Homoeostasis between the GTPase Spg1p and its GAP in the regulation of cytokinesis in *S. pombe*

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Summary

Cytokinesis in *Schizosaccharomyces pombe* begins at mitotic entry, when the site of division is defined by formation of the contractile acto-myosin ring (CAR) at the cell cortex. Contraction of the CAR and formation of the division septum are triggered at the end of mitosis by septation initiation network (SIN) proteins associated with the spindle pole body (SPB). SIN signalling requires activation of the GTPase Spg1p, which is regulated by the bipartite GTPase-activating protein (GAP) Byr4p-Cdc16p. We show that, for Spg1p to associate with the SPB, it must be bound to its GAP or to its mitotic effector, the protein kinase Cdc7p. Analysis of the GAP proteins reveals that the steady-state level of Byr4p reflects that of Spg1p. Furthermore, if the interaction of Byr4p with Spg1p is compromised, the level of Byr4p decreases dramatically. The adaptation of the level of Byr4p to that of Spg1p requires the presence of Cdc16p and is mediated by proteasome-dependent destruction. It requires neither association with the SPB nor an active SIN. We propose a mechanism that limits the amount of the Byr4p-Cdc16p GAP to the amount required to inhibit Spg1p signalling.

Supplementary material available online at
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Key words: Cytokinesis, *S. pombe*, GTPase activating protein (GAP), Proteolysis

Introduction

Coordination of mitosis and cytokinesis is important to assure that each daughter cell receives a full complement of genetic information after division. Premature initiation of cytokinesis can alter the ploidy of the progeny, which might lead to the death of the cell or alter its characteristics. The fission yeast *Schizosaccharomyces pombe* has proved to be an excellent model system for studying the coordination of cytokinesis with other cell cycle events. Fission yeast cells grow mainly by elongation at their tips. They divide by forming a medial division septum, which cleaves the cell, producing two daughters of equal size. Assembly and organisation of the contractile actomyosin ring (CAR) occurs in stages throughout mitosis (Wu et al., 2003). At the end of mitosis, the CAR contracts and guides synthesis of the division septum (Ishiguro, 1998). The onset of septation is triggered by a signal transduction pathway called the septation initiation network (SIN) (reviewed by Krapp et al., 2004b; Krapp and Simanis, 2005; Wolfe and Gould, 2005). Fundamental to the SIN are three protein kinases (Cdc7p, Sid1p and Sid2p) and their associated regulatory proteins (Spg1p, Cdc14p and Mob1p, respectively) (Fankhauser and Simanis, 1993; Fankhauser and Simanis, 1994; Guertin et al., 2000; Hou et al., 2000; Salimova et al., 2000; Schmidt et al., 1997; Sparks et al., 1999). The protein kinase Plo1p also regulates SIN signalling in addition to many other mitotic events (Tanaka et al., 2001). SIN proteins are associated with the spindle pole body (SPB), where they bind to a scaffold comprising three coiled-coil proteins – Ppc89p, Sid4p and Cdc11p. The SPB protein Ppc89p binds to Sid4p, which in turn recruits Cdc11p (Chang and Gould, 2000; Krapp et al., 2001; Rosenberg et al., 2006; Tomlin et al., 2002). The N-terminal domain of Cdc11p appears to function as an assembly site for many SIN proteins at the SPB and is crucial for SIN signalling (Krapp et al., 2004a; Morrell et al., 2004).

Spg1p is a Ras-superfamily GTPase (Schmidt et al., 1997), whose nucleotide status is thought to mediate signalling by the SIN. The association of Spg1p with the SPB depends upon Cdc11p (Krapp et al., 2001; Tomlin et al., 2002), with which it interacts in vitro (Morrell et al., 2004). Spg1p is required for the localisation of Cdc7p to the SPB but does not regulate Cdc7p kinase activity (Mehta and Gould, 2006; Sohrmann et al., 1998). Spg1p signalling is regulated negatively by a GTPase-activating protein (GAP) comprising two subunits (Furge et al., 1998) – Byr4p (Song et al., 1996) and Cdc16p (Fankhauser et al., 1993). Cdc16p is a GAP but requires Byr4p, which acts as a scaffold to juxtapose Cdc16p and Spg1p, to stimulate GTP hydrolysis by Spg1p. In the absence of Cdc16p, Byr4p inhibits GTP hydrolysis and release by Spg1p, thus stabilising the active, GTP-bound form of Spg1p (Furge et al., 1998). Deletion analysis of Byr4p has identified a C-terminal domain required for interaction with Cdc16p and multiple regions that interact with Spg1p (Furge et al., 1999).

Spg1p, Byr4p and Cdc16p all localise to the interphase SPB. While Byr4p and Cdc16p are interdependent for localisation, neither of them is required for the localisation of Spg1p (Cerutti and Simanis, 1999; Li et al., 2000; Sohrmann et al., 1998). Increased expression of full-length Byr4p titrates Spg1p off the SPB and inhibits cytokinesis (Li et al., 2000). After entry into mitosis, Cdc16p no longer associates with the SPB (Cerutti and Simanis, 1999), while the Byr4p signal decreases in intensity (Cerutti and Simanis, 1999) or vanishes (Li et al., 2000). At this time, Cdc7p localises to both spindle pole bodies (Sohrmann et al., 1998). In anaphase B, Byr4p and Cdc16p associate with the old SPB, while Cdc7p and Sid1p-Cdc14p are found on the new SPB (Cerutti and Simanis, 1999; Grallert et al., 2004; Li et al., 2000).

The budding yeast *Saccharomyces cerevisiae* counterpart of the SIN, which is known as the mitotic exit network (MEN), regulates both mitotic exit and cytokinesis (for reviews, see Bardin and Amon, 2001; Simanis, 2003; Wolfe and Gould, 2005). The counterparts of Cdc16p and Byr4p in the MEN are Bub2p (Hoyt et al., 1991) and Bfa1p (Lee et al., 1999; Li, 1999), respectively. They act upon the GTPase Tem1p, in a manner analogous to that of Byr4p and Cdc16p upon Spg1p (Geymonat et al., 2002). Increased expression of *BFA1* arrests cells in anaphase, preventing mitotic exit, while increased expression of *BUB2* has no effect (Lee et al., 1999; Li, 1999; Ro et al., 2002). Bub2p, Bfa1p and Tem1p all associate with the daughter-bound SPB during mitosis, where they function as part of a surveillance mechanism, which assures that correct orientation of the spindle along the mother-daughter axis has been achieved before mitotic exit and cytokinesis (reviewed by Cooper and Nelson, 2006). They associate with the SPB by binding to the scaffold protein Nud1p, the counterpart of Cdc11p (Gruneberg et al., 2000); Bub2p, but not Bfa1p, binds directly to the scaffold protein Nud1p (Gruneberg et al., 2000).

Here, we have examined the behaviour of the GAP proteins Byr4p and Cdc16p through the cell cycle and in mutants that alter SIN signalling. Our data indicate that, in the presence of Cdc16p, the steady-state level of Byr4p is closely correlated with that of Spg1p, suggesting a mechanism to maintain an equilibrium between Spg1p and its GAP. We also demonstrate that association of Spg1p with the SPB requires the presence of either the GAP Byr4p-Cdc16p or its effector Cdc7p.

Results

Analysis of the steady-state levels of the SIN GAP proteins Byr4p and Cdc16p

Synchronous cell populations were generated by *cdc25-22* arrest-release and the steady-state levels of Byr4p and haemagglutinin (HA)-tagged Cdc16p (Cdc16-HAp) were examined by western blotting. Cdc16-HAp was present throughout mitosis and cytokinesis (Fig. 1A). Inactivation of most SIN proteins did not alter the steady-state level of Cdc16p by more than a factor of 2.5 (supplementary material Fig. S1A,B).

To study Byr4p, we raised a polyclonal antiserum, which recognised a broad band of ~90 kDa after purification, which was absent in protein extracts from *mob1-R4 byr4::ura4*$^{+}$ cells. Additional, slower migrating bands were observed in cells expressing a GST-Byr4p fusion protein (supplementary material Fig. S2A). Together, these data demonstrate that the antiserum is specific for Byr4p. Byr4p was present throughout mitosis and cytokinesis (Fig. 1B). However, as cells progressed through mitosis, the band observed in G2 cells became broader, with both slower- and faster-migrating forms of Byr4p appearing during anaphase and septum formation (Fig. 1B). Previous studies have shown that Byr4p is a phosphoprotein (Song et al., 1996). We therefore treated a mitotic protein extract with phosphatase. The majority of these bands collapsed into the fastest-migrating form, suggesting that phosphorylation accounts for a significant part of the changes in migration of Byr4p (Fig. 1B) and that Byr4p undergoes cell-cycle-dependent changes in phosphorylation.

Next, we examined the steady-state level and phosphorylation status of Byr4p after inactivation of various SIN components, including those that have been identified since the original study of Byr4p (Song et al., 1996). Faster-migrating forms of Byr4p were observed in *cdc16-116*, consistent with previous studies (Song et al., 1996); this was accompanied by an increase in the steady-state level of Byr4p (Fig. 2A). Furthermore, after inactivation of SIN scaffold components such as Cdc11p (two alleles) or Sid4p, the slower-migrating form of Byr4p was absent, suggesting that complete phosphorylation of Byr4p requires SPB localisation. By contrast, inactivation of core SIN components such as Cdc7p, Sid1p, Sid2p or Mob1p resulted in small (factor of <3) changes in the level of Byr4p; multiple forms of the protein could be observed in these backgrounds (Fig. 2A). Unexpectedly, we found that the steady-state level of Byr4p was decreased by more than a factor of ten in *spg1-B8*, compared with wild-type cells (Fig. 2A). This was observed at both the permissive and restrictive temperatures (Fig. 5A). The level of Byr4p also decreased in the *sid4-SA1 spg1-B8*

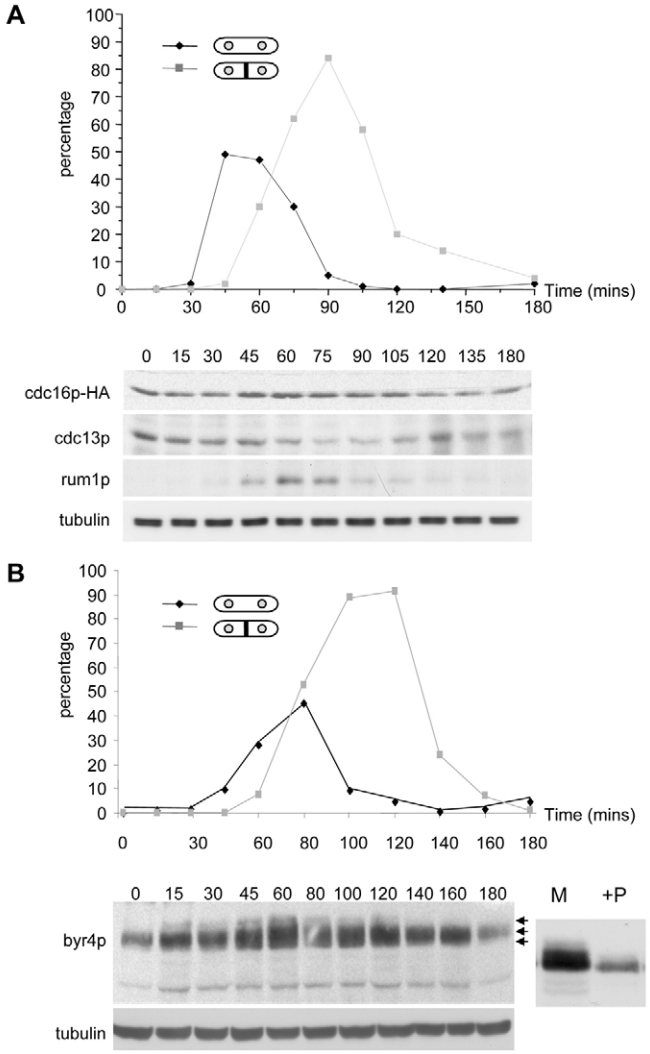

Fig. 1. Analysis of the steady-state levels of Cdc16p and Byr4p through the cell cycle. Cells of the genotypes indicated below were synchronised by *cdc25-22* arrest-release. Cells were collected at the indicated times (minutes after release), fixed and stained with DAPI and Calcofluor. The percentage of cells in anaphase or septation was determined. Western analysis was performed on lysates of the different strains with the indicated antibodies. (A) *cdc25-22 leu1::cdc16-HA(ura4*$^{+}$*) cdc16*$^{+}$. (B) *cdc25-22 byr4*$^{+}$. The three arrows to the right of the western blot in B indicate the different forms of Byr4p seen in mitosis (upper and lower) and the interphase form of the Byr4p (middle). The western blot on the right is part of the 60 minute protein sample treated with alkaline phosphatase (+P) or alkaline phosphatase in the presence of phosphatase inhibitors (M).

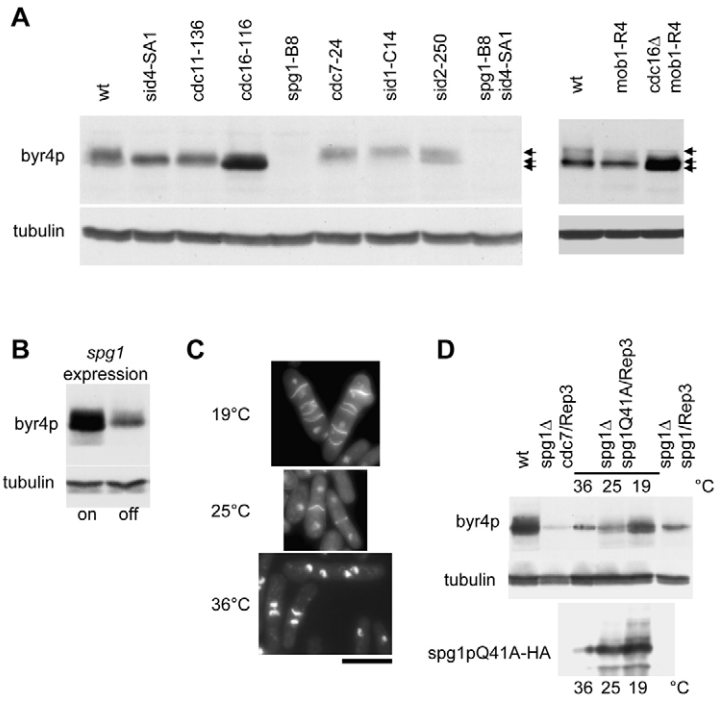

Fig. 2. Analysis of the steady-state level of Byr4p in SIN mutants. (A) The indicated mutants were grown in YE medium to exponential phase and incubated for 4 hours at 36°C. Protein extracts were analysed by western blotting with the indicated antibodies. (B) *leu1::nmt1-spg1*(*ura4*$^+$) *ura4-D18* cells were grown in EMM2 plus leucine in the presence of 2 μM thiamine (off) or without thiamine for 18 hours at 29°C (on) to induce *spg1* expression. Cells overexpressing *spg1* showed a multiseptated phenotype. Protein extracts were prepared, and western blots were probed with the indicated antibodies. The three arrows indicate the most rapidly migrating form, which predominates in *cdc16-116*, and two others that represent different states of phosphorylation. How these relate to the forms observed in Fig. 1 will be the subject of future experiments. (C) The mutant *spg1-Q41A-HA* was expressed from pREP3 in a *spg1::ura4*$^+$ background, in the presence of 2 μM thiamine at 25°C, and cells were then shifted to 19°C for 9 hours or to 36°C for 5 hours. Cells were fixed and stained with DAPI and Calcofluor. Bar, 10 μm. (D) Protein extracts from the experiment shown in C, as well as from wild-type and *spg1::ura4*$^+$ cells rescued by expression of either pREP41-*cdc7* (without thiamine) or pREP3-*spg1* (grown in the presence of thiamine) were analysed by western blotting with the indicated antibodies. The bottom panel shows the steady-state level of Spg1p-Q41A–HA at the indicated temperatures.

mutant (Fig. 2A) and the *cdc11-123 spg1-B8* mutant (data not shown), when Spg1p and Byr4p do not associate with the SPB (Chang and Gould, 2000; Krapp et al., 2001; Li et al., 2000). Therefore, we conclude that the decrease in the steady-state level of Byr4p in *spg1-B8* requires neither active SIN signalling nor association of Byr4p with the SPB.

The steady-state level of Byr4p is affected by Spg1p

As the steady-state level of Byr4p decreased in *spg1-B8*, we analysed the effect of increased expression of *spg1*. Cells carrying an integrated copy of *spg1*, expressed from the full-strength *nmt1* promoter at *leu1* (Schmidt et al., 1997), were induced to express *spg1*; western blotting showed that the steady-state level of Byr4p increased in these cells (Fig. 2B), which were multiseptated (data not shown). This suggested that the level of Byr4p might reflect that of Spg1p. To test this further, we used a novel allele of *spg1*, generated by alanine scanning mutagenesis of *spg1* (A.K. and V.S., unpublished). This allele, *spg1*-Q41A, was expressed as an HA-tagged fusion protein from a plasmid in a *spg1::ura4*$^+$ background. The relevant property of this mutant protein in the context of this study is that its steady-state level changes with temperature. At 25°C, the cells resemble wild-type cells. At 19°C, more Spg1p is present and the cells are multiseptated. Finally, at 36°C, the level of the mutant Spg1p decreases significantly, and the cells display a typical SIN phenotype (Fig. 2C,D). Western blotting of Byr4p showed that its level reflected that of Spg1p-Q41A (Fig. 2D), indicating furthermore that the effect upon Byr4p levels is not specific to *spg1-B8*. Next, we analysed the effect of the complete absence of Spg1p upon the level of Byr4p. Previous studies have shown that the essential function of *spg1* can be substituted by increased expression of *cdc7* (Schmidt et al., 1997). Therefore, we examined the steady-state level of Byr4p in *spg1::ura4*$^+$ cells expressing either *cdc7* or *spg1* from a plasmid, at levels that permit colony formation. We found that the level of Byr4p was very low in *spg1::ura4*$^+$ cells expressing *cdc7*, compared with *spg1::ura4*$^+$ cells expressing *spg1* (Fig. 2D), indicating that Byr4p levels are very low in the absence of Spg1p. These results demonstrate that the steady-state level of Byr4p reflects that of Spg1p.

The interaction of Spg1p with Byr4p is compromised in *spg1-B8*

To examine the interaction of Byr4p and Spg1p, we constructed a strain expressing GFP-Byr4p and Spg1p-GST, each from the gene's own promoter, as the sole copy in the cell (see Materials and Methods). Protein extracts were prepared and passed over a glutathione-agarose column. The vast majority of Spg1p-GST was retained on the column, with a small amount present in the flow-through. Elution with reduced glutathione released Spg1p-GST from the column (Fig. 3A). GFP-Byr4p reflected this distribution. As a control, in extracts from cells in which *spg1* was untagged, Byr4p was present only in the flow-through, demonstrating that the binding to the column depends upon association with Spg1p. These data suggest that the majority of Byr4p in cells is complexed with Spg1p.

To investigate whether the reduced level of Byr4p in the *spg1-B8* mutant could be restored to normal by the presence of a wild-type copy of *spg1*, we constructed strains that had two copies of *spg1*, one GFP tagged, expressed from the *spg1* promoter and inserted at *leu1*; the other at the native locus. Both the GFP-tagged wild-type and the GFP-tagged mutant spg1 proteins were expressed to similar levels (Fig. 3B). We observed that, when at least one wild-type copy of *spg1* was present (*leu1::spg1-GFP spg1*$^+$, or *leu1::spg1-B8-GFP spg1*$^+$, or *leu1::spg1-GFP spg1-B8*), the steady-state levels of Byr4p resembled those in wild-type cells. By contrast, when only the *spg1-B8* allele was present, either at the *spg1* locus or GFP-tagged at the *leu1* locus on a *spg1*-null background, the steady-state level of Byr4p was very low (Fig. 3B). These data suggested to us that the decrease in the level of Byr4p in *spg1-B8* could occur because the interaction of Byr4p with Spg1p was compromised. To examine whether this was the case, we

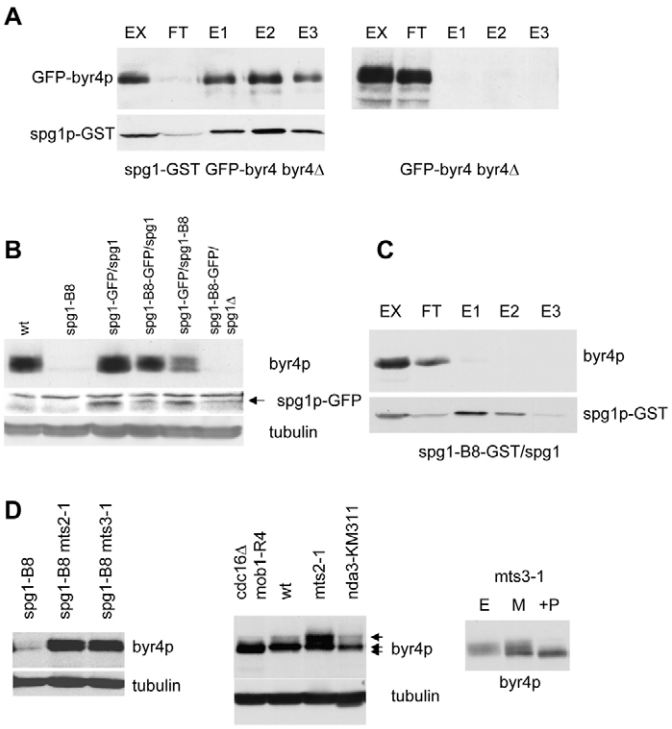

Fig. 3. Byr4p is degraded if its interaction with Spg1p is compromised. (A) Protein extracts (EX) were prepared from the indicated strains and passed over a glutathione-agarose column. The flow-through (FT) and three successive eluates (E1, E2, E3) from the column were analysed using antibodies against Byr4p or GST. (B) Protein extracts were prepared from the indicated strains, grown at 25°C and western blots were probed with antibodies against Byr4p, GFP and tubulin. The relevant genotypes of the strains are shown above the panel. When two copies of *spg1* are present, the first indicates the gene inserted into *leu1*, the second the state of the native *spg1* locus. (C) Protein extracts from the indicated strain were analysed as described in A. (D) Cells from the indicated strains were shifted from exponential growth in YE medium at 25°C to 36°C for 4 hours before harvesting the cells. Protein extracts were prepared and western blots were probed with the indicated antibodies. The western blot on the right of panel D shows the *mts3-1* sample untreated (E), treated with alkaline phosphatase (+P) or with alkaline phosphatase in the presence of phosphatase inhibitors (M). The arrows indicate the positions of the three forms as defined in Fig. 2A.

constructed a strain expressing both Spg1-B8p–GST and Spg1p (see Materials and Methods). Glutathione-agarose chromatography of protein extracts prepared from these cells demonstrated that, even though Spg1-B8p–GST bound to the column, the majority of Byr4p did not (Fig. 3C), indicating that the interaction of Byr4p with Spg1p is compromised in the *spg1-B8* mutant.

Byr4p is degraded by the proteasome in *spg1-B8*

To investigate whether the low level of Byr4p in *spg1-B8* resulted from proteolytic degradation of Byr4p, we compromised proteasome function using the mutants *mts2-1* and *mts3-1* (Gordon et al., 1993). Extracts prepared from *spg1-B8 mts2-1* or *spg1-B8 mts3-1* mutants both showed significant increases in the steady-state level of Byr4p compared with that in *spg1-B8* alone (Fig. 3D). It is noteworthy that the steady-state level of Byr4p was also higher in the *spg1*$^+$ proteasome mutants, suggesting that Byr4p turns over by means of the proteasome even in *spg1*$^+$ cells (Fig. 3D). We also observed the accumulation of a slower-migrating form of Byr4p in the proteasome mutants. Treatment of these samples with phosphatase transformed Byr4p to a more rapidly migrating form (Fig. 3D), demonstrating that phosphorylated forms of Byr4p accumulate in the proteasome mutants.

Degradation of Byr4p in *spg1-B8* requires the interaction of Byr4p with Cdc16p

Analysis of deletion mutants of Byr4p has defined a number of functionally important domains. The C-terminus, amino acids 595–665, is required for binding to Cdc16p. Several regions of Byr4p between amino acids 1-595 interact with Spg1p (Furge et al., 1999). As point mutants of Byr4p that block interaction with either Spg1p or Cdc16p are not available at present, we evaluated the importance of these interactions by studying the effect of overexpression of these Byr4p fragments in a *byr4*$^+$ background.

Increased expression of Byr4p(1-595), which can interact with Spg1p, but not with Cdc16p (Furge et al., 1999), produced a SIN phenotype (Fig. 4A). Localisation of SIN proteins in Byr4p(1-595)-overexpressing cells showed that both Spg1p-GFP and Cdc16p-GFP failed to associate with the SPB (Fig. 4B). As Spg1p is required for the SPB association of both Cdc7p and Sid1p (Guertin et al., 2000; Sohrmann et al., 1998), the SIN cannot signal the onset of septum formation and the cells become multinucleated. Increased expression of this fragment resulted in degradation of full-length Byr4p, probably as Byr4p can no longer interact with Spg1p (Fig. 4E). Byr4p degradation in this background was also dependent upon a functional proteasome (Fig. 4E).

Increased expression of Byr4p(595–665) produced multiseptated cells (Fig. 4C). Localisation of SIN proteins showed that Spg1p-GFP, Cdc7p-GFP and GFP-Sid1p were associated with the spindle pole bodies, at all stages of the cell cycle, whereas Cdc16p and full-length Byr4p failed to localise to the SPB (Fig. 4D), These data are consistent with hyper-activation of the SIN and the multiseptated phenotype (Fig. 4C). Western blotting (Fig. 4E) revealed that the steady-state level of Spg1p changed by a factor of less than two in response to increased expression of this fragment (supplementary material Fig. S2B). By contrast, the level of full-length Byr4p increased upon overexpression of Byr4p(595-665), and the rapidly migrating forms of Byr4p were most abundant, as observed in *cdc16-116*. These data are consistent with the view that increased expression of the Cdc16p-binding domain of Byr4p titrates Cdc16p away from full-length Byr4p. As Byr4p and Cdc16p are interdependent for localisation, full-length Byr4p can no longer associate with the SPB to regulate Spg1p, resulting in constitutive SIN signalling.

As the steady-state level of Byr4p increased in *cdc16-116* (Fig. 2A), and also upon overexpression of Byr4p(595-665) (Fig. 4E), we considered the possibility that degradation of full-length Byr4p requires Cdc16p. To test this hypothesis, we used the strain *mob1-R4 cdc16::ura4*$^+$, in which loss of Cdc16p is rescued by the *mob1-R4* mutant (Fournier et al., 2001). Western blotting revealed that the steady-state level of Byr4p was elevated in *mob1-R4 cdc16::ura4*$^+$ compared with wild-type cells (Fig. 2A). Crucially, Byr4p was no longer degraded in the triple mutant *cdc16::ura4+ mob1-R4 spg1-B8* (Fig. 5A), indicating that degradation of Byr4p that is not complexed to Spg1p requires Cdc16p. The *mob1-R4* mutant alone did not affect the steady-state level of Byr4p (Fig. 2A). Note that the mutant *mob1-R4 spg1-B8* is synthetically lethal at all temperatures (Salimova et al., 2000), which precludes analysis of the effects of this combination upon Byr4p. To investigate this further, we examined the effects of increased expression of Byr4p fragments upon the level of Byr4p in *mob1-R4 cdc16::ura4*$^+$ cells.

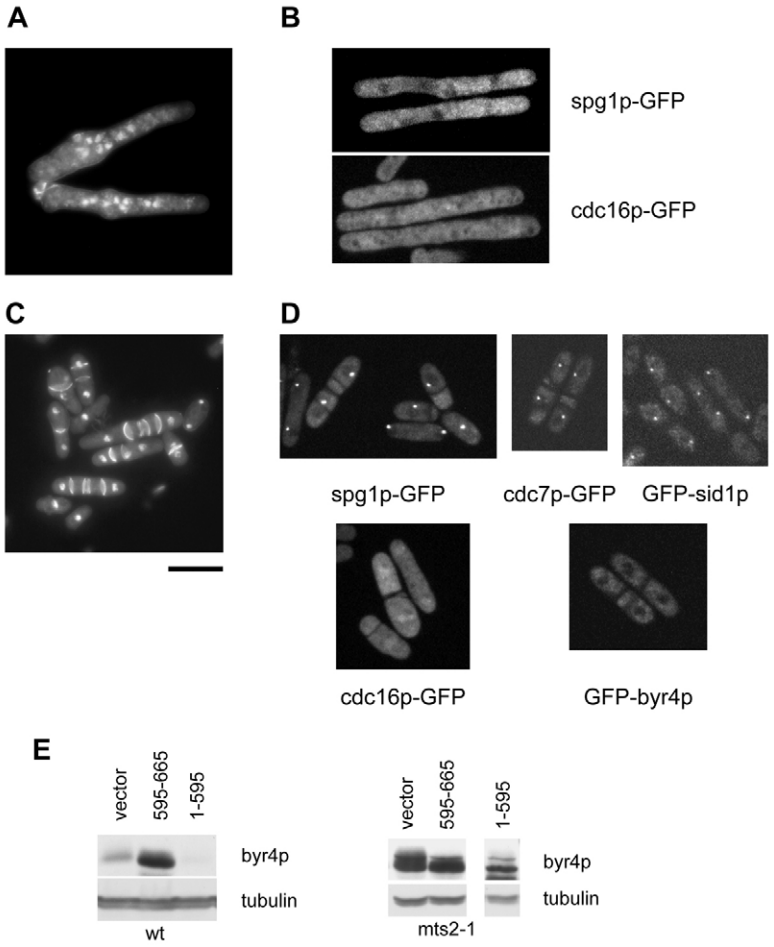

Fig. 4. Analysis of the effects of increased expression of domains of *byr4*. (A) Expression of the *byr4*(1-595) fragment from the *nmt1* promoter was induced by growth in the absence of thiamine for 20 hours at 25°C in EMM2 medium. Cells were fixed and stained with DAPI and Calcofluor. (B) The *byr4*(1-595) fragment was expressed from the *nmt1* promoter as in A. Cells also expressed the indicated tagged GFP-SIN fusion protein. The GFP signals were photographed in living cells. (C) Expression of the *byr4*(595-665) fragment from the *nmt1* promoter was induced by growth in the absence of thiamine for 20 hours at 25°C in EMM2 medium. Cells were fixed and stained with DAPI and Calcofluor. (D) The *byr4*(595-665) fragment was expressed from the *nmt1* promoter as in C, in cells expressing the indicated tagged GFP-SIN fusion protein. The GFP signals were photographed in living cells. (E) Protein extracts were prepared from either wild-type cells grown at 25°C (left panel) or *mts2-1* cells incubated at 36°C for 4 hours (right panel), in which expression of the indicated fragment of *byr4* from the *nmt1* promoter had been induced, as described above. One lane, showing the effects of a truncation not discussed in this paper, has been removed from the gel. Bar, 10 μm (panels A-D).

As shown in Fig. 5B, overexpression of Byr4p(1-595) and Byr4p(595-665) in this strain did not increase or decrease, respectively, the steady-state level of full-length Byr4p, as it does in wild-type cells (Fig. 4E). Finally, we examined the effect of expressing fragments of Byr4p in *spg1-B8*. We found that increased expression of Byr4p(595-665), which would compete for binding to Cdc16p, prevented degradation of full-length Byr4p (Fig. 5C). By contrast, Byr4p(1-595), which cannot interact with Cdc16p, did not (Fig. 5C). Together, these data are consistent with the view that degradation of Byr4p requires its interaction with Cdc16p.

Association of Spg1p with the SPB requires interaction either with its GAP, Byr4p-Cdc16p, or its effector, Cdc7p

Overexpression of the Spg1-binding domain of Byr4p showed that neither Cdc16p nor Spg1p can localize to the SPB in the absence of full-length Byr4p (Fig. 4B). The loss of Cdc16p from the SPB is likely to reflect the interdependence of Byr4p and Cdc16p for localisation (Cerutti and Simanis, 1999; Li et al., 2000). However, the loss of Spg1p-GFP from the SPB was unexpected as loss of either Byr4p-Cdc16p or Cdc7p does not affect Spg1p association with the SPB (Li et al., 2000; Sohrmann et al., 1998). This result led us to hypothesise that the association of Spg1p with the SPB might require the presence of one of its binding partners, the GAP or the effector Cdc7p. We therefore examined the effect of loss of both Cdc7p and GAP function upon Spg1p localisation. In *mob1-R4 byr4::ura4*$^+$ and *mob1-R4 cdc16::ura4*$^+$ cells that lack GAP function, Spg1p-GFP and Cdc7p-GFP both associated with the SPB throughout the cell cycle, while, as expected, Cdc16-GFP and GFP-Byr4 did not (Fig. 6A,B). Likewise, SPB localization of Spg1p-GFP was not compromised in *cdc7-A20 cdc16*$^+$ cells grown at the restrictive temperature (Fig. 6A). However in *cdc7-A20 cdc16::ura4*$^+$ cells (Fournier et al., 2001), Spg1p-GFP was associated with the SPB in the multiseptated cells at 25°C, when Cdc7p is functional, but not in multinucleated cells at 36°C (Fig. 6C), when Cdc7-A20p activity is reduced (Fankhauser and Simanis, 1994). These data indicate that binding of Spg1p-GFP to the SPB requires it to associate with either the GAP or Cdc7p. As the mutant Cdc7-A20p-GFP localises to the SPB at 25°C, but not at 36°C (Fig. 6D), we cannot at present distinguish whether the Spg1p-GFP SPB association in the absence of the GAP requires the kinase activity of Cdc7p, interaction with Cdc7p or both. The same results were obtained using similar strains based on the *cdc7-24* allele (data not shown).

Discussion

We have examined the steady-state levels of Cdc16p and Byr4p, which together comprise the GAP for the GTPase Spg1p that mediates signalling through the *S. pombe* SIN. We have shown that both components are present in interphase cells and throughout mitosis and cytokinesis. Byr4p undergoes significant changes in its phosphorylation state: both slower- and faster-migrating forms of the protein are present from the beginning of mitosis until cytokinesis. Previous studies have shown that the budding yeast *S. cerevisiae* Byr4p counterpart Bfa1p also undergoes changes in

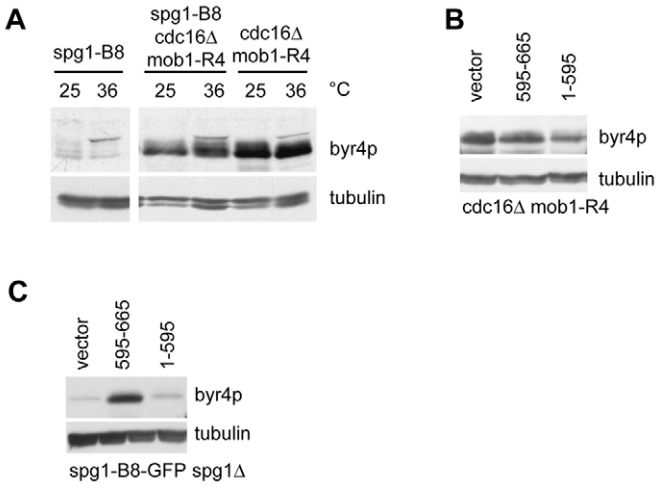

Fig. 5. Degradation of Byr4p requires Cdc16p. (A) Protein extracts were prepared from the indicated strains grown exponentially at 25°C or shifted to 36°C for 4 hours before harvesting. Extracts were analysed on a western blot probed with the indicated antibodies. Two lanes to the right of the *spg1* sample have been deleted from the image of the gel as they show the effects of a mutant not discussed in this study. (B) Protein extracts were prepared from *cdc16::u4+ mob1-R4*, in which expression of the indicated fragment of *byr4* from the *nmt1* promoter had been induced for 20 hours at 25°C in EMM2 medium. Extracts were analysed on a western blot probed with the indicated antibodies. (C) Protein extracts were prepared from *leu1::spg1-B8-GFP spg1::u4+* cells in which expression of the indicated fragment of *byr4* from the *nmt1* promoter had been induced for 16 hours at 25°C in EMM2 medium before shifting to 36°C for 5 hours. Extracts were analysed on a western blot probed with the indicated antibodies.

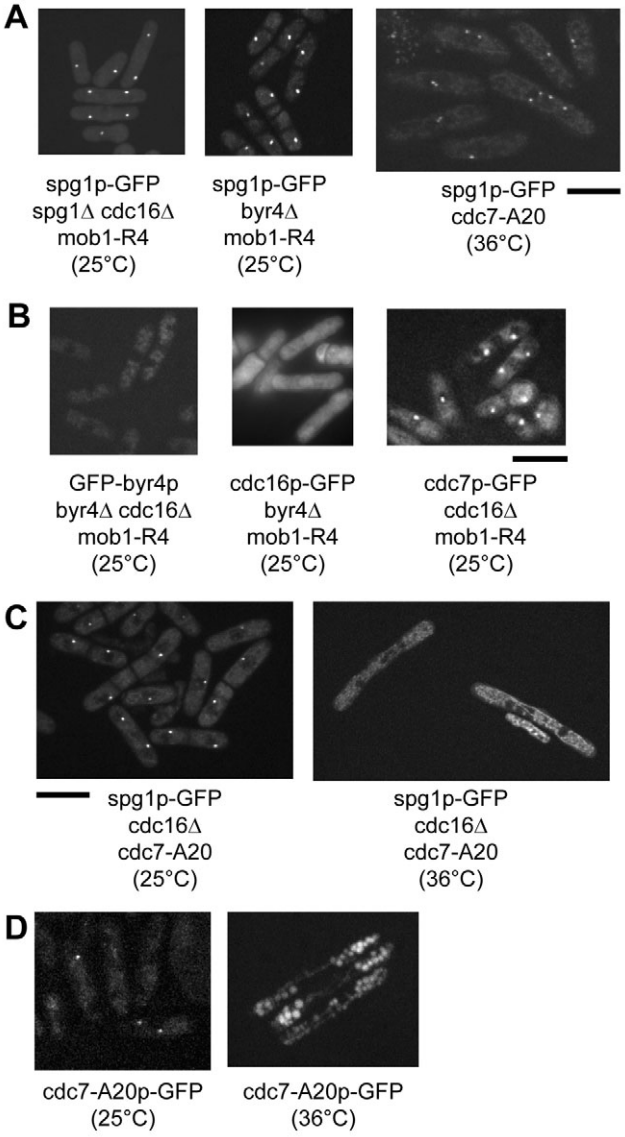

Fig. 6. Analysis of the localisation of Spg1p-GFP in SIN mutant backgrounds. The indicated strains were observed at either 25°C or after a shift to 36°C for 4 hours. The GFP signals were photographed in living cells maintained at 36°C. Bars, 10 μm.

phosphorylation during mitosis (Hu et al., 2001; Pereira et al., 2002), suggesting that phosphorylation of Bfa1p might be a conserved regulatory mechanism. The most rapidly migrating forms of Byr4p accumulate when it fails to associate with the SPB, for example in *cdc16-116* cells, and complete phosphorylation is precluded in the SIN scaffold mutants, suggesting that SPB association is required for the most highly phosphorylated forms of Byr4p to be generated. A full understanding of the role played by phosphorylation in regulating Byr4p function awaits mapping and mutagenesis of the phosphorylation sites.

We have also demonstrated that, in the absence of both the Byr4p-Cdc16p GAP and Cdc7p, Spg1p-GFP can no longer be detected at the SPB. These data imply that, for Spg1p to associate with the SPB, it must be bound to its GAP, or its mitotic effector, Cdc7p. As the mutant Cdc7-A20-GFP fails to associate with the SPB at the restrictive temperature, it is possible that the kinase activity, but not binding per se to Spg1p, is required for localization of Spg1p to the SPB. These data appear inconsistent with the observation that Cdc11p and Spg1p can interact in vitro (Morrell et al., 2004); however, it is possible that, in vivo, this interaction requires either a conformational change in Spg1p induced by its binding to either the GAP or the effector or a modification of Cdc11p.

We have shown that, in vivo, the vast majority of Byr4p is complexed to Spg1p and that the level of Byr4p decreases significantly if its interaction with Spg1p is compromised. This does not reflect the interdependence of Cdc16p and Byr4p for association with the SPB as Byr4p levels still decreased in the *spg1-B8 sid4-SA1* mutant. The adaptation of the Byr4p level to that of Spg1p requires Cdc16p, as shown by analysis of mutants lacking Cdc16p or overexpression of Byr4p fragments that displace Cdc16p from the GTPase-GAP complex. We propose that the equilibrium between Spg1p and its GAP is maintained by promoting the degradation of Byr4p if it binds to Cdc16p in the absence of Spg1p. Keeping the level of free GTPase in the cell low during interphase reduces the chances of Spg1p binding to its effectors during interphase. Furthermore, eliminating excess GAP complexes prevents competition with trimeric Spg1p-Cdc16p-Byr4p complexes for binding to the SPB, thus allowing efficient activation of Spg1p at mitotic entry.

The model proposed here imposes certain constraints upon assembly of the trimeric GAP-Spg1p complex if energetically wasteful and futile Byr4p-Cdc16p interactions are to be minimised. A simple means to avoid this would be if Byr4p interacted with higher affinity with Spg1p than with Cdc16p, or that its binding to Cdc16p is favoured only when a Byr4p-Spg1p complex has been

formed. Alternatively, chaperones might mediate orderly assembly of the GAP-Spg1p complex.

In contrast to overexpression of either *spg1* (Schmidt et al., 1997) or *byr4* (Song et al., 1996), an excess of *cdc16* does not affect cytokinesis or cell cycle progression (supplementary material Fig. S1C,D). The lack of effect on SIN signalling and cytokinesis in these cells can be explained by considering two observations from previous studies. First, Spg1p and Cdc16p can only interact productively in the presence of Byr4p (Furge et al., 1998) and, second, as Cdc16p cannot associate with the SPB in the absence of Byr4p (Cerutti and Simanis, 1999; Li et al., 2000), free Cdc16p will not compete with the Spg1p-Byr4p-Cdc16p complex for binding to the SPB. The steady-state level of Byr4p was unaltered in cells overexpressing *cdc16* (supplementary material Fig. S2C). This observation is consistent with our model as most of the Byr4p is complexed with Spg1p (Fig. 3A), and thus increased levels of Cdc16p should not lead to increased degradation of Byr4p.

Previous studies have noted that ablation of one member of a protein complex can affect the stability of the other proteins. For example, studies of kinetochore formation in *S. cerevisiae* have revealed that one of the key scaffolding subunits of the CBF3 complex, p58, is unstable unless complexed to other components of the complex. It has been proposed that this serves as an editing mechanism to prevent the formation of ectopic kinetochores (Russell et al., 1999). We believe that elimination of excess Byr4p performs a similar homoeostatic role in regulating cytokinesis. As Spg1p and Cdc16p can only interact functionally in the presence of Byr4p, the scaffold is a logical point at which to apply regulation to the GAP complex to maintain homoeostasis between the GAP and the GTPase it regulates.

The mechanism that triggers elimination of Byr4p that is not complexed to Spg1p will be the subject of future studies. However, as we have found that phosphorylated forms of Byr4p accumulate in proteasome mutants, it is possible that phosphorylation will play a role in promoting Byr4p degradation. In *S. cerevisiae*, it has been found that Bfa1p binds to Skp1p, an adaptor for the SCF complex (Kim et al., 2006), although this does not seem to alter the stability of Bfa1p. The finding that Byr4p levels also increase in *spg1*$^+$ cells if the function of the proteasome is compromised suggests that the homoeostasis mechanism proposed here also operates in cells where SIN function is not perturbed.

Materials and Methods

Yeast methods

Established methods were used for growth and manipulation of *S. pombe* (Moreno et al., 1991). Cells were grown in yeast extract (YE) or EMM2 minimal medium, supplemented as required with the appropriate amino acids (50 μg/ml). Transformations were performed by the lithium acetate method (Okazaki et al., 1990). Cells were synchronised by *cdc25-22* arrest release (Moreno et al., 1989). Phosphatase treatment of extracts was performed as described previously (Krapp et al., 2001).

Plasmid constructions and genetic manipulations

Standard techniques were used for molecular biology (Sambrook et al., 1989). Because of the proximity of the *rps13* gene (Fankhauser et al., 1993; Marks and Simanis, 1992), the *cdc16* promoter and ORF was fused to the 12CA5 (HA) epitope and inserted at the *leu1* locus. The Cdc16p-HA fusion protein is functional and rescues a *cdc16::ura4*$^+$ null allele (data not shown). For the generation of GFP or GST fusion proteins integrated at the *leu1* locus, we first generated GFP-IntA and GST-IntA by inserting a PCR product containing either the GFP or the GST coding region flanked at the 5′ and 3′ ends by the *Pst*1, *Sal*I, *Bam*HI and *Nhe*I, *Sma*I and *Sac*I restriction sites, respectively, into pInt5 (Fankhauser and Simanis, 1994). To clone an N-terminally GFP tagged *byr4* expressed under the control of its own promoter, a PCR fragment containing the promoter region (nucleotides –635 to –1) was cloned into *Pst*I-*Sal*I of GFP-IntA, while the *byr4* coding region was inserted after PCR amplification as a *Sma*I-*Sac*I fragment. This plasmid was then integrated into *leu1* as a *Not*I fragment. This tagged, integrated copy is functional and rescues *byr4::ura4*$^+$ (data not shown). A C-terminal GST-tagged *spg1* was constructed by oligonucleotide-mediated tagging as described previously (Bahler et al., 1998). To generate the *spg1*$^+$ or *spg1-B8* tagged at its C-terminus with GFP, expressed from the *spg1* promoter, a PCR product spanning the *spg1* promoter plus coding region (nucleotides –611 to +594) was produced on genomic DNA prepared from either wild-type (972) or *spg1-B8* cells and cloned as a *Pst*I-*Sal*I fragment into GFP-IntA. As before, the targeting cassette was excised as a *Not*1 fragment and integrated into *leu1*. This tagged, integrated copy could rescue *spg1::ura4*$^+$, as demonstrated by crossing. In the case of the *spg1-B8* allele, cells were able to grow at the permissive, but not the restrictive, temperature. The Spg1-GST fusion protein expressing vector was constructed in the same way using the GST-IntA plasmid.

The *byr4* truncation expression pRep3-based vectors (Basi et al., 1993) were generated as follows. A PCR fragment spanning the Cdc16p binding domain of Byr4p (amino acid 595 to 665) was cloned as a *Bam*HI-*Sma*I fragment into pRep3. An ATG codon was inserted at the 5′end. In the case of the Spg1p-binding domain of Byr4p (amino acids 1-595), the PCR product was inserted as a *Sma*I fragment into pRep3.

To express *byr4* in *Escherichia coli*, a *byr4 Xba*I-*Bgl*II fragment, spanning amino acids 1-345, was treated with Klenow fragment and cloned into the *Sma*I site of pGEX-3X to generate Byr4-*Bgl*II-pGEX.

Protein expression and purification and antibody production

E. coli carrying the Byr4-*Bgl*II-pGEX expression plasmid were grown in 2YT for 3.5 hours in the presence of ampicillin at 37°C and then induced by addition of 0.3 mM IPTG for 3.5 hours at 37°C. After freezing on dry ice, the cell pellet was resuspended in 20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 1% Triton-X-100 and protease inhibitors (1% aprotinin, 50 μg/ml leupeptin and 1 mM PMSF). Extracts were clarified by centrifugation after sonication. The GST-Byr4 fusion protein was purified with glutathione-sepharose (Sigma). After elution with 10 mM glutathione, the protein was dialyzed against 100 mM Na-phosphate pH 8.0. Purified GST-Byr4 protein (1 mg) was sent for antibody generation to 'l' élevage scientifique des Dombes', France and two bleeds were obtained. The antibody was then purified on a CNBr-sepharose 4B column (Pharmacia) to which purified GST-Byr4 proteins had been linked.

Binding assays and western blot analysis

Protein extracts from *S. pombe* were obtained as described previously (Moreno et al., 1991). Spg1p-GST pulldown assays were performed by passing soluble extracts on glutathione-sepharose (Sigma). Columns were then washed with ten bed-volumes of protein extraction buffer [50 mM HEPES, pH 8.0, 5 mM EDTA, 1 mM EGTA, 150 mM NaCl, 60 mM β-glycero-phosphate, 0.1 mM Na-ortho-vanadate, 1 mM DTT, 1% NP-40 supplemented with a cocktail of protease inhibitors (Boehringer)]. Complexes were eluted with three bed-volumes of 10 mM glutathione. For western blotting, proteins were separated on a 10% SDS-PAGE gel for analysis of Cdc16p-GFP, Spg1p-GFP, Spg1p-HA, Cdc13p or Rum1p, and on a 7% SDS-PAGE gel for analysis of Byr4p, GFP-Byr4p or tubulin. Proteins transferred to nitrocellulose membranes (Schleicher and Schuell) were probed with 12CA5 for HA-tagged proteins, TAT-1 for tubulin and polyclonal antibodies against GFP, Cdc13p, Rum1p or Byr4p.

Microscopy

DAPI Calcofluor staining was performed on cells that had been fixed with 70% ethanol, as described previously (Balasubramanian et al., 1997; Moreno et al., 1991). Examination of GFP-tagged proteins in living cells was performed using a Zeiss axiovert 200 microscope equipped with a confocal scanner unit model CSU10 (Yokogawa Electric Corporation), a coolSNAPHQ camera (Photometrics) and a 63× 1.4 NA plan-apo objective. Images were collected using Metamorph software (Universal Imaging).

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