Caenorhabditis elegans PIG-1/MELK Acts in a Conserved PAR-4/LKB1 Polarity Pathway to Promote Asymmetric Neuroblast Divisions

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ABSTRACT Asymmetric cell divisions produce daughter cells with distinct sizes and fates, a process important for generating cell diversity during development. Many *Caenorhabditis elegans* neuroblasts, including the posterior daughter of the Q cell (Q.p), divide to produce a larger neuron or neuronal precursor and a smaller cell that dies. These size and fate asymmetries require the gene *pig-1*, which encodes a protein orthologous to vertebrate MELK and belongs to the AMPK-related family of kinases. Members of this family can be phosphorylated and activated by the tumor suppressor kinase LKB1, a conserved polarity regulator of epithelial cells and neurons. In this study, we present evidence that the *C. elegans* orthologs of LKB1 (PAR-4) and its partners STRAD (STRD-1) and MO25 (MOP-25.2) regulate the asymmetry of the Q.p neuroblast division. We show that PAR-4 and STRD-1 act in the Q lineage and function genetically in the same pathway as PIG-1. A conserved threonine residue (T169) in the PIG-1 activation loop is essential for PIG-1 activity, consistent with the model that PAR-4 (or another PAR-4-regulated kinase) phosphorylates and activates PIG-1. We also demonstrate that PIG-1 localizes to centrosomes during cell divisions of the Q lineage, but this localization does not depend on T169 or PAR-4. We propose that a PAR-4-STRD-1 complex stimulates PIG-1 kinase activity to promote asymmetric neuroblast divisions and the generation of daughter cells with distinct fates. Changes in cell fate may underlie many of the abnormal behaviors exhibited by cells after loss of PAR-4 or LKB1.

ASYMMETRIC cell divisions produce daughter cells with distinct fates, a process important for generating cell diversity in organisms as different as bacteria, plants, and animals. In *Caenorhabditis elegans*, asymmetric divisions of neuroblasts often produce cells fated to die. The posterior daughter of the Q cell (Q.p) neuroblasts, for example, divide to produce a neuronal precursor and a cell that dies, but how this fate asymmetry is generated in these divisions is poorly understood. PIG-1 regulates asymmetric neuroblast divisions that generate apoptotic cells by controlling spindle positioning, myosin distribution, and daughter cell fate (Cordes *et al.* 2006; Ou *et al.* 2010). PIG-1 is the sole *C. elegans* ortholog of MELK (maternal embryonic leucine zipper kinase), a serine/threonine kinase that has been implicated in many developmental processes including stem cell renewal, apoptosis, cell cycle progression, and spliceosome assembly (Davezac *et al.* 2002; Vulsteke *et al.* 2004; Nakano *et al.* 2005; Lin *et al.* 2007; Jung *et al.* 2008). MELK and PIG-1 represent a subgroup of a large family of serine/threonine kinases that include molecules like PAR-1 and SAD-1, which regulate cell polarity, and AMPKs, which regulate metabolic processes (Bright *et al.* 2009). These family members are often regulated directly by the LKB1 kinase (Lizcano *et al.* 2004).

Loss of the tumor suppressor LKB1 causes Peutz-Jeghers syndrome, a disease in humans that is characterized by polyp formation in the gastrointestinal tract and predisposition for certain types of cancer (Jeghers *et al.* 1949; Hemminki *et al.* 1998; Jenne *et al.* 1998; Giardiello *et al.* 2000). LKB1 encodes a highly conserved serine/threonine kinase that activates several downstream kinases by phosphorylating a conserved threonine residue in their activation loops (Lizcano *et al.* 2004). One key substrate of LKB1 is AMPK, a master regulator of metabolism. LKB1 and its orthologs (PAR-4 in *C. elegans*) can also regulate polarity through other AMPK-related kinases such as SAD and MARK kinases. SAD-A and SAD-B

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mediate the effects of LKB1 in axon specification of cultured rat hippocampal neurons (Barnes *et al.* 2007). In early divisions of the *C. elegans* embryo, PAR-4-dependent phosphorylation of PAR-1, a MARK ortholog, leads to asymmetric segregation of cell fate determinants (Watts *et al.* 2000; Narbonne *et al.* 2010). By contrast, PAR-1 acts upstream of LKB1 in *Drosophila* oocyte polarity (Martin and St. Johnston 2003).

LKB1 is found in a complex with the pseudokinase STRAD and the adaptor MO25. The association of these two cofactors with LKB1 promotes its kinase activity, stability, and nuclear-to-cytoplasmic translocation (Baas *et al.* 2003; Boudeau *et al.* 2003; Dorfman and Macara 2008). Indeed, the crystal structure of the heterotrimeric complex suggests the binding of STRAD and MO25 locks LKB1 in its active conformation (Zeqiraj *et al.* 2009). Excess expression of both LKB1 and STRAD leads to cell-autonomous polarization of single isolated epithelial cells (Baas *et al.* 2004) and axon specification in developing neurons (Shelly *et al.* 2007). Despite these requirements for STRAD, LKB1 has also been shown to have STRAD-independent functions in *C. elegans* (Kim *et al.* 2010; Narbonne *et al.* 2010).

An *in vitro* study found that, although most AMPK family kinases tested can be phosphorylated and activated by LKB1, one notable exception is MELK (Lizcano *et al.* 2004). MELK exhibits a high basal activity, and the addition of LKB1 does not enhance its kinase activity (Lizcano *et al.* 2004). Nevertheless, the conserved threonine residue in the activation loop that is the target of LKB1 in other kinases is essential for MELK kinase activity (Lizcano *et al.* 2004; Beullens *et al.* 2005). These data suggest that MELK is activated through autophosphorylation of its activation loop residue *in vitro*. Whether the *C. elegans* MELK ortholog PIG-1 is activated independently of PAR-4/LKB1 is unknown.

Here we provide evidence that the *C. elegans* orthologs of LKB1, STRAD, and MO25 are involved in the asymmetric cell division of the Q.p neuroblast lineage. Genetic interactions between *strd-1*, *par-4*, and *pig-1* suggest that they act in the same pathway. Our structure-function analysis suggests that both the N-terminal kinase and the C-terminal kinase-associated 1 domains of PIG-1 are important for its function.

Materials and Methods

Nematode strains and genetics

Nematodes were cultured as previously described (Brenner 1974). N2 Bristol was the wild-type strain used in this study, and strains were maintained at 20° except for strains containing *par-4* or *par-1*, which were maintained at 15°. The following alleles and transgenes were used in this study:

LGI: *zdIs5[Pmec-4::gfp, lin-15(+)]* (Clark and Chiu 2003), *gmIs88[Pmab-5::pig-1::gfp; pRF4(rol-6(su1006))*; *Pdpy-30::NLS::DsRed2]* results from spontaneous integration of *gmEx394* (Cordes *et al.* 2006).

LGII: *mop-25.2(ok2073)* (Caenorhabditis Genetics Center), *mop-25.2(tm3694)* (National Bioresource Project of Japan), and *rrf-3(pk1426)* (Simmer *et al.* 2002).

LGIII: *strd-1(ok2283)*, *strd-1(rr91)* (Narbonne and Roy 2009), and *rdvIs1[Pegl-17::myristoylated mcherry; Pegl-17::mcherry::TEV-S::his-24; Pegl-17::mig-10::YFP; pRF4 (rol-6(su1006))]* (Ou *et al.* 2010).

LGIV: *pig-1* alleles used include *gm280* and *gm301* (Cordes *et al.* 2006), *ced-3(n717)*, and *ced-3(n2436)* (Shaham *et al.* 1999)

LGV: *par-4(it57ts)* (Watts *et al.* 2000) and *par-1(zu310ts)* (Kemphues *et al.* 1988)

LGX: *sad-1(ky289)* (Crump *et al.* 2001), *aak-2(gt33)* (Lee *et al.* 2008), and *gmIs87[Pmab-5::pig-1(T169D)::gfp; Pmyo-2::mcherry]*

For phenotypic analyses, L4-stage hermaphrodites from *it57*ts and *zu310*ts, and control strains were cultured at 15° and transferred to 25° 24 hr later when they carried embryos. Their progeny were examined at L3–L4 stage. Strains containing *strd-1* mutations were also scored at 25°. All the other strains were scored at 20° unless otherwise noted.

RNA interference

RNA interference (RNAi) was performed using the bacterial feeding method as described (Timmons and Fire 1998; Kamath *et al.* 2001). In all experiments, worms were grown on plates supplemented with 25 mM Carbenicillin and 1 mM IPTG. The RNAi cultures were prepared by inoculating bacterial strains in LB with 25 mM Carbenicillin for 15 hr at 37°, followed by addition of 6 mM IPTG and incubation for another hour at 37°. Bacterial strains used to inactivate genes by feeding were obtained from the library designed by the Ahringer lab (Fraser *et al.* 2000).

Molecular biology and germline transformation

The *Pmab-5::par-4::mcherry* and *Pmab-5::strd-1::gfp* were generated by the multi-site Gateway strategy (Invitrogen). To be more specific, *par-4* cDNA was amplified from pJH1139 (*Punc-25::par-4*; a kind gift from Mei Zhen). *strd-1* cDNA was amplified from worms carrying *Pstrd-1::strd-1(cDNA)::gfp* (MR1494; a kind gift from Richard Roy). *gmEx669* and *gmEx673* were generated by injecting *Pmab-5::par-4::mcherry* into *zdIs5 [Pmec-4::gfp]* hermaphrodites at 75 ng/μl with 3 ng/μl *Pmyo-2::mcherry (pCFJ90)*. *gmEx677* and *gmEx682* were generated by injecting *Pmab-5::strd-1::gfp* into *zdIs5[Pmec-4::gfp]* hermaphrodites at 50 ng/μl with 6 ng/μl *Pmyo-2::gfp*.

Pmab-5::pig-1(T169A)::gfp, *Pmab-5::pig-1(T169D)::gfp* and *Pmab-5::pig-1(KAΔ)::gfp* constructs were generated by modifying *Pmab-5::pig-1::gfp* (Cordes *et al.* 2006) with PCR-based mutagenesis (QuikchangeII XL, Agilent Technologies). The following primers were used: for T169A, GATAAGCACAATTTGGATGCGTGTTGTGGATCTCCG and its reverse complement; for T169D, GTATTGATAAGCACAATTTGGATGACTGTTGTGGATCTCCGGCC and its reverse complement; and for KAΔ, GATGGAAGTTCCTTGCGACATTCCCGGGATTGGCCAAAGGACCCA and its reverse complement. *gmEx610*, *gmEx611*, and *gmEx612* were generated by injecting *Pmab-5::pig-1(T169A)::gfp* into N2 hermaphrodites at

10 ng/μl with 3 ng/μl *Pmyo-2*::*mcherry (pCFJ90)*. *gmEx613*, *gmEx614*, and *gmEx615* were generated by injecting *Pmab-5*::*pig-1(T169D)*::*gfp* into N2 hermaphrodites at 10 ng/μl with 3 ng/μl *Pmyo-2*::*mcherry (pCFJ90)*. *gmEx614* was integrated into the genome by gamma-ray mutagenesis, resulting in the stably transmitting strain *gmIs87*. *gmEx637*, *gmEx639*, and *gmEx640* were generated by injecting *Pmab-5*::*pig-1(KAΔ)*::*gfp* into *zdIs5[Pmec-4*::*gfp]* hermaphrodites at 10 ng/μl with 50 ng/μl of pRF4 and 50 ng/μl of plasmid *Pdpy-30*::*NLS*::*DsRed2* (Cordes *et al.* 2006).

To generate *Pmab-5*::*pig-1(KAΔ)*; *K40A*::*gfp*, mutagenesis was performed using *Pmab-5*:: *pig-1 (KAΔ)*::*gfp* as a template. The primer AATCAAAAGGTGGCCATCGCCATAATA GATAAGAAGCAGCTTGGA and its reverse complement was used. *gmEx641* was generated by injecting *Pmab-5*::*pig-1 (KAΔ)*; *K40A*::*gfp* into *zdIs5[Pmec-4*::*gfp]* hermaphrodites at 10 ng/μl with 50 ng/μl of pRF4 and 50 ng/μl of plasmid *Pdpy-30*::*NLS*::*DsRed2* (Cordes *et al.* 2006). Germline transformation was performed by direct injection of various plasmid DNAs into the gonads of adult wild-type animals as described (Mello *et al.* 1991).

Analysis of neuroblast daughter size

The daughters of the Q.p neuroblast were identified using *ayIs9* [*Pegl-17*::*gfp*] (Branda and Stern 2000) and *rdvIs1[Pegl-17*::*myristoylated mcherry]* (Ou *et al.* 2010). We measured cell area in a single plane of focus. These cells are extremely flat, and thus measurements of cell area are good estimates of cell size. We calculated cell area by circumscribing the cell and measuring its interior area with Openlab software (Improvision). We averaged two measurements per cell.

Fluorescence microscopy

For fluorescence microscopy, L4 to young adult hermaphrodite animals were anesthetized with 1% sodium azide, mounted on agar pad, and observed with a Zeiss Axioskop2 microscope.

Results

C. elegans orthologs of LKB1, STRAD, and MO25 regulate asymmetric cell division of the Q.p neuroblast

All *C. elegans* neurons arise from asymmetrically dividing neuroblasts. For example, the Q.p neuroblasts divide to produce a neuronal precursor and a cell that is fated to die (Figure 1A). The neuronal precursor then divides to generate the mechanosensory neuron AVM or PVM and the interneuron SDQR/L (Figure 1A). Mutations in genes that regulate these divisions may transform the fate of the apoptotic cell Q.pp to that of its sister Q.pa. This transformation can result in the production of extra A/PVM and SDQ neurons if Q.pp survives and divides (Figure 1B). However, no extra neurons will be produced if the Q.pp dies (Figure 1C). One such gene is *pig-1*, which encodes a serine/threonine kinase of the AMPK family (Cordes *et al.* 2006). Such cell fate genes are distinct from proapoptotic genes, which, when mutated, allow Q.pp to survive but not divide (Figure 1D). However, the penetrance of the extra neuron phenotype in cell fate mutants such as *pig-1* can be enhanced by removing the function of proapoptotic genes such as *ced-3*, possibly because cell death masks the cell fate transformations (Figure 1E) (Cordes *et al.* 2006).

PIG-1 belongs to a superfamily of kinases that are directly regulated by the LKB1 kinase. Given that LKB1, along with its binding partners STRAD and MO25, has been shown to regulate polarity in different contexts (Jansen *et al.* 2009), we asked whether the *C. elegans* orthologs of these genes are involved in regulating the Q.p division. *C. elegans* encodes a single LKB1 ortholog called *par-4*, a single STRAD ortholog called *strd-1*, and three MO25 homologs called *mop-25.1*, *mop-25.2*, and *mop-25.3*. Based on sequence homology, MOP-25.1 is paralogous to MOP-25.2 (69% identity and 84% similarity) and is more distantly related to MOP-25.3 (20% identity and 45% similarity). Using an integrated *Pmec-4*::*gfp* transgene to visualize the AVM and PVM mechanosensory neurons, we observed a small but significant number of extra A/PVMs in the temperature-sensitive *par-4(it57*ts) mutant, and two putative null *strd-1* mutants, *ok2283* and *rr91* (Figure 1E). We reasoned that, as for *pig-1*, these mutants may display a weak phenotype because the underlying cell fate transformation is masked by cell death (Cordes *et al.* 2006). To test this hypothesis, we removed the function of *ced-3*, which encodes a caspase in the canonical programmed cell death pathway (Yuan *et al.* 1993), from the *par-4* or *strd-1* mutant backgrounds. Consistent with this hypothesis, *ced-3* interacted synergistically with both *par-4* and *strd-1* (Figure 1E). We obtained similar results when we scored the number of extra SDQs in these strains (data not shown). RNAi of the three *mop-25* genes in an RNAi-sensitive *rrf-3* background (Simmer *et al.* 2002) produced a mild extra neuron phenotype for *mop-25.2*, and this phenotype was further enhanced in a weak *ced-3* mutant background [*rrf-3*; *ced-3(n2436)*] (Figure 1E). RNAi of the two other *mop-25* genes failed to produce a phenotype in either a wild-type or a *ced-3* mutant background (not shown). Simultaneous RNAi to both *mop-25.1* and *mop-25.3* also failed to generate a phenotype, and double or triple RNAi experiments failed to enhance the *mop-25.2* phenotype (data not shown). Two existing alleles of *mop-25.2* (*ok2073* and *tm3694*), however, did not produce a significant extra neuron phenotype either alone or in a *ced-3*-sensitized background (data not shown). Given that both alleles of *mop-25.2* produce maternal effect lethality, a maternal contribution of *mop-25.2* from the heterozygous mothers may mask a role of this gene in the Q.p division. The RNAi experiments suggest that *mop-25.2* is the principal MO25 homolog that functions in the Q.p lineage division.

PAR-4 and STRD-1 regulate daughter cell-size asymmetry in the Q.p division

C. elegans neuroblast divisions that produce a cell fated to die generate daughter cells of different sizes (Frank *et al.* 2005; Cordes *et al.* 2006; Hatzold and Conradt 2008). In the

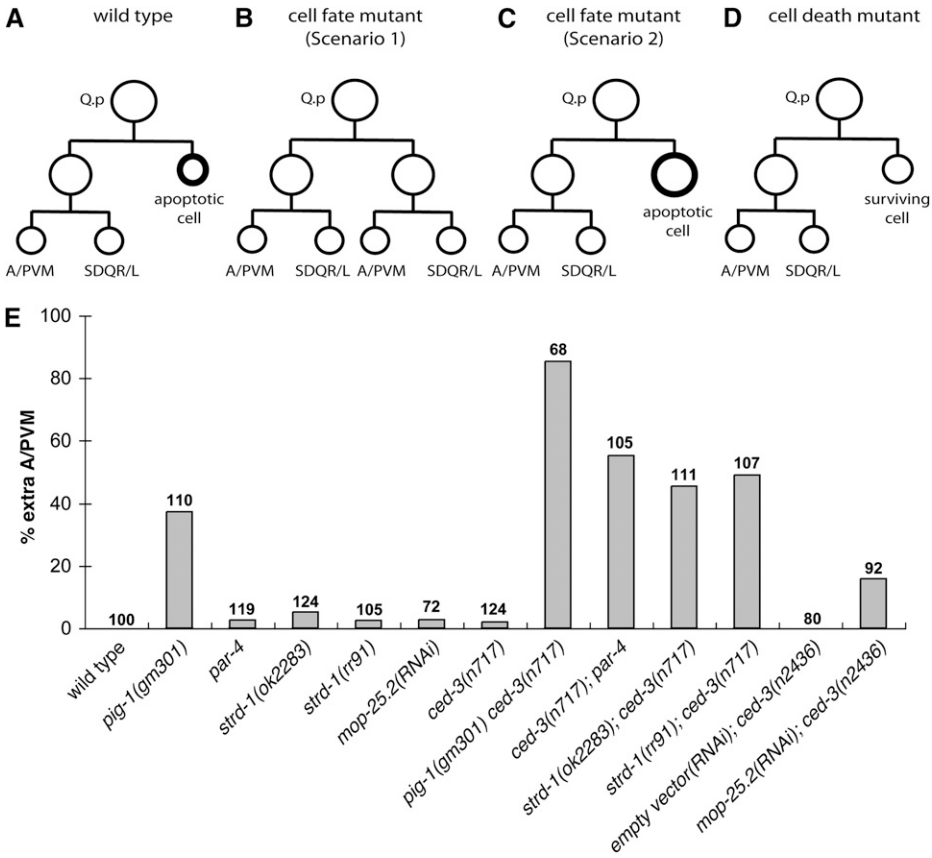

Figure 1 *C. elegans* orthologs of MELK, LKB1, STRAD, and MO25 regulate asymmetric cell division of the Q.p neuroblast lineage. (A) A schematic diagram of the Q.p lineage. In wild type, the Q.p neuroblast divides asymmetrically to produce Q.pa, a neuronal precursor, and Q.pp, which is destined for apoptosis. (B) In a cell-fate mutant, the fate of the apoptotic cell Q.pp is transformed to that of its sister Q.pa, and this transformation can result in the production of extra A/PVM and SDQ neurons if Q.pp survives and divides. (C) No extra neurons will be produced in a cell-fate mutant if Q.pp dies. (D) In a cell-death mutant, Q.pp is allowed to survive, but it will not divide. (E) Mutations in *C. elegans* orthologs of LKB1 (*par-4*), STRAD (*strd-1*), and MO25 (*mop-25.2*) interacted synergistically with *ced-3* to produce extra A/PVMs. The number of lineages scored is indicated above the bars for each genotype. The extra A/PVMs were visualized with an integrated transgene *zdls5[Pmec-4::gfp]*. The empty feeding vector control is L4440.

Q.p division, the mitotic precursor is approximately four times larger than its apoptotic sister due to asymmetric positioning of the mitotic spindle, an asymmetry that requires PIG-1 (Cordes *et al.* 2006; Ou *et al.* 2010). In *par-4* and *strd-1* mutants, the Q.p daughter cells were more equivalent in size (Figure 2A). This phenotype was also more severe in *pig-1* mutants (Cordes *et al.* 2006).

PAR-4 and STRD-1 function in the same pathway as PIG-1 and promote other PIG-1-dependent asymmetries

The observation that the *strd-1(rr91)*; *par-4(it57*ts*)* double mutant died during embryogenesis, even at the permissive temperature for *par-4(it57*ts*)* (Kim *et al.* 2010; Narbonne *et al.* 2010), precluded us from addressing whether these two genes act genetically in the same pathway to promote the asymmetric division of Q.p. However, *pig-1*; *par-4* and *strd-1*; *pig-1* double mutants were viable, allowing us to ask whether *par-4* and *strd-1* are likely to act in the same pathway with *pig-1*. Neither the *par-4* nor the *strd-1* mutation enhanced the extra A/PVM phenotype caused by a strong *pig-1* allele *gm301* (Figure 3A). We did not put *par-4* or *strd-1* in a *ced-3*; *pig-1* background because the percentage of the extra cell phenotype in *ced-3*; *pig-1(strong)* is near 85%, making any enhancement difficult to observe (Cordes *et al.* 2006). One concern is that the *par-4* or *strd-1* mutants lack a penetrant extra A/PVM phenotype and hence might be unable to enhance a *pig-1* mutant. To test whether these mutations could have an effect in a *pig-1* mutant background, we constructed *pig-1*; *par-4* and *strd-1*; *pig-1* double mutants with the weak *pig-1* allele *gm280*. The ability of the *par-4* and *strd-1* mutations to enhance the extra neuron phenotype of this *pig-1* allele shows that loss of *par-4* and *strd-1* can have an effect in a *pig-1* mutant background. The genetic interactions suggest that *par-4*, *strd-1*, and *pig-1* act in the same pathway to regulate the Q.p lineage (Figure 3A).

To determine whether *par-4* and *strd-1* have roles in other asymmetric neuroblast divisions, we examined another lineage regulated by PIG-1—the PLM lineage (Cordes *et al.* 2006). We found that a mutation in *strd-1*, but not *par-4*, enhanced the number of extra PLMs in a *ced-3*-sensitized background. In addition, a mutation in *strd-1* enhanced the extra neuron phenotype of a weak but not a null *pig-1* mutant (Figure 3B), consistent with *strd-1* and *pig-1* functioning in the same genetic pathway to regulate the PLM lineage.

A recent report showed that PIG-1, PAR-4, STRD-1, and two of the MOP25 homologs affect several caspase-independent cell deaths (Denning *et al.* 2012) (see *Discussion*), further supporting our hypothesis that these genes act together in the same pathway.

AMPK family kinase PAR-1 may function with PIG-1 in the Q.p division

LKB1 and its orthologs regulate polarity by activating both AMPK and AMPK-related kinases (Jansen *et al.* 2009). In particular, both SAD and MARK kinases have been implicated in neuronal polarization of cultured hippocampal neurons

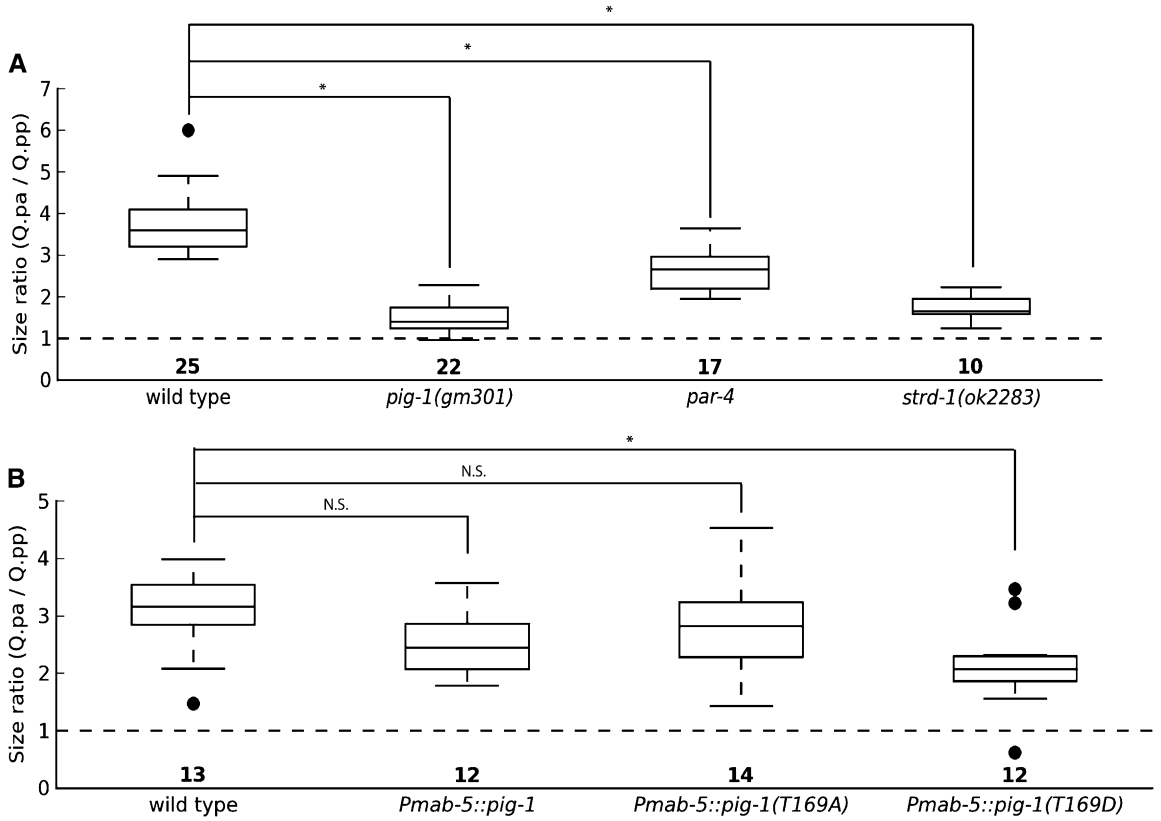

Figure 2 PAR-4 and STRD-1 regulate daughter cell size asymmetry in the Q.p division. In the Q.p division, the mitotic precursor Q.pa is three to four times larger than its apoptotic sister Q.pp (Cordes *et al.* 2006; Singhvi *et al.* 2011). The transcriptional reporters *ayIs9 [Pegl-17::gfp]* (A) and *rdvIs1 [Pegl-17::myristoylated::mcherry]* (B) were used to identify and measure the size of the daughters of the Q.p neuroblast. Ratios of the Q.p daughter cell sizes are depicted as boxplots. The number of divisions scored is indicated above each genotype. Solid circles indicate outliers of the distribution. (A) Mutations in *par-4* and *strd-1* disrupted the cell-size asymmetry of daughter cells of Q.p neuroblast. *pig-1* data (Cordes *et al.* 2006) are shown here for comparison. (B) A transgene expressing PIG-1(T169D), but not one expressing full-length PIG-1 or PIG-1(T169A), disrupted the cell-size asymmetry of daughter cells of the Q.p neuroblast. All of the analyzed transgenes are in the wild-type background. $*P < 0.001$ (Student's *t*-test).

(Kishi *et al.* 2005; Chen *et al.* 2006). We wondered whether *C. elegans* orthologs of these AMPK family kinases work in concert with PIG-1 to regulate the Q.p division. *C. elegans* has two orthologs of AMPK catalytic subunits called AAK-1 and AAK-2, one ortholog of SAD called SAD-1 and one ortholog of MARK called PAR-1. RNAi against *aak-1* and loss-of-function mutations in *aak-2*, *sad-1*, or *par-1* failed to generate a significant extra neuron phenotype in a *ced-3*-sensitized background (data not shown). Simultaneous *aak-1* and *aak-2* RNAi also failed to generate a significant phenotype in a *rrf-3*; *ced-3* background (data not shown). Furthermore, *sad-1* and *par-1* did not enhance the strong *pig-1(gm301)* mutant (Figure 3A). A *par-1* mutation, by contrast, enhanced the extra cell phenotype of a weak *pig-1(gm280)* mutant. These results suggest that a MARK, but not an AMPK or a SAD kinase, regulates the Q.p division (Figure 3A).

par-4 and strd-1 act in the Q lineage

Given that *par-4*, *strd-1*, and *pig-1* act genetically in the same pathway and that *pig-1* acts in the Q lineage (Cordes *et al.* 2006), we tested whether *par-4* and *strd-1* also act in the Q lineage. We expressed cDNAs of both genes from the promoter of the Hox gene *mab-5*, which drives expression in the left Q.p lineage (PVM) but not in the right Q.p lineage (AVM) (Salser and Kenyon 1992). Expression of a wild-type *pig-1* cDNA from this promoter rescued the extra PVM but not the extra AVM defect of *pig-1* mutants, suggesting that *pig-1* acts in the Q lineage (Cordes *et al.* 2006) (Figure 4A). Expression of a mcherry-tagged *par-4* cDNA from the *mab-5* promoter partially rescued the extra PVM phenotype but not the AVM phenotype of *ced-3*; *par-4* double mutants (Figure 3C). We obtained a similar result with a GFP-tagged *strd-1* cDNA (Figure 3C). Our tagged *par-4* and *strd-1* transgenes showed a diffusely uniform localization in the cells of the Q lineage (data not shown), possibly due to excess expression. Partial rescue could reflect inappropriate levels of *par-4* and *strd-1* expression, reduced function of the tagged proteins, or a role of these genes outside of the Q lineage. The ability of both transgenes to partially rescue indicates that PAR-4 and STRD-1 act in the Q lineage.

Conserved threonine residue in the activation loop is essential for PIG-1 activity

LKB1 is a highly conserved serine/threonine kinase that activates AMPK family kinases by phosphorylating

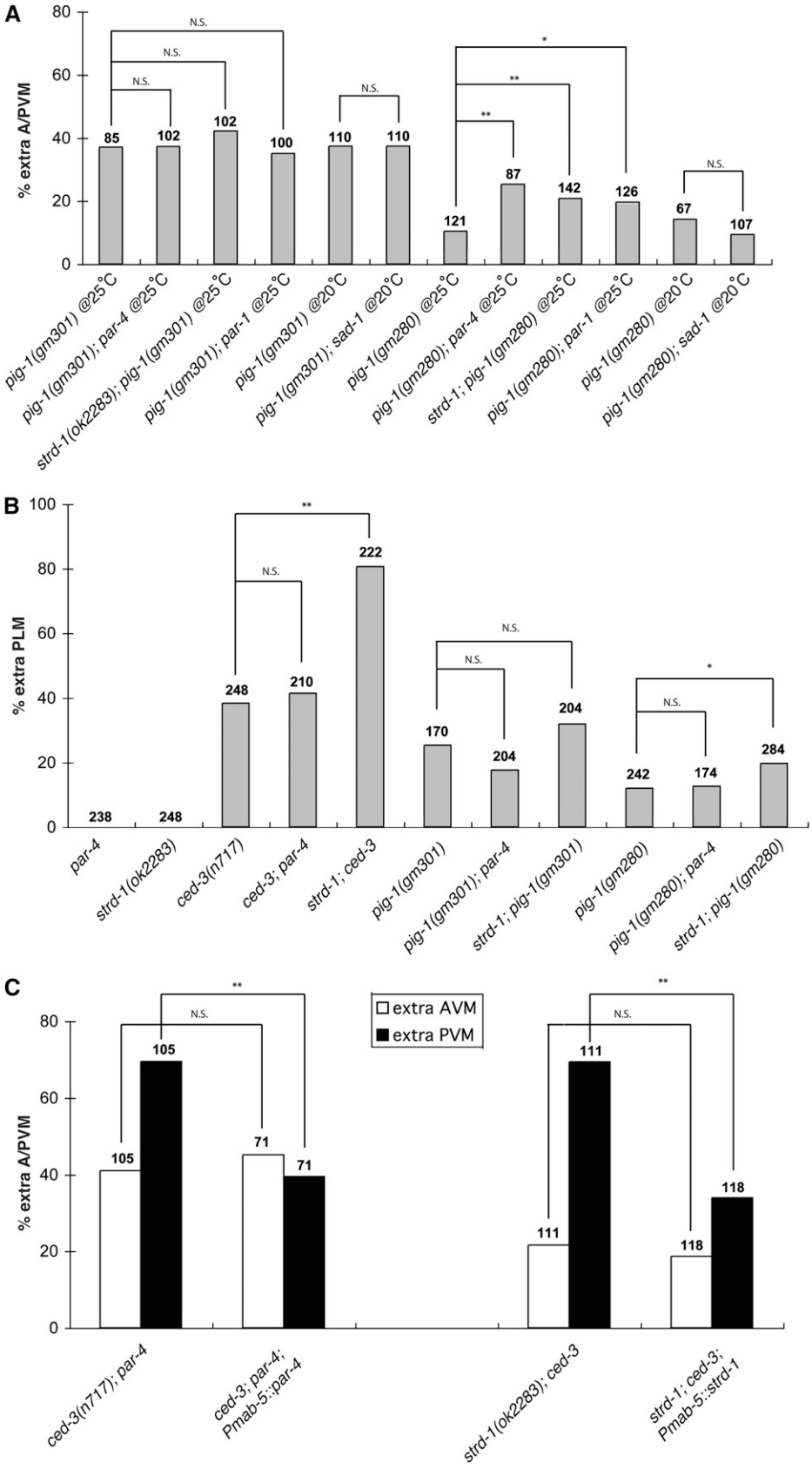

Figure 3 PAR-4 and STRD-1 act in the Q lineage to promote the Q.p division and function in the same pathway as PIG-1. (A) Mutation of *par-4*, *strd-1*, and *par-1* enhanced the extra neuron phenotype caused by the weak *pig-1* allele *gm280*, but not by the strong *pig-1* allele *gm301*. Mutation of *sad-1* failed to enhance either *pig-1(gm301)* or *pig-1(gm280)*. Neither *pig-1* allele is temperature-sensitive for the extra A/PVM phenotype. (B) A mutation in *strd-1*, but not in *par-4*, enhanced the number of extra PLMs in a *ced-3*-sensitized background. In addition, a mutation in *strd-1* enhanced the extra neuron phenotype of a weak but not a strong *pig-1* mutant. (C) Expression of the *par-4* or *strd-1* cDNA from the *mab*-5 promoter partially rescued the extra PVM but not the AVM defect of *ced-3*; *par-4* and *ced-3*; *strd-1*, respectively. The frequency of extra AVMs (open bars) and PVMs (solid bars) is presented separately. We generated two transgenic lines for *Pmab-5*::*par-4*::*mcherry* and two for *Pmab-5*::*strd-1*::*gfp*. Each of the lines for a particular construct was tested and gave similar results. Data for only one line of each type are presented. Number of lineages scored for each genotype is provided. N.S., not significant; $**P < 0.005$; $*P < 0.05$ (Fisher's exact test).

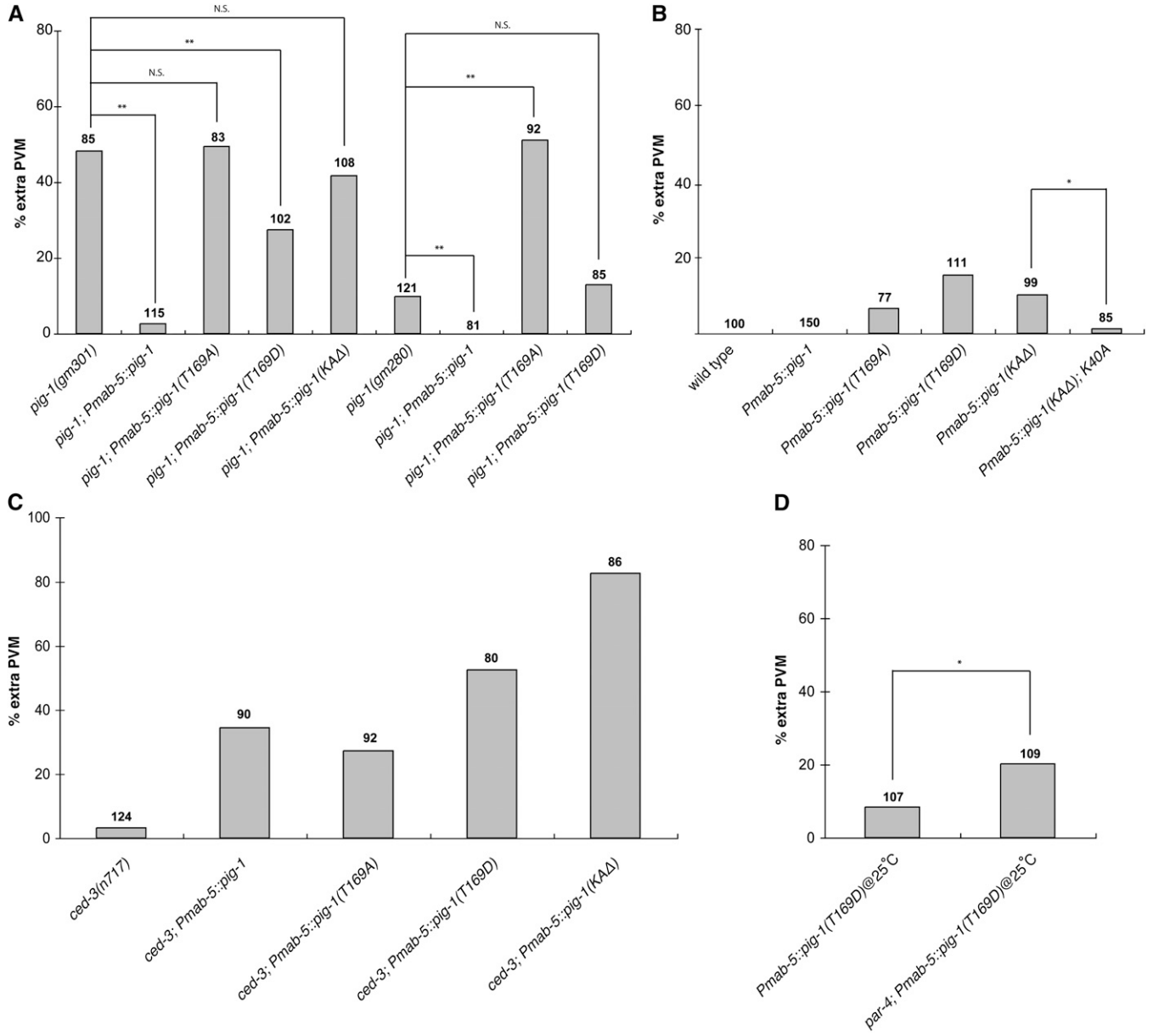

Figure 4 The conserved threonine residue in the activation loop and the kinase-associated 1 domain (KA1) of PIG-1 are essential for its activity. (A) A transgene expressing PIG-1(T169A) failed to rescue the extra neuron phenotype of the strong *pig-1* allele *gm301* and enhanced the extra neuron phenotype of the weak *pig-1* allele *gm280*. A transgene expressing PIG-1(T169D) partially rescued the extra neuron phenotype of *pig-1(gm301)*. A PIG-1 transgene lacking the KA1 domain [PIG-1(KAΔ)] failed to rescue the extra neuron phenotype of *pig-1(gm301)*. (B) Various PIG-1 transgenes induced extra neurons in a wild-type background. (C) A *ced-3* mutation enhanced the extra neuron phenotype of various PIG-1 transgenes. We generated three transgenic lines for PIG-1(T169A), three for PIG-1(T169D), three for PIG-1(KAΔ), and one for PIG-1(KAΔ); K40A. Each of the lines for a particular construct was tested and gave similar results. Data for only one line of each type are presented. Wild-type and mutant PIG-1 proteins were tagged with GFP. All four PIG-1 proteins were expressed at similar levels, so the failure of the PIG-1(T169A) or PIG-1(KAΔ) expression to rescue the extra neuron phenotype of a *pig-1* mutant does not result from inappropriate PIG-1 levels. (D) A mutation in *par-4* does not suppress the extra PVM phenotype in animals expressing PIG-1(T169D). Number of lineages scored for each genotype are provided. N.S., not significant; $^{**}P < 0.005$; $^{*}P < 0.05$ (Fisher's exact test).

a conserved threonine residue in their activation loops (Lizcano *et al.* 2004). While this conserved threonine is essential for MELK kinase activity, MELK autophosphorylates this residue *in vitro* (Lizcano *et al.* 2004; Beullens *et al.* 2005), suggesting that LKB1 does not regulate MELK kinase activity. To test whether this conserved threonine (T169) is equally essential for PIG-1 activity, we generated two variants by introducing point mutations into a transgene containing a *pig-1* cDNA: a nonphosphorylatable form, *pig-1(T169A)*, and a phosphomimetic form, *pig-1(T169D)*. We drove expression of these *pig-1* mutants from the *mab-5* promoter. As expected, when expressed from the *mab-5*

promoter, neither PIG-1(T169A) nor PIG-1(T169D) rescued the extra AVM phenotype of a *pig-1* mutant (data not shown). *Pmab-5*::*pig-1(T169A)*::*gfp* also failed to rescue the PVM phenotype of a *pig-1* mutant (Figure 4A), indicating that the threonine residue is important for PIG-1 activity.

We observed a partial but significant rescue of the extra PVM phenotype from transgenes expressing PIG-1(T169D) (Figure 4A). Partial rescue could result from PIG-1 activity that is not regulated properly. Consistent with this idea, PIG-1 (T169D) induced extra PVMs in the wild-type background (Figure 4B), and this phenotype was further enhanced in a *ced-3*-sensitized background (Figure 4C). Unlike the expression of the full-length PIG-1, PIG-1(T169D) also caused the daughter cells of Q.p to divide more symmetrically (Figure 2B). To our surprise, PIG-1(T169A) also produced an extra PVM phenotype in the wild-type background (Figure 4B) even when it showed no activity in our rescue assay (Figure 4A) and failed to alter the daughter cell size asymmetry of the Q.p division (Figure 2B). Given that excess expression of the full-length PIG-1 did not generate an extra neuron phenotype by itself (Figure 4B), the extra neuron phenotype caused by expression of PIG-1(T169A) suggests that it acts as a dominant negative. The observation that PIG-1(T169A) enhanced the extra neuron phenotype of the weak *pig-1* allele *gm280* (Figure 4A) supports this interpretation. By contrast, PIG-1(T169D) failed to enhance *pig-1(gm280)*. The differences in the behavior of the T169D and T169A transgenes in the strong and weak *pig-1* mutant backgrounds support the hypothesis that these changes result in distinct effects on PIG-1 function. As with *pig-1* loss, neither of the transgenes resulted in missing neurons.

Activated PIG-1 retains function in the absence of PAR-4

Because MELK autophosphorylates the conserved threonine that LKB1 phosphorylates in other AMPK family kinases, PAR-4 could regulate PIG-1 through residues other than the conserved threonine (T169). To test this possibility, we asked whether a *par-4* mutation can suppress the extra PVM phenotype in animals expressing PIG-1(T169D). The lack of suppression (Figure 4D) suggests that PAR-4 regulates PIG-1 through the conserved theronine residue (T169).

Kinase-associated 1 domain of PIG-1 is essential for its activity

The C-terminal kinase-associated 1 (KA1) domains of AMPK superfamily kinases can bind to membrane lipids (Moravcevic *et al.* 2010). In addition, the KA1 domains of these kinases have been implicated in auto-inhibition of their N-terminal kinase domain (Elbert *et al.* 2005). To address whether the KA1 domain of PIG-1 regulates its function, we generated a construct in which this domain was deleted from the full-length *pig-1* cDNA (KAΔ). PIG-1(KAΔ) expressed from the *mab-5* promoter failed to rescue the extra PVM phenotype of a *pig-1* mutant (Figure 4A), suggesting that its KA1 domain is essential for PIG-1 activity. PIG-1(KAΔ) also induced extra PVMs in a wild-type background, and this phenotype was largely abrogated when we mutated the key catalytic lysine residue in the kinase domain (KAΔ; K40A) (Figure 4B). These observations suggest that deregulated kinase activity is primarily responsible for the extra neuron phenotype in animals expressing PIG-1(KAΔ).

PIG-1 localizes to centrosomes during cell divisions of the Q lineage

Xenopus MELK (called pEg3) in cultured cells is normally broadly distributed in the cytoplasm during interphase, but a portion of the protein becomes enriched near the cortex during anaphase and telophase of mitosis (Chartrain *et al.* 2006). Furthermore, pEg3 has recently been reported to localize to the cleavage furrow in early *Xenopus* embryonic divisions (Le Page *et al.* 2011). We previously found PIG-1 to distribute broadly in the cytoplasm of nondividing cells without apparent membrane localization (Cordes *et al.* 2006). We wondered whether PIG-1 would display a different localization pattern during cell divisions and found that PIG-1::GFP localized to the centrosomes during cell divisions of the Q lineage (Figure 5A). To test whether the phosphorylation status of the threonine in the activation loop affects this localization, we examined the expression patterns of PIG-1(T169A)::GFP (data not shown) and PIG-1 (T169D)::GFP (Figure 5B) during Q lineage divisions. We found that both transgenes showed a similar expression pattern to wild-type PIG-1, suggesting that activation loop phosphorylation of PIG-1 is not important for its centrosomal localization. We obtained a similar result with PIG-1 (KAΔ) (data not shown). As *par-4* functions genetically in the same pathway as *pig-1*, we wondered whether PAR-4 localizes PIG-1 to the centrosomes. We found that *par-4* reduction had no effect on PIG-1 centrosomal localization (data not shown).

Discussion

C. elegans orthologs of LKB1, STRAD, and MO25 function with PIG-1/MELK to regulate the Q.p neuroblast division

The heterotrimeric complex composed of LKB1 kinase, STRAD pseudokinase, and MO25 adaptor is a conserved polarity regulator in epithelial cells and neurons (Jansen *et al.* 2009). We find that *C. elegans* orthologs of LKB1 (PAR-4), STRAD (STRD-1), and MO25 (MOP-25.2) regulate the Q.p neuroblast division. Many *C. elegans* neuroblasts, including the Q.p neuroblasts, divide to produce a larger neuronal precursor and a smaller cell that dies, but how this fate and size asymmetry are generated in these divisions is poorly understood. In the Q.p division, previous studies have shown that mutations in *pig-1*, which encodes a kinase orthologous to MELK, and *cnt-2*, which encodes a GTPase-activating protein (GAP) of Arf GTPases, result in daughter cells that are more equivalent in size and transform the fate

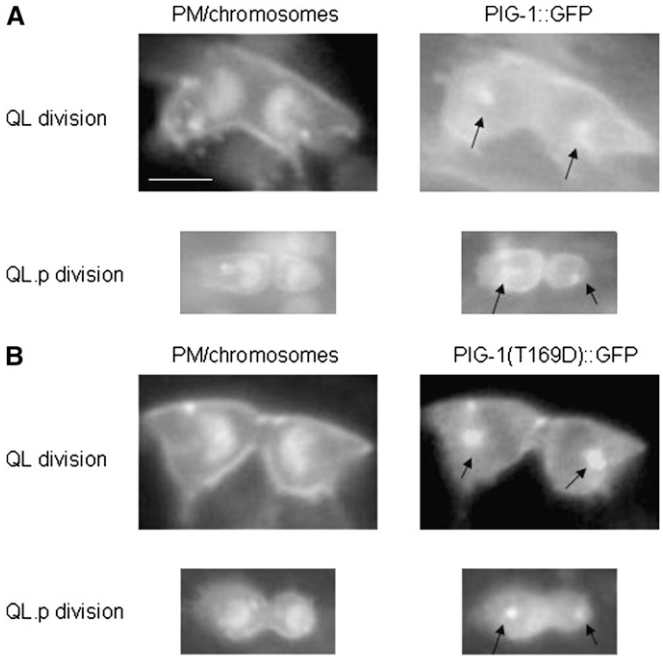

Figure 5 PIG-1 localizes to centrosomes during cell divisions of the Q lineage. (A) Representative fluorescence micrographs showing plasma membrane (PM) and chromosomes (left panels) and PIG-1::GFP (right panels) in QL or QL.p divisions from animals carrying *rdvIs1* and *gmIs88 [Pmab-5::pig-1::gfp]*. (B) Representative fluorescence micrographs showing PM and chromosomes (left panels) and PIG-1(T169D)::GFP (right panels) in QL or QL.p divisions from animals carrying *rdvIs1* and *gmIs87 [Pmab-5::pig-1(T169D)::gfp]*. Arrows indicate the centrosomes, which are judged by morphological criteria (Ou *et al.* 2010). For example, during the anaphase of Q.p divisions, chromosomes (labeled by *rdvIs1*) that are pulled apart appear to wrap around the centrioles (labeled by γ-tubulin::GFP) (Ou *et al.* 2010). The GFP signals from PIG-1::GFP or PIG-1(T169D)::GFP are consistent with their location near the centrioles, possibly in the pericentriolar material. Scale bar, 3 μm.

of the apoptotic cell to that of its sister, resulting in the production of extra neurons (Cordes *et al.* 2006; Singhvi *et al.* 2011). We found that the daughter cells of the Q.p neuroblast are more symmetric in size in *par-4* and *strd-1* mutants (Figure 2A). Furthermore, mutations in *par-4* and *strd-1* and RNAi against *mop-25.2* generated a significant extra neuron phenotype in a *ced-3*-sensitized background (Figure 1E). RNAi against *mop-25.2* did not have as strong a phenotype as mutations in *par-4* or *strd-1* in a *ced-3* background, possibly due to incomplete knockdown of *mop-25.2*. Alternatively, the other two *C. elegans* MO25 homologs, *mop-25.1* and *mop-25.3*, could provide overlapping functions with *mop-25.2*, although the inability of these two MO25 homologs to enhance *mop-25.2* in our triple RNAi experiment argues against this possibility. However, the lack of enhancement of the *mop-25.2* phenotype could also reflect ineffective RNAi. Yet another possibility is that MOP-25.2 facilitates but is not essential for PAR-4 activity. Our observations indicate that PAR-4, STRD-1, and MOP-25.2 are new regulators of the asymmetric division of Q.p.

Cell fate regulators PIG-1 and CNT-2 appear to act in the same pathway in the Q.p lineage (Cordes *et al.* 2006; Singhvi *et al.* 2011). Given that MELK, the vertebrate ortholog of PIG-1, belongs to the family of kinases (AMPK-related kinase family) that can be phosphorylated and activated by LKB1 (Lizcano *et al.* 2004), we asked whether PAR-4 and STRD-1 function in the same pathway as PIG-1. Consistent with this hypothesis, *par-4* and *strd-1* mutations enhanced the extra neuron phenotype of a weak but not a null *pig-1* mutant (Figure 3A). This hypothesis requires that all three genes—*pig-1*, *par-4*, and *strd-1*—act in the same cell, which is supported by our rescue experiments using the *mab-5* promoter (Figure 3C).

The effects of these mutations on other lineages also support the hypothesis that *pig-1*, *par-4*, and *strd-1* act together. For example, we showed that *pig-1* and *strd-1* regulate the PLM lineage (Figure 3B), suggesting that these genetic interactions are not specific for the Q.p lineage. Moreover, Denning *et al.* (2012) recently reported that *pig-1*, *par-4*, and *strd-1* act together to regulate the death of a subset of cells that die early in the *C. elegans* embryo. These cells die by a caspase-independent mechanism and are shed from the embryo in caspase-deficient mutants. Loss- or reduction-of-function of *pig-1*, *par-4*, *strd-1*, or the two *mop-25* homologs (*mop-25.1* and *mop-25.2*) suppresses the cell-shedding phenotype, supporting our hypothesis that these genes act together in the same pathway. The observation that the expression of cell adhesion molecules are affected in *pig-1* mutants led Denning *et al.* (2012) to propose that PIG-1 mediates cell shedding by regulating the expression of cell adhesion molecules in the shed cells. While the role of PIG-1 in cell adhesion can explain the effects of *pig-1* loss on shedding, we propose that this effect is a secondary consequence of the shed cells being transformed into their sisters in these mutants. This interpretation is consistent with the phenotypes observed in multiple cell divisions of *pig-1* mutants (Cordes *et al.* 2006; Ou *et al.* 2010; also see below).

Relationship between cell size and cell fate in the Q.p division

One of the best-studied systems for understanding the mechanisms of asymmetric cell division is the *C. elegans* one-cell embryo. In this division, the conserved PAR proteins control both the positioning of the spindle and the asymmetric localization of determinants to set up the anterior–posterior axis of the embryo (Goldstein and Macara 2007). The Q.p division is similar to the first embryonic division in that both divisions produce a larger anterior cell and a smaller posterior cell, an asymmetry generated by the posterior displacement of the mitotic spindle (Goldstein and Macara 2007; Ou *et al.* 2010).

In *C. elegans* neuroblast divisions, we and others (Hatzold and Conradt 2008; Singhvi *et al.* 2011) have noted a correlation between cell size and cell fate: the more symmetric the cell division, the more severe the extra neuron

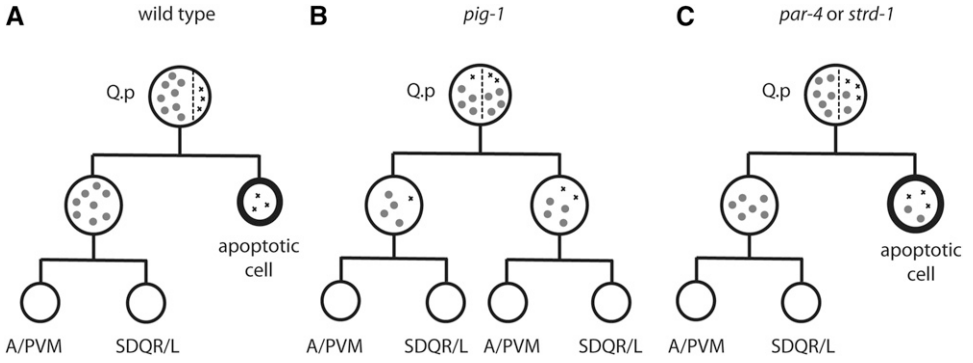

Figure 6 A model for how *pig-1*, *par-4*, and *strd-1* mutants regulate the Q.p lineage. (A) In wild-type animals, the Q.p neuroblast has a posteriorly displaced cleavage plane (vertical dashed line). We propose that anteriorly localized neuronal fate determinants (shaded circles) and posteriorly localized cell-death determinants ("x's") specify the fates of the Q.p daughters. The neuroblast divides to produce a large anterior daughter cell that inherits the neuronal fate determinants and becomes a precursor and a small posterior cell that inherits the death-fate determinants and dies. (B) In *pig-1* mutants, the neuroblast has a centrally localized cleavage plane and more uniformly distributed determinants. Both daughters of the neuroblast inherit the determinants, leading to the production of two neuronal precursors. (C) In *par-4* or *strd-1* mutants, the neuroblast has a centrally localized cleavage plane, but the determinants are still asymmetrically localized. This results in the apoptosis of the posterior daughter because it inherits enough cell-death determinants or because it does not inherit enough neuronal-fate determinants.

phenotype. However, the correlation is not perfect. In the Q.p lineage, for example, *pig-1* mutations result in a higher penetrance of extra neurons than *par-4* or *strd-1* mutations, even though *pig-1* and *strd-1* mutations cause similar cell-size asymmetry defects. One possible explanation for this discrepancy is that while all three genes regulate the asymmetric positioning of the mitotic spindle, *pig-1* functions in a *par-4/strd-1*-independent manner to cause asymmetric segregation of neuronal fate determinants to one of the daughter cells (Figure 6) (Cordes *et al.* 2006). In this model, both the posterior position of the spindle and the segregation of neuronal and death fate determinants regulate the asymmetry of the Q.p division (Figure 6A). The loss of PIG-1 function results in a neuroblast with a centrally positioned spindle and a more uniform distribution of neuronal fate determinants, leading to the production of two neuronal precursors (Figure 6B). Loss of PIG-1, however, does not eliminate all asymmetry since the posterior cell often dies in *pig-1* mutants. The model proposes that a loss of PAR-4 or STRD-1 function causes the neuroblast to divide more symmetrically, but the determinants are still more or less properly segregated (Figure 6C). In this case, the posterior daughter undergoes apoptosis because it still inherits determinants that specify the apoptotic fate or it does not inherit enough neuronal fate determinants.

There are other possible explanations why the extra neuron and cell size phenotypes of the *par-4* mutants are weaker than those of *pig-1* mutants. If PIG-1 is a PAR-4 target, additional kinases could act in parallel to PAR-4 to activate PIG-1. Another possibility is that PAR-4 is essential for PIG-1 function but that the temperature-sensitive *par-4* mutation only reduces *par-4* activity, failing to reveal a more prominent role in the Q.p division.

The cell-size and cell-fate phenotypes of the mutants suggest that STRD-1 may have PAR-4-independent functions in regulating spindle positioning: *strd-1* has a more severe cell-size asymmetry defect than *par-4*, yet *strd-1*; *ced-3* and *ced-3*; *par-4* display comparable extra neuron phenotypes. Recent reports have found that STRAD functions with kinases other than LKB1 to regulate cell polarity in *C. elegans* and mammalian cells (Kim *et al.* 2010; Eggers *et al.* 2012).

LKB1 and AMPK-related kinases play essential roles in polarity. The asymmetric localization of the AMPK family member PAR-1 requires PAR-4, consistent with the hypothesis that PAR-4 regulates PAR-1 activity, and this regulation is likely to be direct as PAR-4 promotes PAR-1 phosphorylation (Narbonne *et al.* 2010). SAD-1 is another AMPK-related kinase that regulates polarity (Crump *et al.* 2001; Hung *et al.* 2007), and both SAD-1 and PAR-4 regulate neuronal polarity (Kim *et al.* 2010). While LKB1 has been linked to the regulation of energy metabolism through activation of AMPK (Steinberg and Kemp 2009), these kinases can nevertheless regulate polarity: LKB1 activates AMPK under starvation conditions to polarize single epithelial cells in culture (Lee *et al.* 2007), and *Drosophila* AMPK null mutants display defects in epithelial and neuroblast polarity (Lee *et al.* 2007; Mirouse *et al.* 2007; Andersen *et al.* 2012). While these members of the AMPK family can function with LKB1 to regulate polarity, our genetic experiments only implicate PAR-1 in the Q.p division. PIG-1 and PAR-1 may function together with PAR-4 in this asymmetric division. It is noteworthy that *pig-1* has recently been shown to significantly enhance the embryonic lethal phenotype of *par-1* mutants (Morton *et al.* 2012). Our results and those of Morton *et al.* suggest that PIG-1 and PAR-1 could act together in multiple asymmetric divisions.

Regulation of PIG-1 activity

LKB1 activates AMPK family kinases by phosphorylating a conserved threonine residue in their activation loops (Lizcano *et al.* 2004). By contrast, MELK autophosphorylates this residue (Beullens *et al.* 2005), but, like the other AMPK family kinases, this threonine is essential for kinase activity (Lizcano *et al.* 2004; Beullens *et al.* 2005). Our rescue experiments suggest that phosphorylation of this conserved threonine (T169) is essential for PIG-1's function in the Q.p division. The importance of this residue in PIG-1 was

recently highlighted in caspase-independent cell deaths that occur in the embryo (Denning *et al.* 2012). The observation that a mutation in *par-4* failed to suppress the extra neuron phenotype in PIG-1(T169D) suggests that this transgene still retains function in the absence of PAR-4. This threonine could be autophosphorylated as it is in MELK, or it could be phosphorylated by PAR-4 similar to the phosphorylation of AMPK family members by LKB1. Our genetic results, however, argue against PAR-4 functioning downstream of PIG-1 as in the relationship between *Drosophila* LKB1 and PAR-1 in oocyte polarity (Martin and St. Johnston 2003).

Our transgene experiments suggest that PIG-1(T169A) acts as a dominant negative and that PIG-1(T169D) possesses deregulated activity. PIG-1(T169A) might sequester a limiting factor needed for PIG-1 activation. How PIG-1 (T169D) functions is unclear. The phenotype could be caused by excess PIG-1 activity or the inappropriate temporal or spatial regulation of PIG-1 activity. In either case, the effect is the same as removing PIG-1 function: a disruption of Q.p polarity and the production of extra neurons.

Excess full-length PIG-1 did not generate an extra neuron phenotype, suggesting that PIG-1's activity is normally regulated. Indeed, MELK is intricately regulated: the C-terminal kinase-associated 1 domain can bind to its N-terminal kinase domain, and this interaction may affect both the kinase activity and the localization of the protein (Beullens *et al.* 2005; Chartrain *et al.* 2006). Removal of the C terminus enhances the kinase activity of MELK *in vitro* (Beullens *et al.* 2005). A similar auto-inhibitory mechanism was observed in the yeast PAR-1 homologs Kin1p and Kin2p (Elbert *et al.* 2005). It has also recently been reported that the conserved basic residues in the kinase-associated 1 domain (KA1) of AMPK family kinases, including MELK, can bind to anionic phospholipids in yeast cells (Moravcevic *et al.* 2010), but the physiological relevance of this interaction remains to be elucidated. *Xenopus* MELK (called pEg3) in cultured cells is broadly distributed in the cytoplasm during interphase, but a portion of the protein becomes enriched near the cortex during anaphase and telophase of mitosis (Chartrain *et al.* 2006). Deletion of the pEg3 N terminus causes the C terminus to localize to the cell periphery independently of cell cycle progression, indicating that the N terminus plays an inhibitory role in pEg3 cortical localization (Chartrain *et al.* 2006). An emerging model from these observations proposes that the mutual inhibition between the N-terminal kinase domain and the C-terminal KA1 domain keeps MELK in an inactive state until some yet-unidentified upstream signals relieve its auto-inhibition. Consistent with this idea, the "open" form of pEg3 has recently been reported to be at the cleavage furrow in early *Xenopus* embryonic divisions (Le Page *et al.* 2011).

We could not tell with certainty whether PIG-1 localizes transiently to the membrane or cleavage furrow during cell divisions. However, we reliably detected both wild-type and the activated form of PIG-1 at the centrosomes (Figure 5), an expression pattern that was not previously reported for its homologs. This localization does not require PAR-4, nor does it require activation loop phosphorylation or the KA1 domain of PIG-1. This intriguing expression pattern could be relevant to the two proposed roles for PIG-1 in asymmetric cell division, namely the positioning of the spindle and the segregation of cell fate determinants (Figure 6) (Cordes *et al.* 2006). As structures that nucleate microtubules, centrosomes can affect spindle positioning. A number of studies also suggest that centrosomes can specify cell fates (reviewed in Knoblich 2010). In the asymmetric cell division of budding yeast, for example, the old centriole (called the spindle pole body) always segregates with the mother cell while the newly born centriole goes to the bud cell. Given that PIG-1 localizes to both centrosomes (instead of being asymmetrically localized to one of them) in the Q.p division, it remains to be determined how this localization contributes to this asymmetric division. Members of the AMPK kinase family such as AMPK and MARK4 have been shown to localize to centrosomes, although their function there remains elusive (Trinczek *et al.* 2004; Vazquez-Martin *et al.* 2009). It is noteworthy that CDC25B, a substrate of MELK and a phosphatase that promotes G2/M progression by removing inhibitory phosphate groups from the cyclin-dependent kinase CDC2, localizes to centrosomes (Mirey *et al.* 2005). It is possible that PIG-1 regulates *C. elegans* homologs of CDC25B in the Q lineage.

To address whether the KA1 domain of PIG-1 is equally essential for its function, we generated PIG-1 transgenes lacking the KA1 domain (KAΔ). We found that the transgenes failed to rescue a *pig-1* mutant, suggesting that the KA1 domain is indispensable for PIG-1 function in the Q.p division. These transgenes also induced extra neurons in the wild-type background, an effect that requires the kinase activity of PIG-1. By contrast, the KA1 domain of PAR-1 (a PIG-1 related kinase) is not essential for PAR-1 activity (Motegi *et al.* 2011). It has been proposed that the ability for PAR-1 to form gradients both at the cortex and in the cytoplasm is important for establishing asymmetry of cell-fate determinants in the one-cell *C. elegans* embryo (Griffin *et al.* 2011). We found PIG-1 to localize strongly to the centrosomes and weakly in the cytoplasm of dividing cells, so it remains to be determined whether PIG-1 has functions in these locations.

The structure–function analysis presented above indicates that both the N-terminal kinase domain and the C-terminal KA1 domain of PIG-1 are important and regulate its activity. Identification of PIG-1 targets will be essential not only for understanding how PIG-1 regulates asymmetric cell division, but also for helping us to better understand how PIG-1's activity is regulated. Both the MAP kinase ASK1 and Smads have been shown to be targets of murine MELK (Jung *et al.* 2008; Seong *et al.* 2010). Perhaps the *C. elegans* homologs of these molecules or new molecules identified in our genetic screens will provide insights into PIG-1 function. In conclusion, our observation that PAR-4 and PIG-1 function together in *C. elegans* asymmetric cell division

suggests that LKB1 and MELK could regulate stem cell divisions in vertebrates. Changes in cell fate may underlie many of the abnormal behaviors exhibited by cells after loss of PAR-4 or LKB1.

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