Hob3p, the fission yeast ortholog of human BIN3, localizes Cdc42p to the division site and regulates cytokinesis

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Cdc42 GTPase is required for polarization in eukaryotic cells, but its spatial regulation is poorly understood. In *Schizosaccharomyces pombe*, Cdc42p is activated by Scd1p and Gef1p, two guanine-nucleotide exchange factors. Two-hybrid screening identified Hob3p as a Gef1p binding partner. Hob3p is a BAR domain-containing protein ortholog of human Bin3. Hob3p also interacts directly with Cdc42p independently of Gef1p. Hob3p, Cdc42p and Gef1p form a complex, and Hob3p facilitates Gef1p–Cdc42p interaction and activation. Hob3p forms a ring in the division area, similar to that of Gef1p. This localization requires actin polymerization and Cdc15p but is independent of the septation initiation network. Hob3p is required for the concentration of Cdc42p to the division area. The actomyosin ring contraction is slower in *hob3*Δ than in wild-type cells, and this contributes to its cytokinesis defect. Moreover, this report extends previous evidence that human Bin3 suppresses the cytokinesis phenotype of *hob3*Δ cells, showing that Bin3 can partially recover the GTP-Cdc42p level and its localization. These results suggest that Hob3p is required to recruit and activate Cdc42p at the cell division site and that this function might be conserved in other eukaryotes.

Introduction

Schizosaccharomyces pombe is a highly polarized yeast growing by elongation of its ends and dividing by medial septation. Cytokinesis begins with the assembly of a contractile actomyosin medial ring attached to the cell membrane (Feierbach and Chang, 2001; Guertin *et al*, 2002). Most of the

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components of this ring are conserved among animal cells (Glotzer, 2005). The septation initiation network (SIN) triggers the contraction of the actin ring and the consecutive addition of new membranes and synthesis of the primary septum in coordination with the nuclear cycle (Balasubramanian *et al*, 2004; Krapp *et al*, 2004; Wolfe and Gould, 2005).

BAR (Bin–Amphiphysin–Rvs) domain-containing proteins are a well-conserved family that includes Rvs proteins in yeast, and amphiphysins and BIN proteins in mammals. BAR family members integrate signal pathways that regulate membrane dynamics, F-actin cytoskeleton, and nuclear processes (Peter *et al*, 2004; Zimmerberg and McLaughlin, 2004; Ren *et al*, 2006). There are three main functions associated with the BAR domain: (1) sensing and/or induction of membrane curvature at endocytic sites (Peter *et al*, 2004; Zimmerberg and McLaughlin, 2004), (2) binding to small GTPases (Habermann, 2004), and (3) transcriptional repression properties (Elliott *et al*, 1999; Miaczynska *et al*, 2004).

Saccharomyces cerevisiae RVS161 and *RVS167* genes, coding for two BAR domain-containing proteins, were isolated in a screening for mutations causing reduced viability upon nutrient starvation. Rvs161p and Rvs167p regulate endocytosis, vesicle traffic, F-actin organization, and cell polarity (Ren *et al*, 2006). These two proteins form heterodimers through their respective BAR domains. Rvs167p contains other domains and interacts with many proteins that have well-characterized roles in the actin cytoskeleton organization, including Abp1p, Acf2p, Act1p, Las17p, Sla1p, Sla2p, and Srv2p (Ren *et al*, 2006). In addition, *RVS167* shows synthetic lethal or synergistic negative growth interactions with *MYO1*, *MYO2*, and *ACT1* as well as with *SLT2* and *KRE6* (Breton *et al*, 2001). Rvs161p is required for actin repolarization upon salt stress and is associated with lipid rafts (Balguerie *et al*, 2002).

S. pombe also has two BAR domain proteins, Hob1p, structurally similar to Rvs167/amphiphysin (Routhier *et al*, 2003), and Hob3p, similar to human BIN3 and Rvs161p (Routhier *et al*, 2001). Surprisingly, neither Hob1p nor Hob3p is required for endocytosis, unlike Rvs167p and Rvs161p. *hob1+* deletion causes totally different phenotypes than those produced by the deletion of the budding yeast *RVS167*. *hob1*Δ cells are slightly elongated, fail to arrest upon nutrient starvation, and are hypersensitive to genotoxic stress. These defects were complemented by human *BIN1* (Routhier *et al*, 2003). *S. pombe* cells lacking Hob3p are multiseptated and the F-actin patches are distributed randomly around the cell. Interestingly, the actin localization defects of *hob3*Δ mutants were rescued by human *BIN3* and only partially rescued by *RVS161* (Routhier *et al*, 2001). These findings suggest that *hob3+* and *BIN3* are orthologs, and have diverged from *RVS161* during evolution.

Cdc42 GTPase is a key molecule in establishing cell polarity in eukaryotic cells. Like other GTPases, Cdc42p acts as a molecular switch, cycling between an inactive (GDP-bound) and an active (GTP-bound) state. The local activation of Cdc42p at the growth site is required for actin polymerization and growth establishment (Etienne-Manneville, 2004). Fission yeast Cdc42p is essential for cell proliferation and also for maintenance of the rod-like cell morphology (Miller and Johnson, 1994). Cdc42p is mainly localized to the medial region and growing tips of the cell. It also localizes to the periphery and internal membranes (Merla and Johnson, 2000). Cdc42p is activated by two GTPase nucleotide exchange factors (GEFs): Scd1p, required to maintain the apical growth (Chang *et al*, 1994); and Gef1p, which plays a role in cytokinesis and the switch to bipolar growth (Coll *et al*, 2003; Hirota *et al*, 2003). The scaffold protein Scd2p allows the interaction between Cdc42p, the exchange factor Scd1p, and the p21-activated kinase Shk1p that regulates apical growth (Chang *et al*, 1999). As mentioned, Cdc42p localizes to the medial region, but a possible role of Cdc42p in *S. pombe* cytokinesis has not been described. Additionally, it is not known if other scaffold proteins, different from Scd2p, would cause the local activation of Cdc42p in the division area. Here we describe the interaction of Gef1p and Cdc42p with the BAR domain-containing protein Hob3p. This protein concentrates Cdc42p to the medial region and it is also necessary for the Gef1p-mediated Cdc42p activation. We also show that overexpression of Bin3 can substitute for Hob3p, suggesting that they have a function conserved among eukaryotes.

Results

Hob3p interacts with Gef1p

It has been shown that Gef1p is a Cdc42-specific exchange factor that participates in the regulation of cytokinesis (Coll *et al*, 2003; Hirota *et al*, 2003). Gef1p contains a Dbl homology (DH) domain responsible for their exchange activity, but lacks a pleckstrin homology (PH) domain. Gef1p does not interact with any of the known proteins from the Cdc42p signaling pathway that interact with Scd1p, such as Ras1p, Scd2p, Shk1p, or Shk2p (Hirota *et al*, 2003; our unpublished results). In order to identify the possible proteins of the Gef1p-Cdc42p signaling pathway, we performed a two-hybrid screen using Gef1p as bait, and 12 positive clones were obtained. Six of them contained the sequence coding for Hob3p, a BAR domain-containing protein, ortholog of human Bin3 and *S. cerevisiae* Rvs161p (Routhier *et al*, 2001). To map the Gef1p interaction with Hob3p, different Gef1p coding fragments were used as bait with pAS2-*hob3*$^+$. *gef1* constructs lacking the sequence coding the C-terminal 243 amino-acid residues failed to associate with Hob3p, suggesting that this region is required for the interaction with Hob3p (Figure 1A).

To verify the interaction between Gef1p and Hob3p, we performed co-immunoprecipitation experiments using cells transformed with the plasmid pREP-GST-Gef1p and we showed that GST-Gef1p co-precipitated with Myc-Hob3p expressed from the endogenous *hob3*$^+$ promoter (Figure 1B). Additionally, we demonstrated that Hob3-GST and Gef1-HA, both expressed from the endogenous promoters, co-immuno-
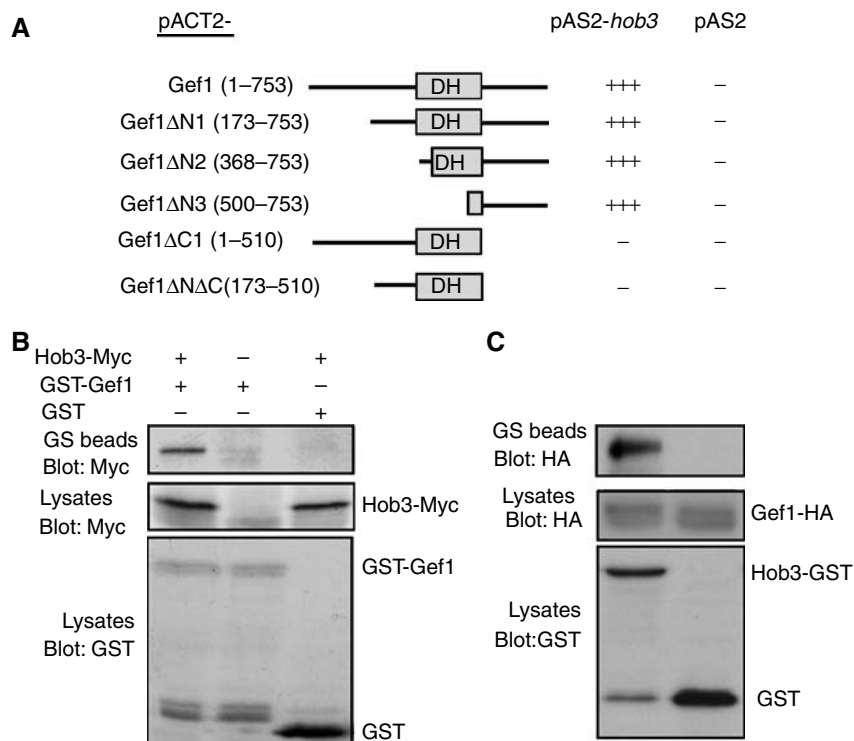

Figure 1 Gef1p interacts with Hob3p. (**A**) Two-hybrid analysis of the interactions between different fragments of Gef1p (pACT2) and Hob3p (pAS2). (**B**) Cells carrying endogenous *hob3-myc* were transformed with pREP1-*GST-gef1*$^+$ and grown in the absence of thiamine for 12 h. Cell extracts were pulled down with GS beads and the precipitates were probed with anti-Myc antibody (top). Extracts were also analyzed by Western blot using anti-Myc (9E10) or anti-GST antibodies (bottom panels). (**C**) Extracts from cells carrying endogenous *gef1-HA* and *hob3-GST* were pulled down with GS beads and the precipitates were probed with anti-HA antibody. Extracts from *gef1-HA* cells transformed with the plasmid pREP-GST were used as negative control.

precipitated (Figure 1C). Together, these results indicate that Gef1p and Hob3p physically associate in *S. pombe* cells.

To see if Hob3p was required for Gef1p localization, we analyzed Gef1-GFP in *hob3Δ* cells, and observed that Gef1-GFP localized to the septation area, as in wild-type cells (data not shown). Therefore, Hob3p was not necessary for Gef1p localization. We also looked for any genetic interaction between *gef1*[+] and *hob3*[+]. The absence of Gef1p causes an increase in septating cells (Coll *et al*, 2003), and aggravates the phenotype of other cytokinesis mutants (our unpublished results). Elimination of Hob3p causes an increase in septating cells and the appearance of some multiseptated cells. *hob3Δ gef1Δ* double mutant was viable at all temperatures and the percentage of cells with one or more septa was similar to *hob3Δ* (40% septating cells; 9% multiseptated cells; 7% elongated cells at 32°C, $n = 550$, for *hob3Δ* cells and 42% septating cells; 9% multiseptated cells; 8% elongated cells at 32°C, $n = 720$ for the *hob3Δ gef1Δ* cells). These results suggest that Hob3p and Gef1p are in the same signaling pathway.

Hob3p interacts with Cdc42p

Hob3p belongs to the BAR domain-containing protein family, and some members of this family can bind to small GTPases (Habermann, 2004). Hence, Hob3p could be an effector of Cdc42p, interacting with this GTPase when activated by Gef1p. To test this possibility, we analyzed whether Hob3p also binds to Cdc42p. Co-precipitation experiments were performed using extracts from cells expressing *GST-hob3* and *HA-cdc42* from their respective endogenous promoters. Interestingly, Hob3p co-precipitated with Cdc42p in both wild-type and *gef1Δ* cells (Figure 2A); therefore, Gef1p is not required for this interaction. Additionally, co-precipitation experiments in cells transformed with either the constitutively active *cdc42G12V* allele or the dominant-negative *cdc42T17N* allele showed that Hob3p interacted with Cdc42p independent of its activation state (Figure 2B). Taken together, these results suggest that Hob3p is not a Cdc42p effector but directly associates with this GTPase.

As Gef1p, Hob3p, and Cdc42p interact with each other, we considered the possibility that Hob3 might facilitate Gef1p–Cdc42p interaction. Therefore, we analyzed if the three proteins coexisted in a complex. Cells expressing *hob3-GFP* and *HA-cdc42* from the respective endogenous promoters were transformed with the pREP-*GST-gef1* plasmid. Moderate expression of the plasmid was induced and co-precipitation of the three proteins by pull-down with gluthation-Sepharose beads was successful (Figure 2C). These experiments suggest that a complex of Gef1p–Hob3p–Cdc42p may be present *in vivo*. To further investigate if the three proteins coexist in a complex, extracts from *hob3-GFP HA-cdc42* and *hob3Δ HA-cdc42* cells transformed with the pREP-GST-gef1 plasmid were fractionated by gel filtration (Figure 2D). HA-cdc42p has a predicted mass of 23 kDa; however, the elution position of HA-cdc42p from the column was that expected for a protein of much bigger size. Similarly, Hob3-GFP (predicted mass of 57 kDa) and GST-gef1p (predicted mass of 110 kDa) appear in fractions expected for proteins of bigger size. A small portion of the three proteins co-fractionated, suggesting that they might form part of a complex of 250–300 kDa. Most Cdc42p appears in higher molecular weight fractions, probably because in a logarithmic culture only 15–20% of the cells are septating and

Cdc42p also forms complex with other signaling proteins, such as Scd1p and Scd2p, at the growing tips. In the absence of Hob3p, GST-Gef1p shifted toward lower molecular weight, and HA-cdc42p appears more spread throughout the column (fractions 21–28). The absence of Hob3p affects Cdc42p and Gef1p mobility, further supporting that they form part of the same complex.

Hob3p is necessary for the Gef1p-dependent activation of Cdc42p

We next analyzed whether Hob3p could be necessary for the Gef1p interaction with Cdc42p. Gluthation-Sepharose (GS) beads pull-down of cell extracts from wild-type and *hob3Δ* cells endogenously expressing *HA-cdc42* and *GST-gef1* were performed. HA-Cdc42p was present in both wild-type and *hob3Δ* cell pull-downs, but the level in *hob3Δ* cells was half that of the wild-type cells (Figure 3A). These results suggest that Hob3p facilitates the interaction of Gef1p with Cdc42p, although is not absolutely required for it. To further investigate whether Hob3p was necessary for Gef1p activation of Cdc42p, we analyzed the amount of active GTP-bound HA-Cdc42p in wild-type, *hob3Δ*, *gef1Δ*, and *gef1Δhob3Δ* double mutant cells. As expected, the levels of GTP-Cdc42p in *gef1Δ* cells were 30% that of wild-type cells. The lack of Hob3p also decreased the levels of GTP-Cdc42p to 45% that of wild-type cells (Figure 3B). Interestingly, *hob3Δ gef1Δ* cells had GTP-Cdc42p levels similar to *gef1Δ* cells (Figure 3B), suggesting that Hob3p and Gef1p are in the same Cdc42 activating pathway. Additionally, whereas the overexpression of Gef1p considerably increased GTP-Cdc42p, Hob3p overexpression did not increase the active Cdc42p levels (Figure 3C). These results suggest that Hob3p is not a Cdc42-GEF, but is necessary for Cdc42p activation. To test genetically whether Hob3p was necessary for the function of Gef1p, we made the *hob3Δ scd1Δ* double mutant strain. The double mutant *gef1Δscd1Δ* lacking both Cdc42p activators is lethal (Coll *et al*, 2003; Hirota *et al*, 2003). Therefore, if Hob3p were necessary for Gef1p function, *hob3Δ scd1Δ* would be lethal as well. *scd1*[+] gene was replaced by *KanMX6* in a *hob3*[+]/*hob3*::*ura4*[+] diploid strain and 44 tetrad spore analysis showed that the *ura*[+] spores (*hob3Δ*) that germinated and proliferated were never resistant to kanamycin (*scd1Δ*). The spores *Kan*[r] *ura*[+] germinated and underwent two or three cell divisions before arresting as small rounded cells (Figure 3D), similar to the spores of the double mutant *gef1Δ scd1Δ* (Coll *et al*, 2003). Taken together, these results indicate that Hob3p facilitates Gef1p–Cdc42p interaction and is necessary for Cdc42p activation mediated by Gef1p; consequently, Hob3p function is essential in the absence of Scd1p, the other Cdc42p activator.

Hob3p forms a ring at the medial region and localizes to patches at the growing poles

To gain further insight into the Hob3p relationship with Cdc42p and Gef1p, we first examined the subcellular localization of Hob3-GFP. The genomic locus of *hob3*[+] was tagged with the *GFP* gene fused in-frame to the 3′ end of *hob3*[+] open reading frame (ORF). Hob3-GFP was observed in the medial region (Figure 4A, left). Confocal microscopy demonstrated that Hob3p formed a ring in that region (Figure 4A, right). To analyze Hob3p localization during septation, we constructed the double mutant *cdc25-22 hob3-GFP* and synchronized cells in G2 phase by arrest at 36.5°C for 4 h. Cells were returned to
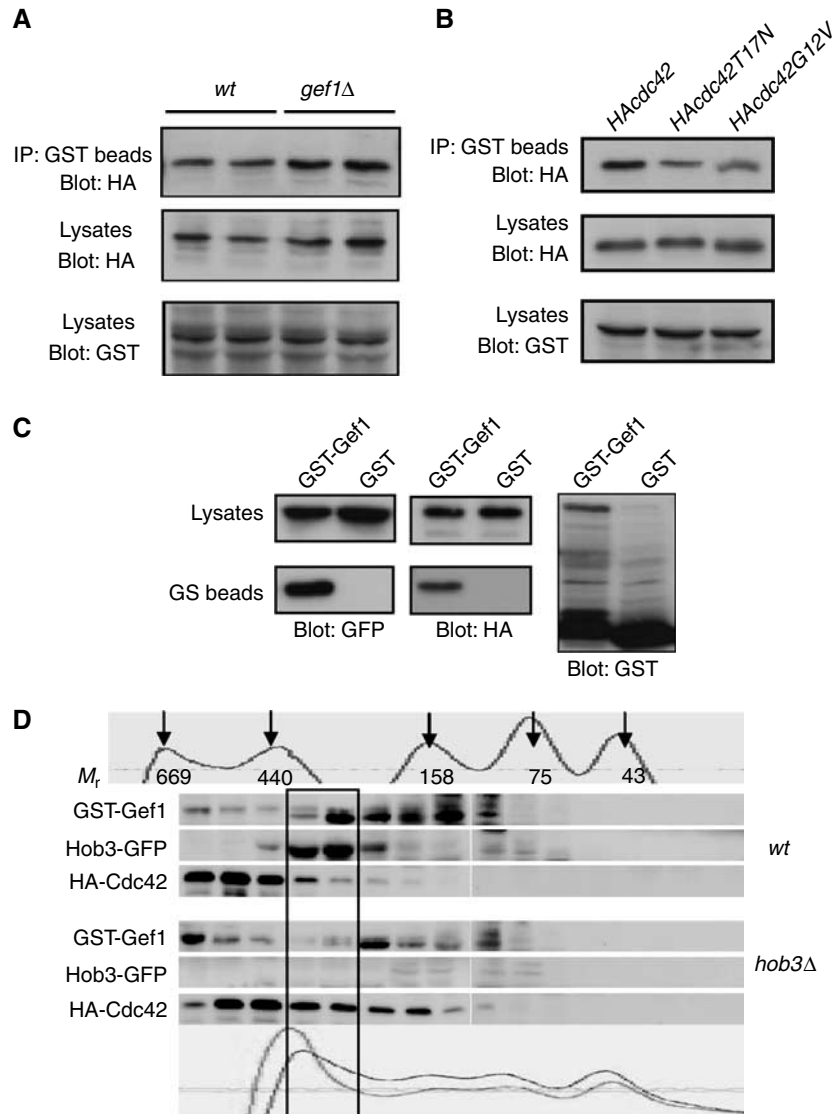
Figure 2 Hob3p interacts with Cdc42p and Gef1p. (**A**) Extracts from wild-type and *gef1Δ* cells carrying *hob3-GST* and *HA-cdc42* expressed under their endogenous promoters were pulled down with GS beads and the precipitates were probed with anti-HA antibody (top). Extracts were assayed for expression of Hob3 and Cdc42 by Western blot analysis using anti-GST or anti-HA (12CA5) antibodies (bottom). (**B**) Cells expressing endogenous *hob3-GST* were transformed with pREP42-*HA-Cdc42*, pREP42-*HA-Cdc42G12V*, or pREP42-*HA-Cdc42T17N* and grown in the absence of thiamine for 12 h. Cell extracts were pulled down with GS beads and the precipitates were probed with anti-HA antibody. Hob3-GST and plasmid expression was analyzed by Western blot. (**C**) Gef1p, Cdc42p and Hob3p form an *in vivo* complex. *hob3-GFP-* and *HA-cdc42-* expressing cells were transformed with either pREP-*GST-gef1*[+] or pREP-*GST* and grown in the absence of thiamine for 12 h. Cell extracts were immunoprecipitated with GS beads and blotted with anti-GFP antibody for the presence of Hob3-GFP (left), stripped and blotted with anti-HA antibody to detect HA-cdc42 (right). (**D**) *hob3-GFP* and *hob3Δ* cells expressing *HA-cdc42* were transformed with pREP-*GST-gef1*[+] and grown in the absence of thiamine for 12 h. Cell extracts were subjected to size-exclusion chromatography (Superdex 200) and fractions were analyzed by SDS–PAGE and blotted with the corresponding antibodies. Fractions 21–36 are shown. A portion of GST-Gef1p, Hob3-GFP, and HA-cdc42p co-migrated in 250–300 kDa molecular mass fractions (box). The standard molecular weight proteins and the UV absortion chromatograms of the extracts (*wt* cells darker and *hob3Δ* cells lighter) are shown in the top and bottom panels, respectively.

permissive temperature (25°C) and samples were taken at different time points to observe Hob3-GFP and calcofluor staining. Hob3-GFP appeared in the medial region before the septum was formed (60 min), remained during the ring contraction and septum formation, and disappeared by cell separation (Figure 4B).

Hob3p was also localized as patches at one or both poles in non-septating cells. *cdc10-129* mutant cells expressing *hob3-GFP* were used to see if Hob3-GFP patches accumulate in the single growing pole. Cells were grown at 25°C and shifted to 36.5°C. At this temperature, *cdc10-129* cells arrest in G1 and

grow only through the old tip. In those cells, Hob3-GFP patches accumulate in the growing pole (Figure 4C). We also examined whether Gef1p was required for proper localization of Hob3p, but we did not observe any difference in Hob3-GFP localization in *gef1Δ* cells compared to wild-type cells (Figure 4D). Therefore, Hob3p and Gef1p do not depend on each other to localize correctly.

Hob3p localization is actin dependent
It has been described that the absence of Hob3p causes mislocalization of actin patches (Routhier *et al*, 2001). To see
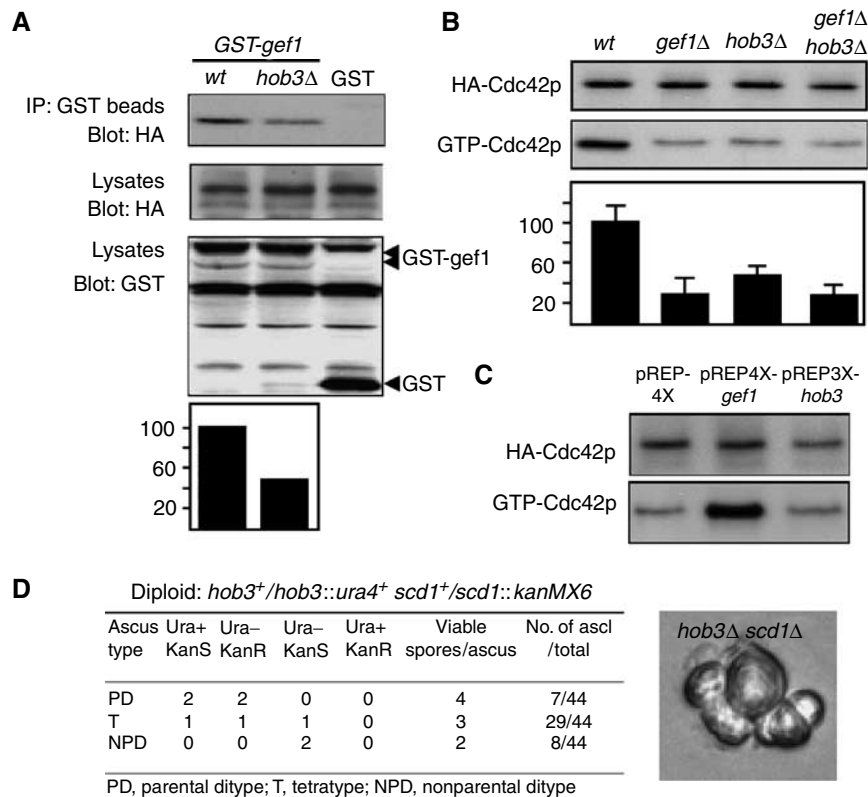
Figure 3 Hob3p is required for Cdc42p activation mediated by Gef1p. (**A**) Extracts from wild-type and *hob3Δ* cells carrying *GST-gef1*[+] and *HA-Cdc42* expressed under their own promoters were pulled down with GS beads and the precipitates were probed with anti-HA antibody. Extracts were assayed for expression of HA-Cdc42 and GST-gef1 by Western blot. Data were quantified and presented as percentage relative to the control extracts run in the same experiment. (**B**) *HA-cdc42*-expressing wild-type, *gef1Δ*, *hob3Δ*, and *hob3Δ gef1Δ* cells were analyzed for total HACdc42p by Western blot using 12CA5 anti-HA monoclonal antibody and for GTP-bound HA-Cdc42p by pulling down the cell extracts with GST-CRIB beads and blotting against 12CA5. Data were quantified and presented as percentage relative to the total HA-Cdc42 controls. (**C**) Wild-type cells transformed with pREP4X, pREP3X-*hob3*, or pREP4X-*gef1*[+] were analyzed for total HA-Cdc42p and for GTP-bound HA-Cdc42p as in (B). (**D**) Tetrad analysis of the diploid strain *hob3*[+]/*hob3*::*ura4*[+] *scd1*[+]/*scd1*::*KanMX6* and a micrograph of *hob3Δscd1Δ* microcolonies formed after spore germination.

whether Hob3p patches contained actin, we examined rhodamine–phalloidin actin staining and Hob3-GFP simultaneously. Hob3-GFP patches overlapped some of those recognized by rhodamine–phalloidin, suggesting that Hob3p localizes to some actin patches at the growth poles (Figure 5A). Hob3-GFP ring also seemed to colocalize with the actin ring (Figure 5B). However, the overlap is not total. It has been described that Gef1p localizes to a contractile ring that resides immediately outside of the contractile actomyosin ring (Coll *et al*, 2003; Hirota *et al*, 2003). It is possible that Hob3p localizes to the same Gef1p ring. We constructed a strain carrying *gef1-GFP* and *hob3-RFP* and, although both proteins are difficult to visualize, the results showed that there was extensive overlapping between Hob3-RFP and Gef1-GFP in a contractile ring during cytokinesis (Figure 5C). Gef1p ring depends on the integrity of the contractile ring and it is dispersed by Latrunculin A treatment (Hirota *et al*, 2003). We examined whether Hob3p localization was also dependent on actin polymerization by promoting the disassembly of actin using 20 μM Latrunculin A. After 5 min with Latrunculin A, most actin was depolymerized, and Hob3-GFP, either from the middle region or from the growing pole patches, was dispersed to a diffuse cytoplasmic fluorescence (Figure 5D). Therefore, Hob3p localization is actin dependent.

Hob3p localization to the division area is dependent on Cdc15p but not on the SIN pathway

To further investigate how Hob3p localization is regulated, we analyzed Hob3-GFP in *cdc15-140* mutant strain. Cdc15p is an FCH domain-containing protein necessary for the actomyosin ring formation (Fankhauser *et al*, 1995). *cdc15-140* cells are impaired in the assembly of the actomyosin ring when grown at 36.5°C (Carnahan and Gould, 2003). Hob3-GFP was not recruited to the medial region in *cdc15-140* cells grown at the restrictive temperature. By contrast, Hob3-GFP patches at the cell poles were still observed at 36.5°C (Figure 6A). When *cdc15-140* cells were switched from 36.5°C to the permissive temperature, Hob3-GFP was observed in the medial region of all septating cells (Figure 6A). These results suggest that the actomyosin ring could serve as a spatial landmark to target Hob3p to the division site.

We also analyzed Hob3-GFP in *cdc11-119* mutant cells to see if Hob3p localization required the SIN, essential for the formation of the division septum after assembly of the actomyosin ring (Wolfe and Gould, 2005). Hob3-GFP localized properly in *cdc11-119* cells grown at the restrictive temperature (Figure 6B). These observations indicate that Hob3p localization is not dependent on the SIN pathway.
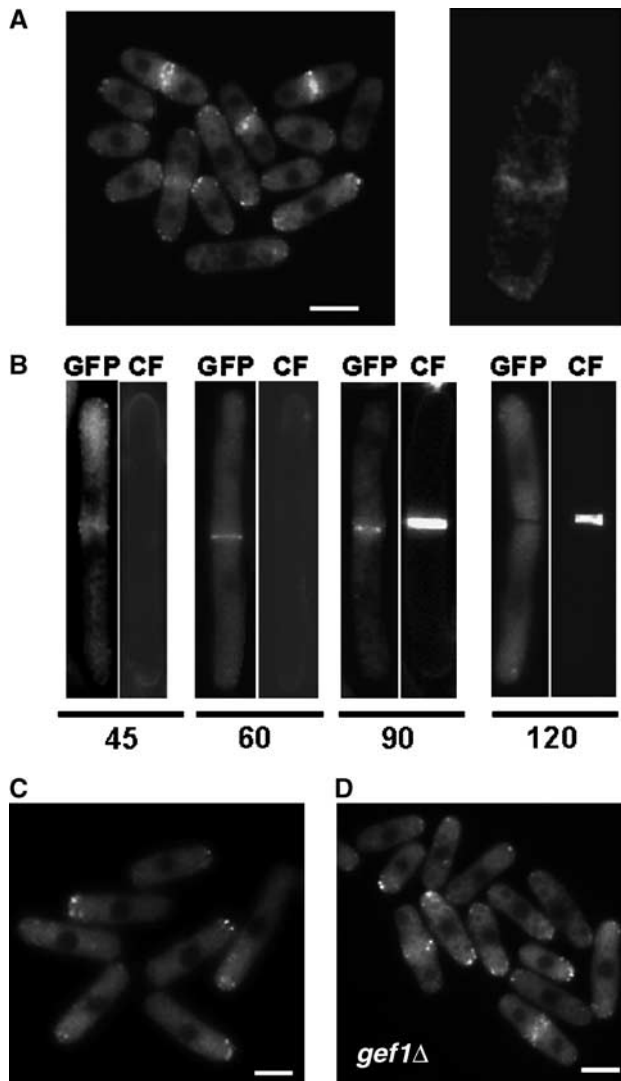
Figure 4 Hob3p localizes to the division area and patches at the growing poles. (**A**) Fluorescence microscopy of *hob3-GFP* cells grown to mid-log phase (left panel) and examined by confocal microscopy (right panel). (**B**) *cdc25-22 hob3-GFP* cells were grown at 25°C to log phase, then shifted to 36.5°C for 4 h to block cells in G_2, and shifted back to 25°C. Cells were taken at different time points, stained with calcofluor, and examined for GFP and calcofluor fluorescence (CF). (**C**) *cdc10-129 hob3-GFP* cells were grown at 25°C to log phase, then shifted to 36.5°C and observed after 4 h. (**D**) *gef1Δ hob3-GFP* cells grown to mid-log phase. The bar corresponds to 5 μm.

Hob3p is required to localize Cdc42p to the division area

It has been speculated that Gef1p and Scd1p may play a key role not only in activating Cdc42p but also recruiting it to the septation site (Hirota *et al*, 2003). As Hob3p is necessary for Gef1p function, we considered the possibility that Hob3p was required to localize Cdc42p to the division area. We constructed a strain carrying endogenously GFP-tagged Cdc42p. Cells with GFP-Cdc42p were viable although slightly misshapen, indicating that GFP-Cdc42p was not completely functional. Endogenous Cdc42p localization was similar to that described using Cdc42p overexpressed from a plasmid (Merla and Johnson, 2000). It localizes to the periphery of the cell and to internal membranes, with a higher concentration at the growth poles and the division area, where it first forms a

ring that closes to form a disc (Figure 7 and Supplementary movie S1). Similar localization was observed in cells lacking either Scd1p or Gef1p, although the GFP-Cdc42 signal in the latter was weaker and non-uniform (Figure 7). By contrast, in cells lacking Hob3p, we could not observe GFP-Cdc42p concentrated at the division area. Cdc42 was still mildly visible at the growth poles (Figure 7 and Supplementary movie S2). *S. pombe* contains another protein from the BAR family, Hob1p, ortholog of human Bin1 (Routhier *et al*, 2003). We examined GFP-Cdc42p localization in *hob1Δ* cells to see if the other protein containing a BAR domain could also regulate Cdc42p. However, GFP-Cdc42p was perfectly localized to the division site in *hob1Δ* cells (Figure 7 and Supplementary movie S3), suggesting that the effect on Cdc42p localization is specific to Hob3p. Western blot analysis of GFP-Cdc42p in total cell extracts showed that the lack of GFP-Cdc42p staining in the septum area of *hob3Δ* cells was not due to a decrease in the total amount of Cdc42p (data not shown). Taken together, these results indicate that Hob3p localizes to the actin ring and is specifically required to localize Cdc42p to the division site.

Hob3p collaborates in the constriction of the actomyosin ring

The main morphologic defect in *hob3Δ* cells was an increase in septating cells and the appearance of some multiseptated cells (Routhier *et al*, 2001). These cytokinesis problems might be due to the mislocalization of Cdc42p. However, a particular role of this essential GTPase in *S. pombe* cytokinesis has not been demonstrated. We therefore asked whether other proteins required for cell separation were properly localized in *hob3Δ* mutant cells. In particular, we analyzed the following: Mid2p, a fission yeast anillin homolog that regulates septin organization (Berlin *et al*, 2003; Tasto *et al*, 2003); Rho4p, a GTPase that regulates septum degradation (Santos *et al*, 2003, 2005); Sec8p and Sec10p from the exocyst complex, involved in the targeting of enzymes responsible for septum cleavage (Wang *et al*, 2002); Eng1p and Agn1p, the glucanases responsible for the degradation of the primary septum and the lateral cell wall respectively (Martin-Cuadrado *et al*, 2003; Garcia *et al*, 2005); and Bgs1p and Bgs4p, identified as putative (1–3)β-D-glucan synthase catalytic subunits responsible for the biosynthesis of the primary and secondary septum, respectively (Cortes *et al*, 2002, 2005). All the proteins analyzed were perfectly localized to the septum area in *hob3Δ* cells (Supplementary Figure 1). Additionally, we analyzed the cell wall composition of cells lacking Hob3p and found no differences with respect to wild-type cells (data not shown). Therefore, we considered the possibility that the cytokinesis defect observed in *hob3Δ* cells could be caused by the absence of Cdc42p in the division area. In budding yeast, Cdc42p is necessary for the correct septin ring organization (Gladfelter *et al*, 2002). Although we did not observe any difference in the Mid2-GFP localization of *hob3Δ* cells, we analyzed localization of GFP-Spn1p in these cells. There was no difference in septin localization with respect to wild-type cells (see Supplementary Figure 2). Additionally, we quantified the phenotypes of *hob3Δ*, *spn3Δ*, and *spn1Δ* together with the double mutants *hob3Δ spn3Δ* and *hob3Δ spn1Δ* at 25 or 36°C. We observed additive effects in the double mutants lacking any of the septins and Hob3p (see Supplementary Figure 2). These results suggest that
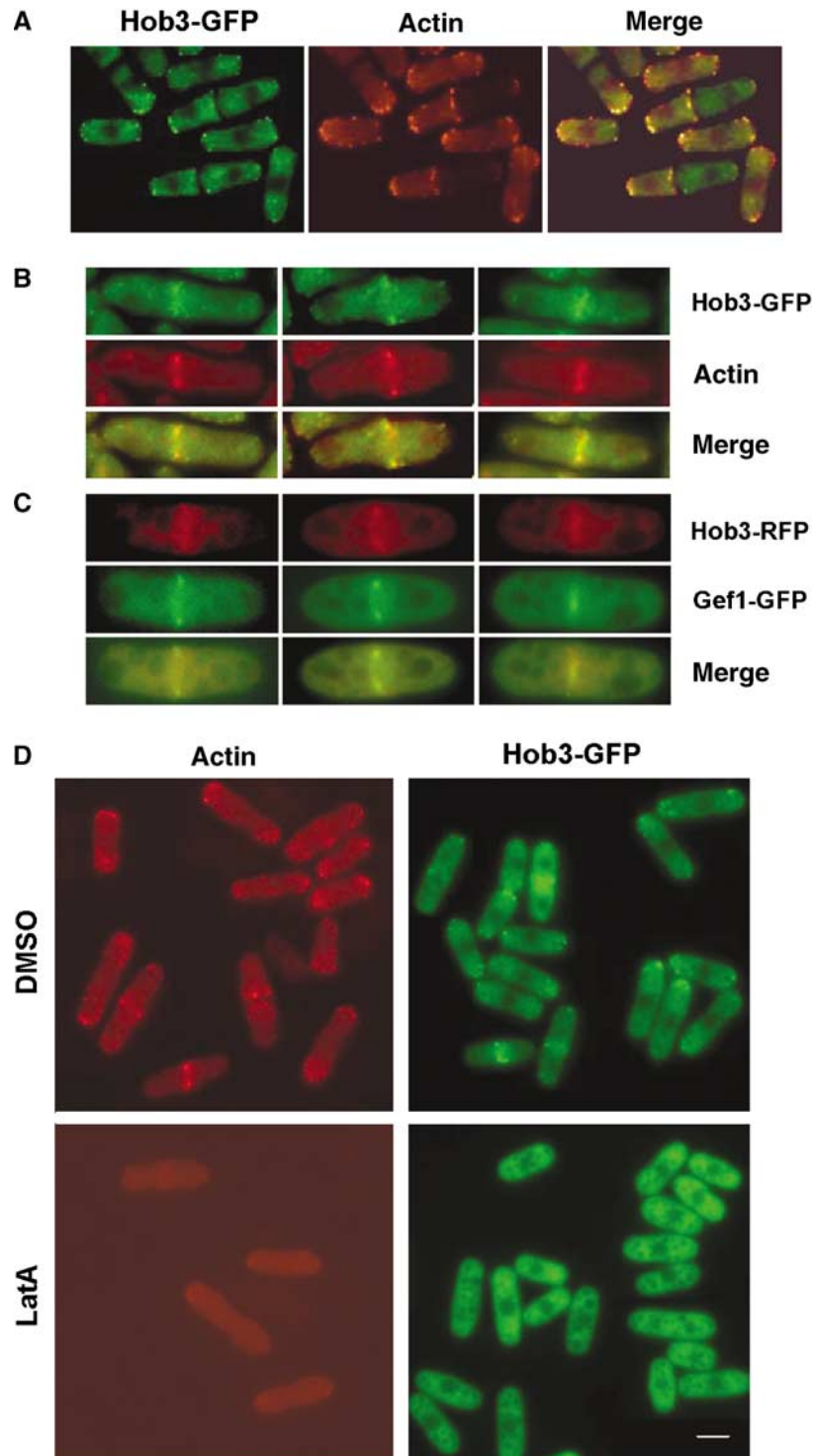
Figure 5 Hob3p localization depends on actin polimerization. (**A**) Colocalization of Hob3p and F-actin. *hob3-GFP* cells, grown to mid-log phase were stained with rhodamine–phalloidin and examined by fluorescence microscopy. (**B**) Three cells are shown in more detail to see the contractile rings. (**C**) Colocalization of Hob3-RFP and Gef1-GFP. Three cells are shown in detail to see the contractile rings. (**D**) Localization of Hob3-GFP depends on F-actin integrity. Fluorescence microscopy of cells grown to mid-log phase and incubated for 5 min in the presence of 1% dimethyl sulfoxide (DMSO) (top) or 20 μM Latrunculin A (LatA) bottom). Cells of the same culture were fixed and stained with rhodamine–phalloidin to observe actin localization. The bar corresponds to 5 μm.

Hob3p plays an additional role to the septins in cell separation during cytokinesis.

Cdc42p causes actin rearrangements in animal cells and in *S. cerevisiae* (Etienne-Manneville, 2004). In budding yeast, Cdc42p recruits and activates the Arp2/3-activating complex formed by Bee1p/Las17p (the ortholog of mammalian WASp) and Vrp1p, which allows the local assembly of actin filaments. It has been proposed that actin polymerization is required for ring assembly and constriction (Pelham and Chang, 2002; Wu *et al*, 2006). We therefore studied if the
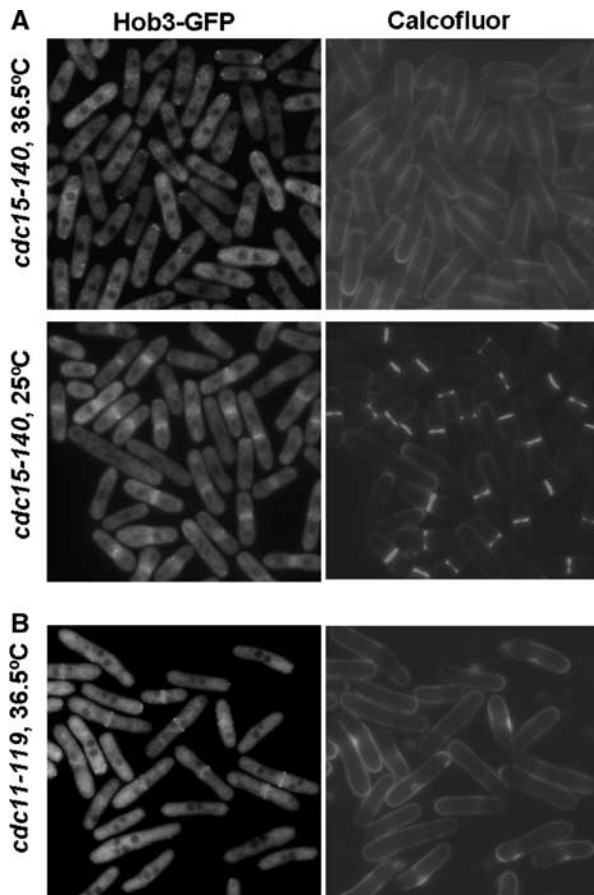
Figure 6 Hob3p localization to the division area depends on the actomyosin ring and is independent of the SIN pathway. (**A**) Fluorescence microscopy of *cdc15-140 hob3-GFP*. Cells were grown at 25°C to log phase, then shifted to 36.5°C for 4 h to block cells at cytokinesis (upper panel) and shifted back to 25°C (lower panel). (**B**) Fluorescence microscopy of *cdc11-119 hob3-GFP* cells grown at 25°C to log phase, then shifted to 36.5°C for 6 h. Cells were stained with calcofluor before observation.

separation defect of *hob3Δ* cells was due to a delay in ring assembly or constriction that could be caused by the lack of Cdc42p. The rates of ring formation and closure in wild-type and *hob3Δ* cells were analyzed by time lapse at 25°C using two GFP-tagged ring proteins, Cdc4p (myosin light chain) and Cdc15p. *hob3Δ* cells assembled the ring, as observed with GFP-cdc4p in cells stained with calcofluor. However, the initiation of ring constriction was delayed, and constriction lasted longer than in wild-type cells (Figure 8A). We also used confocal microscopy in cells carrying both Cdc15-GFP and GFP-tagged histone H3 (Hht2-GFP), to observe simultaneously the nuclear position and the ring constriction. As shown in Figure 8C, the ring closure rate in *hob3Δ* cells was slower than that in the wild-type strain. The constriction rate calculated at 25°C ($n = 10$ cells of each type in three independent experiments) was slower in *hob3Δ* (70 ηm/min) than in wild-type cells (92 ηm/min) (Figure 8D). The total delay accumulated in ring constriction was, approximately, 15 min in each cycle. This delay accumulated during several cycles could cause the increase in septating cells observed in *hob3Δ* cell cultures. A similar phenotype is observed in the *arp2*-1 mutant, where septated cells accumulate when grown at the restrictive temperature (Morrell *et al*, 1999).

Human BIN3 can recover Cdc42 localization and activity in cells lacking Hob3p

It has been described that ectopic overexpression of human Bin3 suppresses cell morphology and actin patch polarization defects due to the absence of Hob3p (Routhier *et al*, 2001). We therefore tested if Bin3 can also complement the *hob3Δ* defects in localization and activation of Cdc42p that might in turn be the cause of the morphologic phenotype observed in these cells.

The localization of endogenous GFP-Cdc42p was examined in *hob3Δ* cells transformed with pREP41-*HA-BIN3*. As shown in Figure 9A, ectopic expression of human Bin3 partially restored the concentration of Cdc42p in the septum area that was lost in *hob3Δ* cells. More important, Bin3 was able to partially recover the levels of active GTP-bound Cdc42p, which were considerably lower in *hob3Δ* cells (Figure 9B). These results suggest that Hob3p function in Cdc42p localization and activation is also conserved in human Bin3.

Discussion

Most Rho GEFs contain a DH responsible for their exchange activity. These DH modules are located upstream of a PH domain, and the lipid-binding properties of this domain play a critical role in the localization of the protein and in the regulation of the DH domain activity (Hoffman and Cerione, 2002). In *S. pombe*, the Cdc42-GEF Scd1p contains the tandem DH–PH domains. In its signaling pathway, the SH3-containing protein, Scd2p, acts as a scaffold upon which Cdc42p, Scd1p, and the p21-activated kinase Shk1p assemble to regulate the apical growth and the mating response (Chang *et al*, 1999). Interestingly, Gef1p, the other fission yeast Cdc42-GEF, does not have a PH domain downstream of the DH domain, and does not interact with Scd2p (Hirota *et al*, 2003; our unpublished results). Instead, Gef1p interacts with Hob3p through the C-terminal region, downstream of the DH domain. Hob3p is structurally similar to *S. cerevisiae* Rvs161p and human Bin3 (Routhier *et al*, 2001). All of them are the simplest members of the BAR domain-containing protein family, containing a single BAR domain (Habermann, 2004). A possible function of this BAR protein could be to help in Gef1p localization, acting like a PH domain. However, Gef1p localization is not altered in *hob3Δ* cells. We therefore considered the possibility that Hob3p was required for the interaction of Gef1p and Cdc42p and analyzed whether Hob3p was interacting with Cdc42p, which was the case. Cdc42–Hob3 interaction was independent of Gef1p, and independent of the Cdc42p activation state, indicating that Hob3p was not a Cdc42p effector. We have also shown that the three molecules Gef1p, Hob3p, and Cdc42p form a complex that could be detected by co-immunoprecipitation.

Two possible roles for Hob3p, not mutually exclusive, arise from these results: (a) Hob3p is required to localize Cdc42 to the division area where it can be activated by Gef1p and (b) Hob3p is required for Gef1p–Cdc42p interaction and activation. The two roles are supported by several facts: (1) the level of Gef1p–Cdc42p co-immunoprecipitated decreased around 50% in the absence of Hob3p; (2) the level of active GTP-bound Cdc42p is considerably lower in *hob3Δ* cells, although Hob3p is not a GEF; (3) the decrease in GTP-bound Cdc42p caused by the simultaneous lack of Gef1p
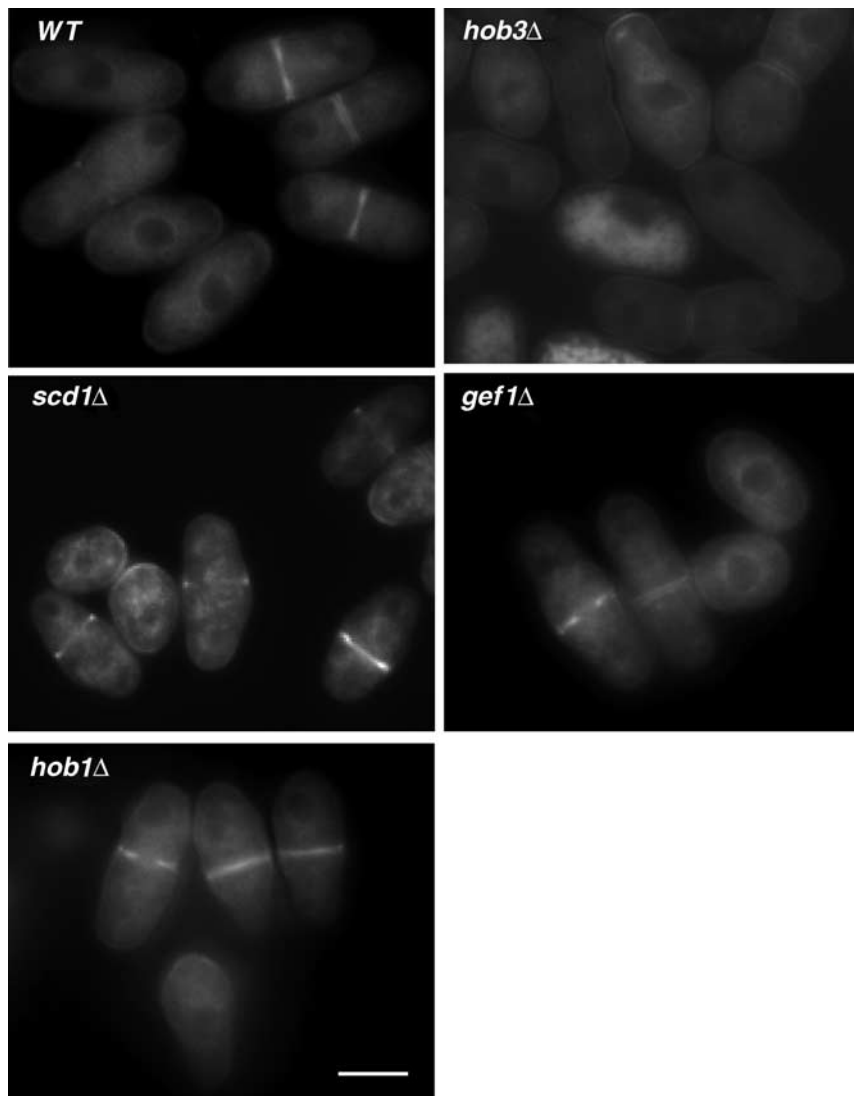
Figure 7 Hob3p is required for Cdc42p localization to the division area. GFP fluorescence micrographs of wild-type, *hob3Δ*, *scd1Δ*, *gef1Δ*, and *hob1Δ* cells with the endogenous *cdc42* gene tagged with *GFP*. The bar corresponds to 5 µm.

and Hob3p is similar to that caused by the lack of Gef1p alone; (4) a *hob3Δscd1Δ* double mutant strain, like a *gef1Δscd1Δ* strain, is not viable. Altogether, these results suggest that Hob3p is required for the activity of Gef1p. In mammalian cells, Tuba is a Cdc42-GEF with a DH domain upstream of a BAR domain that is required for the activation of Cdc42p (Salazar *et al*, 2003). This BAR may functionally replace the PH domain that typically follows a DH domain. Gef1p–Hob3p together could be similar to the protein Tuba in mammalian cells: Gef1p would bind and activate Cdc42p with the help of Hob3p. Interestingly, Scd2p acts as a Cdc42p adaptor but only binds activated Cdc42-GTP (Endo *et al*, 2003), suggesting that Scd2p might be important for the interaction of Cdc42p and some possible effectors but not for its activation. In fact, the double mutant strain *gef1Δscd2Δ* or *hob3Δscd2Δ* is not lethal (our unpublished results), corroborating that Scd2p is not required for the Scd1p-mediated Cdc42p activation.

The possible role of Hob3p as a protein required to localize Cdc42p to the division area would also imply that Hob3p is necessary for the function of Gef1p, as this GEF mainly

localizes to the division area. It has been speculated that Gef1p and Scd1p may play a key role not only in activating Cdc42p but also in recruiting this GTPase to the septation site (Hirota *et al*, 2003). However, our results suggest that neither Gef1p nor Scd1p is required to localize Cdc42p to the septum, and that it is Hob3p that recruits this GTPase to the division area. In *S. cerevisiae*, the spatial control of Cdc42p activity is not only achieved through GEF recruitment but also by recruitment of itself to the site of polarized growth (Wedlich-Soldner *et al*, 2003). Localization of GFP-Cdc42p fusion proteins showed that, during cytokinesis, Cdc42p clusters at the division region before actomyosin ring contraction in *S. cerevisiae* (Richman *et al*, 2002). Although *S. pombe* Cdc42p mainly localizes to the division site, a particular Cdc42p role in cytokinesis has not been shown. Hob3p participates in cytokinesis, and our results suggest that it might act in this process by recruiting Cdc42p, thus providing a novel mechanism for the spatial regulation of Cdc42p signaling pathway. The fact that the cytokinesis defect of *hob3Δ* cells is more severe than that of *gef1Δ* cells suggests that Scd1p can substitute for Gef1p and might also activate
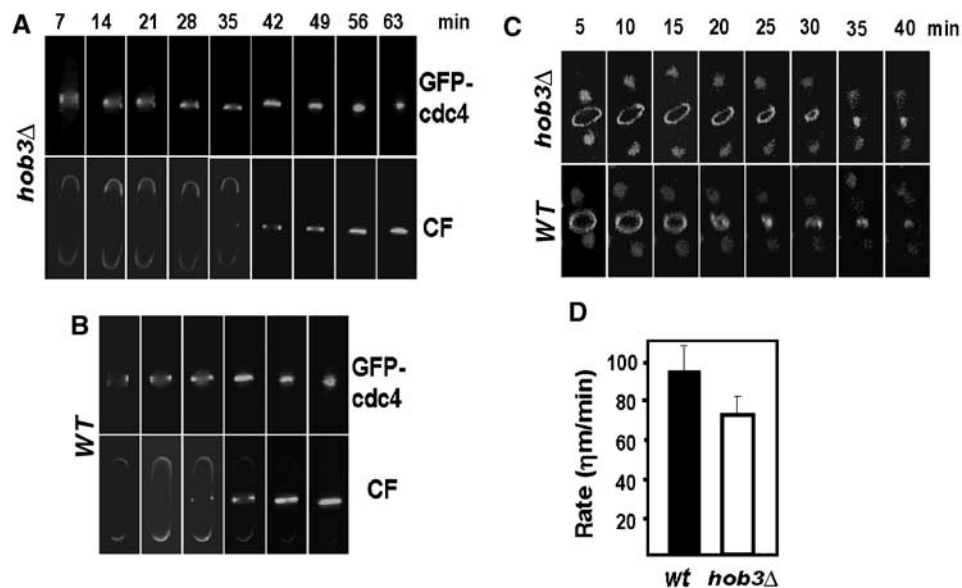
Figure 8 Ring constriction during cytokinesis is delayed in *hob3*Δ cells. Analysis of contractile ring closure at 25°C. Time-lapse fluorescence microscopy of GFP-cdc4 in (**A**) *hob3*Δ and (**B**) wild type cells stained with calcofluor. (**C**) Time-lapse images of Hht2-GFP and GFP-cdc15 in wild-type and *hob3*Δ cells examined by confocal microscopy. (**D**) Rate of ring constriction at 25°C of wild-type ($n = 10$) and *hob3*Δ cells ($n = 10$) calculated from the time-lapse confocal images in three independent experiments.
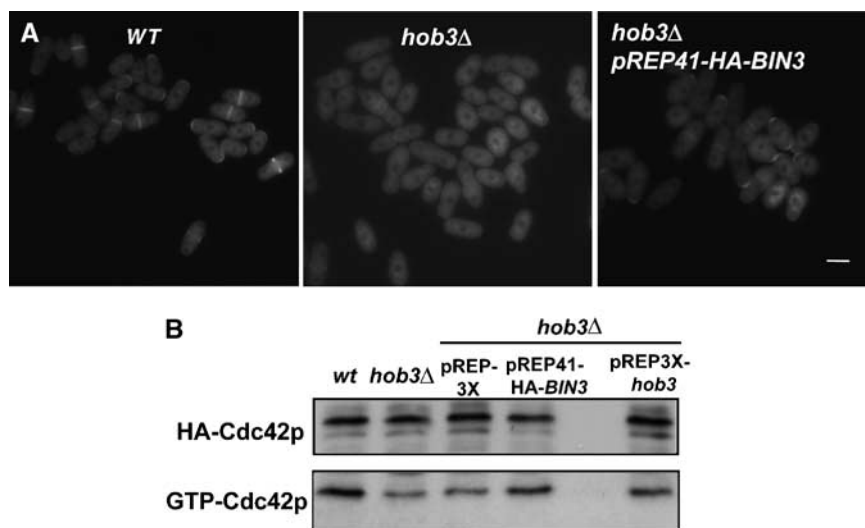

Figure 9 Bin3 can rescue the Cdc42p localization to the division area and activation in cells lacking Hob3p. (**A**) GFP fluorescence micrographs of wild-type, *hob3*Δ, and pREP41-*HA-BIN3*-transformed *hob3*Δ cells carrying the endogenous *cdc42* gene tagged with *GFP*. The bar corresponds to 5 μm. (**B**) *HA-cdc42* expressing wild-type, *hob3*Δ, and *hob3*Δ cells transformed with pREP3X, pREP41-*HA-BIN3*, or pREP3X-*hob3* were analyzed for total HACdc42p by Western blot using 12CA5 anti-HA monoclonal antibody and for GTP-bound HACdc42p by pulling down the cell extracts with GST-CRIB beads and blotting against 12CA5.

Cdc42p during cytokinesis (Hirota *et al*, 2003), or that Hob3p is playing additional roles in cytokinesis besides recruiting Cdc42p. Other molecules participating in cell separation are perfectly localized in *hob3*Δ cells, and the cell wall has no differences in composition with respect to the wild type (data not shown). Therefore, one obvious hypothesis is that the absence of Cdc42p in *hob3*Δ cells causes the cytokinesis defect. Interestingly, Hob1p, the other *S. pombe* BAR domain-containing protein, localizes to actin patches at the cell ends during growth and to the medial site during cell division (Huang *et al*, 2005). However, *hob1*+ deletion failed to implicate Hob1p in cytokinesis (Routhier *et al*, 2003). Additionally, we have shown that Cdc42p localized to the

medial region in cells lacking Hob1p. Therefore, the role of Hob3p regarding Cdc42p localization seems to be specific to this protein.

How does Cdc42p participate in cytokinesis? One possibility would be that Cdc42p organizes the septin cytoskeleton into a ring, as in *S. cerevisiae* (Gladfelter *et al*, 2002). *S. pombe* septin ring is required for cell separation. However, Mid2p or the septin Spn1p localization in *hob3*Δ cells is not altered. Moreover, the phenotype of mutants lacking any of the septins and Hob3p is more severe than that of the single mutants, suggesting that they play different roles in cytokinesis. Another possibility would be that Cdc42p participates in the actin polymerization needed for the assembly and/or

closure of the contractile ring. Thus, the absence of Cdc42p could be causing the slower ring closure observed in cells lacking Hob3p, and the increase in the time cells spend in cytokinesis could raise the percentage of septating cells in *hob3*Δ cultures. In *S. cerevisiae*, Cdc42p regulates the proper spatio-temporal activation of the Arp2/3 complex, which regulates actin polymerization (Lechler *et al*, 2001). Cdc42p also activates Arp2/3 in animal cells via the Wiskott–Aldrich syndrome proteins (WASPs) (Pollard and Borisy, 2003). These targets of Cdc42p mediate most of its cytoskeletal effects (Bompard and Caron, 2004). In *S. pombe*, Wsp1p (a WASP homolog) has been implicated in the regulation of F-actin polymerization, and *wsp1*Δ cultures have an increased percentage of septating cells (Lee *et al*, 2000). *arp2/3* mutant strains also have an increased percentage of septating cells at the restrictive temperature (Morrell *et al*, 1999). Previous works have shown evidence that Arp2/3 complex participates in the assembly of contractile ring filaments (Pelham and Chang 2002; Carnahan and Gould, 2003). However, more recent experiments show that the Arp2/3 complex and its activators do not contribute to the assembly of the contractile ring but participate in the initiation of ring constriction, the rate of ring constriction, the timing of septum formation, the rate of ring disassembly, and the timing of cell separation. All these processes were delayed slightly in strains lacking Myo1p or Wsp1p or in the cold-sensitive *arp1-c1* mutant (Wu *et al*, 2006). The lack of Hob3p causes delay in the same processes, suggesting that Hob3p, and probably Cdc42p, participates in cytokinesis through the activation of Arp2/3 actin polymerization.

Human Bin3 is a widely expressed BAR protein of unknown function, structurally similar to Hob3p and *S. cerevisiae* Rvs161p. Interestingly, Bin3 but not Rsv161p expression completely complements the morphological defects of *hob3*Δ cells (Routhier *et al*, 2003), and we have shown here that Bin3 is able to partially relocalize Cdc42p to the division area and increase its activity in those cells, suggesting that the Hob3p function is fully conserved in eukaryotes. Does Bin3 function as a Cdc42p adaptor for cytokinesis in human cells? Clearly, future studies are required to characterize the possible role of Bin3 and Cdc42p in the mammalian cell cytokinesis.

Materials and methods

Fission yeast strains, media, and techniques
Standard *S. pombe* media and genetic manipulations were employed (Moreno *et al*, 1991). All the strains used were isogenic to wild-type strains *972 h⁻* and *975 h⁺*, and are described in Supplementary Table I. The strains were constructed by either random spore germination method or tetrad dissection. Cells were usually grown in rich medium (YES) or minimal medium (EMM) supplemented with the necessary requirements.

Plasmids and strain construction
The *nmt* promoter-containing vectors pREP3X, pREP4X, and pREP1-GST (Forsburg, 1993) were used for the overexpression of *gef1⁺* or *hob3⁺*. For the two-hybrid analysis, the *gef1⁺* ORF was cloned into the NcoI–BamHI sites of pAS2 and used as bait to screen an *S. pombe* cDNA library constructed in the pACT plasmid (generous gift from Dr SJ Elledge). *S. cerevisiae* Y190 (*MATa gal4 gal80 his3 trp1-901 ura3-52 leu2-3,-112 URA3::GAL−<lacZ, lys2::GAL (UAS)−<HIS3 cyhʳ*) cells were transformed and grown on plates without leucine, tryptophan, and histidine, and supplemented with 40 mM 3-aminotriazole. β-Galactosidase activity was analyzed in the transformant colonies as described (Coll *et al*,

2003). To map the interaction between Gef1p and Hob3p, different *gef1⁺* fragments were cloned into the pACT2 plasmid.

To delete *hob3⁺*, *hob1⁺*, and *scd1⁺* from the *S. pombe* genome, the whole ORF was replaced with the *KanMX6* gene or the *ura4⁺* gene by PCR-based gene targeting as described (Bähler *et al*, 1998). Stable transformants were selected and sporulated. Dissected tetrads were screened by PCR or Southern blot for the appropriate gene replacement. A genomic version of *hob3⁺* with the *GFP*, the *GST*, the *mRFP*, or the *Myc* epitope coding sequences fused at the end of the ORF was generated as described (Bähler *et al*, 1998). A genomic version of *cdc42⁺* with the *GFP* or the HA epitope coding sequences fused at the beginning of the ORF was generated by inserting in a Bluescript plasmid 500 bp of the 5′ *cdc42* flanking sequences, the *GFP* or *HA* sequence, the *cdc42* ORF, the *KanMX6* gene, and 500 bp of the 3′ *cdc42* flanking sequence. The insert was transformed into a homozygous *leu1-32, ura4-D18* diploid strain and stable transformants were selected and screened by PCR for the appropriate gene replacement.

The entire *gef1⁺* sequence was fused in-frame to the 3′-end of the *GST* sequence in the pREP-GST vector. Similarly, *cdc42⁺*, *cdc42G12V*, and *cdc42T17N* were fused in-frame to the 3′-end of HA epitope coding sequence in the pREP42-HA-N vector. Expression of the proteins was induced by growing the cells transformed with these plasmids in the absence of thiamine for 12 h. The entire *gef1-GST* and *gef1-HA* fusions were cloned under the control of the endogenous *gef1⁺* promoter using the integrative plasmid pJK148 in a *gef1*Δ strain. The entire *BIN3* sequence was amplified from a human cDNA library using the appropriate primers, sequenced, and cloned into pREP41-HA-N vector.

Immunoprecipitation
Extracts from 1×10^8 cells expressing the different tagged proteins were obtained as described (Arellano *et al*, 1997), using 200 µl of lysis buffer (20 mM Tris–HCl pH 8.2, 2 mM EDTA, 137 mM NaCl, 0.5% NP-40, 10% glycerol), containing 100 µM *p*-aminophenyl methanesulfonyl fluoride, leupeptin, and aprotinin). The cell extracts were incubated with glutathione beads for 2 h at 4°C. The beads were washed four times with lysis buffer and resuspended in sample buffer. Proteins were separated by SDS–PAGE, transferred to an Immobilon-P membrane (Millipore Corp.), and blotted to detect GST-, HA-, GFP-, or Myc-fused epitopes with the corresponding antibodies and the ECL detection kit (Amersham Corp). Total amounts of proteins were monitored in cell extract aliquots (30 µg of total protein) used directly for Western blot.

Gel filtration
Gel filtration was performed as described (Edwards *et al*, 1999) except that Superdex 200 GL 10/300 column was used. Extracts were prepared as described (Arellano *et al*, 1997) from 2×10^9 cells in 50 mM Na-phosphate buffer pH 7 carrying 150 mM NaCl, 0.1% NP-40 (Sigma), 10% glycerol, 0.5 mM dithiothreitol, 5 mM EGTA, 60 mM β-glycerophosphate, 0.1 mM NaF, 1 mM *p*-aminophenyl methanesulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). The column was equilibrated with two column volumes (CV = 24 ml) of water and two volumes of 50 mM Na-phosphate buffer pH 7 carrying 150 mM NaCl, 0.1% NP-40, 60 mM β-glycerophosphate, and 0.1 mM NaF. The column was calibrated using the standard protein mixture HMW kit (Amersham Corp). A 1.5 mg portion of total protein in 100 µl volume was injected and the column flow rate was 0.4 ml/min. Fractions (0.5 ml) were collected and their UV absortion was recorded. Fractions 18–36 were TCA (20%) precipitated, washed with acetone, and analyzed by Western blot as described for immunoprecipitation.

In vivo analysis of GEF activity
The expression vector pGEX-CRIB (PAK-Cdc42 binding domain) (Manser *et al*, 1998) was used to transform *Escherichia coli*. The fusion proteins was produced according to the manufacturer's instructions and immobilized on glutathione-Sepharose 4B beads (Pharmacia).

The amount of GTP-bound Cdc42p was determined using a pull-down assay as described (Coll *et al*, 2003). Briefly, extracts from wild-type, *gef1*Δ, or *hob3*Δ strains carrying endogenously expressed *HA-cdc42* were obtained as described (Arellano *et al*, 1997), using 500 µl of lysis buffer (50 mM Tris pH 7.5, 20 mM NaCl, 0.5% NP-40, 10% glycerol, 0.1 mM DTT, 1 mM NaF, 2 mM Cl₂Mg, containing 100 µM *p*-aminophenyl methanesulfonyl fluoride, leupeptin, and

aprotinin). A 2 mg portion of the cell extracts was incubated with 10 µg of GST-CRIB protein coupled to glutathione-Sepharose beads for 2 h, washed four times, and blotted as described with 12C5A mAb. Total HA-Cdc42p levels were monitored in whole-cell extracts (30 µg of total protein) that were used directly for Western blot.

Microscopy techniques

For calcofluor staining, exponentially growing *S. pombe* cells were harvested, washed, and resuspended in water with calcofluor at a final concentration of 0.1 mg/ml for 5 min at room temperature. After washing with water, cells were observed. Actin staining was performed with rhodamine–phalloidin. To disintegrate F-actin, Latrunculin A dissolved in DMSO at 20 mM was added to *S. pombe* cultures to a final concentration of 20 µM.

Cell samples were observed using a Leica DMRXA microscope equipped for Nomarski optics and epifluorescence, and photographed with a Photometrics Sensys CCD camera. Confocal microscopy was performed on a Leica TCS SL spectral confocal microscope and the images were analyzed with the Leica Confocal Software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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