Etd1p is a novel protein that links the SIN cascade with cytokinesis

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In animal cells, cytokinesis occurs by constriction of an actomyosin ring. In fission yeast cells, ring constriction is triggered by the septum initiation network (SIN), an SPB-associated GTPase-regulated kinase cascade that coordinates exit from mitosis with cytokinesis. We have identified a novel protein, Etd1p, required to trigger actomyosin ring constriction in fission yeasts. This protein is localised at the cell tips during interphase. In mitosis, it relocates to the medial cortex region and, coincident with cytokinesis, it assembles into the actomyosin ring by association to Cdc15p. Relocation of Etd1p from the plasma membrane to the medial ring is triggered by SIN signalling and, reciprocally, relocation of the Sid2p–Mob1p kinase complex from the SPB to the division site, a late step in the execution of the SIN, requires Etd1p. These results suggest that Etd1p coordinates the mitotic activation of SIN with the initiation of actomyosin ring constriction. Etd1p peaks during cytokinesis and is degraded by the ubiquitin-dependent 26S-proteasome pathway at the end of septation, providing a mechanism to couple inactivation of SIN to completion of cytokinesis.

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Introduction

Cytokinesis in *Schizosaccharomyces pombe* is similar to that observed in animal cells and is achieved through the use of an actomyosin-based contractile ring (Balasubramanian *et al*, 2004; Glotzer, 2005). Actomyosin ring assembly is a spatio-temporally regulated process that starts with the determination of the division plane by Mid1p, a protein that shuttles from the nucleus to cell cortex to form a broad band overlying the position of the nucleus before mitosis (Sohrmann *et al*,

1996; Paoletti and Chang, 2000). Upon entry into mitosis, myosin (Myo2) and its regulatory light chains (Rlc1p, Cdc4p) are recruited to this broad band (Motegi *et al*, 2004). This is followed by the arrival of Cdc12p, which nucleates actin polymerization, and Cdc15p, which is also required for actin ring assembly (Carnahan and Gould, 2003; Kovar *et al*, 2003; Wu *et al*, 2003). This primary broad band, composed of actin and myosin together with many other ring components, is compacted prior to anaphase B into a thinner mature ring (Wu *et al*, 2003). After ring assembly, actin patches become enriched along side the actomyosin ring. F-actin patch localisation is cell cycle dependent. During interphase, actin patches accumulate at the growing ends of the cells, while at the onset of mitosis, patches assemble at the medial region, where they might be required for septum synthesis (Marks *et al*, 1986; Pelham and Chang, 2001).

By the end of anaphase B, once the two sets of chromosomes have been segregated, the septum initiation network (SIN) triggers ring contraction and septum deposition behind the contracting ring. A number of genes are required for SIN function, including *spg1/sid3*, *cdc7*, *cdc11*, *sid4*, *cdc14*, *sid1*, *sid2* and *cps1* (Simanis, 2003). The absence of any of these proteins allows normal actomyosin ring assembly but cells fail to initiate ring constriction. All these components function in a conserved GTPase-regulated protein kinase cascade located at the spindle pole body (SPB)—the yeast equivalent of the centrosome of higher eukaryotes (Ding *et al*, 1997; McCollum and Gould, 2001).

The nucleotide binding state of Spg1p, a small GTPase of the Ras superfamily, is of key importance in SIN activity. In interphase cells, Spg1p is in the GDP-bound inactive form. As the mitotic spindle forms during metaphase, Spg1p becomes GTP-bound and active at both SPBs until anaphase B, when it reconverts to the inactivated (GDP-bound) form at one of the two SPBs (Schmidt *et al*, 1997; Cerutti and Simanis, 1999). The protein kinase Cdc7p is asymmetrically recruited to the SPB that maintains the activated form of Spg1p (Sohrmann *et al*, 1998) and Sid1p–Cdc14p binds to this activated SPB. The recruitment of Sid1p–Cdc14p to the SPB depends on inactivation of the Cdc2p/Cdc13p (Cdk1/cyclinB) protein kinase complex at the end of anaphase (Guertin *et al*, 2000). The SIN signal is then transduced to the division site by the Sid2p–Mob1p protein kinase complex. This complex relocates from the SPB to the cell division site and triggers medial ring constriction and septation (Salimova *et al*, 2000; Hou *et al*, 2004). Cell wall material is deposited behind the constricting ring by the septum synthesis machinery, which includes Cps1p/Drc1p/Bgs1p, the catalytic subunit of β 1-3 glucan synthase (Liu *et al*, 1999).

Regulation of Spg1p is critical for the control of the SIN. Cells that overexpress *spg1* undergo multiple rounds of septation but never complete cell separation (Schmidt *et al*, 1997). The GTPase-activating proteins (GAPs) Byr4p and Cdc16p negatively regulate Spg1p by promoting nucleotide

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hydrolysis, which converts active GTP-bound Spg1p to the inactive GDP-bound form (Furge *et al*, 1998). GTPases are also activated by guanine nucleotide exchange factors (GEFs) (Schmidt and Hall, 2002), although GEFs for Spg1p have not yet been identified. In budding yeast, the equivalent pathway to the SIN is known as the MEN (mitotic-exit network) (Bardin and Amon, 2001). In this yeast, the Tem1 GTPase (Spg1p in *S. pombe*) is negatively regulated by the GAP complex composed of Bfa1 and Bud2 (Cdc16p-Byr4p in fission yeast), while the putative GEF Lte1 activates it (Bardin and Amon, 2001).

A number of regulatory mechanisms are known to coordinate SIN activity with mitosis (Krapp *et al*, 2004). In addition to signalling cytokinesis, the SIN regulates the localisation of Clp1p/Flp1p, a conserved Cdc14-like phosphatase. Clp1p/Flp1p is released from the nucleolus during mitosis by SIN signalling, and the non-nucleolar Clp1p/Flp1p antagonises Cdc2p/Cdc13p (CDK) activity, which is inhibitory to SIN. This feedback loop connecting SIN with CDK activity ensures that SIN is active only when CDK activity drops (Cueille *et al*, 2001; Trautmann *et al*, 2001). Dma1p, a spindle checkpoint protein, prevents cytokinesis during spindle checkpoint arrest by inhibiting SIN activity, thereby providing another mechanism by which mitotic exit is coordinated with SIN signalling (Guertin *et al*, 2002b).

As in the case of mitotic events, the SIN should be coordinated with cytokinetic events, but regulatory mechanisms connecting SIN with initiation, progression or exit from cytokinesis are poorly understood. By using ethanol as a temperature-independent conditional method, we identified Etd1p, a novel protein essential for cytokinesis (Jimenez and Oballe, 1994). Characterisation of this protein revealed a novel mechanism connecting SIN activity with cytokinesis.

Results

Etd1p is a new protein required for cytokinesis

Many genes required for cytokinesis in yeasts have been identified in screenings for temperature-sensitive mutants, but not all genes yield thermo-sensitive mutant alleles. To identify new genes, we used ethanol as a conditional system that operates independently of temperature. Two large groups of mutants were isolated as ethanol-sensitive mutants (*ets*) and ethanol-dependent mutants (*etd*) (Jimenez and Oballe, 1994). One of the mutants belonging to the latter group, *etd1-1*, produced elongated and multinucleate cells without a septum under restrictive conditions (absence of ethanol), a characteristic phenotype of *S. pombe* cells defective in cytokinesis (Figure 1A and B).

The *etd1* gene encodes an uncharacterised 391-amino-acid polypeptide (Jimenez and Oballe, 1994). Etd1p sequence is not conserved in other eukaryotes and conventional sequence analysis failed to provide any information about its putative function. To determine whether *etd1* was essential, one copy of the *etd1* gene was replaced with the *ura4* gene in a diploid *S. pombe* strain. Spores deleted for *etd1* (*etd1Δ*) germinated and accumulated multiple nuclei without septation, an identical phenotype to that of *etd1-1* mutant cells under restrictive conditions (Figure 1C). Thus, *etd1* encodes an essential new protein required for cytokinesis in fission yeast.

Figure 1 *etd1* is an essential gene required for cytokinesis. (A) *etd1-1* is a conditional mutant that requires ethanol for growth. Wild-type and *etd1-1* cells were grown at 25°C in YE medium containing 6% ethanol (permissive condition) and then replica-plated to medium without ethanol (restrictive condition). (B) *etd1-1* cells grown under permissive (+ ethanol) and restrictive conditions (–ethanol) for 4, 8 and 12 h were fixed and stained with DAPI to visualise DNA and calcofluor to visualise cell wall material. (C) One copy of the *etd1* open reading frame (ORF) was replaced by *ura4* (scheme) to construct a deletion allele (*etd1Δ*) in a diploid strain. Tetrad dissection of asci from this *etd1Δ/etd1+* diploid strain is shown (left panel). *etd1Δ* spores were germinated in medium lacking uracil (middle panel) and the *etd1Δ* spores fixed and stained with DAPI (right panel). (D) Strong expression of *etd1* also impairs cytokinesis. *etd1-1* mutant cells expressing *etd1* (cDNA) under the *nmt1* thiamine-repressible promoter were grown in minimal medium containing thiamine. To determine the phenotype associated with overexpression of *etd1*, these cells were washed three times with thiamine-free medium and finally resuspended in medium with or without thiamine for 18 h at 25°C. Cells were fixed and stained with DAPI and calcofluor. Bars: 3 μm.

To further analyse the function of *etd1*, we examined the effect of increased expression of the *etd1* gene. A cDNA encoding *etd1* was cloned in the pREP3X vector under the control of the thiamine-repressible *nmt1* promoter (Maundrell, 1993). The pREP3X-*etd1* plasmid was transformed into *etd1-1* mutant cells and in wild-type cells. Under repressed conditions (+thiamine), the *nmt1:etd1*

construction provided sufficient Etd1p expression to rescue the lethal phenotype of the *etd1-1* mutant (see Figure 1D). Under derepressed conditions (–thiamine), Etd1p overproduction generated elongated and multinucleate cells in both *etd1-1* mutant and wild-type backgrounds (Figure 1D and data not shown). Thus, the phenotypic defect caused by an excess of this protein, suggesting that Etd1p functions in a stoichiometric protein complex.

To better understand the role of Etd1p in cytokinesis, we examined the localisation of the protein in *S. pombe* cells. A strain was constructed that expressed an Etd1p-GFP fusion from the thiamine-repressible *nmt41x* promoter. A single

copy of the construction was integrated at the *leu1* locus. Under expression conditions, the resulting Etd1p-GFP fusion protein was functional and able to complement the lethality of the *etd1-1* mutant and the *etd1* null allele. In interphase cells, Etd1p-GFP was located at the cell cortex and was more concentrated at the cell tips (Figure 2A, cell 1). In early anaphase, Etd1p-GFP became concentrated in the medial region of the cell cortex as a broad band (Figure 2A, cell 2) and finally as a ring late in anaphase before septation (Figure 2A, cell 3). At the time of septum formation, Etd1p-GFP spread into the cell (Figure 2A, cell 4) and once the primary septum is formed, it appears as a double layer at the cell equator (Figure 2A, cell 5). Finally, during cell

Figure 2 Etd1p localises to the division site at the end of mitosis. (A) Low-level expression of Etd1p-GFP (driven by the *nmt41x* promoter) was used to analyse the *in vivo* localisation of Etd1p. Cell 1 is in interphase, cell 2 is in early anaphase, cell 3 in late anaphase, cell 4 is undergoing septation, cell 5 formed the primary septum and cell 6 completed cytokinesis. GFP images (upper panels) and phase contrast (lower panels) are shown. Asterisks indicate localisations of Etd1p in cells 1 and 2. (B) Cell wall digested with novozyme and cell wall-free spheroplasts observed by fluorescence microscopy. Etd1p-GFP was associated with the plasma membrane. (C, D) *cdc25-22* cells expressing Etd1p-GFP were synchronised by a *cdc25-22* block-release protocol after 4 h of incubation at 36°C. Time-lapse images of living cells were collected every 5 min for 2 h after release at 25°C, using a confocal microscope. Two stacks of images were captured, one with a step size of 1 µm between focal planes (C) and the other of 0.3 µm serial sections to reconstruct three-dimensional images of the cell (D). (E) Time-lapse images in Etd1p-GFP *Cdc7p*-GFP living cells were used to determine precisely the transition of Etd1p from the cell tip to the cell centre during interphase (cell 1), entry into mitosis (cell 2)—as determined by *Cdc7p* association to the SPB—and initiation of anaphase (cell 3)—according to SPB segregation. (F) Localisation (asterisks) of Etd1p-GFP or *Cdc15p*-GFP—actomyosin ring marker—in *nda3*-arrested cells in metaphase (*nda3-KM311* mutant background, incubated for 3 h at the restrictive temperature of 20°C). Cells incubated at the permissive temperature are shown as a control (32°C). In arrested cells, *Cdc15p* was always found in the medial ring while Etd1p was mainly in the cell tips (about 82%, cell 1) and a small fraction was at the middle of the cell (about 18%, cell 2). (G) Analysis of the relocation of Etd1p-GFP from a broad band (cell 1) to a compact ring (cell 2), in relation to *Cdc7p*-GFP as described in panel E.

separation, Etd1p-GFP signal disappeared from the middle of the cell (Figure 2A, cell 6). An identical localisation was observed when the Etd1p-GFP fusion was expressed under its own promoter on a multicopy plasmid or from its normal chromosomal locus, although fluorescence was almost undetectable in this latter case (data not shown). In spheroplasts lacking the cell wall, Etd1p remained associated with the cell periphery, suggesting that this protein is associated with the cell cortex or anchored to the cell membrane (Figure 2B).

To better characterise the cell cycle dynamics of Etd1p localisation, *cdc25-22* mutant cells expressing Etd1p-GFP were synchronised by a temperature block-release protocol. This procedure causes a G2-phase cell cycle block at the restrictive temperature after which entry into mitosis is synchronously induced by shifting the culture to the permissive temperature. In G2 cells, Etd1p-GFP was observed at the cell tips (Figure 2C and D, time 0) and prior to anaphase began to relocate to the cell centre, about 15–20 min after the release (Figure 2C). Between 20 and 40 min after the release, Etd1p-GFP was observed as a broad band at the cell cortex in the middle region of the cell (Figure 2C, time 20–40 min; Figure 2D, time 35 min). At late anaphase, Etd1p compacted into a ring at the site of cytokinesis (Figure 2C and D, time 55 min). After this, Etd1p was observed in the region of septum formation, spreading in a centripetal manner into the cell as the actomyosin ring contracted (Figure 2D, time 75 min). This observation suggests that it is probably associated with the ingressing plasma of the cleavage furrow. Between 75 and 95 min after the release, coinciding with septum formation, Etd1p-GFP was observed along both sides of the septum (Figure 2C and D, time 80–95 min). Finally, after degradation of the primary septum, and simultaneous to cell separation, the Etd1p-GFP signal decreased strikingly (Figure 2C, time 100–115 min). Overall, localisation and dynamics of Etd1p agree with a role of this protein in cytokinesis.

Dynamics of SPBs can be used to determine more precisely the cell cycle-dependent localisation of Etd1p. Cdc7p is particularly useful to this end because this protein binds to the SPB at the G2–M transition and, after SPB duplication, it remains associated with only one of the two poles of the spindle during anaphase B (Sohrmann *et al*, 1998). Using Cdc7p-GFP, we found that Etd1p-GFP initiated its localisation at the cell centre after association of Cdc7p to the SPB and before SPB duplication (metaphase), being more concentrated as the SPB duplicated and initiated separation (anaphase A) (Figure 2E, cells 2 and 3 respectively). In agreement with this observation, only a reduced fraction of cells showed Etd1p in the medial cortex region in metaphase-arrested cells by using the *nda3-KM311* mutation (Figure 2F). In these *nda3-KM311*-arrested cells, the actomyosin ring was already formed, as revealed by the medial ring component Cdc15p-GFP (Figure 2F), indicating that Etd1p arrives to the middle cortex after medial ring assembly. At the time that Cdc7 is seen on only one SPB, in anaphase B, Etd1p compacted to a tight ring (Figure 2G, cell 2).

Thus, Etd1p localises to the cell tips during interphase, relocates to the cell centre shortly coincident with the metaphase to anaphase transition, after actomyosin ring assembly, and compacts to a medial ring during anaphase B (see Supplementary data, Movie 1).

Figure 3 Assembly of Etd1p-GFP into the actomyosin ring. (A) Localisation of Etd1p-GFP in *mid1*-deleted cells (*mid1Δ*) and wild-type cells (*wt*). (B) Medial ring *cdc8-110* and *cdc15-140* thermo-sensitive mutant cells expressing Etd1p-GFP were grown to mid-exponential phase at 25°C; half of the culture was shifted to 36°C for 4 h, and living cells were photographed. (C) Protein extracts prepared from cells expressing Etd1p-GFP, Cdc15p-13Myc or both were immunoprecipitated with anti-Myc antibodies; the immunoprecipitates were run on SDS-PAGE gels and probed with anti-Myc and anti-GFP antibodies. Western blots of total extracts were also probed with anti-Myc and anti-GFP antibodies to check the levels of tagged proteins.

Etd1p is essential for actomyosin ring constriction

To determine whether Etd1p associates to the actomyosin ring, we examined its location in different types of actomyosin ring mutants. Mid1p is a key factor for the central positioning of the cytokinetic ring (Sohrmann *et al*, 1996). The location and dynamics of Etd1p and Mid1p at the cell centre are very similar (Celton-Morizur *et al*, 2004); however, in *mid1*-deleted cells, Etd1p-GFP localised at the randomly positioned actomyosin rings found in these mutant cells (Figure 3A). Therefore, medial ring components rather than Mid1p could be involved in the medial ring localisation of Etd1p. Cdc8p tropomyosin is an essential protein required to form F-actin rings (Balasubramanian *et al*, 1992; Arai *et al*, 1998). We therefore analysed the localisation of Etd1p-GFP in *cdc8-110* mutant cells and found that, at the restrictive temperature of 36°C, Etd1p never formed a ring (Figure 3B, upper panels). The *S. pombe* Cdc15p is also required for medial ring formation during cytokinesis (Fankhauser *et al*, 1995; Carnahan and Gould, 2003). Overexpression of *cdc15* is sufficient to drive medial actin recruitment in G2-arrested cells, indicating that Cdc15p plays a key role in the establish-

ment of the medial actomyosin ring. In *cdc15-140* mutant cells under restrictive conditions (Fankhauser *et al*, 1995), we also failed to detect Etd1p-GFP localised as a ring (Figure 3B, lower panels). In both *cdc8-110* or *cdc15-140* mutants at the restrictive conditions, Etd1p-GFP remained at the cell tips or as a diffuse central band in mitotic cells but it was not assembled into the medial ring. Therefore, we conclude that Etd1p requires the actin ring for its proper localisation to the division site.

Recruitment of Etd1p into the medial ring could take place through an interaction with Cdc15p. This is based on the fact that cells expressing Cdc15p tagged with HA or GFP in combination with HA-tagged Etd1p showed a synthetic lethal *cdc* phenotype (data not shown). To investigate a possible physical interaction between Etd1p and Cdc15p, strains carrying a plasmid expressing Etd1p-GFP (tagged at the N-terminus), Cdc15p-Myc (tagged at the N-terminus) or both were constructed. Protein extracts were prepared from these strains and the association between these proteins was determined in co-immunoprecipitation experiments. As shown in Figure 3C, anti-Myc immunoprecipitates contained Etd1p-GFP, demonstrating that Etd1p interacts physically with Cdc15p. Similarly, Cdc15p was detected in anti-GFP immune complexes (data not shown). Thus, Etd1p may localise to the actomyosin ring by association with Cdc15p.

Cytokinesis in *S. pombe* cells requires actomyosin ring assembly and F-actin patch rearrangement from the cell tips to the division site. Cdc15p is involved in both processes (Fankhauser *et al*, 1995). F-actin cables are also involved in

the formation of the actomyosin ring (Arai and Mobuchi, 2002). To determine whether Etd1p has a role in any of these events, we analysed the assembly of Cdc15p-GFP, the formation of F-actin cables and dynamics of Crn1p-GFP (coronin), a marker for actin patches (Pelham and Chang, 2001). As shown in Figure 4A–C, neither of these processes was affected in *etd1-1* mutant cells, suggesting that Etd1p functions downstream of Cdc15p and F-actin patch recruitment in cytokinesis. However, in *etd1-1* mutant cells, the medial ring marked with Cdc15p-GFP seems to fail constriction.

To better determine a role of Etd1p in actomyosin ring constriction, we used the myosin regulatory light chain (encoded by the *rlc1* gene) tagged with GFP as a ring marker (Le Goff *et al*, 2000). Time-lapse images of *rlc1-GFP* in wild-type and *etd1-1* living cells progressing from G2 to cytokinesis were obtained. In wild-type cells, the actomyosin ring assembled early during mitosis (in metaphase) and initiated constriction late in anaphase, between 30 and 35 min after assembly (Figure 4D, upper panels). In *etd1-1* mutant cells, actomyosin ring assembled as in wild type, but the ring failed to constrict and finally collapsed (Figure 4D, lower panels, and Supplementary data, Movie 2A and B). Thus, Etd1p is not required for actomyosin ring assembly, but is essential for ring contraction.

A role of Etd1p in SIN signalling

Actomyosin ring contraction requires proper ring assembly and the activation of the SIN. Since *etd1-1* mutant cells assembled a normal medial ring, we wondered whether

Figure 4 Actomyosin ring is assembled in the *etd1-1* mutant but fails to contract. (A) Assembly of Cdc15p-GFP—a key component of the actomyosin ring—was imaged in living wild-type (upper panels) or *etd1-1* mutant cells under the restrictive condition (lower panels) by time-lapse microscopy at 5 min intervals on a single focal plane with a step size of 0.3 μ m. (B) Rhodamine-conjugated phalloidin was used to stain F-actin structures in *etd1-1* mutant cells under the restrictive condition. Actin cables and patches are indicated. (C) The *S. pombe* coronin homologue *crn1* tagged with GFP was used as a marker of F-actin patches. Living cells were imaged as described in panel A. (D) The myosin regulatory light-chain Rlc1p tagged with GFP was used as a marker of the actomyosin ring. Living cells were imaged as described in panel A.

Figure 5 Effects of Etd1p on SIN signalling. (A) Localisation of Mob1p-GFP to the division site depends on Etd1p. Mob1p-GFP was imaged in live wild-type cells (upper panels) or *etd1-1* mutant cells (lower panels) under restrictive conditions (–ethanol) by time-lapse microscopy at 5 min intervals on a single focal plane. (B) Etd1p is required to maintain SIN active. Cdc7p-GFP was imaged by time-lapse microscopy at 5 min intervals on a single focal plane in living wild-type cells (upper panels) and *etd1-1* mutant cells (lower panels) under restrictive conditions (–ethanol). Fluorescence intensity was quantified (arbitrary units) and represented for each SPB (uSPB, up; dSPB, down). (C) Hyperactivation of SIN does not bypass the requirement of Etd1p for Sid2p-GFP localisation. Sid2p-GFP was localised in cells with (–thiamine) or without Etd1p (+thiamine) under a normal (25°C) or hyperactive (34°C) SIN cascade. Cells of the *etd1Δ nmt81x:etd1 sid2-GFP cdc16-116* strain expressing *etd1* were grown to mid-exponential phase at 25°C. Thiamine was added to half of the culture, and after 2 h, half of each subculture was shifted to 34°C. Sid2p-GFP was observed in living cells after 4 h. Bar: 3 μm.

Etd1p might be required for SIN signalling. In fact, Etd1p-deficient cells resembled *sin* mutants (see Figure 1). The protein kinase complex Sid2p–Mob1p functions at a late stage of the SIN pathway by transmitting the signal from the SPB to the medial ring to initiate cytokinesis (Salimova *et al*, 2000; Hou *et al*, 2004). We produced Mob1p-GFP and Sid2p-GFP constructions and determined that, as previously described (Salimova *et al*, 2000; Hou *et al*, 2004), Mob1p-GFP localised to both SPBs during mitosis and at the division site during septation in wild-type cells (Figure 5A, upper panels). In Etd1p-deficient cells, the SPB localisation of Mob1p-GFP remained unaffected, but notably, Mob1p-GFP did not relocate to the division site (Figure 5A, lower panels). We were unable to construct a *sid2-GFP etd1-1* double mutant due to negative genetic interaction between these two alleles (data not shown). However, in a strain deleted for *etd1* (*etd1Δ*) kept alive by expressing *etd1* from the weak *nmt81x* promoter (*etd1Δnmt81x:etd1*), we observed that the localisation of Sid2p-GFP to the cleavage site also required Etd1p (see below). We thus conclude that Etd1p is required for the transduction of the Sid2p–Mob1p signal from the SPB to the division site.

Since Etd1p associates to the actomyosin ring and is required for SIN signalling, this new protein might be a ring component required for the recruitment of Sid2–Mob1 com-

plexes to this structure, that is, a downstream element of the SIN cascade localised at the medial ring. If this were the case, activation of upstream elements of SIN should take place normally in *etd1*-mutant cells, and similarly, ectopic activation of the SIN alone should not bypass the requirement of Etd1p to relocate Sid2p or Mob1p from the SPB to the actomyosin ring.

To analyse upstream activation of SIN, we studied the localisation of Cdc7p-GFP in *etd1-1* mutant cells by time-lapse microscopy. The Spg1p GTPase localises to the SPBs throughout the cell cycle. In interphase cells, Spg1p is GDP-bound, but upon entry into mitosis, it converts into the GTP-bound form. Spg1p is then active at both SPBs until anaphase B, when it converts back into the inactive GDP-bound form at one of the two SPBs. Cdc7p only binds the active (GTP-bound) form of Spg1p (Sohrmann *et al*, 1998). Thus, Cdc7p is an excellent marker for monitoring upstream activation of the SIN cascade. As shown in Figure 5B (upper panels), under permissive conditions for *etd1-1*, Cdc7p-GFP appeared at both SPBs at the initiation of mitosis and only at one SPB as cells progressed through anaphase until the completion of cell division. Cdc7p-GFP also localised to both SPBs in early mitosis in *etd1-1* cells under restrictive conditions, indicating that the initial activation of Spg1p does not require Etd1p. However, in these Etd1p-deficient cells, Cdc7p-GFP signal

Figure 6 (A) Effects of SIN on Etd1p localisation. *sid2-250* and *cdc16-116* thermo-sensitive mutant cells expressing Etd1p-GFP were grown to mid-exponential phase at 25°C; half of the culture was shifted to 36°C for 2 h, and live cells were imaged. Asterisks indicate the main cellular location of Etd1p-GFP. In *sid2-250* cells at 36°C in which SIN is inactive, Etd1-GFP accumulates at the cell membrane overlying the division site, and relocates to the cell tips, but it is not localised in the medial ring. In *cdc16-116* cells at 36°C, Etd1p localises to the multiple septa originated by SIN hyperactivation. (B) Effects of the *etd1-1* mutation on the localisation of sterol-rich membrane domains in comparison to the effects of *sid2-250* (sin mutant) and *cdc8-110* (actomyosin ring mutant) thermo-sensitive mutants. Cells were incubated at their respective restrictive conditions for 2 h, stained with filipin and imaged (wild-type cells were also used).

rapidly decayed early in anaphase (Figure 5B, lower panels), suggesting that Etd1p is somehow necessary to maintain Spg1p activity during anaphase until the completion of cytokinesis. Quantification of the fluorescence intensity at the SPBs reinforces this observation (see Figure 5B).

To determine whether this premature Spg1p inactivation in cells lacking Etd1p is the only reason for the failure of SIN signalling through Mob1p-Sid2p, we maintained the SIN active with a *cdc16-116* mutation and investigated the localisation of Mob1p-GFP and Sid2p-GFP in an *etd1Δ nmt81x:etd1* background. Under repressed conditions at 25°C (+ T), these cells mimicked the *sin* defect of a deficiency in Etd1p and were unable to localise Sid2p-Mob1p proteins to the cell division site (see Figure 5C, upper panels, for the case of Sid2p-GFP). Upon inactivation of Cdc16p in these cells (at 34°C), frequent unseptated postmitotic cells were found (Figure 5C, lower panels, for Sid2p-GFP). Similar results were obtained by activation of the SIN by overexpressing *plp1* or *spg1*. These results indicate that Etd1p functions downstream of Spg1p in SIN signalling.

To elucidate the role of Etd1p in SIN signal transduction, we analysed the localisation of Etd1p-GFP in different mutants of the SIN pathway. Interestingly, we found that in

cdc7^{ts} and *sid2^{ts}* mutant cells, Etd1p-GFP failed to localise to the medial ring at the restrictive temperature. Instead, these mutant cells accumulated Etd1-GFP in a broad band at the plasma membrane overlying the site of cytokinesis (Figure 6A, upper panel, and data not shown). These observations indicate that relocation of Etd1p from the cell cortex to the actomyosin ring requires Spg1p activation. In agreement with this observation, Etd1p-GFP localised to the multiple septa produced by the ectopic activation of SIN (Figure 6A, lower panels, for *cdc16-116* mutants). Hence, relocation of Etd1p from the cell cortex to the ring is a downstream step of the SIN pathway required for signalling the initiation of cytokinesis.

In *S. pombe*, sterol-rich membrane domains and Etd1p follow similar cell cycle dynamics. In *sin* mutants, these sterol-rich membrane domains accumulate in a specific pattern, which is different from that observed in medial ring mutants (Takeda *et al*, 2004; Figure 6B). Interestingly, patterns in *etd1-1* and *sid2-250* mutant cells are identical (Figure 6B). Thus, although Etd1p is located at the cell cortex and the medial ring, this protein behaves more like a SIN-signalling protein than a structural component of the cytokinetic machinery.

As described in Figure 1D, overproduction of Etd1p caused a SIN-defective phenotype similar to that of *etd1-1* mutants. The above properties described for Etd1p-deficient cells also occurred in Etd1p-overproducing cells (see Supplementary Figure 1 for Crn1p-GFP, Rlc1p-GFP and Cdc7p-GFP), suggesting that the amount of Etd1p is critical in the control of cytokinesis in fission yeasts.

The amount of Etd1p is cell cycle regulated

As shown in Figure 2, the cellular localisation of Etd1p is regulated in a cell cycle-dependent manner. To study whether its expression was also regulated in a cell cycle-dependent manner, we determined the levels of Etd1p and *etd1* mRNA in a synchronous culture, using a strain in which the single chromosomal copy of *etd1* had been tagged with three copies of HA (see Materials and methods). Cells were synchronised using the *cdc25-22* block-release protocol, as described above. Etd1p-HA levels were sharply periodic, rising to a peak at the end of anaphase, shortly after the peak of binucleated cells (Figure 7A and B, 60 and 60–75 min), and decreasing at 90–105 min when most of the cells had completed septation (Figure 7A and B). The kinetics of *etd1* mRNA accumulation was similar to that described for Etd1p, increasing briefly before accumulation of the protein (see Figure 7B). The results of this experiment show that *etd1* mRNA and protein levels fluctuate during the cell cycle, peaking during actomyosin constriction and septation.

To investigate whether the decay in Etd1p levels after septation was due to rapid degradation by the proteasome pathway, we determined Etd1p levels in the *mts3-1* temperature-sensitive mutant, which is defective in subunit 14 of the 26S proteasome (Gordon *et al*, 1996). The amount of Etd1p was higher in the *mts3-1* strain at the restrictive temperature than in the wild-type strain or *mts3-1* at 25°C (Figure 7C, upper panels). This result suggests that the proteasome degradation pathway might be involved in the regulation of Etd1p levels.

In the *mts3-1* mutant at 36°C, Etd1p high molecular weight bands were observed after a longer exposure of the films

Figure 7 The abundance of *etd1* mRNA and protein levels were followed by respective Northern and Western blot analyses in a *cdc25-22* block-release experiment as described in Figure 2. RNA and protein samples were collected every 15 min along two consecutive cell cycles after the release to 25°C. (A) Samples were scored for separation of the nuclei and the appearance of the division septum to verify the synchrony of the culture. (B) Proteins were separated by SDS-PAGE and the Western blot was probed with either anti-HA (12CA5) or anti-Cdc2p (C2) antibodies (upper panels). A Northern blot was probed for *etd1*; rRNA was stained with bromophenol blue as a loading control prior to hybridisation (lower panels). (C) Etd1p is polyubiquitinated and stabilised in the *mts3-1* mutant defective in the 26S proteasome. *etd1-HA* or *etd1-HA mts3-1* strains were grown at 25°C to mid-exponential phase in YE and then shifted to 36°C for 4 h. Protein extracts were collected at times of 0, 1, 2 and 4 h after the shift and the levels of Etd1p and Cdc2p (as a loading control) were determined by Western blot using anti-HA (12CA5) or anti-Cdc2p (C2) antibodies (upper panels). To determine Etd1p ubiquitination, His₆-ubiquitin was expressed from the *nmt1* promoter in *mts3-1 etd1-HA* mutant cells for 16 h at 25°C and then shifted to 36°C for 4 h. The His₆-ubiquitin conjugates were purified in Ni²⁺-NTA columns and analysed by Western blot using 12CA5 antibodies.

(data not shown), which could represent ubiquitin conjugates. To test this possibility, we expressed a His₆-tagged version of ubiquitin in *mts3-1* cells and determined the presence of Etd1-ubiquitin conjugates. Extracts were purified using Ni²⁺-NTA resin (Treier *et al*, 1994), separated on a polyacrylamide gel and then Western blotted with anti-HA antibodies. Etd1p high molecular bands were detected in *mts3-1* mutants at the restrictive temperature (Figure 7C, lower panels). These results indicate that Etd1p is polyubiquitinated and degraded through the ubiquitin-dependent 26S-proteasome pathway.

Discussion

Cellular localisation of Etd1p

In this study, we identified a novel protein, Etd1p, required for constriction of the actomyosin ring in fission yeast (see Figure 4). This protein resides associated with the cell membrane (Figure 2B). According to its hydrophilic amino-acid profile, Etd1p is a soluble protein (data not shown), suggesting that this plasma membrane association may be mediated by interactions with other membrane proteins or by post-translational modifications.

During the cell cycle, Etd1p relocates from the cell tips to the medial cortex region in the metaphase-anaphase transition, and it translocates to the actomyosin ring as the cells initiate cytokinesis in anaphase B (Figure 2). In mutant cells defective in medial ring assembly or SIN activity, Etd1p fails to assemble into a ring (Figures 3 and 6), indicating that recruitment of Etd1p to the actomyosin ring requires ring components and SIN signalling. In these mutants, Etd1p relocated normally from the cell tips to the centre, indicating that this relocation of Etd1p occurs by an independent mechanism to that involved in its assembly into the actomyosin ring. The localisation pattern of sterol-rich membrane domains is regulated in a cell cycle-dependent manner (Wachtler *et al*, 2003), following a similar dynamics to that described for Etd1p (Figures 2 and 3). It is possible that these membrane domains could play a role in the mechanism regulating cell cortex association of Etd1p and its relocation from the tips to the cell centre. As shown in this report, Etd1p associates to Cdc15p (Figure 3C), a key element of the actomyosin ring, which has been recently shown to be required for the proper localisation of the sterol-rich domains at the cell centre (Takeda *et al*, 2004).

Once in the medial cell cortex, the recruitment of Etd1p into the ring requires the actomyosin ring and is triggered by SIN activation (Figures 3 and 6). The fact that signalling of the Sid2p-Mob1p protein kinase complex from the SPB to the division site requires Etd1p (Figure 5), the association of Etd1p with Cdc15 (Figure 3C) and the synthetic lethality found in double *etd1-1 sid2-250* mutants suggests that Etd1p, Cdc15p and Sid2p-Mob1p are closely regulating the initiation of septum formation and ring constriction. In a simple model, Etd1p could be a scaffold protein that associate to Cdc15p to facilitate the recruitment of Sid2p-Mob1p to the division site; however, the requirement of Etd1p is suppressed by certain morphogenetic mutants (Jimenez and Oballe, 1994), suggesting that Etd1p could play a regulatory role in ring constriction rather than being an integral scaffold component of the contractile ring. The role of the *S. pombe* Cdc42p GTPase and other cell polarity proteins in Etd1p localisation and function is currently under study.

Connecting SIN with ring constriction

The SIN cascade coordinates mitosis and cytokinesis. While mechanisms coordinating karyokinesis (mitosis) with SIN activity are well established (Bardin and Amon, 2001; Guertin *et al*, 2002b), little is known about how cytokinetic events (initiation, progression and exit) are connected with this regulatory cascade. Wild-type cells septate only once per cell cycle and this is controlled by SIN regulation. Therefore, when cytokinesis is initiated in response to SIN signalling, a feedback mechanism must operate in wild-type cells to

maintain SIN activity during ring constriction and to halt it once septation is completed. Etd1p is a good candidate for the coordination of ring constriction with SIN activity: recruitment of this protein to the actomyosin ring is triggered by SIN activity, it accumulates at the medial ring during cytokinesis, is required for the execution and maintenance of SIN signal-

Figure 8 A model for the function of Etd1p in the coordination of SIN activity and actomyosin ring constriction. (A) During interphase, Etd1p localises to the cell cortex, mainly at the cell tips. (B) Early in anaphase, Etd1p relocates to the middle of the cell following actomyosin ring assembly (which occurs in metaphase). (C) Late in anaphase, SIN activity triggers the recruitment of Etd1p to the actomyosin ring, possibly by association with Cdc15p. The actomyosin ring initiates constriction coupled to septum formation by the septum synthesis machinery (SSM). (D) Etd1p levels increase during ring constriction, and the protein accumulates behind the constricting ring. The accumulation of Etd1p maintains the SIN active during the progression of ring constriction and septation. (E) Etd1p is degraded by the proteasome pathway at the end of septation and contributes to coordinate exit from cytokinesis and inactivation of SIN.

ling and is degraded at the end of septation. A model for this regulatory circuit is schematised in Figure 8 and discussed below.

The initiation of actomyosin ring constriction requires relocation of Etd1p to the division site. This process only occurs when actomyosin ring has been assembled and mitosis triggers initial SIN activity. Thus, Etd1p integration into the actin ring may ensure that the ring components and mitotic events take place normally before ring constriction is initiated. In *Saccharomyces cerevisiae*, it has been proposed that Tem1p becomes active when it is exposed to Lte1 during the migration of the nucleus to the bud at anaphase. This mechanism ensures that one end of the spindle, and hence one set of chromosomes, has traversed the bud neck and entered the daughter cell before triggering exit from mitosis by activating the MEN (Bardin *et al*, 2000). In *S. pombe*, a rod-shaped cell that divides by fission, constriction of the medial ring triggered by the SIN at anaphase ensures that each cell will receive a nucleus in the course of a normal division. Like Lte1 in the bud, Etd1p localisation at the medial ring provides an excellent means by which to coordinate ring constriction and SIN activity.

Once at the division site, progression of ring contraction is accompanied by increasing levels of Etd1p (see Figure 7), which colocalises with new membrane surface behind the ring as it constricts (see Figure 2). Since Spg1p becomes inactive prematurely in the absence of Etd1p (Figure 5B), the accumulating Etd1p at the contracting ring could have a role in a feedback loop maintaining SIN activity during the process.

Protein degradation by the proteasome pathway has been shown to regulate processes such as chromosome segregation by degrading securin (Funabiki *et al*, 1996), or exit from mitosis by degrading mitotic cyclins (Glutzer *et al*, 1991). Etd1p is the first component of the SIN known to be ubiquitinated and degraded via the proteasome during each cell cycle (Figure 7C). Since the lack of Etd1p impedes SIN signalling (Figure 5), we suggest that the proteolytic degradation of Etd1p at the end of cytokinesis ensures SIN inactivation once ring constriction and septation are completed.

S. pombe divides by medial fission through the use of an actomyosin contractile ring analogous to the cleavage furrow in animal cells (Balasubramanian *et al*, 2004). The protein described in this study provides a novel function that sheds light on a new mechanism coordinating initiation, progression and exit of cytokinesis with SIN activation in the fission yeast. It is clear that some of the components of the SIN have homologues in higher eukaryotes (Guertin *et al*, 2002a). Etd1p does not appear to have sequence homologues in other eukaryotes; however, the identification of *etd1* functional homologues may be helpful in understanding how mammalian cells connect the SIN cascade and cytokinesis.

Materials and methods

Fission yeast strains and methods

Strains used in this study are listed in Supplementary Table I. Growth conditions and strain manipulations were as described previously (Moreno *et al*, 1991). Medium containing 6% ethanol was made up as described by Jimenez and Oballe (1994). Yeast transformation was carried out using the lithium acetate transformation protocol (Norbury and Moreno, 1997). Experiments in liquid culture were carried out in minimal medium (EMM)

supplemented as required, starting with a cell density of $2-4 \times 10^6$ cells/ml, corresponding to the mid-exponential phase growth. To prepare spheroplasts, growing cells were washed with water, resuspended in 0.65 M KCl containing 1 mg/ml novozyme (at about 10^7 cells/ml) and incubated at 30°C until spheroplasts were formed (for 20–30 min).

Induction of synchrony was accomplished by arrest–release of a *cdc25-22* mutant. Cells were grown to mid-exponential phase at 25°C and then shifted to 36°C for 4 h. The culture was cooled rapidly to 25°C by introducing it into an ice-cold water bath. Samples were taken every 15 min for analysis of RNA and protein. For regulated *nmt* expression, described methods were used (Maundrell, 1993). Repression was achieved by adding thiamine, and *nmt*-repressed cells analysed 12–15 h after the addition. Thiamine was removed for *nmt*-driven expression and cells analysed 18–20 h after induction.

etd1 deletion

The *etd1Δ* null allele was generated by a PCR-based approach as described previously (Bahler et al, 1998). The *S. pombe ura4⁺* gene was amplified by PCR using the KS-*ura4⁺* plasmid as template and the primers 5'-ATGGGTGACGCGTACAGTCTTTCTCTACGCAAT AATTCTTTCTTTAATTTAGCTTAAGAGAAATATAGATTTACGAGTCGC CAGGGTTTCCCAGTCACGAC-3' and 5'-ATTGAAATTATCCTAAGC GAAATTTTGACACTTTGGATAACAAATAGATACTTCCCATTCATTAA TGGATTAAATAGCCAGCGGTAACAAATTTACACAGGA-3' (the 5' ends are targeting sequences corresponding to 80 nucleotides immediately upstream and downstream of *etd1* ORF and the 3' ends are 24 nucleotides corresponding to sequences on either side of pBlueScript). A diploid was constructed by mating the *h⁺leu1-32 ura4-d18 ade6-M210* and *h⁻leu1-32 ura4-d18 ade6-M216* strains. This diploid was transformed with the PCR-amplified fragment, and the *ura4⁺* transformants were checked for the correct integration of the DNA fragment in the *etd1* locus by Southern blot analysis. A heterozygous diploid was sporulated and the spores were germinated in YE or YE + 6% ethanol at different temperatures between 25 and 36°C; after tetrad dissection, two spores were able to grow and two spores died under all conditions analysed.

In order to obtain a conditional deficiency for *etd1*, an *etd1Δ nmt81x:etd1* strain was constructed, where *etd1* expression is regulated under the weak repressible *nmt81X* promoter. The *PstI*–*EcoRI* fragment of *nmt81x:etd1* was subcloned into *PstI*–*SacI* sites of pJK148. The resulting plasmid was linearised with *NruI* and integrated at the *leu1* locus in the *etd1::ura4/etd1 ade6-M210/ade6-M216 ura4-d18/ura4-d18 leu1-32/leu1-32* strain. Diploid cells were selected in EMM with adenine and thiamine. The sporulated cells were digested with β-glucuronidase and selected in EMM supplemented with adenine.

Construction of Etd1-HA

Etd1-HA was generated by a PCR-based approach as described by Bahler et al (1998). Primers 5'-TAGATTTTCGATGCGTCCATGCTAC CAGGGCTAAACGATCATGTGCATTATTTAATGTCCCACTCCAACCTA TTATTACACCGGATCCCGGGTTAATTAA-3' and 5'-AAGCTTCTTAA GATGCAGAGTATAAAAAGGTTGTTAATGGCCAGTTTCACCTTTACT TGCTATCCACCAATGCGAAGAATTCGAGCTCGTTTAAAC-3' were used to amplify 3HA-Kan from pFA6a-3-HAkanMX6. The PCR-amplified fragment was transformed into a wild-type *h⁻972* haploid strain. Stable single-copy integrants were selected and checked for the correct integration of the DNA fragment at the *etd1* locus by PCR and Southern blot.

Construction of Etd1p-GFP

The pREP41-EGFP-Etd1 plasmid was created by cloning the *NdeI*–*SmaI* fragment containing the *etd1* ORF generated by PCR from genomic DNA using the primers 5'-ATTTGCGCATATGGGAGTTGTA

TACCG-3' and 5'-TAGATACTTCCCCGGGCCATTAATG-3' in the vector pREP41-EGFP (Craven et al, 1998) digested with *NdeI* and *SmaI*. A *pJK148nmt41x.EGFP-etd1* strain was created by PCR amplification from pREP41-EGFP-*etd1* and subcloning into *PstI*–*EcoRI* sites of pJK148. The resulting plasmid was linearised with *NruI* and integrated in the *S. pombe leu1* locus by homologous recombination.

Protein extracts and Western blots

Total protein extracts were prepared from 3×10^8 cells as described previously (Daga et al, 2003). For Western blot analysis, 100 μg of total protein extract was run on a 12% SDS–PAGE gel, transferred to nitrocellulose and probed with anti-HA 12CA5 (0.15 μg/ml) monoclonal antibodies. Goat anti-mouse antibodies conjugated to horseradish peroxidase (Amersham) (1:3500) were used as secondary antibodies. As loading controls, rabbit affinity-purified anti-Cdc2p C2 antibodies (1:500) were used. Immunoblots were developed using the Super Signal kit (Pierce).

Co-immunoprecipitation analysis of Etd1p-GFP and Cdc15p-Myc

Total protein extracts were prepared from 3×10^8 cells using HB buffer (Moreno et al, 1991). Cell extracts were spun at 4°C in a microfuge for 15 min, and protein concentrations were determined. A 2–3 mg portion of total soluble protein was subjected to immunoprecipitation by consecutive incubation with the monoclonal anti-Myc 9E10 (1 μg) for 1 h on ice, and protein G-Sepharose (Pharmacia-Biotech) for 30 min at 4°C in a rotating wheel. Immunoprecipitates were washed six times with 1 ml of HB buffer. Immunoprecipitates were resolved on 10% SDS–polyacrylamide gels, blotted and probed with anti-Myc 9E10 (1:1000) or anti-GFP (1:1000) antibody.

RNA preparation and Northern blots

RNA from cells was prepared by lysis with glass beads in the presence of phenol (Moreno et al, 1991). RNA gels were run in the presence of formaldehyde, transferred to GeneScreen Plus (NEN, Dupont) and probed with the *etd1* ORF according to the manufacturer's instructions.

Microscopy

DNA, septum material and F-actin were visualised using DAPI, calcofluor and rhodamine-phalloidin respectively. Staining with rhodamine-conjugated phalloidin was performed as described (Marks et al, 1986). Sterol-rich membrane domains were localised in live cells by filipin staining (Takeda et al, 2004). Filipin (Sigma F9765) was used at a final concentration of 5 μg/ml (in 1% DMSO). Cells were examined using a Leica fluorescence microscope equipped with a plan Apo $\times 100$ lens. Three-dimensional confocal images were obtained with a confocal Zeiss microscope. For time-lapse experiments, cells were imaged in Z-series every 5 min with a step size of 0.3 μm between focal planes.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

- Arai R, Mobuchi I (2002) F-actin ring formation and the role of F-actin cables in the fission yeast *Schizosaccharomyces pombe*. *J Cell Sci* **115**: 887–898
- Arai R, Nakano K, Mabuchi I (1998) Subcellular localization and possible function of actin, tropomyosin and actin-related protein 3 (Arp3) in the fission yeast *Schizosaccharomyces pombe*. *Eur J Cell Biol* **76**: 288–295

- Bahler J, Wu JQ, Longtine MS, Shah NG, McKenzie A, Steever AB, Wach A, Philippsen P, Pringle JR (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14**: 943–951
- Balasubramanian MK, Bi E, Glotzer M (2004) Comparative analysis of cytokinesis in budding yeast, fission yeast and animal cells. *Curr Biol* **14**: R806–R818

- Balasubramanian MK, Helfman DM, Hemmingsen SM (1992) A new tropomyosin essential for cytokinesis in the fission yeast *S. pombe*. *Nature* **360**: 84–87
- Bardin AJ, Amon A (2001) Men and sin: what's the difference? *Nat Rev Mol Cell Biol* **2**: 815–826
- Bardin AJ, Visintin R, Amon A (2000) A mechanism for coupling exit from mitosis to partitioning of the nucleus. *Cell* **102**: 21–31
- Carnahan RH, Gould KL (2003) The PCH family protein, Cdc15p, recruits two F-actin nucleation pathways to coordinate cytokinetic actin ring formation in *Schizosaccharomyces pombe*. *J Cell Biol* **162**: 851–862
- Celton-Morizur S, Bordes N, Fraissier V, Tran PT, Paoletti A (2004) C-terminal anchoring of mid1p to membranes stabilizes cytokinetic ring position in early mitosis in fission yeast. *Mol Cell Biol* **24**: 10621–10635
- Cerutti L, Simanis V (1999) Asymmetry of the spindle pole bodies and spg1p GAP segregation during mitosis in fission yeast. *J Cell Sci* **112**: 2313–2321
- Craven RA, Griffiths DJ, Sheldrick KS, Randall RE, Hagan IM, Carr AM (1998) Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe*. *Gene* **221**: 59–68
- Cueille N, Salimova E, Esteban V, Blanco M, Moreno S, Bueno A, Simanis V (2001) Flp1, a fission yeast orthologue of the *S. cerevisiae* CDC14 gene, is not required for cyclin degradation or rum1p stabilisation at the end of mitosis. *J Cell Sci* **114**: 2649–2664
- Daga RR, Bolanos P, Moreno S (2003) Regulated mRNA stability of the Cdk inhibitor Rum1 links nutrient status to cell cycle progression. *Curr Biol* **13**: 2015–2024
- Ding R, West RR, Morphey DM, Oakley BR, McIntosh JR (1997) The spindle pole body of *Schizosaccharomyces pombe* enters and leaves the nuclear envelope as the cell cycle proceeds. *Mol Biol Cell* **8**: 1461–1479
- Fankhauser C, Reymond A, Cerutti L, Utzig S, Hofmann K, Simanis V (1995) The *S. pombe* cdc15 gene is a key element in the reorganization of F-actin at mitosis. *Cell* **82**: 435–444
- Funabiki H, Yamano H, Kumada K, Nagao K, Hunt T, Yanagida M (1996) Cut2 proteolysis required for sister-chromatid separation in fission yeast. *Nature* **381**: 438–441
- Furge KA, Wong K, Armstrong J, Balasubramanian M, Albright CF (1998) Byr4 and Cdc16 form a two-component GTPase-activating protein for the Spg1 GTPase that controls septation in fission yeast. *Curr Biol* **8**: 947–954
- Glotzer M (2005) The molecular requirements for cytokinesis. *Science* **307**: 1735–1739
- Glotzer M, Murray AW, Kirschner MW (1991) Cyclin is degraded by the ubiquitin pathway. *Nature* **349**: 132–138
- Gordon C, McGurk G, Wallace M, Hastie ND (1996) A conditional lethal mutant in the fission yeast 26 S protease subunit mts3 + is defective in metaphase to anaphase transition. *J Biol Chem* **271**: 5704–5711
- Guertin DA, Chang L, Irshad F, Gould KL, McCollum D (2000) The role of the sid1p kinase and cdc14p in regulating the onset of cytokinesis in fission yeast. *EMBO J* **19**: 1803–1815
- Guertin DA, Trautmann S, McCollum D (2002a) Cytokinesis in eukaryotes. *Microbiol Mol Biol Rev* **66**: 155–178
- Guertin DA, Venkatram S, Gould KL, McCollum D (2002b) Dma1 prevents mitotic exit and cytokinesis by inhibiting the septation initiation network (SIN). *Dev Cell* **3**: 779–790
- Hou MC, Guertin DA, McCollum D (2004) Initiation of cytokinesis is controlled through multiple modes of regulation of the Sid2p–Mob1p kinase complex. *Mol Cell Biol* **24**: 3262–3276
- Jimenez J, Oballe J (1994) Ethanol-hypersensitive and ethanol-dependent cdc- mutants in *Schizosaccharomyces pombe*. *Mol Gen Genet* **245**: 86–95
- Kovar DR, Kuhn JR, Tichy AL, Pollard TD (2003) The fission yeast cytokinesis formin Cdc12p is a barbed end actin filament capping protein gated by profilin. *J Cell Biol* **161**: 875–887
- Krapp A, Gulli MP, Simanis V (2004) SIN and the art of splitting the fission yeast cell. *Curr Biol* **14**: R722–R730
- Le Goff X, Motegi F, Salimova E, Mabuchi I, Simanis V (2000) The *S. pombe* rlc1 gene encodes a putative myosin regulatory light chain that binds the type II myosins myo3p and myo2p. *J Cell Sci* **113**: 4157–4163
- Liu J, Wang H, McCollum D, Balasubramanian MK (1999) Drc1p/Cps1p, a 1,3-beta-glucan synthase subunit, is essential for division septum assembly in *Schizosaccharomyces pombe*. *Genetics* **153**: 1193–1203
- Marks J, Hagan IM, Hyams JS (1986) Growth polarity and cytokinesis in fission yeast: the role of the cytoskeleton. *J Cell Sci Suppl* **5**: 229–241
- Maundrell K (1993) Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene* **123**: 127–130
- McCollum D, Gould KL (2001) Timing is everything: regulation of mitotic exit and cytokinesis by the MEN and SIN. *Trends Cell Biol* **11**: 89–95
- Moreno S, Klar A, Nurse P (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol* **194**: 795–823
- Motegi F, Mishra M, Balasubramanian MK, Mabuchi I (2004) Myosin-II reorganization during mitosis is controlled temporally by its dephosphorylation and spatially by Mid1 in fission yeast. *J Cell Biol* **165**: 685–695
- Norbury C, Moreno S (1997) Cloning cell cycle regulatory genes by transcomplementation in yeast. *Methods Enzymol* **283**: 44–59
- Paoletti A, Chang F (2000) Analysis of mid1p, a protein required for placement of the cell division site, reveals a link between the nucleus and the cell surface in fission yeast. *Mol Biol Cell* **11**: 2757–2773
- Pelham RJ, Chang F (2001) Role of actin polymerization and actin cables in actin-patch movement in *Schizosaccharomyces pombe*. *Nat Cell Biol* **3**: 235–244
- Salimova E, Sohrmann M, Fournier N, Simanis V (2000) The *S. pombe* orthologue of the *S. cerevisiae* mob1 gene is essential and functions in signalling the onset of septum formation. *J Cell Sci* **113** (Part 10): 1695–1704
- Schmidt A, Hall A (2002) Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev* **16**: 1587–1609
- Schmidt S, Sohrmann M, Hofmann K, Woollard A, Simanis V (1997) The Spg1p GTPase is an essential, dosage-dependent inducer of septum formation in *Schizosaccharomyces pombe*. *Genes Dev* **11**: 1519–1534
- Simanis V (2003) Events at the end of mitosis in the budding and fission yeasts. *J Cell Sci* **116**: 4263–4275
- Sohrmann M, Fankhauser C, Brodbeck C, Simanis V (1996) The dmfl1/mid1 gene is essential for correct positioning of the division septum in fission yeast. *Genes Dev* **10**: 2707–2719
- Sohrmann M, Schmidt S, Hagan I, Simanis V (1998) Asymmetric segregation on spindle poles of the *Schizosaccharomyces pombe* septum-inducing protein kinase Cdc7p. *Genes Dev* **12**: 84–94
- Takeda T, Kawate T, Chang F (2004) Organization of a sterol-rich membrane domain by cdc15p during cytokinesis in fission yeast. *Nat Cell Biol* **6**: 1142–1144
- Trautmann S, Wolfe BA, Jorgensen P, Tyers M, Gould KL, McCollum D (2001) Fission yeast Clp1p phosphatase regulates G2/M transition and coordination of cytokinesis with cell cycle progression. *Curr Biol* **11**: 931–940
- Treier M, Staszewski LM, Bohmann D (1994) Ubiquitin-dependent c-Jun degradation *in vivo* is mediated by the delta domain. *Cell* **78**: 787–798
- Wachtler V, Rajagopalan S, Balasubramanian MK (2003) Sterol-rich plasma membrane domains in the fission yeast *Schizosaccharomyces pombe*. *J Cell Sci* **116**: 867–874
- Wu JQ, Kuhn JR, Kovar DR, Pollard TD (2003) Spatial and temporal pathway for assembly and constriction of the contractile ring in fission yeast cytokinesis. *Dev Cell* **5**: 723–734