

Polo kinases regulate *C. elegans* embryonic polarity via binding to DYRK2-primed MEX-5 and MEX-6

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Polo kinases are known key regulators of cell divisions. Here we report a novel, non-cell division function for polo kinases in embryonic polarity of newly fertilized *Caenorhabditis elegans* embryos. We show that polo kinases, via their polo box domains, bind to and regulate the activity of two key polarity proteins, MEX-5 and MEX-6. These polo kinases are asymmetrically localized along the anteroposterior axis of newly fertilized *C. elegans* embryos in a pattern identical to that of MEX-5 and MEX-6. This asymmetric localization of polo kinases depends on MEX-5 and MEX-6, as well as genes regulating MEX-5 and MEX-6 asymmetry. We identify an amino acid of MEX-5, T₁₈₆, essential for polo binding and show that T₁₈₆ is important for MEX-5 function in vivo. We also show that MBK-2, a developmentally regulated DYRK2 kinase activated at meiosis II, primes T₁₈₆ for subsequent polo kinase-dependent phosphorylation. Prior phosphorylation of MEX-5 at T₁₈₆ greatly enhances phosphorylation of MEX-5 by polo kinases in vitro. Our results provide a mechanism by which MEX-5 and MEX-6 function is temporally regulated during the crucial oocyte-to-embryo transition.

KEY WORDS: *Caenorhabditis elegans*, MEX-5, Embryo, Polarity, Polo kinase

INTRODUCTION

During *Caenorhabditis elegans* embryogenesis, anteroposterior (AP) polarity is established by a hierarchy of PAR proteins (for a review, see Kemphues, 2000). Many of these PAR proteins are asymmetrically localized at the cortex along the AP axis. The asymmetric distribution of PAR proteins determines the position of the first mitotic spindle and the asymmetric localization of key cytoplasmic determinants (for reviews, see Cowan and Hyman, 2004; Lyczak et al., 2002). This results in two daughters that are different in both size and developmental fate: the larger anterior somatic cell, AB, and the smaller posterior germline blastomere, P1. P1 then undergoes a series of three more asymmetric divisions, each giving rise to a germline blastomere (sequentially P2 to P4, termed the P lineage) and a corresponding somatic cell (Fig. 1).

PAR proteins regulate spindle position via a G-protein signaling pathway (Colombo et al., 2003; Hess et al., 2004), and cytoplasmic polarity via two maternally supplied proteins, MEX-5 and MEX-6 (Schubert et al., 2000). MEX-5 and MEX-6 are closely related proteins and are both preferentially localized toward the anterior cytoplasm of the one-cell embryo and are enriched in the somatic daughter after the division of each germline blastomere (Cuenca et al., 2003; Schubert et al., 2000). Whereas *mex-5* mutants exhibit 100% embryonic lethality, *mex-6* mutant embryos are 100% viable with no observable defects (Schubert et al., 2000). However, many molecular defects in *mex-5* mutant embryos are dramatically enhanced when *mex-6* is also mutated or depleted, suggesting partially redundant functions for these two genes (Schubert et al., 2000). For simplicity, unless specifically noted, we will use MEX-5/6 to refer to MEX-5 and MEX-6.

One major function of MEX-5/6 is to restrict the localization of maternally supplied germline proteins, such as PIE-1, POS-1 and MEX-1, to germline blastomeres (Guedes and Priess, 1997; Mello et al., 1996; Schubert et al., 2000; Tabara et al., 1999). In the one-cell embryo, as MEX-5/6 become asymmetrically localized anteriorly, PIE-1 becomes localized posteriorly (Cuenca et al., 2003; Mello et al., 1996; Schubert et al., 2000). After cell division, PIE-1 is enriched in P1, and this pattern reiterates in each subsequent P-lineage division. The small amount of PIE-1 segregated to the somatic sister after each division is degraded by a ZIF-1-containing CUL-2 E3 ligase complex (DeRenzo et al., 2003; Reese et al., 2000). Both asymmetric distribution of PIE-1 before division, as well as asymmetric degradation after division, require the function of MEX-5/6 (DeRenzo et al., 2003; Schubert et al., 2000). MEX-5/6 are themselves also substrates for this ZIF-1-containing E3 ligase complex (DeRenzo et al., 2003).

Before meiosis II, high levels of both PIE-1 and MEX-5/6 proteins are detected uniformly throughout the cytoplasm of oocytes and one-cell embryos (Fig. 1) (Cuenca et al., 2003; Schubert et al., 2000). This suggests that localization of PIE-1 by MEX-5/6 is a developmentally regulated event that initiates following meiosis II. However, it is not clear what regulates the function of MEX-5 or MEX-6, or even what their biochemical activity as it relates to PIE-1 localization might be. MEX-5/6 each contain two TIS-11-like zinc fingers, a protein domain shown to function in RNA-binding (Pagano et al., 2007; Schubert et al., 2000). It is not known whether RNA-binding is required for MEX-5/6 regulation of PIE-1 localization or degradation. It has been suggested that PAR-1 protein, which regulates MEX-5/6 asymmetric localization, is also required independently for their activity (Cuenca et al., 2003).

Asymmetric localization of PIE-1 in the one-cell zygote, as well as degradation in the soma, also requires the DYRK2 kinase, MBK-2 (Pellettieri et al., 2003). MBK-2 activation occurs at meiosis II and is accompanied by changes in its subcellular localization, and in vivo phosphorylation of two known substrates, MEI-1 and OMA-1 (Nishi and Lin, 2005; Pellettieri et al., 2003; Shirayama et al., 2006; Stitzel et al., 2006). Depletion of *mbk-2* abolishes PIE-1 asymmetry and PIE-1 degradation, but not MEX-5 asymmetry (Pellettieri et al.,

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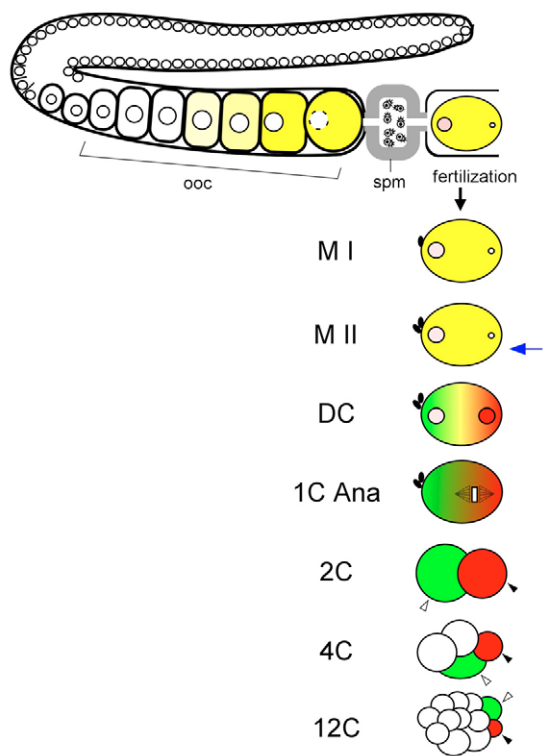


Fig. 1. Schematic diagram of MEX-5 and PIE-1 proteins in a wild-type *C. elegans* hermaphrodite gonad, oocytes and early embryos. Germ nuclei (white circles) are in a syncytial cytoplasm of the gonad arm before being enclosed by membranes to form developing oocytes. The oocyte adjacent to the spermatheca undergoes maturation, and is then immediately ovulated and fertilized. Small black ovals: polar bodies. Nuclei are only shown in one-cell embryos. The levels of MEX-5 and PIE-1 proteins are indicated by shades of green and red, respectively. Yellow indicates the presence of both MEX-5 and PIE-1. The blue arrow indicates the proposed time for MBK-2 activation. Embryo images are oriented with the anterior pole to the left, with closed and open arrowheads pointing to the germline blastomere and its somatic sister, respectively. 1C Ana, first mitotic anaphase; 2C, two-cell; 4C, four-cell; 12C, 12-cell; DC, decondensation; MI, meiosis I; MII, meiosis II; ooc, developing oocytes; spm, spermatheca.

2003), suggesting that regulation of PIE-1 by MBK-2 is either independent of MEX-5 function or downstream of MEX-5 localization.

Polo and polo-like kinases (PLKs) are serine/threonine kinases that regulate diverse processes during cell division, including centrosomal duplication and maturation, DNA damage checkpoint activation, mitotic onset, bipolar spindle formation, Golgi fragmentation and assembly, chromosome segregation, and cytokinesis (Glover et al., 1998; Nigg, 1998). In addition, PLKs have also been shown to function in a number of other cell biological processes (Eckerd et al., 2005; Ma et al., 2003; Seeburg et al., 2005; Takai et al., 2005; Wang et al., 2007). All PLKs possess an N-terminal kinase domain and a noncatalytic, C-terminal polo box domain (PBD), composed of two related polo boxes, each approximately 70–80 residues long (Clay et al., 1993; Lee et al., 1998). Both domains are essential for PLK function in vivo. The PBD binds preferentially to phosphorylated serine or threonine at the consensus sequence S-[pS/pT]-P/X (Elia et al., 2003a), and is crucial for in vivo spatial and temporal subcellular

localization of polo kinases (Lee et al., 1998; Lee et al., 1999; Qi et al., 2006; Seong et al., 2002). Structural studies indicate that the PBD binds intramolecularly to its own kinase domain (Elia et al., 2003b), inhibiting both kinase activity and the ability of PLK to bind phosphopeptides (Elia et al., 2003b; Jang et al., 2002; Lee and Erikson, 1997; Mundt et al., 1997). Upon binding of the PBD to a phosphorylated peptide substrate, kinase activity is elevated (Elia et al., 2003b). The PBD alone is better than the full-length PLK in binding the phosphopeptide substrate (Elia et al., 2003a).

In *C. elegans*, there are three genes that encode polo-like kinases, *plk-1*, *plk-2* and *plk-3*, with PLK-3 protein being significantly more diverged (Chase et al., 2000a; Chase et al., 2000b; Ouyang et al., 1999). Depletion of *plk-1* by RNAi results in defects in nuclear envelope breakdown, meiotic chromosome segregation and cytokinesis, producing embryos arrested at the one-cell stage without cleavage, consistent with a role in cell division (Chase et al., 2000b). The functions of *plk-2* and *plk-3* remain unclear, as no abnormality was observed in embryos depleted of either gene alone by RNAi (Rual et al., 2004).

Here we report a novel function for PLKs in embryonic cytoplasmic polarity. We show that PLK-1 and PLK-2 interact with both MEX-5 and MEX-6 in yeast and exhibit an asymmetric cytoplasmic localization similar to MEX-5/6 in early embryos. Both interaction in yeast and asymmetric localization in embryos require the PBD of PLK-1 and PLK-2 as well as amino acid T₁₈₆ of MEX-5. In addition, PLK-1 and PLK-2 are required for MEX-5/6 function in vivo, including regulating PIE-1 polarity in the one-cell embryo and degradation in somatic cells. We show that MBK-2 is required for PLK-1 cytoplasmic polarity in vivo, and that MBK-2 primes for PLK-1 at the T₁₈₆ site in vitro. Our results provide a mechanism by which MEX-5/6 function is temporally regulated during the crucial oocyte-to-embryo transition.

MATERIALS AND METHODS

Strains

N2 was used as the wild-type strain. Genetic markers are: LGI, *par-6(zu222)*; LGII, *mex-6(pk440)*, *lin-5(ev571)*; LGIII, *par-2(lw32)*, *par-3(it71)*, *pie-1(zu154)*, *mpk-1(ga111)*, *unc-119(ed3)*; LGIV, *mex-5(zu199)*, *mbk-2(pk1427)*, *par-5(it55)*, *let-99(or204)*, *unc-30(e191)*; LGV, *par-1(zu310)*, *par-4(it57)*. The OMA-1::GFP strain, TX189, was previously described (Lin, 2003). Transgenic strains were generated by microparticle bombardment (Praitis et al., 2001) of the respective plasmids: *P_{pie-1gfp}::mex-5 T₁₈₆A* in TX672(*teIs35*) and TX673(*teIs36*); *P_{pie-1gfp}::plk-1^{PBD}* in TX773(*teIs65*), TX774(*teIs66*) and TX775(*teIs67*); *P_{pie-1gfp}::plk-1^{FL}* in TX787(*teIs70*). For a given transgene, multiple transgenic lines were analyzed and showed identical patterns. GZ336 (Leidel and Gonczy, 2003), JH227 (Reese et al., 2000) and JH1448 (*axEx1125*) (Cuenca et al., 2003) contain *P_{pie-1gfp}::plk-1^{FL}*, *P_{pie-1gfp}::pie-1* and *P_{pie-1gfp}::mex-5* transgenes, respectively.

Plasmid construction

All expression clones used in this study were generated using the Gateway technology (Invitrogen) and have been confirmed by sequencing. Germline expression constructs were generated by introducing corresponding cDNAs into the destination vector pID3.01B under the control of the *pie-1* promoter (Reese et al., 2000). MBK-2 and PLK-1 kinases were N-terminally tagged with maltose binding protein (MBP) and FLAG, respectively (Nishi and Lin, 2005). Full-length MEX-5 and MEX-6 were tagged with MBP, whereas truncated MEX-5 and MEX-6 were tagged with 6× His, all at the N-terminus. All site-directed mutagenesis was performed using the Quick Change Site-directed Mutagenesis Kit (Stratagene). The kinase dead mutations used were Y₂₃₇A for MBK-2 and N₁₆₆A for PLK-1. Details regarding the cloning of these plasmids are available upon request.

Protein purification and in vitro kinase assays

MBP-tagged MBK-2, OMA-1, MEX-5 and MEX-6 were expressed in and purified from Rosetta (DE3) pLysS (Novagen) cells as described previously (Nishi and Lin, 2005). His-tagged MEX-5 and MEX-6 fragments were purified using Ni²⁺ columns (Clontech) according to the manufacturer's instructions.

FLAG-tagged PLK-1 and MBK-2 were expressed in and purified from HEK293T cells as described previously for FLAG-tagged GSK-3 (Nishi and Lin, 2005). MBK-2 (25 mM HEPES pH 7.6, 5 mM MgCl₂, 5 mM MnCl₂, 0.5 mM DTT, 10 mM β-glycerol phosphate, 30 nM ATP and 0.5 μCi [γ-³²P] ATP), *H.s* DYRK2 (same as for MBK-2 except 50 μM ATP), *H.s*Cdk-1 (50 mM HEPES pH 7.6, 10 mM MgCl₂, 1 mM DTT, 20 mM β-glycerol phosphate, 50 μM ATP and 0.5 μCi [γ-³²P] ATP), and PLK-1 (same as for *H.s*Cdk1 except 10 mM β-glycerol phosphate, 1 mM NaVO₄) kinase assays were performed at 25°C for 20 minutes.

For the tandem kinase reaction, MBP::MEX-5 was preincubated with *H.s*DYRK2 (Upstate/Millipore) in the presence of 500 μM ATP (37°C for 30 minutes), pulled down with amylose resin (New England Biolabs), washed extensively, eluted with 50 mM maltose and added to the second kinase reaction with *H.s*PLK-1 (Cell Signaling Technology) (20°C for 20 minutes). Products were separated by SDS-PAGE and Coomassie stained. Incorporation of ³²P was visualized by autoradiography of dried gels.

Generation of PLK-2 antibody

Rabbit Anti-PLK-2 antibody (Bethyl Laboratory) was generated against a peptide corresponding to the first 16 amino acids of PLK-2, a region significantly diverged from PLK-1.

RNA interference

RNAi for *pie-1* was performed by injection. For *plk-1* and *plk-2*, similar results were obtained with feeding and injection RNAi. Feeding RNAi was performed at 25°C by placing L2 worms onto RNAi bacteria and scoring 24–30 hours later (Timmons and Fire, 1998). Because *plk-2(RNAi)* alone has no phenotype, a mild *plk-1(RNAi);plk-2(RNAi)* effect was obtained by diluting *plk-1(RNAi)* bacteria with *plk-2(RNAi)* bacteria to various ratios. The *mex-6*-specific RNAi clone contains sequence corresponding to amino acids 1–75 of the MEX-6 protein, chosen for sequence divergence relative to MEX-5. The *plk-1* 3'UTR feeding RNAi clone contains a 350 bp sequence beginning 5 bp downstream of the stop codon.

Rescue assay

Transgenic strains carrying *axEx1125(P_{pie-1gfp::mex-5})* or *teIs36(P_{pie-1gfp::mex-5} T_{186A})* were crossed to JJ1238 [*unc-30(e191) mex-5(zu199) IV/nT1 (IV;V)*] and F2 *unc-30(e191) mex-5(zu199)* progeny that expressed GFP were singled out to 25°C plates and assayed for the number of viable progeny produced. Each *unc-30(e191) mex-5(zu199)* animal produced approximately 150 embryos in our assay. *teIs35* is an integrated transgene and animals carrying one or two copies exhibit different expression levels. Only animals carrying two copies of *teIs35* were scored for rescue. Rescued lines were placed on *mex-6*-specific RNAi plates and resulting embryos analyzed. RNAi with the *mex-6*-specific RNAi clone results in no abnormal or dead embryos from wild-type N2 animals, but 100% dead embryos that resemble *mex-5(zu199);mex-6(ah6)* mutant embryos from *mex-5(zu199)* animals.

The *plk-1* 3'UTR RNAi clone does not interfere with the expression of GFP::PLK-1 or GFP::PLK-1^{PBD} and results in near 100% dead embryos in wild-type N2 that exhibit a phenotype resembling a weak phenotype obtained by RNAi with *plk-1* coding sequence (double nucleated blastomeres). No significant rescue was observed with either GFP::PLK-1^{FL}-, or GFP::PLK-1^{PBD}-expressing worms.

Immunofluorescence

Immunofluorescence protocols for antibodies specific for PLK-1 (Lin et al., 1998), PLK-2 (Lin et al., 1998), PIE-1 (Mello et al., 1996), MEX-5 (Schubert et al., 2000) and GFP (Molecular Probes, rabbit A-11122) (Lin et al., 1998) are as described. Dilutions used: PLK-1, 1/2000; PLK-2, 1/500; PIE-1, 1/10; MEX-5, no dilution; GFP, 1/200. We could not reproducibly detect nuclear PIE-1 signal in one- and two-cell wild-type embryos by anti-PIE-1 antibody. All experiments showing nuclear PIE-1 in one-cell embryos utilized a GFP::PIE-1-expressing strain.

Analysis of embryos and imaging

Imaging of immunofluorescence and live embryos was performed using an Axioplan microscope equipped with epifluorescence, differential interference contrast (DIC) optics, and a MicroMax-512EBFT CCD camera as described previously (Rogers et al., 2002).

Yeast two-hybrid assay

Yeast two-hybrid assays were performed using the GAL4-based transcription system in AH109 (Clontech), with PLK-1 and PLK-2 expressed as baits and MEX-5 and MEX-6 expressed as preys. Transformants were spotted onto SD-Leu-Trp-His plates containing various concentrations of 3-amino triazole (3AT, 0–50 mM), and their growth at 30°C 3 days after spotting was scored and imaged. A higher 3AT concentration represents a more stringent assay and normally detects a stronger interaction.

RESULTS

Cytoplasmic PLK-1 is asymmetrically localized reciprocal to germline proteins in early *C. elegans* embryos

An antibody to PLK-1 was previously shown to localize to centrosomes and chromosomes (Chase et al., 2000b). With this antibody, we observed, in addition to the reported localization patterns, strong cytoplasmic staining in newly fertilized embryos following the completion of meiosis (Fig. 2A–C). This cytoplasmic staining was asymmetric along the AP axis, with the highest levels at the anteriormost portion of the embryo (Fig. 2B,C). After the first mitotic division, anti PLK-1 cytoplasmic staining was higher in the somatic cell AB than in the germline blastomere P1 (Fig. 2D). This asymmetric pattern, that is, asymmetric cytoplasmic localization before division and enrichment in the somatic cell after division, reiterated at the three subsequent germline blastomere divisions (Fig. 2E,F, and data not shown). The level of cytoplasmic staining was dynamic throughout the cell cycle, decreasing during mitosis when a high level of staining was observed on centrosomes and chromosomes. This made it difficult to compare the level of cytoplasmic staining in later cells that have very different cell division rates. Therefore, the results reported here focus on embryos up to the four-cell stage.

A GFP reporter containing the PLK-1 polo box domain recapitulates PLK-1 localization by immunofluorescence

We believe that the observed asymmetric cytoplasmic staining represented PLK-1 localization in vivo for the following three reasons. First, in *plk-1(RNAi)* embryos, the cytoplasmic staining, as well as centrosomal and chromosomal stainings, were not detected (0%, *n*=30; not shown), indicating that all observed stainings were *plk-1*-dependent. Second, we generated an antibody against PLK-2 and detected a similar, albeit much fainter, asymmetric staining pattern (Fig. 2M,N). PLK-1 and PLK-2 have a partially redundant function (see below), which can be attributed to this cytoplasmic asymmetry. Third, fusion proteins in which GFP was fused to the PLK-1 PBD (GFP::PLK-1^{PBD}, amino acids 340–648) recapitulated the anti-PLK-1 staining pattern, including centrosomal, chromosomal and asymmetric cytoplasmic stainings (Fig. 2G–L). GFP fused to full-length PLK-1 (GFP::PLK-1^{FL}), however, did not recapitulate the anti-PLK-1 staining pattern. As previously reported, a GFP::PLK-1^{FL} reporter exhibited only weak centrosomal and chromosomal signals (Leidel and Gonczy, 2003). We found that while we could obtain transgenic lines expressing higher levels of GFP::PLK-1^{FL}, still

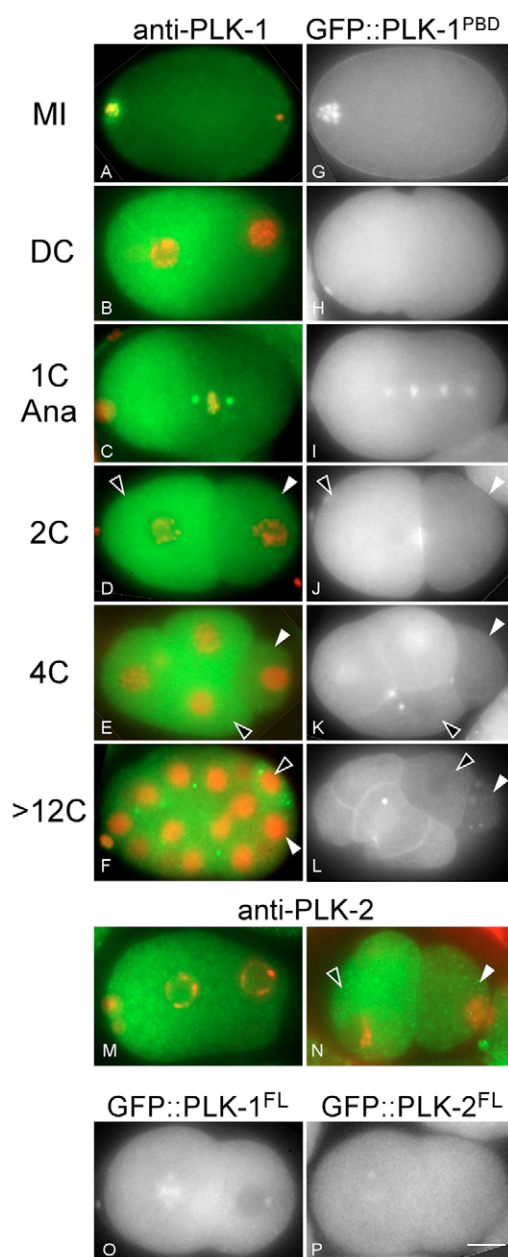


Fig. 2. PLK-1 cytoplasmic asymmetry in early *C. elegans* embryos. Merged immunofluorescence micrographs of DAPI staining (red) with anti-PLK-1 (A-F) and anti-PLK-2 (M,N) staining (green). (G-L) GFP fluorescence of GFP::PLK-1^{PBD}. (O,P) GFP fluorescence of GFP::PLK-1^{FL}. (Q,R) GFP fluorescence of GFP::PLK-2^{FL}. Closed and open arrowheads indicate the germline blastomere and its somatic sister, respectively. Stages of embryos in A-L are marked to the left; M, 1C; N-P, 2C. Scale bar: 10 μ m.

only a small proportion of GFP fluorescence was associated with centrosomes and chromosomes. In only a small percentage of embryos (~5%, $n > 200$), we could detect that the cytoplasmic GFP is slightly higher in the somatic cell compared with the germline blastomere. Our results suggest that the subcellular localization of PLK-1 in early *C. elegans* embryos is mediated through the PBD domain. We will refer to this asymmetric cytoplasmic staining as PLK-1 cytoplasmic asymmetry or simply PLK-1 asymmetry.

PLK-1 asymmetry is dependent on *mex-5* and *mex-6*, as well as genes functioning upstream of *mex-5/6*

The temporal and spatial localization patterns of cytoplasmic PLK-1 resembled those reported previously for MEX-5/6 (Schubert et al., 2000), suggesting a possible functional relationship. We found that MEX-5/6 are redundantly required for cytoplasmic PLK-1 asymmetry. Simultaneous depletion of both *mex-5* and *mex-6* resulted in a fully penetrant defect in the observed PLK-1 asymmetry ($n \geq 300$, Fig. 3C,M,Q,R). Depletion of *mex-6* alone by either genetic mutation or RNAi did not result in any observable defect in PLK-1 polarity ($n > 200$, Fig. 3B,L). Inactivation of *mex-5* alone by either genetic mutation or RNAi resulted in a weaker, but still scorable, PLK-1 asymmetry ($n > 200$, Fig. 3A,K,R). However, MEX-5 localization remained asymmetric in *plk-1(RNAi)* embryos (see more below and Fig. 7), suggesting that MEX-5/6 regulates the localization of PLK-1 and not vice versa.

Localization of MEX-5/6 to the anterior cytoplasm depends on PAR proteins (Cuenca et al., 2003; Schubert et al., 2000). We observed a loss of PLK-1 cytoplasmic asymmetry in all *par* mutants tested: *par-1* ($n = 38$), *par-2* ($n = 25$), *par-3* ($n = 41$), *par-4* ($n = 15$), *par-5* ($n = 22$) and *par-6* ($n = 7$) (Fig. 3D-I,R). In embryos depleted of *pie-1*, a gene downstream of MEX-5/6, PLK-1 cytoplasmic asymmetry was unaffected ($n > 100$, Fig. 3J). Importantly, the centrosomal and chromosomal localization of PLK-1 during mitosis was not disrupted in mutants with abolished PLK-1 cytoplasmic asymmetry ($n > 100$, Fig. 3N-R and data not shown).

Heterotrimeric G proteins also regulate polarity in early *C. elegans* embryos (Colombo et al., 2003; Hess et al., 2004). They are asymmetrically activated at the cortex and are required for the asymmetric positioning of the spindle. The asymmetric activation of G proteins also depends on PAR proteins, but is independent of MEX-5/6. We found no evidence for an involvement of genes in the G-protein signaling pathway in PLK-1 cytoplasmic asymmetry. We observed wild-type PLK-1 asymmetry in *goa-1(RNAi)*; *gpa-16(RNAi)* (Colombo et al., 2003), *lin-5(ev571tsw)* (Lorson et al., 2000) or *let-99(or204ts)* (Tsou et al., 2002) embryos ($n > 30$ for each strain; data not shown).

Taken together, these results demonstrate that regulation of PLK-1 asymmetry lies downstream of MEX-5/6 but upstream of PIE-1. In addition, PLK-1 asymmetry, like MEX-5 asymmetry, is established independently of G-protein signaling.

PLK-1 and PLK-2 physically interact with MEX-5, and this interaction depends on T₁₈₆ of MEX-5 and the polo box domain of PLK

The similar cytoplasmic asymmetry exhibited by PLK-1 and MEX-5/6 in early embryos, as well as the dependency of PLK-1 asymmetry on MEX-5/6, suggests a physical interaction that polarizes cytoplasmic PLK-1 in vivo. Indeed, we detected interaction between MEX-5 or MEX-6, and PLK-1 or PLK-2, using yeast two-hybrid assays (Fig. 4A). The polo box domains of PLK-1 and PLK-2 are both necessary and sufficient for this interaction. No interaction was observed using the N-terminal, kinase domain and only weak interaction was detected with full-length PLK-1 or PLK-2. For simplicity, unless otherwise noted, we will refer to PLK-1 and PLK-2 as PLK-1/2.

The crystal structure of human PLK1 shows that W₄₁₄ and V₄₁₅ of the first polo box, and H₅₃₈ and K₅₄₀ of the second polo box, are crucial for phosphopeptide binding. Amino acid changes W₄₁₄A/V₄₁₅A or H₅₃₈A/K₅₄₀M in human PLK1 abolished phosphopeptide binding without altering the structure of the polo

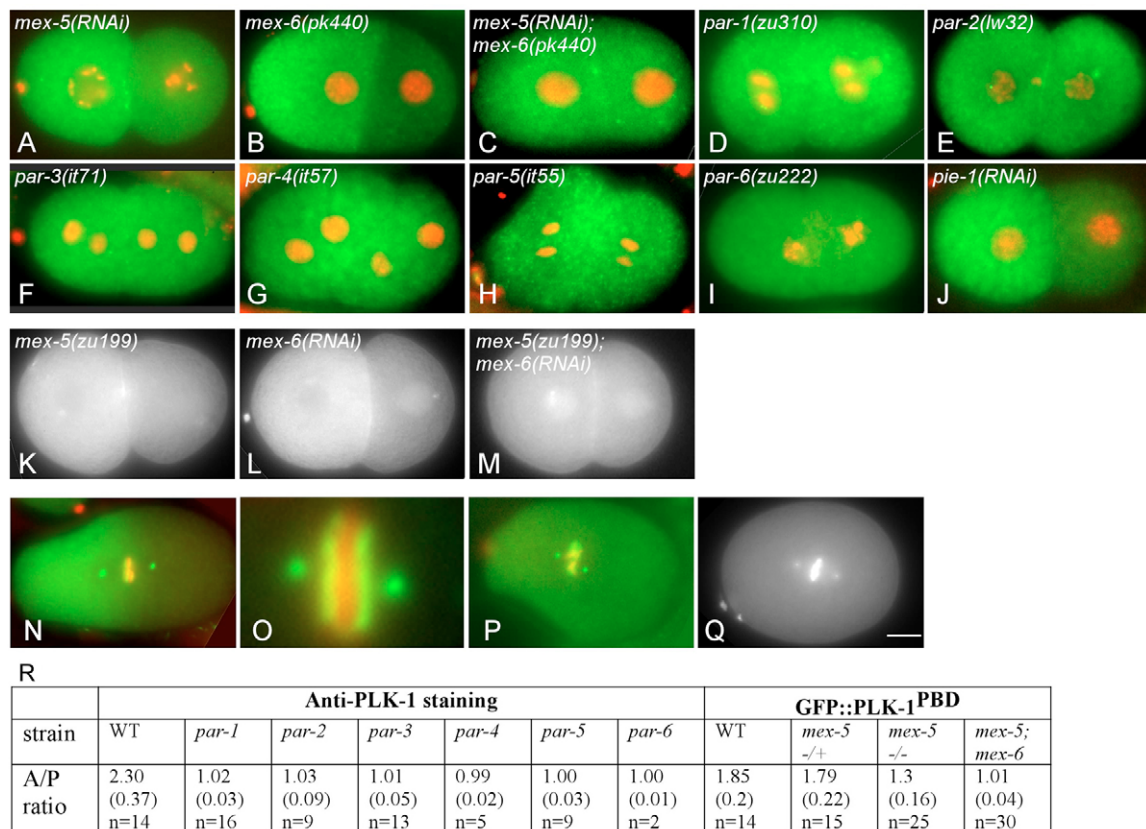


Fig. 3. PLK-1 cytoplasmic asymmetry is regulated downstream of MEX-5/6 and upstream of PIE-1 in *C. elegans*. Micrographs of two- to four-cell stage mutant or RNAi embryos (indicated at top). (A-J) Merged immunofluorescence of anti-PLK-1 (green) and DAPI staining (red). (K-M) GFP fluorescence of GFP::PLK-1^{PBD}. (N-P) Centrosomal and chromosomal anti-PLK-1 staining in one-cell wild-type (N) and *par-2(lw32)* (P) embryos. A higher magnification photo of wild-type mitotic chromosomes is shown in O. (Q) Centrosomal and chromosomal GFP::PLK-1^{PBD} signal in one-cell *mex-5(zu199);mex-6(RNAi)* embryos. (R) Quantification of PLK-1 asymmetry. The ratio represents the relative intensity of anterior and posterior portions of one-cell embryos, or somatic versus germline blastomeres in later stage embryos. Numbers in parentheses are standard deviations. *n*, number of embryos measured. Scale bar: 10 μ m in all, except 2.5 μ m in O.

domain (Elia et al., 2003b; Lee et al., 1999; Seong et al., 2002). The corresponding changes to *C. elegans* PLK-1^{PBD} (W₄₁₇A/V₄₁₈A and H₅₄₂A/K₅₄₄M) also abolished binding with MEX-5/6 in yeast (Fig. 4A), suggesting that PLK-1 binds to MEX-5/6 in yeast via phosphorylated epitopes. Sequence analysis of MEX-5 identified a putative polo docking site (TSSTPL) surrounding threonine 186 (threonine 190 of MEX-6). Changing MEX-5 T₁₈₆ or MEX-6 T₁₉₀ to alanine abolished or dramatically reduced the interaction between MEX-5/6 and the two PBDs in the yeast two-hybrid assay (Fig. 4A). We detected no interaction between MEX-5 T₁₈₆A with either PLK-1^{PBD} or PLK-2^{PBD}. A weak interaction, detected only on 20 mM but not 50 mM 3AT plates, was observed between MEX-6 T₁₉₀A and PLK-1^{PBD} or PLK-2^{PBD} (Fig. 4A and data not shown). These results support a model whereby PLK-1/2 bind to MEX-5/6 through their polo box domains, resulting in PLK-1/2 asymmetry in the cytoplasm of early *C. elegans* embryos.

T₁₈₆ is important for MEX-5 function in vivo

To investigate whether PLK-1 binding affected MEX-5 function, we first asked whether T₁₈₆ is important for MEX-5 function in vivo. We generated two independently integrated transgenic lines that expressed GFP::MEX-5 in which T₁₈₆ was mutated to alanine (GFP::MEX-5 T₁₈₆A), and in both cases obtained the same result. As predicted, T₁₈₆ was not required for MEX-5 localization in vivo.

The temporal and spatial expression patterns of GFP::MEX-5 T₁₈₆A were indistinguishable from that of wild-type GFP::MEX-5 (Fig. 4B). *mex-5(zu199)* is a maternal-effect-lethal mutation (Schubert et al., 2000). Worms homozygous for *zu199* can grow to adulthood but produce dead embryos. We assayed the ability of MEX-5 T₁₈₆A to rescue the embryonic lethality of embryos produced by *mex-5(zu199)* mothers. We scored the number of viable larvae produced by *mex-5(zu199)* mothers that express no transgenic protein, GFP::MEX-5 or GFP::MEX-5 T₁₈₆A in embryos. We observed that 0% (*n*=62), 38% (*n*=94) and 4% (*n*=48) of *mex-5(zu199)* worms expressing no transgenic protein, GFP::MEX-5 and GFP::MEX-5 T₁₈₆A, respectively, produced viable larvae (Fig. 4C). The percentages of viable larvae per mother were low, with no more than 22% for GFP::MEX-5-expressing, and lower than 5% for GFP::MEX-5 T₁₈₆A-expressing *mex-5(zu199)* worms. Despite weak rescue, however, embryos expressing either transgenic protein could grow up to adulthood and rescue lines could be maintained. This result suggests that T₁₈₆ is important, but not essential, for the function of MEX-5 in vivo.

We also assayed the function of GFP::MEX-5 T₁₈₆A by examining its ability to regulate asymmetric PIE-1 distribution before, and degradation after, cell division. Both wild-type MEX-5 and MEX-6 can polarize PIE-1 before division. Pre-division PIE-1 polarity is abolished only when both *mex-5* and *mex-6* are mutated

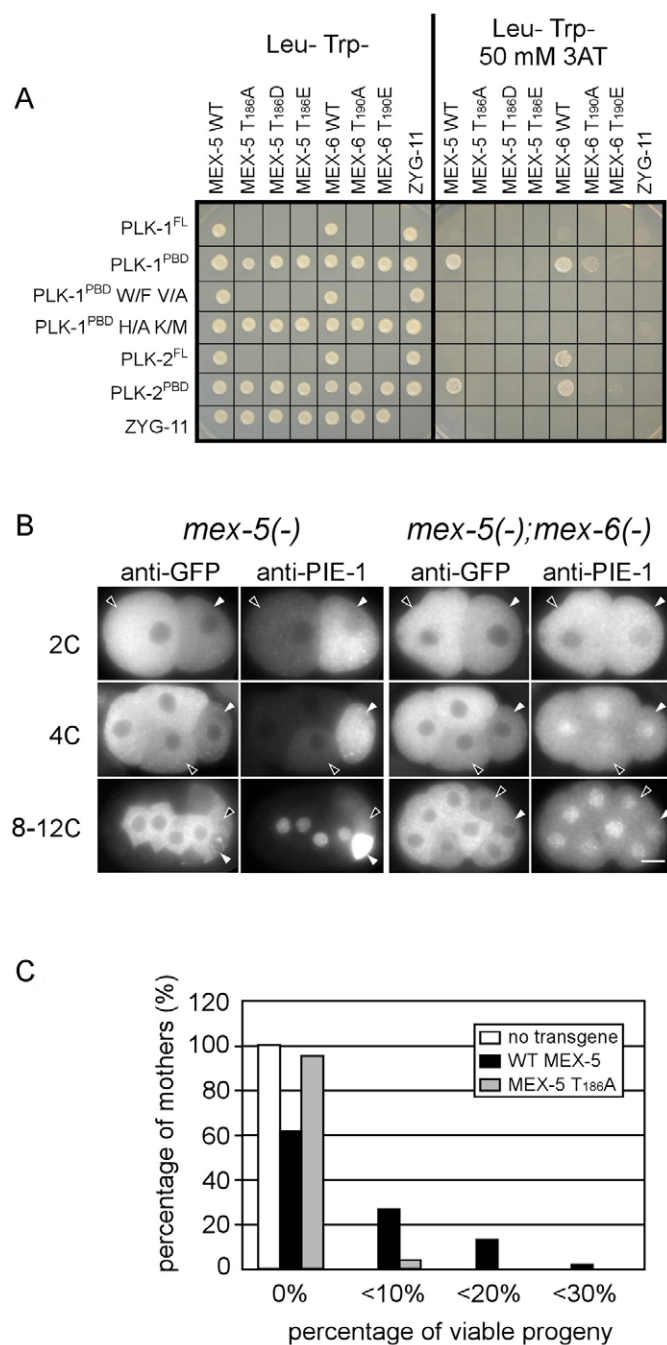


Fig. 4. T₁₈₆ is required for interaction with PLK-1^{PBD} and is important for MEX-5 function. (A) Yeast two-hybrid assays.

Duplicated strains were incubated on leucine and tryptophan double dropout medium (Leu- Trp-) either without 3AT (left) or with 50 mM 3AT (right). Each strain tested contains a bait plasmid expressing the protein indicated along the top and a prey plasmid expressing the protein indicated to the left. An unrelated protein, ZYG-11, was used as a negative control for both bait and prey. H/A K/M: H₅₄₂A, K₅₄₄M. (B) Immunofluorescence micrographs of anti-GFP and anti-PIE-1 stainings in *mex-5(zu199)*, or *mex-5(zu199);mex-6(RNAi)* embryos expressing GFP::MEX-5 T₁₈₆A. Stages indicated to the left. Closed and open arrowheads indicate the germline blastomere and its somatic sister, respectively. (C) Rescue of *mex-5(zu199)* lethality by GFP::MEX-5 versus GFP::MEX-5 T₁₈₆A, as scored by percentage of viable embryos produced per *mex-5* homozygote mother. Scale bar: 10 μ m.

or depleted (Schubert et al., 2000). By contrast, MEX-5 has a predominant function versus MEX-6 in regulating PIE-1 degradation in somatic cells. PIE-1 degradation is defective in at least some somatic cells in 58% of *mex-5(zu199)* and 0% of *mex-6(ah6)* single mutant embryos, but 100% of *mex-5(zu199);mex-6(ah6)* double mutant embryos (DeRenzo et al., 2003; Schubert et al., 2000). We show that expressing wild-type GFP::MEX-5 in *mex-5(zu199)* embryos reduced the degradation defect to 15% ($n=150$). By contrast, approximately 50% ($n=150$) *mex-5(zu199)* embryos expressing GFP::MEX-5 T₁₈₆A still exhibited a PIE-1 degradation defect (Fig. 4B). The weak rescue by GFP::MEX-5 T₁₈₆A of the PIE-1 degradation defect is consistent with its weak rescue of embryonic viability. When *mex-6* was depleted by RNAi in *mex-5(zu199)* embryos expressing GFP::MEX-5 T₁₈₆A, defects in both pre-division PIE-1 polarity ($n>200$) and PIE-1 degradation in somatic cells ($n>300$) were enhanced to 100%. It has been shown that MEX-5/6 function was required for their own degradation in somatic cells (DeRenzo et al., 2003). We observed that while GFP::MEX-5 T₁₈₆A was polarized properly, its degradation was completely abolished in *mex-5(zu199);mex-6(RNAi)* embryos (Fig. 4B). The preceding rescue result and these molecular analyses demonstrate that GFP::MEX-5 T₁₈₆A has a dramatically reduced MEX-5 function.

MBK-2 is required for GFP::PLK-1^{PBD} cytoplasmic polarity in vivo and phosphorylates T₁₈₆ in vitro

To identify the kinase that phosphorylates MEX-5 at T₁₈₆, priming MEX-5 for PLK-1 phosphorylation in vivo, we assayed the localization of GFP::PLK-1^{PBD} in embryos depleted of candidate kinases. The putative polo docking site surrounding T₁₈₆ resembles the consensus sequence for phosphorylation by the CMGC superfamily of kinases, which includes cyclin-dependent kinases, MAP kinases, GSK3 and the dual specificity CLK and DYRK kinases (Elia et al., 2003a). In *C. elegans*, there are over 30 kinases in this superfamily, and therefore we focused on the CMGC kinases known to be required for early *C. elegans* embryogenesis (Boxem et al., 1999; Kamath et al., 2003; Pang et al., 2004; Pellettieri et al., 2003; Quintin et al., 2003; Schlesinger et al., 1999; Simmer et al., 2003). We examined the localization of GFP::PLK-1^{PBD} in *cdk-1(RNAi)*, *mpk-1(gall1ts)*, *mpk-1(RNAi)*, *gsk-3(RNAi)* and *mbk-2(RNAi)* embryos. Weak RNAi (see Materials and methods) was performed for *cdk-1* and *mbk-2* in order to allow embryonic divisions, as strong depletion of either gene results in embryos arresting at the one-cell stage (Boxem et al., 1999; Pellettieri et al., 2003).

We detected no defect in GFP::PLK-1^{PBD} cytoplasmic polarity when either *gsk-3* or *mpk-1* was inactivated ($n>300$; Fig. 5). However, in *cdk-1(RNAi)* and *mbk-2(RNAi)* embryos, we observed a loss of GFP::PLK-1^{PBD} cytoplasmic asymmetry without observable defect in the asymmetric localization of MEX-5 ($n>150$; Fig. 5). *mbk-2(RNAi)* embryos were affected most severely, exhibiting a nearly fully penetrant polarity defect. This result suggests that MBK-2 and CDK-1 function in PLK-1 polarity downstream of MEX-5 localization, a property consistent with a priming kinase. We believe that the *cdk-1(RNAi)* phenotype is indirect for the following two reasons. First, it has been shown that CDK-1 is required for MBK-2 activation (Stitzel et al., 2006). Therefore, *cdk-1(RNAi)* would also affect processes requiring MBK-2 activity. We observed a similar abolishment of PLK-1 polarity in embryos depleted by RNAi of *cul-2*, another gene required for MBK-2 activity (Stitzel et al., 2006) (data not shown). Second, we detected no phosphorylation of MEX-5 in our assay using human

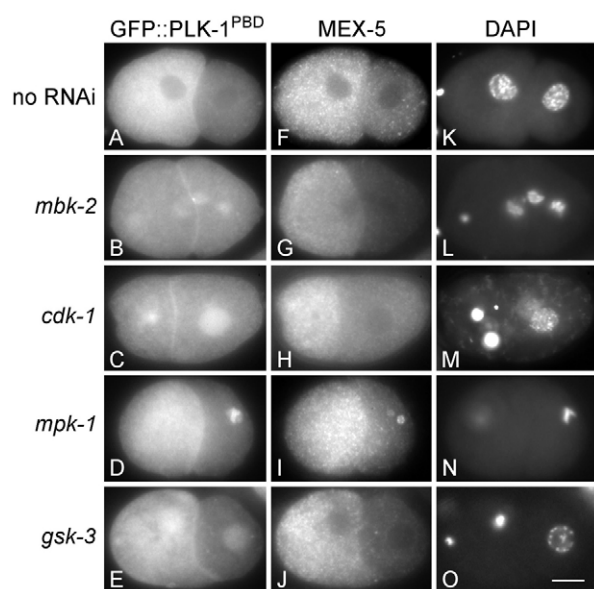


Fig. 5. MBK-2 and CDK-1 are required for the cytoplasmic asymmetry of PLK-1^{PBD} but not MEX-5 in *C. elegans*.

Immunofluorescence micrographs of anti-GFP (A–E), anti-MEX-5 (F–J) and DAPI staining (K–O) in two-cell wild-type (no RNAi), *mbk-2(RNAi)*, *cdk-1(RNAi)*, *mpk-1(RNAi)* or *gsk-3(RNAi)* embryos expressing GFP::PLK-1^{PBD} as indicated. Scale bar: 10 μm.

CDK1/cyclin (Qi et al., 2006) (data not shown), whereas MBK-2 can phosphorylate MEX-5 at T₁₈₆ in vitro (Fig. 6A). T₁₈₆ is not the only MEX-5 residue phosphorylated by MBK-2 in our assay, although it is the only residue within a fragment of MEX-5 encompassing amino acids 117–270. When T₁₈₆ was mutated to either aspartate or alanine within MEX-5_{117–270}, phosphorylation by MBK-2 was abolished. Taken together, these results support MBK-2 being the priming kinase that phosphorylates MEX-5 at T₁₈₆.

Prior phosphorylation at T₁₈₆ dramatically enhances in vitro phosphorylation of MEX-5 by polo kinase

Many proteins that provide a polo docking site also serve as polo substrates (Lowery et al., 2005). Therefore, we asked whether PLK-1 phosphorylates MEX-5, and if so, whether PLK-1-dependent phosphorylation is affected by the phosphorylation state at T₁₈₆ of MEX-5. We assayed phosphorylation of MEX-5 by PLK-1 either with or without prephosphorylation at T₁₈₆. We found that human DYRK2 (*HsDYRK2*) phosphorylated T₁₈₆ in MEX-5_{117–270} with the same specificity as *C. elegans* MBK-2 purified from mammalian tissue culture cells, but did so much more efficiently (Fig. 6; not shown). Under optimal conditions, nearly 100% of MEX-5_{117–270} was shifted to a slower migrating band in a polyacrylamide gel (Fig. 6A; not shown).

We performed a tandem kinase assay, first with *HsDYRK2* and excess cold ATP, followed by PLK-1 and radioactive ATP, to ask whether prior phosphorylation of MEX-5 at T₁₈₆ enhanced subsequent phosphorylation by PLK-1. We used human PLK1 (*HsPLK1*) here because of its superior activity over *C. elegans* PLK-1 isolated from mammalian tissue culture cells. We detected a robust and specific phosphorylation of MEX-5 by *HsPLK1* (as assayed by the incorporation of radioactive ATP) that was dependent on both T₁₈₆ and *HsDYRK2* (Fig. 6B). When MEX-5 T₁₈₆A or no

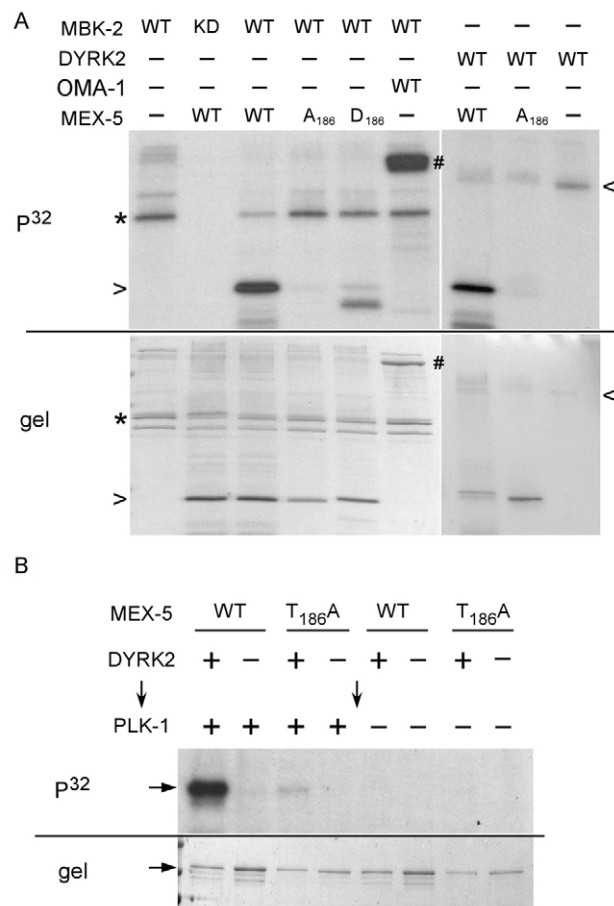
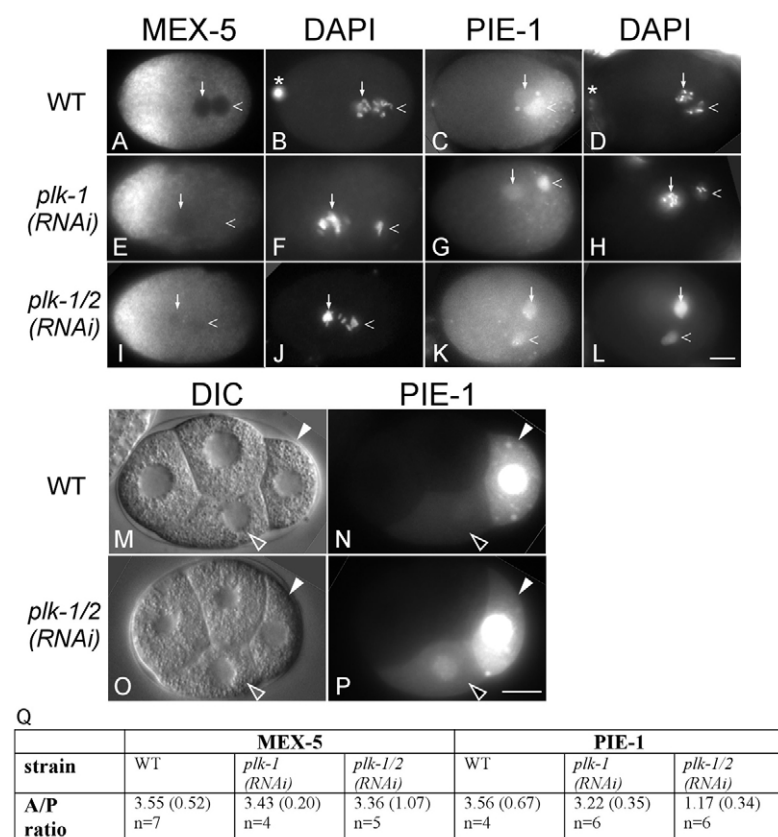


Fig. 6. MBK-2/DYRK2 phosphorylates MEX-5 at T₁₈₆ and primes for PLK-1 in *C. elegans*. (A) Kinase assay conducted using indicated substrates and kinases. MEX-5 used here is the fragment containing amino acids 117–270. The positions of MEX-5^{117–270} (>), OMA-1 (#) and DYRK2 (<) are indicated. Assays with *HsDYRK2* and MBK-2 were exposed differentially to get comparable signals for wild-type MEX-5^{117–270}. (B) Tandem kinase assays using MEX-5^{FL} WT or T₁₈₆A as substrate, first with *HsDYRK2* (cold ATP), followed by *HsPLK1* (³²P-γATP). The arrow marks the position of MEX-5^{FL}. Upper panel: ³²P incorporation visualized by autoradiography. Lower panel: Coomassie-stained gel. *, a contaminating band co-purified with MBK-2; A₁₈₆, T₁₈₆A; D₁₈₆, T₁₈₆D; DYRK2, *HsDYRK2*; KD, kinase dead; WT, wild type.

HsDYRK2 was used, only very low levels of radioactive MEX-5 were detected (Fig. 6B). These results suggested that: (1) prephosphorylation of MEX-5 by *HsDYRK2* dramatically enhances phosphorylation of MEX-5 by *HsPLK1*; and (2) although *HsDYRK2* phosphorylated multiple sites on MEX-5, only phosphorylation at T₁₈₆ is important for *HsPLK1* docking and phosphorylation of MEX-5. This result strongly supports the conclusion that MBK-2 phosphorylates MEX-5 at T₁₈₆, priming for PLK-1 interaction and phosphorylation in vivo.

PLK-1 and PLK-2 are required for MEX-5 and MEX-6 function in vivo

We also characterized localization of MEX-5 and PIE-1 in embryos depleted of *plk-1*, *plk-2* or both. We observed three phenotypes associated with *plk-1(RNAi)*, depending on the strength of the RNAi effect, which we classified as either strong, medium or mild. Strong



RNAi resulted in sterile adults. Medium RNAi resulted in embryos arrested at the one-cell stage without cleavage, as previously reported (Chase et al., 2000b). These embryos failed to complete meiosis and did not have polar bodies. Mild RNAi resulted in a very penetrant phenotype (~80% of embryos examined, $n > 100$) of double-nucleated blastomeres, probably the result of failure in oocyte pronucleus nuclear envelope breakdown (Chase et al., 2000b). These defects were not enhanced when *plk-2* was also depleted (Chase et al., 2000b). We observed that the one-cell arrested embryos that resulted from medium strength *plk-1/2* RNAi still exhibited asymmetrically localized GFP::MEX-5 (Fig. 7A,B,E,F,I,J,Q). This result is consistent with our above observation that MEX-5 T₁₈₆ (and presumably PLK-1 binding) was not required for MEX-5 localization, but regulates MEX-5 function in vivo. However, we could not rule out that a low level of PLK-1/2 remains in our medium-strength RNAi embryos that is sufficient to polarize GFP::MEX-5.

We then asked whether MEX-5 function is jeopardized in embryos when *plk-1*, *plk-2* or both were depleted. We assayed three phenotypes that are normally associated with low MEX-5/6 function. First, polarization of PIE-1 in the one-cell zygote requires MEX-5/6 function (Schubert et al., 2000). We asked therefore whether PIE-1 is polarized properly along the AP axis when *plk-1*, *plk-2* or both are depleted in embryos by performing RNAi in either wild type or a GFP::PIE-1-expressing strain. We observed that both endogenous PIE-1 and GFP::PIE-1 were not polarized in embryos derived from *plk-1*(RNAi);*plk-2*(RNAi) animals, but both appeared normally polarized in embryos depleted of *plk-1* or *plk-2* alone (Fig. 7C,D,G,H,K,L,Q; data not shown).

Second, MEX-5/6 activity has also been correlated with the extent of PIE-1 accumulation in pronuclei (Cuenca et al., 2003). In the wild-type one-cell embryo, PIE-1 is detected in the sperm-derived

Fig. 7. PLK-1 and PLK-2 are required for MEX-5 and MEX-6 function in *C. elegans*.

(A-L) Immunofluorescence micrographs of anti-MEX-5 and anti-GFP staining and corresponding DAPI staining in one-cell embryos expressing GFP::PIE-1. (A-D) Wild type (no RNAi). (E-H) *plk-1*(RNAi). (I-L) *plk-1*(RNAi);*plk-2*(RNAi). The arrows and arrowheads point to the oocyte- and sperm-derived pronuclei, respectively. Because of defective meiosis, the oocyte pronucleus has a higher DNA content than the sperm pronucleus in *plk-1*-depleted embryos. (M,O) DIC and (N,P) fluorescence micrographs of GFP::PIE-1 in four-cell wild-type (M,N) or mild *plk-1*(RNAi); *plk-2*(RNAi) (O,P) embryos. Closed and open arrowheads indicate the germline blastomere and its somatic sister, respectively. (Q) Quantification of MEX-5 and PIE-1 cytoplasmic asymmetry in one-cell wild-type, *plk-1*(RNAi), and *plk-1*(RNAi);*plk-2*(RNAi) embryos. See Fig. 3R legend for details. Scale bar: 10 μ m. *, polar bodies.

pronucleus, but is not detected, or is detected at a very low level, in the oocyte-derived pronucleus (Fig. 7C,D). Although the significance of this low PIE-1 protein in the oocyte-pronucleus is not known, it has been shown that it requires MEX-5/6 function. In genetic backgrounds in which MEX-5/6 activity is low, PIE-1 was detected in both pronuclei (Cuenca et al., 2003). We observed that embryos derived from *plk-1*(RNAi);*plk-2*(RNAi), but not *plk-1*(RNAi) or *plk-2*(RNAi) animals, often exhibited a high and equal level of nuclear GFP::PIE-1 in both pronuclei (Fig. 7G,H,K,L; data not shown), consistent with *plk-1*(RNAi);*plk-2*(RNAi) embryos having reduced MEX-5/6 activity.

Third, MEX-5/6 function is required for the degradation of PIE-1 in somatic cells (DeRenzo et al., 2003; Schubert et al., 2000). To assay for the activity of MEX-5 in the degradation of PIE-1 in somatic cells requires that embryos undergo divisions beyond the four-cell stage. With mild *plk-1/2*(RNAi), we obtained embryos that developed further than the one-cell stage, permitting the assay of PIE-1 levels in somatic cells. We observed a slightly higher level of PIE-1 in somatic cells in many *plk-1*(RNAi);*plk-2*(RNAi) embryos (Fig. 7M,N,O,P). The defect is not as strong as in *mex-5*(RNAi);*mex-6*(RNAi) embryos, but is consistent with a decrease in MEX-5/6 activity.

All together, our analyses are consistent with a reduced MEX-5/6 activity in *plk-1*(RNAi);*plk-2*(RNAi) embryos.

DISCUSSION

We report here a novel role for polo kinases PLK-1 and PLK-2 in regulating embryonic cytoplasmic polarity. We show that PLK-1/2 co-localize with and enhance the function of MEX-5/6 in vivo by binding, via their PBDs, to MBK-2-primed MEX-5/6. The developmentally regulated MBK-2 activity provides a precise temporal control for the interaction between PLK-1/2 and MEX-5/6 and the onset of MEX-5/6 function.

Partial redundancy between PLK-1 and PLK-2

While polo kinases are key regulators in cell divisions, they also have been shown to regulate processes unrelated to cell division. Polo kinases regulate synapse remodeling in the mammalian nervous system by promoting the degradation of spine-associated Rap guanosine triphosphatase-activating protein (SPAR) (Kauselmann et al., 1999; Pak and Sheng, 2003; Seeburg et al., 2005). In addition, a recent paper showed that polo phosphorylates Pon, which is important for asymmetric localization of Numb and inhibition of progenitor self-renewal in *Drosophila* neuroblasts (Wang et al., 2007). Our results here demonstrate another novel, non-cell division role for polo kinases – regulation of cytoplasmic polarity in early *C. elegans* embryos. We show here that, in addition to cytokinesis and chromosome segregation, PLK-1 has a redundant role with PLK-2 in regulating MEX-5/6 function. The subcellular localization of PLK-2, although detected only weakly with our PLK-2 antibody, resembles that of PLK-1. Therefore the partial redundancy of PLK-1 and PLK-2 cannot be explained by their subcellular localization patterns. We propose that PLK-1 and PLK-2 function together in vivo, with PLK-1 contributing more than PLK-2 to net PLK activity. In this scenario, depletion of *plk-1* alone by RNAi would reveal defects only in those processes requiring highest PLK activity levels (such as cytokinesis and chromosome segregation). Processes requiring a low PLK activity (such as MEX-5/6 activation) would not be affected unless both *plk-2* and *plk-1* are depleted.

The partial redundancy between PLK-1 and PLK-2 is reminiscent of that between MEX-5 and MEX-6 (Schubert et al., 2000). This similarity suggests the possibility that PLK-1 regulates MEX-5, whereas PLK-2 regulates MEX-6 activity. However, our data argue against this possibility. First, both PLK-1 and PLK-2 bind to both MEX-5 and MEX-6 in yeast. Second, the asymmetric localization pattern of PLK-1 and GFP::PLK-1^{PBD} was disrupted only when both *mex-5* and *mex-6* were depleted or mutated. Therefore, we propose that PLK-1 and PLK-2 regulate both MEX-5 and MEX-6 in vivo and the difference in *plk-1(RNAi)* and *plk-2(RNAi)* phenotypes is probably due to their different expression levels in vivo.

How might PLK-1 and PLK-2 regulate MEX-5 and MEX-6 function?

Currently there are two models, not mutually exclusive, for how the PBD regulates in vivo substrate phosphorylation by polo kinase (reviewed by Lowery et al., 2005). In the ‘processive phosphorylation’ model, PBD binding brings the kinase domain to the bound protein, which polo phosphorylates at another site. In the ‘distributive phosphorylation’ model, PDB binding brings the kinase domain to the vicinity of its substrate(s), which is not the same protein to which the PBD is bound. How might PLK-1/2 enhance MEX-5/6 function? It is possible that PLK-1/2 enhances MEX-5/6 function by directly phosphorylating them (progressive phosphorylation model), thereby changing the binding affinity and/or specificity of MEX-5/6 toward RNA targets or interacting proteins. Alternatively, it is possible that MEX-5/6 serve simply as a support for PLK-1/2, which provides MEX-5/6 with an acquired enzymatic activity (distributive phosphorylation model). Phosphorylation has been shown to facilitate substrate recognition for many proteasome-mediated protein degradations (for a review, see Harper, 2002). PLK-1/2 might phosphorylate substrates, ZIF-1 or another component in the E3 ligase complex (DeRenzo et al., 2003) that degrades PIE-1, POS-1 and MEX-1, allowing their spatially and temporally regulated degradation. Experiments are underway to distinguish between these two models.

PLK-1/2 and MEX-5/6 interaction

Our results demonstrate that MBK-2 phosphorylates T₁₈₆ of MEX-5 (and T₁₉₀ of MEX-6), providing a docking site for PLK-1/2, and that PLK-1/2 binding enhances MEX-5/6 function. We believe that phosphorylation at T₁₈₆ promotes, but is not absolutely required for, PLK-1 binding. First, weaker binding was still detected between PLK-1^{PBD}/PLK-2^{PBD} and MEX-6 T_{190A} in yeast. We assume that the observed strong binding with wild-type MEX-5/6 was facilitated by a yeast kinase phosphorylating T₁₈₆ and T₁₉₀ in vivo. The candidate yeast kinases include CDC28, the CDK1 homolog, and YAK1, which shares weak similarity to MBK-2. Second, while prephosphorylation at T₁₈₆ by MBK-2 dramatically enhances MEX-5 phosphorylation by *Hs*PLK1, we still detected a low level of radioactive MEX-5 with MEX-5 T_{186A} or in the absence of *Hs*DYRK2. Third, T₁₈₆ is not essential for all MEX-5 function, as some, albeit few, embryos expressing only GFP::MEX-5 T_{186A} survive, whereas embryos completely devoid of MEX-5 do not. This is consistent with other studies showing that priming phosphorylations enhance binding and phosphorylation of polo substrates by polo kinases, but are not absolutely required (Jackman et al., 2003; Kumagai and Dunphy, 1996; Nakajima et al., 2003; Yarm, 2002; Zhou et al., 2003).

The observation that full-length PLK-1/2 did not recapitulate the subcellular localization pattern observed with antibody staining is intriguing. One possible explanation is that overexpressed GFP::PLK-1^{FL} exceeds the available phosphorylated docking sites. We do not believe this to be the case, because the level of GFP was observed to be equal, if not higher, in GFP::PLK-1^{PBD}-expressing lines. Alternatively, in vivo localization of PLK1/2 might be subjected to another level of regulation, in addition to the availability of phosphorylated docking sites. It has been shown that the mutual inhibitory interaction between the PBD and the kinase domain of PLK1 results in an approximately ten-fold reduction in phosphopeptide binding (Elia et al., 2003b). It is possible that a regulation that weakens this mutual inhibitory interaction is required in vivo and that GFP::PLK-1^{FL} and GFP::PLK-2^{FL} were not regulated properly. Consistent with this possibility, we showed that GFP::PLK-1^{FL} did not rescue embryos depleted of endogenous PLK-1 (Materials and methods).

Priming by MBK-2

For most proteins that provide a phosphorylated polo docking site, cyclin-dependent kinases (CDK) have been shown to be the priming kinase (van Vugt and Medema, 2005). Here we show that MBK-2 is the priming kinase for PLK-1 at MEX-5 T₁₈₆ both in vitro and in vivo and that CDK-1 regulates MEX-5/6 and PLK-1/2 interaction probably indirectly through regulating MBK-2. The finding that MBK-2 is the priming kinase for PLK-1 at T₁₈₆ is somewhat surprising. First, DYRK kinases have not been previously shown to prime for polo kinases. Second, the amino acid sequence surrounding MEX-5 T₁₈₆ (LTSSTP) does not constitute a typical MBK-2 consensus site, which is R(X)₁₋₃/S/TP (Campbell and Proud, 2002; Himpel et al., 2000). Our in vitro data suggest that MBK-2 phosphorylates at least one more site on MEX-5, in addition to T₁₈₆. This additional site is likely to be T₃₉₀, because its surrounding sequence (RATTP) resembles the MBK-2 consensus phosphorylation site. However, our data shown in Fig. 6 demonstrate clearly that only phosphorylation at T₁₈₆ by *Hs*DYRK2 primes for *Hs*PLK1 in vitro.

Although at first glance MBK-2 seems an unlikely kinase to prime for PLK-1 at MEX-5 T₁₈₆, it makes excellent sense with respect to early *C. elegans* embryogenesis. PLK-1 is detected in

oocytes and is active during meiotic divisions, regulating chromosome segregation and nuclear envelope breakdown. However, MEX-5-dependent processes are first observed only following completion of both meiotic divisions. As MBK-2 activation does not occur until meiosis II, MBK-2 phosphorylation-dependent PLK-1 binding would provide a precise developmental regulation of MEX-5 function, ensuring that MEX-5 does not become prematurely activated.

Upon activation at meiosis II, MBK-2 has been shown to phosphorylate three other proteins, MEI-1, OMA-1 and OMA-2, promoting their developmentally regulated degradation (Nishi and Lin, 2005; Shirayama et al., 2006; Stitzel et al., 2006). OMA-1 and OMA-2 are closely related Tis11 zinc-finger-containing proteins that were initially identified as being redundantly required for oocyte maturation (Detwiler et al., 2001). Precisely timed degradation of OMA-1 (and probably OMA-2) is crucial for embryonic development (Detwiler et al., 2001; Lin, 2003; Nishi and Lin, 2005; Shirayama et al., 2006). We, and others, have shown that phosphorylation of OMA-1 by MBK-2 occurs primarily at T₂₃₉ (Nishi and Lin, 2005; Shirayama et al., 2006; Stitzel et al., 2006). Interestingly, the OMA-1 sequence surrounding T₂₃₉ also resembles the consensus polo domain interaction sequence S-[pS/pT]-P. Consistent with a model that PLK-1 regulates OMA-1 via an MBK-2 primed phosphorylation site, our previous studies have suggested that MBK-2 phosphorylation not only regulates OMA-1 degradation but might also regulate its function (Nishi and Lin, 2005). As DYRK2 kinases have been implicated in many biological processes, our findings suggest the possibility that additional non-mitotic functions for polo kinases will be identified in worms and other developmental systems.

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