A *Drosophila melanogaster* homologue of *Caenorhabditis elegans par-1* acts at an early step in embryonic-axis formation

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In *Drosophila melanogaster* and *Caenorhabditis elegans*, development of the germ-line lineage depends upon cytoplasmic granules localized to the posterior pole of the zygote, which are present in germ-line cells throughout development[1,2]. In *D. melanogaster*, polar granules are components of the pole plasm assembled at the posterior pole during oogenesis, and in *C. elegans* P granules accumulate at the posterior pole of one-cell embryos. Mutations in *D. melanogaster* pole-plasm components such as OSKAR result in defects in embryonic patterning and germ-line specification[2]; mutations in *C. elegans* polarity proteins, such as the Ser/Thr kinase PAR-1, that result in mispositioning of P granules, cause defects in embryonic patterning and cell-fate determination[3]. Here we show that a *D. melanogaster* homologue of PAR-1 becomes asymmetrically localized as polarity is established, and that mutations affect-

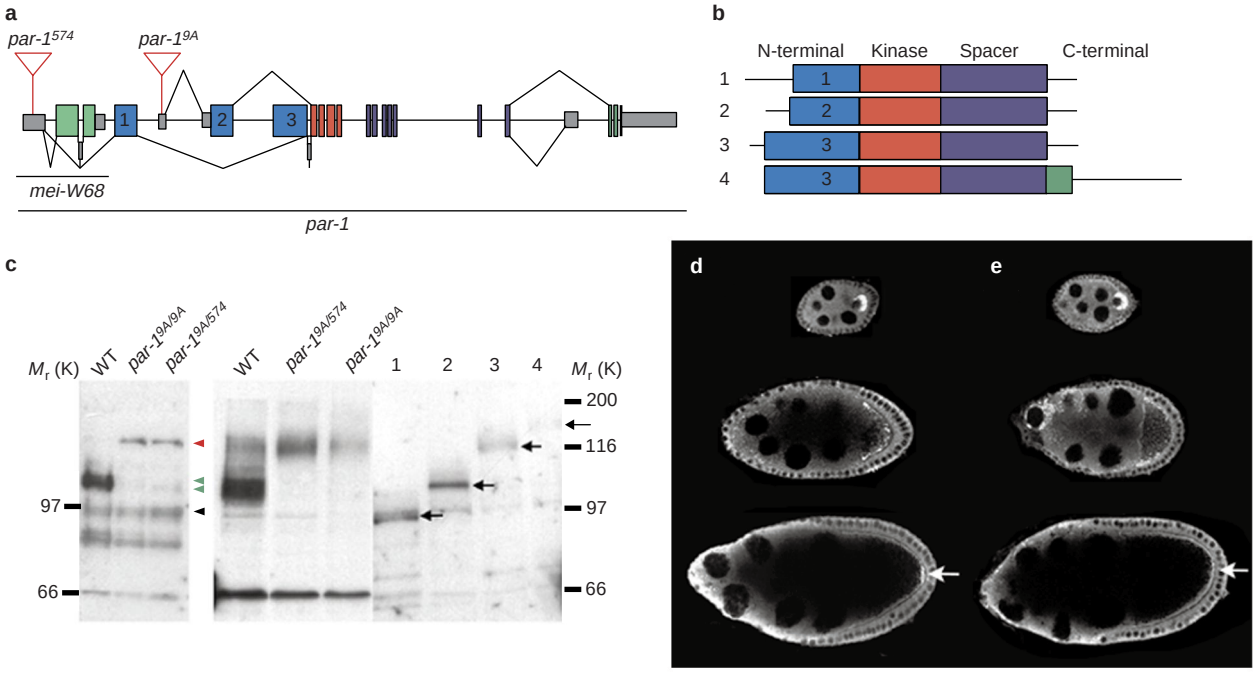

Figure 1 Molecular analysis of *D. melanogaster par-1*. **a,** Schematic representation of the *par-1* locus. Thin horizontal lines represent introns; exons encoding the following are shown: grey, 5′ and 3′ untranslated regions; blue, N-terminal domains; red, kinase domains; purple, spacer sequences; green, conserved C-terminal domain. The *meiW68* locus is highlighted and the coding region of *mei-W68* is shown in green. Approximate locations of P elements *par-1*^{574} (l(2)k05603) and *par-1*^{9A} (l(2)k06323) are indicated. Alternative splicing patterns are indicated by bent lines, all other exons are invariantly spliced into transcripts in their linear order. **b,** Domain compositions of *par-1* transcripts. Colour coding as in **a**; domains are also labelled (top). Thin horizontal lines represent 5′ and 3′ untranslated regions. **c,** Western blots of wild-type and mutant ovary extracts and of the *in vitro* translated *Drosophila par-1* cDNAs shown in **b** (lanes 1–4). Blots were probed with two different affinity-purified antibodies against *Drosophila* PAR-1 (left and right panels, antibody 1; middle panel, antibody 2; gifts from J. Knoblich). Red arrow highlights the PAR-1 isoform that exhibits increased expression in mutants; green arrows indicate isoforms that are strongly suppressed in mutants; black arrow indicates the isoform that seems to be unaffected. A 66K protein does not correspond to any of the isolated cDNAs. An 80K doublet (left panel) is recognized by pre-immune serum. **d, e,** Expression of *Drosophila* PAR-1 during oogenesis in wild-type (**d**) and *par-1*-mutant (**e**) ovaries. Top, PAR-1 is localised to the posterior of the oocyte during early oogenesis in both wild-type and *par-1*-mutant egg chambers. Middle, at stage 8, PAR-1 is uniformly distributed along the oocyte cortex. Expression is also visible in nurse cells and follicle cells. Bottom, during stage 9, PAR-1 is concentrated at the posterior of wild-type oocytes (arrow). In *par-1* mutants, however, PAR-1 is not concentrated at the posterior pole (arrow), but is evenly distributed along the cortex.

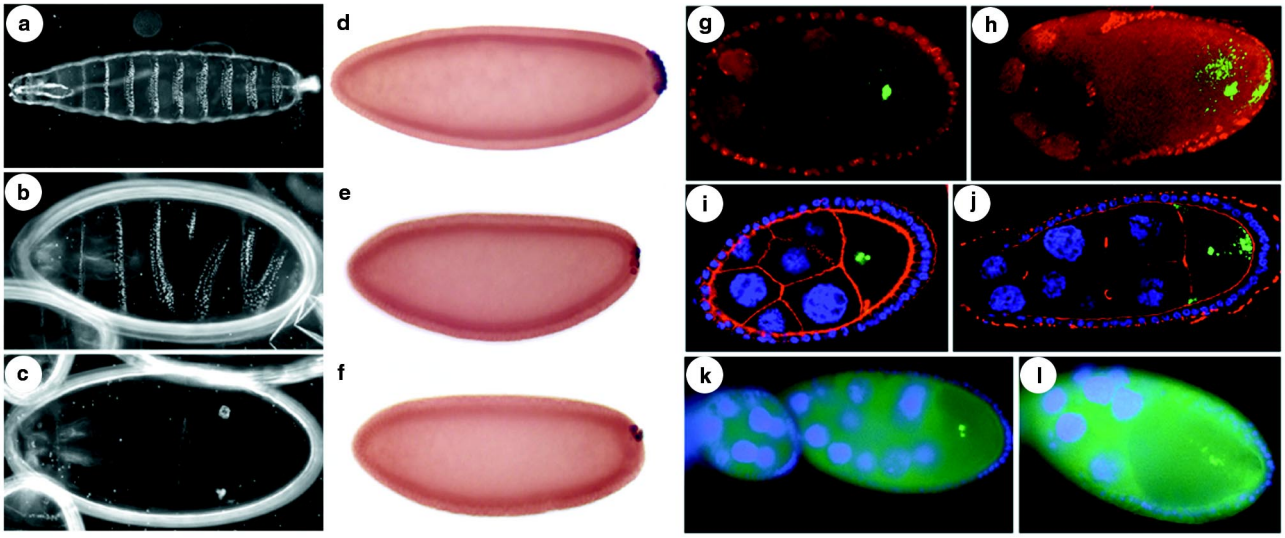

Figure 2 *Drosophila par-1* mutants exhibit defects in abdominal patterning, pole-cell formation and pole-plasm positioning. **a–c,** Embryonic-patterning phenotypes in *par-1*-mutant embryos range from normal segmentation (**a**) to complete absence of abdominal segments (**c**). **d–f,** Whereas some mutant embryos develop several pole cells (as shown by staining with anti-Vasa antibody) comparable to those of the wild type (**d**), the number of pole cells formed is markedly reduced in most mutant embryos (**e, f**). **g, h,** *oskar* mRNA (green; DNA shown in red) is fully (**g**) or partially (**h**) mislocalized to the middle of the oocyte in *par-1*-mutant egg chambers. **i, j,** The pole-plasm component STAUFEN (stained with anti-STAUFEN antibody in green; DNA shown in blue; actin shown in red) follows the distribution of *oskar* mRNA in *par-1*-mutant ovaries. **k, l,** OSKAR protein (stained with anti-OSKAR antibody in green; DNA shown in blue) accumulates ectopically in the middle of the oocyte (**k**) and fails to be fully restricted to the posterior pole of the oocyte (**l**) in *par-1* mutants.

ing its expression perturb posterior patterning, germ-line development and posterior localization of pole-plasm components. We propose that *par-1* has an important and conserved function in establishment of polarity and development of the germ-line lineage in both of these species.

We identified a *D. melanogaster* homologue of *par-1*, a member of the PAR-1/MARK/KIN1 kinase gene family (*Drosophila par-1*). Alternative splicing and alternative use of promoters generate at least four different transcripts from this locus (Fig. 1a, b). Proteins predicted from those transcripts contain the conserved kinase domain (red) and the non-conserved spacer domain (purple) typical of this protein family, but differ in their amino termini (N1, N2 and N3; blue) and in inclusion of the conserved carboxy-terminal domain (green). Antibodies raised against part of the spacer region recognized several proteins in wild-type ovary extracts, three of which (relative molecular masses (M_r) 97K, 110K and 116K) correspond to *in vitro* translated products of identified complementary DNAs 1, 2 and 3 (Fig. 1c).

Analysis of the distribution of *Drosophila* PAR-1 in ovaries revealed that it is present in the spectrosome and early fusome of the germarium (data not shown) and becomes enriched in the oocyte during early oogenesis (Fig. 1d, top). During stages 5–8 of oogenesis, the protein is uniformly distributed along the oocyte cortex (Fig. 1d, middle), and at stage 9, it is enriched at the posterior pole, although it can be detected at a low level throughout the oocyte cortex (Fig. 1d, bottom). PAR-1 is also expressed in follicle cells (Fig. 1d).

To determine the role of *Drosophila* PAR-1, we identified P-element insertions within the genomic region encompassing the *par-1* locus and the overlapping *mei-w68* locus. Two insertions, l(2)k06323 ($par\text{-}1^{9A}$) and l(2)k05603 ($par\text{-}1^{574}$) affected expression of PAR-1 isoforms (Fig. 1a, c). Both P elements caused reductions in levels of isoforms of M_r~110K and led to increased levels of an isoform of M_r~116K. Excision of the $par\text{-}1^{9A}$ P element restored the wild-type expression pattern, demonstrating that these changes were caused by insertion of the P element and confirming the identity of the affected proteins as PAR-1 isoforms (see Supplementary Information). In *par-1*-mutant egg chambers, distribution of PAR-1 was found to be altered (Fig. 1e). In stage-9 oocytes, PAR-1 enrichment at the posterior pole was reduced in these mutants; instead the protein appeared evenly distributed along the cortex (Fig. 1e, bottom). These changes in PAR-1 distribution presumably reflect changes in the amounts of the various PAR-1 isoforms, as detected by western blotting (Fig. 1c).

Females harbouring the $par\text{-}1^{9A}/par\text{-}1^{9A}$ and $par\text{-}1^{9A}/par\text{-}1^{574}$ mutations were found to be fertile; however, 20% of embryos produced by these individuals died, exhibiting defects ranging from disturbances in posterior patterning, such as fusion or bifurcation of segments, to complete absence of abdominal segments (Fig. 2b, c). Abdominal patterning in *D. melanogaster* is initiated by assembly of the posterior pole plasm, which is also required for formation of the germ-line precursors, the pole cells[2]. In the majority of *par-1*-mutant embryos, the number of pole cells was significantly reduced (Fig. 2e, f), indicating that pole-plasm formation may be affected by *par-1* mutations.

Pole-plasm assembly is induced by localisation and translational derepression of *oskar* messenger RNA at the posterior pole of the oocyte, creating a localized source of OSKAR protein, which in turn recruits further pole plasm components[4–7]. In 70% of $par\text{-}1^{9A}$-mutant egg chambers at stages 8–10 ($n = 70$), *oskar* mRNA was either fully or partially mislocalized to the middle of the oocyte (Fig. 2g, h). This aberrant distribution of *oskar* mRNA was mirrored by similar defects in localization of STAUFEN, another pole-plasm component (Fig. 2i, j). Moreover, in 20% of stage 8–9 egg chambers ($n = 120$), some or all of the OSKAR protein detected was found in the middle of the oocyte (Fig. 2k, l), indicating that pole-plasm mispositioning may be a cause of defects in abdominal patterning and pole-cell formation.

Thus, *D. melanogaster* PAR-1, like its counterpart in *C. elegans*, has a function in establishing embryonic polarity, and becomes asymmetrically distributed during the period when polarity is specified. Mutations that alter its expression lead to alterations in the distribution of pole-plasm components, reduced numbers of pole cells and defective posterior patterning. Given the nature of the

changes in expression, these defects could result from reduced levels of a critical isoform, inappropriate levels or distribution of an isoform, or a combination of the above.

Drosophila PAR-1 may influence polarity through the cytoskeleton. Localization of *oskar* mRNA to the posterior pole requires both microtubule-dependent and microfilament-dependent processes[8,9]. The idea that *Drosophila* PAR-1 regulates microtubules is supported by the fact that mammalian PAR-1 homologues exhibit microtubule-destabilizing activities[10]. In *C.elegans*, however, there is no evidence of a role for microtubules in *par-1*-dependent processes, and PAR-1 protein has been shown to interact with non-muscle myosin, an actin motor[11]. Furthermore, proper asymmetric localization of P granules is resistant to microtuble-depolymerizing drugs but sensitive to microfilament-depolymerizing agents[12]. It is therefore also possible that *Drosophila* PAR-1 acts through a microfilament-dependent mechanism, such as anchoring *oskar* mRNA and/or OSKAR protein to the posterior pole. Indeed, maintenance of pole-plasm components at the posterior pole of the *D. melanogaster* embryo depends on an intact actin cytoskeleton[13]. Previous studies have shown fundamental differences in the early development of *D. melanogaster* and *C. elegans* with respect to establishment of embryonic polarity[14,15]. In spite of these marked differences, PAR-1 and *Drosophila* PAR-1, members of the PAR-1/MARK/KIN1 family of Ser/Thr kinases, are distributed asymmetrically at the cell periphery in oocytes and in early embryos of *D. melanogaster* and *C. elegans*, respectively. This finding raises the possibility that at the heart of these two widely divergent systems lies a conserved mechanism to initiate cell polarity and germ-line development. □

Methods

Molecular biology and western blotting.

We carried out a polymerase chain reaction (PCR), using the following degenerate primers, designed against the highly conserved PAR-1 C-terminal domain, to obtain a partial *Drosophila par-1* clone: forward, 5′-CCGGAATTCGTNCA(AG)TGGGA(AG)ATGGA; reverse, 5′-ACCGGATCC(AT)NGC(AGT)AT(AG)TT(CT)TT(AG)AA. *par-1* cDNA 1 corresponds to expressed-sequence tag (EST) GH10312 from the Berkeley Drosophila Genome Project (BDGP) EST project, and was identified by BLAST searches, 3′-end sequencing and restriction analysis. *par-1* cDNAs 2 (3.8 kilobase (kb)) and 3 (3.9 kb; Fig. 1b) were obtained by screening an oligonucleotide-dT-primed oogenesis library (a gift from A. Spradling) with a DNA probe derived from the predicted spacer sequences. Library screens were carried out as described[16]. *par-1* cDNA 4 was amplified from total ovarian RNA (purified with RNAClean) with primers specific for the predicted start and stop codons of N-terminal exon N3 and the last C-terminal exon of *par-1*, respectively. In addition to these cDNA clones, we also examined the sequences of other partial clones that show further alternative splicing within the spacer region.

P elements in and around the *Drosophila par-1* locus were mapped using sequence-tagged site STS data available from the BDGP and computer-assisted alignment of the flanking genomic sequences isolated by inverse PCR against a database of *D. melanogaster* genomic sequences, obtained from the BDGP public sequence repository. In the case of P element l(2)k06323 (*par-1*^{hk}), we obtained the flanking genomic sequences by inverse PCR and mapped them by *in situ* hybridization to polytene chromosomes, confirming the BDGP data.

Western blotting was carried out as described[17]. *In vitro* translation of cDNAs in reticulocyte lysates was carried out using the TNTcoupled system (Promega).

Drosophila strains and manipulations.

The following *Drosophila* strains were used in this study: l(2)k06323/CyO and l(2)k05603/CyO (Bloomington *Drosphila* Stock Center, Bloomington, Indiana) and Oregon R (wild-type). A lethal mutation that was not associated with the *Drosophila par-1* locus in l(2)k06323 was removed by recombination with wild-type chromosome II. Remobilization of the l(2)k06323 P element led to complete reversion of the oogenesis phenotype in the majority of cases, demonstrating that the observed phenotypes were due to the P-element insertion.

Antibody preparation.

An N-terminal hexahistidine (6×His) fusion construct containing a 309-base-pair (bp) *Pst*I fragment from the spacer domain was cloned into the bacterial expression vector pQE30. The resulting 130-amino-acid 6×His fusion protein was affinity-purified under native conditions using Ni–NTA resin (Qiagen Ic, Valencia, CA) according to the manufacturer's instructions, concentrated by centrifugation using Centriprep and Centricon 3 concentrators (Amicon, Beverly, Massachusetts) and used to immunize two rabbits according to standard protocols.

For western analysis, antibody against *Drosophila* PAR-1 was affinity-purified by batch incubation with sepharose beads coupled to a glutathione-*S*-transferase (GST)-tagged fragment of the *Drosophila* PAR-1 spacer region (*Pst*I fragment) expressed in *Escherichia coli*. Affinity-purified antibody was eluted with 100 mM glycine, pH 2.5, into 4.6 ml 1.5 M Tris pH 8.8 and 10 ml 3 M KCl, and dialysed overnight against PBS. For *in situ* analysis of *Drosophila* PAR-1 distribution, antiserum against *Drosophila* PAR-1 was pre-adsorbed by overnight incubation with 2 volumes of 0–16-h-old wild-type embryos in 450 ml PBT Triton 0.1% at 4 °C.

Detection of proteins and nucleic acids in whole mounts.

For whole-mount antibody staining, ovaries were dissected in Grace's medium (Sigma), fixed in 4% formaldehyde for 20 min, washed twice (10 min each) in PBT and blocked for 1.5 h in PBS supplemented with 1% Triton and 0.5% BSA. Ovaries were then incubated overnight with primary antibody (rabbit anti-STAUFEN, 1:2000, and rabbit anti-OSKAR, 1:3000, rabbit anti-*Drosophila* PAR-1 pre-adsorbed, 1:500 final dilution) in PBS with 0.3% Triton and 0.5% BSA. After two 20-min washes in PBS containing 0.3% Triton and 0.5% BSA, ovaries were blocked in PBS containing 0.1% Triton and 10% normal goat serum for 2.5 h and then incubated with a fluorescein-conjugated anti-rabbit (1:500; Amersham) or rhodamine-conjugated anti-mouse (1:500; Amersham) secondary antibody and, optionally, with rhodamine-conjugated phalloidin (1:200) in PBS with 0.1% Triton (PBT) for 2 h. Ovaries were then washed twice (15 min each) in PBT, incubated for 5 min with PBT and 4′-6-diamidino-2-phenylindole (DAPI; 1:2,500) and washed again three or four times in PBT. After overnight equilibration in *N*-propylgallate, ovaries were mounted and analysed by confocal (Leica) or light (Zeiss Axiophot) microscopy. Embryos were fixed and stained with a rat anti-VASA antibody (1:4000), following standard procedures.

In situ hybridizations using RNA probes were carried out as described[18]. An RNA probe corresponding to the full-length RNA was transcribed from a plasmid containing *oskar*[4] cDNA and was fluorescently labelled as described[18].

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