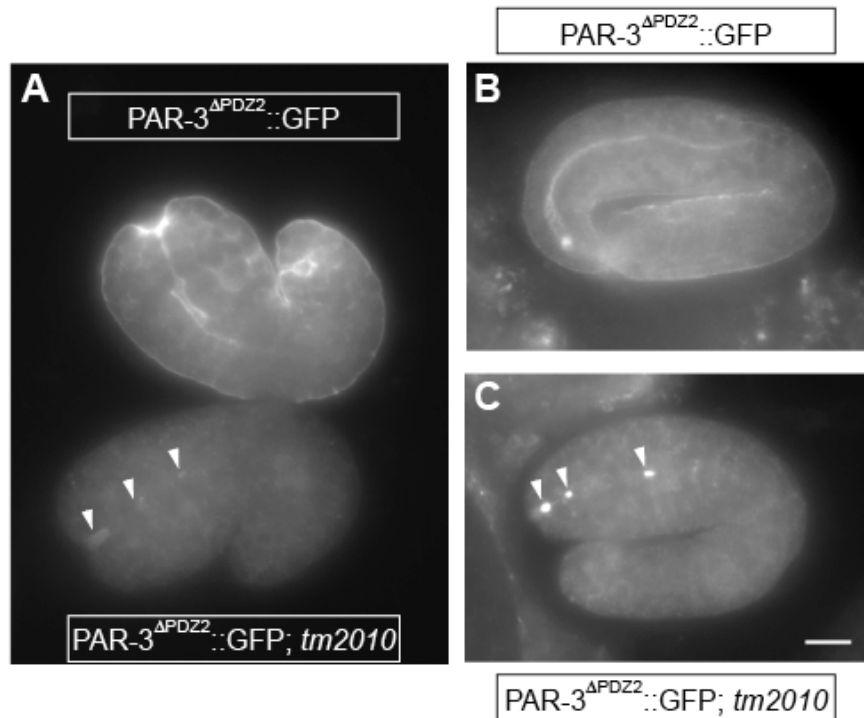
Figure 2.8. PAR-3$^{\Delta PDZ2}$::GFP in late *par-3(tm2010)* embryos.

1.5-fold stage embryos (A) and 2-fold stage embryos (B, C) expressing PAR-3$^{\Delta PDZ2}$::GFP stained with anti-GFP antibody. Note that PAR-3$^{\Delta PDZ2}$::GFP shows apical localization in the developing pharynx, gut and rectum in *par-3(+)* embryos (A upper embryo; B), but is undetectable at the cortex of most cells of *par-3(tm2010)* embryos except developing pharynx where it forms aggregates (arrowheads) in or near the lumen (A lower embryo; C).

in mammalian cultured cells (Benton and St Johnston, 2003a; Feng et al., 2007; Mizuno et al., 2003). To investigate the role of CR1 in *C. elegans*, we first tested whether CR1 of worm PAR-3 mediates oligomerization. Using the yeast-two-hybrid system, we found that CR1 of *C. elegans* PAR-3 was indeed capable of self–association. We found that deletion of aa 1-68, which is specific to worm PAR-3, did not block PAR-3 self-association, although three other small deletions in CR1 (Δ69-82, Δ109-119; Δ122-132) each abolished this property (data not shown). Recently the structure of the CR1 (NTD) domain of mammalian Par3 has been solved (Feng et al., 2007) and two point mutations (equivalent to V80D and D138K in *C. elegans* PAR-3) were identified as being able to disrupt CR1 oligomerization without significantly affecting its overall structure. We introduced the deletion Δ(69-82) and the double point mutation V80D, D138K into full length PAR-3::GFP (PAR-3$^{\Delta(69-82)}$::GFP and PAR-3$^{V80D, D138K}$::GFP respectively) and generated lines expressing these constructs to assess the requirement for oligomerization of PAR-3 *in vivo*.

Neither PAR-3$^{\Delta(69-82)}$::GFP nor PAR-3$^{V80D, D138K}$::GFP localized normally in early *par-3(+)* embryos. PAR-3$^{\Delta(69-82)}$::GFP displayed a diffuse signal in the cytoplasm and no sign of asymmetry was detected in the one-cell stage embryos (n>50, figure 6A). In embryos after pronuclear meeting, PAR-3$^{V80D, D138K}$::GFP behaved indistinguishably from PAR-3$^{\Delta(69-82)}$::GFP (n>50, Fig. 2.9C); however, among thirty-one very early embryos expressing PAR-3$^{V80D, D138K}$::GFP, five exhibited a very weak transient cortical signal which clears from the posterior pole then disappears from the cell periphery during pronuclear migration (Fig. 2.9D). However, in late *par-3(+)* embryos and larvae, both PAR-3$^{\Delta(69-82)}$::GFP and PAR-3$^{V80D, D138K}$::GFP localized similarly to wild-type PAR-3::GFP; they accumulate at apical surfaces of cells in pharynx, intestine, vulva, and somatic gonad (Fig. 2.9E, F and data not shown).

Figure 2.9. PAR-3^{CR1Δ(69-82)}::GFP and PAR-3^{V80D, D138K}::GFP in *par-3(+)* embryos and larvae.

(A-D) Fluorescence images of *par-3(+)* embryo expressing PAR-3^{CR1Δ(69-82)}::GFP (A), PAR-3^{V80D, D138K}::GFP (C, D) and no transgene (B). Note that PAR-3^{V80D, D138k}::GFP is cytoplasmic in most early *par-3(+)* embryos (C), but shows weak and transient cortical localization occasionally (D). Arrowhead points to the weak cortical signal. (E-F) Fluorescence images of *par-3(+)* larvae expressing both PAR-3^{CR1Δ(69-82)}::GFP (E) and PAR-3^{V80D, D138K}::GFP (F). Arrows point to the vulva; bracket indicates the pharynx.

PAR-3^{CR1Δ(69-82)}::GFP

negative control

A

B

PAR-3^{V80D, D138K}::GFP

PAR-3^{V80D, D138K}::GFP

C

D

PAR-3^{CR1Δ(69-82)}::GFP

PAR-3^{V80D, D138K}::GFP

E

F
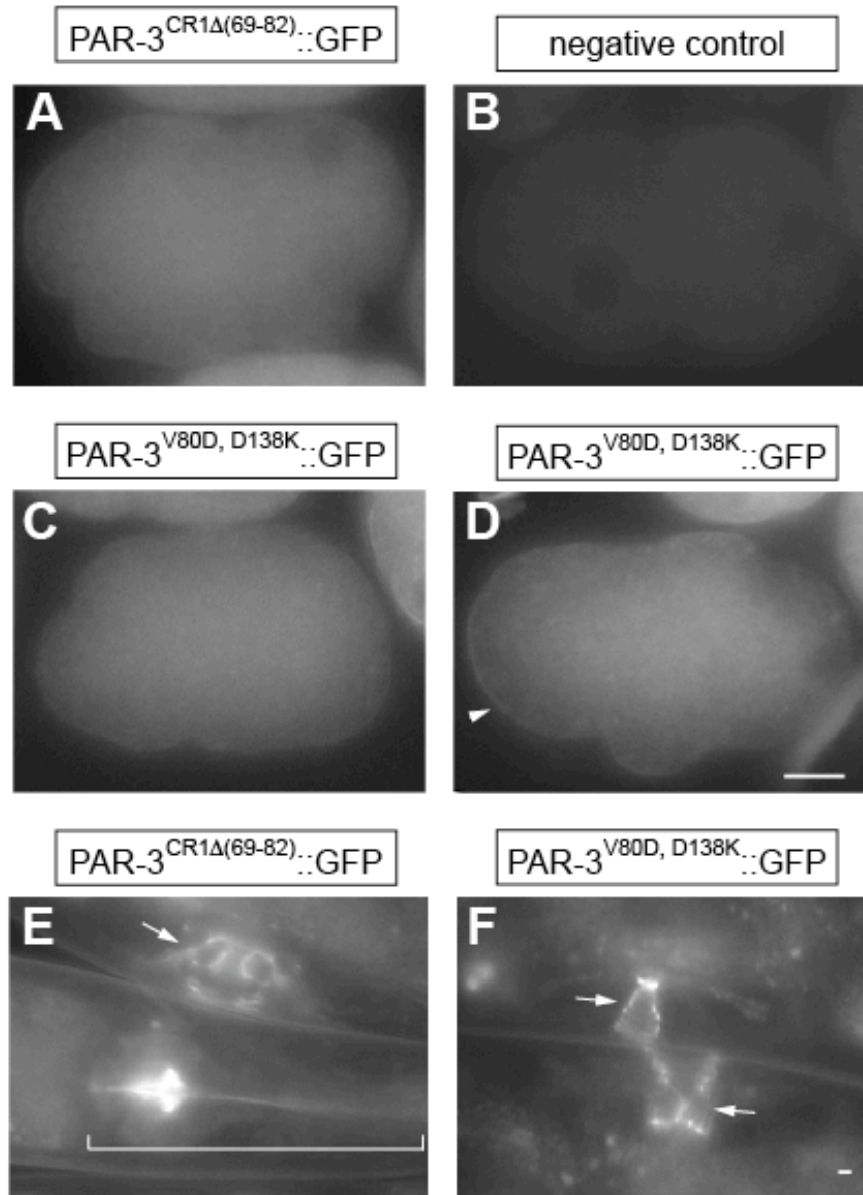
In *par-3(it71)* embryos, both PAR-3$^{\Delta(69-82)}$::GFP and PAR-3$^{V80D, D138K}$::GFP distribution is similar to *par-3(+)* embryos (data not shown). Consistent with its failure to localize, PAR-3$^{\Delta(69-82)}$::GFP failed to rescue the maternal-effect lethality of *par-3(it71)* (Fig. 2.3A), indicating that aa 69-82 are essential for maternal PAR-3 function. PAR-3$^{V80D, D138K}$::GFP showed partial and variable rescue --8.6% to 40.4% of the offspring survived and grew to fertile adults. The rescue variability occurs both between and within lines, and appears to be specific to the PAR-3$^{V80D, D138K}$::GFP construct only (Fig. 2.3A). Two possible explanations for the weak rescue by PAR-3$^{V80D, D138K}$::GFP can be drawn: the mutations did not abolish the ability of PAR-3 to form oligomers, or the CR1 domain has a function in addition to oligomer formation that monomers can facilitate.

Surprisingly, both PAR-3$^{\Delta(69-82)}$::GFP and PAR-3$^{V80D, D138K}$::GFP were capable of rescuing the larval-lethality of *par-3(tm2010)* efficiently (Fig. 2.3B). Together with the observation that both constructs localized properly in zygotic tissues, these results suggest that CR1 is required for PAR-3 to localize and function in early embryogenesis, but it is dispensable for late embryogenesis and postembryonic development.

PKC-3 phosphorylates PAR-3 at a conserved serine

In mammals, aPKC, the homologue of *C. elegans* PKC-3, can bind and phosphorylate mPar3 both *in vitro* and *in vivo* (Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Nagai-Tamai et al., 2002; Suzuki et al., 2001). The single phosphorylation target of mPar3 is serine 827, although binding to aPKC requires serine 829 (Nagai-Tamai et al., 2002); the equivalent serines in *C. elegans* PAR-3 are S863 and S865. To investigate whether PAR-3 is a target of *C. elegans* PKC-3 *in vitro*, we carried out kinase assays using purified *C. elegans* proteins (see Materials

and Methods). We divided PAR-3 into five pieces and tested whether any of them could be phosphorylated by His-PKC-3 *in vitro*. Except for a fragment containing amino acids 383 to 758, which we were unable to express, we found that only the PAR-3 fragment containing amino acids 678 to 935, which includes the *C. elegans* region corresponding to the aPKC binding and phosphorylation site in mammals, could be phosphorylated by wild-type PKC-3 (Fig. 2.10A). PKC-3^{K266A}, a kinase-dead form of PKC-3, failed to phosphorylate PAR-3, indicating that PAR-3 is specifically phosphorylated by PKC-3 in our assay (Fig. 2.10A).

Conversion of the putative target, S863, to alanine completely abolished the phosphorylation by His-PKC-3 but conversion of S865 to alanine had no effect. Thus *C. elegans* PKC-3 phosphorylates PAR-3 at conserved serine S863 *in vitro*.

Phosphorylation at S863 in PAR-3 is not required in early embryogenesis, but is important for later development

To investigate the *in vivo* significance of PKC-3 phosphorylation, we mutated S863 to alanine to block phosphorylation or to glutamic acid to mimic constitutive phosphorylation, and then generated transgenic worms expressing PAR-3^{S863A}::GFP and PAR-3^{S863E}::GFP. We found that in both *par-3(+)* and *par-3(it71)* embryos, PAR-3^{S863A}::GFP and PAR-3^{S863E}::GFP were able to localize to the anterior cortex like wild-type PAR-3::GFP (Figure 2.10B-D and data not shown). Moreover, both PAR-3^{S863A}::GFP and PAR-3^{S863E}::GFP exhibited robust rescue of *par-3(it71)* (Figure 2.3A). To test if S865 could serve as a redundant phosphorylation site *in vivo*, we generated PAR-3^{S863AS865A}::GFP and found that this double mutant also localized properly and rescued *par-3(it71)* efficiently (Fig. 2.3A; Fig. 2.10E). These results suggest that phosphorylation of PAR-3 on S863 or S865 is not essential for early embryogenesis in

C. elegans. We did, however, note a difference between these two constructs. PAR-3^{S863A}::GFP appeared to have a much stronger cortical signal than PAR-3^{S863E}::GFP or wild-type PAR-3::GFP in the early embryos (Fig. 2.11). This difference is quite consistent among more than three independent lines for each construct.

To test whether there is a zygotic requirement for phosphorylation of PAR-3 by PKC-3, we crossed both PAR-3^{S863A}::GFP and PAR-3^{S863E}::GFP into the *par-3(tm2010)* strain. In contrast to the results showing rescue of *par-3(it71)*, PAR-3^{S863A}::GFP showed poor ability to rescue the larval-lethality in *par-3(tm2010)*, but PAR-3^{S863E}:GFP was able to rescue *par-3(tm2010)* efficiently (single tail t-test, p<0.005; Figure 3B). We checked offspring from the *tm2010/qC1 III* mothers expressing either PAR-3^{S863A}::GFP or PAR-3^{S863E}::GFP and examined the localization of the transgene. For PAR-3^{S863E}::GFP, all embryos (n=103) were indistinguishable from wild-type PAR-3::GFP staining patterns (data not shown). Furthermore we were able to isolate *par-3(tm2010)/par-3(tm2010); par-3^{S863E}::gfp* lines that produce fertile progeny, confirming that PAR-3 ^{S863E}::GFP is functional during zygotic development.   For PAR-3^{S863A}::GFP, we could detect no obvious defects in distribution or levels of the mutant protein in any of the 62 embryos we checked (data not shown). Normal localization of PAR-3^{S863A}::GFP may result from perdurance of maternal PAR-3 loaded by the heterozygous mothers, or may indicate that S863A impairs PAR-3 function in some way other than by affecting its apical localization. We conclude that PKC-3 phosphorylation is required for *C. elegans* PAR-3 function in late embryogenesis or post-embryonic development or both, but not in early embryos.

Phosphorylation at two potential 14-3-3 binding sites is not essential for PAR-3 function in *C. elegans*.

PAR-5 is a *C. elegans* 14-3-3 protein and restricts PAR-3 distribution to the

Figure 2.10. PAR-3 phosphorylation at S863 in *par-3(it71)* embryos.

(A) *in vitro* PKC-3 kinase assay with a portion of PAR-3(aa 678-932) and the mutated variants PAR-3^{S863A}::GFP, PAR-3^{S863E}::GFP and PAR-3^{S863AS865A}::GFP as substrate. PKC-3^{K266A}, the kinase-dead form of PKC-3, was used as negative control. (B-E) Anti-PAR-3 antibody stained *par-3(it71)* embryos that express PAR-3::GFP (B), PAR-3^{S863A}::GFP (C), PAR-3^{S863E}::GFP (D) and PAR-3^{S863AS865A}::GFP (E).

A

PKC-3 | PKC-3^{K266A}

WT S863A S865A S863AS865A WT S863A S865A S863AS865A

36kD

29kD

21kD

← PAR-3 (678-932)
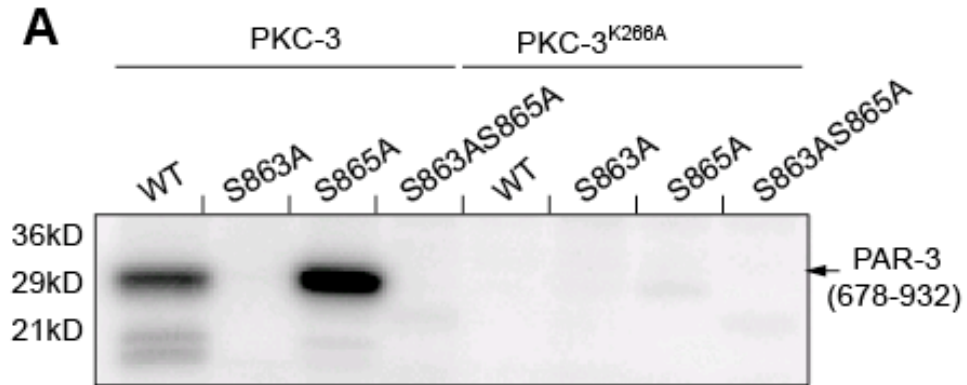
B PAR-3::GFP; *it71*
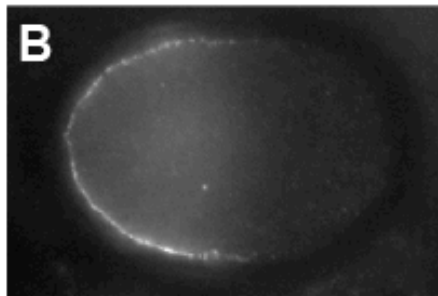
C PAR-3^{S863A}::GFP; *it71*
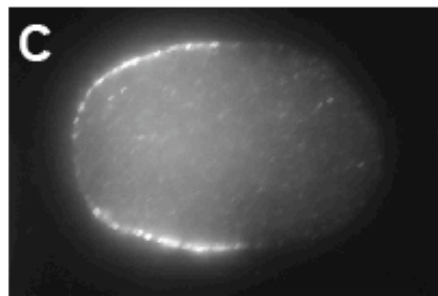
D PAR-3^{S863E}::GFP; *it71*

E PAR-3^{S863AS865A}::GFP; *it71*

anterior in one-cell embryos (Cuenca et al., 2003; Morton et al., 2002). Previous studies in *Drosophila* and mammals suggest that PAR-3 binds to 14-3-3 proteins directly and this interaction requires the phosphorylation of a conserved serine S950 (Benton and St Johnston, 2003b; Hurd et al., 2003a; Izaki et al., 2005). To assess the physiological significance of this phosphorylation in *C. elegans*, we mutated S950, as well as S251, another residue that may be involved in this interaction, to alanines singly and in combination (Benton and St Johnston, 2003b; Hurd et al., 2003a; Izaki et al., 2005). However both PAR-3^{S950A}::GFP and PAR-3$^{S251A, 950A}$::GFP function properly throughout development (summarized in Fig. 2.5). This may be explained by the observation that PAR-3 has multiple putative PAR-5 binding sites, and the point mutations we made may not be sufficient to abolish the interaction *in vivo*.

DISCUSSION

PAR-3 is a highly conserved scaffold protein that functions in a variety of cellular events such as asymmetric cell division, epithelial polarization, directional cell migration and neuronal specification (Goldstein and Macara, 2007). In *C. elegans*, PAR-3 is essential for anterior-posterior polarity in the early embryo (Cuenca et al., 2003; Etemad-Moghadam et al., 1995; Kemphues et al., 1988; Tabuse et al., 1998; Watts et al., 1996) and for processes in later embryonic (Nance et al., 2003) or early larval development (this report) and later larval development (Aono et al., 2004). Here we report results of an analysis of the function of PAR-3's conserved protein domains in living animals. We find that in spite of the overall structural conservation among animals, the requirements for specific PAR-3 domains appear to be stage and species-specific.

Figure 2.11. PAR-3::GFP, PAR-3^{S863A}::GFP, PAR-3^{S863E}::GFP and PAR-3^{S863AS865A}::GFP in *par-3(it71)* embryos. Fluorescence images of one-cell *par-3(it71)* embryos expressing PAR-3::GFP (A), PAR-3^{S863A}::GFP (B),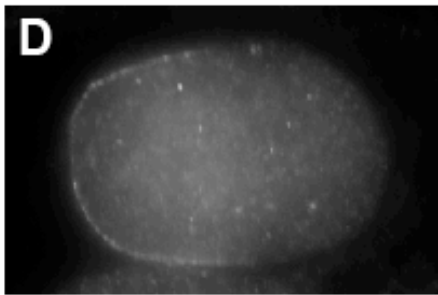 PAR-3^{S863E}::GFP (C) and PAR-3^{S863AS865A}::GFP (D). All images were taken and processed under the same conditions.
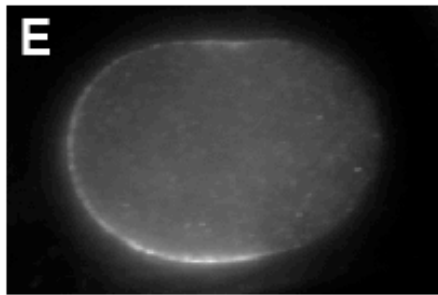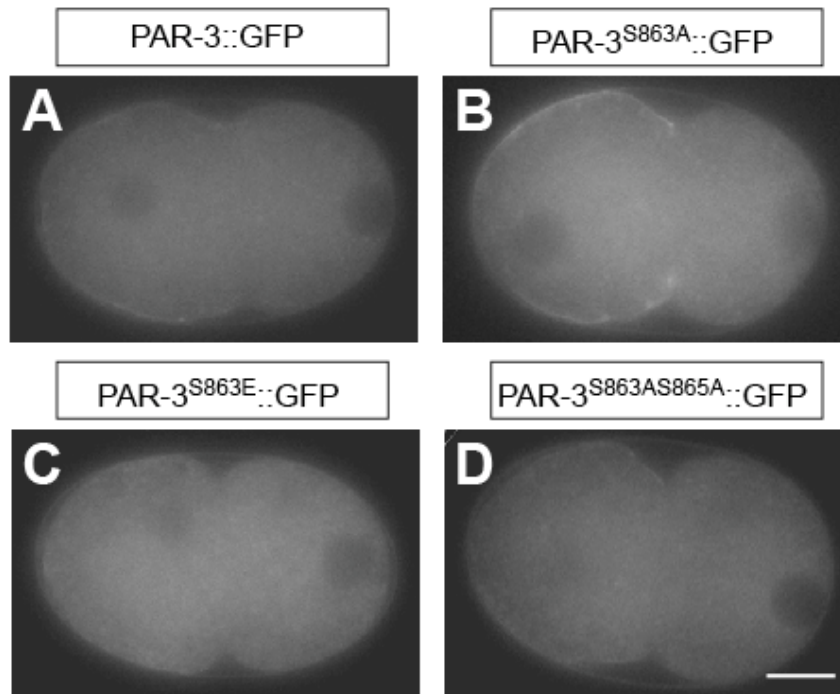
The role of PAR-3 PDZ domains

PDZ domains are 80-90 amino acid-long modules, forming a barrel-like structure consisting of 5-6 β-strands and 2 α-helices (Nourry et al., 2003; Sheng and Sala, 2001). PDZ domains can bind the C-terminus, internal peptides, other PDZ domains of their client proteins or phosphatidylinositol moieties (Roh and Margolis, 2003; Tonikian et al., 2008). PAR-3 has three PDZ domains, so it is reasonable to suppose that this protein may organize large complexes via these PDZ domains. Surprisingly, we found that although deletion of PDZ2 rendered the protein non-functional, deletion of either PDZ1 or PDZ3 had no effect on the ability of the mutated protein to rescue loss-of-function mutations of *par-3*.

The PDZ1 domain of mPar3 or Bazooka has been shown to bind to various proteins including mPar-6, JAM-1, nectins, Inscuteable, and p75 to regulate junction formation in epithelial cells, asymmetric division in neuroblasts and myelination in hippocampal cells (Chan et al., 2006; Ebnet et al., 2001; Itoh et al., 2001; Joberty et al., 2000; Lin et al., 2000; Schober et al., 1999; Takekuni et al., 2003; Wodarz et al., 1999). The *in vitro* interaction between PAR-3 and PAR-6 has been verified in many species including *C. elegans* (J. Li and K. K., manuscript in preparation), although the consequence of this binding remains unclear (Gibson and Perrimon, 2003). In one study, overexpressed mPar6 can perturb epithelial polarity, and mutations in mPar6 that reduce mPar3-mPar6 interaction (KPLG167-170AAAA) abolished this activity (Joberty et al., 2000). However the same mutations can also abolish the interaction between mPar6 and Pals1, therefore making it difficult to interpret the results (Hurd et al., 2003b). In another study, mPar-3 binding to mPar6 is dispensable for tight junction (TJ) assembly in polarizing MDCK cells (Chen and Macara, 2005). Because of this conserved interaction and because PAR-6 and PAR-3 are mutually required for stable localization to the cell cortex of early embryos, our finding that deletion of

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PDZ1 had no apparent effect on PAR-3 function in *C. elegans* was unexpected, although it is consistent with results from our parallel analysis of the PAR-6 PDZ domain. Point mutations in the PAR-6 PDZ domain that block binding of PAR-3 PDZ1 and PAR-6 PDZ *in vitro* have no effect on the PAR-6 function in *C. elegans* (J. Li and K.K. manuscript in preparation).

Limited research has been reported on PAR-3 PDZ2 and PDZ3 domains until recently, when the structure of mPar3 PDZ2 and PDZ3 domains were solved and their roles in mammalian epithelial polarization were examined (Feng et al., 2008; Wu et al., 2007). mPar3 PDZ2 shows high affinity to phosphatidylinositol lipids, but the physiological significance in epithelial polarization is still controversial (Chen and Macara, 2005; Wu et al., 2007); in one study, PDZ2 is not required for mPar3 to restore TJ assembly in mPar3-depleted MDCK cells (Chen and Macara, 2005), whereas another study showed that mPar3 with a PDZ2 deletion fails to localize and function properly in MDCK cells (Wu et al., 2007). We found that PAR-3 PDZ2 is absolutely required for *C. elegans* early embryogenesis and later development. Although the sequence of *C. elegans* PAR-3 PDZ2 domain is not strikingly similar to its mammalian homologues, it does contain a cluster of positively charged amino acids (H512, H555, K557, R597) with spacing similar to that proposed to mediate the electrostatic interaction between mPar3 PDZ2 and phospholipid membranes (K458, R504, K506, R546). It is possible then that *C. elegans* PAR-3 associates with the cell periphery through PDZ2-lipid interaction. Because deleting PDZ2 does not completely dissociate PAR-3 from the cell periphery in early embryos, this putative interaction with phospholipid cannot be the sole mechanism responsible for PAR-3 cortical localization. Indeed, the association of PAR-3 with the cortex in the early embryo is also dependent upon an intact actomyosin network (Severson and Bowerman, 2003).

We found that PAR-3 PDZ3 is dispensable in *C. elegans* in spite of its clear

role in other animals. For example, in mammals, PTEN, the phosphatase that generates PtdIns(4,5)P$_2$, binds directly to mPar3 PDZ3 and this interaction is important for membrane enrichment of PTEN and epithelial polarity (Feng et al., 2008). PDZ3 is also required for mPar3 to concentrate at TJ and to control TJ assembly in polarizing MDCK cells (Chen and Macara, 2005).

The role of PAR-3 CR1 domain

The CR1 domain of PAR-3 is highly conserved in all PAR-3 homologues and mediates PAR-3 oligomerization both *in vitro* and *in vivo* (Benton and St Johnston, 2003a; Feng et al., 2007; Mizuno et al., 2003). mPar3 lacking CR1 shows diffuse cellular distribution in MDCK cells, and overexpression of CR1 delays the formation of functional TJs (Feng et al., 2007; Mizuno et al., 2003). In *Drosophila*, deletion of CR1 disrupts Bazooka apical localization and strongly compromises its function in follicular epithelial cells (Benton and St Johnston, 2003a). We find that in *C. elegans,* intact CR1 is critical for PAR-3 function and cortical localization in early embryos, but not in late embryos and larvae, suggesting that the later function of PAR-3 is independent of CR1-mediated oligomerization. Whether PAR-3 can function in a monomeric form or form oligomers through other regions needs further analysis.

The role of phosphorylation of PAR-3

The CR3 region of PAR-3 is highly conserved from worms to mammals (73% identical). In mammals, aPKC binds to the CR3 region of mPar3 directly and phosphorylates serine 827 both *in vitro* and *in vivo* (Lin et al., 2000; Nagai-Tamai et al., 2002). However, the physiological significance of this phosphorylation is not clear. One study showed that overexpression of an S827A mutant, but not wild-type mPar3, significantly inhibits TJ reformation in polarizing MDCK cells (Nagai-Tamai

et al., 2002). In another study, however, mPar3 can function properly in epithelial polarization independent of aPKC (Chen and Macara, 2005). In our study, we found that in *C. elegans,* the phosphorylation of PAR-3 by PKC-3 does not markedly affect PAR-3 function in early embryogenesis. The phosphorylation may play a subtle role, however, because blocking the phosphorylation consistently resulted in higher levels of cortical PAR-3. In contrast, in late embryogenesis or post-embryonic development, phosphorylation at S863 is required for PAR-3 function. The phosphorylation appears to be permissive rather than regulatory because the phospho-mimic mutation can function as well as wild-type PAR-3.

In flies and mammals, Bazooka and mPar3 can bind to 14-3-3 proteins in a phosphorylation-dependent manner to regulate epithelial polarization (Benton and St Johnston, 2003b; Hurd et al., 2003a). We found that blocking the phosphorylation of PAR-3 at two conserved 14-3-3 (PAR-5) binding sites also had no effect on localization or function of the protein. It is possible that in *C. elegans* additional putative PAR-5 binding sites have assumed the role of the two conserved sites that we tested.

The role of PAR-3 C-terminal region

The C-terminal region of PAR-3 does not contain any recognizable domain structures, but plays important roles in polarity establishment in mammalian neurons and epithelia (Chen and Macara, 2005; Nishimura et al., 2004; Nishimura et al., 2005; Zhang and Macara, 2006). Several studies have revealed that motifs in the C-terminal region are essential for mPar3 to localize properly and to recruit effectors, such as Tiam1, a RacGEF protein (Chen and Macara, 2005; Nishimura et al., 2005). We found that PAR-3 lacking the C-terminal region is still able to associate with the cell periphery in late embryos and developing larvae. These differences are consistent with

the significant sequence difference between worm PAR-3 and its vertebrate homologues (Etemad-Moghadam et al., 1995; Lin et al., 2000; von Trotha et al., 2006).

In summary, our results revealed differential requirements for the conserved domains of PAR-3 in early embryogenesis and larval development. Although PAR-3, PAR-6 and PKC-3 function co-dependently, direct binding between PAR-3 and PAR-6 appears not to be essential, and a requirement for PKC-3 phosphorylation may be dynamic throughout worm development. Interestingly, PAR-3 may function as a monomer or oligomer at different developmental stages, since CR1, the self-association domain, is not required for zygotic development. These findings illustrate the dynamic complexity of PAR-3 interactions and regulation in different developmental contexts to control cell polarity.

ACKNOWLEDGEMENTS

CHAPTER THREE

SUMMARY AND FUTURE DIRECTIONS

3.1 Summary

This thesis describes the work that I have done to better understand the mechanisms by which PAR-3 localizes and functions throughout worm development. The PAR-3 protein plays important roles in cell polarity in many animals. In *C. elegans*, it is a critical component of the anterior PAR system that regulates cell polarity in the early embryo (Kemphues et al., 1988) and is essential for processes in late embryonic or larval development (Aono et al., 2004; Nance et al., 2003; Totong et al., 2007) et al 2007. To understand how PAR-3 localizes and functions during worm development, I introduced targeted mutations and deletions into PAR-3::GFP and examined their localization and function in different developmental stages. I found that the phosphorylation of PAR-3 by PKC-3 at S863 is not essential in early embryogenesis, but is important for PAR-3 function in later stages. Among the three PDZ domains, surprisingly only PDZ2 is essential for function. Neither PDZ1, the PAR-6, nor PDZ3, reported to bind PTEN in mammals and flies, are essential for localization or function. However, PDZ2 is required for stable accumulation of PAR-3 at the cell periphery in early embryos and at apical surface in pharyngeal and intestinal epithelial cells. Furthermore PAR-3 proteins lacking PDZ2 are unable to rescue either the maternal or zygotic PAR-3 function. CR1, the self-oligomerization domain, is required for PAR-3 cortical distribution and function only during early embryogenesis. Disruption of PAR-3 self-association appears dispensable for zygotic development. All these results indicate that in spite of the conservation of PAR-3's overall structure, the requirements for specific PAR-3 domains appear to be organism and stage specific.

3.2. Future directions

3.2.1. To identify PAR-3 PDZ2 interacting molecules

PDZ2 is absolutely required for worm PAR-3 to achieve stable cortical localization and to polarize the early embryo. However, the molecule(s) that interacts with PDZ2 to link PAR-3 to the cell periphery is still not known. The PDZ2 domain of mammalian Par3 displays high affinity to phosphotidylinositol lipid membranes and this interaction is essential for Par3 to localize at TJs in MDCK cells (Wu et al., 2007). Although the sequence of worm PAR-3 PDZ2 is not strikingly similar to its mammalian counterpart, the positive cluster responsible for the electrostatic interaction to the lipid membrane is still present, raising the possibility that worm PAR-3 associates with the cell periphery through PDZ2-lipid interaction. Lipid-binding assays, which can test the direct interaction between the purified protein and synthetic lipid components, will be able to tell us if this interaction is also conserved in worms. If yes, replacing PDZ2 with another lipid-binding motif such as PLCζ pH domain will test whether PDZ2 contributes to PAR-3 localization and function solely by recruiting it to the membrane. If not, yeast-two-hybrid assays can be performed to screen for putative PDZ2 binding partners that participate in localizing PAR-3.

3.2.2. To test if PAR-3 functions independent of PAR-6/PKC-3

Emerging evidence indicate that PAR-3 might execute distinct roles independent of PAR-6/PKC-3 during worm development (Beers and Kemphues, 2006; Hung and Kemphues, 1999; Tabuse et al., 1998; Totong et al., 2007). In chapter 2, I presented evidence that direct interaction between PAR-3 with PAR-6 or PKC-3 is dispensable for worm development, as PAR-3 mutants with presumably disrupted PAR-3-PAR-6 binding (PDZ1Δ), or PAR-3-PKC-3 interaction (S863E,

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S863AS865A), or PKC-3 phosphorylation are normal in terms of localization and function. However, none of the PAR-3 mutants mentioned above presumably could completely distrupt the whole complex. For example, PAR-3 PDZ1Δ in *par-3(it71)* embryos presumably cannot recruit endogenous PAR-6 by itself, but it is still able to interact with PKC-3, and PKC-3 can bring PAR-6 to this complex via its PB1 domain. It will be interesting to test if PAR-6/PKC-3 maintains cortical PAR-3 by being physically part of the complex, or by some other pathways. Analysis of the behavior of PAR-3$^{PDZ1\Delta S863E}$::GFP (or PAR-3$^{PDZ1\Delta S863AS865A}$::GFP) in the absence of endogenous PAR-3 may help to answer the question since these mutants are likely to be "PAR-6/PKC-3-free" PAR-3. In addition, *in vitro* experiments will be necessary to test if these mutations can truly abolish the interaction of PAR-6/PKC-3 with PAR-3.

Since neither PDZ1 and PDZ3 are necessary for PAR-3 localization or function, it also raises the possibility that these two domains may act redundantly. It will be helpful if PDZ1ΔPDZ3Δ double mutant can be generated to test this hypothesis.

3.2.3. To identify other par-3 isoforms and regulatory elements for par-3 expression

Several lines of evidence imply that mulitple par-3 isoforms exist and they may differ in the 5'-end sequence as well as the expression pattern and timing. It will help us to better understand how PAR-3 polarizes cells in different contexts if we could identify other isoforms. (1) 5'RACE-PCR (rapid amplification of cDNA ends)-PCR can be used to isolate the 5′ end sequences of cDNA. (2) the 4th intron of genomic par-3 is 3.6kb long, which lies in the middle of CR1 domain, raising the possibility for bearing an alternative promoter in this region. In addition, this idea is also supported by the fact that two maternal-effect-lethal par-3 mutants, *par-3(it71)* and *par-3(t1591),* both bear nonsense mutations upstream of the 4th intron. To test the

alternative promoter hypothesis, we can fuse par-3 intron4 with GFP, and check if the 4th intron itself can drive the expression of GFP in live worms. (3) I've observed that all *par-3^{CT}::gfp* animals I checked (n=24 lines) have much stronger zygotic GFP signal than that of any other par-3 mutant, indicating that there might be negative regulatory elements residing in the 5' genomic region. A promoter bashing experiment might help to elucidate the mechanism underlying the expression of PAR-3 in zygotic tissues.

3.2.4. *To identify molecules that work with PAR-3 to regulate actomyosin network*

In mammals, the C-terminus of PAR-3 recruits Tiam1, the RacGEF, to control cell polarization through Rac-mediated regulation of cytoskeleton (Chen and Macara, 2005; Nishimura et al., 2005). In *C.elegans* zygote, PAR-3 is also required to generate sustainable cortical flow (Munro et al., 2004). It raises the possibility that PAR-3 may also recruit regulators of actomyosin meshwork to promote cytoskeleton rearrangement. One of the candidates is ECT-2, the putative RhoGEF protein. Despite its important role in early polarity establishment, ECT-2 has also been shown to interact with both Par3 and Par6 in mammalian cells (Liu et al., 2004). More candidates might be identified with the help of fast-growing bioinformatics and powerful reverse genetics. For example, most GEFs contain a PH (pleckstrin homology) domain and a C-terminal DH (Dbl homology) domain (Rossman et al., 2005; Schmidt and Hall, 2002). There are 70 DH members in the human genome, 23 in *Drosophila*, and 18 in *C. elegans* (Rossman and Sondek, 2005). In addition, another subgroup of Rho-GEFs has been recently characterized, which bear two DH homology regions, called DHR1 and DHR2, instead of the classical DH catalytic domain (Meller et al., 2005; Nishikimi et al., 2005). Investigation on the roles of potential worm GEFs

and GAPs might also shed light on our understanding of the mechanism by which the

PARs polarize the worm embryos.

APPENDIX

This appendix consists of two parts. The first part describes my attempt to identify different PAR-3 isoforms and to identify a *par-3* null mutant. The second part reports the RNAi analysis of *C.elegans* homolgues of some human PAR interacting proteins and potential worm interactors from a large-scale yeast-two-hybrid assay .

A.I. Investigation of PAR-3 zygotic isoforms and *par-3* null mutants

A.1.1. 5'RACE-PCR

5′ RACE-PCR is a technique to obtain the 5' sequence of an mRNA of interest. It first uses an anti-sense primer that recognizes a known sequence in the mRNA of interest, then synthesizes the first strand of cDNA by reverse transcription reaction. DNA polymerase is used to generate the second strand of cDNA and RNAse H is used to degrade mRNA template. Following cDNA synthesis, a double-stranded adaptor whose sequence is known is added to the 5'ends of the cDNA by T4 DNA ligase. A regular PCR reaction is then carried out to amplify the product.

Synchronized *glp-4(ts)* L1 worms were cultured at 25°C until L3-L4 stage. RNA extraction was performed according to the Trizol procedure, and the poly-A$^+$ RNA was prepared by FastTrack mRNA isolation kit (Invitrogen). Marathon cDNA amplification kit (Clontech) was used for 5' RACE-PCR. Sequencing of two putative *par-3* isoforms revealed that both started exactly from the 6th exon (aa93). However, both of them lack SL1 or SL2 attached at the 5' end, indicating that they may reflect truncated mRNA of cDNA instead of intact isoforms.

A.1.2. Complementation test of 7 lethal mutations

All previously isolated *par-3* alleles are strict maternal-effect-lethal mutants (Etemad-Moghadam et al., 1995; Kemphues et al., 1988). *par-3 (it71)* contains a

nonsense mutation in exon 3 and showed no detectable protein in early embryos (Etemad-Moghadam et al., 1995; Kemphues et al., 1988). However, PAR-3 accumulates normally in epithelial cells of the digestive tract and somatic gonad, indicating that *it71* is not a null allele (Aono et al., 2004). To check if any of available mutants could be *par-3* null, I checked 7 lethal mutations in the region where *par-3* is mapped (Stewart et al., 1998) for complementation with the maternal effect lethal mutation *par-3(it71)*. I crossed *par-3(it71)* males to hermaphrodites of *let-718, let-780, let-797, let-798, let-799, let-809 and let-810*, then checked the viability of worms laid from *it71/let* mothers. All of the *let* mutants mentioned above were able to complement the maternel-effect-lethality of *par-3(it71),* indicating that none of these embryonic or larval lethal mutations is a mutation in *par-3*.

A.1.3. Generation of par-3(tm2010)/qC1

We obtained a *par-3* deletion allele *(tm2010)* from the National Bioresource Project. *tm2010* contains a 409bp-long deletion in *par-3* including part of intron 6 and exon 7, and the homozygotes of *tm2010* mostly die as L1 larvae. K. Kemphues outcrossed *tm2010* with N2, then I sequenced the deletion region and balanced it with qc1 balancer. The mutation fails to complement *par-3(it71)* and can be rescued by a wild-type *par-3::gfp* transgene, indicating that *tm2010* carries a zygotic *par-3* mutation and the expression of PAR-3 in late embryogenesis or/and postembryonic development is required for viability.

A.1.4. Verification of par-3(it136)

par-3(it136) is a unique *par-3* allele, since *it136* homozygotes are embryonic lethal instead of maternal-effect-lethal, and the maternal mutations can not be

separated from the embryonic lethal one (C. Kirby). To verify this observation, I

checked the segregation of the progeny of 2 lines:

lon-1(e0185)par3(it136)/qc1III;rol-6(e187) F3 phage INT

lon-1(e0185)par3(it136)/qc1III

Both strains segregate about a quarter fertilized dead embryos (24.9% n=389,

and 23.8% n=302, respectively). This result indicates that *par-3(it136)* may carry two

closely-linked mutations since the exogenous wild-type PAR-3 cannot rescue the

embryonic lethality. Complementation test with *tm2010* can be performed to

determine if *t136* is a null allele.

A.II. Investigation of potential PAR-3 interactors

A.2.1. Analysis of mammalian PAR-3 interacting proteins

Brajenovic and colleagues carried out a TAP (tandem affinity purification)

assay to identify mammalian proteins that co-purified with PAR proteins in

mammalian epithelial cells (Brajenovic et al., 2004). This study confirmed many

known interactors of the PAR proteins and reported other novel interactions. J. Li, M.

Beers and I collaborated in the identifcation of the *C.elegans* homologues for each of

the PAR-interacting proteins and determining the phenotypes resulting from RNAi-

mediated depletion of the proteins from early embryos.

dsRNA was made by *in vitro* transcription and then injected into 15-20 young

adult worms. The eggs were allowed to incubate for 12-16 hours at 25°C and then

were scored for viability and terminal phenotype if lethality was observed. Time-lapse

microscopy was also used to determine if the RNAi caused any defects in early

embryonic development. A summary of these data is shown in Table A.1. Only genes

previously known to have roles in polarity or microtubule function in the early embryo

gave significant amounts of embryonic lethality.

A.2.2. Analysis of worm PAR-3 interacting proteins

In 2004, The Vidal group carried out a genome-wide mapping of protein-protein interactions, defining a *C. elegans* "interactome" (Li et al., 2004). 3024 worm proteins that relate to multicellular functions were used as baits and more than 4000 interactions were identified from high-throughput, yeast two-hybrid screens. Independent affinity purification assays were used to validate the overall quality of the data set. Together with previously identified interactions, the Worm Interactome map contains approximately 5500 interactions.

I checked most of the PAR-related protein partners, and repeated the RNAi of some of them to see if any of them plays a role in early embryogenesis. The results are summarized in Table A.2.

Table A.1 human PAR network analysis

human ID	worm ID	bait	RNAi	Remarks on human prey
SQDTM1	T12G3.1	aPKC	WT	Drosophila REF(2) like
	Y32B12A.1		WT	Zinc finger
USP9	F37B12.4	Par1	~10% Ste	Ubiquitin C-terminal hydrolase
NIPSNAP2	K02D10.1	aPKC	WT	4-nitrophenylphosphatase
C6orf69	R05F9.1	Par1	WT	transport, BTB/POZ domain
FLJ20645	F13H8.2	Par6	Gro	Mitochondrial, WD domain
LOC342684	C34E11.3	Par1	WT	Kinetichore-associated
	C23H4.6		WT	Transport, ABC domain
STRADa	C24A8.4	Par4	WT	Ser/Thr kinase
	T19A5.2a		WT	GCK-3 family of ste20 kinase
	C45B11.1b		WT	Rho-binding domain, Ste20 family
LOC55580	Y51A2D.15	Par3	Egl	ER->Glogi transport
KIAA0802	T10G3.5	Par1	~20%Lvl	Vesicular transport
USP7	H19N07.2	Par1	WT	MATH domain, apoptosis
FLJ20643	F32D8.6	Par6	~20%Lvl	Vesicular transport

Table A.2. Analysis of worm interactome

bait	name	prey	RNAi	remarks
par-3	F54E7.3a	pkc-3	Emb	
		F42A10.3	WT	Indolehyl amine N-methyl transferase
		T11B7.4	WT	PDZ-LIM domain, cell adhesion
		par-6	Emb	
par-6	T26E3.3	par-3	Emb	
		H06I04.1	WT	Rho associated GTPase
		ZK849.2	WT	Golgi-associated, PDZ domain
		pkc-3	Emb	
		pkc-2	WT	
		F53B3.1	Ste	Pyrophosphatase, zic finger
par-5	M117.2	K08B4.1	Lvl	Transcription factor in Wnt pathway
		Y51H4A.8	WT	transposase
		zyg-8	Emb	Concentrated with spindles
		C53D6.6	WT	transposase
pkc-3	F09E5.1	par-6	Emb	
		par-3	Emb	
		num-1	WT	Numb homologue, endocytic transport
nmy-2	F20G4.3	F31C3.2	WT	Nucleotidyl transferase

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