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The role of PAR-1 in regulating the polarised microtubule cytoskeleton in the *Drosophila* follicular epithelium

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SUMMARY

The PAR-1 kinase plays a conserved role in cell polarity in *C. elegans*, *Drosophila* and mammals. We have investigated the role of PAR-1 in epithelial polarity by generating null mutant clones in the *Drosophila* follicular epithelium. Large clones show defects in apicobasal membrane polarity, but small clones induced later in development usually have a normal membrane polarity. However, all cells that lack PAR-1 accumulate spectrin and F-actin laterally, and show a strong increase in the density of microtubules. This is consistent with the observation that the mammalian PAR-1 homologues, the MARKs, dramatically reduce the number of microtubules, when overexpressed in tissue culture cells. The MARKs have been proposed to destabilize microtubules by inhibiting the stabilizing activity of the Tau family of microtubule-associated proteins. This is not the case in *Drosophila*, however, as null mutations in the single *tau* family member in the genome have no effect on the microtubule organisation in the follicle cells. Furthermore, PAR-1 activity stabilises microtubules, as microtubules in mutant cells depolymerise much more rapidly after cold or colcemid treatments. Loss of PAR-1 also disrupts the basal localisation of the microtubule plus ends, which are mislocalised to the centre of mutant cells. Thus, *Drosophila* PAR-1 regulates the density, stability and apicobasal organisation of microtubules. Although the direct targets of PAR-1 are unknown, we suggest that it functions by regulating the plus ends, possibly by capping them at the basal cortex.

Key words: PAR-1, Tau, Follicle cells, *Drosophila*

INTRODUCTION

Apicobasal polarity is essential for the formation and morphogenesis of epithelia, and for their function as selective permeability barriers between different compartments of the body. Much of our knowledge of this process comes from work on the polarisation of cultured mammalian cells, such as Madin Darby canine kidney (MDCK) cells, and from genetic analysis of *Drosophila* epithelia, particularly the primary embryonic epithelium and the follicular epithelium that surrounds the developing oocyte (Tepass et al., 2001; Yeaman et al., 1999). This has shown that polarity is induced by external cues, such as adhesion to the extracellular matrix through integrins, and cadherin-dependent adhesion between cells. These interactions lead to the partitioning of the cell membrane into apical and basolateral domains that accumulate different sets of membrane proteins, which become separated by the formation of a series of cell junctions along the apicobasal axis of the lateral membrane (Müller, 2000).

These membrane asymmetries are propagated to other cellular compartments. In the *Drosophila* follicular epithelium, for example, the cortical spectrin cytoskeleton becomes polarised into an apical domain that is composed of β_{heavy} spectrin and α-spectrin, and a basolateral domain that contains β-spectrin/α-spectrin complexes (Lee et al., 1997). Actin also becomes enriched in the apical cortex of these cells, as in other epithelia (Baum et al., 2000; Mooseker, 1985). The microtubule cytoskeleton is also polarised to form an array of very stable microtubules (MTs) that run parallel to the apicobasal axis, with their minus ends at the apical membrane and their plus ends oriented toward the basal membrane (Bacallao et al., 1989; Bre et al., 1990; Clark et al., 1997). In mammalian cells, at least, the distinct membrane domains are further reinforced by sorting in the secretory pathway that delivers different sets of proteins and lipids to the apical and basolateral domains (Keller and Simons, 1997). Many epithelial cells also mediate polarised transcytosis to transport extracellular factors from one side of the epithelium to the other (Mostov et al., 2000).

Although the steps in the establishment of epithelial polarity are well characterised, little is known about how the initial extracellular cues are transduced to polarise the different components of the cell. One group of proteins that appear to play an essential role in this process are the PAR proteins,

which were originally identified because they are required for the anterior-posterior polarity of the *C. elegans* zygote (Kemphues et al., 1988). Three of these proteins, PAR-3, atypical Protein Kinase C (aPKC) and PAR-6, form a conserved protein complex that localises to the anterior cortex of the one cell zygote, where they are required for the asymmetry of the first cell division (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Tabuse et al., 1998; Watts et al., 1996). The *Drosophila* homologues of PAR-3 (Bazooka), PAR-6 and aPKC localise to a sub-apical region in epithelial cells to define the position of the most apical junction, the zonula adherens, and loss of any of these proteins leads to a loss of polarity (Kuchinke et al., 1998; Muller and Wieschaus, 1996; Petronczki and Knoblich, 2001; Wodarz et al., 2000). The complex shows a similar localisation to the most apical junction in mammalian epithelia, in this case the tight junction, and overexpression of kinase-dead aPKC disrupts the localisation of the tight junction proteins and causes the mislocalisation of apical membrane proteins (Izumi et al., 1998; Suzuki et al., 2001).

The conserved serine/threonine kinase PAR-1 has also been implicated in cell polarity in several contexts (Böhm et al., 1997; Guo and Kemphues, 1995; Shulman et al., 2000; Tomancak et al., 2000). PAR-1 localises to the posterior of the *C. elegans* zygote in a complementary pattern to the PAR-3/PAR-6/aPKC complex, and is required for the asymmetric positioning of the mitotic spindle and the posterior localisation of the P granules (Guo and Kemphues, 1995). *Drosophila* PAR-1 is required for anteroposterior polarisation of the oocyte at two stages of oogenesis. Null mutations in *par-1* block the formation of a microtubule organising centre (MTOC) at the posterior cortex of the early oocyte, resulting in the loss of oocyte fate (Cox et al., 2001a; Huynh et al., 2001b). The oocyte is specified normally in hypomorphic *par-1* mutants, but the repolarisation of the oocyte MT cytoskeleton that defines the anteroposterior axis of the embryo is disrupted (Shulman et al., 2000; Tomancak et al., 2000). In mid-stage oocytes, an unidentified signal from the posterior follicle cells induces the disassembly of the original posterior MTOC, and leads to the formation of a new MT array, in which most MTs are nucleated from the anterior with their plus ends extending towards the posterior pole (Cha et al., 2001; Clark et al., 1994; Clark et al., 1997; Theurkauf et al., 1992). In *par-1* mutants, MTs are found more evenly around the oocyte cortex, and the plus ends are focussed on the centre of the oocyte rather than the posterior (Benton et al., 2002; Shulman et al., 2000). As a consequence, *oskar* mRNA is mislocalised to the middle of the oocyte, and the resulting embryos therefore lack an abdomen and germline. Thus, the principal function of PAR-1 in *Drosophila* axis formation appears to be to organise a polarised MT array.

Several results indicate that PAR-1 is also required for epithelial polarity. A mouse PAR-1 homologue, EMK, localises to the basolateral membrane of polarised MDCK cells, and overexpression of EMK lacking the kinase domain causes these cells to lose their columnar morphology and be extruded from the monolayer (Böhm et al., 1997). PAR-1 shows a similar localisation to the basolateral cortex of *Drosophila* follicle cells, and removal of PAR-1 from these cells results in defects in epithelial organisation and the mislocalisation of membrane proteins (Cox et al., 2001a; Shulman et al., 2000). As is the case in the oocyte, PAR-1 appears to play a particularly important role in regulating the MT cytoskeleton in epithelial cells, as mutant follicle cells have been reported to lack MTs (Cox et al., 2001a).

The mammalian PAR-1 homologues, MARK1 and MARK2, also regulate MTs, and are thought to act by phosphorylating the microtubule associated proteins (MAPs), Tau, MAP2 and MAP4 (Drewes et al., 1995; Illenberger et al., 1996). These MAPs contain three to four copies of a conserved MT-binding domain (MTBD), and bind along the length of MTs to stabilise and stiffen them (Chapin and Bulinski, 1992). Tau and MAP2 are highly expressed in neurons, and localise to axons and dendrites, respectively, whereas MAP4 is expressed more widely (Matus, 1991). MARKs phosphorylate a conserved KXGS motif in the MTBDs of these proteins, which inhibits their binding to MTs (Drewes et al., 1997; Illenberger et al., 1996). MARK activity should therefore decrease MT density. Consistent with this, overexpression of the MARKs leads to hyperphosphorylation of these MAPs, and causes a breakdown of the MT cytoskeleton (Drewes et al., 1997; Ebneth et al., 1999). Thus, the MARKs seem to have the opposite effect on MTs to *Drosophila* PAR-1, which is required to maintain the MT array (Cox et al., 2001a). This apparent difference between the function of the mammalian and *Drosophila* kinases prompted us to analyse how *Drosophila* PAR-1 regulates MT organisation in epithelial follicle cells, and to investigate the role of Tau family of MAPs in this process.

MATERIALS AND METHODS

Fly stocks

The following fly stocks were used in this work:
w; FRT-G13-par-1^{W3}/CyO (Shulman et al., 2000)
y,w,hs-flp; FRT-G13-nlsGFP/CyO (Luschnig et al., 2000)
w; FRT-G13-par-1$^{\Delta 16}$ /CyO (Cox et al., 2001a)
w; UASp-par-1(N1S)-GFP/TM3,Sb (Huynh et al., 2001b)
Gal4 follicle cell driver: w; E4 (Queenan et al., 1997)
w; 133.4 Nod:lacZ (Clark et al., 1997)
w; KZ503 Kin:lacZ (Clark et al., 1994)
w; FRT-82B-Df(3R)MR22(tau)/TM3, Ser act:GFP (this work)
w,hs-flp; FRT-82B-nlsGFP (Chou and Perrimon, 1996).

Follicle cell clones

Follicle cell clones were generated by the FLP/FRT technique (Chou et al., 1993; Chou and Perrimon, 1996), using the FRT-G13-nlsGFP chromosome. Clones were induced by heat-shocking third instar larvae or adult females at 37°C for 2 hours on two consecutive days. Female were dissected 1 day after the last heat-shock.

Colcemid treatment

Flies were starved for 5 hours and then fed with 200 μg/ml colcemid (Sigma) mixed with some dry yeast for 16 hours, and dissected immediately.

Cold shock

Females were kept on ice for 1 hour and dissected either immediately or after a recovery time at room temperature.

Fluorescence quantification

Quantification of the intensity of the GFP and the α-Tubulin staining was measured using the Laser Pix4 software (BioRad) (Cha et al., 2002).

Cloning of *Drosophila tau*

tau cDNAs were isolated from the Berkeley *Drosophila* Genome Project (BDGP) adult head (GH) ZAPII cDNA library using a PCR product spanning the genomic region encoding the Tau MTBD as a probe, following standard procedures.

Antibody production and western analysis

The Tau antibody was raised in rabbits against a 6×His tagged C-terminal fragment of Tau-A (amino acids 183-375), and affinity purified using a purified MBP fusion of the same fragment, following standard procedures (Harlow and Lane, 1988; Huang and Raff, 1999). Embryo extracts for SDS-PAGE/western blotting were prepared by boiling and homogenising 12-18 hours embryos in Laemmli sample buffer.

tau mutant generation

EP(3)3597 and *EP(3)3203* were identified from the BDGP P element disruption project collection database. To generate deletions uncovering the *tau* locus, we induced P element-mediated male recombination (Preston et al., 1996), between the *EP(3)3203* chromosome and a homologue bearing the flanking visible markers *ebony* (*e*) and *claret* (*ca*). From 14,000 progeny, we identified one *e ca*$^{+}$ recombinant chromosome (MR22), which was homozygous lethal but retained the original *EP(3)3203* insertion. Using inverse PCR, we determined the presence of a deletion between the 5′ end of *EP(3)3203* and position 144091 in genomic contig AE003761. This deletion, *Df(3R)MR22(tau)*, was recombined on to the FRT 82B chromosome to generate homozygous clones.

Microtubule-spin down assay

Twelve- to 18-hour-old embryos were homogenised in an equal volume of C-buffer (50 mM HEPES, pH 7.6, 1 mM $MgCl_2$, 1 mM EGTA) with a Complete Protease Inhibitor cocktail (Roche). The extract was centrifuged for 1 hour at 100,000 ***g***. Dithiothreitol and GTP were added to the supernatant to 1 mM final concentration, and this was split into two equal aliquots. To one aliquot, Taxol was added to 10 μm, to polymerise the tubulin, whereas only buffer was added to the other. The supernatants were warmed to 25°C for 5 minutes to allow polymerisation to initiate, and then shifted to 4°C for a further 15 minutes. The supernatants were layered onto a 2 volume cushion of C-buffer with 50% sucrose and this was centrifuged at 100,000 ***g*** for 10 minutes. Both supernatant and pellet were resuspended in Laemmli sample buffer and analysed by SDS-PAGE and western blotting.

Generation and analysis of transgenic lines

The *pUASp-tau-A:mGFP6* transgene contains the full-length *tau-A* ORF, lacking the STOP codon, upstream of the GFP variant, mGFP6, in the pUASp vector (Rørth, 1998; Schuldt et al., 1998). Transgenic lines were generated by standard methods and crossed to *nanos-GAL4:VP16*. Females were dissected in 10S Voltaleff oil (Atochem) and viewed under an inverted confocal microscope.

Kinase assay

In vitro kinase assays were performed as previously described (Benton et al., 2002). The MBP:Tau-A MTBD (amino acids 144-375) substrate was expressed in and purified from bacteria. The mutant variant (‘KXGA’), containing the mutations S184A, S243A, S275A and S305A in the four KXGS motifs, was generated by oligonucleotide-directed mutagenesis.

Staining procedures

Females were fattened for 24 hours and the ovaries dissected in PBT (PBS + 0.1% Tween), fixed for 10 minutes or 20 minutes with 8% or 4% paraformaldehyde/PBT respectively, washed three times for 10 minutes with PBT, blocked with PBT-10 (PBT + 10% BSA) for 1 hour and incubated with the antibody in PBT-1 (PBT + 1% BSA) overnight. After several washes with PBT for 2 hours, the ovaries were incubated with the secondary antibody for 4 hours. They were finally washed three times with PBT for 15 minutes and mounted in Vectashield (Vector). All steps were performed at room temperature. Primary antibodies were used as follows: mouse anti-Armadillo (7A1) (1/200) (Riggleman et al., 1990); rat anti-*D*E-Cadherin (1/2000) (Oda et al., 1994); mouse anti-Crumbs (cq4) (1/50) (Tepass et al., 1990); rabbit anti-β-gal (1/2000, ICN Pharm, Cappel); mouse anti-β-PS-Integrin CF6G11 (1/3) (Brower et al., 1984); mouse anti-Notch^{ICD} (C179C6) (1/1000) (Fehon et al., 1991); rat anti-Neurotactin BP106 (1/40) (Hortsch et al., 1990); rabbit anti-nPKC (1/500, Santa Cruz Biotechnology); rabbit anti-α-Spectrin (1/500) (Byers et al., 1987); rabbit anti-β-Spectrin (1/200) (Byers et al., 1989); mouse anti-β_{heavy} spectrin (1/200) (Thomas and Kiehart, 1994); mouse anti-α-Tubulin DM1A (1/500, Sigma). FITC- and Red Texas-conjugated secondary antibodies (Molecular Probes) were used at 1/100 dilution. Actin staining was performed with Rhodamine-conjugated phalloidin (Molecular Probes). All confocal micrographs were collected using a BioRad MRC1024 scan head mounted on a Nikon E800 microscope.

RESULTS

par-1 null clones cause partially penetrant defects in apicobasal polarity

par-1 null mutations are homozygous lethal, and we therefore used the FLP/FRT technique to generate homozygous mutant clones for the null alleles, *par-1*^{W3} and *par-1*$^{\Delta 16}$, which were marked by the absence of nuclear GFP expression (Luschnig et al., 2000; Xu and Rubin, 1993). We only obtained a low frequency of large *par-1* mutant clones, compared with that of the sibling twin spot clones, suggesting that many of the cells either die or are lost from the epithelium. The large clones that survive show penetrant defects in apicobasal polarity. The apical Bazooka/PAR-6/ aPKC complex is not localised, as shown by aPKC staining, and the mutant cells often fail to form a coherent single-layered epithelium (Fig. 1A). Smaller clones, which are presumably induced after the epithelium has formed, display a much lower frequency of defects which are usually milder: 74% of clones showed an apical localisation of aPKC, 12% showed a marked reduction in this localisation and 14% show no apical enrichment at all (n=42 clones) (Fig. 1B and data not shown). The penetrance of these defects tends to increase with the size of the clone and the stage of oogenesis, but even single mutant cells can show a complete delocalisation (Fig. 1C).

We also examined other markers for membrane polarity, including the apical transmembrane proteins, Crumbs, Notch and Neurotactin, the zonula adherens components, *D*E-cadherin and Armadillo, and the basolateral transmembrane protein, β-PS Integrin, which mediates attachment of epithelial cells to the basement membrane (Bateman et al., 2001; Quaranta, 1990; Tepass et al., 2001). In all cases, the proteins were delocalised in only a proportion of small clones (Fig. 1C,D and data not shown). Mutant clones also caused occasional defects in the organisation of the follicular epithelium without disrupting apicobasal polarity: single mutant cells often failed to span the epithelium (Fig. 4D), and small clones sometimes led to the formation of more than one layer of cells (Fig. 1D).

As membrane polarity appears largely normal in small *par-1* clones, we next asked whether the cortical spectrin cytoskeleton is affected. As is the case for the membrane markers, the majority of mutant cells in small late clones show

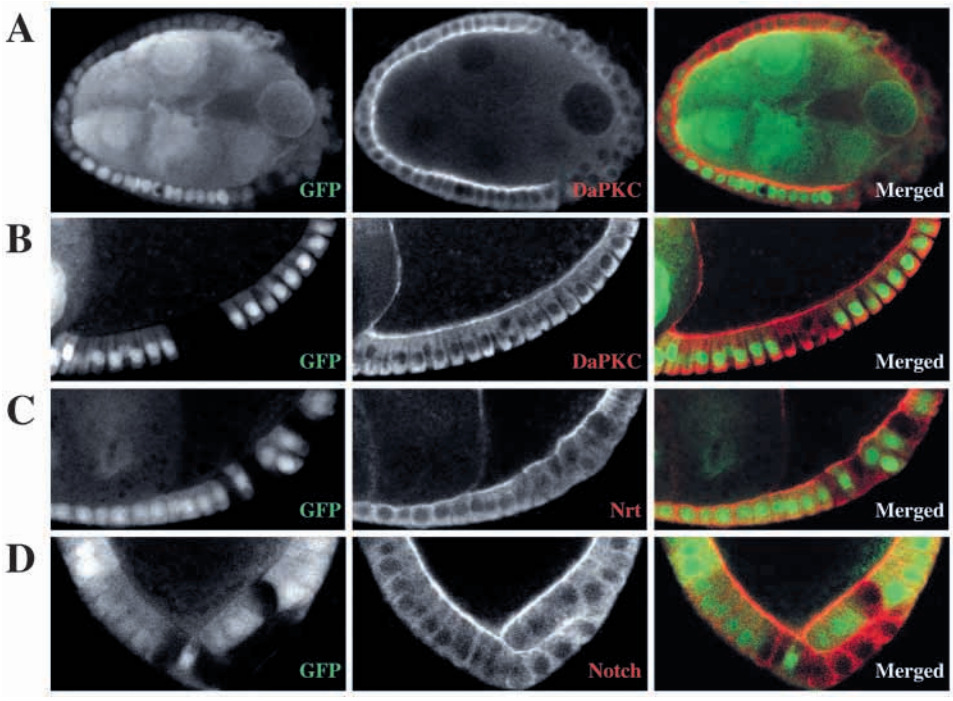

Fig. 1. Loss of PAR-1 causes partially penetrant defects in follicle cell polarity. In all the figures, the follicular epithelium is shown with its apical side (which faces the oocyte) towards the top of the picture and its basal side towards the bottom. (A) Stage 6 egg chamber containing a *par-1* mutant clone induced early in oogenesis, marked by the loss of nuclear GFP (in this and all subsequent figures of clones, GFP is shown in the first column, and is shown in green in the merged images in the third column). Mutant cells lose their epithelial organisation, and fail to localise DaPKC apically (centre panel: red in merged image). (B) A stage 10a egg chamber containing a smaller clone induced later in oogenesis, showing normal epithelial organisation and DaPKC localisation. Note that the nuclei are no longer in a consistent position in mutant cells. (C) Stage 9 egg chamber containing three mutant cells stained for the apical marker Neurotactin (Nrt). Most mutant cells in small clones show a wild-type apical localisation of Nrt (top right mutant cell), but some cells show reduced localisation (middle) or no localisation at all (bottom left). (D) Stage 9 egg chamber containing a small mutant clone stained for Notch, which localises apically as in wild type, even when the mutant cells form a double layered epithelium.

a normal localisation of β_{heavy} spectrin to the apical cortex, and of β-spectrin to the basolateral cortex (Fig. 2A and data not shown). However, all mutant cells show a marked increase in the amount of β-spectrin staining along the lateral cortex (Fig. 2A). Because β-spectrin binds to actin, we also examined the actin cytoskeleton of mutant cells by staining with rhodamine-phalloidin. Mutant cells show an increase in the amount of F-actin along the lateral cortex, compared with the adjacent cells (Fig. 2B,C). Thus, PAR-1 is required to limit β-spectrin and F-actin recruitment to the lateral cortex in all cells, even when other aspects of apicobasal polarity are normal.

PAR-1 regulates the density, stability and polarity of MTs

As the *par-1* phenotypes in anteroposterior axis formation of the oocyte are caused by an alteration in the organisation of the MTs, we analysed the arrangement of the MTs in *par-1* mutant follicle cells, using the optimised procedure for preserving MTs in *Drosophila* described by Theurkauf (Theurkauf, 1994). All mutant cells show an increase in the density of MTs compared with their neighbours (Fig. 3A). Quantification of the fluorescence intensity reveals that *par-1* mutant cells show nearly twice the level of microtubule staining as wild-type follicle cells (Fig. 3D). As this phenotype is fully penetrant, regardless of

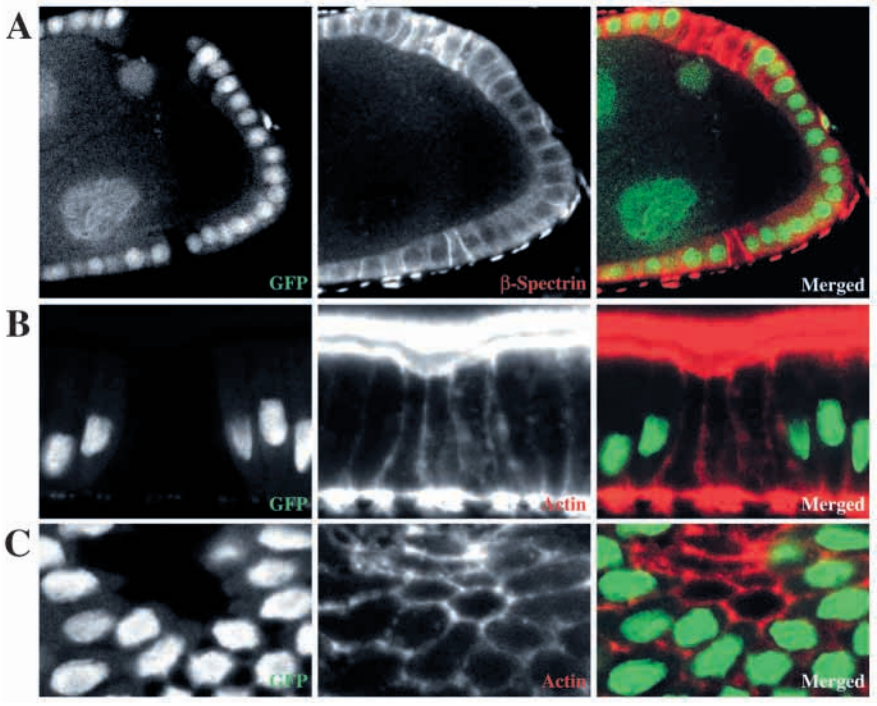

Fig. 2. β-spectrin and F-actin are enriched laterally in *par-1* clones. (A) β-Spectrin localisation in a stage 9 egg chamber containing two small *par-1* clones. β-Spectrin still localises to the lateral cortex of mutant cells, but is present in higher amounts than in wild-type cells. (B,C) Small clones in stage 10 egg chambers in which F-actin has been labeled with rhodamine-phalloidin. Mutant cells show an increase in F-actin along the lateral cortex. (B) Sagittal view. (C) Horizontal view at the level of the nuclei. GFP, green; β-Spectrin or Actin, red.

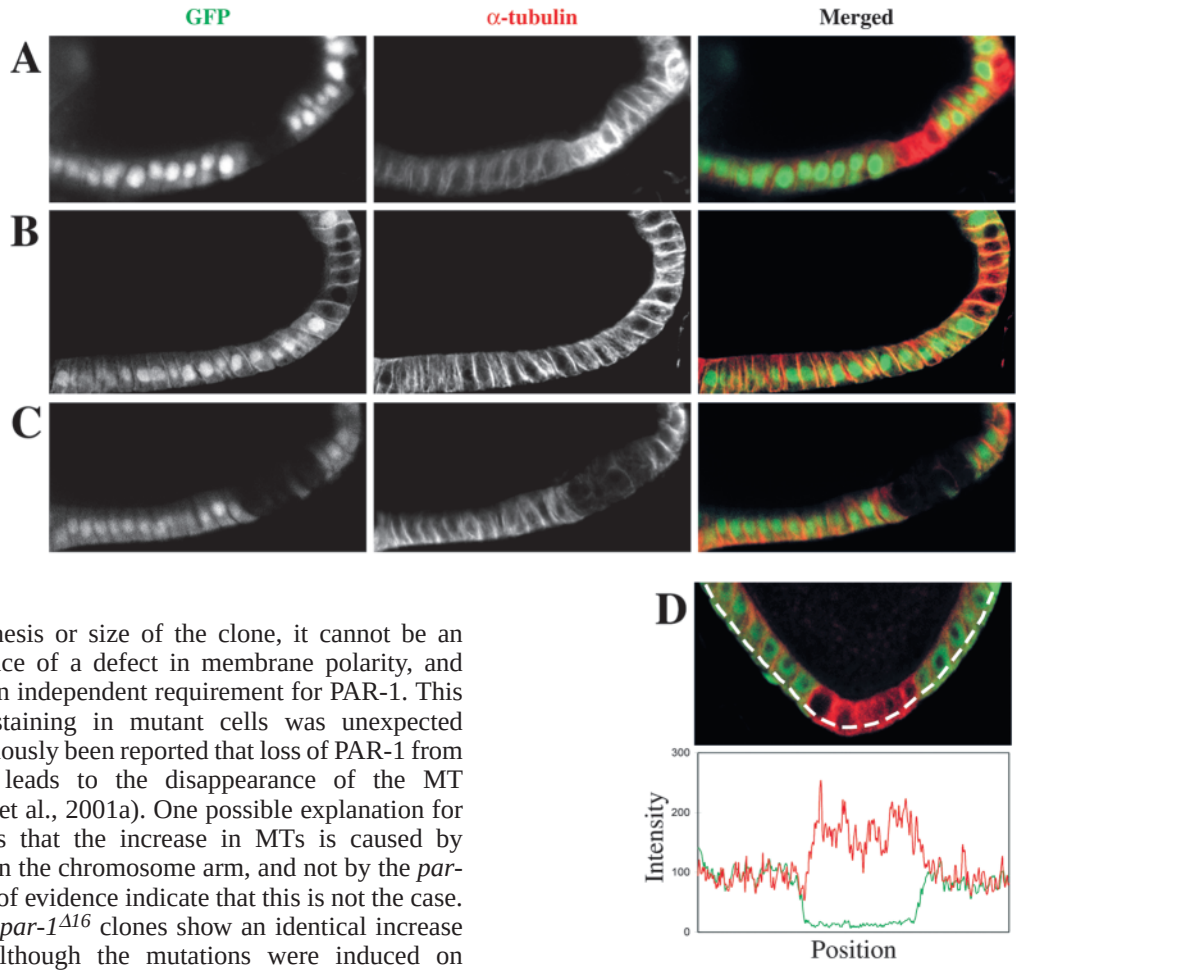

Fig. 3. MT density is increased in all *par-1* mutant clones. (A) A stage 10 egg chamber containing two small *par-1* mutant clones, fixed under optimised conditions for preserving MTs (8% PFA) and stained for α-Tubulin. The mutant cells contain more MTs than the adjacent wild-type cells. (B) Expression of a GFP-PAR-1 fusion protein rescues this phenotype. The mutant cells that express the transgene can be identified by the presence of lateral GFP-PAR-1 signal and by the loss of nuclear GFP, and show a MT network that is similar to that in the neighbouring wild-type cells. (C) Under standard fixation conditions (4% PFA), *par-1* clones appear to lack MTs, suggesting that MTs are less stable than in wild type. (D) Quantification of the intensity of the GFP and the α-Tubulin staining in wild-type and mutant follicle cells. Fluorescent signal was measured along the broken white line using the Laser Pix4 software (BioRad). The *par-1* mutant cells, which are marked by the decrease in the GFP fluorescence (green line), show twice as much microtubule staining (red line) as the wild-type cells. GFP, green; α-Tubulin, red.

the stage of oogenesis or size of the clone, it cannot be an indirect consequence of a defect in membrane polarity, and therefore reflects an independent requirement for PAR-1. This increase in MT staining in mutant cells was unexpected because it has previously been reported that loss of PAR-1 from the follicle cells leads to the disappearance of the MT cytoskeleton (Cox et al., 2001a). One possible explanation for this discrepancy is that the increase in MTs is caused by another mutation on the chromosome arm, and not by the *par-1* allele. Two lines of evidence indicate that this is not the case. First, *par-1*^{W3} and *par-1*$^{\Delta 16}$ clones show an identical increase in MT density, although the mutations were induced on different chromosomes. Second, this phenotype can be rescued by expressing a GFP-PAR-1 transgene in the mutant cells (Fig. 3B).

An alternative explanation for the opposite effects of the removal of PAR-1 on MTs in our experiments and those of Cox et al. is that they are a consequence of different fixation procedures. We therefore repeated these experiments using the standard fixation with 4% formaldehyde that is normally used for ovary immunostaining, instead of the 8% formaldehyde fixative used for preserving MTs. Although this change has little effect on the MTs in wild-type/heterozygous cells, the adjacent homozygous mutant cells now appear to contain almost no MTs (Fig. 3C). These results suggest that the MTs in mutant cells are extremely unstable and depolymerise rapidly under slower fixation conditions, whereas the MTs in wild-type follicle cells are insensitive to the method of fixation.

To confirm that the MTs are less stable in mutant cells, we examined their resistance to treatments that promote MT depolymerisation. The MTs in follicle cells are extraordinarily resistant to disassembly, as they are still present after more than 24 hours exposure to the MT-depolymerising drug, colcemid. By contrast, *par-1* mutant follicle cells fixed under optimised conditions lack visible MTs after short colcemid treatments (Fig. 4A). The disassembly of dynamic MTs is also promoted by cold, and we therefore kept females on ice for 1 hour before fixing their ovaries with the optimised protocol. The mutant cells again appear to lack MTs, whereas the MTs in the non-mutant cells become fuzzy (Fig. 4B). This depolymerisation is reversible, because MTs reappear in mutant cells if the females are allowed to recover from the cold shock for 5 minutes (Fig. 4C). After 10 minutes recovery, mutant cells show a higher density of MTs than the adjacent heterozygous cells, as they do in the absence of cold shock (Fig. 4D). Thus, PAR-1 is required to stabilise the MTs in cells, and its removal leads to the formation of more MTs that are less stable.

In epithelial cells, most MTs exhibit a uniform apicobasal polarity, with their minus ends localised at the apical

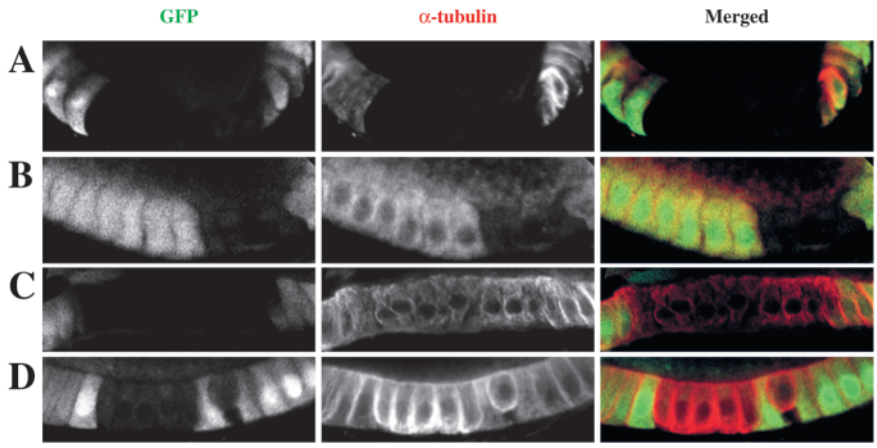

Fig. 4. Loss of PAR-1 destabilises MTs. (A) A *par-1* clone in a stage 10 egg chamber that has been treated with the MT-depolymerising drug, colcemid, and then fixed under optimised conditions and stained for α-Tubulin. The *par-1* mutant cells lack MTs, whereas the wild-type cells show a similar MT organisation to untreated cells. (B) Cold shock for 1 hour leads to the disappearance of the MTs in *par-1* clones. Note that the cold shock also reduces the nuclear localisation of nls-GFP, and partially disperses the cortical bundles of MTs in wild-type cells. (C) Cold shock followed by 5 minutes of recovery at room temperature. The MTs have started to re-grow in mutant cells, but are still less dense than in the wild-type cells. (D) Cold shock followed by 10 minutes of recovery at room temperature. The mutant cells now contain more MTs than wild-type cells, as in untreated ovaries. GFP, green; α-Tubulin, red.

membrane and their plus ends extending towards the basal membrane (Bacallao et al., 1989; Bre et al., 1990; Mogensen et al., 1989). This is also the case in epithelial follicle cells and can be visualised using motor proteins as markers for the plus and minus ends of MTs (Clark et al., 1997). A Kin:β-gal fusion protein containing the motor domain of the plus end-directed MT motor, Kinesin 1, accumulates at the basal side of the cell, whereas a Nod:β-gal fusion protein localises apically. Nod:β-gal shows an identical localisation to the apical membrane in *par-1* mutant follicle cells as in wild-type cells, indicating that the distribution of minus ends is not dramatically affected (Fig. 5A). By contrast, the plus end marker, Kin:β-gal, accumulates in the centre of mutant cells, most probably around the nucleus (Fig. 5B). High magnification views of MTs in follicle cells show the MTs extending from the apical to the basal cortex, with a lower density along the basal membrane. By contrast, *par-1* mutant follicle cells show a high density of MTs along the basal cortex (Fig. 5C).

PAR-1 does not act through *Drosophila* Tau

The defects in MT organisation in *par-1* mutant clones in both the germline and the follicle cells prompted us to investigate whether PAR-1 functions in a similar way to the MARK kinases, which have been proposed to regulate MT stability by phosphorylating the Tau family of MAPs (Drewes et al., 1995; Illenberger et al., 1996). There is a single gene in the 'complete' *Drosophila* genome sequence that encodes the MTBD characteristic this family of MAPs, which has been named *tau* (Heidary and Fortini, 2001). We identified cDNAs that correspond to two transcripts, *tau-a* and *tau-b*, which share six common exons, but splice to alternative final exons 7a and 7b (Fig. 6A). In addition, the large first intron of *tau* contains another gene transcribed from the opposite strand, which encodes the ribosomal protein S10. The *tau* transcripts are predicted to encode proteins of 361 (Tau-B) and 375 (Tau-A) amino acids, similar in length to human tau, and differ only in their C termini. Both isoforms contain five copies of the MTBD motif of the tau-family MAPs, four of which contain the consensus phosphorylation site of the MARK kinases (KXGS) (Fig. 6B).

An affinity-purified antibody against the MTBD of Tau, recognises three bands of 50-60 kDa on western blots of embryonic extracts (Fig. 6C). These bands correspond to Tau, as all three are absent in extracts of homozygous *tau* mutant embryos (see below) (Fig. 6C). To test whether *Drosophila* Tau binds MTs, we performed a MT-spin down assay, in which embryonic extracts were incubated with tubulin in the presence or absence of the MT-stabilising drug taxol. After fractionation through a sucrose cushion, almost all Tau sediments with polymerised tubulin, indicating a strong affinity for MTs (Fig. 6D). We also generated transgenic flies expressing GFP-tagged Tau in the germline. The Tau:GFP fusion protein displays a

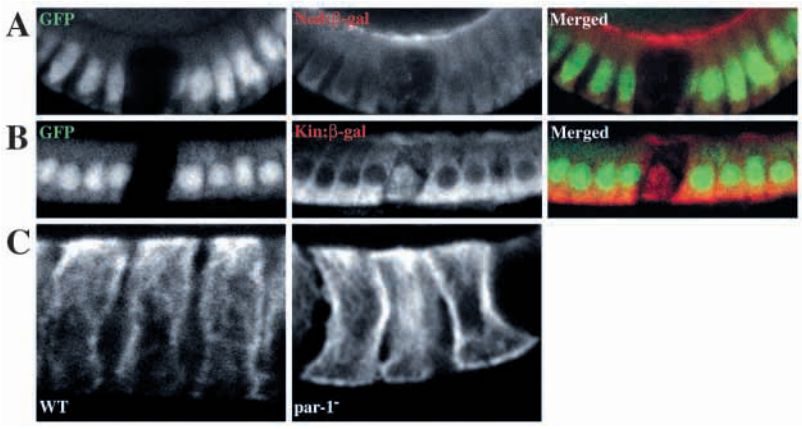

Fig. 5. The loss of PAR-1 alters the distribution of MT plus-ends. (A) A *par-1* clone in a stage 10 egg chamber in which the MT minus end marker, Nod:β-Gal, is expressed in the follicle cells. Nod:β-Gal localises apically in both mutant and wild-type cells. (B): A *par-1* clone in a stage 10 egg chamber in which the MT plus-end marker, Kin:β-Gal, is expressed in the follicle cells. Kin:β-Gal localises basally in wild-type cells, but accumulates in the centre of mutant cells. GFP, green; β-Gal, red. (C) α-Tubulin stainings in wild-type follicle cells (WT) show MTs extending from the apical to the basal cortex, with a lower density along the basal membrane. *par-1* mutant follicles cells (par-1^{-}) show some MTs along their basal membrane.

very similar distribution to that observed in MT stainings of fixed ovaries, and is indistinguishable to that of a bovine tau:GFP fusion protein, which has been used extensively as a live reporter of MT distribution in *Drosophila* (Fig. 6E) (Micklem et al., 1997). Thus, *Drosophila* Tau associates with MTs in extracts and in vivo, like its mammalian counterparts.

To examine whether Tau is a substrate for PAR-1, we performed in vitro kinase assays, in which immunoprecipitated PAR-1 was incubated with bacterially expressed Tau in the presence of labelled ATP. PAR-1 phosphorylates Tau in this assay, but this is not significantly affected by replacing the serines with alanine in all four of the KXGS motifs within the Tau MTBD (Fig. 6F). These results indicate that, although Tau is a substrate for PAR-1 in vitro, this phosphorylation is not at the same regulatory sites as described for the MARK kinases.

To address the role of Tau in MT organisation, we sought to identify mutant alleles of the gene. Two P element insertions, EP(3)3203 and EP(3)3597, have been recovered in the first intron of the *tau* locus, close to the start of the *S10* gene (Fig. 6A). Embryos homozygous for either insertion show no detectable Tau protein on western blots, indicating that they are both strong *tau* mutants, and we have therefore renamed them $tau^{EP(3)3203}$ and $tau^{EP(3)3597}$. Surprisingly, homozygotes of both alleles are viable and fertile, and display no obvious morphological or behavioural defects. Moreover, the organisation of MTs in both follicle and germline cells is indistinguishable from wild type (data not shown). As it is

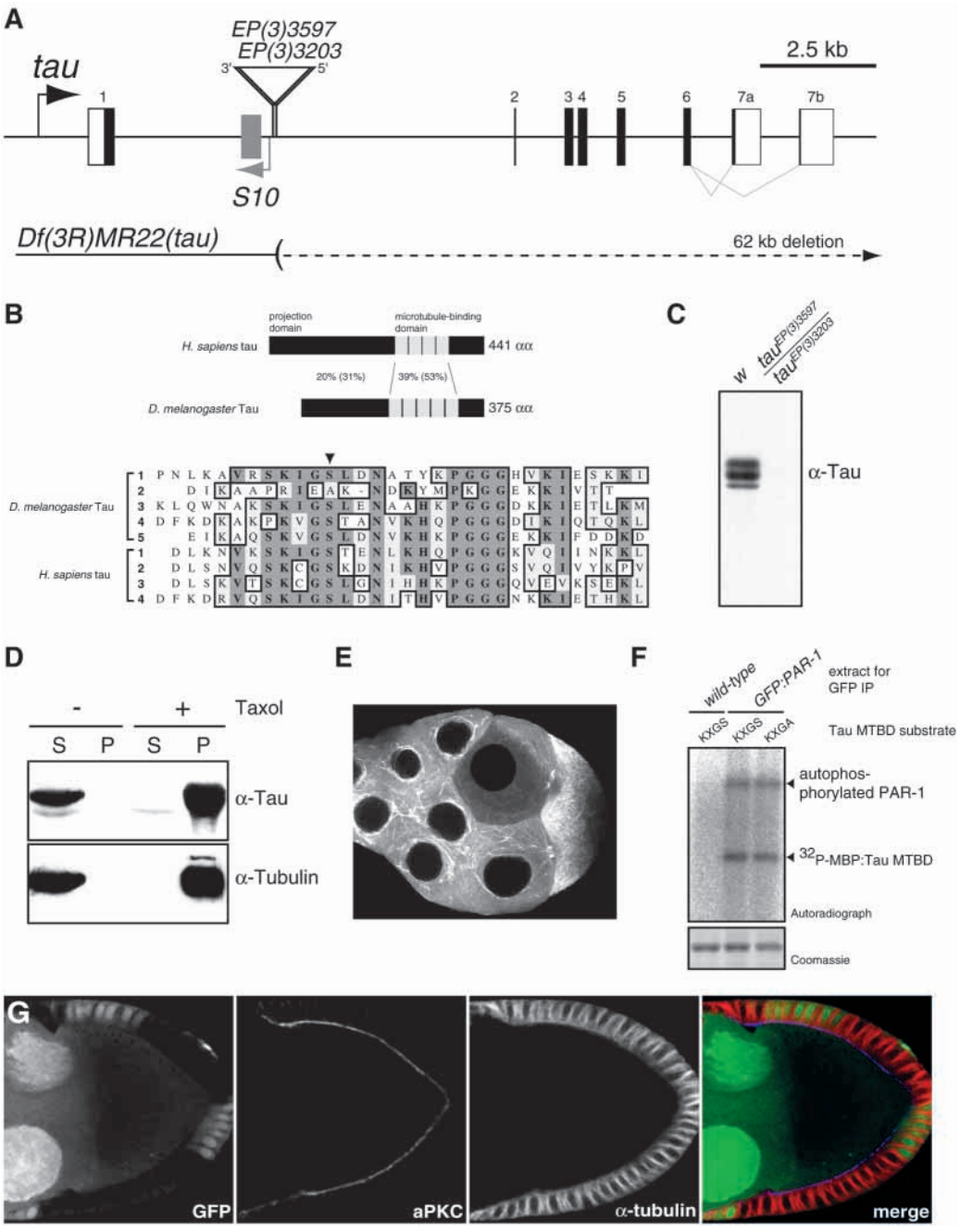

Fig. 6. Identification and characterisation of *Drosophila* Tau as a candidate PAR-1 substrate. (A) Organisation of the *tau* locus, showing the intron/exon structure of *tau* (UTRs in white) and the location of the *S10* gene and the EP element insertions within the first intron. The position of the deficiency uncovering *tau*, *Df(3R)MR22(tau)* is shown below. (B) Domain structure of human and *Drosophila* Tau, illustrating the percent identity (similarity) between the N-terminal projection domains and the MTBD repeats (in grey); an alignment of these repeats is shown below. The putative PAR-1 target serine within the KXGS motif is conserved in four of the five *Drosophila* repeats (arrowhead). (C) Western blot of 12-18 hour and *tau* mutant embryos probed with an antibody raised against the MT-binding domain of *Drosophila* Tau. (D) MT spin-down assay, revealing co-sedimentation of Tau with Taxol-induced polymerised Tubulin in the pellet (P) fraction. In the absence of Taxol, both remain in the supernatant (S). (E) MT localisation of Tau:GFP in a living *Drosophila* ovary. (F) PAR-1 kinase assay with GFP:PAR-1 immunoprecipitated from ovarian extracts and MBP:Tau MTBD substrates, containing (KXGS) or lacking (KXGA) the four putative PAR-1 target sites. (G) Stage 10 egg chamber containing two large mutant clones for *Df(3R)MR22(tau)* stained for DaPKC (blue) and α-tubulin (red). DaPKC and α-tubulin localise normally in *tau* mutant clones.

possible that the EP elements do not completely abolish Tau expression, we used transposase-mediated male recombination to generate deletions that remove the *tau* locus, but not *S10*. One recombinant, Df(3R)MR22, is a 62 kb deletion extending distally from EP(3)3203 that removes almost all of the *tau* locus, including the exons encoding the MTBD (Fig. 6A). Germline and follicle cell clones of Df(3R)MR22 also display no MT defects (Fig. 6G and data not shown). Consistent with this, we observed no Tau expression in the follicle cells. Thus, Tau is apparently dispensable in *Drosophila* and is not required for MT organisation during oogenesis.

DISCUSSION

The PAR proteins were originally identified because they are required for anteroposterior axis formation in *C. elegans*, but it is becoming increasingly clear that they play a conserved role in the generation of cell polarity in many contexts. All of the identified *Drosophila* PAR proteins are required for the early anterior-posterior polarisation of the *Drosophila* oocyte (Benton et al., 2002; Cox et al., 2001a; Cox et al., 2001b; Huynh et al., 2001a; Huynh et al., 2001b; Martin and St Johnston, 2003). Furthermore, four PAR proteins, the Bazooka (PAR-3)/PAR-6/aPKC complex and the *Drosophila* PAR-4 homologue, LKB1, play essential roles in the apicobasal polarisation of epithelia (Knust, 2000; Martin and St Johnston, 2003). Our results demonstrate that this is also the case for PAR-1.

Although *par-1* mutants produce similar polarity phenotypes in the follicle cells to mutants in the components of the Bazooka/PAR-6/aPKC complex, they are not identical. *par-1* clones cause a complete disruption of polarity only when induced early in the follicle cell lineage. The smaller clones that arise later in oogenesis often show little or no reduction in the localisation of apical and basolateral markers, and usually remain as a single layer of cells. By contrast, even late clones of *bazooka* or *aPKC* produce penetrant epithelial defects (Cox et al., 2001b) (R.B. and D.St.J., unpublished). This difference is also apparent in the embryo, where loss of zygotic *bazooka*, *PAR-6* or *aPKC* disrupts epithelial organisation (Kuchinke et al., 1998; Muller and Wieschaus, 1996; Petronczki and Knoblich, 2001; Wodarz et al., 2000). *par-1* homozygous embryos, however, display no obvious epithelial polarity phenotype, although it is not possible to remove the maternal PAR-1 completely (Shulman et al., 2000; Sun et al., 2001). The reason for low penetrance of polarity defects in smaller *par-1* follicle clones is unclear, but similar differences between early large clones and smaller late clones have been observed for *crumbs*, *discs lost* and *lkb1* (Martin and St Johnston, 2003; Tanentzapf et al., 2000). One possibility is that these genes are required for the initial formation of the follicular epithelium, but not for its maintenance. This seems unlikely to be the case for *par-1*, however, because small clones containing only one or two cells can sometimes show strong apicobasal polarity defects. A more likely explanation is that the low penetrance of this phenotype in small clones is due to the perdurance of the PAR-1 that was present at the time when the clones were induced. In support of this, the penetrance of the polarity defects of *par-1* clones increases with clone size and the stage of oogenesis, as one would expect if the protein is gradually degraded over time, and is diluted out by cell division.

In contrast to apicobasal membrane polarity, the density, the stability and the organisation of MTs are disrupted in all *par-1* clones, regardless of their size or the stage of oogenesis. This is likely to represent a distinct function of the kinase from its other roles in cell polarity, because it is much more sensitive to a reduction in activity. The effects of PAR-1 on MT density are consistent with results on the mammalian PAR-1 homologues, MARK1 and MARK2. Our experiments show that removal of PAR-1 causes an increase in the density of MTs in each cell, whereas overexpression of MARK1 or MARK2 in unpolarised tissue culture cells causes most MTs to disappear (Drewes et al., 1997; Ebneth et al., 1999).

The MARKs have been proposed to regulate the MT cytoskeleton by phosphorylating Tau family MAPs, thereby inhibiting them from binding to and stabilising MTs. Although PAR-1 does phosphorylate *Drosophila* Tau in vitro, *tau* null mutations are viable and fully fertile and have no effect on the arrangement of MTs in either the follicle cells or the oocyte. Therefore, this mechanism cannot account for its function in organising the MTs in the follicle cells. The viability of *tau* mutants is surprising, given the many functions ascribed to Tau in human neurons (Lee et al., 2001). It does have a precedent, however, as *tau* knockout mice are viable, have a morphologically normal nervous system, and display only defects in MT stability and organisation in small-calibre axons (Harada et al., 1994). The mild phenotype of *tau* in mice has been proposed to be due to functional redundancy with the closely related MAP2, but this cannot be the case in *Drosophila*, which does not contain a MAP2 homologue. It may be redundant with other types of MAP, however, and the best candidate is Futsch, which has significant structural and functional homology to mammalian MAP1B (Hummel et al., 2000; Roos et al., 2000). MAP1B appears to have functional overlap with both Tau and MAP2 in mammals, because mice that are homozygous for null mutations in *map1b* and *tau*, or *map1b* and *map2*, show defects in axonal elongation, neuronal migration and MT organisation that are much more severe than in mice lacking only one of these genes (Takei et al., 2000; Teng et al., 2001).

Another compelling argument that PAR-1 regulates MTs by a different mechanism from that proposed for the MARKs is that it is required to stabilise rather than destabilise MTs, at least in epithelial cells. The MTs in follicle cells are among the most stable in nature, because they are almost completely resistant to cold or to prolonged colchicine treatments (Gutzeit, 1986; Theurkauf, 1992) (this study). By contrast, the MTs in *par-1* mutant cells appear to be highly dynamic as: (1) they disappear after brief colchicine treatments; (2) they depolymerise at 4°C, but re-grow in a few minutes after return to 25°C; (3) they are lost during fixation, if the fixative is too weak. Indeed, the instability of the MTs may explain the discrepancy between our results and those of Cox et al. (Cox et al., 2001a), as most MTs in mutant cells disappear during fixation with 4% formaldehyde, but not with 8% formaldehyde, even though the two fixatives preserve the MTs in wild-type cells equally well.

The opposite effects of PAR-1 and the MARKs on MT stability may indicate that these closely related kinases have evolved to fulfil distinct functions in invertebrates and

mammals. It is also possible, however, that this reflects the different experimental approaches and cell-types that have been used to examine their activities. The MARKs have been assayed by over-expressing them in CHO cells, which are a transformed line of rapidly dividing, undifferentiated and unpolarised cells. By contrast, we have examined the loss-of-function phenotype of PAR-1 in post-mitotic follicle cells, which are highly polarised and differentiated epithelial cells. The two cell types also have very different microtubule cytoskeletons. In CHO cells, microtubules are nucleated from a central centrosome, and are presumably reasonably dynamic, because they disassemble at each mitosis, whereas the follicle cells lose their centrosomes when they form a columnar epithelium, and nucleate a very stable apicobasal array of microtubules. It would therefore be interesting to test the effects on MT stability of disrupting the function of PAR-1 homologues in more similar mammalian cell-types, such as polarised MDCK cells.

In addition to its effect on stability, PAR-1 is required to maintain the normal organisation of the MTs. The MT arrangement in the follicle cells is typical of a polarised epithelium, with the minus ends associated with the apical membrane, and the plus ends at the basal side of the cell (Gonzalez et al., 1998; Mogensen, 1999; Mogensen et al., 1989). The arrangement of minus ends appears to be largely unchanged in *par-1* mutant cells, but a marker for the plus ends, Kin:β-gal, accumulates in the centre rather than the basal region of the cell. This phenotype is very similar to that observed in *par-1* mutant oocytes, in which the plus ends become abnormally focussed in the centre of the oocyte, rather than at the posterior, and there is an increase in the density of MTs around the cortex (Shulman et al., 2000). Thus, PAR-1 may regulate the MTs in the same way in the two cell types, and most probably acts primarily on the plus ends. PAR-1 may also have some effect on the distribution of the minus ends of MT in the oocyte, as *bicoid* mRNA, which is believed to be transported towards minus ends, is found around the lateral cortex of mutant oocytes, rather exclusively at the anterior (Benton et al., 2002). Although we cannot rule out the possibility that there is also an effect on the minus ends in mutant follicle cells, this is not detectable using Nod:β-gal as a marker.

It seems paradoxical that the loss of PAR-1 should increase the density of MTs in follicle cells, while decreasing their stability, but one possible explanation is suggested by studies in mammalian cells on populations of stable MTs that are marked by detyrosinated α-tubulin (Bulinski and Gundersen, 1991; Webster et al., 1987). These MTs are resistant to nocadazole-induced depolymerisation, and fail to incorporate new tubulin subunits, leading to the proposal that they are capped at their plus ends in a way that prevents both the addition and loss of tubulin (Infante et al., 2000; Schulze and Kirschner, 1987; Webster et al., 1987). Thus, it is possible that PAR-1 stabilises the MTs in the follicle cells by capping plus ends when they reach the basal cortex, thereby preventing them from either growing or shrinking. If the conditions inside the cell favour MT polymerisation, the loss of the PAR-1-dependent cap would allow the plus ends to continue to grow once they reach the basal cortex. This could account for both the increase in MT density and the redistribution of plus ends to the centre of mutant cells. However, the uncapped MTs would rapidly shrink under conditions that favour MT depolymerisation, such as cold or colchicine treatment, explaining why the MTs disappear in mutant cells.

par-1 null clones also show fully penetrant and cell-autonomous increases in the recruitment of β-spectrin and actin to the lateral cortex. Like the microtubule phenotype, these effects appear to be independent of the defect in apicobasal membrane polarity, as the latter is much less penetrant. These phenotypes may therefore reflect a third distinct function of the kinase. It is also possible, however, that the MT defects are a consequence of the changes in actin organisation or vice versa. In this context, it is interesting to note that Rho family GTPases, which are major regulators of the actin cytoskeleton, have also recently been found to control the capping of MT plus ends at the leading edge of migrating cells (Gundersen, 2002). The Rac and Cdc42 effector, IQGAP, interacts with the plus end binding protein, CLIP170, to stabilise MTs transiently, whereas the Rho effector, mDia, leads to the formation of more stable MTs, perhaps through the plus-end binding protein EB1 (Cook et al., 1998; Fukata et al., 2002; Palazzo et al., 2001). Given that PAR-1 does not appear to function through the obvious candidate, Tau, it would be interesting to test whether this kinase acts through either of these pathways to regulate MT organisation in epithelial cells.

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