MTOC formation during mitotic exit in fission yeast

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SUMMARY

Microtubules polymerise from nucleation templates containing γ tubulin. These templates are generally concentrated in discrete structures called microtubule organising centres (MTOCs). In *Schizosaccharomyces pombe*, an equatorial MTOC (EMTOC) forms mid-way through anaphase B and then disassembles during the final stages of cell separation. We show that the EMTOC was generated by recruiting γ tubulin to the equatorial F-actin ring before it constricted to cleave the cell in two during cytokinesis. The EMTOC was not a continuous ring. It had a variable structure ranging from a horseshoe to a number of short bars. EMTOC integrity depended upon the integrity of the F-actin but not the microtubule cytoskeleton. EMTOC assembly required the activity of both the septation-inducing network (SIN) that regulates the onset of cytokinesis and the anaphase-promoting complex. Activation of the SIN in interphase cells induced F-actin ring formation and contraction and the synthesis of the primary septum but did not promote EMTOC assembly. In contrast, overproduction of the polo-like kinase, Plo1, which also induced multiple rounds of septation in interphase cells, induced EMTOC formation. Thus, the network governing EMTOC formation shared many of the regulatory elements that control cytokinesis but was more complex and revealed an additional function for Plo1 during mitotic exit.

Key words: Mitosis, γ Tubulin, MTOC, Cytokinesis, *Schizosaccharomyces pombe*

INTRODUCTION

Microtubules are filamentous polymers composed of repeating α and β tubulin heterodimers. Microtubules polymerise from complexes that contain another tubulin, γ tubulin. In most cells γ tubulin complexes are associated with discrete structures called microtubule organising centres (MTOCs) (Hagan et al., 1998). Alternative forms of acentrosomal microtubule nucleation occur in several systems, such as higher plants, some tissue culture cell lines and a number of meiotic systems (Hyman and Karsenti, 1998). The ability to organise microtubule arrays in the absence of a discrete MTOC in these systems may represent the activity of dispersed γ-tubulin-containing microtubule nucleation templates that can be organised by motor proteins. Alternatively it may arise from the promotion of tubulin assembly by stabilising microtubule-associated proteins (Hyman and Karsenti, 1998).

The microtubule nucleating capacity of the centrosome increases dramatically upon commitment to mitosis (Kuriyama and Borisy, 1981; Snyder and McIntosh, 1975). Visualisation of γ-tubulin–GFP fusions in living cells suggest that this increase arises from increased recruitment of γ tubulin complexes to mitotic centrosomes (Khodjakov and Rieder, 1999b). Mitotic recruitment of γ tubulin is likely to occur through the modification of existing MTOC proteins or the association of mitosis-specific proteins with this organelle. In *Drosophila*, recruitment of Asp protein appears to enhance the nucleation capacity of stripped centrosomes in vitro, suggesting that Asp may influence γ TURC (γ tubulin ring complex) complexes at the centrosome (Avides and Glover, 1999). In the budding yeast *Saccharomyces cerevisiae*, the Spc110p and Spc72p components of the spindle pole body (SPB) recruit γ tubulin complexes to the SPB (Knop and Schiebel, 1997; Knop and Schiebel, 1998). Functional equivalents have not been identified in higher eukaryotes, although compelling data supporting the existence of a molecule related to Spc110 (Tassin et al., 1997). It has recently been reported that Spc110 shares a conserved 'PACT' domain with mammalian pericentrin and mammalian AKAP450 (Flory et al., 2000; Gillingham and Munro, 2000). As this domain targets these two proteins to the centrosome, and pericentrin has been reported to associate with γ tubulin (Dictenberg et al., 1998), AKAP450/pericentrin is the prime candidate for an anchor for γ tubulin complexes.

Although the proteins required to nucleate microtubules are being identified, the mechanisms by which changes in the microtubule-nucleating capacity of the MTOC are regulated remain unclear. It is also unclear how these changes are coordinated with cell cycle progression to generate specific microtubule arrays. The extensive knowledge of cell cycle controls and dramatic changes in microtubule organisation that accompany mitotic commitment and exit in *Schizosaccharomyces pombe* make it an ideal model system for studying MTOC regulation and biogenesis.

There are three distinct MTOCs in fission yeast; two are present during mitotic growth whereas the other is specific for the sexual phase of the life cycle (Hagan, 1998; Hagan and Petersen, 2000; Petersen et al., 1998). The principle MTOC, the spindle pole body (SPB), is functionally equivalent to the centrosomes of higher eukaryotes. The fission yeast SPB undergoes cell-cycle-dependent changes in both nucleation competence and localisation, relative to the nuclear envelope

(Ding et al., 1997; Masuda et al., 1992). At the end of mitosis, cytoplasmic microtubules are nucleated from the SPBs and a region at the cell equator to generate a post anaphase array (PAA) (Hagan and Yanagida, 1997; Hagan, 1998; Hagan and Hyams, 1988; Horio et al., 1991; Vardy and Toda, 2000).

The equatorial region is also the site where an F-actin ring forms upon commitment to mitosis (Marks and Hyams, 1985). This ring persists until late anaphase when a signal from the septation-inducing network (SIN) promotes its constriction (Le Goff et al., 1999). Spg1, a small GTP-binding protein of the Ras superfamily, lies at the top of the SIN (Schmidt et al., 1997). Spg1 activity is regulated by a two part GAP protein complex composed of Cdc16 and Byr4 (Furge et al., 1998). The protein kinases Cdc7, Sid1 and Sid2 and the products of *mob1*$^+$, *cdc11*$^+$, *cdc14*$^+$, and *sid4*$^+$ act in this network (Balasubramanian et al., 1998; Chang and Gould, 2000; Fankhauser and Simanis, 1993; Fankhauser and Simanis, 1994; Guertin et al., 2000; Salimova et al., 2000; Schmidt et al., 1997; Sparks et al., 1999). None of these proteins is required for F-actin ring assembly, but they are required to drive the use of this ring for cytokinesis and septation. Other aspects of cell cycle progression proceed normally if the SIN does not function (Balasubramanian et al., 1998; Le Goff et al., 1999; Nurse et al., 1976).

We have used indirect immunofluorescence microscopy and an array of yeast mutants with specific defects in cell cycle progression to show that the equatorial MTOC (EMTOC) is an incomplete ring structure whose integrity requires F-actin but not microtubules. We describe a role for the SIN and the anaphase-promoting complex (APC/C) in the regulation of EMTOC formation and describe data that suggest that these regulatory steps maybe controlled by the polo-like kinase Plo1.

MATERIALS AND METHODS

Cell culture and strains

The strains used in this study are listed in Table 1. Cells were cultured in yeast extract media (YES) or appropriately supplemented EMM2 minimal media, as described in Moreno et al. (Moreno et al., 1991). 2 μM Thiamine was added to minimal EMM2 media to repress the *nmt1*$^+$ promoter (Maundrell, 1990). Hydroxy urea (HU) was added from a 1.2 M stock solution in water to a final concentration of 12 mM. A 20 mM stock solution of Latrunculin-A (LAT-A; a generous gift from K. Ayscough) in DMSO was added to a final concentration of 20 μM. Thiabendazole (TBZ; Sigma) was made as an 40 mg ml^{-1} stock solution in DMSO. Synchronous populations of cells were generated by centrifugal elutriation using a Beckman JE 5.0 rotor.

Generation of anti-γ tubulin antibodies

Full-length fission yeast γ tubulin protein on plasmid pTS251 was expressed in *Escherichia coli* BLR21 containing plysS$^+$ (Stearns et al., 1991). After induction, γ tubulin was recovered from insoluble inclusion bodies and used in conjunction with Titermax Gold adjuvant (Stratatech) to generate polyclonal antibodies, which were affinity purified using bacterial γ tubulin immobilised on nitrocellulose (Harlow and Lane, 1988).

Indirect immunofluorescence microscopy

Standard anti-α-tubulin immunofluorescence procedures were used to stain microtubules (Hagan and Hyams, 1988). For detection of γ-tubulin, Dmf1/Mid1 and Pk glutaraldehyde was omitted from the fixation step. Anti-Sad1 AP9.2, TAT1 (anti-α tubulin), anti-Mid1/Dmf1 and MAb 336 anti-Pk antibodies were used as described previously (Craven et al., 1998; Hagan and Yanagida, 1995; Sohrmann et al., 1996; Woods et al., 1989). Anti-γ-tubulin antibodies were concentrated 10-fold with macrosep filtration columns (Flowgen, UK) and diluted 1/3 or 1/2. Actin was visualised in cells fixed in –80°C methanol using the N350 monoclonal antibody (Lin, 1981) or in formaldehyde-fixed cells with rhodamine-conjugated phalloidin (Sigma) (Marks and Hyams, 1985). DAPI (4,6-diamidine-2-phenyl-indole) and calcofluor staining were as previously described (Moreno et al., 1991; Toda et al., 1981).

Table 1. Strains used in this study

Strain number	Genotype	Source
IH109	h$^-$ *cut4.533*	Hirano et al., 1986
IH111	h$^-$ *cdc14.118*	Nurse et al., 1976
IH119	h$^-$ *cut8.563*	Hirano et al., 1986
IH127	h$^-$ *cdc11.132*	Nurse et al., 1976
IH132	h$^-$ *cdc25.22*	Nurse et al., 1976
IH154	h$^-$ *cut9.665 leu1.32*	Hirano et al., 1986
IH365	h$^-$ *ura4.d18 leu1.32*	Lab collection
IH366	h$^+$ *ura4.d18 leu1.32 his2*	Lab collection
IH738	h$^-$ *cdc7.A20 ura4.d18*	Fankhauser and Simanis, 1994
IH758	h$^-$ *dmf1.6*	Sohrmann et al., 1996
IH1083	h$^-$ *ura4.d18 leu1.32* pCT134	This study
IH1291	h$^-$ *leu1*::pINT5 dma1	Murone and Simanis, 1996
IH1297	h$^-$ *cdc16.116 ura4.d18*	Nurse et al., 1976
IH1387	h$^-$ *nda3.1828 ura4.d18 leu1.32*	Radcliffe et al., 1998
IH1469	h$^+$ *sid1.239 ura4.d18 leu1.32 ade6.M216*	Balasubramanian et al., 1998
IH1470	h$^+$ *sid2.250 ura4.d18 leu1.32 ade6.M216*	Balasubramanian et al., 1998
IH1471	h$^-$ *sid4.A1 ura4.d18 leu1.32 ade6.M216*	Balasubramanian et al., 1998
IH1548	h$^-$ *cdc25.22 leu1.32* pREP1plo1	Ohkura et al., 1995
IH1554	h$^-$ *ura4.d18 leu1.32* pREP41GPkC	This study
IH1556	h$^-$ *spg1.B8 ura4.d18 leu1.32*	Schmidt et al., 1997
IH1740	h$^-$ *spg1.B8 cdc7.A20 ura4.d18 leu1.32*	Tanaka et al., 2001

Microscopy

Images were obtained using a Hamamtsu SIT camera and NIH image software or a Quantix Photometrics CCD camera with Metamorph software (Universal Imaging). Spindle and cell lengths were determined with Metamorph. A Deltavision system (Applied Precision) was used in conjunction with Softworks software to produce 3D reconstructions of deconvolved serial optical slices through EMTOC-containing cells.

RESULTS

γ tubulin antibodies recognise the SPB and EMTOC

Two polyclonal anti-γ-tubulin antisera were generated by using bacterially expressed *S. pombe* γ tubulin as an immunogen. Affinity-purified antibodies from these sera (IH1 and IH2) recognised a band of approximately 50 kDa on immunoblots of wild-type cell extracts (data not shown). The relative intensity of this band increased in extracts from a strain containing *tug1*$^+$/*gtb1*$^+$ on a multicopy plasmid, and an additional band was observed at 60 kDa upon expression of a plasmid-borne version of *tug1*$^+$/*gtb1*$^+$ that was fused to an epitope tag (data not shown).

Immunofluorescence microscopy of wild-type cells with a mixture of IH1 and IH2 antibodies revealed that γ tubulin concentrated at the sites of microtubule nucleation, the SPBs,

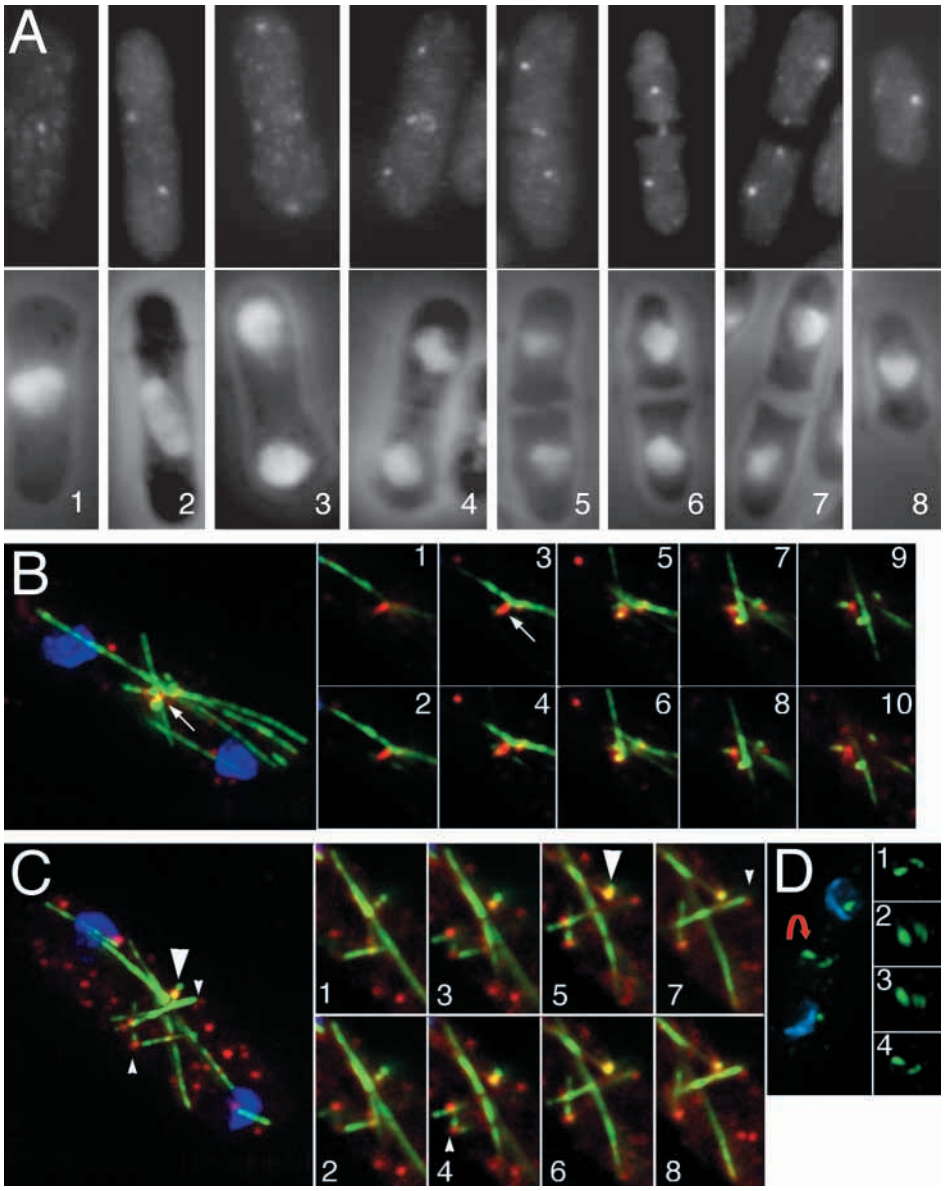


Fig. 1. Immunolocalisation of γ tubulin. (A) γ tubulin localisation and cell cycle progression. Cells are arranged in order of mitotic progression from G_2 (1) through mitosis (2,3) to septation (4-7), and back into G_2 once more (8). γ tubulin was found on the SPB throughout the cell cycle, appeared at the cell equator during anaphase B (3) and persisted (cells 4-7) until septation was complete (compare 7 and 8). Note that fission yeast do not extrude a structure containing γ tubulin as mammalian cells do with a mid-body, instead they retain medial γ tubulin and divide it between the two new daughter cells (7). (B,C) Wild-type cells were stained with a mixture of rabbit anti-γ-tubulin (red) and mouse anti-α-tubulin (green) antibodies and then processed to stain with DAPI (blue) before imaging. Optical sections were processed by the application of deconvolution algorithms. In each panel, the left hand figure shows a projection of all of the images into a single plane of view, whereas the smaller numbered panels show sections through the series that produced this image from top (1) to bottom (2). (B) Not all regions of the EMTOC that contain γ tubulin staining are associated with the ends of microtubules (arrow). (C) The upper large arrowhead shows the EMTOC. The two small arrowheads highlight cortical spots of γ tubulin staining that are associated with microtubule ends. (D) 3D deconvolution of γ tubulin staining shows that the EMTOC is not a continuous ring. The left hand panel shows a projection of a cell that contains an EMTOC, whereas the right hand, numbered, panels show four snap shots, as a 3D reconstruction of this structure was rotated through 360° in the direction indicated by the red arrow. The EMTOC in this cell is composed of two crescents of γ tubulin staining that face each other from opposite sides of the cell equator.

throughout the cell cycle and at the cell equator from mid-anaphase B until cell separation (Fig. 1). Identical patterns were seen when cells were fixed with formaldehyde or immersed in cold solvent and stained using either antibody alone or both mixed together (data not shown). Similarly, localisation of epitope-tagged γ tubulin gave the same distribution as the polyclonal antibodies. The absence of equatorial γ tubulin staining during cytokinesis in *cut* mutants (see below) provided further confirmation that the antibodies specifically recognised γ tubulin rather than a component of the F-actin ring. We conclude that IH1 and IH2 recognise *S. pombe* γ tubulin and stain the SPB throughout the cell cycle and the EMTOC at the cell equator towards the end of mitosis.

EMTOC formation and cell cycle progression

The precise timing of EMTOC formation was established by comparing the presence of equatorial γ tubulin staining to nuclear morphology and the extent of septation. Mitotic nuclei are led towards the cell tips during anaphase by the SPBs (Fig. 1A, cell 2). When the spindle disassembles, the SPB moves around and leads the nucleus back to a region that will become the middle of the daughter cell (Fig. 1A, cell 4) (Hagan and Yanagida, 1997). Comparing the relative locations of the SPB and chromatin showed that the EMTOC formed before the end of anaphase B (Fig. 1A). In cells that were dividing, with an average length of 12.5 μm, the EMTOC formed when the spindle had reached 9.5 μm (Fig. 2). The diameter of the EMTOC decreased during cytokinesis (Fig. 1A, cells 3-7). Just before cell separation, γ tubulin dots were observed immediately interior to the septum in each daughter cell (Fig. 1A, cell 7). γ tubulin was restricted to the SPBs once septation was complete (Fig. 1A, cell 8).

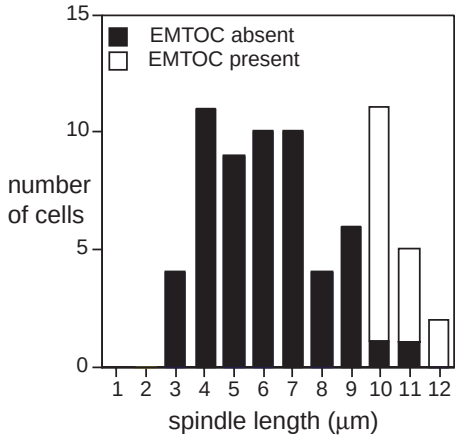


Fig. 2. The EMTOC appears towards the end of anaphase B. Wild-type cells (IH365) were cultured to mid-log phase in YES media at 25(C before processing for immunofluorescence microscopy. The spindle and cell length were measured. Because the cell structure shrinks back from the cell wall during preparation, the spindle length figures were calculated by multiplying measured spindle length by the length of the cell wall shell and dividing by the length of the shrunken cell structure.

EMTOC structure

Focusing the microscope through the EMTOC, suggested that it may not be a continuous structure (Fig. 1A, cell 4). We therefore used confocal and 3D deconvolution microscopy to reconstruct 3D models of 33 EMTOCs. In none of these cases was the EMTOC a complete ring. The structure was highly variable, ranging from a horseshoe shape (Fig. 1A, cell 4) through to discrete crescents around the cell equator (Fig. 1D). At later stages the structure sometimes seemed to have a helical pitch as it stretched between the two cells (data not shown). The variability in the structure of the EMTOC may account for the variability in the patterns of microtubules of the PAA. We therefore generated 3D reconstructions of α and γ tubulin distribution in the same cells (Fig. 1B,C). This staining showed that not all regions of the EMTOC were associated with microtubules at any one point in time (Fig. 1B, panel 3 arrow). In addition it revealed cortical γ tubulin foci (Fig. 1C, small arrowheads) that were close to, but not part of, the main body of the EMTOC (Fig. 1C, large arrowhead), yet they were associated with the ends of microtubules. Localisation to the end of a microtubule would be consistent with a role for γ tubulin in microtubule nucleation. This may suggest that the other spots seen in the cells at this time may represent free, inactive, γ tubulin complexes.

The EMTOC and the medial F-actin ring

The localisation of F-actin and γ tubulin and the frequency of division-related events were monitored in a culture that had been synchronised with respect to cell cycle progression showed that F-actin rings formed much earlier than the EMTOC (Fig. 3D).

We used two approaches to determine whether the F-actin ring and the EMTOC appear at the cell equator by a similar mechanism or whether each independently associates with the midpoint of the cell. In the first approach we examined γ tubulin in *dmf1.6* cells, which form misplaced and misshapen F-actin rings PAAs and septa (Chang et al., 1996; Sohrmann et al., 1996). The EMTOC did not form at the equator in these mutant cells but re-located, along with F-actin and the septation machinery, to the aberrant septation sites where it nucleated microtubules (Fig. 3B,C).

Table 2. The EMTOC is dispersed following depolymerisation of F-actin

Treatment	% EMTOCs	% F-actin rings
DMSO added 90 minutes after shift to 25°C and 15 minutes before fixation at 105 min	38	18
200 μM LAT-A added 90 minutes after shift to 25°C and 15 minutes before fixation at 105 minutes	0	0

We next asked whether disruption of the equatorial F-actin ring would affect EMTOC structure. The design of this experiment was complicated because F-actin appears to be vital for growth in *S. pombe*, and cells have to attain a critical cell volume before they will commit to mitosis (Marks and Hyams, 1985; Mitchison and Nurse, 1985). Thus, F-actin disruption by addition of Latrunculin-A (LAT-A) to interphase cells would make it impossible to judge the effect of F-actin depolymerisation on EMTOC formation by inhibiting growth and thus commitment to mitosis when the EMTOC forms. Cells were therefore temporarily arrested at the G_2/M boundary without blocking cell growth by incubating *cdc25.22* cells at 36°C for 255 minutes (Hagan and Hyams, 1988; Nurse et al., 1976). Upon return to the permissive temperature of 25°C these cells exceeded the critical size threshold for division and so immediately entered mitosis. 90 minutes after returning the culture to 25°C, 40% of the cells had progressed to the point in anaphase B when the EMTOC forms (Fig. 3E, arrowhead). The addition of LAT-A to these cells led to the disappearance of both F-actin rings and EMTOCs from a sample fixed 15 minutes later (Fig. 3E, smaller arrow) (Table 2). As both were still present in control cultures treated with DMSO alone, these manipulations show that F-actin is essential for the structural integrity of the EMTOC. The excellent synchrony in this culture also showed that the frequency of cells with Dmf1/Mid1 rings (Fig. 3F, open circles) declined as the number of cells with an EMTOC increased (Fig. 3F, diamonds).

Microtubules and EMTOC formation

Minus-end-directed motors capture randomly nucleated microtubules and move towards the γ tubulin complexes at the minus ends of these tubes to organise arrays that resemble those nucleated from an MTOC (Hyman and Karsenti, 1998). If a minus-end-directed microtubule motor protein were associated with the medial F-actin ring in *S. pombe*, it would move to the minus end of microtubules until it encountered the γ tubulin cap. If the motor was anchored at the ring, the result would be a ring-like array of γ tubulin at the equator. If this were the case, γ tubulin should not be seen at the equator when cells septate in the absence of microtubules. We exploited the temperature-sensitive *nda3.1828* β tubulin strain (Radcliffe et al., 1998) to test this possibility. Microtubules are highly unstable at the restrictive temperature in cells bearing this mutation. Like other *S. pombe* cells with defective interphase microtubule function, tip extension is only delocalised and not blocked by the loss of microtubules. The cells therefore enter

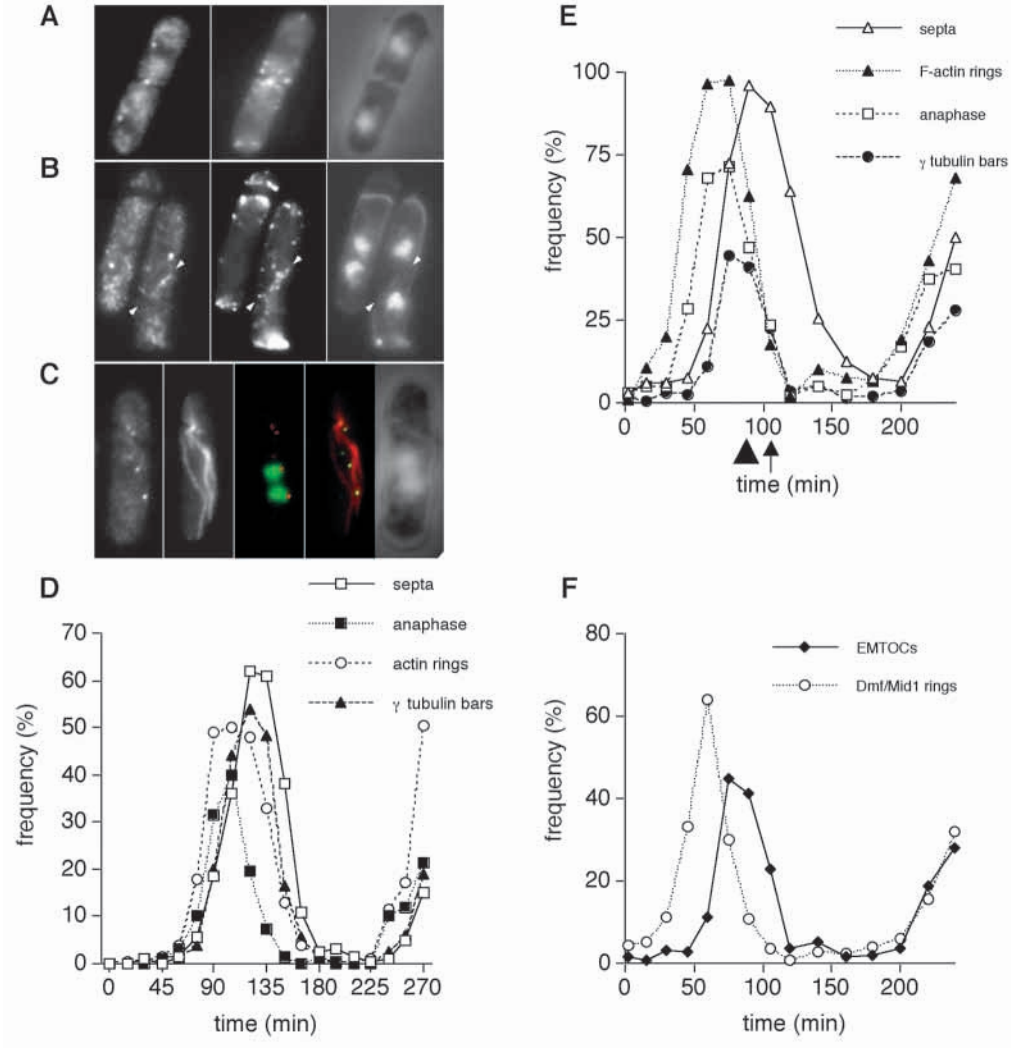


Fig. 3. The EMTOC and the F-actin ring. Wild-type (A) or *dmf1.6* cells (B,C) were fixed and stained with antibodies specific to γ tubulin, F-actin (A,B) or α tubulin (C). (A) γ tubulin (left), actin (middle) and a DAPI/phase contrast image of a wild-type cell during the late stages of cytokinesis. (B) γ tubulin (left), actin (middle) and a DAPI image of a *dmf1.6* cell during cytokinesis at 36°C. The arrows indicate that the EMTOC forms along the sloping F-actin ring. (C) A *dmf1.6* cell during cytokinesis at 36°C. From left to right: γ tubulin; α tubulin; γ tubulin (red) and chromatin (green); γ tubulin (green) and α tubulin (red); combined DAPI/phase contrast image. Note the microtubule nucleation from the asymmetrically located EMTOC. (D) A wild-type (IH365) culture was synchronised with respect to cell cycle progression by centrifugal elutriation and the indicated features were scored as the population underwent a synchronous cell division. Cells were maintained at 25°C throughout the experiment. The proportion of cells with F-actin rings increased before the appearance of cells with EMTOCs and calcofluor-stained septa, which increased simultaneously. The F-actin ring and EMTOC disappeared together at the end of mitosis when F-actin ring staining was replaced by more general dot-like staining at the junction of the two separating cells. (E,F) An exponentially dividing culture of *cdc25.22* cells was shifted to the restrictive temperature for 225 minutes and returned to the permissive temperature (at 0 minutes) to generate a synchronous mitosis. Indicators of mitotic progression in an untreated control culture are shown in the graph. Cells were sampled and treated with LAT-A dissolved in DMSO to inhibit F-actin polymerisation, or the solvent DMSO alone just after the peak of EMTOC formation (arrowhead). Both of these cultures were fixed and stained 15 minutes later to visualise γ tubulin and F-actin (arrow). F-actin rings were not observed after LAT-A addition to the media (Table 2, *n*=400 cells). Similarly, EMTOCs were observed in the control cells but not in those exposed to LAT-A (*n*=400 cells). Anaphase was scored as cells in which the SPBs are on diametrically opposite sides of the two daughter nuclei. (F) The frequency of cells containing Dmf1/Mid1 rings showed a sharp decline as EMTOCs appeared.

mitosis when they have exceeded the critical size threshold. As the defect arising form this particular tubulin mutation does not activate a checkpoint response at 36°C, even when all the microtubules are absent, the cells continue through mitosis and septate. We therefore assessed the requirement for microtubules during EMTOC formation by asking whether γ tubulin associated with the F-actin ring during this cytokinesis.

We adopted two measures to ensure that microtubules were completely absent from the cells at the restrictive temperature and an additional control to confirm that this apparent absence was not due to processing artifacts. An asynchronous *nda3.1828* culture was incubated in an ice/salt bath for 10 minutes to completely depolymerise the microtubules. During this incubation the anti-microtubule drug thiabendazole was

Table 3. EMTOC formation is not inhibited by the abolition of microtubules

	EMTOCs	Cuts	Cuts with EMTOCs	Spindles	Cytoplasmic microtubules?
nda3.1828	7.5%	5%	2%	5%	No
nda3.1828+DMSO	8.5%	2%	1.5%	3.5%	No
nda3.1828+300 μg ml^{-1} TBZ	12.5%	12.5%	12.5%	0	No
Control wild-type cells mixed with *nda3.1828*+300 μg ml^{-1} TBZ	n/d	n/d	n/d	4%	In some cells

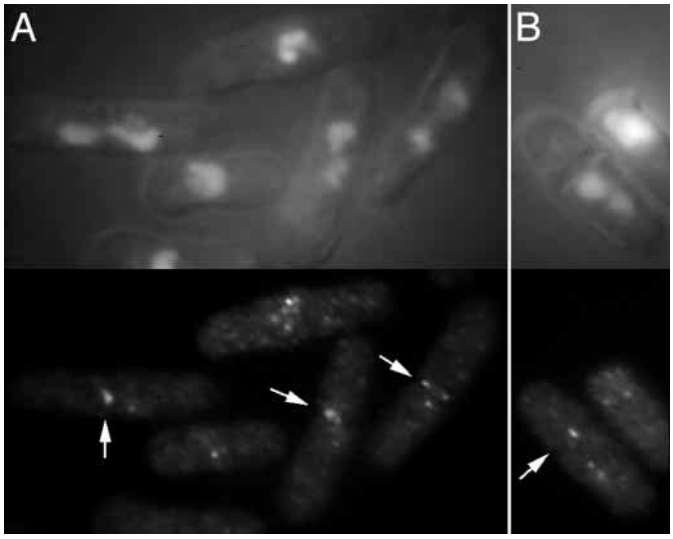


Fig. 4. EMTOC formation does not require microtubules. *nda3.1828* cells were manipulated by cold shock followed by treatment with 300 μg ml^{-1} TBZ at 36°C, as described in the text before staining for γ tubulin (lower panels) and chromatin (DAPI/DIC) (upper panels). EMTOC formation (arrows) accompanied septation, as septation occurred in the absence of microtubules.

added to the culture to a final concentration of 300 μg ml^{-1} and the culture was then incubated at 36°C. 90 minutes later cells were prepared for immunofluorescence microscopy. Before fixation the cultures were split into two, and untreated wild-type cultures were mixed with one of the aliquots seconds before all samples were processed to stain microtubules, γ tubulin and DNA. Although microtubules were absent in the drug-treated mutant culture, they were present in the dividing, wild-type cells of the mixed samples. We conclude that the inability to see microtubules in the drug-treated cultures reflected a genuine absence rather than a processing artifact. EMTOCs formed as these cells underwent their inappropriate septation event and decondensed their chromosomes to regenerate the diffuse chromatin staining seen in interphase cells (Fig. 4) (Table 3). Thus the EMTOC is a bona fide MTOC rather than a motor-generated coalescence of microtubule ends.

Table 4. EMTOC formation and SIN activity

Strain	Frequency of EMTOCs
Wild-type	11%
spg1.B8+wild-type	0% *spg1.B8* 11% wild-type
cdc7.A20+wild-type	0% *cdc7.A20* 11% wild-type
cdc11.132	0%
cdc14.118	0%
sid1.239	0%
sid2.250	0%
sid4.A1	0.5%
spg1.B8 cdc7.A20	0%
cdc16.116 cells septating in G_1 phase	0%

The table indicates the frequency of cells that have an EMTOC after incubation of an asychronous culture for five hours at the restrictive temperature (*n*=200 for each sample). Where indicated, wild-type cells were mixed with mutant cells prior to fixation in order to ensure that an absence of EMTOCs in mutants was not due to a processing problem.

SIN activity is required for EMTOC formation

The F-actin ring forms during prophase and constricts towards the end of anaphase B. The constriction of the ring and the subsequent formation of a primary septum is regulated by the SIN (Le Goff et al., 1999). The SIN becomes active midway through anaphase B when a number of SIN components are found on one of the two SPBs (Cerutti and Simanis, 1999; Guertin et al., 2000; Sohrmann et al., 1998). The coincidence between EMTOC appearance and monopolar distribution of SIN components suggested that the EMTOC may form as a consequence of SIN activation. If so, one would predict that the EMTOC would not form when SIN components were defective. We therefore stained cells that contained temperature-sensitive mutations in all of the known SIN components with γ tubulin antibodies. In every case, γ tubulin staining of SPBs was unaffected, but EMTOCs were not seen (Table 4). Equatorial γ tubulin staining was also absent when the SIN was inhibited by overproduction of the spindle assembly checkpoint component Dma1 (data not shown)

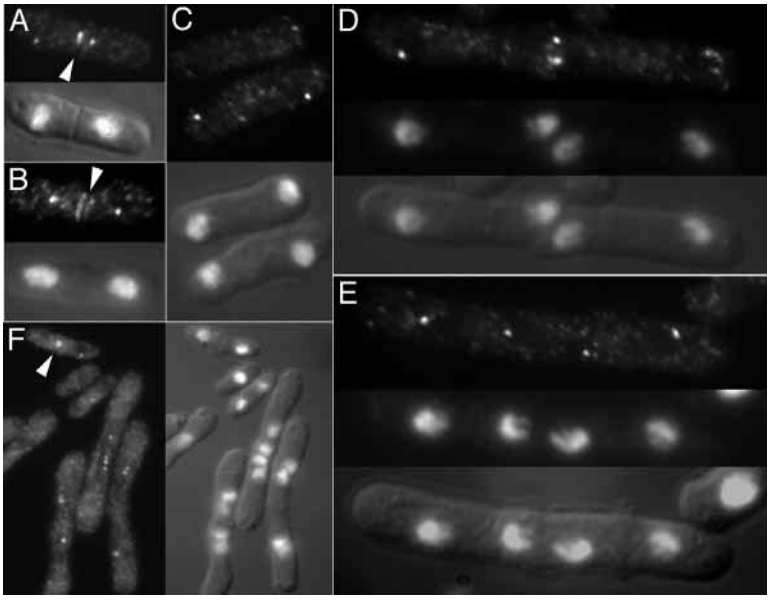


Fig. 5. EMTOC formation requires SIN function. *spg1.B8 cdc7.A20* double mutant cells were grown to early log-phase at 25°C before the culture temperature was shifted to 36°C. Panels show γ tubulin (upper panels in A-E and left in F) and DAPI/DIC images (lower panels in A-E and right in F) of the same fields of cells. EMTOCs clearly formed at 25°C (arrowheads A, B) but were not seen in the first (C) or second division (D,E) at 36°C. The profile of the nuclei in C and D show that the cells are in late anaphase B when the EMTOC would be expected to have already formed. Similarly, the nuclear profiles in E are those of a cell in which the spindle has just depolymerised. Cells at this stage should also have an EMTOC. (F) A mixed culture of *cdc7.A20* and wild-type cells was processed for γ tubulin staining five hours after the temperature of the culture had been changed from 25°C to 36°C. EMTOCs were not observed in the long multi-nucleate mutant cells but were seen in the wild-type control cells (arrowhead).m

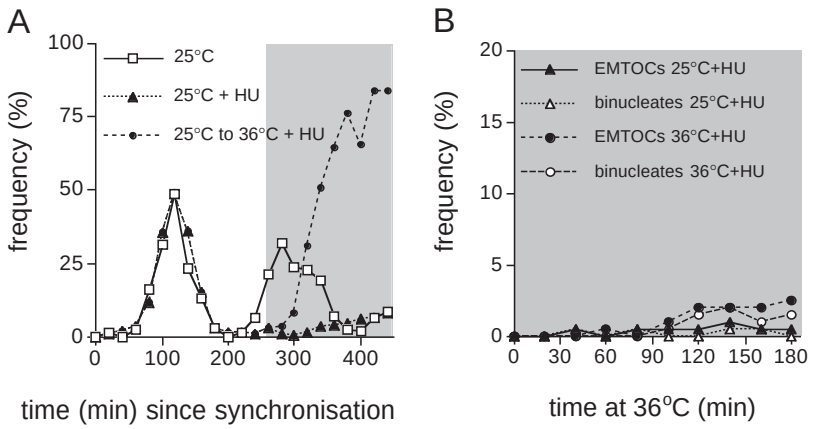


Fig. 6. Inappropriate activation of the SIN induces cytokinesis but not EMTOC formation. (A) *cdc16.116* cells were cultured in YES at 25°C then size selection was used to generate a synchronous population of late S phase/early G_2 cells and the frequency of septa (A) or EMTOCs (B) was scored. The culture was split into two. One portion was constantly cultured at 25°C to monitor the cell cycle synchrony by following the septation index (A, open squares). Hydroxyurea (HU) was added to the second culture 60 minutes after elution, and it was cultured at 25°C for 260 minutes. At this time this second culture was split into two and one aliquot incubated at 36°C (A, filled circles) and the other maintained at 25°C (A, filled triangles). The grey area of the graph represents the timing of the temperature shift. Panel A shows the septation profile of each portion of the culture as indicated on the graph. (B) Failure of EMTOC formation in HU-arrested cells lacking Cdc16 function at 36°C. EMTOC formation in HU-arrested *cdc16.116* cells at 36°C (filled circles) was not significantly higher than in those that retained Cdc16 function at 25°C (filled triangles), despite septum formation in over 75% of the cells following Cdc16 inactivation (filled circles in A). The frequency of binucleate cells was scored to gauge the efficacy of the S phase arrest. A minority of cells had leaked through the checkpoint arrest and re-entered the cell cycle and executed mitosis (open triangles and circles), and many of these cells contained EMTOCs (filled triangles and circles). The EMTOCs that were seen are therefore likely to be due to leak through the cell cycle arrest rather than arising from the activation of the SIN by shift to 36°C.

(Murone and Simanis, 1996). Fig. 5 shows staining of anaphase B *spg1.B8 cdc7.A20* double mutant cells at 25°C and 36°C. EMTOCs were present in late anaphase B cells at 25°C (Fig. 5A, arrowhead) but not 36°C (Fig. 5C-E). When cultures that contained both wild-type and SIN mutant cells were stained, EMTOCs were only seen in the short binucleate wild-type cells and not the multi-nucleate *cdc7.A20* cells (Fig. 5F, arrow). This showed that the inability to detect EMTOCs in these strains was due to the absence of the structure rather than repeated fixation artifacts. We conclude that a functional SIN was required for EMTOC formation.

Inappropriate activation of SIN is not sufficient to drive EMTOC formation

Given that an active SIN was required for EMTOC formation, we next asked whether SIN activation was sufficient to drive EMTOC formation. The SIN was activated in interphase-arrested cells using established procedures (Cerutti and Simanis, 1999). Small G_2 *cdc16.116* cells, which harbour a temperature-sensitive mutation in the Spg1 GAP protein, were grown at the permissive temperature (25°C) for 60 minutes. The culture was then divided into three parts and HU was added to two of the three cultures. After the first round of septation (Fig. 6A, 260 minutes), the temperature of one of the two S-phase-arrested cell cultures was increased to 36°C to abolish Cdc16 function (Cerutti and Simanis, 1999). The second was left at 25°C as a control. EMTOC formation was associated with the first, normal, mitosis that occurred after synchronisation but not with the septation event that followed the temperature shift to 36°C. 80% of the cells in this shifted batch of the culture formed a septum. As septation is always preceded by actin-ring formation (LeGoff et al., 1999), it can be assumed that these cells formed an actin ring that contracted as a primary septum formed. Only 2% formed an EMTOC (Fig. 6B). As a similar number of EMTOCs and septa formed in the control culture, which was maintained at 25°C (Fig. 6B), these infrequent EMTOCs in the 36°C culture are due to cells that have leaked through the HU-induced cell cycle checkpoint arrest to execute a defective, but properly co-ordinated, mitosis. Because these leak-through septation events resemble a normal mitosis, septation is associated with the formation of an EMTOC. The lack of EMTOC formation during septation in the 80% of the culture that is septating in S phase revealed a strong distinction between the regulation of the formation of the F-actin ring and the EMTOC: although induction of the SIN in interphase-arrested cells can drive F-actin ring formation, F-actin ring constriction and septum formation, it does not induce EMTOC formation.

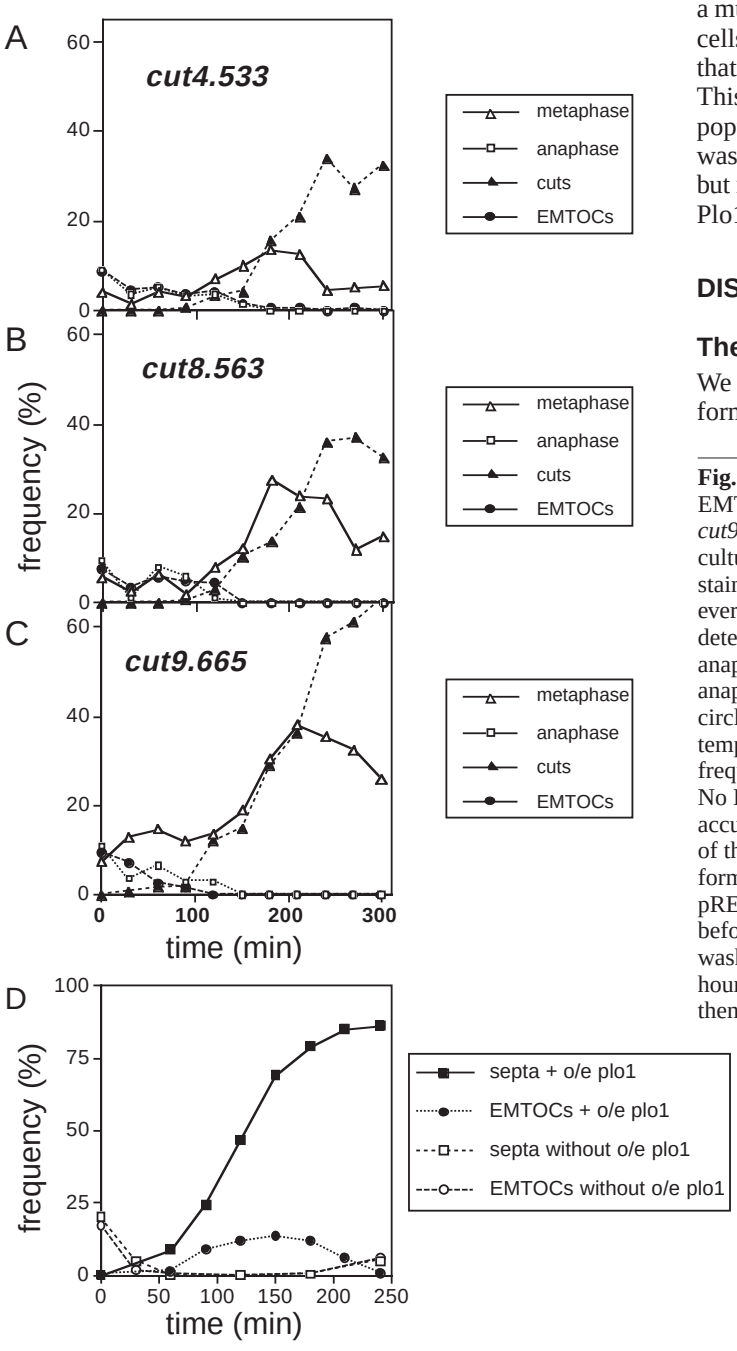
APC/C activity is required for EMTOC formation

As SIN activity was required, but was not sufficient, for EMTOC formation, we reasoned that other regulatory inputs must also be required. A good candidate for a trigger that ultimately facilitates EMTOC formation is the destruction of an inhibitor following conjugation to ubiquitin by the anaphase-promoting complex (APC). The APC/C plays a crucial role in regulating the metaphase-to-anaphase transition and mitotic exit by targeting key molecules for proteolysis (Zachariae and Nasmyth, 1999). We therefore examined the possibility that the APC/C may also be involved in EMTOC regulation by studying conditional mutations in the APC/C components Cut4 and Cut9. At the restrictive temperature, these cells were unable to progress from metaphase to anaphase but executed a normal cytokinesis, which often randomly cleaved the undivided chromatin (Hirano et al., 1986; Yamada et al., 1997; Yamashita et al., 1996). EMTOCs (Fig. 7A,C, filled circles) did not form in *cut4.533* and *cut9.665* at 36°C despite normal progression through cytokinesis to septation to produce the ‘cut’ phenotype (Fig. 7A,C, closed triangles). EMTOC formation was similarly blocked in *cut8.563* cells at 36°C (Fig. 7B). The *cut8*$^+$ gene product is required to localise the proteosome to the nuclear periphery. In *cut8.563* mutants, mitosis is blocked at the metaphase-to-anaphase transition but the F-actin ring forms, constricts and a septum is formed (Samejima and Yanagida, 1994; Tatebe and Yanagida, 2000). Thus EMTOC formation, but not the formation and function of the F-actin ring, requires

a proteolytic event that is regulated by the APC/C activity in addition to its requirement for an active SIN.

Overproduction of the Plo1 kinase promotes EMTOC formation

Overproduction of the polo-related kinase Plo1 activates the SIN and induces F-actin ring formation and constriction and primary septum synthesis in interphase cells (Mulvihill et al., 1999; Ohkura et al., 1995; Tanaka et al., 2001). Data from diverse systems suggest that polo kinases activate the APC/C (Glover et al., 1998; Nigg, 1998). Given the requirement for both the SIN and APC/C during EMTOC formation, we determined the effect of Plo1 overproduction in interphase cells upon EMTOC formation.



Plo1 was induced in *cdc25.22* cells at 25°C. 14 hours later small G_2 cells were isolated and incubated at the restrictive temperature of 36°C. This regimen induced actin ring formation and contraction and the formation of a primary septum (Ohkura et al., 1995) (Fig. 7D). It also induced EMTOC formation, however, there was a significant difference between septation and EMTOC formation. Septa continued to accumulate throughout the induction and reached over 80% by six hours (Fig. 7D, filled squares). In contrast, EMTOCs appeared only transiently, peaking around 13% at 150 minutes, before declining to 0 at 250 minutes (Fig. 7D, filled circles). EMTOCs were never seen in cells that had already septated. This indicated that the EMTOC appeared transiently and that it only appeared on the first actin ring that was induced by Plo1 overproduction. Because *plo1*$^+$ was being over-expressed from a multi-copy plasmid, the variation in plasmid number between cells meant that Plo1 protein accumulated to the critical level that will induce septation at different rates in different cells. This natural variation in absolute levels throughout the population meant that the profile of the EMTOC formation plot was broad. We conclude that EMTOCs formed during the first, but not subsequent rounds, of septation events were induced by Plo1 overproduction.

DISCUSSION

The EMTOC forms during mitotic exit

We have used immunolocalisation of γ tubulin to monitor the formation of the EMTOC. The EMTOC forms at the cell

Fig. 7. Anaphase-promoting complex function is required for EMTOC formation. (A-C) Asynchronous *cut4.533*, *cut8.563* and *cut9.665* cultures were grown at 25°C, and the temperature of the culture was then shifted to 36°C at 0 minutes. Cells were fixed and stained with antibodies to and γ tubulin or the SPB component Sad1 every 30 minutes. 200 cells were scored at each time point to determine the frequency of metaphase spindles (open triangles), anaphase (open squares), septation occurring in the absence of anaphase (cuts, filled triangles), and EMTOC formation (filled circles). After approximately 160 minutes at the restrictive temperature, cells began to accumulate in metaphase, and the frequency of cells in anaphase and cells with an EMTOC declined. No EMTOCs were observed after 160 minutes, despite an accumulation of cut cells indicating a successful cytokinesis in any of these strains. (D) Overexpression of *plo1*$^+$ induced EMTOC formation in interphase cells. A culture of *cdc25.22* cells containing pREP1*plo1*$^+$ was grown in EMM2 containing thiamine at 25°C before being split into two. One of the two cultures was extensively washed and re-inoculated to EMM2 medium lacking thiamine for 15 hours to induce *plo1*$^+$ overexpression. Centrifugal elutriation was then used to isolate small G_2 cells from both cultures, and the cells were immediately shifted to 36°C to inactivate Cdc25 and maintain cells in G_2 phase. Different cytological features were monitored in both cultures as shown in the legend. Cells with septa rapidly accumulated in these G_2-arrested cells when Plo1 was overproduced (filled squares). The frequency of cells containing EMTOCs peaked around 150 minutes and declined to 0 by 250 minutes (filled circles). In the control culture, septation (open squares) and EMTOC formation (open circles) were negligible. The few cells in this control culture in which *plo1*$^+$ expression was repressed had binucleate EMTOCs, indicating that they had leaked through the G_2/M arrest and executed a normal mitosis.

equator during late anaphase. It starts off as a broad structure that constricts as cytokinesis ensues, until it becomes the small punctate structure reported by Horio et al. (Horio et al., 1981). The discontinuous nature of γ tubulin staining in the EMTOC is consistent with the incomplete microtubule ring of Alp4 reported by Vardy and Toda and the discontinuous ring of α tubulin staining reported by Pichova et al. (Pichova et al., 1995; Vardy and Toda, 2000). Simultaneous localisation of γ and α tubulin revealed that in some cases it is only a discrete region of the EMTOC that nucleates microtubules. This may account for the apparently punctate nature of the microtubule foci of the post-anaphase array (Hagan and Hyams, 1988). Dual labeling of α and γ tubulin also showed microtubules ending at cortical dots of γ tubulin that were not part of the main body of the EMTOC. These structures may be analagous to the small, amorphous, cytoplasmic microtubule nucleating structures described by Horio et al. (Horio et al., 1991). Although γ tubulin complexes are known to cap free minus ends (Wiese and Zheng, 2000), the appearance of this arrangement so soon after the spindle depolymerises suggests that these may well be nucleation sites. It is not clear, however, whether they are structures that have been released from the EMTOC, structures that are soon to fuse to the maturing EMTOC or whether they will remain independent of the EMTOC.

The dispersal of the EMTOC when the F-actin ring was depolymerised suggested that the EMTOC forms by recruitment of γ tubulin complexes to the F-actin ring. Consistent with earlier reports that the PAA of microtubules is displaced in *mid1.366* mutants (Chang et al., 1996), we found that the EMTOC colocalised with the misplaced F-actin ring in a *dmf1.6* strain. Thus the EMTOC appears to form through the association of γ tubulin complexes with elements of the F-actin ring.

The absence of SPB markers such as Spg1, Cut12, and Sad1 (Bridge et al., 1998; Hagan and Yanagida, 1995; Sohrmann et al., 1998) from the EMTOC underlines the inherent differences between the EMTOC and the SPB. The EMTOC is reminiscent of acentrosomal MTOCs that form during nuclear division in other systems. The mid-body in animal cells and the preprophase band and phragmoplasts of higher plants all form at the site of cytokinesis and lack many of the classic spindle-pole components. It is not clear whether the midbody is a true MTOC that nucleates microtubules de novo during telophase or whether it is assembled from spindle microtubules shed by the centrosomes. Anti-parallel sliding of these microtubules would bring the γ tubulin caps at their minus ends together to generate a focus of γ tubulin in the middle of the cell (Khodjakov and Rieder, 1999a; Mastronarde et al., 1993). However, the inhibition of midbody formation by addition of anti-γ-tubulin antibodies to anaphase cells (Julian et al., 1993) argues that microtubule release is not the sole mechanism of midbody formation. Several facts also argue against EMTOC arising as a result of sliding forces within the central spindle. The first is that the EMTOC can form in cells that lack any microtubules. By definition, these cells do not have a central spindle that could slide in an anti-parallel fashion to create an organelle in the central overlap region. Secondly, even if a structure did form as a result of events in the central spindle, this spindle is contained within a sheath of nuclear envelope, and so there is a physical barrier that would stop it from influencing events at the plasma membrane (Tanaka and Kanbe, 1986). Finally all of the microtubules are attached to the spindle pole at this stage of division (Ding et al., 1993). Thus, there are no free microtubules to slide over each other to create this structure. Rather than being influenced by the central spindle, EMTOC behaviour strongly reflects the behaviour of the actin ring, so it would seem that it is forming by recruitment of γ-tubulin-containing complexes to the F-actin ring rather than from any influence of the central spindle. Therefore when the central spindle has wandered half way along the cell, the EMTOC still forms at the middle of the cell where the actin ring is cleaving the cell in two, rather than in the spindle overlap zone (Hagan and Hyams, 1988).

Regulating EMTOC formation

γ tubulin staining of strains with conditional mutations in components of the SIN indicated that this network is required for EMTOC formation towards the end of anaphase B. The conversion of Spg1, the SPB-bound G protein that lies at the top of this network, into its GTP-bound form at the beginning of mitosis results in a physical association with Cdc7 kinase that recruits Cdc7 to the SPB (Sohrmann et al., 1998). A number of changes in markers of SIN activity then occur during the later stages of anaphase B. For example, Cdc7 staining becomes monopolar, Sid1 is recruited to one SPB and the kinase Sid2 associates with the F-actin ring (Guertin et al., 2000; Sohrmann et al., 1996; Sparks et al., 1999). Similarly, epitope-tagged versions of the Spg1 GAP protein, Cdc16, show enhanced affinity for the SPB that lacks Cdc7 (Cerutti and Simanis, 1999).

One of the first signs of cytokinesis, and so presumably of SIN activation, is the disassociation of Dmf1/Mid1 from the F-actin ring (Bähler et al., 1998; Sohrmann et al., 1998). Data from highly synchronised mitoses show a remarkable correlation between loss of Dmf1/Mid1 from the ring and EMTOC formation. Taken with our inability to detect equatorial γ tubulin and Dmf1/Mid1 in the same cells (data not shown), these data suggest that there is a change in ring structure or regulation that results in Mid1 loss and EMTOC formation. We consider the simple model that Dmf1/Mid1 mobilisation exposes a docking site for a γ tubulin complex unlikely because we did not see premature EMTOC formation in *dmf1.6* cells at the restrictive temperature (data not shown). Nor did we see EMTOC formation in *dmf1.6 sin^{ts}* double mutants (data not shown). A more attractive possibility is that Dmf1/Mid1 dispersal and EMTOC formation are mediated by the same effector that is an integral part or target of the SIN. However, activation of the SIN alone was insufficient to drive EMTOC formation, even though it did induce repeated rounds of the changes in F-actin ring behaviour associated with septation. This indicated that additional regulatory pathways must control EMTOC formation. The targeting of one or more proteins for destruction by ubiquitination by the APC/C is one of these. Strains harbouring conditional mutations in the APC/C components Cut9 and Cut4 or the proteosome-targeting protein Cut8 undergo a normal cytokinesis and yet do not form an EMTOC. APC/C activity is known to target Cdc13/cyclinB and the securin Cut2 for destruction (Funabiki et al., 1996). EMTOC formation in *cut2* mutants and in strains expressing non-degradable Cut2 indicates that targeting of Cut2 for destruction by APC/C is not required for EMTOC

formation (data not shown). APC/C must therefore target Cdc13/cyclin B or a presently unidentified molecule for destruction to promote EMTOC formation. Loss of MPF activity can be a key factor in facilitating cytokinesis (He et al., 1997); however, APC/C mutants eventually undergo cytokinesis. Because an EMTOC does not form during these leak-through cytokinesis events, it would appear that the specific APC/C mutant alleles selected in the *cut* mutant screen (Hirano et al., 1986) have sufficient residual activity to eventually drive MPF inactivation below a cytokinesis threshold without enabling sufficient proteolytic activity to permit the proteolysis event that is required for EMTOC formation.

The link between APC/C and SIN activity in EMTOC regulation identified here bears a striking resemblance to the crosstalk between the APC/C and the mitotic exit network (MEN) in budding yeast (Shirayama et al., 1999). The MEN is equivalent to the SIN. Highly related molecules function at similar points in each network (Balasubramanian et al., 2000). The similarity between the two pathways is highlighted by the ability of *S. pombe* Cdc7 to complement a temperature-sensitive mutation in the homologous *S. cerevisiae* MEN component, Cdc15p (Fankhauser and Simanis, 1994). The sole essential role for *S. cerevisiae* APC^{cdc20} is to direct the destruction of Pds1p and Clb5p. Pds1p destruction releases Cdc14p from the nucleolus (Shirayama et al., 1999). Cdc14p is a phosphatase that apparently forces cells out of mitosis by dephosphorylating MPF substrates (Visintin et al., 1998). Cdc14p release is also regulated by the activity of the MEN (Shou et al., 1999; Visintin et al., 1999). Thus both the APC/C and MEN impinge upon Cdc14. The requirement for the function of all components of the SIN to enable EMTOC formation suggests that an analogous final SIN effector could control EMTOC formation.

Plo1 and EMTOC formation

The polo-like kinase Plo1 seems to form a bridge between the two pathways regulating EMTOC formation. Like SIN activation, overproduction of Plo1 in interphase cells induces F-actin ring formation, F-actin ring constriction and primary septum synthesis. However, unlike activation of the SIN, overproduction of Plo1 also drives EMTOC formation on the first actin ring that it induces. As overproduction of Plo1 activates the SIN (Tanaka et al., 2001), and yet SIN activation alone can not drive EMTOC formation, Plo1 overproduction must stimulate EMTOC formation by doing more than simply activating the SIN. Polo-like kinases appear to regulate APC/C activity in a number of different systems (Glover et al., 1998; Nigg, 1998). Most significantly the budding yeast polo-like kinase Cdc5p appears to promote mitotic exit through the activation of APCCdh1/Hct1 and the concomitant destruction of Clb2 (Charles et al., 1998; Shirayama et al., 1999). The relationship between Plo1 and the *S. pombe* APC/C awaits investigation, but the data presented here are consistent with Plo1 functioning as an APC/C regulator. Plo1 may therefore trigger EMTOC formation by activating both the APC/C and the SIN (Fig. 8A, red). An alternative model is suggested by studies of centrosome function in *Drosophila*. Recent data show that polo kinase regulates the centrosomal recruitment of Asp that is associated with the enhanced microtubule-nucleation capacity of salt stripped centrosomes in cell extracts (Avides and Glover, 1999; do Carmo Avides et al., 2001). Plo1 could therefore play a more direct role in controlling EMTOC formation in fission yeast. As such, it could either operate independently of the SIN and APC/C (Fig. 8B, yellow) or it could act as a downstream effector that is dependent upon the activity of both pathways (Fig. 8B, blue).

A final aspect of Plo1 regulation of EMTOC formation that bears mention is the fact that Plo1 overproduction was only able to promote EMTOC formation during the first of a repeated series of cytokinesis events. This raises the interesting possibility that EMTOC formation may need to be licensed by cell cycle progression through mitosis or START before it can occur once again.

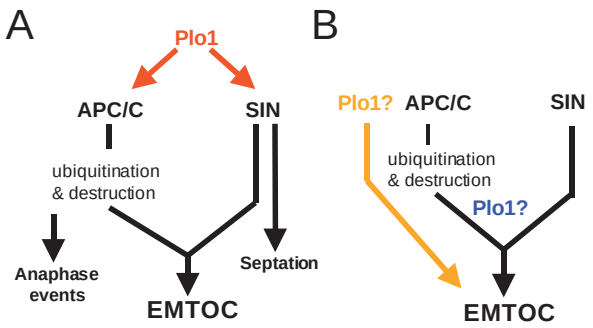


Fig. 8. Regulation of EMTOC formation. The formation of the EMTOC requires the activity of both the SIN and APC/C. It can also be induced by overexpression of *plo1*$^{+}$ but not by activation of the SIN. These observations and data from other studies, such as the ability of Plo1 overexpression to activate the SIN, would be consistent with Plo1 acting upstream of both the APC/C and the SIN (red – for details see text) (A). We favour this model over the alternative possibilities (B) that Plo1 acts independently of both of these pathways (yellow) or functions downstream of either SIN or APC (blue).

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