Differential requirements for STRAD in LKB1-dependent functions in C. elegans

Patrick Narbonne¹, Vincent Hyenne², Shaolin Li¹, Jean-Claude Labbé²,³ and Richard Roy¹,*

SUMMARY
The protein kinase LKB1 is a crucial regulator of cell growth/proliferation and cell polarity and is the causative gene in the cancer-predisposing disease Peutz-Jeghers syndrome (PJS). The activity of LKB1 is greatly enhanced following its association with the Ste20-like adapter protein STRAD. Unlike LKB1 however, mutations in STRAD have not been identified in PJS patients and thus, the key tumour suppressive role(s) of LKB1 might be STRAD independent. Here, we report that Caenorhabditis elegans strd-1/STRAD mutants recapitulate many phenotypes typical of par-4/LKB1 loss of function, showing defects during early embryonic and dauer development. Interestingly, although the growth/proliferation defects in severe par-4 and strd-1 mutant dauers are comparable, strd-1 mutant embryos do not share the polarity defects of par-4 embryos. We demonstrate that most of par-4-dependent regulation of germline stem cell (GSC) quiescence occurs through AMPK, whereby PAR-4 requires STRD-1 to phosphorylate and activate AMPK. Consistent with this, even though AMPK plays a major role in the regulation of cell proliferation, like strd-1 it does not affect embryonic polarity. Instead, we found that the PAR-4-mediated phosphorylation of polarity regulators such as PAR-1 and MEX-5 in the early embryo occurs in the absence of STRD-1. Thus, PAR-4 requires STRD-1 to phosphorylate AMPK to regulate cell growth/proliferation under reduced insulin signalling conditions, whereas PAR-4 can promote phosphorylation of key proteins, including PAR-1 and MEX-5, to specify early embryonic polarity independently of STRD-1. Our results therefore identify a key strd-1/STRAD-independent function of par-4/LKB1 in polarity establishment that is likely to be important for tumour suppression in humans.

KEY WORDS: AMPK, LKB1, PAR-1, Peutz-Jeghers syndrome, Polarity, STRAD, C. elegans

INTRODUCTION
Peutz-Jeghers syndrome (PJS) is an autosomal dominant disorder that predisposes affected individuals to tumour formation in several organs (Giardiello et al., 1987; Jeghers et al., 1949; Peutz, 1921; Spigelman et al., 1989). Mutations within the LKB1 locus (also known as STK11) have been found associated with this disease (Hemminki et al., 1998; Jenne et al., 1998), although the role of this gene in the aetiology of PJS is still not clear. LKB1 encodes a highly conserved serine/threonine kinase, and nearly all mutations in LKB1 that have been identified in PJS patients are predicted to disrupt its kinase activity (Alessi et al., 2006; Launonen, 2005). Thus, LKB1 is likely to act as a tumour suppressor through phosphorylating one or several target proteins.

LKB1 phosphorylates and activates AMPK (Hawley et al., 2003; Woods et al., 2003), as well as 12 other kinases of the AMPK family in vitro (Lizcano et al., 2004). Some of these LKB1 targets have been found to regulate cell growth/proliferation and/or cell polarity in various model systems. Indeed, AMPK was initially characterised as a master cellular metabolic regulator (Hardie, 2007), which also affects growth by phosphorylating TSC2 and consequently inhibiting the TOR (target of rapamycin) pathway in response to energetic stress (Inoki et al., 2003). Interestingly, the loss of AMPK activity also results in polarity defects during energy-stress conditions in Drosophila epithelia (Lee et al., 2007; Mirouse et al., 2007). Likewise, the MARK kinase PAR-1 requires LKB1-dependent phosphorylation to regulate cell polarity (Alessi et al., 2006; Martin and St Johnston, 2003; Wang et al., 2007), and PAR-1 also regulates growth/metabolism (Bessone et al., 1999; Hurov and Piwnica-Worms, 2007). The LKB1-dependent activation of SAD-A/B kinases (also known as BRSK1 and BRSK2), conversely, induces polarization and axonal outgrowth in undifferentiated neurites (Kishi et al., 2005; Shelly et al., 2007). LKB1 also phosphorylates the tumour suppressor dual phosphatase PTEN in vitro (Mehenni et al., 2005), although the effect of this modification on PTEN activity has not been well characterised and PTEN affects cell growth/proliferation and cell polarity in many organisms (Brazil et al., 2004; Lee et al., 2001; Martin-Belmonte et al., 2007; von Stein et al., 2005).

The tumour suppressor function of LKB1 might thus be conferred through its ability to negatively regulate cell growth/proliferation and positively regulate polarity establishment/maintenance by phosphorylating one or several of these downstream targets. Interestingly, mutations in PTEN also result in predisposition to cancer and an increased frequency of hamartomatous tumours: multiple benign growths that are also common in patients with tuberous sclerosis and PJS (Wirtzfeld et al., 2001). Thus, the aetiology of the related cancer-predisposing syndromes resulting from germline mutations in PTEN, TSC1/2 or LKB1 might be related to their common functions in regulating cell growth/proliferation and polarity in potentially overlapping pathways.

The kinase activity and subcellular localization of LKB1 are modified upon its association with two cofactors, a Ste20-related adapter protein called STRAD and MO25, which together form the highly active heterotrimeric complex (Baas et al., 2003; Boudeau et al., 2003; Boudeau et al., 2004; Hawley et al., 2003). Surprisingly, however, mutations in STRAD or MO25 are not associated with any
predisposition to cancer or PJS (Alhropou et al., 2005; de Leng et al., 2005). There are two isoforms of STRAD (α and β) in humans, both of which can enhance LKB1 activity (Boudeau et al., 2003) and thus contribute to the suppression of GSC quiescence. Alternatively, the functions of LKB1 in tumour suppression could be independent of STRAD. Consistent with this, the purified LKB1 kinase can still phosphorylate protein targets in the absence of both STRAD and MO25, albeit with less efficiency (Baas et al., 2003; Boudeau et al., 2003).

In Caenorhabditis elegans, the LKB1 orthologue par-4 regulates both cell growth/proliferation and cell polarity and is essential for appropriate partitioning of developmental determinants during the first cell division in the zygote (Kemphues et al., 1988; Watts et al., 2000). Its precise role in establishing the anterior and posterior domains of the first asymmetric division is still unclear, but par-4 mutants demonstrate defects in segregating P granules, ribonucleoprotein complexes that function as germ line determinants, while also affecting division timing in the second zygotic cell division (Morton et al., 1992). In addition to this role in early axis specification in C. elegans, par-4/LKB1 induces quiescence in the GSC population in response to environmental stress, partially through activating AMPK (Narbonne and Roy, 2006). Indeed, mutations that affect either AMPK or LKB1, as well as PTEN signalling, cause germline hyperplasia in the dauer larva, a long-lived juvenile stage that renders animals highly resistant to harsh environmental conditions while maintaining them in a developmentally quiescent state (Narbonne and Roy, 2006).

To better understand how the LKB1 signalling cascade ultimately regulates cell proliferation in C. elegans, we combined forward and reverse genetic strategies to isolate and characterise new genes that regulate GSC quiescence during dauer development. We describe here the roles of strd-1, aak-1/2 and aakb-1/2, the C. elegans orthologues of STRADα (there is no clear homologue of STRADβ in C. elegans), and of the two AMPK α-catalytic and β-regulatory subunit isoforms, respectively, in the regulation of GSC quiescence and early embryonic development. We show that strd-1 cooperates with par-4 to ensure GSC quiescence during environmental stress, while also affecting cortical integrity during early embryonic development, indicating that PAR-4 and STRD-1 are implicated in both developmental processes. However, we show that the requirement for strd-1 differs in each situation. Indeed, although the severity of the GSC proliferation defects of par-4 and strd-1 mutants are similar, the establishment of the polarity axis in the zygote and viability of the embryos are essentially not compromised in strd-1 mutants. Thus, our results together suggest that PAR-4 requires STRD-1 to activate AMPK and induce GSC quiescence under nutrient stress, whereas the essential function of PAR-4 that consists in activating PAR-1 and other targets to regulate embryonic polarity, is largely independent of STRD-1. The latter strd-1/STRAD-independent function of par-4/LKB1 is likely to be crucial for tumour suppression in humans.

**MATERIALS AND METHODS**

*C. elegans* genetics

Nematodes were grown using standard procedures on NGM (Brenner, 1974) or MYOB plates (Church et al., 1995), fed E. coli of the strains OP50 or HB101 and maintained at 15°C unless otherwise mentioned. The Bristol N2 strain was used as wild type.

The following alleles, transgenes and rearrangements were used: LG III: daf-2(e1370), qsl-56[lag-2::GFP, unc-119(+)j] animals were mutagenized with EMS and screened for mutants with dauer germline hyperplasia (Narbonne and Roy, 2006). Three alleles were isolated (rr88, rr91, rr92) and out-crossed four times prior to subsequent analysis. rr88, rr91 and rr92 all showed moderate linkage to daf-2 (9.1 M.U.) on LGII. Complementation analysis indicated that rr91 and rr92 were allelic. Linkage analysis was used to map rr88 and rr91 between unc-69 (+2.3 M.U.) and dpy-18 (+8.7 M.U.) on LGII with rr91 to the left of rr88.

Within this interval, we identified two strong candidates. Among these, aakb-2 (Y47D3A.15) at +7.6 M.U. on LGII, which is a *C. elegans* AMP-activated kinase beta regulatory subunit homologue. We identified a single base pair substitution (GC to AT) at position 1 of the predicted codon 88 in rr88, thereby introducing a premature stop codon (Narbonne and Roy, 2009). We cloned a segment of genomic DNA from wild-type animals containing the aakb-2 gene flanked by ~850 bp upstream and 2 kb downstream sequence, and micro-injected it (20 ng/ul) together with pRF4[rol-6(sa1006)] (Mello and Fire, 1995) into daf-2(rr88) mutants. Three independent lines were obtained, in each of which the dauer germline hyperplasia of rr88 mutant dauer animals was partially rescued (data not shown). As repetitive transgenes are generally silenced in the *C. elegans* germ line (Kelly et al., 1997), complete transgenic rescue was not expected.

Between unc-69 and dpy-18 and to the left of aakb-2, located at +5.75 M.U., resides strd-1, the closest *C. elegans* homologue of the Ste20-related adapter protein (STRAD) α, a cofactor for optimal LKB1 activity in mammalian cells (Baas et al., 2003; Boudeau et al., 2003; Hawley et al., 2003). As par-4/LKB1 is involved in the regulation of dauer-dependent GSC quiescence (Narbonne and Roy, 2006), we performed RT-PCR of strd-1 for both alleles, which were found to carry typical EMS-induced GC to AT substitutions (see Fig. S1 in the supplementary material) (Narbonne and Roy, 2009). To test whether the impairment of strd-1 causes dauer germline hyperplasia, we performed strd-1(RNAi) on daf-2 mutants. This indeed consistently caused germline hyperplasia, which was, however, less severe than in the rr91 or rr92 mutants (data not shown), probably owing to a weak strd-1(RNAi) response.

**Mapping and cloning of rr88, rr91, rr92 and r111**

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**rr111** was isolated from a similar additional screen that used a daf-7(e1372); qsl-56[lag-2::GFP, unc-119(+)j] background. Moderate linkage to daf-7 was found and both aakb-2 and strd-1 were directly sequenced. aakb-2 was found to be of wild-type sequence, but strd-1 carried a GC to AT substitution at position 1 of the donor splice site of intron 1 that resulted in cryptic mRNA splicing (see Fig. S1 in the supplementary material).

**RNA interference**

Double-stranded RNA was either synthesized from pJH573 (Fire et al., 1998) and injected into animals or RNAi was induced by feeding animals on individual recombinant bacterial clones following IPTG induction of dsRNA (Fraser et al., 2000).

**Antibody production and immunoprecipitation**

To generate anti-STRAD-1 antibodies, the strd-1(rr91) C. elegans strain was used as wild type. Polyclonal antibodies recognizing the GST::STRAD-1 fusion protein were produced in rabbits and the antisera were affinity purified and used at a dilution of 1:500 for immunoblotting or 1:25 for immunofluorescence.

To generate anti-PAR-4 antibodies, the full-length par-4 cDNA was amplified by PCR, cloned into plasmids pDEST15 and pDEST17 (Invitrogen) to produce GST- and His-tagged proteins, respectively. Polyclonal antibodies against GST-PAR-4 were produced in two rats (Covance) and antisera were affinity purified using His-PAR-4. The antibodies were used 1:25 in immunofluorescence.

Immunoprecipitation was performed as previously described (Liao et al., 2004).
Western blot analysis
Worm samples were mixed with 5× sample buffer (250 mM Tris pH 6.8, 500 mM DTT, 10% SDS, 0.5% BBP, 50% glycerol) and lysed by five cycles of –80°C freeze/100°C thaw. Lysates were electrophoresed on a 10% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was incubated either with rabbit polyclonal anti-STRD-1, rabbit polyclonal anti-PAR-1 (Guo and Kemphues, 1995), rabbit monoclonal anti-AMPK (Cell signalling technology), mouse anti-α-tubulin (Sigma), rabbit polyclonal anti-P-PAR-1 (Wang et al., 2007), or rabbit polyclonal anti-P-MEX-5 (Tenlen et al., 2008) primary antibodies. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies were used.

Microscopy
For the visualization of early embryonic development in live specimens, embryos were obtained from gravid hermaphrodites and mounted on a coverslip coated with 1% poly-L-lysine in 2 μL of egg buffer (Edgar, 1995). Time-lapse images were acquired by a Zeiss HRM camera mounted on a Zeiss Axioimager Z1 microscope, and the acquisition system was controlled by Zeiss Axiovision software (Carl Zeiss Canada). Images were acquired at 10-second intervals using a Plan Apochromat 63×/1.4 NA objective.

For immunofluorescence analysis, embryos were fixed in methanol and stained as previously described (Labbé et al., 2004). Embryos were stained for P granules (mouse OIC1D4 1:300, Developmental Studies Hybridoma Bank, University of Iowa, USA), and PAR-6 protein [rabbit anti-PAR-6 1:150 (Hyenne et al., 2008)]. Secondary antibodies were Alexa546-coupled anti-rabbit or anti-mouse secondary antibodies were used.

RESULTS

AMPK requirements for dauer-dependent GSC quiescence
In a genetic screen designed to identify factors required for GSC quiescence during dauer development in C. elegans, we previously recovered a dominant-negative allele of the catalytic α2 subunit of AMPK (aak-2) (Narbonne and Roy, 2006). RNAi of the other AMPK catalytic α1 subunit aak-1 revealed that both subunits function additively to regulate GSC quiescence. Similar dauer germine hyperplasia phenotypes were also observed in mutants of the C. elegans orthologues of the tumour suppressors PTEN (daf-18) and LKB1 (par-4) (Narbonne and Roy, 2006). We extended the screen to identify further loci that affect GSC quiescence. Two independent loci were identified on linkage group (LG) III that gave rise to par-4-like germine hyperplasia in dauer animals (Fig. 1). One of these alleles (rr88) affects a C. elegans orthologue of an AMPK regulatory β-subunit, which we hereafter refer to as aakb-2 (see Fig. S1 in the supplementary material). The mutant allele is predicted to produce a truncated protein that lacks part of the conserved glycogen-binding domain and the carboxyl-terminal domain deemed critical for association with the other AMPK subunits (Iseli et al., 2005; Polekhina et al., 2005). The molecular nature and the determination of the cell/nuclei counts in the hyperplastic germ lines of aakb-2(rr88) mutants indicate that this mutation is a potent null allele. That is, aakb-2(RNAi) did not enhance hyperplasia of the germ line of aakb-2(rr88) mutant dauers (Table 1; data not shown).

A recently generated deletion allele of aakb-1(tm2658) that is predicted to disrupt part of the glycogen-binding domain (see Fig. S1 in the supplementary material, which allows us to analyze the phenotype of aakb-1; aakb-2 double mutants, in which both of the C. elegans AMPK β-subunits are impaired. These animals showed a dauer germine hyperplasia phenotype that was slightly more pronounced than that of aakb-2(rr88) animals and very similar (P=0.38) to that of aak-1; aak-2 double mutants that are catalytically null for AMPK (Table 1). This suggests that the AMPK catalytic subunits cannot phosphorylate their relevant downstream effectors to establish GSC quiescence in the absence of a functional β-subunit. Furthermore, our data indicate that aakb-2 is the main β-subunit that is required to inhibit GSC proliferation during dauer development, whereas aakb-1 only has an accessory, yet non-negligible, role in this regulation (Table 1).

par-4 requires strd-1 to establish GSC quiescence
In addition to aakb-2(rr88), we identified three other non-complementing alleles also located on LGIII that disrupt the establishment of GSC quiescence during dauer development. These alleles affect the C. elegans orthologue of STRADα (strd-1), a component of the heterotrimeric LKB1 kinase complex that greatly enhances LKB1 activity in vitro (Baas et al., 2003). All of these strd-1 alleles (rr91, rr92 and rr111) are recessive loss-of-function mutations that cause germine hyperplasia in dauer larvae, a phenotype reminiscent of par-4/LKB1 mutants (Fig. 1; Table 1). strd-1(rr92) is a typical EMS-induced (G727A) transition that affects the region adjacent to the highly conserved pseudokinase domain, whereas in strd-1(rr91), a similar transition (C964T) introduces a premature stop codon that disrupts the two last exons of the protein (see Fig. S1 in the supplementary material). Finally, a third allele, strd-1(rr111), contains a mutation at the donor splice site of intron 1 (GT to AT) (see Fig. S1 in the supplementary material). This results in the production of cryptically spliced mRNA variants, the most abundant of which...
lacks the last 13 base pairs of exon 1, causing a frameshift and a premature stop codon, thus disrupting the production of STRD-1 protein (see Fig. S1 in the supplementary material; data not shown).

Based on the degree of hyperplasia observed in each of the mutants, strd-1(rr91) and strd-1(rr111) are similar and considerably more severe than strd-1(rr92) (Fig. 1; Table 1). Two recently generated strd-1 deletion alleles, (ok2275) and (ok2283), allowed us to confirm the null phenotype of strd-1. Both of these deletions are predicted to produce similarly truncated proteins that end after exon 3, in which most of the conserved pseudokinase domain is deleted (see Fig. S1 in the supplementary material). Correspondingly, the dauer germline hyperplasia of strd-1(ok2275) and strd-1(ok2283) is similar and also statistically indistinguishable from that of strd-1(rr91) or strd-1(rr111), thus suggesting that strd-1(rr91, rr111) mutations are very strongly hypomorphic and potentially null (Table 1).

If par-4 requires strd-1 to induce GSC quiescence, it would be expected that par-4- and strd-1-null mutants should show a similar degree of hyperplasia, and that reducing par-4 activity in a strd-1-null mutant background would not further enhance the observed dauer germline hyperplasia. Consistent with this, the germline hyperplasia typical of strd-1-null mutant dauer larvae is similar and slightly more severe than that of par-4(it57) strong hypomorphic mutant animals, and is also comparable to that of strd-1(rr92), par-4(it57) double mutants (Table 1). strd-1(rr91); par-4(it57) double mutants die during embryogenesis, even at the permissive temperature (15°C) for par-4(it57), and therefore we did not evaluate germline hyperplasia in these animals. Our data nonetheless suggest that the presumptive null germline phenotypes of strd-1 and par-4 are similar, and are likely to resemble that of strd-1(rr91, rr111, ok2275, ok2283) or strd-1(rr92); par-4(it57) animals. strd-1 and par-4 therefore participate in the same genetic pathway, wherein PAR-4 requires the presence of STRD-1 to appropriately establish GSC quiescence during dauer development.

### Table 1. Success of embryonic development and germline proliferation during dauer formation

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>% maternal effect embryonic lethality † (n)</th>
<th>Number of germ cells in dauer‡ (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0 (440)</td>
<td>31.4±4.3 (25)</td>
</tr>
<tr>
<td>aak-1(tm1944)</td>
<td>0.3 (326)</td>
<td>61.4±9.2 (25)</td>
</tr>
<tr>
<td>aak-2(k524)</td>
<td>0.8 (517)</td>
<td>60.2±7.2 (25)</td>
</tr>
<tr>
<td>aak-1(tm1944); aak-2(ok524)</td>
<td>0.4 (230)</td>
<td>182.1±18.5 (20)</td>
</tr>
<tr>
<td>aakb-1(tm2685)</td>
<td>0.2 (569)</td>
<td>36.5±4.2 (25)</td>
</tr>
<tr>
<td>aakb-2(rr88)</td>
<td>1.3 (446)</td>
<td>150.0±29.9 (25)</td>
</tr>
<tr>
<td>aakb-2(rr88); aakb-1(tm2685)</td>
<td>1.0 (303)</td>
<td>183.9±18.4 (20)</td>
</tr>
<tr>
<td>par-4(it57ts)</td>
<td>4.1 (1076)</td>
<td>128.1±13.0 (25)</td>
</tr>
<tr>
<td>aak-1(tm1944); par-4(it57ts); aak-2(ok524)</td>
<td>2.8 (252)</td>
<td>240.0±15.0 (5)</td>
</tr>
<tr>
<td>par-1(1z310ts)</td>
<td>0.5 (418)</td>
<td>32.3±4.3 (25)</td>
</tr>
<tr>
<td>par-1(b274)</td>
<td>1.0 (307)</td>
<td>22.0±3.2 (25)</td>
</tr>
<tr>
<td>strd-1(rr91)**</td>
<td>7.1 (434)</td>
<td>148.0±24.7 (45)</td>
</tr>
<tr>
<td>strd-1(rr92)</td>
<td>1.1 (523)</td>
<td>86.4±13.4 (25)</td>
</tr>
<tr>
<td>strd-1(rr111)</td>
<td>2.2 (504)</td>
<td>139.3±20.4 (15)</td>
</tr>
<tr>
<td>strd-1(ok2275)‡</td>
<td>1.4 (214)</td>
<td>153.6±20.1 (25)</td>
</tr>
<tr>
<td>strd-1(ok2283)**</td>
<td>2.5 (395)</td>
<td>153.5±26.8 (45)</td>
</tr>
<tr>
<td>strd-1(rr91); par-4(it57ts)</td>
<td>100 (807)</td>
<td>ND</td>
</tr>
<tr>
<td>strd-1(rr92); par-4(it57ts)</td>
<td>84.4 (334)</td>
<td>149.6±20.6 (20)</td>
</tr>
<tr>
<td>strd-1(rr91); par-1(1z310ts)</td>
<td>90.9 (419)</td>
<td>128.1±13.3 (7)</td>
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<tr>
<td>strd-1(rr92); par-1(1z310ts)</td>
<td>21.8 (591)</td>
<td>81.0±11.0 (25)</td>
</tr>
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</table>

*Genotype includes daf-2(e1370ts) in all strains. †For data at 25°C, animals were upshifted at the L4 stage and eggs that were laid within the next 48 hours were scored.

**The maternal effect embryonic lethality of strd-1(rr91) and strd-1(ok2283) was also verified in a daf-2(e1) background: 7.0% (n=329) and 2.9% (n=105) at 15°C versus 9.5% (n=654) and 22.7% (n=440) at 25°C, respectively. ††Complete genotype includes daf-2(e1370ts) and aak-1(tm1944); par-4(it57ts); aak-2(ok524).
In C. elegans, there are two catalytic AMPK subunits (aak-1 and aak-2) that act additively to inhibit GSC proliferation during dauer development downstream of par-4 (Narbonne and Roy, 2006) (Fig. 1; Table 1). A deletion allele of the aak-1 gene (tm1944) allowed us to study the effect of the removal of all AMPK catalytic activity during C. elegans early embryonic development. Unlike in mammals or Drosophila, aak-1; aak-2 AMPK catalytic null (as well as aakb-1; aakb-2 double mutants) animals are viable and fertile, even in conditions of reduced insulin-like signaling, or following starvation (Table 1; data not shown). Furthermore, none of the AMPK mutant embryos, or any combinations thereof, showed defects in the segregation of P granules (data not shown). Moreover, mutations in the AMPK α catalytic subunits, which have highly penetrant effects on GSC quiescence, do not enhance the embryonic lethality of a temperature-sensitive par-4 loss of function at the permissive temperature (Table 1). We therefore conclude that par-4/LKB1 does not establish embryonic polarity through aak/AMPK in C. elegans.

**par-4 does not require strd-1 to establish embryonic polarity**

Because STRAD was shown to enhance the kinase activity of LKB1 in tissue culture (Baas et al., 2003) and because our findings suggest that PAR-4 requires STRD-1 to phosphorylate its downstream target (AAK-2) and establish GSC quiescence, we wondered whether STRD-1 is required for all par-4 functions. Thus, we were curious whether

Table 2. Early phenotypes of C. elegans embryos*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Blebbing at 1st cytokinesis</th>
<th>Posterior P granule localization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15°C</td>
<td>25°C</td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>par-4(it57ts)</td>
<td>5% (20)</td>
<td>5% (20)</td>
</tr>
<tr>
<td>strd-1(rr91)</td>
<td>88% (17)</td>
<td>100% (13)</td>
</tr>
<tr>
<td>strd-1(rr91); par-4(it57)</td>
<td>24% (25)</td>
<td>100% (20)</td>
</tr>
<tr>
<td>par-4(zu310)</td>
<td>100% (21)</td>
<td>ND</td>
</tr>
<tr>
<td>strd-1(rr91); par-4(zu310ts)</td>
<td>17% (18)</td>
<td>13% (8)</td>
</tr>
<tr>
<td></td>
<td>100% (24)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Phenotypes were determined either by time-lapse analysis of developing embryos (blebbing) or by immunofluorescence on fixed specimens (P granule localization). The values correspond to the percentage of embryos displaying the given phenotype. The number in parentheses corresponds to the number of embryos scored for each phenotype. ND, not determined; ts, temperature-sensitive allele.
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Data not shown). This indicates that properly localized in segregate to the presumptive anterior and posterior regions of wild-type embryos. Furthermore, we found that the PAR-6 protein and P granules, which undergo early development relatively normally (see Movie 1 in the supplementary material). We did not observe gross abnormalities characteristic of par-4 mutants during the first or the second cell division of these embryos.

During the early phases of embryonic development similar to wild type and temperature-sensitive par-4 mutants do not display defects that are reminiscent of par-4 mutants. strd-1 (rr91, rr911, ok2275, ok2283) mutant fertilized embryos that lack both maternal and zygotic strd-1, however, all hatch at a relatively high frequency, even if insulin signalling is reduced or if the temperature is raised to 25°C (Table 1). Indeed, time-lapse images of developing embryos revealed that strd-1(rr91) mutant embryos execute the invariant events typical of early embryonic development similar to wild type and undergo early development relatively normally (see Movie 1 in the supplementary material). We did not observe gross abnormalities during the first or the second cell division of these embryos. Furthermore, we found that the PAR-6 protein and P granules, which segregate to the presumptive anterior and posterior regions of wild-type embryos respectively (Kemphues and Strome, 1997), were properly localized in strd-1-null mutant embryos (Table 2; Fig. 3E-H; data not shown). This indicates that strd-1 mutants do not display strong defects in embryonic polarity and infer that, in contrast to GSC quiescence, par-4 functions largely independently of strd-1 to ensure the correct asymmetric segregation of developmental determinants during the early phases of C. elegans embryonic development.

The PAR-4 protein is uniformly enriched at the cortex of the one-cell embryo, as well as at the cortex of every cell that arises following cell division (Watts et al., 2000) (Fig. 3A-D). To determine the expression pattern and localization of STRD-1 during C. elegans embryonic development, we raised antibodies against STRD-1. Our antibodies recognize an ~44 kDa protein in wild-type, mixed-stage C. elegans extracts that is lost in strd-1 mutants rr91, rr111, ok2275 and ok2283 (Fig. 2B). Using immunofluorescence, we found that STRD-1 is enriched at the cell cortex in the early embryo (Fig. 3A-D). The STRD-1 signal is reduced in strd-1(rr92) and lost in strd-1 mutants rr91, rr111, ok2275 and ok2283 (Fig. 3E-H). To independently confirm the endogenous localization of STRD-1, we generated a transgenic line expressing a STRD-1::GFP fusion driven by the strd-1 operon promoter using the Mos1-mediated single-copy insertion strategy (Frokjaer-Jensen et al., 2008). STRD-1::GFP is enriched at the cortex of every cell in early transgenic embryos as well as at the cortex in the germ line of developing larvae and adults (see Fig. S2 in the supplementary material). Overall, STRD-1 localization seemed very similar to the localization pattern that has been described for PAR-4 (Watts et al., 2000), and double staining confirmed that the two proteins co-localize at the cell cortex in early C. elegans embryos (Fig. 3A-D). To determine whether the two proteins interact, we immunoprecipitated STRD-1 from wild-type and strd-1(ok2275) lysates using rabbit anti-STRD-1 antibodies and probed the precipitates on a western blot with rat anti-PAR-4 antibodies. PAR-4 was detected specifically in the wild-type precipitate (Fig. 2C), indicating that PAR-4 and STRD-1 associate in a complex in vivo.

Interestingly, we find that strd-1 is not required for the cortical localization of PAR-4 during embryonic development, whereas par-4 is required for the cortical localization of STRD-1 (Fig. 3I,J). Moreover, STRD-1 levels are reduced in par-4 mutant animals (Fig. 2B), suggesting that the interaction with PAR-4 stabilises STRD-1. Therefore, although STRD-1 co-localizes with PAR-4 in the early embryo and could thus contribute to cortical PAR-4 functions, PAR-4 is still present at the cortex in the absence of STRD-1 and this is sufficient to ensure the establishment of the early polarity axis.

**strd-1 and par-4 mutants share non-essential functions during early embryonic divisions**

Although par-4 does not require strd-1 to establish the polarity axis in the early C. elegans embryo, we wondered whether strd-1 contributes, even weakly, to the early events of embryonic development, which could explain the low percentage of embryonic lethality observed in these mutants (Table 1). Although strd-1 single mutants are largely viable, detailed examination of strd-1 mutant embryos did reveal some phenotypic abnormalities that are shared with par-4 mutant embryos. In addition to the essential polarity defects attributed to par-4 impairment, a reduction in par-4 activity notably causes cortical ‘blebbing’ in the anterior blastomere during the first cytokinesis, and this defect is also seen at high penetrance in strd-1 mutants (see Movie 1 in the supplementary material). Indeed, this blebbing phenotype is rarely observed in wild-type embryos, but occurs in 100% of par-4(it57) or strd-1(rr91) embryos grown at 25°C (Fig. 4; Table 2). Furthermore, this defect was also observed in the temperature-sensitive par-4(it57) embryos grown at permissive temperature, albeit at a reduced frequency and intensity, suggesting that this defect is linked to a reduction of par-4 activity. Blebbing was proposed to result

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**Fig. 3. STRD-1 and PAR-4 co-localize in the early C. elegans embryo.** (A-D) STRD-1 (left, green) co-localizes with PAR-4 (middle, red) at the cell cortex (arrows) during early embryonic development. The right-most column shows a merged image with DNA (blue) stained with DAPI. P granules (punctate pattern) are non-specifically labelled by our polyclonal anti-STRD-1 antibodies (see Fig. S3 in the supplementary material). A, early one-cell; B, late one-cell; C, two-cell; D, four-cell stage. (E-I) Cortical STRD-1 is absent in (E) strd-1(rr91), (G) strd-1(rr111), (H) strd-1(ok2283) [strd-1(ok2275) were identical; not shown], and is reduced in (F) strd-1(rr92) embryos. Cortical localization of STRD-1 is lost in (I) daf-2(e1370); par-4(it57) embryos raised at 25°C (note the uniform repartition of the non-specific P granule staining, a characteristic of par-4 mutants). Cortical localization of PAR-4 is maintained in (J) strd-1(ok2283) embryos. Four-cell stage embryos are shown. Arrows indicate the cell cortex. Scale bar: 20 μm.
permissive temperature of 15°C (and embryo length is ~50 μm). Between 12 and 20 embryos were scored for each genotype and error bars correspond to the s.d. of the mean. (G) Difference in cell division timing (in seconds) between AB and P1 in embryos of various genotypes. Between 12 and 20 embryos were scored for each genotype and error bars correspond to the s.d. of the mean. strd-1(rr92); par-4(it57) double mutant embryos divide significantly faster than par-4(it57) embryos at the permissive temperature of 15°C (P = 4x10^{-4}, t-test), and strd-1(rr91); par-1(zu310) double mutant embryos divide significantly faster than par-1(zu310) embryos at 15°C (P = 3x10^{-7}, t-test).

from an uncoupling of the cytoplasmic membrane and the underlying actin meshwork (reviewed by Sheetz et al., 2006). Thus, the data suggest that par-4 regulates the integrity of the early embryonic cortex in a strd-1-dependent manner.

Temperature-sensitive par-1 mutants, conversely, also show a blebbing phenotype like strd-1 and par-4 mutant embryos, but at a much lower frequency, and this is not affected by temperature, suggesting that this phenotype is not related to the essential function of par-1 (Table 2). Consistent with this, only 25% of par-1(RNAi) (n=12) embryos showed a weak blebbing phenotype at 25°C (data not shown). Thus, it seems that par-4 and strd-1 function together to prevent these defects in cortical integrity during cytokinesis, whereas par-1 does not have a predominant role in this process.

To address the importance of the mechanism that regulates cortical integrity during early embryonic development when polarity is weakly compromised, we assessed whether weak par-4 or par-1 mutations could enhance the embryonic lethality phenotype of strd-1 mutant animals. When maintained at the permissive temperature (15°C), par-4(it57) or par-1(zu310), as well as strd-1(rr91, rr92) animals hatch at a high frequency. However under the same conditions strd-1; par-4(it57) and strd-1; par-1(zu310) double mutants show substantially decreased embryonic viability, whereby strd-1(rr91) is more severe than strd-1(rr92), resulting in fully penetrant maternal effect embryonic lethality in a par-4(it57) background (Table 1). Thus, strd-1 function becomes essential when polarity is weakly compromised. Consistent with this, time-lapse analysis of developing embryos revealed that the blebbing defect observed in strd-1 mutant embryos was enhanced when the activity of either par-1 or par-4 was weakly compromised (Table 2). Furthermore, disruption of strd-1 enhanced the abnormalities in cell division timing between the AB and P1 blastomeres in par-1 or par-4 mutants (Fig. 4G). Other phenotypes, such as spindle positioning and orientation, as well as the localization of PAR-6 protein and P granules were unaffected in double mutants (Table 2; data not shown). These data suggest that the integrity of the early embryonic cortex is regulated by a pathway that requires both strd-1 and par-4, whereas the essential asymmetric segregation of embryonic determinants occurs in a par-4- and par-1-dependent manner. Therefore, although these different processes that take place during early embryonic development seem to be regulated through divergent pathways, they are interrelated and together clearly affect the viability of the embryo.

**strd-1 is not required for par-4-dependent phosphorylation of PAR-1**

In human cells in culture, as well as in *Drosophila*, LKB1 phosphorylates and activates the MARK kinase PAR-1 at a conserved site in the activation loop (equivalent to Thr^{325} in C. **Phosphorylation events and/or positive regulation. Bars indicate negative regulation.**
**elegans** PAR-1) (Lizcano et al., 2004; Wang et al., 2007). We therefore hypothesized that in the early *C. elegans* embryo, cortical PAR-4 might be required to phosphorylate and activate PAR-1, which is enriched at the posterior cortex (Guo and Kemphues, 1995), to asymmetrically segregate early fate determinants. Consistent with this, we found that par-4 is required for the phosphorylation of PAR-1 at Thr\(^{325}\) in vivo, as phospho-PAR-1 is absent in par-4(ts53) catalytic null animals (Fig. 2D). Surprisingly however, PAR-1 remains highly phosphorylated in *strd-1*-null mutants, as well as at the restrictive temperature in two par-4 temperature-sensitive mutants (Fig. 2D). Thus, PAR-4-dependent phosphorylation of PAR-1 is robust and, unlike AAK-2 phosphorylation, it does not require *strd-1*.

**strd-1 is not required for par-4- and par-1-dependent phosphorylation of MEX-5**

Following the initial asymmetric segregation of the PAR proteins, the zinc finger protein MEX-5 accumulates in the anterior cytoplasm of the early embryo, such that embryos depleted of PAR proteins fail to localize MEX-5 (Schubert et al., 2000; Tenenhaus et al., 1998). Recent findings have demonstrated that phosphorylation of MEX-5 at a crucial residue (Ser\(^{458}\)) within its C-terminal domain is essential for its asymmetric distribution and interestingly, PAR-4 and PAR-1 are both required for this phosphorylation (Tenlen et al., 2008). As both PAR-1 and PAR-4 activities are required for MEX-5 phosphorylation, we reasoned that if PAR-4 does not require STRD-1 to phosphorylate and activate PAR-1, *strd-1* should equally be dispensable for the phosphorylation of MEX-5 at Ser\(^{458}\). Western blot analysis using anti-phospho-MEX-5 antibodies revealed that MEX-5 is still highly phosphorylated at Ser\(^{458}\) in *strd-1* mutants, even at 25°C (Fig. 2E). This result confirms that both par-4 and par-1 have *strd-1*-independent functions, further supporting the hypothesis that PAR-4 directly phosphorylates and activates PAR-1 independently of STRD-1.

**DISCUSSION**

Using genetic analysis we have identified and characterised new alleles of genes that function with PAR-4/LKB1 in establishing GSC quiescence during dauer formation in *C. elegans*. We have shown that the germline hyperplasia phenotype of *strd-1*-null mutants is comparable to that of strong *par-4* alleles, and that these genes do not act in an additive manner, suggesting that they function in a single linear pathway, potentially as a single molecular complex. In addition, we provide biochemical evidence that PAR-4 interacts with and requires STRD-1 to phosphorylate AAK-2 at its activation loop during dauer development. Thus, we propose that PAR-4 forms a complex with STRD-1 that phosphorylates and activates AAK-2 to induce GSC quiescence during dauer development. AAK-1 is likely to be activated during dauer development through a similar mechanism. We have further demonstrated that each of the catalytic AMPK subunits also require the β-subunit aakb-2 or, to a lesser extent, aakb-1, to inhibit GSC proliferation during dauer formation (Fig. 5). A second function of aak-2, that is not shared with aak-1, is to ensure the long-term survival of dauer larvae by inhibiting lipolysis in the hypodermis. In this case, aak-2 requires either one of the two β-subunits (Narbonne and Roy, 2009). Therefore, multiple AMPK complexes seem to carry partially overlapping roles in different tissues. Whether this is due to differential expression patterns of the AMPK subunits, or to functional differences between the possible complexes, however, remains unclear.

We have further shown that despite the fact that they are relevant STRD-1-dependent PAR-4 targets, the *C. elegans* orthologues of AMPK α- and β-subunits, are not involved in regulating cell polarity, at least not in the early embryo. This contrasts with observations made both in mouse and *Drosophila* reporting that catalytically active AMPK is required for embryonic development and also to maintain epithelial polarity under energetic stress (Lee et al., 2007; Mirouse et al., 2007). Nonetheless, a more thorough set of assays might reveal subtle polarity defects in *C. elegans* AMPK mutants. Consistent with this, aak-2 mutants show modest motility and also to maintain epithelial polarity under energetic stress (Lee et al., 2008). Yet, it seems most probable that AMPK did not originally evolve as a regulator of polarity, but might have progressively been co-opted for this novel function in increasingly complex organisms.

In early *C. elegans* embryos, mutations in par-4 or par-1 similarly result in the loss of the asymmetric distribution of the early fate determinants, suggesting that they function in the same process (Guo and Kemphues, 1995; Kemphues et al., 1988; Watts et al., 2000). PAR-4 is present in the cytoplasm and is enriched and evenly distributed at the cortex of all the cells of the germ line and of the early embryo (Watts et al., 2000). PAR-1, conversely, is first detected evenly distributed at the periphery of the germ cells during meiosis, becoming asymmetrically localized to the posterior cortex of embryos upon fertilization (Guo and Kemphues, 1995). In *Drosophila* and mammals, LKB1 was shown to directly phosphorylate and activate several kinases of the AMPK family, including the microtubule affinity regulating kinase (MARK) PAR-1/Par1b/EMK1 (Lizcano et al., 2004). Based on genetic and cell biological data in *C. elegans*, as well as on biochemical data from other systems, the most widely accepted hypothesis is that PAR-4 directly phosphorylates and activates PAR-1 at the posterior cortex in the early embryo, where their expression overlaps, and this would be necessary for the segregation of the germ line determinants to the posterior pole. We have shown that par-4 is indeed required for the activating phosphorylation on PAR-1. This phosphorylation might, however, not be the sole essential function of PAR-4 as par-4(ts) mutants maintain significant phosphorylation of PAR-1, despite the observed complete embryonic lethality at the restrictive temperature. It is thus possible that PAR-4 carries out several essential functions during the establishment of the A/P axis. Most importantly, we have shown that *strd-1* is not required for the PAR-4-dependent activating phosphorylation on PAR-1. Consistent with this, PAR-4 remains enriched at the cell cortex even in the absence of STRD-1 and, most convincingly, *strd-1*-null mutants do not share the fully penetrant maternal effect embryonic lethality typical of *par-4* and *par-1* mutants. Furthermore, we provide evidence that there is sustained *par-4* and *par-1* activity in the absence of *strd-1* because MEX-5 Ser\(^{458}\) is still highly phosphorylated in these mutant embryos. Thus, in contrast to the case in the dauer germline, PAR-4 does not require association with STRD-1 to set up the polarity axis in the early embryo, at least in part owing to its ability to phosphorylate and hence activate or induce the phosphorylation and activation of PAR-1 and MEX-5 (Fig. 5).

Nonetheless, weak *par-4*-like early embryonic phenotypes were observed in *strd-1* mutants. Notably, *strd-1* mutant embryos showed membrane blebbing at the anterior blastomere during the first cytokinesis, and *strd-1* also enhanced the AB and P\(_1\) blastomere division timing defects of weak *par-4* and *par-1* mutants. It is not well defined how defects in these gene products result in the observed defects in cortical integrity. As *par-1* is not necessary to prevent the blebbing phenotype downstream of *par-4*, the data suggest that PAR-4 requires STRD-1 to phosphorylate additional
substrate(s) in the early embryo, to ensure proper cortical integrity (Fig. 5). Further elucidation of how PAR-4 and STRD-1 affect this process will be facilitated by the identification of the target substrates that mediate these events.

The mechanisms through which cells elicit change often impinge on key effector molecules that commonly include protein kinases. Yet still unresolved is how individual protein kinases can affect numerous distinct cellular processes in a manner that is both highly specific and robust. LKB1 is one such kinase that plays pivotal roles in the appropriate regulation of cell polarity and cell/tissue growth during development. Taken together, our findings indicate that STRD-1 and PAR-4 work as a unit in many, but not all, cellular contexts. The depressive germine quiescence phenotype of strd-1 mutants corresponds to a strong loss of par-4 function, whereas the embryonic phenotypes correspond to a weak, or partial loss of par-4 activity. One explanation for this finding is that the function of STRD-1 could be to maintain high-levels of PAR-4 kinase activity, whereas the PAR-4 effector(s) that polarizes the early embryo (i.e. PAR-1) might be a better substrate for PAR-4 phosphorylation, such that it is phosphorylated even when par-4 kinase activity is low. This, however, seems unlikely because we did not observe a marked reduction in PAR-1 and MEX-5 phosphorylation in the absence of STRD-1. Alternatively, the association of PAR-4 with STRD-1 could induce conformational changes such that the complex would acquire novel properties including an enhanced affinity for a given set of substrates. It is also possible that a second adapter protein activates PAR-4 and compensates for STRD-1 during embryogenesis but, in its absence, this adapter becomes limiting when par-4 function is compromised. Nonetheless, our data suggest that PAR-4 is capable of phosphorylating specific substrates independently of STRD-1 (i.e. PAR-1) but acquires the ability to phosphorylate additional targets upon association with STRD-1 (i.e. AAK-1,2).

That STRAD or AMPK have not been directly implicated in PJS or in any form of cancer suggests that the most robust LKB1-dependent processes, those that require only minimal LKB1 activity, might indeed be the crucial hinge pins that underlie the aetiology of PJS. Our data hint that these might include LKB1-meditated events in polarity establishment and further emphasizes the importance of uncovering the identities of these important targets. How other genes associated with hamartoma-producing syndromes that are similar to PJS might converge on common substrates to regulate such decisions will be of considerable interest to better understand how their impairment translates to tumour growth.

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Competing interests statement
The authors declare no competing financial interests.

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