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

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Abstract

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 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 CR1, the PAR-3 self-oligomerization domain, is required for PAR-3 cortical distribution and function only during early embryogenesis and that PDZ2 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. We also show that phosphorylation at S863 by PKC-3 is not essential in early embryogenesis, but is important in later development. Surprisingly neither PDZ1 not nor PDZ3 are essential for localization or function. Our results indicate that the different domains and phosphorylated forms of PAR-3 can have different roles during *C. elegans* development.

Keywords

cell polarity; PAR-3; phosphorylation; PDZ domain

INTRODUCTION

Acquisition of cell polarity is a critical process for specifying body axis and maintaining distinct organ function in metazoan development. The PAR (partitioning 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

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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, 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). Levels of 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 a conserved N-terminal domain called CR1, which mediates PAR-3 oligomerization both *in vitro* and *in vivo* (Benton and St Johnston, 2003a; Feng et al., 2007; Mizuno et al., 2003) three PDZ domains in tandem (PDZ1, PDZ2, PDZ3) followed by a region called CR3 containing a conserved PKC-3 binding site (Figure 1A) (Etemad-Moghadam et al., 1995) Izumi et al. 1998). 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. Mutant strains used in this study are KK653, *unc32(e189)par-3(it71)/qC1 III* (Etemad-Moghadam et al., 1995), SS104, *glp-4(bn2*ts) (Beanan and Strome, 1992) 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 which contains genomic *par-3* and 3962 base pairs of upstream regulatory sequences (gift from Dr. Jeremy Nance (Nance et al., 2003)). Mutations or deletions were constructed by site-directed mutagenesis (Quickchange kit, Stratagene) or recombinant PCR. In most cases, internal deletions could be constructed without deleting any intronic sequences, which could potentially contain regulatory elements. However, deletion of PDZ2 required that we delete all of intron 7 as well. All constructs included the wild-type *unc-119* gene as a 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 although we recovered lines with stable long-term maternal expression for most of our constructs, maternal expression was lost in *par-3^{ΔNT}::gfp* lines within 6 weeks after the lines were generated. In all other cases, we selected for subsequent analysis only lines that expressed the mutated fusion protein at least to the same level as wild type protein as assayed by immunofluorescence.

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

We recovered integrated homozygous transgenic lines that express mutated variants of PAR-3::GFP both maternally and zygotically and 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 constructs, 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, which were plated individually and allowed to lay eggs. Unc-32 hermaphrodites will produce viable progeny only if *it71* recombines away from *unc-32* or if the transgene rescues. Because recombination away from the marker is rare as determined by control crosses lacking transgenes, rescue is easily distinguishable by the high frequency of Unc-32 animals that give progeny. In addition, we confirmed rescue by showing that production of progeny correlated with GFP expression in the pharynx and developing embryos of the Unc-32 worms.

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 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 embryos and larvae to be viable in the F2; if the transgene fails to rescue, we expect ¾ of the embryos and larvae to be viable. Therefore

we define full zygotic rescue as 93.75% survival to adult, and no rescue as 75% survival. The percentage rescue was determined by the following formula: (X-75%)/(93.75%-75%), X=scored viability. Note that due to undercounting of embryos, the level of rescue can exceed 100%. 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 homozygous for *par-3^{S863A}::gfp (itIs182), par-3$^{\Delta NT}$::gfp (itIs195), par-3$^{\Delta CT}$::gfp (itIs200)* and *par-3$^{\Delta PDZ2}$::gfp (itIs232)* respectively. *For each strain,* we examined GFP distribution in the alimentary tract of a mixture of 50 or more bean, comma, 1.5-fold, 2-fold and 3-fold stage embryos from the *tm2010/qC1* mothers. One fourth of the embryos are expected to be homozygous for *tm2010*. Counts verified that one fourth of the progeny died as embryos or arrested near the L1 to L2 molt. If the mutated fusion proteins localized normally, 100% of the examined embryos exhibited normal localization; if not, 25% showed an abnormal distribution. *par-3(tm2010)/qC1* strains homozygous for wild-type *par-3::gfp* (*itIs179)* and rescuing construct *par-3^{S863E}::gfp(itIs166)* served as controls.

Microscopy

Observations of live embryos were made on a Leica DM RA2 microscope with a 63× 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. Confocal images were collected on a Leica TCS SP2 system with a Leica DMRE-7 microscope and an HCX PL APO 63× oil immersion lens. Images were processed using the Leica Confocal SP2 software program and Adobe PhotoShop.

RT-PCR

mRNAs were extracted from L3-L4 N2 or *glp-4(bn2*ts) worms using the FastTrack mRNA isolation kit (Invitrogen). RT-PCR reactions were performed using the First Strand DNA synthesis kit and the pdN$_{(6)}$ primer it provided (Biosciences). PCR primers used for amplification of the diagnostic fragments are: Primer (B)5′-acagttggtcaactagcagacgcagc-3′; Primer (C)5′-atgcataacggtcgtggtggtcg-3′; Primer(ctrl1)5′-gagacgcaggtggtatgcgcaatg-3′; Primer (ctrl2) 5′-acacgcatcggctataatttcagcac-3′; Primer (R) 5-gctcggcgagcttcttctcaacttc-3′. All procedures were performed according to the manufacture's protocols. PCR products were then cloned into TOPO vector (Invitrogen) and sequenced.

Western blots

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. anti-PAR-3 primary antibody was diluted 1:1000 and HRP-conjugated secondary antibody (Jackson ImmunoResearch) was diluted 1:5000.

Immunostaining

Embryos were fixed in methanol following previously published procedures (Guo and Kemphues, 1995). The following primary antibodies and dilutions were used: anti-PAR-3 mouse monoclonal (Nance et al., 2003) 1:70, anti-GFP goat polyclonal (Rockland Immunochemicals) 1:400, anti-PAR-2 rabbit polyclonal antibody (Boyd et al, 1996) 1:15, anti-PAR-6 rabbit polyclonal antibody (Hung and Kemphues, 1999) 1:30, anti-PKC-3 rat polyclonal antibody (Aono et al. 2004) 1:30. Primary antibodies were detected by Alexa

Fluor 488 labeled goat anti-mouse (Invitrogen), Cy3 labeled goat anti-mouse at 1:200 or 1:250, Cy 3 labeled donkey anti-goat 1:400 or Cy3 labeled donkey anti-rabbit or anti-rat at 1:200 (Jackson ImmunoResearch Laboratories, Inc.). Unless indicated otherwise, immunostaining observations were based on the analysis of more than 10 embryos at the appropriate stage.

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 but one 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. 1A). In contrast to most 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.

Previous studies suggested that the maternal-specific alleles were due to mutations within a region of the 5′ end of the mRNA (F54E7.3a or b) that were not included in a putative alternative transcript that was expressed only in late embryonic or larval stages or both (Aono et al., 2004). Indeed, a short transcript, F54E7.3c, is predicted and has been partially confirmed (Wormbase, release WS207; Fig. 1A). To test whether this transcript is expressed post-embryonically we performed RT-PCR using mRNA isolated from a mix of L3 and L4 N2 worms. Diagnostic fragments of F54E7.3b and F54E7.3c were amplified by primers B and R and C and R respectively (Fig. 1B, lanes B and C) and confirmed by sequencing. In addition, both primer ctrl2 which targets sequences immediately 5′ of the predicted start codon of F54E7.3c, ATG of F, and primer ctrl1 which targets a more 5′ region of intron 4, failed to amplify any product (Fig. 1A, lanes ctrl2 and ctrl1), indicating that it is likely that F54E7.3c reading frame initiates from the predicted start codon. The long transcript must be maternal because strictly maternal mutations affect only the long transcript. The long transcript also appears to be transcribed zygotically because L3 and L4 *glp-4(bn2ts)* hermaphrodites grown at restrictive temperature, which have severely reduced germ lines (Beanan and Strome, 1992), show the same levels of both transcripts as wild type (Supplemental Figure S1). We did not determine whether the short transcript is expressed maternally; if it is expressed, it is not capable of substituting for the long form.

The predicted protein product of F54E7.3c substitutes 71 novel amino acids for the first 107 amino acids of F54E7.3b and results in a disruption of the CR1 domain in the protein product of F54E7.3c, replacing the first 38 amino acids of the 83 amino acid domain. Blast search revealed no homology to the novel amino acids coded by the F54E7.3c message in Par-3 proteins other than in *C. elegans* and *C. briggsae*.

Both the N-terminal and C-terminal portions of PAR-3 contain information required for cortical accumulation

To identify the core sequences 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. For these and all subsequent transgene constructs we mutated the full length genomic DNA within pJN102 (Nance et al., 2004; see materials and methods). We first made reciprocal constructs missing either C-terminal (ΔCT) or N-terminal (ΔNT) portions of PAR-3 fused to GFP: *par-3$^{\Delta CT}$::gfp* (Δ aa 809-1379) and *par-3$^{\Delta NT}$::gfp* (Δ aa 1-809), 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 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 endogenous 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 *par-3(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, wild-type PAR-3::GFP displayed a weak signal but an identical distribution to endogenous PAR-3 protein as reported previously (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 entire cortex of the anterior cell, AB, as well as the anterior cortex of the posterior cell, P1 (Fig. 2A, E; Supplementary Movie S1). In L4 larvae, PAR-3::GFP localized to apical surfaces of pharyngeal and vulval epithelial cells (Fig. 3A, B). In addition, both *par-3(it71)* and *par-3(tm2010)* were rescued by PAR-3::GFP (Fig. 4A, B), indicating that this fusion protein functions normally throughout development.

Neither PAR-3$^{\Delta CT}$::GFP nor PAR-3$^{\Delta NT}$::GFP behave like wild-type PAR-3. When expressed in *par-3(+)* embryos, PAR-3$^{\Delta CT}$::GFP localizes to the anterior cortex of early embryos, although high levels of protein are present in the cytoplasm (compare Fig. 2B to 2A). However, in early *par-3(it71)* embryos, little PAR-3$^{\Delta CT}$::GFP was detected at the cortex and the cytoplasmic GFP signal was higher than for the wild-type transgene (compare Fig. 2F to 2E). The truncated protein is not completely incapable of cortical accumulation, however, because cortical protein is detectable after the 16-cell stage, in both the *par-3(+)* and *par-3(it71)* backgrounds (Fig. 2C, 2G). Since *par-3(it71)* embryos lack maternal PAR-3, and zygotic PAR-3 does not express until the 300–400 cell stage (Nance et al., 2003), this late cortical localization is likely a result of gradual accumulation of PAR-3$^{\Delta CT}$::GFP rather than recruitment of the truncated protein via interaction with zygotically expressed wild-type PAR-3. It is also possible that this delayed cortical accumulation indicates that different mechanisms localize PAR-3$^{\Delta CT}$::GFP in one-cell and >16-cell stage embryos. In spite of this weak localization, PAR-3$^{\Delta CT}$::GFP failed to rescue the maternal-effect-lethality of *par-3(it71)* (Fig. 4A). 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.

Zygotically expressed PAR-3$^{\Delta CT}$::GFP showed the same distribution as zygotically expressed wild-type PAR-3:GFP either in *par-3(+)* (Fig. 3D, E) or *par-3(tm2010)* (Fig. 3F) genetic backgrounds. In spite of this normal distribution, PAR-3$^{\Delta CT}$::GFP failed to rescue *par-3(tm2010)* (Fig. 4B), indicating a requirement for amino acids 809-1379 for PAR-3 zygotic function.

In *par-3(+)* embryos, maternally-expressed PAR-3$^{\Delta NT}$::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. 2D, H; 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$^{\Delta NT}$::GFP by Western blot (Supplementary Figure S2). Compared to wild-type PAR-3::GFP, which displayed restricted apical localization in epithelial tissues (Fig. 3A, B), zygotically expressed PAR-3$^{\Delta NT}$::GFP was diffuse in all epithelial tissues examined irrespective of the presence of endogenous wild type PAR-3 (Fig. 3G, H, I). Consistent with its failure to localize cortically and apically, PAR-3$^{\Delta NT}$::GFP also failed to rescue *par-3(it71)* and *par-3(tm2010)* (Fig. 4).

Overall, these results indicate that the first 808 amino acids of PAR-3 (ΔCT), contain information sufficient for cortical localization but not for proper function, and that amino acids 809 to 1379 (ΔNT) 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 transgenic lines expressing any fragment smaller than PAR-3$^{\Delta CT}$::GFP. Therefore we took an alternative approach by making targeted deletions of conserved domains (summarized in Table 1). We started our analysis by deleting each of the PDZ domains. We generated lines expressing constructs PAR-3$^{\Delta PDZ1}$::GFP (Δ aa 383-463), PAR-3$^{\Delta PDZ2}$::GFP (Δ aa 515-584), PAR-3$^{\Delta PDZ3}$::GFP (Δ aa 659-738) and examined the expression level and distribution of the GFP-tagged transgenes. We were able to generate lines with levels of accumulation of fusion protein similar to that of wild type fusion proteins. 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 Table 1). Occasionally we observed *par-3(+)* embryos expressing PAR-3$^{\Delta PDZ2}$::GFP that showed *par-3(it71)*-like phenotypes 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. 5A, B, C) and rescued the progeny from homozygous mothers to near wild-type viability (Fig. 4A). In contrast, in the absence of endogenous wild type PAR-3, PAR-3$^{\Delta PDZ2}$::GFP deviated from wild type, forming sparse and large cortical puncta (Fig. 5D). These embryos retained high levels of GFP signal in the cytoplasm indicating that failure to rescue was not likely to be due to reduced expression of the mutant protein (Fig. 5D; Fig. 6E, M). Embryos fell into two categories. In 20 of the 28 embryos we examined either via live imaging or in fixed specimens, cortical puncta were extremely sparse and showed no obvious asymmetry (Supplementary Movie S2, the right embryo). Eight of the embryos had larger numbers of cortical puncta which occasionally showed some asymmetry during the early phase of the first cell cycle (Fig. 5D; Supplementary Movie S2, the left embryo); 7 of 24 fixed embryos showed numbers of cortical puncta similar to the embryo in the movie. In both classes of

embryos, the cortical puncta disappear at metaphase and return during prophase of the next cell cycle (Fig. 5, E, F; Supplementary Movie S2).

We next asked how deletion of PDZ2 affects the ability of PAR-3 to co-localize with PAR-6 and PKC-3. In *par-3::gfp; par-3(it71)* embryos, PAR-3 puncta co-localize extensively with PAR-6 (Fig. 6A-D; supplementary Fig. S3) and PKC-3(Fig. 6I-L; supplementary Fig. S3). However, the large puncta containing PAR-3$^{\Delta PDZ2}$::GFP fail to co-localize with PAR-6 (n= 15 embryos) or PKC-3 (n= 15 embryos). Indeed, PAR-6 and PKC-3 are not enriched at the cortex at all in these embryos (Fig. 6F, H, N, P; supplementary Fig. S3).

Consistent with these defects, PAR-3$^{\Delta PDZ2}$::GFP transgenes failed to rescue *it71* mutants (Figure 4A) and the embryos exhibited phenotypes typical for loss of maternal PAR-3: cytoplasmic flow was attenuated, spindle displacement failed, and the first division was equal (n=27). 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)/+* worms and scored progeny for viability. Based on survival rates we concluded that 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 4B). To verify the unexpected result suggesting that PDZ1 and PDZ3 were not required and to determine whether the surviving worms were sterile or exhibited any morphical or behavior abnormality, we constructed stable lines that were homozygous for *tm2010* and carried the rescuing transgene. Homozygous *tm2010* worms rescued by PAR-3$^{\Delta PDZ1}$::GFP or by PAR-3$^{\Delta PDZ3}$::GFP exhibit variable amounts of embryonic and larval lethality. For example, among the progeny of 20 fourth generation *tm2010* homozygotes expressing PAR-3$^{\Delta PDZ1}$::GFP, viability to adult ranged from 10% to 89%; among progeny of 20 fourth generation *tm2010* homozygotes expressing PAR-3$^{\Delta PDZ3}$::GFP viability ranged from no survivors (two cases) to 96% survivors. Most worms that survive to adulthood, however, exhibit wild type morphology and behavior and produce normal broods of viable progeny. We cannot distinguish to what extent the variable level of lethality is due to compromised protein function vs. variable expression of the transgenes.

As stated above, the PAR-3$^{\Delta PDZ2}$::GFP fusion protein failed to rescue *tm2010* mutants. We examined the localization of 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 be homozygous for *tm2010*. 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. 7A, C) while the remaining embryos showed normal localization (n=30/38; Fig. 7A, 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 (conserved region 1), also called NTD (N-terminal domain), 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 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 (Supplementary Fig. S4). 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 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(+)* or *par-3(it71)* embryos. PAR-3$^{\Delta(69-82)}$::GFP displayed a diffuse and uniform signal in the cytoplasm (n>50, figure 8A). In embryos after pronuclear meeting, PAR-3$^{V80D, D138K}$::GFP behaved indistinguishably from PAR-3$^{\Delta(69-82)}$::GFP (n>50, Fig. 8C); 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. 8D). In contrast to their behavior in early embryos, in late *par-3(+)* and *par-3(tm2010)* 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. 8E, F and data not shown).

Consistent with its failure to localize in early embryos, PAR-3$^{\Delta(69-82)}$::GFP failed to rescue the maternal-effect lethality of *par-3(it71)* (Fig. 4A), 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. This variability in extent of rescue occurs both between and within lines, and appears to be specific to the PAR-3$^{V80D, D138K}$::GFP construct only (Fig. 4A). Two possible explanations for the weak rescue by PAR-3$^{V80D, D138K}$::GFP can be drawn: the mutations do 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.

To determine the importance of PAR-3 self-oligomerization to the distribution of other PAR proteins in early embryos, we examined the localization of PAR-6 and PAR-2 in *par-3(it71)* embryos carrying PAR-3::GFP or PAR-3$^{V80D, D138K}$::GFP. In *par-3(it71); par-3::gfp* embryos, PAR-6 and PAR-2 localized reciprocally at the anterior and posterior cortex (Fig. 9A, C). However, when PAR-3::GFP was substituted by PAR-3$^{V80D, D138K}$::GFP, little PAR-6 was detected at the cell periphery, and PAR-2 expanded into the anterior domain (Fig. 9B, D), indicating that the oligomerization function of PAR-3 is necessary for PAR-6 localization and PAR-2 restriction in early embryos.

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. 4B). Together with the observation that both PAR-3$^{\Delta(69-82)}$::GFP and PAR-3$^{V80D, D138K}$::GFP localized properly in the rescued larvae, these results suggest that CR1 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 partially 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 the fragment containing amino acids 383 to 678, 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. 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. 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 10B–D, Supplementary Movie S3). Moreover, both PAR-3^{S863A}::GFP and PAR-3^{S863E}::GFP exhibited robust rescue of *par-3(it71)* (Fig. 4A). 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. 4A; Fig. 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 (Supplementary Movie S3). This difference is quite consistent among at least 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 lethality of *par-3(tm2010)*, but PAR-3^{S863E}:GFP was able to rescue *par-3(tm2010)* efficiently (single tail t-test, p<0.005; Fig. 4B). We checked offspring from the *tm2010/qC1 III* mothers expressing either PAR-3^{S863A}::GFP or PAR-3^{S863E}::GFP (n=62 and 103 respectively) and examined the localization of the transgene. All embryos (of which ¼ should be homozygous for *tm2010*) 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. 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 conserved 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 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 on a potential PAR-1 target site (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, singly and in combination with S251, another potential PAR-1 target that may be involved in this interaction, to alanines (Benton and St Johnston, 2003b; Hurd et al., 2003a; Izaki et al., 2005). However PAR-3[S950A]::GFP and PAR-3[S251A, 950A]::GFP localize asymmetrically throughout development and rescue *it71*. We did not test PAR-3[S950A]::GFP for rescue of *tm2010*, but PAR-3[S251A, 950A]::GFP rescues *tm2010* (Figure 4 and Table 1). Because we could identify at least 10 additional putative PAR-5 binding sites, our results do not rule out a role for PAR-1 or PAR-5 in regulating PAR-3.

DISCUSSION

PAR-3 is a highly conserved scaffold protein that functions in a variety of polarized 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) early larval (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.

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 nonfunctional, deletion of either PDZ1 or PDZ3 had little or 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* (Li et al., 2010), 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 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* (Li et al., 2010).

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 although PAR-3 lacking PDZ2 retains weak ability to become enriched at the cortex, it is unable to recruit PAR-6 or PKC-3, suggesting either that PDZ2 plays some direct role in recruiting one or both of these proteins or that proper cortical association or concentration of PAR-3 is required for formation of stable complexes.

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. Our verification of the expression of an alternative mRNA that has a disrupted and presumably non-functional CR1 domain is consistent with our functional data and with the existence of maternal-specific mutant alleles. Mutations like *it71* that affect only the large mRNA are able to support zygotic development because the short mRNA can function without an intact

CR1 domain, but fail to provide the maternally provided function in early embryos because the short mRNA, even if it is produced in embryos, would generate a protein lacking a functional CR1 domain. Whether this alternative form of PAR-3 can function in a monomeric form or forms oligomers through via a mechanism other than CR1 multimerization needs further investigation.

The role of phosphorylation of PAR-3

The CR3 region of PAR-3 is highly conserved from worms to mammals (Izumi et al., 1998). 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-mimetic mutation can function as well as wild-type PAR-3. A recent report from Drosophila is consistent with our results and provides insight into a possible basis for these results. Phosphorylation at S890 of Bazooka (PAR-3), the analogous position to *C. elegans* S863, is required in epithelial cells to exclude PAR-3 from the apical domain, but appears not be required in the Drosophila oocyte (Eurico Morais-de-Sá et al., 2010). Larval lethality in *C. elegans par-3* mutants is likely to be due to defects in polarized epithelial cells (Achilleos et al., 2010). Thus, the difference in dependency on phosphorylation likely reflects differences in the precise role of PAR-3 in epithelial cells vs. its role in the early embryo.

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 asymmetric distribution 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 in its mammalian homologues the region plays important roles in polarity establishment in 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 late embryonic or 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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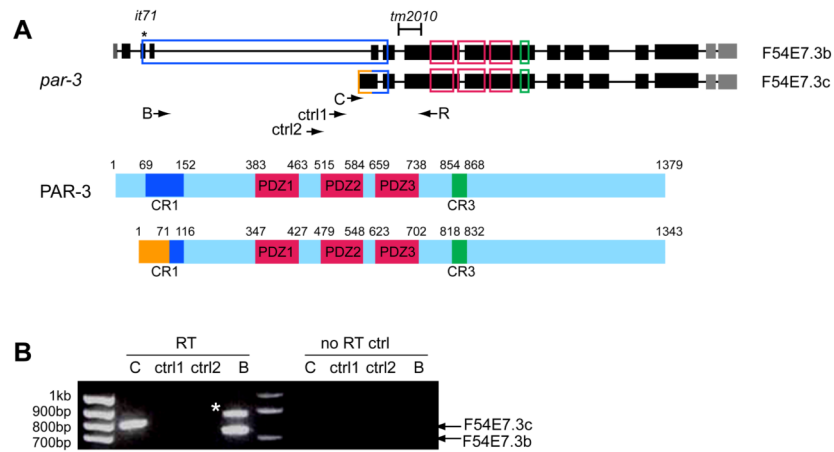
Figure 1. Structure of the *par-3* gene and its transcripts

(A) Schematic drawing of the *par-3* gene (upper row) and PAR-3 protein structure (lower rows). *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 open rectangles denote the genomic regions corresponding to CR1, PDZ1, 2, 3 and CR3 domains respectively. The blue and orange open rectangle indicates the sequences encoding CR1 (blue) and coding sequences unique to F54E7.3c that disrupt the CR1 domain (orange). 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) Results of RT-PCR reactions showing diagnostic segments of F54E7.3b (Lane B) and F54E7.3c (Lane C) amplified from mRNA isolated from wild type mixed L3 and L4 stage larvae and sequenced (see text). The asterisk indicates a spuriously amplified segment of bacterial RNA.
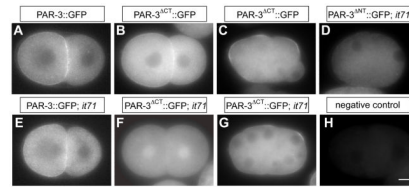
Figure 2. PAR-3$^{\Delta CT}$::GFP and PAR-3$^{\Delta NT}$::GFP in early embryos
Fluorescence images of PAR-3::GFP, PAR-3$^{\Delta CT}$::GFP and PAR-3$^{\Delta NT}$::GFP in *par-3(+)* embryos (A–C) and in *par-3(it71)* embryos (D–G). (H) shows an embryo with no transgene under the same microscopy conditions. In this and all figures, anterior is to the left of the embryo and the scale bar is approximately 10µm. The transient enrichment of GFP signal at the time of nuclear envelope localization in (F) is a common occurrence of GFP fusion proteins.
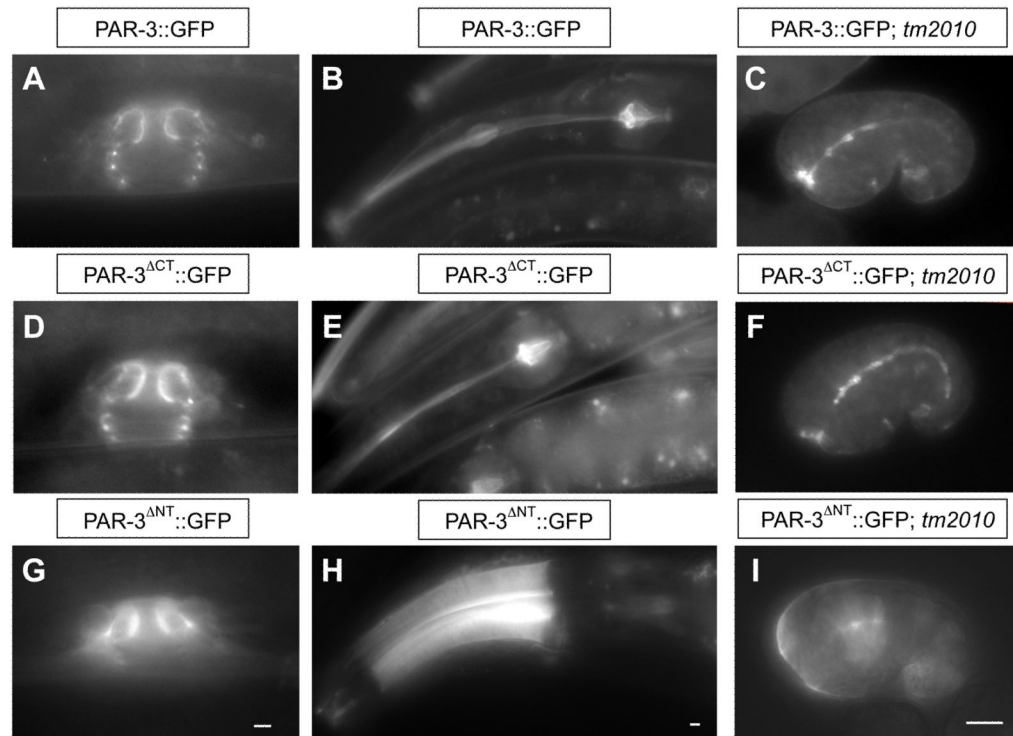
Figure 3. PAR-3$^{\Delta CT}$::GFP and PAR-3$^{\Delta NT}$::GFP in late *par-3(+)* embryos, developing larvae and *par-3(tm2010)* embryos

Fluorescence images of *par-3(+)* larvae (A, B, D, E, G, H) and representative comma stage progeny of *par-3(tm2010)/qC1* embryos (C, F, I) expressing PAR-3::GFP (A–C), PAR-3$^{\Delta CT}$::GFP (D–F) and PAR-3$^{\Delta NT}$::GFP (G–I). (A, D, G), vulva; (B, E, H), pharynx. Note that we could not determine the genotypes of the embryos in C, F and I, but all embryos from the *par-3(tm2010)/qC1* mothers exhibited the protein distributions shown.

Figure 4. 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. Rescue can appear greater than 100% due to undercounting of laid eggs.

Figure 5. Effect of PDZ domain deletions on PAR-3 protein distribution
One-cell *par-3(it71)* embryos that express PAR-3::GFP (A), PAR-3$^{\Delta PDZ1}$::GFP (B), PAR-3$^{\Delta PDZ3}$::GFP (C) and PAR-3$^{\Delta PDZ2}$::GFP (D–F) stained with anti-PAR-3 antibody only (A–C) or co-stained with anti-PAR-3 and anti-PAR-6 antibody (D–I). (A–C) are low resolution confocal images taken at the middle plane of the cell and (D–I) are mid focal plane wide-field microscope images from a live embryo (see Supplemental Movie 2).
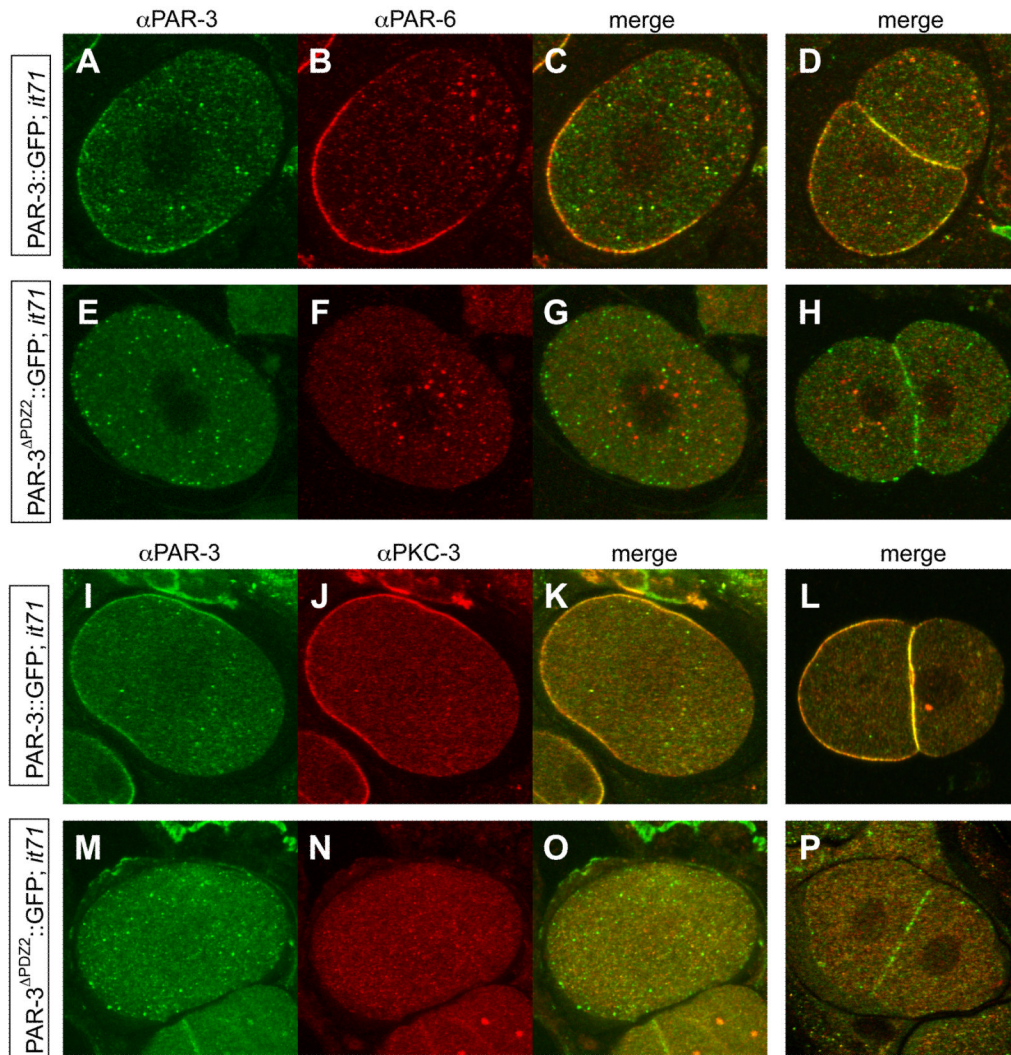
Figure 6. PAR-3$^{\Delta PDZ2}$::GFP fails to associate with PAR-6 or PKC-3

(A–H) Confocal sections of one and two-cell stage embryos of the indicated genotypes doubly labeled with anti-PAR-3 and anti-PAR-6 antibodies. The anti-PAR-6 positive larger puncta in the posterior of the embryos expressing PAR-3::GFP and clustered around the nuclei in the embryos expressing PAR-3$^{\Delta PDZ2}$::GFP are P granules which are stained by this rabbit polyclonal antibody. (I–P) Confocal sections of one and two-cell stage embryos of the indicated genotypes doubly labeled with anti-PAR-3 and anti-PKC-3 antibodies. Panels E-G and M-O are projections of six adjacent sections; all other panels are single sections.
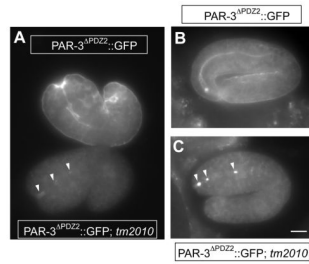
Figure 7. PAR-3^{ΔPDZ2}::GFP in late *par-3(tm2010)* embryos

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). Note that the *tm2010* genotype is inferred because embryos of this phenotype occur as ¼ of the progeny of *tm2010/+* mothers.
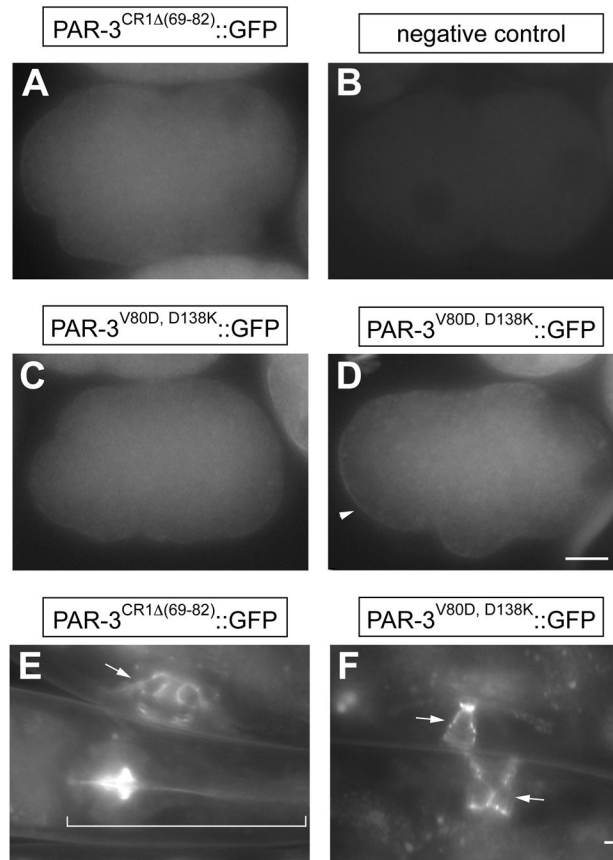
Figure 8. PAR-3^{CR1Δ(69-82)}::GFP and PAR-3^{V80D, D138K}::GFP in *par-3(+)* embryos and larvae
(A–D) Fluorescence images of *par-3(+)* embryo expressing PAR-3^{CR1Δ(69-82)}::GFP (A),
PAR-3^{V80D, D138K}::GFP (C, D) and no transgene (B). Note that PAR-3^{V80D, D138K}::GFP is
cytoplasmic in most early *par-3(+)* embryos (C), but occasionally shows weak and transient
cortical localization (D). Arrowhead points to the weak cortical signal. (E–F) Fluorescence
images of *par-3(+)* larvae expressing PAR-3^{CR1Δ(69-82)}::GFP (E) and
PAR-3^{V80D, D138K}::GFP (F). Arrows point to the vulva; bracket indicates the pharynx.
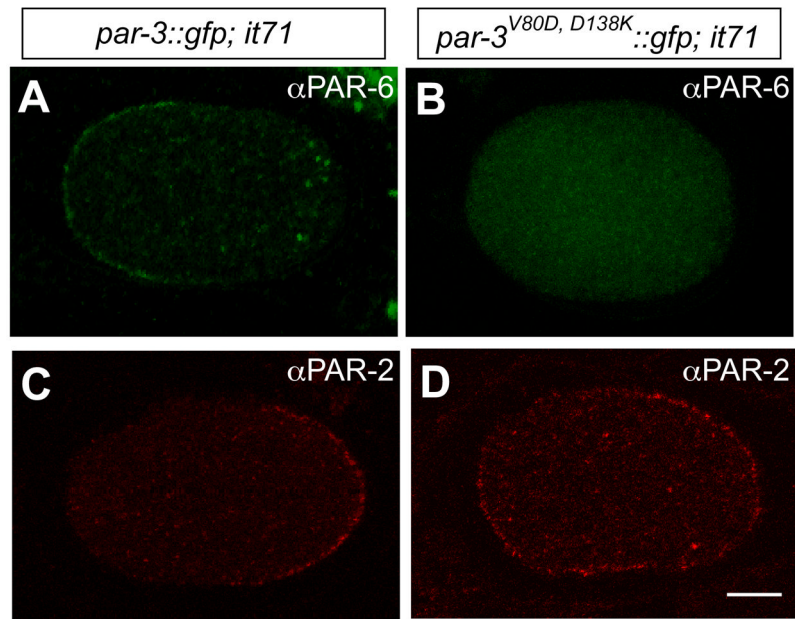
Figure 9. PAR-6 and PAR-2 in *par-3^V80D, D138K ::gfp; par-3(it71)* embryos
Fluorescence images of PAR-6 (green) and PAR-2 (red) in *par-3::gfp; par-3(it71)* embryos
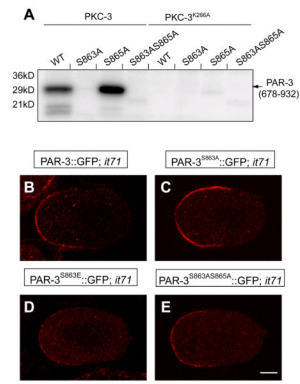(A, C) and *par-3^V80D, D138K ::gfp; par-3(it71)* embryos (B, D).

Figure 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).

Table 1

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".