

pavarotti encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis

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Mutations in the *Drosophila* gene *pavarotti* result in the formation of abnormally large cells in the embryonic nervous system. In mitotic cycle 16, cells of *pav* mutant embryos undergo normal anaphase but then develop an abnormal telophase spindle and fail to undertake cytokinesis. We show that the septin Peanut, actin, and the actin-associated protein Anillin, do not become correctly localized in *pav* mutants. *pav* encodes a kinesin-like protein, PAV-KLP, related to the mammalian MKLP-1. In cellularized embryos, the protein is localized to centrosomes early in mitosis, and to the midbody region of the spindle in late anaphase and telophase. We show that Polo kinase associates with PAV-KLP with which it shows an overlapping pattern of subcellular localization during the mitotic cycle and this distribution is disrupted in *pav* mutants. We suggest that PAV-KLP is required both to establish the structure of the telophase spindle to provide a framework for the assembly of the contractile ring, and to mobilize mitotic regulator proteins.

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Cytokinesis is the ultimate act of mitosis, whereby segregated daughter nuclei are partitioned into two separate cells. In eukaryotes, this is accomplished by an actin-myosin contractile ring that forms around the cell equator during mitosis and constricts inwards at telophase (Schroeder 1972). In higher eukaryotes the correct positioning and assembly of the contractile ring requires the mitotic spindle, although the mechanism by which this occurs is a matter of controversy (Swann and Mitchison 1956; Zhang and Nicklas 1996). The classical experiments of Rappaport (1961) suggest that the spindle poles are sufficient to stimulate cytokinesis. By generating horseshoe-shaped single cell echinoderm embryos containing two mitotic spindles, not only were cleavage furrows formed in the central region of the mitotic spindles, but an extra furrow was formed equidistant between two asters lacking any intervening chromosomes or spindle microtubules. Similar phenomena have been observed in cultured mammalian cells, and although this does occur infrequently it suggests that this mechanism may act universally (Eckley et al. 1997; Rieder et al. 1997). Two mechanisms have been put forward to demonstrate how the spindle poles might induce cleavage furrows. In the

first model, the poles signal directly to the equatorial cortex of the cell, perhaps by the action of astral microtubules (Devore et al. 1989). Alternatively, the astral relaxation model (White and Borisy 1983) proposes that spindle poles induce a relaxation of the cell cortex nearest to the poles, leading to a tension differential between the poles and the equator that results in equatorial contraction. However, this model has been discredited recently by force measurements showing that tension increases at the equator without a concomitant decrease at the cell poles (Burton and Taylor 1997).

On the other hand, there is growing evidence that the central spindle plays an essential role in the positioning and assembly of the contractile ring. The central spindle is composed of a dense network of overlapping antiparallel microtubules that forms between the separating daughter nuclei during anaphase (Mastronarde et al. 1993). In cultured cells induced to develop multipolar spindles as result of treatment with low concentrations of the microtubule-destabilizing drug colcemid, the formation of a cleavage furrow absolutely depends on the presence of central spindle microtubules that are required throughout cytokinesis (Wheatley and Wang 1996). Creation of a barrier between the central spindle and the cell cortex prevents cleavage, indicating that a signal for cytokinesis may emanate from the central spindle (Cao and Wang 1996). Manipulation of grasshopper neuroblast spindles results in the formation of a

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cleavage furrow when the spindle is moved close to the cell cortex (Kawamura 1977).

In addition to the contribution made by the spindle, several proteins, known as the chromosomal passenger proteins, dissociate from chromosomes at the metaphase-anaphase transition to be deposited at the cell equator. The inner centromere proteins (INCENPs), for example, transfer to the central spindle and the cell cortex and are necessary for completion of cytokinesis (Eckley et al. 1997; Earnshaw and Cook 1991), whereas TD-60 forms a disc structure in the position of the metaphase plate anticipating the cleavage plane (Andreassen et al. 1991). This disc is proposed to link the central spindle to the cell cortex (Martineau et al. 1995).

In *Drosophila*, mutational analysis has identified several genes encoding structural elements of the contractile ring essential for cytokinesis, such as myosin light chain, cofilin, profilin, and Peanut, a homolog of the yeast septins (Karess et al. 1991; Neufeld and Rubin 1994; Gunsalus et al. 1995; Giansanti et al. 1996). Recently, cloning of the gene defective in *KLP3A* mutants identified a kinesin-like protein (KLP) as having a role in cytokinesis in male meiosis (Williams et al. 1995). KLPs are microtubule motor proteins responsible for many of the dynamic aspects of mitosis such as centrosome separation, spindle assembly, and anaphase movement of chromosomes (Moore and Endow 1996).

In this paper we describe the phenotype of lethal mutations in the essential gene *pavarotti* (*pav*), which encodes a fly homolog of the mammalian mitotic kinesin-like protein-1 (MKLP-1). MKLP-1 has been suggested to play a role in anaphase B spindle pole elongation based on in vitro motility assays (Nislow et al. 1992). However, we show that in *pav* mutants anaphase B occurs normally but cytokinesis is defective, due to a disruption of the central spindle structure and subsequent failure to assemble a contractile ring. In mitosis, the KLP encoded by *pav* (PAV-KLP) first associates with the centrosomes and then becomes localized at the spindle equator at late anaphase. We also show that PAV-KLP associates with Polo kinase and is required for its localization to the centrosome and central spindle. The Polo-like kinases (plks) are known to be required for centrosome function: Mutations in the *Drosophila* gene *polo* and its fission yeast homolog *plp1* lead to spindle defects including the formation of monopolar structures (Sunkel and Glover 1988; Lamazares et al. 1991; Ohkura et al. 1995). Injection of antibodies against human plk also result in the formation of monopolar structures in HeLa cells (Lane and Nigg 1996). An additional role for plks in cytokinesis is suggested from the late mitotic phenotype of fission yeast *plp1* and its budding yeast counterpart *cdc5* (Hartwell et al. 1973; Ohkura et al. 1995). Multiple septation has been shown to result from *plp1*⁺ overexpression in fission yeast, or the expression of mammalian Plk in budding yeast (Ohkura et al. 1995; Lee and Erikson 1997). In light of the association of Polo kinase with PAV-KLP, we discuss whether the plks might also have a role in cytokinesis in metazoan cells that has been obscured by the earlier requirement for mitosis.

Results

pav embryos show defects at cytokinesis

Preliminary characterization of the phenotype of *pav* mutants by staining embryos with the neuronal marker mAb 22C10 indicated the formation of fewer and larger neurons than usual in the embryonic development of the PNS, and an absence of support cells (Salzberg et al. 1994). To investigate whether the large cells seen in *pav* mutant embryos could arise from cell division defects, we looked for mitotic defects in *pav* mutants from cycle 14 (the first cycle under the control of the zygotic genome) onward through cycles 15 and 16 (Materials and Methods). We could detect no differences between wild-type and *pav* embryos in cycle 14 or 15, but in cycle 16 we observed that although mitosis appeared to be normal within the ventral epidermis, interphase nuclei were frequently closely associated in pairs in both P-element and EMS-induced alleles of *pav* (Fig. 1B). The boundaries of the cells of the embryo shown in this field are defined using an antibody against α -spectrin, a membrane-bound structural protein. It can be seen that the cell membrane does not invaginate during telophase in *pav* embryos as it does in wild type (Fig. 1A). Despite this failure of cytokinesis, nuclei appear to enter interphase 17 successfully, as nuclear laminae reform around both nuclei in binucleate cells (Fig. 1D). Such binucleate cells are never seen in wild type (Fig. 1C).

In addition to the cytokinesis defects that can be observed in essentially all cells at cycle 16, we also observed other mitotic defects at a very low frequency in *pav*^{B200} and *pav*^{M187} embryos but not in the P-element alleles. The affected cells show multipolar spindles and multiple centrosomes that are always associated with what appear to be tetraploid masses of chromatin (data not shown). This would suggest that in the *pav*^{M187} and *pav*^{B2004} alleles, binucleate cells are formed that can undergo another round of mitosis with double the usual number of centrosomes. This could occur if the maternal supply of *pav* gene product were to become exhausted in some cells during cycle 15. Such cells would fail to undertake cytokinesis but then proceed to attempt a sixteenth division, the last cell cycle for the majority of epidermal cells.

pav cells show defects in the central spindle at telophase, and septin and actin rings fail to form

To address how *pav* mutations might affect cytokinesis, we examined the structure of the mitotic spindle in mutant embryos. The morphology of the spindle was indistinguishable from wild type in the mitotic stages up until late anaphase. We observe no differences between wild-type and *pav* mutants in the degree of elongation of the spindle as cells progress from metaphase to late anaphase (see legend to Fig. 2). However, in *pav* mutants the distinctive morphology of the central spindle is substantially changed at telophase so that it appears to contain fewer bundles of microtubules (Fig. 2). In addition, whereas peripheral spindle microtubules (i.e., those mi-

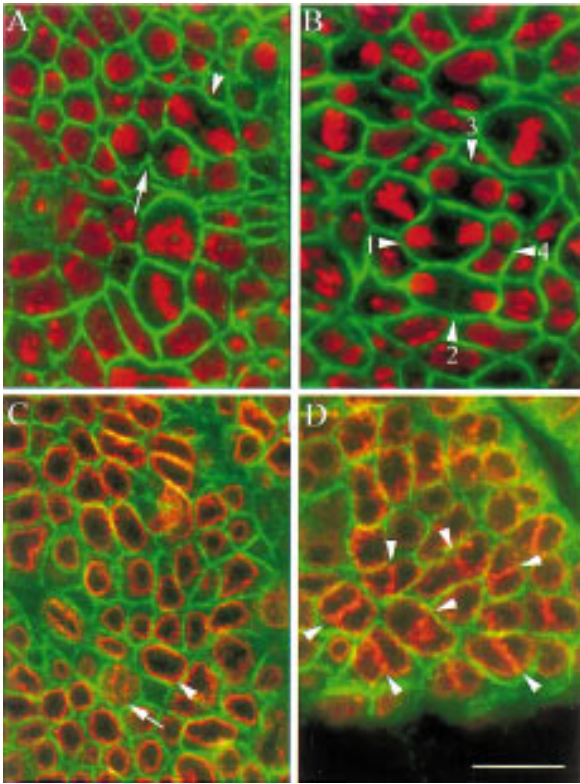


Figure 1. *pav* embryos show cytokinesis defects at cycle 16. (A) A wild-type embryo at cycle 16 stained to reveal α -spectrin (green) and DNA (red). The contraction of the cleavage furrow can be seen in cells in late anaphase (arrowhead) and telophase (arrow). (B) Cells in homozygous *pav*^{B200} embryos do not develop this constriction in cycle 16. Arrowheads 1–4 show the development of binucleate cells at progressively later stages from anaphase through telophase. (C) A wild-type embryo at cycle 16 stained to reveal lamin A (red) and α -spectrin (green). There is only one nuclear lamina per cell (arrowhead), which is dismantled during mitosis (arrow). (D) Homozygous *pav*^{B200} embryo at cycle 16, with many cells containing two nuclear laminae (arrowheads). Scale bar, 10 μ m.

cro-tubules emanating from the centrosome that contact the cell cortex) are seen to become constricted in wild type, this appears not to occur in the *pav* mutant.

We also examined the localization of Peanut, Actin, and Anillin, proteins known to be required for cytokinesis. We first looked at the formation of the septin ring using antibodies to the *Drosophila* septin, Peanut. The septin family of proteins was originally identified in *Saccharomyces cerevisiae* as required for bud neck formation and subsequently shown to be present in most eukaryotes (Longtine et al. 1996). Although *peanut* has been shown to be required for cytokinesis in *Drosophila*, its exact role is unknown (Neufeld and Rubin 1994). Recent results suggest that it may mediate attachment between the actin network and the spindle (Glotzer and Hyman 1996). We observe that in wild-type cellularized embryos, Peanut protein is confined to the cell surfaces, with punctate spots of the protein at the site of the in-

tercellular bridges remaining from the previous division (Fig. 3A). At late anaphase, Peanut accumulates in the equatorial region of the cell and appears at the contractile ring at telophase. In *pav* embryos, Peanut staining is generally weaker and more delocalized than in wild type, although some cells appear to have a near normal surface distribution of the protein. However, although areas of punctate Peanut staining can sometimes be seen in apparent association with the plasma membrane midway between the telophase nuclei, Peanut never appears at the site of the contractile ring (Fig. 3D).

We also examined *pav* mutants to determine whether Actin or Anillin localized at the contractile ring and if cleavage furrows would form. In wild-type cellularized embryos, Actin is present around the surface of the cell throughout the cell cycle and accumulates in the equatorial region of the cell during telophase before forming the contractile ring (Fig. 3B). The Actin binding protein

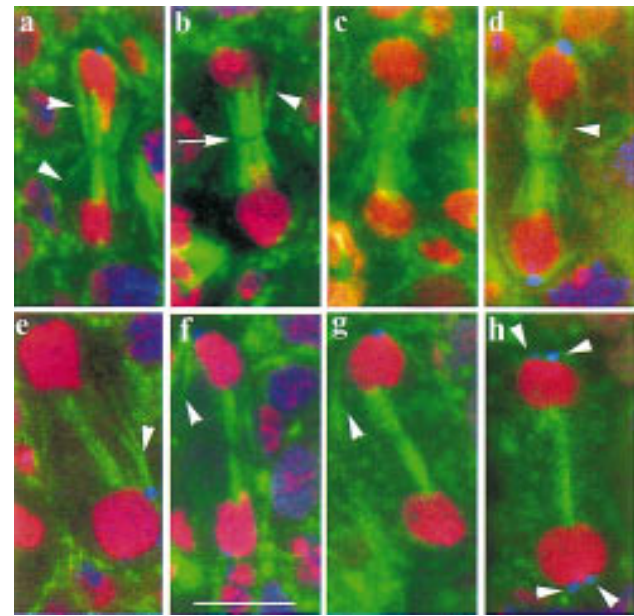
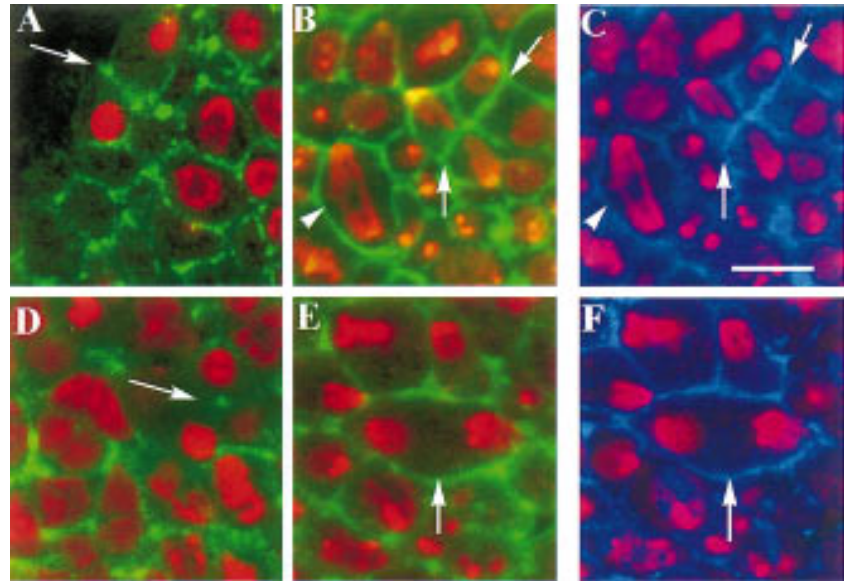


Figure 2. *pav* embryos show abnormal central spindles. A selection of confocal images showing telophase spindles from cycle 16 wild type (a–d) and *pav* (e–h) embryos stained to reveal β -tubulin (green), DNA (red), and the centrosomal antigen CP190 (blue). The wild-type spindle at telophase comprises a dense network of microtubules extending between the separated nuclei. Peripheral microtubules taper inward at the spindle equator due to the action of the contractile ring (arrowheads in a–d). A clear gap between the two halves of the spindle is also apparent (arrow in b). In contrast, the peripheral microtubules in *pav* telophases fail to taper inward (arrowheads in e–g) and the microtubules of the central spindle are generally more compact (a–h). (h) A rare telophase cell with four centrosomes and large nuclei. Measurements of centrosome–centrosome distances at metaphase and anaphase revealed no differences between wild type and *pav* mutants. Spindle lengths were $5.46 \pm 0.46 \mu\text{m}$ ($n = 32$) wild-type metaphase; $9.41 \pm 1.00 \mu\text{m}$ ($n = 43$) wild-type anaphase; $5.43 \pm 0.41 \mu\text{m}$ ($n = 10$) *pav* metaphase; $10.22 \pm 0.96 \mu\text{m}$ ($n = 17$) *pav* anaphase. Scale bar, 10 μ m.

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Figure 3. Neither Peanut protein, Actin, or Anillin becomes correctly localized at the end of mitosis in *pav* embryos. Wild-type (A–C) and *pav* (D–F) cycle 16 embryos were stained to reveal DNA (red) and either Peanut (A and D, green), Actin (B and E, green), or Anillin (C and F, blue). In wild type (A), Peanut is distributed beneath the cell surface, and at anaphase it accumulates near the nascent contractile ring (arrow) and remains associated with this region through telophase (arrow). In *pav* embryos (D) Peanut protein is variable in distribution and more diffuse than in wild type. Peanut is notably absent from the equatorial region of the cell during telophase (arrow). Dividing cells from wild-type embryos (B and C) accumulate actin (B) in the equatorial region at late anaphase (short arrow) through telophase (long arrow). The arrowhead shows a mid-anaphase figure, before this accumulation has occurred. The distribution of Anillin (C) at the end of mitosis is very similar to that of Actin. In addition, anillin shows faint nuclear staining during interphase. In *pav* embryos, neither Actin nor Anillin localize to the equatorial region during telophase (arrows in E and F, respectively). Scale bar, 10 μ m.



Anillin is thought to be involved in regulating or organizing the contractile domains of the Actin cytoskeleton (Field and Alberts 1995). It is found in the nucleus of wild-type cellularized embryos during interphase but is relocalized to the cortex during mitosis and to the cleavage furrow at cytokinesis (Fig. 3C). It appears at the cleavage furrow slightly preceding Actin. In *pav* mutant embryos, there is no indication of the accumulation of either Actin or Anillin at the position that the contractile ring and cleavage furrow normally form. However, the distribution of these proteins at other stages of the cell cycle appears to be unaffected (Fig. 3E,F).

pav encodes a KLP with homology to MKLP1

A route toward the cloning of the *pav* gene was provided by the finding of P-element-induced alleles *pav*^{934/13} and *pav*^{973/8} in a collection of third chromosome P-element insertion mutants (Deak et al. 1997; Salzberg et al. 1997). One of these mutants, *pav*^{934/13}, reverted to wild type following mobilization of the P element under dysgenic conditions, indicating that the P element is likely to be responsible for the mutation (Salzberg et al. 1997; Y. Inoue, pers. comm.). We rescued sequences flanking the P insertion at 64B-2-10 that detected 3.2-kb cDNA clones in 0- to 4-hr and 0- to 8-hr embryonic cDNA libraries (Brown and Kafatos 1988). DNA sequencing revealed that the P element lay 191 bp immediately 5' to the first ATG of the ORF within the cDNA. To test whether this candidate gene would rescue *pav* mutations, we introduced a 10.5-kb segment of genomic DNA containing 4 kb of DNA upstream and 3 kb downstream of the tran-

scription unit into the transformation vector pW8 (Klimentz et al. 1987) termed pRC1 in Figure 4. A transformant containing this 10.5-kb fragment is able to rescue two different alleles of *pav* that were tested (see Materials and Methods). To demonstrate that *pav* was rescued by the candidate gene immediately downstream of the P-element insertion, and not by some other gene present in the rescue construct, we made a second construct,

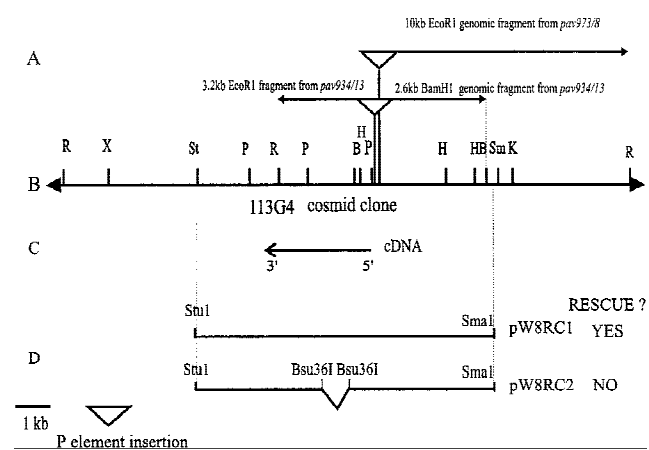


Figure 4. Physical map of the *pav* region. (A) Genomic fragments obtained by plasmid rescue of P-element mutants. (B) Restriction endonuclease cleavage map of a 20.5-kb segment of genomic DNA. Cleavage sites are: *Eco*RI (R); *Xba*I (X); *Stu*I (St); *Pst*I (P); *Hind*III (H); *Sma*I (Sm). (C) Location and polarity of 3.2-kb pNB*pav* cDNA. (D) Transformation constructs to test rescue of *pav* phenotype.

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PRC2, containing an internal 1.3-kb deletion within the coding region of the gene. Two independent transformants carrying this construct were unable to rescue *pav*, confirming the correct identity of the candidate gene.

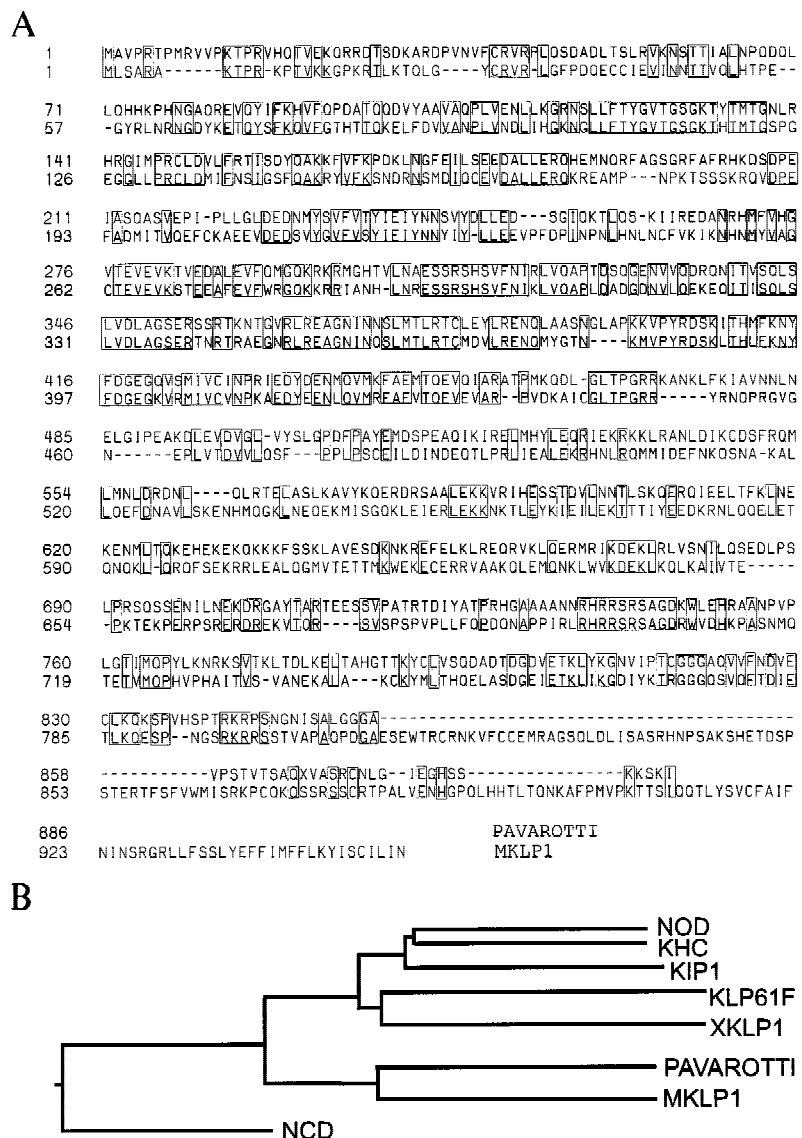
The sequence of a 3.2-kb *pav* cDNA reveals an ORF of 2658 bases coding for an 886-amino-acid protein of 100 kD predicted molecular mass with homology to the KLP family of microtubule motor proteins (Fig. 5). It contains a predicted microtubule motor domain of ~350 amino acids that includes a nuclear localization signal, an ATP binding site, and several motifs common to plus end-directed KLPs. A predicted coiled-coil region lies between residues 500 and 700, whereas residues 700–886 show homology only with the MKLP-1 subfamily of KLPs. PAV-KLP is most similar to this subfamily throughout its entire sequence, showing 34% identity with MKLP-1 as compared to 13% identity to other mitotic KLPs. The highest degree of identity (48%) lies in

the motor and hinge domains (residues 1–450), although patches of identity extend throughout the entire carboxy-terminal region of the protein (residues 700–886), a segment of KLPs known to be highly diverged. The MKLP-1 subfamily of KLPs comprises human MKLP-1 and its Chinese hamster counterpart CHO-1, first identified as a mitotic spindle antigen (Sellitto and Kuriyama 1988; Nislow et al. 1992.)

PAV-KLP associates with the spindle poles early in mitosis and with the spindle interzone from late anaphase

We wished to study the subcellular distribution of the PAV-KLP protein throughout the cell cycle and study its potential association with other molecules. To this end, we raised an antibody against a 450-amino-acid peptide from the central region of the protein (residues 208–686)

Figure 5. Comparison of the PAV-KLP with MKLP-1. (a) The full nucleotide sequence of the pNB*pav* cDNA and corresponding genomic region have been deposited in the EMBL databank. There are five putative initiation ATGs between the site of the P-element insertion and the longest ORF present in pNB*pav*. This ORF, located 191 bp, 3' to the site of the P-element insertion identifies a protein of 886 amino acids with extensive homology to MKLP-1 and a predicted molecular mass of 100 kD. PAV-KLP contains a consensus nuclear localization motif (KTPR) at amino acids 13–17, an ATP binding site (GSGKT) at amino acids 132–137, and two motifs common to all KLPs (SSRSHS and LAGSE) at residues 308–313 and 349–352, respectively. Analysis of the sequence using the Lupas algorithm (Lupas et al. 1991) predicts the formation of coiled-coil regions in the central region of the protein (residues 500–700; data not shown). The comparison of the amino acid sequences of PAV-KLP (*top row*) and MKLP-1 (*bottom row*) was made by Clustal analysis using DNASTAR software (Lasergene). Identical residues are boxed. Both sequences show substantial sequence identity in the first 450 amino acids, corresponding to the conserved motor domain. Regions of sequence identity also extend into the carboxy-terminal domains. Overall, PAV-KLP shows 34.3% identity with MKLP-1. (B) A phylogenetic tree illustrating the sequence homology between mitotic KLPs. PAV-KLP is related more closely to MKLP-1 than any other KLP shown. Other than these two KLPs, the remaining members of this superfamily that are shown are *Drosophila* NOD; *Drosophila* KHC (kinesin heavy chain); *S. cerevisiae* KIP1; *Drosophila* KLP61F; *Xenopus* XKLP1, and *Drosophila* NCD (Yang et al. 1988; McDonald et al. 1990; Zhang et al. 1990; Hoyt et al. 1992; Heck et al. 1993; Vernos et al. 1995).



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expressed in *Escherichia coli*. The antibody recognizes not only the bacterially expressed protein but also one predominant band at ~100 kD in Western blots of *Drosophila* embryo extracts (Fig. 6A). It immunostains wild type and *pav*^{B200/+} and embryos at cycle 16 to give distinct punctate nuclear staining in interphase cells in the ventral epidermis. In mitotic cells, bright bands of staining are seen equidistant from separating telophase nuclei, presumably at the site of the spindle interzone. Bright spots are also seen that appear to correspond to midbodies persisting from earlier divisions (Fig. 6B and see below). In contrast, *pav*^{B200} homozygous mutant embryos lack all nuclear and central spindle staining at cycle 16, except for some punctate spots likely to correspond to maternally contributed protein in remnants of putative midbodies from earlier cycles (Fig. 6C). Thus, the immunostaining is specific for PAV-KLP.

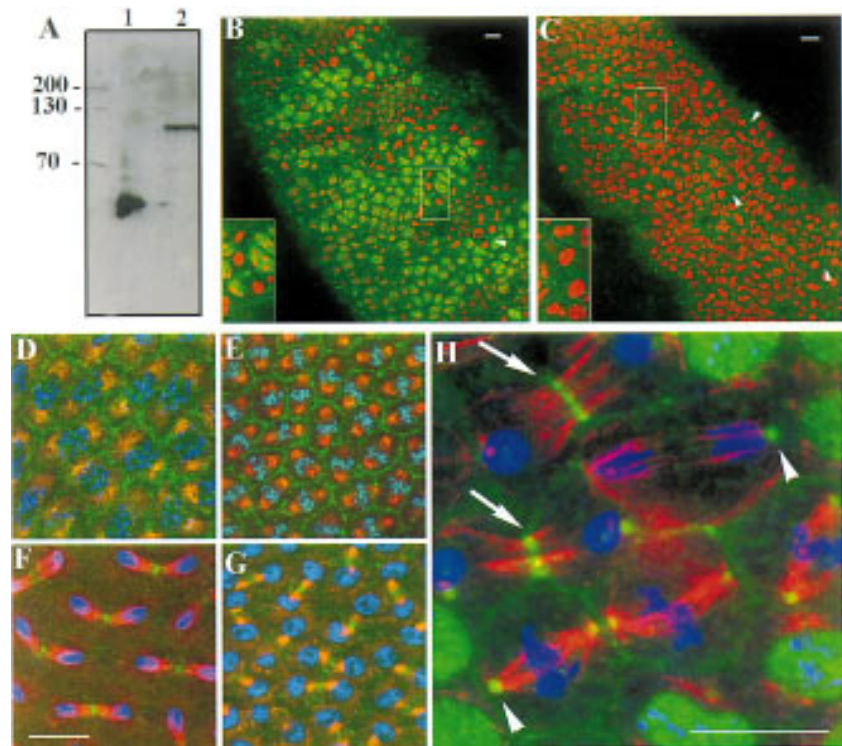
A closer examination of the localization of PAV-KLP revealed that in wild-type syncytial embryos, the protein is diffuse during interphase (Fig. 6D), but during prophase and prometaphase it becomes localized in the ac-

tin-containing furrows that separate nuclei and maintain nuclear spacing (Fig. 6E). During anaphase, the central, interzonal region of the mitotic spindle is stained (Fig. 6F). Anaphases showing staining at both sites are very rarely seen, even though the slight asynchrony between dividing nuclei in the blastoderm embryo allows all stages of anaphase to be observed in a single embryo. At telophase, staining intensifies at the spindle interzone, and as nuclei enter the next interphase the staining becomes diffuse once again (Fig. 6G).

In cellularized cycle 14 embryos (Fig. 6H) staining is nuclear during interphase. It is then possible to detect centrosomal staining in cells at metaphase and anaphase, and at late anaphase the protein begins to concentrate at the central region of the spindle. As telophase progresses, staining remains associated with the spindle during its constriction by the contractile apparatus.

As the septin homolog Peanut has been proposed to connect the central spindle with the contractile ring, we examined its distribution with respect to that of PAV-KLP. At late anaphase, Peanut becomes concentrated at

Figure 6. Subcellular localization of PAV-KLP at division. (A) Western blot analysis of *E. coli* cells induced to express the construct PpavEC2, and 0- to 4-hr *Drosophila* embryo extracts probed with the rabbit antiserum Rb3301. The antiserum recognizes the 52-kD polypeptide against which it was raised (lane 1) and a single protein of ~100 kD in the embryo extracts (lane 2). We were unable to carry out Western analysis on homozygous mutant embryos, as it proved difficult to distinguish these from their heterozygous siblings on the basis of the phenotype revealed by DNA staining alone. We could distinguish homozygotes from heterozygotes carrying a balancer chromosome expressing *lacZ*, but neither immunohistochemical staining methods to detect β -galactosidase preserved proteins sufficiently well for Western analysis. (B) Homozygotes and heterozygotes can, however, be distinguished by immunostaining with the anti-*pav* antibody, even though this antibody is not sensitive enough to detect levels of protein from single embryos on Western blots. *TM6B/TM6B* or *pav*^{B200}/*TM6B* embryos show strong nuclear staining in segmented bands of interphase cells using the antibody Rb3301 (green; DNA staining is in red). Bright regions of staining are also seen equidistant from separating anaphase cells (arrowhead and inset). Smaller punctate staining is also visible scattered throughout the embryo. (C) *pav*^{B200} homozygotes show neither nuclear staining at interphase nor equatorial staining at anaphase using the Rb3301 antibody (arrowheads and inset). However, there are some spots of staining distributed throughout the embryo, likely to correspond to the staining of residual maternally contributed protein. (D–G) Syncytial wild-type type embryos were stained to reveal PAV-KLP with Rb3301 (green), tubulin with monoclonal antibody YL1/2 (red), and DNA with TOTO-3 (blue). At prophase (D), PAV-KLP is diffusely distributed but is beginning to locate to the furrows separating the mitotic nuclei. PAV-KLP localization to the furrows persists through metaphase (E). Upon initiation of anaphase (F), PAV-KLP relocates to the spindle interzone region. At late telophase (G) PAV-KLP staining is concentrated at the midbody region of the spindle, and diffuse staining is reappearing in the cytoplasm. (H) A cellularized embryo at cycle 14 showing cells at various stages of mitosis. PAV-KLP is visible at the centrosomes in metaphase and anaphase cells (arrowheads) but does not appear at the spindle midzone until telophase, where it clearly colocalizes with microtubules (arrows). Interphase cells show punctate nuclear staining.



the spindle interzone, and colocalizes with PAV-KLP (data not shown). At telophase, we found that PAV-KLP remains associated with microtubules at the midbody, whereas peanut staining extends further and is concentrated at either side of the midbody closer to the plasma membrane. Thus, PAV-KLP and Peanut do not fully colocalize at this stage. However, they do colocalize in the previously identified small punctate regions of staining. As Peanut is known to be expelled from the cell at the end of mitosis (Neufeld and Rubin 1994), the presence of PAV-KLP in the same location supports the notion that these spots are remains of midbodies from previous divisions.

The localization of polo protein kinase is disrupted in pav embryos

It has been reported previously that MKLP1 can be detected in an immunoprecipitate of murine plk (Lee et al. 1995). In light of our results this association of the two proteins would be consistent with a role for plks in cytokinesis in metazoans, as has been shown in fission yeast (Ohkura et al. 1995). We therefore carried out immunoprecipitation experiments on extracts of *Drosophila* embryos using antibodies against Polo protein kinase. Western blot analysis indicates that PAV-KLP is present in the Polo immunoprecipitates obtained with either of three different anti-polo monoclonal antibodies but not with antibodies that recognize β -tubulin or the centrosomal component CP190 (Fig. 7). We therefore undertook immunostaining experiments to determine the extent to which these proteins colocalize throughout the

mitotic cycle. Using the mAb 294 anti-Polo antibody we were able to observe that in cells of wild-type embryos, Polo protein kinase localizes at centrosomes early in mitosis and, simultaneously, some staining is detected associated with the metaphase chromosomes (Fig. 8b). The immunostaining of the centrosomes by the anti-Polo antibody is progressively lost during anaphase-telophase concurrently with an increase in the staining of the midbody. By the end of telophase Polo protein seems to be associated only with the midbody. There is extensive colocalization of PAV-KLP to the centrosome and subsequently to the midbody during mitosis (Fig. 8a,b,c). In pav mutant embryos, on the other hand, no centrosome staining is observed either with the anti-PAV-KLP or the anti-Polo antibodies (Fig. 8d,e,f). The midbody staining is also dramatically changed in the mutant embryos, being completely lost with the anti-PAV-KLP antibody and dramatically reduced with the anti-Polo antibody. Thus it would appear that the localization of Polo protein kinase at the spindle poles and in the central spindle requires the function of PAV-KLP.

Discussion

Pav mutant embryos show cytokinesis defects in virtually all dividing cells at cycle 16. As with other genes required for cell cycle progression in *Drosophila*, the lethal phase depends on the role and stability of the wild-type protein, the abundance of its maternal provision to the embryo, and the strength of the mutant allele. The majority of cell cycle proteins are supplied maternally, and the rate at which this supply is exhausted depends on the particular protein. The maternal supply of PAV-KLP appears to be depleted early in development, either by degradation or its expulsion from newly divided cells in the remnants of the midbody. Consequently, the lethal phase occurs in embryonic development leading to the previously noted defects in the PNS, embryonic tissues in which cells continue dividing beyond a sixteenth cell cycle. Lethality at this stage is a consequence of the inability of each mutant allele to produce functional gene product, as all alleles of *pav* show the same lethal phase, whether homozygous or hemizygous against a chromosome deficiency for the region.

As the cell progresses through its division cycle, the distribution of PAV-KLP follows a highly dynamic pattern that is very similar to that of MKLP-1 (Nislow et al. 1990). Both the mammalian and fly proteins show a punctate distribution in the region of the nucleus at interphase. They are present at the centrosomes from metaphase onwards and localize to the spindle midzone at late anaphase and telophase. Many proteins with roles in cytokinesis, including PAV-KLP and Peanut, become concentrated in midbodies, the remnants of which are expelled from the two daughter cells as they separate, and are seen as discrete spots of staining between interphase cells in cellularized embryos.

The pattern of distribution of PAV-KLP in syncytial embryos differs in some respects from that seen in individual cells presumably reflecting the absence of cytoki-

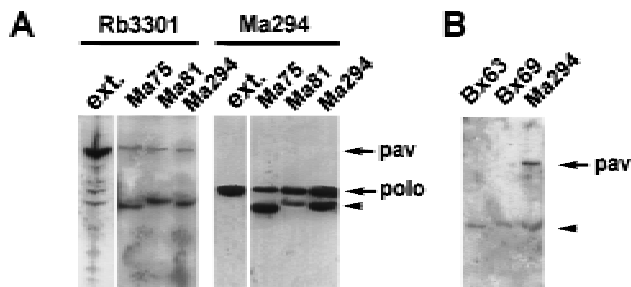
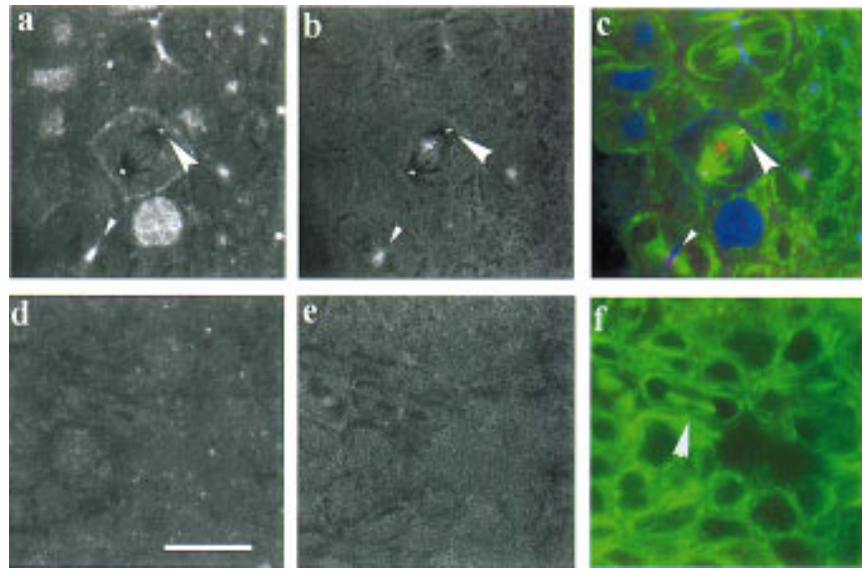


Figure 7. PAV-KLP coimmunoprecipitates with Polo protein kinase. (A) Polo was immunoprecipitated from *Drosophila* embryo extracts using three different antibodies (mAb 75, mAb 81, mAb 294), and the immunoprecipitates were analyzed by Western blot as described in Materials and Methods. An aliquot of the embryo extract was loaded in a separate lane as a control (ext.). The Western blot was first probed with mAb Rb3301 to detect PAV-KLP, and after washing, the membrane was re-probed with mAb 294 to detect Polo protein. (B) A comparison of immunoprecipitates obtained using Bx63, a mAb against the centrosomal-associated protein CP190; an anti- β -tubulin mAb Bx69; and the anti-polo mAb Ma294. The Western blot is probed with the anti-PAV-KLP Rb3301. (A,B) The lower band marked by the arrowhead corresponds to the antibodies used for the immunoprecipitations that are detected by the secondary antibody used in the Western blot.

Figure 8. The colocalization of Polo kinase with PAV-KLP at centrosomes and in the central spindle is disrupted in *pav* mutant embryos. Wild-type (*a-c*) or *pav*^{B200} (*d-f*) cycle 16 embryos stained for either PAV-KLP (*a,d*) or Polo (*b,e*). (*e,f*) Merged images of PAV-KLP (blue), Polo (red), and tubulin (green). In wild type, Polo colocalizes with PAV-KLP at the centrosome (large arrowhead in *a-c*) and the midbody (small arrowhead in *a-c*). In addition, faint Polo staining is apparent at the metaphase plate. However, in *pav* embryos, neither PAV-KLP (*d*) nor Polo (*e*) is present at the centrosomes or midbody of a mutant telophase (arrowhead in *f*).



nesis. The protein still shows a pronounced association with the central region of the spindle at late telophase but then follows a pattern that suggests association with actin or actin-associated molecules. Although cytokinesis does not take place at this stage of development, the organization of the actin cytoskeleton is important in maintaining the distribution of and spacing between the rapidly increasing numbers of syncytial nuclei. Thus, an actin-containing structure analogous to the contractile ring forms at the position of the former metaphase plate, but in interphase is redistributed to the cortex where it will form a “cap” that sits between the nucleus and plasma membrane. Upon entry into mitosis the cortical actin is reorganized into the “pseudocleavage furrows” that form a network capturing individual metaphase figures. In many respects these are analogous to cleavage furrows except that they are formed earlier in the mitotic cycle. PAV-KLP appears to concentrate in this network but is then liberated to associate with the spindle and move to the central region at late anaphase. Foe et al. (1993) have proposed that actin is continually binding to microtubules at this stage and moving toward their plus ends. One might speculate that PAV-KLP could provide the requisite motile force.

Mutations leading to cytokinesis defects have been described in genes encoding two other KLPs in *Drosophila*, *KLP3A*, and *KLP38B* (Williams et al. 1995; Ohkura et al. 1997). It is difficult to make comparisons between the mutant phenotypes, as in the case of *KLP38B*, a cytokinesis phenotype is inferred in larval neuroblasts that attain low levels of polyploidy, and from the observation of binucleate follicle cells in the ovary (Ohkura et al. 1997). *KLP3A* mutants also show a central spindle defect leading to a failure of cytokinesis, but this is seen in male meiosis (Williams et al. 1995). The differing lethal phases of the *pav* and *KLP3A* mutants suggest a specialized role for the two proteins for cytokinesis in different cell types, but an overlapping function of PAV-KLP in

meiosis cannot be ruled out at the present time. Both PAV-KLP and *KLP3A* accumulate in the midbody at telophase, although PAV-KLP has the distinctive attribute of being associated with the centrosome.

pav embryos initially show dramatic defects in the morphology of the central spindle at telophase. The simplest model for PAV-KLP function is that it participates in reorganizing the central spindle region after anaphase B has occurred, in a manner that is vital for assembly of the contractile ring. How the central spindle assists formation of the contractile ring is poorly understood. The septin Peanut, itself essential for cytokinesis in *Drosophila* (Neufeld and Rubin 1994), fails to localize to a ring-like structure in the *pav* mutants. This may be a direct consequence of the disrupted structure of the telophase spindle, as recent work with *Xenopus* shows that septins are microtubule-associated proteins, and that the injection of anti-septin antibodies will inhibit contractile ring function (Glotzer and Hyman 1996). The septins may link the central spindle to the cell cortex, and consequently the delocalization of peanut in *pav* mutants would disrupt the formation of the contractile ring. Anillin and actin also fail to associate with the equatorial region of the cell during anaphase and telophase, and consequently cells fail to divide and become tetraploid.

The role of the spindle poles in cytokinesis is less clear. Do they signal the onset of cytokinesis as suggested by Rappaport's experiments (see introductory section) or, rather, direct the positioning of cell cleavage? In *Drosophila* male meiosis, study of the male sterile mutation *asterless* reveals that in the absence of astral microtubules cell cleavage occurs to completion but in the wrong place (Bonaccorsi et al. 1996). A normal central spindle is seen in these mutants, indicating that in this system the central spindle may play some role in the recruitment of contractile ring components, whereas astral microtubules dictate the position of the cleavage furrow.

Our finding that *pav* is required for cytokinesis seems at odds with previous work suggesting that the mammalian homolog might function during either metaphase or anaphase. In vitro assays demonstrated an ability to cross-link and slide apart antiparallel microtubules, leading to the suggestion of a role in spindle pole separation during anaphase B, whereas the injection of anti-MKLP-1 antibodies into mitotic HeLa cells resulted in a metaphase arrest (Nislow et al. 1990, 1992). The in vitro assay may not be sufficiently specific to dissect the exact role of MKLP-1, as the system lacks centrosomes and the ancillary mechanisms necessary to generate a contractile ring, both of which influence microtubule behavior during cytokinesis. Thus, the ability to cross-link and slide along microtubules in vitro may be a property required of the KLP when associated with contractile ring structures. In any event, the *pav* mutant phenotype shows no indication of a defect in anaphase B, and we find that spindles extend to the same length as in wild-type embryos at late anaphase and telophase. Moreover, the rare binucleate cells that have lost *pav* function in the previous mitosis can perform anaphase perfectly well. Thus, if PAV-KLP does play a role in anaphase spindle elongation or spindle assembly, it would seem that this role is redundant and can be fulfilled by other KLPs. Although MKLP-1 is the most closely related klp to PAV-KLP, the sequence homology outside the conserved motor domain is not particularly high (20%), so there could be nonidentical functions. The idea that KLPs in the same subfamily can have distinct functions in different organisms is not without precedent. For example, the chromokinesin subfamily of DNA-binding KLPs involved in chromosome congression includes *Drosophila* KLP3A, which does not appear to bind DNA and is also required for cytokinesis (Williams et al. 1995). Nevertheless, as the size, domain structure, ability to associate with plks, and dynamic localization of *pav*-KP is so similar to MKLP-1, it would be surprising if it were not a functional homolog.

The presence of PAV-KLP and its mammalian counterparts at the centrosomes and the central region of the spindle raises the possibility that it may transport a signaling molecule required for either centrosome function, cytokinesis, or both. The serine-threonine protein kinase encoded by *polo* is an ideal candidate for such a molecule. *polo* mutants show abnormal centrosome behavior, including the formation of monopolar spindles, a phenotype that is also seen following the microinjection of anti-plk antibodies into mammalian cells (Sunkel and Glover 1988; Llamazares et al. 1991; Lane and Nigg 1996.) In addition to the formation of monopolar spindles, the additional consequence of disruption of the fission yeast homolog is to prevent cytokinesis (Ohkura et al. 1995). It is not clear whether the *polo* mutation results in cytokinesis defects in *Drosophila* as the mitotic cycle is blocked at an earlier stage. Nevertheless, the localization of polo kinase throughout the mitotic cycle would be consistent with a dual role for the enzyme similar to that demonstrated in fission yeast. The association that we have demonstrated between Polo ki-

nase and PAV-KLP leads us to speculate that in addition to a role in assembling the central region of the spindle, PAV-KLP may also be responsible for transporting Polo kinase from one set of centrosome-associated substrates to a second set of substrates in the midzone of the spindle as mitosis progresses.

Materials and methods

Stocks

Stocks were maintained under standard conditions (Roberts 1986). Wild-type embryos used were of the Oregon-R strain. EMS-induced alleles of *pav* were of genotype *yw*; +; *p[w⁺64A pav th cu sr e ca/TM6B-abd lacZ* (Salzberg et al. 1994). P-element-induced alleles of *pav* were of genotype *yw*; +; *p[w⁺ lacZ]/TM6B abd-lacZ* (Deak et al. 1997). The *lacZ* reporter gene under the control of the *abdA* promoter allowed homozygous embryos to be identified by an absence of β -galactosidase staining in the posterior half of the embryo.

Analysis of mutants and immunostaining

The *pav* alleles *pav^{B200}*, *pav^{M187}*, *pav^{M547}*, and *pav^{934/13}* are all embryonic lethal and show cytokinesis defects during mitosis 16, either as homozygotes or as hemizygotes. The *pav^{B200}* allele showed the greatest penetrance of the phenotype and was used for more detailed analysis. Immunostaining of embryos was performed as described previously (Glover and Gonzalez 1993). Freshly prepared 4% paraformaldehyde in buffer B (45 mM KCl, 15 mM NaCl, 10 mM phosphate buffer at pH 6.8) was found to be the best fixative. The following primary antibodies were used: tubulin (YL/12; 1:20; Harlan SeraLabs); CP190 (Rb188; 1:500; Whitfield et al. 1988); lamin A (T47; 1:20; Frasch et al. 1986); spectrin (anti-chicken spectrin; 1:200; Sigma); Peanut [mAb4C9; 1:10; kind gift of G. Rubin (Neufeld and Rubin 1994)]; anillin [Rb1-271; 1:200; kind gift of C. Field (Field and Alberts 1995)]; actin (C4; 1:200; ICN Biomedicals); Pav (Rb3301; 1:100; this study); Polo (mAb M294; 1:10; Tavares et al. 1996). FITC, Texas Red, and Cy5 secondary antibodies were purchased from Jackson Immunochemicals. DNA was stained using either 1 μ g/ml propidium iodide or 5 μ g/ml TOTO-3 (Molecular Probes). Specimens were viewed using either an MRC600 or MRC1024 confocal microscope (Biorad). Images were processed using PhotoShop (Adobe Systems) and printed using a Fargo dye-sublimation color printer.

Cloning *pav*

P elements and flanking genomic sequences were rescued from the strains *pav^{934/13}* and *pav^{973/8}* following DNA digestion with either *EcoRI* or *BamHI* followed by ligation, transformation into *E. coli XL1B* and selection for ampicillin-resistant colonies. This enabled the isolation of a 3.2-kb *EcoRI* fragment and a 2.6-kb *BamHI* fragment flanking both sides of the insertion in *pav^{934/13}*; and a 10-kb *EcoRI* fragment of flanking DNA from one side of the insertion in *pav^{973/8}*. The flanking fragments hybridized in situ to salivary gland chromosome region 64B2-7, the site to which *pav* has been mapped genetically (Salzberg et al. 1994). The 934/13 *EcoRI* fragment detected a number of similar cDNAs in 0- to 4-hr and 0- to 8-hr embryonic cDNA libraries (Brown and Kafatos 1988), kindly provided on a gridded filter by J. Hoheisel. The P-element-flanking fragments and the cDNA identified several cosmids when used as hybridization probes to the European Genome Project cosmid library, of

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which cosmid 113G4 was used as a source of a 10.5-kb *Sma*-*Stu*I fragment (termed RC1) for germ-line transformation experiments.

Germ-line transformation

*Not*I linkers (Stratagene) were ligated to both ends of the 10.5-kb *Sma*-*Stu*I fragment RC1. Following *Not*I digestion, RC1 was first subcloned into pKS and then into the *Not*I site of the pW8 transformation vector pW8 (Klemenz et al. 1987). Transformation was achieved following the injection of this plasmid, pRC1^{w+}, into embryos derived from homozygous *w*⁻; *p*[Δ 2-3] flies following standard procedures. A transformed line was established with the insertion on the third chromosome. This was maintained as a stock balanced against *TM6B*. The transforming P element was mobilized by crossing this stock to one carrying the Δ 2-3 P element on chromosome 3 as a source of transposase. Transposition events to the second chromosome in the resulting *p*[RC1 *w*⁺]/*p*[Δ 2-3] *Sb* *ry* *e* males were detected in a subsequent generation by the generation of *Sb* *w*⁺ males in a *w*⁻ background. Crossing to *Tft*/*CyO* females enabled a balanced stock to be selected. Successful rescue was demonstrated by first crossing this *p*[RC1 *w*⁺]/*CyO* stock to *TM3*/*TM6B* to select *p*[RC1 *w*⁺]; *TM3* progeny, which were sequentially crossed to *pav*^{M547}/*TM6B* and *pav*^{B200}/*TM6B* stocks for the rescue of *pav*^{M547}/*pav*^{B200}. As a negative control, a second construct, pRC2, was made by digestion of pW8RC1 with *Bsu*36I followed by recircularization using blunt end ligation to delete a 1.3-kb *Bsu*36I fragment from within the coding region of the gene. This was transformed into flies using standard procedures to obtain five independent transformant lines. Two lines in which the pW8RC2 construct was carried on the X chromosome were tested for their ability to rescue *pav* by sequential crosses to *pav*^{M547}/*TM6B* and *pav*^{B200}/*TM6B* stocks testing for the capability to generate *w*⁻ *p*[RC2 *w*⁺]; *pav*^{M547}/*pav*^{B200} flies. Neither transformant was capable of rescuing *pav*.

Production of anti-Pav antibodies

An amino-terminally His-tagged protein fragment of PAV-KLP was expressed in *E. coli*, purified, and used to immunize rabbits. A 1.4-kb *Bam*HI-*Pst*I of *pav* cDNA coding for amino acids 208-686 was subcloned into pQE31 (Qiagen) to make pEC2. pEC2 was transformed into *E. coli* M15/*REP4* cells. Induced protein was purified on a Ni-agarose column under denaturing conditions according to the manufacturer's instructions (Qiagen) and then dialyzed in PBS. Protein was injected into rabbits at 1 mg/ml with Freund's adjuvant. After four boosts, serum was obtained that recognized a single band of 100 kD on Western blots of total *Drosophila* embryo extracts.

Immunoprecipitations and Western blotting

Polo-kinase immunoprecipitations were carried out using the three previously described monoclonal antibodies mAb 75, mAb 81, and mAb 294 (Llamazares et al. 1991; Tavares et al. 1996) coupled to magnetic beads coated with sheep anti-mouse IgG (DYNAL). Briefly, 0- to 8-hr *Drosophila* embryos were homogenized in 5 volumes of NEWIP buffer (25 mM Tris at pH 7.5, 1% Triton X-100, 20 mM glycerophosphate, 20 mM NaF, and 5 mM EGTA) supplemented with 1 mM PMSF and 1 μ g/ml aprotinin, pepstatin A, and leupeptin, and centrifuged for 10 min at 14000g to remove cellular debris. The supernatant obtained was thoroughly chilled to 4°C to depolymerize microtubules and incubated with the antibodies coupled to Dyna beads for 1.5 hr at 4°C. As a control an aliquot of the extract was incubated with

Dyna beads not coupled to anti-polo antibodies. The immunoprecipitates were washed four times with NEWIP buffer and resuspended in SDS sample buffer, and the proteins separated on SDS-10% polyacrylamide gels. The proteins were transferred to Immobilon-P membrane (Millipore), and the Westerns were executed as described previously (Tavares et al. 1996) using the antibody Rb3301 diluted 1/250 to detect PAV-KLP, and the antibody mAb 94 diluted 1/5 to detect Polo.

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***pavarotti* encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis**

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