# The protein kinase kin1, the fission yeast orthologue of mammalian MARK/PAR-1, localises to new cell ends after mitosis and is important for bipolar growth

Gerard Drewes*, Paul Nurse

*Cell Cycle Laboratory, Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK*

Received 25 August 2003; accepted 18 September 2003

First published online 7 October 2003

Edited by Giulio Superti-Furga

**Abstract The kin1 protein kinase of the fission yeast *Schizosaccharomyces pombe* is a member of the PAR-1/MARK (partitioning-defective 1/microtubule-associated protein/microtubule affinity-regulating kinase) family important in eukaryotic cell polarity and cytoskeletal dynamics. We show here that kin1 plays a role in establishing the characteristic rod-shaped morphology of fission yeast. Cells in which kin1 was deleted are viable but are impaired in growth, and are rounded at one end or both ends. They are monopolar because after mitosis they fail to activate bipolar growth, and are delayed in cytokinesis, resulting in a high proportion of septated cells often with multiple septa. This phenotype can be partially rescued by heterologous expression of human MARKs, which restore bipolar growth in most cells, but do not correct the delay in cytokinesis. Using chromosomal epitope tagging, we show that kin1p localises to the cell ends, except during mitosis when it disappears from cell ends. After mitosis, kin1p first reappears at the new cell end. Overexpression of kin1 results in a loss of polarity, with partially or fully rounded cells. From these results we suggest that kin1 is required to direct the growth machinery to the cell ends.** 

*Key words:* Fission yeast; Cell polarity; Cytokinesis; Septation; Orthology; kin1; Microtubule-associated protein/microtubule affinity-regulating kinase; Partitioning-defective-1

## 1. Introduction

In eukaryotic cells, microtubules (MTs) provide a dynamic network that is critical for the segregation of chromosomes in mitosis and for cellular morphogenesis, enabling asymmetric cell growth and cell polarity [1–3]. In these processes, MTs function as tracks for regulated movement and positioning of membranous vesicles and organelles [4].

MARKs (microtubule-associated protein/microtubule affinity-regulating kinases) constitute a novel serine/threonine kinase family in mammals that influence MT stability and MT-based transport events by phosphorylating MT-associated proteins [5,6]. Putative orthologues of the mammalian MARKs have been described in fission yeast (kin1 [7]), *Caenorhabditis elegans* (PAR-1 (partitioning-defective 1) [8]), and *Drosophila melanogaster* (dPAR-1 [9,10]). In these organisms, the kin1/PAR-1/MARK kinases are involved in processes related to the establishment of cell polarity. The considerable degree of sequence conservation found in the MARK/PAR-1/KIN family of protein kinases suggests that they may act in an essential conserved signalling pathway governing cell polarity. The fission yeast *Schizosaccharomyces pombe* is a simple model organism for the genetic study of morphogenesis [11]. At mitosis, the cylindrical cells undergo medial fission and enter the next cycle by unipolar growth from the 'old' cell end, followed by the switch to bipolar growth, when a second growth zone is established at the newly formed cell end. In the present study we describe the effect of deletion and overexpression of the protein kinase kin1 on the morphology of fission yeast, the dynamic localisation of kin1 during polar cell growth, and we demonstrate some functional orthology between *S. pombe* kin1 and mammalian MARK kinases.

## 2. Materials and methods

### 2.1. Fission yeast strains and plasmids

Standard methods were used for growth, transformation, and genetic manipulation of *S. pombe* (available at http://www.sanger.ac.uk/PostGenomics/S_pombe/docs/nurse_lab_manual.pdf). If not mentioned otherwise, all cells were grown at 32°C on YE medium with supplements, except for cells carrying plasmids that were grown on minimal EMM2 medium. The pREP1, pREP41, and pREP81 plasmids, which allow different levels of thiamine-repressed expression, were used for kin1 overexpression and for exogenous expression of human and rat MARK cDNAs. pREP81 contains the lowest expression version of the nmt1 promoter, pREP41 contains a medium expression version, and pREP1 contains the strongest (wild-type) version [12]. The kin1 cDNA was subcloned into pREP1 from pDB248-kin1 [13] using *Nde*I, which was introduced by polymerase chain reaction (PCR) at the start codon, and *Xho*I downstream of the termination codon. Rat MARK1 and human MARK2, MARK3, and MARK4 were subcloned into *Nde*I and *Sma*I sites of the pREP polylinker using *Nde*I and *Sma*I introduced by PCR [5].

### 2.2. Gene disruption and gene tagging

The strain Δ*kin1::kanMX6 leu1-32h-* was a kind gift of Dr K. Sawin (Cancer Research UK). In this strain, the *kin1* gene was deleted using homologous recombination as described in [14]. The same method was used for chromosomal tagging of the *kin1*$^+$ gene at the C-terminus with 13 copies of the human c-*myc* epitope, resulting in the strain *kin1*$^+$*::13myc-kanMX6 leu1-32h-*. We also introduced the

*Corresponding author. Present address: Cellzome AG, Meyerhofstrasse 1, 69117 Heidelberg, Germany. Fax: (49)-6221-13757206.
*E-mail address:* gerard.drewes@cellzome.com (G. Drewes).

*Abbreviations:* HA, haemagglutinin; GFP, green fluorescent protein; MARK, Microtubule-associated protein/microtubule affinity-regulating kinase; MT, microtubule; NETO, new end take-off; OETO, old end take-off; PAR, partitioning-defective

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01080-9

c-*myc* epitope tag after the SPBC32C12.03C gene, a putative paralogue which we termed *kin2*, resulting in the strain *kin2*$^{+}$*::13myc-kanMX6 leu1-32h-*.

### 2.3. Microscopy

Live cells were stained with calcofluor at 50 μg/ml in 2.5 mM sodium citrate/5 mM sodium phosphate pH 6.0 containing 50% glycerol and 0.3 mg/ml *p*-phenylenediamine. For indirect immunofluorescence analysis, cells were fixed in culture by incubation for 20 min with 3.7% freshly prepared formaldehyde and 0.2% glutaraldehyde (final concentrations). The formaldehyde was added 30 s prior to the glutaraldehyde. Alternatively, cells were fixed by 5–20 min incubation in −20°C methanol; or by the addition of 10% trichloroacetic acid. All washes were performed in MT-stabilising buffer (PEM buffer; 100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$ pH 6.9). Primary antibodies were anti-tubulin [TAT-1] 1:50 dilution), anti-tip1 1:1000 dilution, and anti-myc 9E10 (Sigma), followed by Alexa 488 goat anti-rabbit or Alexa 546 anti-mouse secondary antibodies (Molecular Probes). Cells were mounted in 50% glycerol, 1 μg/ml DAPI, 0.1 mg/ml paraphenylene in 0.1 M Tris pH 8 on poly-L-lysine-coated coverslips. Immunofluorescence images were acquired with a Axiovert fluorescence microscope (Zeiss) equipped with a 63× oil immersion objective, video CCD camera (Hamamatsu model C5985) connected to an Apple Macintosh G3/400 computer.

## 3. Results

### 3.1. Δkin1 cells are monopolar

Levin and Bishop reported that the disruption of the *kin1*$^{+}$ gene within the catalytic domain causes *S. pombe* cells to grow as spheres or cones, in contrast to wild-type cells, which grow as rods [7]. We re-evaluated the phenotype of the *kin1*$^{+}$ disruption in the strain Δ*kin1::kanMX6*, where the entire *kin1* gene was replaced by a kanamycin resistance cassette. Fluorescence micrographs of living Δ*kin1* cells using the dye calcofluor to stain the cell wall show clear morphological abnormalities (Fig. 1). Δ*kin1* cells exhibit a pronounced delay in cytokinesis: Under conditions of exponential growth, approximately 40% of the cells are undergoing septation, compared to ∼5% in wild-type cells (Fig. 1A). This percentage is retained in the stationary growth phase, where no septated cells are observed in wild-type cells (Fig. 1B). Upon regrowth, these septated cells do not complete cytokinesis but form cells with two or three septa (Fig. 1C). We also visualised MTs by immunofluorescence microscopy and observed a high proportion of cells with a post-anaphase MT array, consistent with a delay in the completion of septation (data not shown). Δ*kin1* cells also show a pronounced defect in cell shape; the cells grow monopolar and, after mitosis, the new cell end fails to activate polarised growth (new end take-off, NETO). As a result, cells often become rounded, leading to a typical rounded or 'cone-shaped' morphology. Finally, Δ*kin1* cells tend to form clumps, probably due to a change in the cell wall that renders them sticky.

### 3.2. Overexpression of kin1p leads to a loss of cell polarity

To check whether overexpression of kin1p would also affect growth polarity, we constructed plasmid pREP1-kin1, which allows the overexpression of the *kin1*$^{+}$ gene under the control of the strong nmt promoter. Wild-type cells were transformed with this plasmid and released from thiamine repression. Cells overexpressing kin1 tend to be more rounded than wild-type cells (Fig. 2). This overexpression phenotype was not dramatic, possibly due to the fact that the kin1p kinase, like mammalian MARKs [15], may require activation by phosphorylation by an upstream kinase to become catalytically active.

### 3.3. Partial rescue of the Δkin1 phenotype by mammalian MARKs

We next asked whether it is possible to rescue the kin1

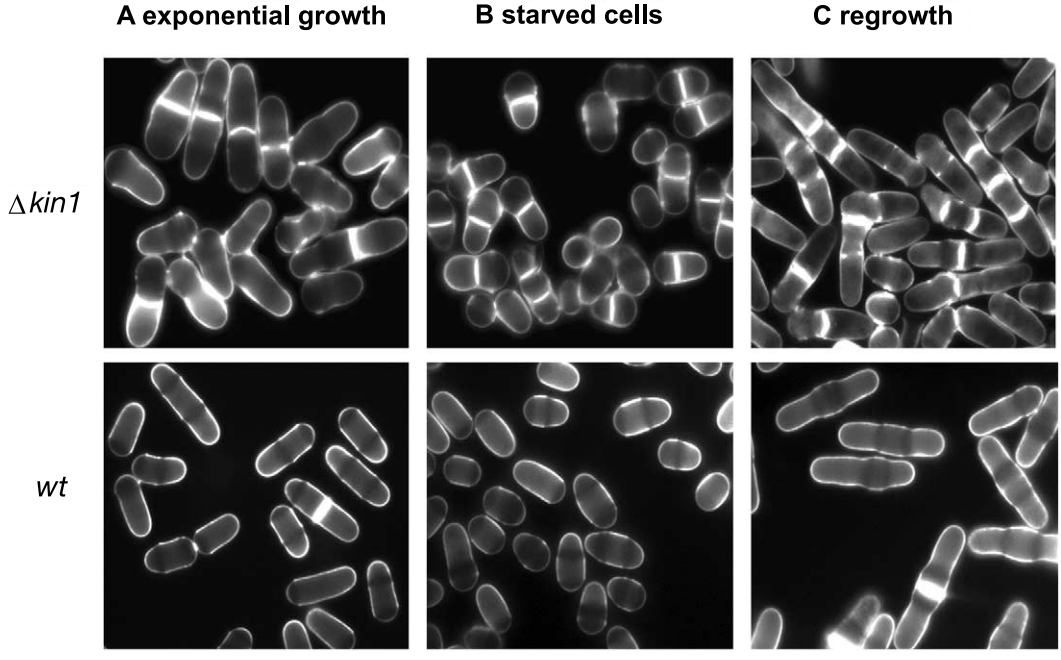


Fig. 1. *S. pombe* cells in which the *kin1* gene has been deleted show distinct morphological abnormalities. The upper panels show cells lacking the *kin1* gene (Δ*kin1::kanMX6*); the lower panels show wild-type cells (972 strain) for comparison. Live cells were stained with the cell wall dye calcofluor. Left panels (A): Cells growing exponentially at 25°C. All Δ*kin1* cells are monopolar and about 40% of the cells are septating, compared to ∼5% in wild-type cells. Middle panels (B): Starved cells. The 40% percentage of septating cells is retained, whereas no septated cells are observed in wild-type cells. Right panels (C): Cells regrowing after starvation. Many septated Δ*kin1* cells do not complete cytokinesis but form cells with two or three septa.

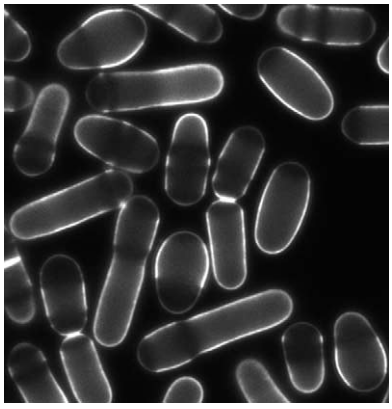

Fig. 2. Overexpression of kin1 affects cell shape. Overexpression of kin1 was achieved from the full-strength nmt1 promoter (pREP1) and live cells were stained with calcofluor 4 h after release from thiamine repression. Cells overexpressing kin1p tend to be more rounded than wild-type cells.

deletion by the ectopic expression of mammalian MARK kinases, which are candidate functional orthologues of fission yeast *kin1*. The study of MARK function in cultured mammalian cells has been hampered by the toxicity of overexpressed MARK. This results in low transfection rates and low viability of transformants [5]. In *S. pombe*, we attempted to solve this problem by the use of plasmid vectors that allow expression under the control of repressible nmt1 promoters with different activity [12]. We found that higher levels of MARK expression, as achieved from the full-strength nmt promoter, are also toxic to *S. pombe* cells, and at first sight it appeared that, even with the low-expression promoters nmt41 and nmt81, no significant rescue was observed, especially because analysis of individual cells was hampered by the tendency to form cell clumps. However, closer inspection revealed that the expression of low levels of MARK1 and MARK2 (under control of the nmt81 promoter) indeed restores bipolar growth to some cells, whereas all of the Δ*kin1* cells are deficient in NETO, the initiation of growth from the new cell end (Fig. 3A–C). Under the conditions of these experiments, MARK4 appeared to be even more toxic and failed to partially rescue the NETO defect (Fig. 3D). However, even in the case of MARK1 and MARK2 the rescue of the NETO defect was only seen in a subset of cells, and the tendency of the cells to form clumps was not reversed.

### 3.4. kin1p but not kin2p localises to cell ends

In polarised cells, mammalian MARKs as well as the putative nematode and fly orthologues show distinct polar localisation patterns [9,10,16–20]. For example, in Madin–Darby canine kidney cells, MARK2 localises at the tight junctions that form the boundary between the apical and the basolateral membrane [21], and in neuroblastoma cells, MARK4 is found at the tip of the neuritic processes (B. Trinczek and G. Drewes, unpublished data). Hence we asked whether kin1p adopts a polarised localisation to affect fission yeast growth polarity. Therefore we constructed epitope-tagged *S. pombe* strains, fusing kin1 at the carboxy-terminus with c-*myc*, haemagglutinin (HA), and green fluorescent protein (GFP) tags by homologous recombination. We found that only the tag consisting of 13 c-*myc* epitopes allowed detection of kin1p by indirect immunofluorescence. The tagged kin1 protein shows a pronounced localisation to one or both cell ends (Fig. 4B). The fusion protein is expressed at a size of 115 kDa as detected by immunoblotting (Fig. 4A).

To analyse whether this localisation is a specific feature of kin1p and not a staining artefact, we performed myc-epitope tagging in an analogous fashion on the closest homologue of *kin1*, the putative paralogue SPBC32C12.03C, which we termed *kin2*. *Kin2* contains the amino-terminal catalytic domain but lacks the carboxyl-terminal 'spacer' and C-terminal KA domains [15]. Indirect immunofluorescence analysis revealed that kin2p does not exhibit localisation to the cell ends but shows a uniform cytoplasmic distribution (Fig. 4D).

### 3.5. kin1p localises to the new cell end after mitosis

From an analysis of the kin1p staining in the *kin1*$^{+}$*::13myc-kanMX6* cells in different stages of growth, it appeared that the localisation of kin1p to the cell ends is lost in mitosis. The signal disappears from cell ends between late anaphase and the completion of cytokinesis (Fig. 4B, cells labelled 'M'). Interestingly, this is the stage where Δ*kin1* cells fail to properly reactivate growth and get delayed or arrested as described above. Some time after the completion of septation, kin1p reappears first at the cell end which is newly formed after cytokinesis. The new cell end can be clearly distinguished from the 'old' end as it is less pointed (Fig. 4B, cells labelled 'N'). Later in interphase, in longer cells, kin1p is mostly found at both cell ends (Fig. 4B, cells labelled 'O'). These results are summarised in the cartoon in Fig. 4C.

The disappearance of kin1p from the cell ends during mitosis is not due to protein degradation, since expression levels of kin1p are constant during mitosis, as assayed by immuno-

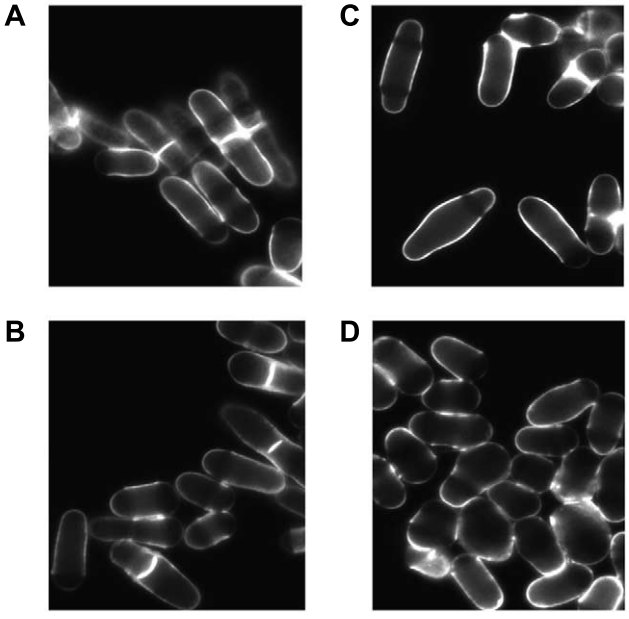


Fig. 3. Ectopic expression of mammalian MARK kinases in Δ*kin1* cells partially restores bipolar growth. Expression of rat MARK1 (A), rat MARK2 (B), human MARK3 (C) and human MARK4 (D) in the Δ*kin1::kanMX6* strain. MARK genes were cloned into pREP81 (weak nmt promoter) and transformed into *S. pombe*. Expression was induced by release from thiamine repression and live cells were stained with calcofluor. Bipolar growth is restored to some cells in panels A–C, and the percentage of cells in septation is lower compared to non-transformed Δ*kin1* cells. However, the tendency of the Δ*kin1* cells to form clumps is not affected.

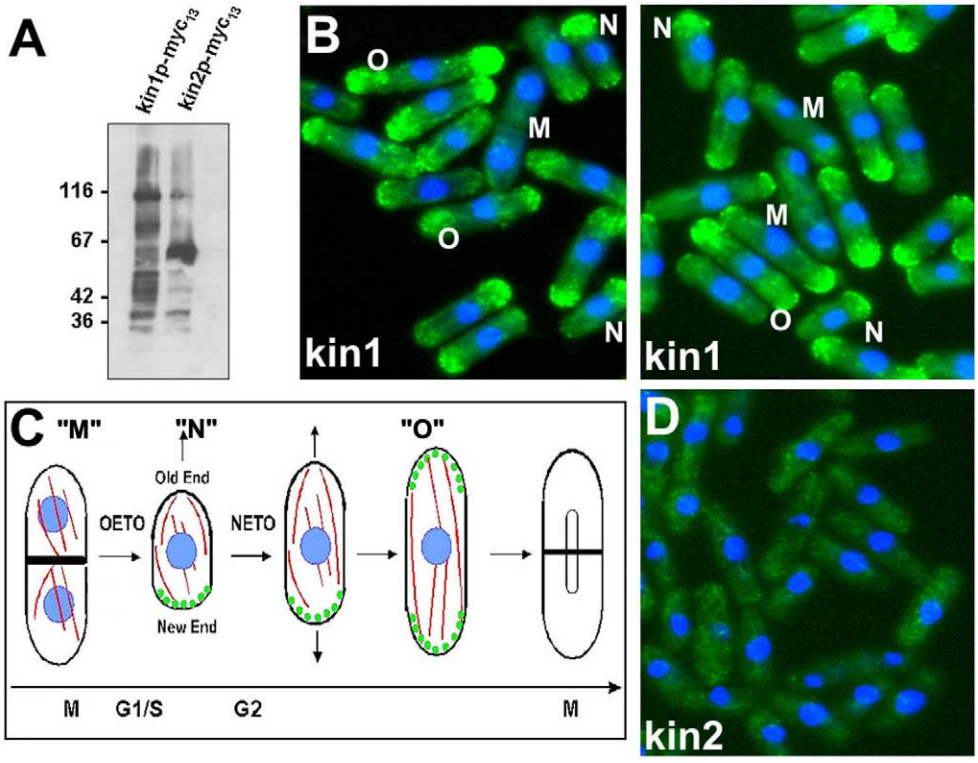


Fig. 4. After mitosis kin1p localises first to the new, and later to both cell ends. A: In the chromosomally tagged cells, kin1p is expressed at a size of 115 kDa and kin2p is expressed at a size of 55 kDa by immunoblot analysis. B: Immunofluorescence micrographs of an epitope-tagged *kin1⁺::13myc-kanMX6* strain. Kin1 is stained in green, nuclei are stained with DAPI (blue). The localisation of kin1p to the cell ends is lost late in mitosis, during late anaphase to cytokinesis (see cells labelled 'M'). Kin1p appears at the cell ends somewhere between the completion of septation and cytokinesis, but always first at the newly formed 'edged' cell end, which is distinguished from the rounded 'old' end (see cells labelled 'N'). Larger cells also show labelling of the old end (see cells labelled 'O'). C: Cartoon summarising the localisation results from the micrographs in panel B. D: Immunofluorescence micrographs of an epitope-tagged *kin2⁺::13myc-kanMX6* strain. Kin2p does not exhibit localisation to the cell ends but shows a cytoplasmic staining.

blotting in a cross of the *kin1⁺::13myc-kanMX6* with a cdc25$^{ts}$ strain that was blocked at 36°C and subsequently cooled, allowing progression through mitosis (data not shown).

### 3.6. *The cell end marker* tea1 *is not affected in Δ*kin1 *cells*

The putative orthologue of kin1 in *Saccharomyces cerevisiae*, termed Kin1, forms a protein complex with the Kelch repeat protein Kel1 ([26], see also http://yeast.cellzome.com/). The likely orthologue of *S. cerevisiae KEL1* in fission yeast is *tea1*, which encodes a cell end marker [22,27]. It is tempting to speculate that kin1p might function by directing the growth machinery to the cell ends and/or regulates the dynamic processes involving the cytoskeletal elements required for growth

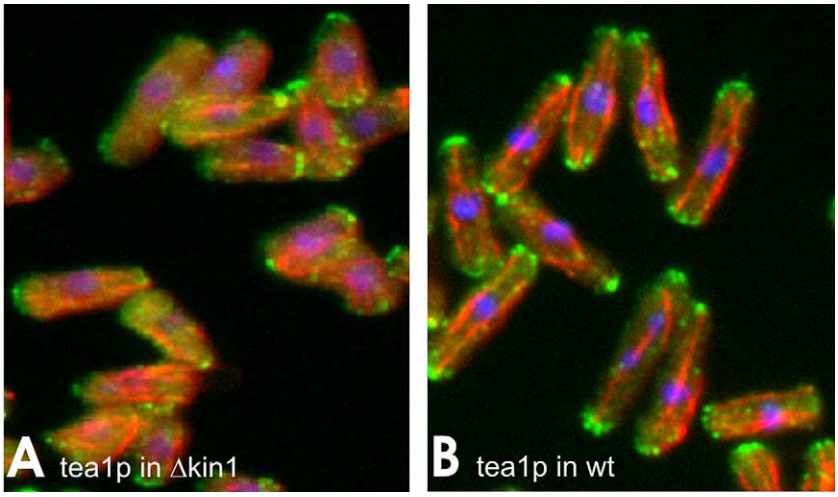


Fig. 5. tea1p localisation is not affected in *Δkin1* cells. A,B: Indirect immunofluorescence staining of *Δkin1::kanMX6* (A) and wild-type *S. pombe* (B) with antibodies against tubulin (red) and tea1p (green). Deletion of *kin1* does not significantly affect the MT array and the cell end marker tea1p.

at the cell end. Hence we asked whether the localisation of tea1p [22] is affected in our kin1 deletion strain. Using immunofluorescence microscopy, we found that the cell end marker tea1p retained proper localisation in most Δ*kin1* cells (Fig. 5A).

## 4. Discussion

Protein kinases of the PAR-1/MARK family play crucial roles in the establishment of cell polarity in organisms as diverse as *C. elegans* [8,19], *D. melanogaster* [9,10,17], and cultured mammalian cells [21,24,25]. Hence it is tempting to speculate that these kinases are true orthologues which operate in a conserved signalling pathway controlling cell polarity. However, the underlying molecular mechanism of the effect on cell polarity in these different organisms remains unclear, as little is known about the upstream regulatory mechanisms and the downstream effects, e.g. the phosphorylation of substrate proteins. In this study, we further characterise kin1p, which constitutes the single member of this protein kinase family in fission yeast. We show that both the loss and overexpression of kin1p interfere with proper cell morphogenesis, and that the localisation of kin1p is not specifically restricted during cytokinesis, but soon afterwards it is redistributed to the newly formed cell end and later in interphase it is found at both cell ends. This is consistent with a scenario where kin1p has a function in directing the growth machinery towards the new cell end, and in the absence of kin1 cells grows only from the old end and remains monopolar. However, it seems that kin1p has an additional function in cytokinesis but not in septation directly, since Δ*kin1* cells are delayed post-anaphase, leading to the accumulation of multi-septated cells.

The *Drosophila* and mammalian members of the PAR-1/MARK kinase family play a role in MT organisation [5,17]. We show that, in fission yeast, the deletion of *kin1* has no dramatic effect on MTs, other than many cells displaying a post-anaphase array as a result of the delayed cytokinesis. The cell cycle-dependent recruitment of kin1p to the cell ends suggests that it may bind in a regulated fashion to an end-marker protein. As described above, kin1p and the end marker tea1p are interlogues; their possible orthologues in budding yeast interact in a protein complex. The positioning of polarised growth sites at the opposite ends of the fission yeast cell depends on the delivery of tea1p to the cell end [22]. We show that the localisation of tea1p is largely preserved in Δ*kin1* cells, which suggests that tea1p functions upstream of kin1p. Another protein dependent on tea1p is the MT end-associated protein tip1p, which is less abundant at the cell ends in *tea1*-deleted cells [23]. Interestingly, it was recently reported that, in *Drosophila*, loss of PAR-1 disrupts the asymmetric localisation of the MT plus ends, which are mislocalised to the centre of mutant cells, and it was suggested that dPAR-1 functions by regulating MT plus ends by capping them at the basal cortex [28].

The fact that *tea1* does not have an orthologue in mammals, and that ectopic expression of the mammalian MARKs was only partially able to rescue the Δ*kin1* phenotype, suggests that despite the high conservation in the kin1/PAR-1/MARK family, the actual molecular mechanisms have diverted during evolution.

*Acknowledgements:* G.D. was supported by a fellowship from the Human Frontier Science Program Organization. We would like to thank Drs Heidi Browning, Damian Brunner and Ken Sawin for invaluable suggestions and support.

## References

[1] Hayles, J. and Nurse, P. (2001) Nat. Rev. Mol. Cell Biol. 2, 647–656.
[2] Howard, J. and Hyman, A.A. (2003) Nature 422, 753–758.
[3] Nelson, W.J. (2003) Nature 422, 766–774.
[4] Bloom, G.S. and Goldstein, L.S. (1998) J. Cell Biol. 140, 1277–1280.
[5] Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E.M. and Mandelkow, E. (1997) Cell 89, 297–308.
[6] Ebneth, A., Drewes, G. and Mandelkow, E. (1999) Cell Motil. Cytoskeleton 44, 209–224.
[7] Levin, D.E. and Bishop, J.M. (1990) Proc. Natl. Acad. Sci. USA 87, 8272–8276.
[8] Guo, S. and Kemphues, K.J. (1995) Cell 81, 611–620.
[9] Shulman, J.M., Benton, R. and St Johnston, D. (2000) Cell 101, 377–388.
[10] Tomancak, P., Piano, F., Riechmann, V., Gunsalus, K.C., Kemphues, K.J. and Ephrussi, A. (2000) Nat. Cell Biol. 2, 458–460.
[11] Brunner, D. and Nurse, P. (2000) Phil. Trans. R. Soc. Lond. B Biol. Sci. 355, 873–877.
[12] Maundrell, K. (1993) Gene 123, 127–130.
[13] Snell, V. and Nurse, P. (1994) EMBO J. 13, 2066–2074.
[14] Bahler, J. et al. (1998) Yeast 14, 943–951.
[15] Drewes, G., Ebneth, A. and Mandelkow, E.M. (1998) Trends Biochem. Sci. 23, 307–311.
[16] Cox, D.N., Seyfried, S.A., Jan, L.Y. and Jan, Y.N. (2001) Proc. Natl. Acad. Sci. USA 98, 14475–14480.
[17] Cox, D.N., Lu, B., Sun, T.Q., Williams, L.T. and Jan, Y.N. (2001) Curr. Biol. 11, 75–87.
[18] Huynh, J.R., Shulman, J.M., Benton, R. and St Johnston, D. (2001) Development 128, 1201–1209.
[19] Guo, S. and Kemphues, K.J. (1996) Curr. Opin. Genet. Dev. 6, 408–415.
[20] Riechmann, V., Gutierrez, G.J., Filardo, P., Nebreda, A.R. and Ephrussi, A. (2002) Nat. Cell Biol. 4, 337–342.
[21] Bohm, H., Brinkmann, V., Drab, M., Henske, A. and Kurzchalia, T.V. (1997) Curr. Biol. 7, 603–606.
[22] Mata, J. and Nurse, P. (1997) Cell 89, 939–949.
[23] Brunner, D. and Nurse, P. (2000) Cell 102, 695–704.
[24] Brown, A.J., Hutchings, C., Burke, J.F. and Mayne, L.V. (1999) Mol. Cell. Neurosci. 13, 119–130.
[25] Biernat, J., Wu, Y.Z., Timm, T., Zheng-Fischhofer, Q., Mandelkow, E., Meijer, L. and Mandelkow, E.M. (2002) Mol. Biol. Cell 13, 4013–4028.
[26] Gavin, A.C. et al. (2002) Nature 415, 141–147.
[27] Hofken, T. and Schiebel, E. (2002) EMBO J. 21, 4851–4862.
[28] Doerflinger, H., Benton, R., Shulman, J.M. and Johnston, D.S. (2003) Development 130, 3965–3975.