Pom1 kinase links division plane position to cell polarity by regulating Mid1p cortical distribution

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Accepted 15 September 2006

Journal of Cell Science 119, 4710-4718 Published by The Company of Biologists 2006

doi:10.1242/jcs.03261

Summary

In fission yeast, Mid1p, a major determinant for division plane position, defines a medial cortical compartment where it recruits myosin II at the onset of mitosis to initiate contractile ring assembly. How Mid1p is restricted to the medial cortex is unknown. We report here that in a *pom1* polarity mutant, which displays a monopolar growth pattern, Mid1p distribution expands towards the non-growing cell tip, uncoupling Mid1p localization from nuclear position. This accounts for the displacement of the contractile ring during mitosis. By contrast, Mid1p localization is normal in a *bud6Δ* strain, indicating that Mid1p misdistribution is not a general consequence of monopolar growth. We conclude that Pom1 kinase acts as a negative regulator of Mid1p distribution, excluding Mid1p from non-growing ends, whereas a Pom1-independent mechanism prevents Mid1p association with growing ends. Our work therefore provides evidence that cell polarity regulators influence the distribution of Mid1p, linking division plane position to cell polarity.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/22/4710/DC1

Key words: Cell division, Cytokinesis, Cell polarity, Cytoskeleton

Introduction

The correct orientation of the division axis, perpendicular to the mitotic spindle axis, is crucial for the successful outcome of mitosis. In animal cells, the division plane is dictated by signals emanating from the mitotic spindle ensuring a direct coupling of both orientations. This mechanism enables animal cells to adapt the orientation of the division plane to late changes in spindle orientation and provides a mean to control division plane orientation by spindle rotation events during the development of multicellular organisms (for reviews, see D'Avino et al., 2005; Glotzer, 2005; Piekny et al., 2005). In yeasts, which have a rigid cell wall and a stereotyped organization, orientation of these two axes is defined independently of each other, according to the cellular pattern: in budding yeast the division plane coincides with the budding site and the spindle is aligned along the mother-bud axis; in fission yeast, the spindle orientates along the long axis of the cell and division takes place perpendicular to this axis, in the middle of the cell, at the position of the nucleus (for reviews, see Chang and Nurse, 1996; Balasubramanian et al., 2004; Wolfe and Gould, 2005). In this organism, the displacement of nuclei by using cell centrifugation or optical tweezers recently revealed that the nucleus can actively influence the position of the division plane (Daga and Chang, 2005; Tolic-Norrelykke et al., 2005).

A major factor for division plane definition in fission yeast is the nuclear shuttling protein Mid1p (Chang et al., 1996; Sohrmann et al., 1996; Paoletti and Chang, 2005), which forms a band at the medial cortex during interphase (Paoletti and Chang, 2000). Mid1p concentrates in this band at the onset of mitosis, upon nuclear export (Bahler et al., 1998; Paoletti and Chang, 2000), and is thought to specify the division plane position by recruiting myosin II to the medial cortex, thereby initiating the assembly of the contractile ring in the middle of the cell (Wu et al., 2003; Motegi et al., 2004). The recruitment of myosin II is mediated by an interaction between Mid1p and the C-terminus of the tail of myosin II heavy chain Myo2p, and is temporally regulated by dephosphorylation of Myo2p (Motegi et al., 2004). Myosin II recruitment is followed by the recruitment of other ring components including the formin Cdc12p and the PCH protein Cdc15p which trigger the nucleation of an F-actin meshwork (Fankhauser et al., 1995; Chang et al., 1997; Carnahan and Gould, 2003; Wu et al., 2003). Cdc12p and Cdc15p recruitment also depends on Mid1p (Wu et al., 2006). All ring components then compact into a tight ring precisely located in the middle of the cell, that starts contracting in late anaphase (Wu et al., 2003).

How Mid1p binds preferentially to the medial region of the cortex, which is crucial for its function, is not understood. We have recently shown that Mid1p anchorage to the cell cortex depends on a dual binding mechanism (Celton-Morizur et al., 2004). First, the Mid1p N-terminal region is able to form faint cortical spots in the medial region of the cortex, in the vicinity of the nucleus. Second, the Mid1p C-terminal domain interacts with membranes through an amphipathic helix. The interaction is reinforced by a polybasic cluster, which also functions as a NLS when Mid1p is not membrane-bound. Strikingly, like the full-length protein both Mid1p N-terminal and C-terminal domains show preferential anchoring to the medial cortex compared with the cell tips (Celton-Morizur et al., 2004). This suggests that the spatial control of Mid1p distribution is highly regulated and may involve several overlapping mechanisms.

Another factor involved in the definition of the division plane is the DYRK kinase Pom1p (Bahler and Pringle, 1998),

which displays genetic interactions with Mid1p (Bahler and Pringle, 1998). *pom1* mutant cells often form misplaced contractile rings and septa. In addition, *pom1* mutant cells have a monopolar pattern of growth, from the new or the old end, indicating that Pom1p also acts as a regulator of cell polarity. However, how Pom1 kinase regulates the position of the division plane remains unknown. Immunofluorescence experiments have shown that Mid1p is normally associated with the misplaced contractile rings in *pom1* mutant cells (Bahler et al., 1998), but these experiments did not reveal the distribution of Mid1p on the cortex before mitosis, which is crucial to understand how the contractile ring is positioned in these cells.

Since Pom1p localization at the cell tips depends on microtubules and on the polarity factors Tea1p and Tea4p (Bahler and Pringle, 1998; Tatebe et al., 2005), we speculated that Pom1p negatively regulates the association of Mid1p with the cell cortex at cell tips and links Mid1p cortical distribution to the establishment of cell polarity. We therefore determined the localization of Mid1p in *pom1* mutant cells. We indeed found that in these cells, Mid1p distribution on the cortex expands from the medial cortex towards the non-growing tip of the cell and is uncoupled from the position of the nucleus. We propose a model in which Pom1 kinase acts as a negative regulator of Mid1p distribution, excluding it from non-growing cell tips.

Results

Cortical Mid1p expands towards one cell tip in *pom1* mutants

To efficiently detect Mid1p on the cortex during interphase, we fused Mid1p to a quadruple green fluorescent protein (GFP) tag in C-terminus (mid1-4GFP) (Maekawa et al., 2003). In a control *mid1Δ* strain where this construct was integrated at the *leu1* locus and expressed under the control of the *mid1* promoter (strain AP1442, Table 1), mid1-4GFP was expressed like endogenous Mid1p and septa were properly positioned (Fig. 1, see also Fig. 5D,E). The fluorescent signal of mid1-4GFP was brighter but very similar to mid1-GFP signal (Fig. 1A, left) (Paoletti and Chang, 2000). mid1-4GFP also complemented the synthetic lethality of *mid1Δpom1Δ* double deletion. This indicates that the 4GFP tag does not alter Mid1p function or distribution.

In *pom1Δ* cells (Strain AP1502, Table 1), mid1-4GFP cortical distribution was different from in wild-type cells (Fig. 1A, right). First, during interphase, the nuclear staining was reduced as reported previously (Bahler et al., 1998). Second, instead of being restricted to the medial cortex, mid1-4GFP cortical staining expanded towards one cell tip. As shown on fluorescence intensity graphs along the cortex (Fig. 1D), mid1-4GFP spots were often unevenly distributed along the cortex. To analyze the distribution of mid1-4GFP in detail, we counted the number of cells containing mid1-4GFP spots on the medial, lateral and tip cortex on one side of the cell (see scheme in Fig. 1E; a, b and c, respectively). 72% of *pom1Δ* cells displayed Mid1p spots in all three regions (abc cells) compared with 3% in wild-type cells. The distribution of mid1-4GFP spots did not vary during cell-cycle progression, monitored by measuring cell length (Fig. 1E). We also performed an automated detection of mid1-4GFP cortical spots and measured their distance to the medial axis of the cell (Fig. 6A). mid1-4GFP spots were more numerous in *pom1Δ* cells (12 spots per single focal plane in AP1502, n=77 and 7.5 spots per single focal plane in AP1442, n=107 in single focal planes) and very frequently located far from the medial cell axis on one side of the cell: 45% of spots were located more than 2 μm apart from the cell medial axis compared with 17% in wild-type cells; 68% of spot centroids, which represent the mean distribution point of spots in individual cells and whose position is indicative of the asymmetry of spots distribution, were located more than 0.75 μm apart compared with 12% in wild-type cells (Fig. 6B,C).

Similar redistributions of Mid1p were observed in *pom1-1* mutant cells and in kinase-dead *pom1-2* mutant cells (our unpublished observations) (Bahler and Nurse, 2001). Therefore, Pom1p activity is required to prevent Mid1p association with one of the two cell tips. These experiments also indicated that in the *pom1* mutant, the coupling between Mid1p cortical distribution and nucleus position (Paoletti and Chang, 2000; Daga and Chang, 2005) is abolished. To further test this hypothesis, we ultracentrifuged cells in presence of the microtubule-depolymerizing drug MBC to move the nucleus away from the cell center (Khar and Mitchison, 1989; Daga and Chang, 2005) (see supplementary material Fig. S1a,c). Wild-type cells that were kept in MBC-containing medium slowly relocated Mid1p spots to the new cortical region facing the nucleus (see supplementary material Fig. S1b, arrowheads). By contrast, in *pom1Δ* cells, Mid1p failed to relocalize to the cortex facing the nucleus. Accordingly, the percentage of *pom1Δ* cells with cortical spots on one side and the nucleus on the other did not change over time after centrifugation (about 40% of cells with displaced nuclei, see supplementary material Fig. S1d, arrows). We conclude that, in absence of Pom1p activity, the coupling between Mid1p cortical distribution and nucleus position no longer operates.

Mid1p cortical distribution negatively correlates with the pattern of growth of *pom1Δ* cells

One consequence of mid1-4GFP cortical redistribution in *pom1Δ* cells is that some mid1-4GFP spots were localized at

Table 1. List of strains used in this study

AP 1442	*ade6-M216 leu1-32 ura4-D18 mid1Δ::ura4+ h– + pAP221 integrated (pmid mid1-4GFP, leu1+)*
AP 1502	*pom1Δ::ura4 mid1Δ::kanMX4 ura4-D18 leu1-32 ade-M216 h– +pAP221 integrated (pmid mid1-4GFP, leu1+)*
AP 1717	*tea3Δ::kanMX6 mid1Δ::ura4+ ura4D18 leu1-32 h– + pAP221 integrated (pmid mid1-4GFP, leu1+)*
AP 1727	*tea1Δ::ura4+ mid1Δ::kanMX4 ade6-M216 leu1-32 ura4-D18 h– +pAP221 integrated (pmid mid1-4GFP, leu1+)*
AP 1737	*tea3Δ::kanMX6 pom1Δ::ura4 mid1Δ::ura4 ura4D18 leu1-32 ade6-M216 h+ + pAP221 integrated (pmid mid1-4GFP, leu1+)*
AP 1758	*bud6Δ::kanMX6 mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 h– + pAP221 integrated (pmid mid1-4GFP, leu1+)*
AP 1329	*pom1-GFP::kanMX6 ura4-D18 h–*
AP 1760	*pom1-GFP::kanMX6 tea1Δ::ura4+ ura4-D18 ade6-M216 h–*
AP 1823	*pom1Δ::ura4 myo2-GFP::ura4+ ura4-D1 8 leu1-32 h+*

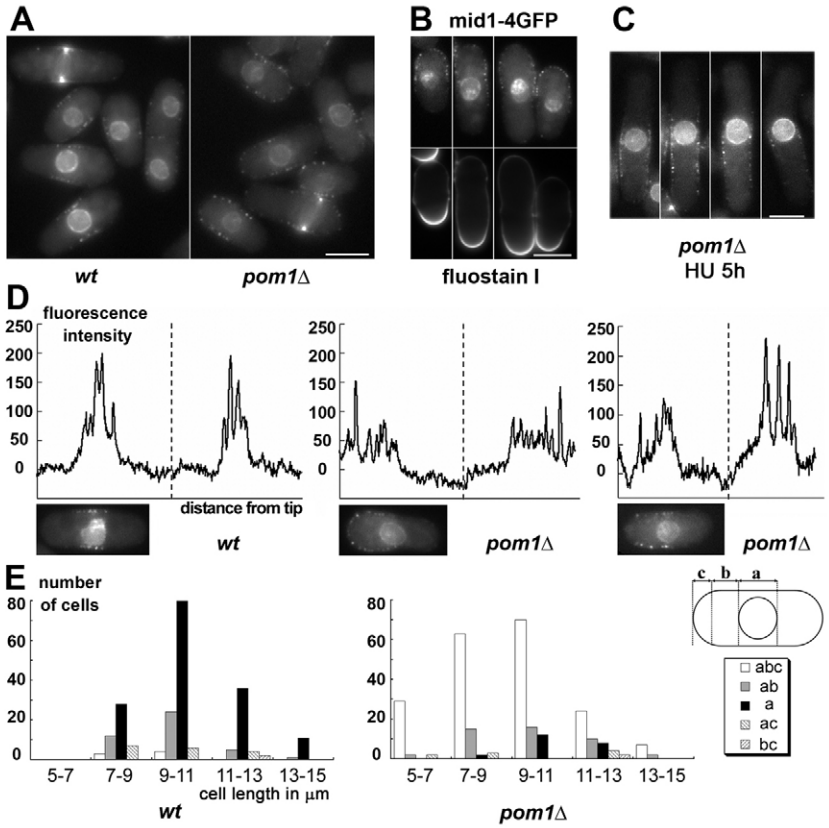

Fig. 1. Cortical mid1-4GFP expands towards the non-growing cell end in *pom1Δ* mutants. (A) GFP fluorescence in living wild-type (left panel-AP 1442) and *pom1Δ* cells (right panel-AP 1502) expressing mid1-4GFP, grown at 25°C. Bar, 5 μm. (B) GFP (upper panel) and fluostain I fluorescence (lower panel) in living *pom1Δ* cells expressing mid1-4GFP. Note that mid1-4GFP is present at the non-growing tip. Bar, 5 μm. (C) Distribution of mid1-4GFP in highly elongated *pom1Δ* cells obtained by treatment with 11 mM HU for 5 hours at 25°C. Note that HU treatment increases the pool of nuclear mid1-4GFP. mid1-4GFP spots are less abundant in the tip region compared with the medial or lateral cortex. Bar, 5 μm. (D) mid1-4GFP fluorescence profile along the cortex in wild-type (left panel) and *pom1Δ* cells (middle and right panels). Cortical intensity of mid1-4GFP was measured clockwise along the entire cell cortex of cells shown on the bottom, starting at the left cell tip. Dotted lines represent the position of the right cell tip. (E) Distribution of mid1-4GFP cortical spots in wild-type and *pom1Δ* cells. 223 wild-type and 268 *pom1Δ* cells from three independent experiments were analyzed. The presence of one or more mid1-4GFP spots at the medial (a), lateral (b) and tip (c) cortex (see scheme on the right) was scored in each cell, and correlated to cell length to monitor cell-cycle progression. Typical a-, abc- and ab-cells are shown in D (from left to right).

one cell tip, in the same area as the cell polarity regulator Tea1p (Mata and Nurse, 1997), which is normally localized at the two cell tips in *pom1* mutants (Bahler and Pringle, 1998). As *pom1* cells exhibit a monopolar pattern of growth (Bahler and Pringle, 1998), we tested whether the tip containing mid1-4GFP corresponded to a specific tip by using fluostain I, which brightly stains growing tips. mid1-4GFP was consistently found at the non-growing tip (Fig. 1B). This result is of particular interest because it indicates that, in absence of Pom1p a second mechanism may prevent Mid1p association with the growing tip.

Mid1p cortical redistribution accounts for division plane displacement towards the non-growing tip in *pom1Δ* cells

In wild-type cells, ring components are first recruited to the medial cortical compartment defined by Mid1p before compacting into a tight ring (Wu et al., 2003; Wu et al., 2006). This mechanism is thought to allow a precise positioning of the contractile ring in the middle of the cortical region defined by Mid1p. Cortical redistribution and uncoupling of Mid1p from the position of the nucleus in *pom1* mutants may therefore directly affect the position of the division plane during mitosis.

To directly test this hypothesis, we made time-lapse movies of wild-type and *pom1Δ* cells expressing mid1-4GFP. All wild-type cells expressing mid1-4GFP that entered mitosis formed well assembled, centrally placed contractile rings (Fig. 2Aa). By contrast, in the *pom1Δ* mutant, six out of nine cells entering mitosis during the movies formed offset rings that were displaced towards the non-growing cell tip where mid1-4GFP cortical spots were located before mitosis (Fig. 2Ab,c). In these cells, the division plane position did not correspond to the position of the premitotic nucleus (see stars in Fig. 2Ab,c). In the three other cells, contractile rings assembled centrally but they either exhibited shape defects or appeared not to compact fully, as judged from our analysis in a single focal plane (our unpublished observations).

Moreover, we found that in *pom1Δ* cells, myosin II heavy chain Myo2p, whose recruitment to the medial cortex depends on Mid1p (Wu et al., 2003; Motegi et al., 2004), first accumulated asymmetrically on the cortex before compacting into offset rings on the same side of the cell (Fig. 2B). We therefore conclude that the redistribution of Mid1p towards the non-growing cell tip is responsible for the mispositioning of the contractile ring.

Finally, it has been reported that highly elongated *cdc25 pom1Δ* cells that are released from the cell-cycle block form contractile rings and septa at or near the cell middle (Bahler et al., 1998). To understand why this is the case, we analyzed the distribution of Mid1p in elongated *pom1Δ* cells that were obtained by a 5-hour treatment with hydroxyurea (HU) at 25°C (Fig. 1C). As in control cells, mid1-4GFP spots were asymmetrically redistributed towards the non-growing cell tip. The distribution of spots varied from one cell to the other but, generally, the number of spots was reduced at the cell tips compared with the lateral or medial cortex. This reduction in spot number at the cell tip in very long cells might explain why ring-positioning defects are not more dramatic in highly elongated *pom1Δ* cells.

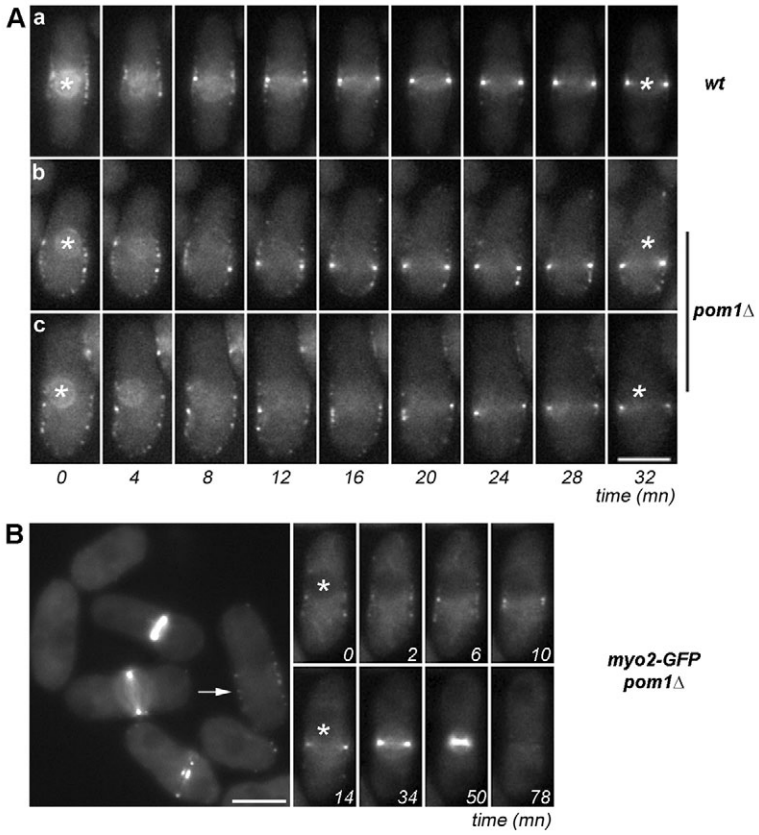

Fig. 2. Contractile ring position is uncoupled from nuclear position in *pom1Δ* mutant. (A) Time-lapse video-microscopy of wild-type (a) or *pom1Δ* cells (b,c) that express mid1-4GFP and were grown at 25°C during entry into mitosis. Cells were imaged for GFP fluorescence every 4 minutes. Note that, in *pom1Δ* cells the contractile ring forms away from the position of the premitotic nucleus (star), closer to the non-growing end where mid1-4GFP spots were distributed before mitosis. Bar, 5 μm. (B) Localization of myo2p in the *pom1Δ* mutant*:* myo2-GFP fluorescence in *pom1Δ* cells grown at 25°C (left) and time-lapse video-microscopy of a similar cell in early mitosis (right). Note the asymmetrical distribution of myo2-GFP cortical spots in early mitosis (arrow). In the early mitotic cell on the right, asymmetrically distributed spots of myo2-GFP (*t*=0-6 minutes) compact into an offset tight ring (*t*=14 minutes) that contracts (*t*=34 and 50 minutes) to form two daughter cells of unequal size (*t*=78 minutes). Star, position of the premitotic nucleus. Bar, 5 μm.

Mid1p cortical distribution is established before NETO in wild type cells

As in wild-type cells Pom1p relocalizes to the newly forming end during septation (Bahler and Pringle, 1998) but its activity only increases when the new end starts growing at new-end take off (NETO) (Bahler and Nurse, 2001), we wondered whether Pom1p increased activity is necessary to remove Mid1p from the cortex of the new cell tip at NETO. However, in wild-type cells, most pre-NETO cells, ranging from 7-9 μm in length (Mitchison and Nurse, 1985), displayed a medial distribution of mid1-4GFP spots on the cortex (56%; Fig. 1E). Similarly, in wild-type cells that expressed mid1-4GFP and were blocked in S phase – before NETO (Mitchison and Nurse, 1985) – by treatment with hydroxyurea (HU), mid1-4GFP cortical staining was still restricted to the central cortex overlying the nucleus, like in non-treated control cells (Fig. 3). We conclude that Mid1p distribution is restricted to the central cortex before NETO in wild-type cells. Pom1p might, therefore, start to act on Mid1p distribution earlier in the cell cycle, before Mid1p activity increases.

Mid1p dissociates normally from the contractile ring and the septum-assembly region in *pom1Δ* cells

To determine more precisely how early in the cell cycle Pom1p starts regulating Mid1p distribution, we recorded time-lapse movies during mitosis exit and septation. These movies show that in *pom1Δ* cells – like in wild type cells – mid1-4GFP spread laterally on the cell cortex surrounding the contractile ring in late anaphase, concomitantly with a reduction in the cortical staining and the reappearance of a nuclear pool of the protein (Fig. 4A).

We also analyzed mid1-4GFP distribution on single images to distinguish Mid1p cortical spots better during late stages of mitosis (Fig. 4B,C). Interestingly, cortical spots of mid1-4GFP were frequently detected at the old end, before or during sister-cell separation in *pom1Δ* cells, whereas they were already centrally located in wild-type cells. In conclusion, Pom1 kinase is not required for the release of Mid1p from the ring or septum region and may act on Mid1p cortical distribution immediately after the exit of mitosis.

Defects in Mid1p cortical distribution are specific to *pom1*-deficient cells

Since Mid1p cortical distribution in *pom1Δ* cells correlated negatively with the monopolar pattern of growth, we analyzed whether this defect was specific to *pom1* mutants or whether it could be observed in other strains deficient for bipolar growth.

In the *bud6Δ* strain (Glynn et al., 2001), in which 50% of the cells display a monopolar pattern of growth (Glynn et al., 2001), we observed a completely normal distribution of mid1-4GFP at the medial cortex (Fig. 5A,B; 10% of spots and 10% centroids located >2 μm and >0.75 μm apart from the medial axis, respectively, Fig. 6B,C). Accordingly, septa were normally positioned in this strain (Fig. 5D). This indicates that the defective distribution of Mid1p in *pom1Δ* cells is not a general consequence of their deficiency in establishing bipolar growth.

In *tea3Δ* cells, which grow monopolarly and have septum-position defects (Arellano et al., 2002), mid1-4GFP spots were most often restricted to the medial cortex (48.5±15% a cells instead of 66±11% in wild-type cells, Fig. 5A,B). The number of spots on the lateral cortex was slightly increased compared

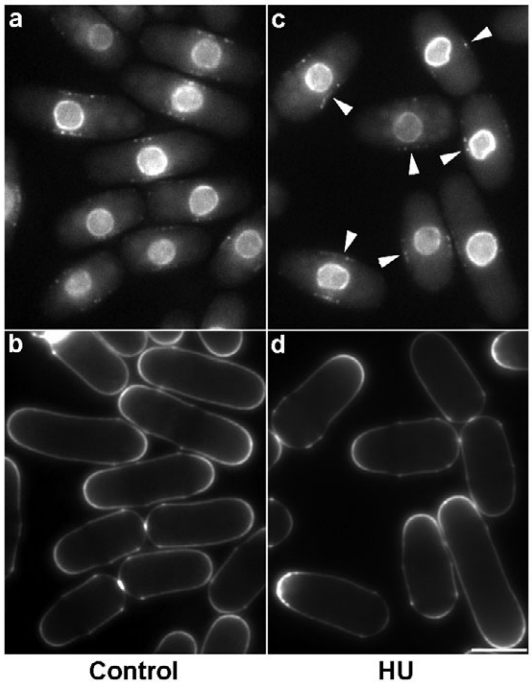

Fig. 3. Mid1p is located at the medial cortex before NETO. GFP fluorescence (a,c) and fluostain I staining of the cell wall (b,d) in wild-type cells expressing mid1-4GFP (AP1442) grown at 25°C, or wild-type cells grown for 3 hours in presence (c,d) or absence (a,b) of 11 mM HU to prevent DNA synthesis and to block cells before NETO. mid1-4GFP spots are medially located in monopolarly growing cells after HU treatment (arrowheads), indicating that Mid1p distribution to the medial cortex is established before NETO. Bar, 5 μm.

with wild-type cells (26% of spots and 22% centroids located >2 μm and >0.75 μm apart from medial axis, respectively, Fig. 6B,C). We also observed mild displacements of nuclei away from the cell center which could participate in the weak mispositioning of septa in this strain (25± 8.5%).

In *pom1Δtea3Δ* double mutants, which form strongly displaced septa at 36°C (Arellano et al., 2002) (our unpublished observations), mid1-4GFP displacement towards the non-growing tip was largely similar to what was observed in *pom1Δ* single mutants when cells were grown at 25°C (77.5±11.5% abc cells; Fig. 5A,B; 49% of spots and 67% centroids located more than 2 μm and 0.75 μm apart from medial axis, respectively; Fig. 6B,C). Some cells displayed strong defects in shape were not considered because their global polarity may be abnormal. Importantly, mid1-4GFP was still absent from the growing tip in *pom1Δtea3Δ* cells (Fig. 5A), indicating that Tea3p is not required to prevent Mid1p association with the growing tip in absence of Pom1p. We were unable to determine mid1-4GFP distribution at 36°C because the mid1-4GFP signal was lost in these conditions of growth (our unpublished observations).

Finally, the *tea1Δ* mutant provides a situation to test whether Pom1p localization to the cell ends is required to regulate Mid1p cortical distribution, because Pom1p localization to the cell ends was reported to be abolished in this background, whereas its kinase activity remained high (Bahler and Pringle, 1998; Bahler and Nurse, 2001). In *tea1Δ* cells, mid1-4GFP spots were frequently present on the lateral cortex of one cell end (50±1.5% ab cells compared with 20±9% in wild-type cells; Fig. 5A,B; 38% of spots and 46% centroids located more than 2 μm and 0.75 μm apart from medial axis, respectively; Fig. 6B,C). However, mid1-4GFP spots were infrequently found in the tip region (25±11% abc cells in *tea1Δ* cells compared with 64±5.5% in *pom1Δ* cells; Fig. 5A,B).

We did not expect a mild redistribution phenotype if Pom1p needs to concentrate at the cell ends to negatively regulate the distribution of Mid1p on the cortex. However, when we reexamined the pom1-GFP distribution in *tea1Δ* cells (Fig. 5C), we consistently observed some staining at the cell tips and in the septum region and, occasionally, on the lateral cortex. The fact that Pom1p does not dissociate fully from the cell tips in *tea1Δ* cells may therefore account for mid1-4GFP partial redistribution and septum mild mispositioning defects observed in this strain (40±10% compared with 5.5± 3% in wild-type cells and 90.5±0.3% in *pom1Δ* cells, Fig. 5D).

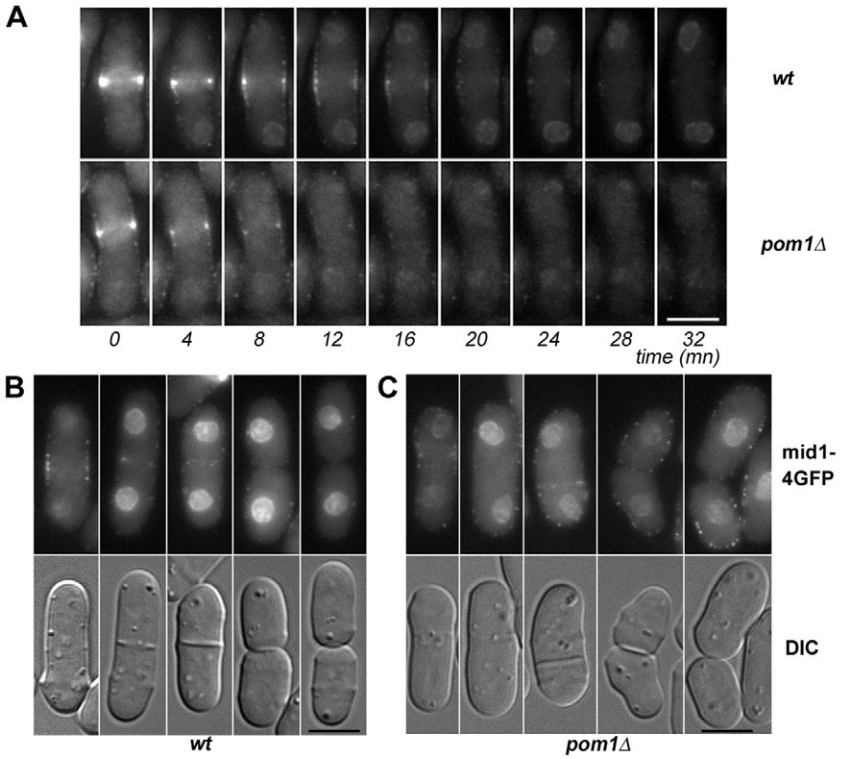

Fig. 4. Distribution of mid1-4GFP in late mitosis in wild-type and *pom1Δ* cells. (A) Time-lapse video-microscopy of wild-type (a) or *pom1Δ* cells (b-c) expressing mid1-4GFP grown at 25°C in late mitosis. Bar, 5 μm. (B,C) Distribution of mid1-4GFP cortical spots in living wild-type (B) and *pom1Δ* cells (C) during late anaphase and septation. Note that cortical spots of mid1-4GFP are frequently observed at the old end in a *pom1Δ* background. Bar, 5 μm.

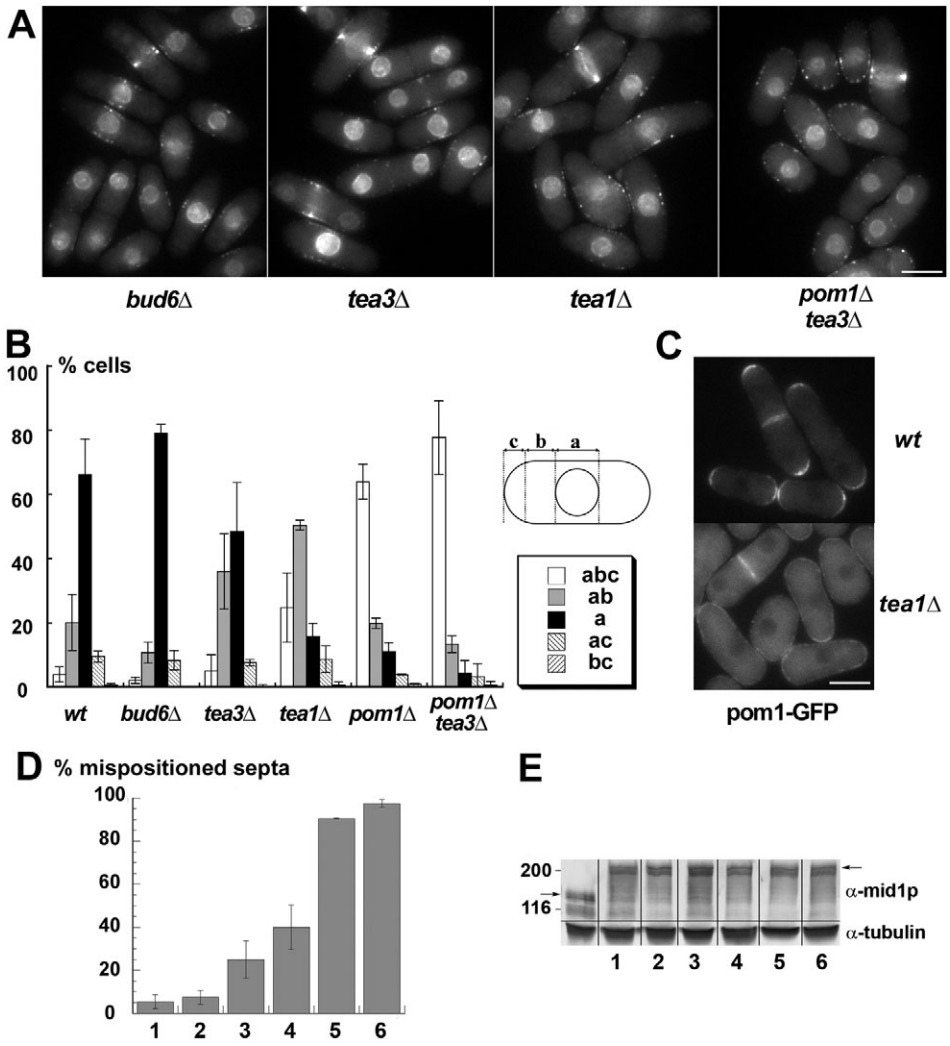

Fig. 5. Distribution of mid1-4GFP in other polarity mutants. (A) GFP fluorescence in live *bud6Δ* (AP1758), *tea3Δ* (AP1717), *tea1Δ* (AP1727) and *pom1Δtea3Δ* (AP1737) strains expressing mid1-4GFP, grown at 25°C. Bar, 5 μm. (B) Distribution of mid1-4GFP spots on the cortex of wild-type, *bud6Δ*, *tea3Δ*, *tea1Δ*, *pom1Δ* and *pom1Δtea3Δ* cells. Presence of one or more spots of mid1-4GFP at the medial (a), lateral (b) and tip (c) cortex was recorded in 300 to 400 cells from three to four independent experiments. Error bars give standard deviation (s.d.). (C) Localization of pom1-GFP in wild-type (AP1329) and *tea1Δ* cells (AP1760) grown at 25°C. In *tea1Δ* cells, pom1-GFP is still consistently detected at the cell tips and in the region of septum formation. Bar, 5 μm. (D) Septum position defects in polarity mutants. Cells were grown at 25°C and stained for septa with fluostain I. The percentage of abnormally placed or oriented septa was recorded in 400 to 800 septated cells from two to four independent experiments. Error bars give s.d. 1, wild type; 2, *bud6Δ*; 3, *tea3Δ*; 4, *tea1Δ*; 5, *pom1Δ*; 6, *pom1Δtea3Δ*. (E) mid1-4GFP expression level in polarity mutants. Western blot analysis of Mid1p [FC418 strain (Celton-Morizur et al., 2004), first lane and left arrow] and mid1-4GFP expression levels (right arrow) in wild type (1), *bud6Δ* (2), *tea3Δ* (3), *tea1Δ* (4), *pom1Δ* (5) and *pom1Δtea3Δ* (6) strains grown at 25°C using affinity purified anti-Mid1p antibody and TAT1 monoclonal antibody against tubulin.

Altogether, these results strengthen our hypothesis that Mid1p distribution on the cortex is specifically controlled by Pom1p and suggest that Pom1 kinase activity may locally inhibit Mid1p association with the cortex at the cell tips.

Discussion

We have found that in *pom1*-deficient cells, Mid1p distribution on the cortex expands from the medial cortex towards the non-growing tip of the cell and is uncoupled from the position of the nucleus. This defect is specific for *pom1*-deficient cells and not a general consequence of monopolar growth because we did not observe it in *bud6Δ* cells. Our analysis of the kinase-dead *pom1-2* mutant (our unpublished results) and of the *tea1Δ* mutant, in which Pom1p is partially delocalized, also reveals that the regulation of Mid1p distribution requires active Pom1 kinase, properly localized at the cell tips. This indicates that Pom1p functions as a negative regulator in the distribution of Mid1p, preventing its association with the cortex at non-growing cell tips (see Fig. 7). Accordingly, we found that Mid1p cortical spots were more numerous in *pom1Δ* cells compared with wild-type cells.

Our observations of Mid1p cortical distribution in wild-type and *pom1Δ* cells during mitosis exit indicate that Pom1p is not necessary for Mid1p dissociation from the contractile ring or from the region of septum formation where Pom1 kinase relocates in late mitosis. Since in *pom1Δ* cells growth resumes at the new end in about half of the cases (Bahler and Pringle, 1998), a defect in the dissociation of Mid1p from the septum region would not have been sufficient to explain a consistent Mid1p redistribution to non-growing tips anyway. In wild-type cells, Pom1p might rather prevent Mid1p distribution to non-growing tips in very early stages of the cell cycle, at the old end, before growth resumes in this region and at the new end until NETO.

Interestingly, we found that in absence of Pom1p, Mid1p is still excluded from the growing tip of the cell. Mid1p distribution is therefore directly linked to the pattern of growth in these cells. This indicates that, in addition to Pom1p, other factors localized at the growing tip and absent from the non-growing tip can negatively regulate Mid1p cortical distribution. In wild-type cells under bipolar growth, Pom1p-dependent and independent mechanisms might constitute overlapping mechanisms preventing Mid1p association with growing tips, whereas Pom1 kinase might act alone at non-growing tips in early phases of the cell cycle (see Fig. 7).

The fact that contractile rings assemble in the region of the

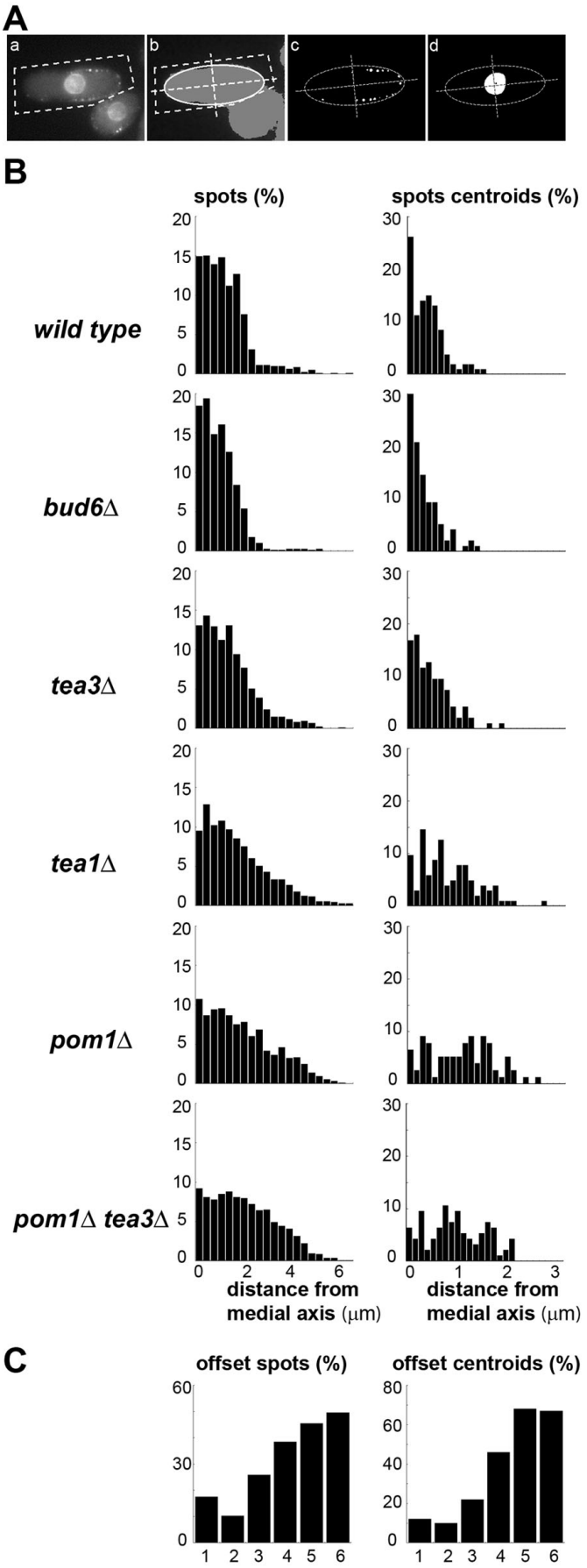

Fig. 6. Automated analysis of the distribution of mid1-4GFP cortical spots. (A) After manual selection (a), and determination of cell medial and long axes by ellipse fitting (b), coordinates of cortical spots (c) and nuclei (d) on cell axes were automatically extracted. (B) Distribution of distances between cortical spots (left) or spots centroids (right) and the medial axis in wild-type (n=107), *bud6Δ* (n=96), *tea3Δ* (n=95), *tea1Δ* (n=103), *pom1Δ* (n=77) and *pom1Δtea3Δ* (n=94) cells expressing mid1-4GFP, grown at 25°C. Spots centroids represent the mean distribution point of spots in individual cells. (C) Percentage of off-set spots (2 μm apart from the medial axis or more) or spots centroids (0.75 μm apart from the medial axis or more) in wild-type (1), *bud6Δ* (2), *tea3Δ* (3), *tea1Δ* (4), *pom1Δ* (5) and *pom1Δtea3Δ* (6) cells expressing mid1-4GFP, grown at 25°C.

cell where Mid1p cortical spots are redistributed before mitosis indicates that, Mid1p misdistribution is responsible for the displacement of the contractile ring towards the non-growing cell tip that has previously been reported in *pom1*-deficient cells (Bahler and Pringle, 1998). These data are consistent with the fact that septum-position defects are generally weaker in *pom1Δ* cells than in *mid1Δ* cells, most septa forming perpendicularly to the long axis of the cell, generally not more than a few micrometers away from the medial axis of the cell (Bahler and Pringle, 1998; Bahler et al., 1998) (our unpublished observations). Indeed, this localization of septa correlates well with the distribution of centroids of Mid1p spots, spread between 0-2 μm apart from the cell medial axis in *pom1Δ* cells (see Fig. 6B). Moreover, we found that in highly elongated *pom1Δ* cells, Mid1p cortical spots are generally less numerous in the tip region compared with the lateral cortex. This might explain why ring-positioning defects are not aggravated upon artificial elongation of *pom1Δ* cells (Bahler et al., 1998).

The reasons for decreased Mid1p staining at the tips in long *pom1Δ* cells need to be clarified. One hypothesis is that Mid1p anchoring to the cortex requires positive signals generated in the medial region of the cell. These positive signals would be sufficient to allow anchoring all along the cortex of non-growing ends of short *pom1Δ* cells but would not reach cell tips in highly elongated cells. The fact that after ultracentrifugation of wild-type cells Mid1p cortical spots relocate in the vicinity of the displaced nucleus in presence of the microtubule depolymerizing drug MBC suggests that such positive signals could be generated by the nucleus or by nucleus-associated structures. However, other parameters, such as the growth activity of the tip in the previous cell-cycle might also affect the distribution of Mid1p at the cell tips because we observed important variations in the distribution of spots along the cortex from one cell to another in highly elongated as well as in short *pom1Δ* cells (see Fig. 1C,D).

It will be important to determine how Pom1 kinase acts on Mid1p to regulate its cortical distribution. One obvious hypothesis is that Pom1 kinase directly phosphorylates Mid1p, thereby regulating its association with the cortex. To test this hypothesis, we performed in vitro Pom1 kinase assays on Mid1p purified from cell extracts and obtained negative results (our unpublished observations). We also produced a triple mutant of Mid1p on the three potential Pom1 phosphorylation sites that we detected on the sequence according to the DYRK2-DYRK3 kinase consensus site

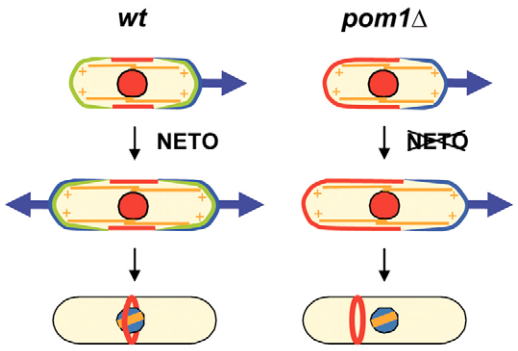

Fig. 7. A model of negative regulation of Mid1p cortical distribution. In wild-type cells, Pom1 kinase (green) is delivered to the cell tips in a microtubule-dependent manner. Before NETO (top), Pom1p excludes Mid1p (red) from the non-growing tip while at the growing tip (blue arrow), other polarity factors (blue) may act in conjunction with Pom1p. These two mechanisms operate at the two growing tips after NETO (middle). This allows the formation of a medial cortical band of Mid1p in early phases of the cell cycle and yields to the formation of a centrally placed contractile ring during mitosis (bottom). In *pom1*-deficient cells, which have a monopolar pattern of growth, Mid1p is only excluded from the growing tips. Mid1p asymmetrical distribution is responsible for the displacement of the contractile ring towards the non-growing tip of the cell during mitosis. Microtubules (orange) are represented during interphase with their plus ends facing cell tips and their minus ends anchored on the nuclear envelope (black circle) and during mitosis (bottom) as they form an intranuclear mitotic spindle.

(Campbell and Proud, 2002) (T34A, S218A, T517A). This mutant was correctly localized at the medial cortex and fully functional (our unpublished observations). Therefore, Pom1p may act indirectly on Mid1p.

In any case, in contrast to what was proposed from the observation that *pom1* and *mid1* have synthetic lethal interactions (Bahler and Pringle, 1998), our data indicate that Pom1p acts in the same pathway as Mid1p, controlling the position of the division plane. The synthetic lethality between the two deletions could either result from combined defects in cell polarity and morphogenesis, and in division plane positioning or from other functions of Pom1p in division plane placement, independent from Mid1p.

A search for additional factors having dual functions in the establishment of cell polarity and division plane placement may reveal intermediary factors through which Pom1p affects Mid1p distribution. It may as well identify the factors necessary to exclude Mid1p from the growing end of the cell independently of Pom1p. One candidate factor was Tea3p (Arellano et al., 2002). However, our analysis showed that Tea3p might only play a minor role in the regulation of Mid1p distribution on the cell cortex and, particularly, that Tea3p is not required to prevent Mid1p association with the growing tip in the absence of Pom1p.

Finally, our work provides the first evidence that the definition of the division plane by Mid1p is influenced by cell-polarity regulators and that Mid1p cortical distribution can be uncoupled from the position of the nucleus in their absence. Since Pom1 kinase is delivered to the cell tips in a microtubule-dependent manner (Bahler and Pringle, 1998), our work indicates that the nucleus controls the definition of the division plane indirectly through its ability to anchor antiparallel microtubule bundles at interphase microtubule organizing centers (iMTOCs) (Tran et al., 2001): microtubules bundles with their minus ends anchored on the nucleus and their plus ends at the cell tips deliver polarity regulators that in turn control Mid1p distribution on the cortex. It thus suggests that in fission yeast, like in animal cells, the microtubule network influences the definition of the division plane. One apparent objection to this model is that the depolymerization of microtubules does not affect Mid1p cortical distribution (Paoletti and Chang, 2000). This objection fails if Mid1p distribution is also regulated by microtubule-independent mechanisms or if the distribution of polarity regulators is multimodal, as recently described for Tea1p (Snaith et al., 2005).

Materials and Methods

Strains and plasmids

All strains used in the present study are isogenic to the wild-type 972 strain and listed in Table 1. Standard *S. pombe* genetic approaches were used (Moreno et al., 1991).

pAP221 plasmid (*pmid* mid1-4GFP *leu1+*) was obtained from pAP146 (Celton-Morizur et al., 2004) by replacing the GFP cassette (*Not*I-*Sma*I fragment) with the 4GFP cassette from pSM1023 (fragment *Not*I-*Bgl*II blunted) (Maekawa et al., 2003) a generous gift from E. Schiebel (ZMBH, Heidelberg, Germany). pAP221 was digested with *Nru*I and transformed into the SP1601 strain (*mid1Δ::ura4+*) (Sohrmann et al., 1996) to obtain strain AP1442. AP1442 was crossed with strain *mid1Δ::KanMX6* (our collection) to obtain strain AP1487 (*mid1Δ::KanMX6 + pAP221 integrated*).

Strains AP1502, AP1717, AP1727, AP1737 and AP1758 were obtained by crossing strains AP1442 with AP1487, and strains derived by genetic crosses with JB110 (*pom1Δ::ura4+*) a generous gift from J. Bähler (Sanger Centre, Hinxton, UK) (Bahler and Pringle, 1998), PN4370 (*tea3Δ::kanMX6*) a generous gift from P. Nurse, (Arellano et al., 2002), PN1690 [*tea1Δ::ura4+*, generous gifts from P. Nurse (Cancer Research UK, London, UK) (Mata and Nurse, 1997)], and AP37 (*bud6Δ::kanMX6 mid1Δ::ura4+*) (Glynn et al., 2001) (our collection). Strain AP1329 was derived from strain JB115 (*pom1-GFP::KanMX6*, a generous gift from J. Bähler) (Bahler and Pringle, 1998), and strain AP1760 was obtained by crossing AP1329 with a strain derived from strain PN1690. Strain AP1823 was obtained by crossing JB110 (*pom1Δ::ura4+*, a generous gift from J. Bähler) (Bahler and Pringle, 1998) with a myo2-GFP strain derived from MBY498 (myo2GFP::ura4+, a generous gift from M. Balasubramanian, Temasek Life Science Laboratory, Singapore).

Cell culture and imaging

All strains were exponentially at 25°C in YE5S under shaking and imaged between slides and coverslips after a short centrifugation (10 seconds at 10,000 ***g***) to concentrate the cells. Imaging was performed as described in Celton-Morizur et al. (Celton-Morizur et al., 2004), with acquisition times of 500 mseconds for time-lapse movies and of 2 seconds for mid1-4GFP or pom1-GFP and tea1-CFP single images. Cell walls were visualized by staining with fluostain I (2 μg/ml, Sigma, St Louis).

Ultracentrifugation of cells

To move the nucleus away from the cell center, 1 ml of wild-type (AP1442) or *pom1Δ* cells (AP 1502) grown exponentially at 25°C in YE5S were treated for 5 minutes with MBC (25 μg/ml, Aldrich, Milwaukee), deposited in a ultra-clear centrifuge tube (Beckman Instruments, Palo Alto) on top of a 1% SeaKem LE agarose cushion (Cambrex, Rockland), and ultracentrifuged for 8 minutes at 100,000 ***g*** in a SW41 swinging rotor (Beckman Instruments, Palo Alto, USA) as described by Daga and Chang (Daga and Chang, 2005). After centrifugation cells were either imaged shortly after a brief centrifugation to concentrate the cells (i.e. 10-20 minutes after the ultracentrifuge stopped running), or shaken at 25°C in medium containing MBC and imaged 2-2.25 hours later.

Cell extracts and western blot analysis

Total cells extracts and western blot analysis were performed as described in Celton-Morizur et al. (Celton-Morizur et al., 2004), using anti-Mid1p antibody (1:150) (Paoletti et al., 2003) and TAT1 monoclonal antibody against tubulin (1:2000, generous gift from Keith Gull (University of Oxford, UK).

Automated analysis of mid1-4GFP cortical spots distribution

For each image, cells were located by user region drawing (see in Fig. 6Aa). Cells were precisely segmented using an automated local threshold inside the regions. The center of the cell was set to the segmented region. The cell orientation α_c was

computed from an ellipse fitting on the region (see in Fig. 6Ab). (X_c, Y_c) and α_c define the major and minor axes of the cell, named long and medial axes respectively in the rest of the manuscript. Nuclei were detected using the MIA software developed on site, which allows the precise detection of objects with various sizes using 'a-trous' wavelet-segmentation algorithms (Starck et al., 1998). Spots were detected outside from the nuclear region with the same software (see Fig. 6Ac,d). Coordinates of spots on the axes of the cell were automatically extracted and analysed for all strains. Coordinates on the long axis directly correspond to the distance between the spots and the medial axis of the cell. The position of spots centroids (or center of mass) was calculated with a similar weight for each spot. Coordinates of spots centroids on the long axis directly correspond to their distance from the medial axis.

We thank V. Doye, I. Loïodice, A-M Tassin and P. T. Tran for critical reading of the manuscript, J. Bähler, M. Balasubramanian, P. Nurse and E. Schiebel for strains and plasmids. This work was supported by ARC (grant no. 3623 to A.P.). S.C.M. received a doctoral fellowship from ARC and a short-term traveling fellowship from Journal of Cell Science.

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