Nak1 interacts with Hob1 and Wsp1 to regulate cell growth and polarity in *Schizosaccharomyces pombe*

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Summary

We have previously reported that Nak1, a group-II germinal center (GC) kinase, is essential for polarized growth in *Schizosaccharomyces pombe*. Here, we provide evidence that Nak1 regulates cell growth and polarity, in part, through its interactions with Hob1 (an Rvs167/amphiphysin homolog) and Wsp1 (Wiskott-Aldrich-syndrome-protein homolog). We found that Nak1, Hob1 and Wsp1 interact physically, and that both Hob1/green-fluorescent-protein (Hob1-GFP) and Wsp1-GFP fusion proteins localized to F-actin patches at growing cell ends and medial division sites. Hob1-GFP was dissociated from patches in cells lacking Wsp1. Also, Hob1 overexpression dissociated Wsp1-GFP from foci, inhibited Wsp1-directed F-actin formation in vitro and partially restored polarity defects associated with Wsp1 overexpression or *nak1* repression. Furthermore, loss of both Wsp1 and Hob1 resulted in rounded cells, slow growth and multiple septae. Together, these observations suggest that Hob1 and Wsp1 cooperate to mediate cell polarity, growth and division. Repression of *nak1* resulted in a random redistribution of Hob1-GFP and Wsp1-GFP foci, and inhibition of Wsp1-directed F-actin formation in vitro. Furthermore, *hob1*Δ and *wsp1*Δ mutants exhibited synthetic growth defects in combination with *nak1* repression, suggesting that Nak1 has redundant functions with Hob1 and Wsp1. Collectively, our results suggest that Nak1 both regulates and cooperates with Hob1 and Wsp1 to promote F-actin formation and polarized cell growth.

Key words: GC kinase, Actin, Cytoskeleton, Rvs167, Amphiphysin, Wiskott-Aldrich-syndrome protein

Introduction

The establishment of cell polarity is fundamental to many cellular processes, including cell morphogenesis, polarized vesicular transport, cell division and motility. In budding yeast, bud emergence is guided by cortical cues established during previous budding events, which recruit Cdc42 to the bud site (Zheng et al., 1995; Chant, 1999; Park et al., 1999). As a key mediator of cell polarity, Cdc42 regulates F-actin formation at polarized sites of growth. Cdc42 might bind directly to formins, which stimulate the formation of unbranched actin filaments comprising actin cables (Evangelista et al., 1997; Evangelista et al., 2002; Pruyne et al., 2002). Formins are also required for Cdc42-mediated recruitment of the Arp2/3 activator Bee1/Las17 [a Wiskott-Aldrich-syndrome protein (WASP) homolog] to polarized sites (Lechler et al., 2001). Cdc42 also binds and activates the p21-activated kinases (PAKs), which phosphorylate and activate the type-I myosins Myo3 and Myo5, which then activate the Arp2/3 complex (Evangelista et al., 2000; Lechler et al., 2001). The activated Arp2/3 complex nucleates and caps actin filaments, enabling the construction of filament networks such as those comprising actin patches (Mullins et al., 1998a; Mullins et al., 1998b; Svitkina and Borisy, 1999). Cortical actin patches are often associated with actin cables near sites of growth and appear to be involved in endocytosis (Pruyne and Bretscher, 2000). The polarized organization of actin cables directs vesicle traffic, which delivers components necessary for growth to emerging buds.

In fission yeast, growth is restricted to the cell ends, producing an elongated, cylindrical cell morphology. Several *Schizosaccharomyces pombe* polarity mutants result in either asymmetric growth (*for3*, *bud6*, *tea1*, *pom1*) or rounded morphology (*cdc42*, *orb2/pak1/shk1*, *orb5*, *orb6*, *mor2*, *scd1*, *nak1*) (Miller and Johnson, 1994; Chang et al., 1994; Verde et al., 1995; Mata and Nurse, 1997; Bahler and Pringle, 1998; Yang et al., 1998; Glynn et al., 2001; Feierbach and Chang, 2001; Hirata et al., 2002; Huang et al., 2003). Although it is not clear how these factors mediate polarity, several of these mutants exhibit actin cytoskeleton defects. The mechanisms that regulate actin structures in fission yeast are not well characterized, but Wsp1 (a Bee1/WASP homolog) and Myo1 (a type-I myosin) have been implicated to regulate F-actin polymerization (Lee et al., 2000; Pelham and Chang, 2002).

Rvs167 and Rvs161, the budding yeast amphiphysin homologs, have been implicated in actin cytoskeleton organization and endocytosis. Rvs167 physically interacts with Rvs161 (Navarro et al., 1997), actin (Act1) (Amberg et al., 1995), Abp1 (Lila and Drubin, 1997), Bee1/Las17 and the Pcl1 and Pcl2 cyclins (Lee et al., 1998; Colwill et al., 1999), and localizes to actin patches (Balguerie et al., 1999). Also, *rvs* mutants exhibit defects in actin organization and endocytosis (Crouzet et al., 1991; Bauer et al., 1993; Durrens et al., 1995; Munn et al., 1995; Sivadon et al., 1995; Dorer et al., 1997; Lee et al., 1998; Lombardi and Riezman, 2001) and genetically interact with other genes linked to the actin cytoskeleton and

vesicular transport (Munn et al., 1995; Revardel et al., 1995; Dorer et al., 1997; Lila and Drubin, 1997; Singer-Kruger and Ferro-Novick, 1997; Breton and Aigle, 1998; Breton et al., 2001). However, deletion of the fission yeast *RVS167* homolog *hob1* failed to implicate Hob1 in cytoskeletal regulation (Routhier et al., 2003).

Five PAK-related kinases have been identified in *S. pombe*: Shk1/Pak1, Shk2/Pak2 and the three germinal-center kinases (GCKs) Sid1, Nak1 and an uncharacterized kinase. Shk1 and Shk2 are activated by Cdc42 and act somewhat redundantly in mediating cell polarity (Marcus et al., 1995; Ottilie et al., 1995; Yang et al., 1998; Sells et al., 1998). By contrast, Sid1 mediates septation during cell division (Balasubramanian et al., 1998; Guertin et al., 2000). We previously reported that Nak1 is essential for cell growth and polarity, and that repression of *nak1* expression resulted in abnormal round morphology, disorganized actin foci, slow growth and cell-division defects (Huang et al., 2003). Here, we provide evidence that Hob1 and Wsp1 are involved in determining cell polarity, and that Nak1 can form complexes with these proteins and is required for their proper localization and function at growing cell ends.

Materials and Methods

Yeast strains and methods

Genotypes of *S. pombe* strains used in this study are listed in Table 1. Methods for yeast culture and genetic analyses were undertaken as described previously (Moreno et al., 1991).

Yeast two-hybrid screen

The *Saccharomyces cerevisiae* L40 (*MATα his3 trip1 leu2 ade2 LYS2::lexA-HIS3 URA3::lexA-lacZ*) strain harboring pLexA-Nak1$^{554\text{-}652}$ was used to screen a pGAD-GH cDNA library (a gift from G. Hannon and D. Beach, Cold Spring Harbor Laboratory, NY) for interacting proteins by the yeast two-hybrid method (Loewith et al., 2000).

Plasmids

Expression plasmids were constructed using the plasmids pAALNHA, pREP3× and pREP4×. pAALNHA contains *LEU2*, *ars1* and the *adh1* promoter sequence flanking a hemagglutinin (HA)-epitope-coding sequence and a polylinker site (Huang et al., 2003). pREP3× and pREP4× contain the strong thiamine-repressible *nmt1* promoter (Forsburg, 1993). pAALNHA-Nak1 has been previously described (Huang et al., 2003). pAALNHA-Hob1, pAALNHA-Hob1$^{1\text{-}281}$ and pAALNHA-Hob1$^{275\text{-}466}$ were constructed by insertion of Hob1-encoding fragments into the *Spe*I/*Bam*HI sites of pAALNHA. pAALNHA-Nak1^{T171A} and pAALNHA-Nak1$^{1\text{-}562}$ have been previously described (Huang et al., 2003). pAALNC-GFP [which encodes green fluorescent protein (GFP)] was constructed by the insertion of a *Xho*I/*Bgl*II fragment encoding GFP (amplified from pREP3×GFPHA) into the *Xho*I/*Bam*HI sites of pAALNHA, replacing the HA-epitope-encoding fragment. pAALNeGFP was constructed by insertion of the amplified fragment encoding GFP into *Xho*I/*Bam*HI sites of pAALNHA. pAALN-Hob1-GFP was constructed by insertion of a fragment encoding Hob1 into the *Spe*I/*Bgl*II sites of pAALNC-GFP. pREP4×Myc was constructed by replacing the *Xho*I/*Spe*I HA-encoding fragment from pREP4×HA with an *Xho*I/*Spe*I fragment (5′-CTCGAGATGGAACAAAAGCTTATTTCTGAAGAAGACTTGGG-CGAGCAGAAACTAATAAGCGAGGAGGATCTGGGAGGACCG-TCGACAACTAGT-3′) encoding two tandem copies of the Myc epitope. pREP4×Myc-Hob1, pREP4×Myc-Hob1$^{1\text{-}281}$, pREP4×Myc-Nak1 and pREP4×Myc-Nak1$^{1\text{-}562}$ were constructed by inserting amplified *Spe*I/*Bgl*II fragments encoding regions of Nak1 or Hob1 into the pREP4×Myc *Spe*I/*Bam*HI sites. pLexA-Nak1$^{554\text{-}652}$ was constructed by inserting an amplified 299 bp fragment encoding the Nak1 C-terminal region (CTR) into the pBTM116 *Bam*HI/*Pst*I sites (Bartel et al., 1993). Other pLexA fusion plasmids listed in Table 2 were constructed by inserting amplified *Eco*RI/*Bgl*II fragments in frame into the pBTM116 *Eco*RI/*Bam*HI sites. VP16 fusion plasmids were constructed by inserting corresponding amplified fragments in frame into pVP16 *Bam*HI/*Not*I sites (Shih et al., 1996).

pAALNC-GFP-Wsp1 was constructed by amplification of the *wsp1* open reading frame from genomic DNA, and insertion into pAALNC-GFP *Spe*I/*Bam*HI sites. pAALNHA-Nak1^{T171A}/Wsp1-GFP was constructed by inserting an *Xma*I Wsp1-GFP-encoding fragment amplified from pAALNC-GFP-Wsp1 into the pAALNHA-Nak1^{T171A}/mycHob1 *Xma*I sites, thereby replacing the mycHob1 coding sequence in the proper orientation behind the *hob1* promoter. pREP3×WspHA and pREP4×MycWsp1 were constructed by insertion of the amplified Wsp1-coding fragment into *Spe*I/*Bam*HI sites in pREP3×C-HA and pREP4×Myc. pREP3×C-His$_6$ was constructed by amplification of a His$_6$-coding fragment from the pET33b vector (Stratagene) using 5′-GATCCTCGAGACTAGTAGC-GGCCGCAGGATCCGGAGGAATGGGCAGCAGCCATCATCAT-CATCATCACTGA-3′ and 5′-GATCAGATCTTCAGTGATGATGA-TGATGATGGC-3′ primers, and insertion into pREP3×HA *Xho*I/*Bam*HI sites. pREP3×C-HAHis$_6$ was constructed by the insertion of the *Spe*I/*Bgl*II HA-epitope-encoding fragment, amplified using 5′-GATCCTCGAGACTAGTAGCGGCCGCAGGATCCGGAGGAATG-TATCCTTATGACGTGCCTGAC-3′ and 5′-GATCAGATCTTGTCG-ACGGTCCTCCCAGGCTG-3′ primers, into pREP3×C-His6 *Spe*I/*Bam*HI sites. pREP3×-WspHAHis$_6$ was constructed by the insertion of the Wsp1-encoding fragment described above into pREP3×C-HAHis$_6$ *Spe*I/*Bam*HI sites. pBTMWsp1 was constructed by the insertion of the *Wsp1* cDNA, amplified from a *S. pombe* cDNA library, into pBTM116 *Eco*RI/*Bam*HI sites. pAALNGFPHA-Atb2 (Huang et al., 2003), encoding GFP-Atb2p (α tubulin 2) was used to visualize microtubule distribution.

hob1 and *wsp1* gene replacement

A 1.8 kb *Hin*dIII fragment containing *ura4* was used to replace a 1872 bp *Hin*dIII fragment containing the *hob1* open reading frame in pGEM-RKO. The 3651 bp *Sph*I-*Not*I fragment from pGEM-RKO was used to replace the endogenous *hob1* gene in SP826 by homologous recombination. Tetrad dissections of h^{90}/h^{+} revertants were performed to obtain the *hob1::ura4* RKO5B and RKO5N strains, and Southern-blot analysis was performed to confirm disruption of the *hob1* locus.

The *wsp1* gene was disrupted with *ura4* by a similar method previously described (Lee et al., 2000). The *ura4*-containing fragment was inserted into pREP3×-WspHA *Bgl*II sites, replacing a 48 bp fragment. A *Spe*I/*Bam*HI fragment containing the *wsp1* integration cassette was used to replace the endogenous *wsp1* gene in the SP826 diploid strain by homologous recombination. Tetrad dissections of h^{90}/h^{+} revertants were performed to obtain the *wsp1*Δ strain WSP3Δ-12b and Southern-blot analysis was performed to confirm disruption of *wsp1*.

wsp1 promoter replacement

The endogenous *wsp1* promoter was replaced with the attenuated thiamine-repressible *nmt1* promoter from pREP81× as follows. A *Kpn*I/*Bam*HI fragment encoding Wsp1 was inserted into pGEM-nmt-nak1 (Huang et al., 2003), producing pGEM-3′nmt-Wsp. The 5′ Wsp1 promoter region was amplified and ligated into *Xho*I/*Nhe*I sites in pGEM-3′nmt-Wsp to produce pGEM-nmt-Wsp1. *Xho*I/*Bgl*II digestion of this construct liberated a *ura4-nmt1-wsp1* integration cassette, which was transformed into the SP200 strain. Proper

Table 1. *S. pombe* strains

Strain	Genotype	Source/reference
SP826	*h⁺/h⁺ leu1-32/leu1-32 ura4-d18/ura4-d18 ade6-M210/ade6-M216*	David Beach*
RL143	*h⁺ leu1-32 ura4-d18 ade6-M210*	Loewith et al., 2000
TYH1	*h⁺ leu1-32 ura4-d18 ade6-M210 ura4-nmt1-nak1*	Huang et al., 2003
SPU	*h⁺ leu1-32 ade6-M210*	Huang et al., 2003
RKO3B-4U	*h⁺ leu1-32 hob1::ura4 ade6-M210*	This study
RKON	*h90 leu1-32 hob1::ura4 ade6-M216*	This study
RKO3A-3U	*h⁺ leu1-32 hob1::ura4 ade6-M216*	This study
RK2N	*h⁺/h90 leu1-32/leu1-32 hob1::ura4/+ ura4-nmt1-nak1/+ ade6-M210/ade6-M216*	This study
RK3A	*h⁺ leu1-32 hob1::ura4 ura4-nmt1-nak1 ade6-M216*	This study
RK2A	*h90 leu1-32 hob1::ura4 ura4-nmt1-nak1 ade6-M210*	This study
RK6B	*h⁺ leu1-32 hob1::ura4 ura4-nmt1-nak1 ade6-M210*	This study
WSPΔ3-12B	*h⁺ leu1-32 wsp1::ura4 ade6-210*	This study
NW5D	*h⁺ leu1-32 wsp1::ura4 ura4-nmt1-nak1 ade6-M216*	This study
NMTWSP40	*h⁻ leu1-32 ura4-nmt1-wsp1 ade6-210*	This study
SP200	*h⁻ leu1-32 ura4-d18 ade6-M210*	This study
RNW2D	*h⁻ leu1-32 ura4-nmt1-wsp1 hob1::ura4 ade6-210*	This study
IRG2	*leu1-32 ade6-210 hob1-GFP-kanMX*	This study
IRG7	*leu1-32 ade6-210 ura4-nmt1-nak1 hob1-GFP-kanMX*	This study
DY201	*leu1-32 ade6-210 cdc10-129 hob1-GFP-kanMX*	This study
DY202	*leu1-32 ade6-210 cdc25-22 hob1-GFP-kanMX*	This study

*Cold Spring Harbor Laboratory, NY.

integration of the *nmt1* promoter was confirmed by Southern-blot analysis.

GFP-tag integration

The genomic *hob1* locus was tagged with GFP by homologous recombination using a DNA fragment amplified from the C-terminal GFP-tagging vector pFA6a-GFP-kanMX (Bahler et al., 1998). The GFP-KanMX module was amplified using the polymerase chain reaction (PCR) and integrated into the wild-type RL143-23A and TYH1 (*nmt1-nak1*) strains to generate the IRG2 (*hob1-GFP*) and IRG7 (*hob1-GFP nmt1-nak1*) strains.

Double mutant strains

RKO5N (*hob1::ura4*) was mated with TYH1 (*nmt1-nak1*) to generate the RK2N diploid strain, tetrads were dissected and 3:1 *ura4+:ura4-* segregants were obtained to produce the *hob1Δ nmt1-nak1* strains RK2A and RK3A. These two strains were mated to produce the diploid homozygous strain RK2A/RK3A-2N, which was sporulated and dissected to produce the haploid strain RK6B (*hob1Δ nmt1-nak1*). NW5D (*wsp1Δ nmt1-nak1*) was produced by mating WSP3Δ-12B (*wsp1Δ*) to RK4D (*nmt1-nak1::ura4*), and 2:2 *ura4* segregants were selected. RNW2D (*hob1Δ nmt1-wsp1*) was generated by mating RKO3A-3U (*hob1Δ*) to NMTWSP40 (*nmt1-wsp1*). Tetrads were dissected and assayed for the *kanMX* on media containing G418. The genotypes of all double mutant strains were confirmed by Southern-blot analysis.

Other methods

Immunoprecipitations and immunoblotting were performed as previously described (Huang et al., 2003). Bead-directed F-actin assays were performed as previously described (Geli et al., 2000; Soulard et al., 2002). Actin and calcofluor staining were performed as previously described (Alfa et al., 1993).

Results

Nak1 associates with Hob1 and Wsp1

We have previously defined the C-terminal 99-residue region of Nak1 (the CTR) as an important regulatory component of Nak1 activity, suggesting that this region might associate with other cell components required for cell polarity (Huang et al., 2003). In order to identify proteins that interact with the Nak1 CTR, we performed a yeast two-hybrid screen for proteins that interact with this region. Of 15 unique potential binding partners obtained, one interacting protein was identified as Hob1, the *S. pombe* homolog of Rvs167.

We further demonstrated that, although LexA-Hob1 associated with VP16-Nak1, it failed to associate with VP16-Nak1 fusions lacking the CTR (Table 2). Furthermore, we found that a VP16-CTR fusion (VP16-Nak1$^{554-652}$) interacted weakly with LexA-Hob1, whereas a fusion with a slightly larger Nak1 C-terminal region (VP16-Nak1$^{481-652}$) interacted more strongly with LexA-Hob1 (Table 2). We then performed coimmunoprecipitation assays to verify the interaction between Nak1 and Hob1. To do this, we co-expressed epitope-tagged proteins from expression plasmids using the *nmt1* promoter. Our results show that Myc-Nak1 immunoprecipitated with HA-Hob1 from extracts of fission yeast co-expressing these proteins, but failed to immunoprecipitate from extracts of control cells expressing Myc-Nak1 alone (Fig. 1A). By contrast, Myc-Nak1$^{1-562}$ (lacking the CTR) failed to immunoprecipitate with HA-Hob1. We also showed that Myc-Hob1 immunoprecipitated with HA-Nak1 from fission-yeast extracts (Fig. 1B). Together, these

Table 2. Yeast two-hybrid tests

LexA fusions	VP16 fusions	β-Galactosidase activity	His⁺ growth
Hob1	Control	–	–
Hob1	Nak1	++	+++
Hob1	Nak1$^{242-652}$	+++	+++
Hob1	Nak1$^{242-562}$	–	–
Hob1	Nak1$^{554-652}$	+	+
Hob1	Nak1$^{481-652}$	+++	+++
Hob1$^{1-414}$	Nak1	+++	+++
Hob1$^{1-281}$	Nak1	+++	+++
Hob1$^{275-466}$	Nak1	–	–
Hob1$^{275-414}$	Nak1	–	–

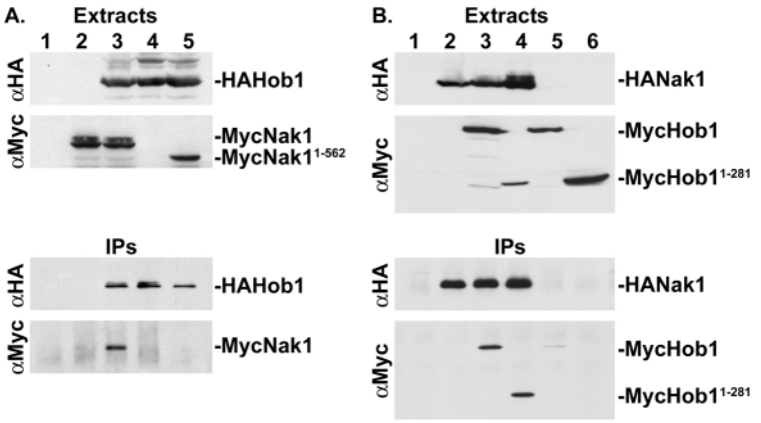


Fig. 1. Hob1 interacts with Nak1. Epitope-tagged proteins were expressed in wild-type (RL143) cells using *nmt1* promoter expression plasmids, and were assayed for expression by western-blot analysis using anti-Myc (9E10) or anti-HA (12CA5) monoclonal antibodies (top). Extracts were immunoprecipitated with anti-HA antibody and equal portions of the immunoprecipitates were probed with anti-HA antibody and anti-Myc antibody (bottom). (A) Control vectors (lane 1), Myc-Nak1 alone (lane 2), HA-Hob1 and Myc-Nak1 (lane 3), HA-Hob1 alone (lane 4), and HA-Hob1 and Myc-Nak$^{1-562}$ (lacking the CTR; lane 5). (B) Control vectors (lane 1), HA-Nak1 (lane 2), HA-Nak1 and Myc-Hob1 (lane 3), HA-Nak1 and Myc-Hob1$^{1-281}$ (lane 4), Myc-Hob1 (lane 5), and Myc-Hob1$^{1-281}$ alone (lane 6).

results indicate that Nak1 and Hob1 associate in *S. pombe*, and that this interaction requires the Nak1 CTR.

Hob1 contains three distinct domains: a conserved N-terminal BAR domain, a variable central linker region and a C-terminal SH3 domain. To determine which region of Hob1 interacts with Nak1, we performed yeast two-hybrid tests for interaction between VP16-Nak1 and LexA fusions with various Hob1 domains (Table 2). Our results demonstrated that LexA fusions containing the BAR domain interacted with VP16-Nak1, whereas fusions lacking the BAR domain failed to interact. We also demonstrated that the Hob1 BAR domain (Myc-Hob1$^{1-281}$) immunoprecipitated with HA-Nak1 from lysates of yeast co-expressing these proteins (Fig. 1B). Thus, the BAR domain is necessary and sufficient for the interaction of Hob1 and Nak1.

Budding yeast Rvs167 has also been shown to bind Bee1/Las17, the WASP homolog (Colwill et al., 1999). Thus, we examined whether fission yeast Nak1 and Hob1 interact with Wsp1, the fission-yeast WASP homolog. Interestingly, we found that myc-Hob1 immunoprecipitated with Wsp1-HA and that myc-Wsp1 immunoprecipitated with HA-Hob1 from extracts of fission yeast expressing these proteins from *nmt1*-promoter-regulated expression plasmids (Fig. 2A,B). Hob1 deletion constructs lacking the conserved BAR domain or the linker/SH3 domain failed to associate with Wsp1, suggesting that these domains are required for this interaction. We also found that myc-Wsp1 immunoprecipitated with HA-Nak1 (Fig. 2C). However, we failed to observe an interaction between LexA-Wsp1 and VP16-Nak1 in a yeast two-hybrid assay. Furthermore, Hob1 overexpression was unable to bridge interactions between LexA-Wsp1 and VP16-Nak1 (data not shown). These results suggest that Nak1 and Wsp1 do not interact directly and that proteins other than Hob1 are required to form a complex containing these proteins. In summary, our results indicate that Wsp1, Hob1 and Nak1 can interact to form complexes in vivo but that other proteins might be involved in the formation of complexes containing Nak1 and Wsp1.

Hob1 localization to cell ends and sites of division is dependent on Nak1

We integrated the GFP coding sequence into the C-terminal end of the genomic *hob1* locus, and we examined the cellular localization of Hob1-GFP in these strains by fluorescence microscopy. We found that Hob1-GFP preferentially localized to patches at sites of cell growth and cell division during the cell cycle as indicated by calcofluor staining (Fig. 3A, left). Furthermore, the Hob1-GFP patches localized to actin patches at the cell ends and the actin ring that forms at the medial region before cell division (Fig. 3A, right).

Before septum formation, Hob1-GFP localized to a band around the cell midpoint (Fig. 3A, top left), similar to band-like patterns representing sterol-rich regions at the cell middle as visualized by filipin staining (Wachtler et al., 2003).

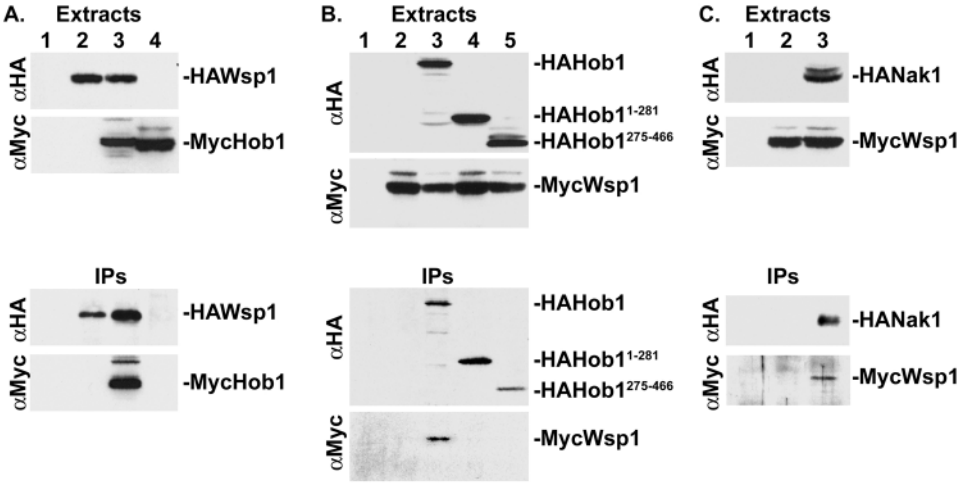


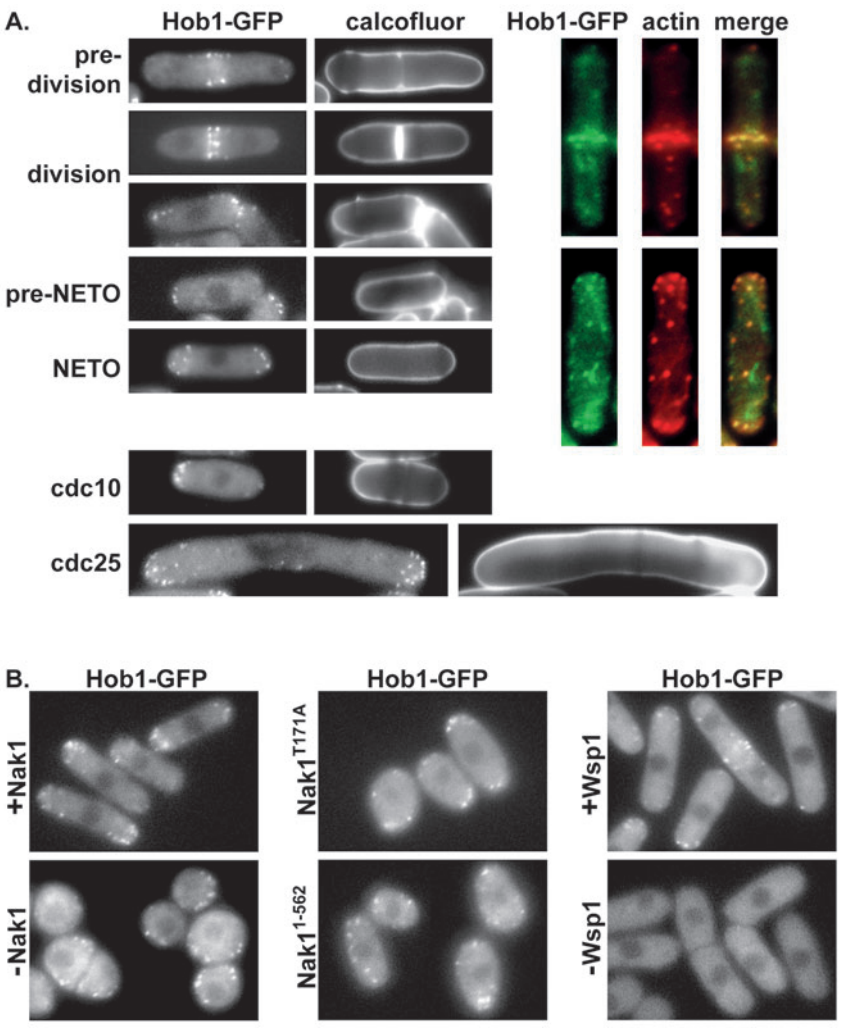
Fig. 2. Nak1 and Hob1 interact with Wsp1. Extracts (top) and anti-HA immunoprecipitates (bottom) from wild-type (RL143) cells expressing HA- or Myc-tagged proteins from *nmt1* promoter expression plasmids were assayed by western-blot analysis using anti-Myc (9E10) or anti-HA (12CA5) monoclonal antibodies. (A) Control vectors (lane 1), Wsp1-HA alone (lane 2), Wsp1-HA and Myc-Hob1 (lane 3), and Myc-Hob1 alone (lane 4). (B) Control vectors (lane 1), Myc-Wsp1 (lane 2), HA-Hob1 and MycWsp1 (lane 3), HA-Hob1$^{1-281}$ and Myc-Wsp1 (lane 4), and HA-Hob1$^{275-466}$ and Myc-Wsp1 (lane5). (C) Control vectors (lane 1), Myc-Wsp1 alone (lane 2) and Myc-Wsp1 and HA-Nak1 (lane 3).

Although this localization pattern is also similar to that observed with the cell-midpoint marker Mid1 (Paoletti and Chang, 2000), *hob1*Δ cells failed to show division defects such as tilted or displaced actin rings like those observed in *mid1* mutants (Chang et al., 1996), suggesting that Hob1 is not essential for cell division processes at the cell medial region.

Shorter cells that have recently divided by cytokinesis grow only at the old end, leaving a dark, unstained region comprising the new pole formed by septation. These cells then subsequently grow at both ends after new-end take-off (NETO) (Mitchison and Nurse, 1985). We found that Hob1-GFP localized to the old growing ends of shorter cells, and to both poles in longer cells after NETO. To verify these observations, we examined the localization of Hob1-GFP in temperature-sensitive cell-cycle mutants arrested before NETO (*cdc10*) and following NETO (*cdc25*) (Fig. 3A, bottom). Indeed, we found that Hob1-GFP localized primarily to the growing cell end in arrested *cdc10* cells and to both poles in the arrested *cdc25* cells. Together, these results indicate that Hob1 localizes to actin patches at the cell ends during growth and to the medial site during cell division.

We also examined whether Nak1 is required for proper Hob1 localization. We constructed a strain containing the integrated thiamine-repressible *nmt1-nak1* allele and the genomically tagged *hob1-GFP* allele, and we found that Hob1-GFP remained localized to patches in this strain following *nak1* repression (Fig. 3B). However, these patches were no longer concentrated at polarized sites and Hob1-GFP often failed to localize to the midpoint in dividing *nak1*-repressed cells. These results indicate that Nak1 function is required for proper Hob1 localization to sites of cell growth and division. Moreover, although the expression of either Nak1$^{1\text{-}562}$ or Nak1^{T171A} in *nak1*-repressed cells partially restored proper localization of Hob1-GFP patches to the cell ends, some Hob1 foci were mislocalized to non-polar sites, suggesting that Nak1 CTR and kinase activity might be important for Hob1 localization (Fig. 3B).

We also examined whether *wsp1* is required for proper localization of Hob1. We generated a strain containing an integrated thiamine-repressible *nmt1-wsp1* allele and the genomically tagged *hob1-GFP* allele. In unrepressed conditions, Hob1-GFP localized properly but, following *wsp1* repression, Hob1-GFP failed to localize to patches (Fig. 3B). Thus, Wsp1 is required for proper localization and association of Hob1-GFP with actin patches. This strain does not exhibit morphological defects similar to *hob1*Δ *wsp1*-repressed cells, indicating that the genomically tagged Hob1-GFP is functional.



Wsp1 localization to cell ends and sites of cell division is dependent on Nak1

We similarly examined the localization of Wsp1-GFP. We note that the expression of Wsp1-GFP complemented *wsp1*Δ salt sensitivity and morphological defects, indicating that Wsp1-GFP is functional (data not shown). We found that Wsp1-GFP localized to actin patches near the cell ends during interphase and to medial sites of division in normally growing cells (Fig. 4A), as has been previously reported (Carnahan and Gould, 2003). However, following *nak1* repression, Wsp1-GFP delocalized from cell ends but remained associated with actin patches, which

Fig. 3. Hob1 localization to sites of cell growth and division requires Nak1. (A) IRG2 (*hob1-GFP*) cells were grown to mid-log phase in selective medium, stained with calcofluor (left) or TRITC-phalloidin (right) and examined by fluorescence microscopy; panels on the right are enlarged. DY201 (*cdc10 hob1-GFP*) and DY202 (*cdc25 hob1-GFP*) cells were grown at 25°C to log phase and then shifted to 36°C for 4 hours to block cells before and after NETO, respectively (bottom, cdc10 and cdc25). (B) IRG7 (*hob1-GFP nmt1-nak1*) cells were grown in the absence (+Nak1) or presence (–Nak1) of 100 μg ml^{-1} thiamine for 18 hours to repress endogenous *nak1* expression. IRG7 cells expressing Nak1^{T171A} and Nak1$^{1\text{-}562}$ from *adh1* promoter expression plasmids were also grown to repress endogenous *nak1* expression (top right). WIR1 (*hob1-GFP nmt1-wsp1*) cells were grown in the absence (+Wsp1) or presence (–Wsp1) of 100 μg ml^{-1} thiamine for 18 hours to repress endogenous *wsp1* expression.

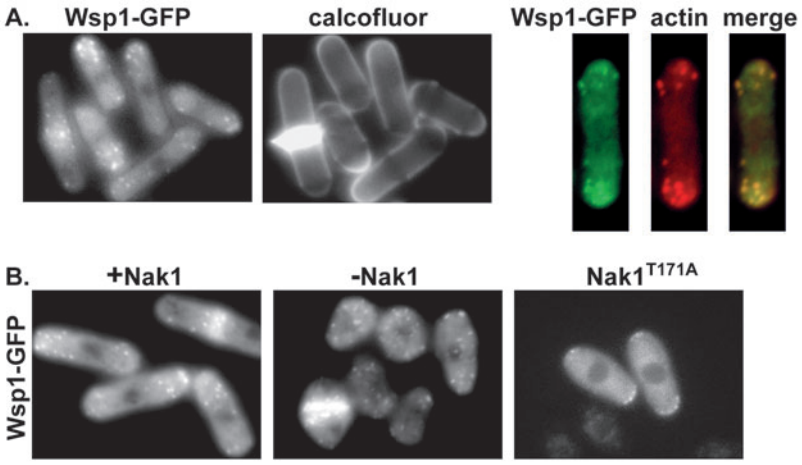


Fig. 4. Wsp1 localization to sites of cell growth requires Nak1. (A) WSPΔ3-12B (*wsp1*Δ) cells expressing Wsp1-GFP from an *adh1* promoter expression plasmid were grown to mid-log phase in minimal medium and stained with TRITC-phalloidin (top) or calcofluor (bottom), and examined by fluorescence microscopy. (B) TYH1 (*nmt1-nak1*) cells expressing Wsp1-GFP were grown in the absence (+Nak1) or presence (–Nak1) of 100 μg ml^{-1} thiamine for 18 hours to repress endogenous *nak1* expression. TYH1 (*nmt1-nak1*) cells coexpressing Wsp1-GFP and $Nak1^{T171A}$ (+$Nak1^{T171A}$) were also examined following endogenous *nak1* repression.

moved to random sites around the cell periphery (Fig. 4B). Thus, Nak1 is required for proper localization of Wsp1 to cell ends, but the association of Wsp1 with actin patches is independent of Nak1. We also found that expression of $Nak1^{T171A}$ in *nak1*-repressed cells restored proper localization of Wsp1-GFP patches to the cell ends, suggesting that a partial reduction of Nak1 kinase activity has little effect on Wsp1 localization (Fig. 4B, bottom left).

Localization of Hob1, but not Wsp1, depends on F-actin integrity

We found that disassembly of actin patches by latrunculin A (LatA) treatment resulted in delocalization of Hob1-GFP from patches and the cell middle during cell division, whereas the disassembly of microtubules by thiabendazole (TBZ) treatment failed to disrupt Hob1-GFP localization (Fig. 5A). By contrast, we observed that neither LatA nor TBZ treatment inhibited the accumulation of Wsp1-GFP to patches (Fig. 5B), similar to Bee1/Las17 localization in budding yeast (Madania et al., 1999). Thus, proper localization of Wsp1 does not appear to depend on intact microtubules or F-actin.

Hob1 overexpression partially suppresses the morphological and growth defects in *nak1*-repressed cells

To investigate further the role of Hob1 in cell growth, we examined the effects of overexpressing Hob1 in normal and *nak1*-repressed cells. We found that overexpression of HA-Hob1 in normal cells did not have a significant effect on cell morphology but partially inhibited growth (Fig. 6A). Furthermore, overexpression of HA-$Hob1^{1-281}$ (the N-terminal BAR domain) failed to have any effect on cell growth, whereas overexpression of HA-$Hob1^{275-466}$ (the linker/SH3 domain) inhibited cell growth only in the presence of high salt concentration (data not shown).

Overexpression of HA-Hob1 or HA-$Hob1^{1-281}$ in *nmt1-nak1* cells partially suppressed the aberrant-rounded morphology associated with *nak1* repression, whereas overexpression of HA-$Hob1^{275-466}$ did not suppress this defect (Fig. 6B, Table 3). Furthermore, overexpression of either HA-Hob1 or HA-$Hob1^{1-281}$ also partially rescued growth inhibition caused by *nak1* repression (Fig. 6C). By contrast, we found that overexpression of $Hob1^{275-466}$ resulted in severe growth inhibition of *nak1*-repressed cells (Fig. 6C). Together, these observations suggest that Hob1 might act in the same pathway as Nak1 or in a parallel pathway to regulate growth and morphology.

Hob1 and Wsp1 cooperate with Nak1 to mediate cell growth and polarity

Although Hob1 overexpression partially suppressed *nak1*-dependent growth and morphological defects, it was unclear how Hob1 might mediate these processes in the context of its association with Nak1 and Wsp1. To examine further the functional relationship of Wsp1, Hob1 and Nak1, we generated *wsp1* and *hob1* deletion strains. Similar to a previous report (Routhier et al., 2003), we found that deletion of *hob1* in fission yeast resulted in no apparent growth, salt-sensitivity or morphological defects (data not shown). Additionally, we did not observe defects in endocytosis or actin distribution in *hob1*Δ strains (data not shown). We found that *nmt1-nak1* strains and *hob1*Δ *nmt1-nak1* double-mutant strains exhibited similar abnormal round morphologies under *nak1*-repressed conditions (data not shown). However, the combination of *hob1*Δ and *nak1* repression resulted in marked growth inhibition on media containing increased salt concentration compared with *hob1*Δ or *nak1*-repressed strains alone, as indicated by a significant reduction in colony size (Fig. 7A). These results indicate that Hob1 and Nak1 act synergistically to permit growth under salt-stress conditions.

Similar to previous reports, we found that *wsp1* deletion resulted in a salt-sensitive phenotype in addition to irregularities in actin distribution (Lee et al., 2000). Although *wsp1* deletion results in an irregular cell morphology, a significant portion of *wsp1*Δ cells are able to maintain two opposing poles (Fig. 7B) (Lee et al., 2000), suggesting that the *nak1*-repressed phenotype is not due entirely to loss of Wsp1 activity.

Nak1 overexpression had little effect in *hob1*Δ or wild-type strains. However, we found that Nak1 overexpression in *wsp1*Δ strains inhibited cell growth and produced a round morphology similar to that observed in *nak1* repression (Fig. 7B,C), suggesting that Nak1 overproduction results in a dominant-negative effect in the absence of Wsp1. We also found that *wsp1* deletion exacerbated growth defects associated with *nak1* repression; *wsp1*Δ *nmt1-nak1* double mutants grew very poorly in *nak1*-repressed conditions compared with *nmt1-nak1* or *wsp1*Δ strains alone (Fig. 7D). We observed no significant difference in cell morphology in *nak1*-repressed *wsp1*Δ strains compared with *nak1*-repressed strains alone (data not shown).

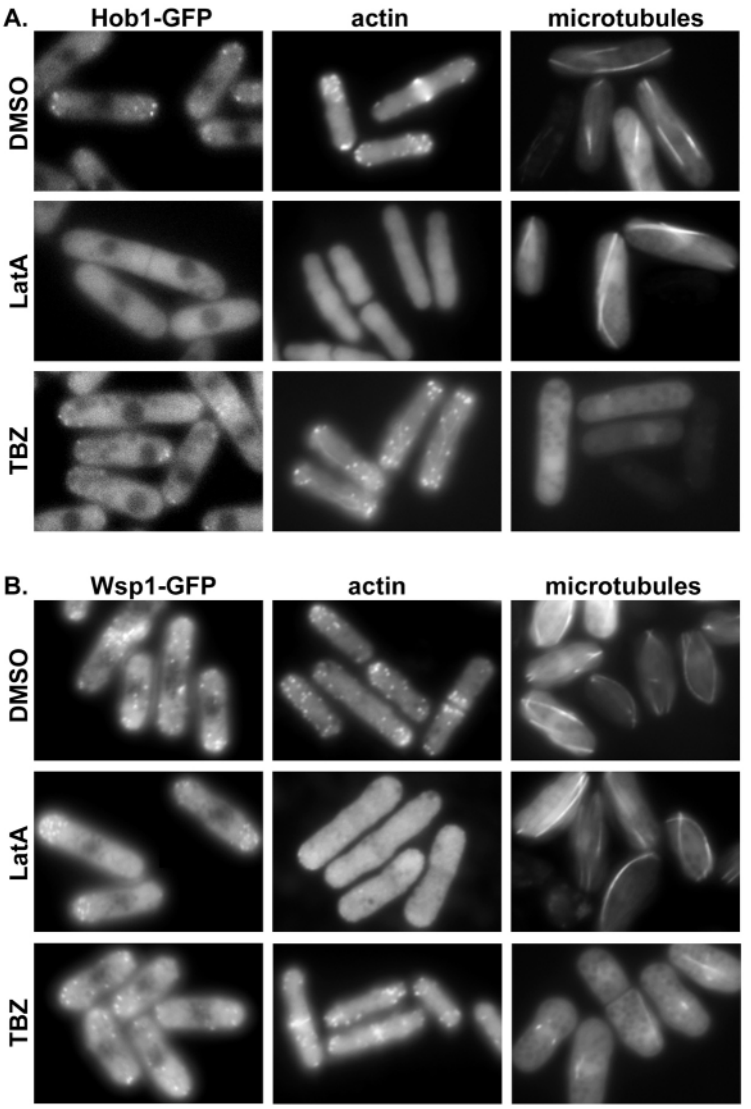


Fig. 5. Localization of Hob1, but not Wsp1, depends on F-actin integrity. (A) IRG2 (*hob1-GFP*) cells (Hob1-GFP and actin panels) or SPU (wild type) cells expressing GFP/α-tubulin (microtubules) were grown to mid-log phase and incubated for 15 minutes in the presence of 1% dimethyl sulfoxide (DMSO) (top), 20 μM LatA and 1% DMSO (LatA, middle) or 100 μM TBZ and 1% DMSO (TBZ, bottom). Some cells were then fixed and stained with TRITC-phalloidin to observe actin localization. (B) Cells expressing Wsp1-GFP were similarly grown to mid-log phase, treated with DMSO, LatA or TBZ, and examined for Wsp1-GFP localization.

These results suggest that Nak1 and Wsp1 act cooperatively to mediate cell growth.

Hob1 and Wsp1 cooperate to mediate cell growth, division and polarity

Additionally, we found that the combination of *wsp1* repression and *hob1* deletion resulted in synthetic defects. The *wsp1*-repressed *hob1*Δ strains grew very poorly (data not shown), and some cells exhibited a multiply septated phenotype and a significant proportion of these cells were round, implying polarity and cell-division defects (Fig. 8A). However, we observed that *wsp1*-repressed cells appeared to be morphologically similar to *wsp1*Δ cells and did not exhibit an aberrantly rounded or multiply septated morphology (data not shown). Thus, it appears that cooperative Wsp1 and Hob1 function is important for normal cell growth, morphology and division.

We also found that Wsp1-GFP overexpression in a wild-type background resulted in a slightly rounded morphology, indicating that an overabundance of Wsp1 can interfere with endogenous polarity mechanisms (Fig. 8B, left). Interestingly, this rounded morphology was corrected by Hob1 overexpression, suggesting that Hob1 can regulate Wsp1 activity to remedy growth and morphology defects caused by Wsp1 overexpression.

Furthermore, we found that Wsp1-GFP dissociated from patches into the cytosol in cells overexpressing Hob1 (Fig. 8B). However, Wsp1-GFP remained associated with patches in cells overexpressing Hob1$^{1\text{-}281}$, which lacks the SH3 domain and fails to interact with Wsp1. These results suggest that Wsp1 localization is sensitive to increased Hob1 levels and that Hob1-mediated Wsp1 delocalization might involve direct interactions between these proteins. Thus, interactions between Wsp1 and Hob1 might be important for their proper function. We also note that Wsp1 localization does not require Hob1, as Wsp1-GFP localized properly to patches in both wild-type and *hob1*Δ strains (data not shown).

Hob1 overexpression or *nak1* repression inhibits Wsp1-mediated F-actin formation in vitro

WASP has been previously shown to activate Arp2/3-mediated F-actin formation through interactions with its C-terminal acidic domain (Symons et al., 1996; Kolluri et al., 1996; Rohatgi et al., 2000). We found that Wsp1-HAHis$_6$-coated NTA-agarose beads were able to recruit F-actin-forming machinery from wild-type cell extracts to produce an F-actin halo around the beads, whereas control beads failed to show F-actin-forming activity under the same conditions (Fig. 9A, Table 4). We also observed no F-actin-forming activity in Wsp1-HAHis$_6$-coated beads in wild-type cell extract in the presence of 10 μM LatA. Interestingly, we also observed that overexpressed myc-Hob1 bound to Wsp1-HAHis$_6$-coated beads and inhibited Wsp1-mediated F-actin formation (Fig. 9B). However, overexpressed myc-Hob1$^{1\text{-}281}$ failed to bind to Wsp1-HAHis$_6$-coated beads (data not shown) and did not inhibit Wsp1 activity, indicating that the linker/SH3 domain, which is required for interaction with Wsp1, is required for this effect (Fig. 9B).

We also found that extracts from *nak1*-repressed strains failed to produce F-actin halos around Wsp1-coated beads, suggesting that Nak1 is required for Wsp1-mediated F-actin polymerization (Fig. 9C). Furthermore, although extracts derived from *nak1*-repressed strains expressing exogenous Nak1 were able to polymerize F-actin on Wsp1-coated beads, extracts derived from *nak1*-repressed strains expressing the partially active Nak1^{T171A} kinase produced little or no F-actin on Wsp1-coated beads. These results suggest that Nak1 kinase activity is important for Wsp1-mediated F-actin formation.

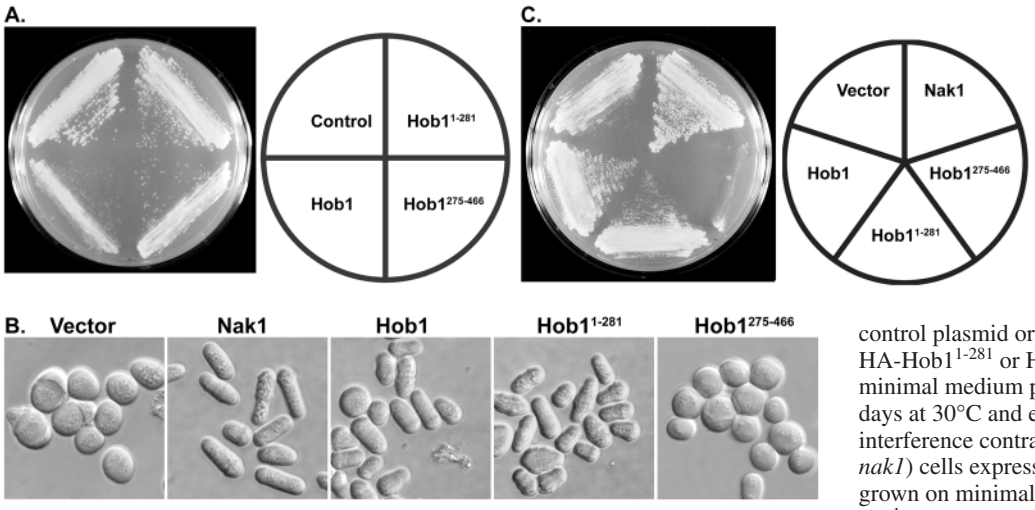


Fig. 6. Hob1 overexpression in wild-type and *nak1*-repressed cells. (A) Wild-type RL143 cells containing a control plasmid or expressing HA-Hob1, HA-Hob1$^{1-281}$ or HA-Hob1$^{275-466}$ from *adh1* promoter expression plasmids were grown on selective medium at 30°C for 3 days. (B) TYH1 (*nmt1-nak1*) cells containing a control plasmid or expressing HA-Nak1, HA-Hob1, HA-Hob1$^{1-281}$ or HA-Hob1$^{275-466}$ were grown on minimal medium plus 100 μg ml^{-1} thiamine for 2 days at 30°C and examined by differential interference contrast microscopy. (C) TYH1 (*nmt1-nak1*) cells expressing the indicated proteins were grown on minimal medium plates containing 100 μg ml^{-1} thiamine for 5 days at 30°C.

Discussion

We have previously showed that Nak1 is required for polarized growth in *S. pombe* (Huang et al., 2003). Herein, we describe a means by which Nak1 might mediate cell growth and polarity, in part through its association with Hob1 and Wsp1. In summary, our results support a model in which Hob1 and Wsp1 localize to actin patches at sites of growth and division, and function cooperatively to promote polarized growth and morphology. Nak1 regulates the localization of Hob1, Wsp1 and associated actin patches to cell ends, and Nak1 and Hob1 regulate Wsp1 activity to promote F-actin formation and polarized growth at these sites. In addition, Nak1, Hob1 and Wsp1 have distinct roles that work cooperatively to regulate growth and bipolar morphology.

Hob1 and Wsp1 regulate cell polarity

The association of Nak1, Hob1 and Wsp1 suggests that the functions of these proteins are related. Furthermore, the localization of Hob1 and Wsp1 to actin patches at growing cell ends and division sites suggest that they are involved in cell polarity and division. However, because *hob1* and *wsp1* deletion strains maintain a bipolar morphology, the role of these components in cell polarity was unclear (Lee et al., 2000; Routhier et al., 2003). Our observations confirm a previous report that *hob1* deletion has little effect on cell growth, morphology, cytoskeletal maintenance and endocytosis

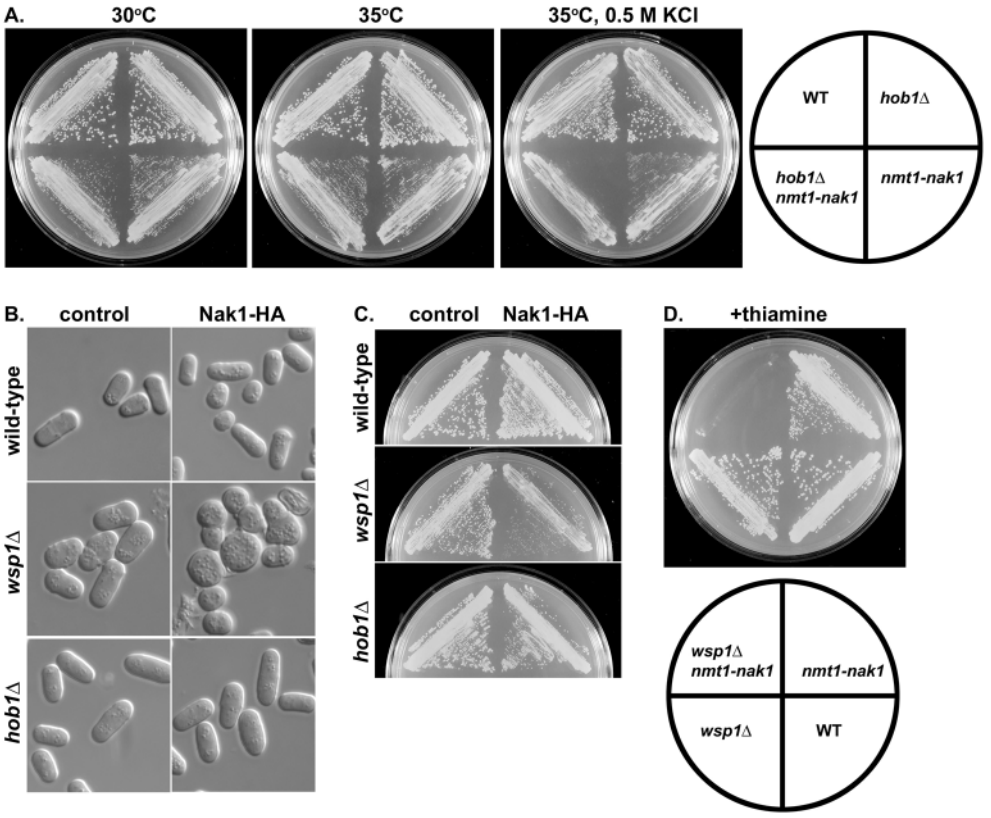


Fig. 7. Hob1 and Wsp1 cooperate with Nak1 to mediate cell growth and polarity. (A) SPU (wild type), RKO3B-4U (*hob1*Δ), TYH1 (*nmt1-nak1*) and RK6B (*hob1*Δ *nmt1-nak1*) strains were grown on minimal medium with 100 μg ml^{-1} thiamine and with or without 0.5 M KCl at the indicated temperature (30°C or 35°C) for 3-5 days. (B,C) Wild-type (SPU), *wsp1*Δ (WSPΔ3-12B) and *hob1*Δ (RKO3B-4U) cells expressing HA alone (control) or Nak1-HA from *nmt1* promoter expression plasmids were grown on minimal medium for 3 days at 30°C and examined (B) or assayed for growth (C). (D) SPU (wild type), WSPΔ3-12B (*wsp1*Δ), TYH1 (*nmt1-nak1*) and NW5D (*wsp1*Δ *nmt1-nak1*) strains were grown on minimal medium with 100 μg ml^{-1} thiamine at 30°C for 3 days.

(Routhier et al., 2003). Nevertheless, our results support a role for Hob1 in mediating growth and cell polarity processes in fission yeast, and suggest that other mechanisms might be functionally redundant with Hob1. Indeed, this is supported by our observations that deletion of *hob1* and *wsp1* in combination resulted in synthetic growth and morphological defects not seen in *wsp1*Δ or *hob1*Δ mutants alone, suggesting that Wsp1 and Hob1 have overlapping functions that regulate cell growth, morphology and division.

Table 3. Suppression of *nak1*-repressed round morphology

Overexpressed protein*	Morphology		
	Normal	Round	Normal (%)
Control	36	231	13.5
Nak1	322	19	94.4
Hob1	582	228	71.9
Hob1$^{1-281}$	341	200	63
Hob1$^{275-466}$	4	346	1.1

*Numbers were derived from *nak1*-repressed cells overexpressing the indicated protein (Fig. 6B).

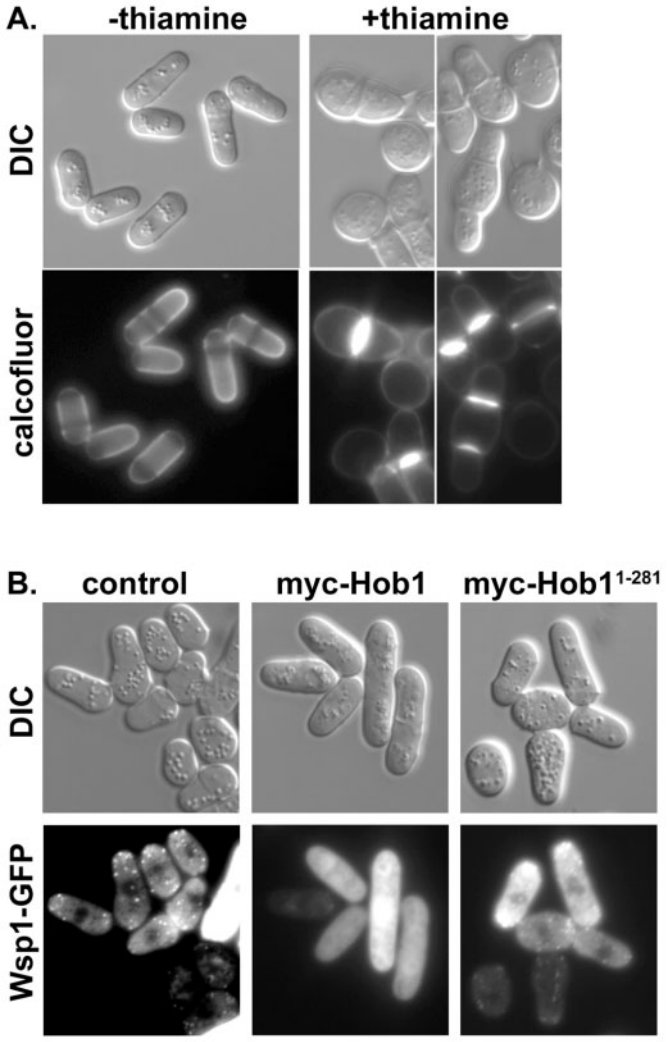


Fig. 8. Hob1 and Wsp1 cooperate to mediate cell growth, division and polarity. (A) RNW2D (*nmt1-wsp1 hob1*Δ) cells were grown to mid-log phase in minimal medium in the absence and presence of 100 μg ml^{-1} thiamaine and stained with calcofluor to observe cell septae. (B) Wild-type (RL143) cells co-expressing Wsp1-GFP and the myc epitope alone (control, left), myc-Hob1 (middle) or Hob1$^{1-281}$ (right) were grown in minimal medium and viewed for cell morphology and Wsp1-GFP localization.

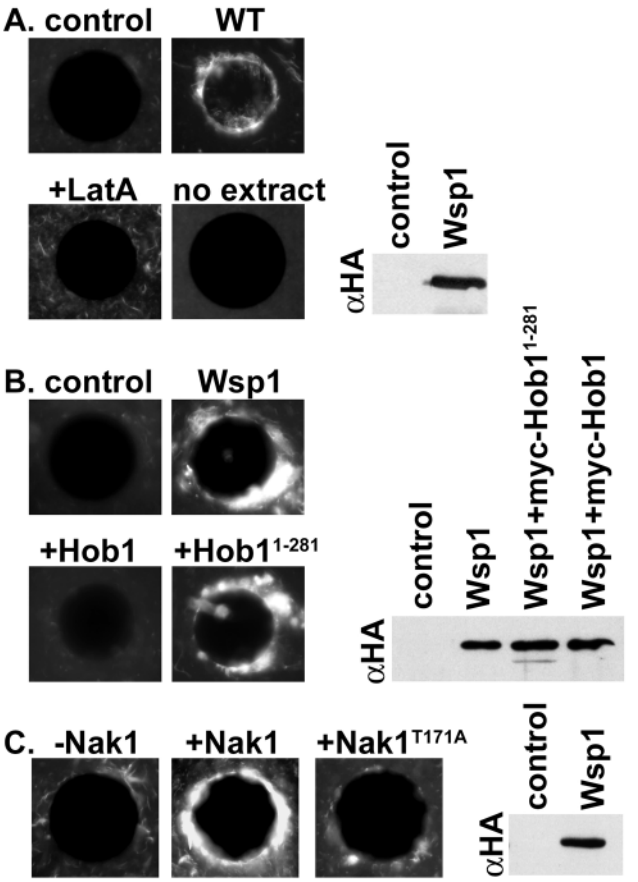


Fig. 9. Hob1 overexpression or *nak1* repression inhibits Wsp1-mediated F-actin formation in vitro. (A) NTA-agarose beads were either coated with Wsp1-HAHis$_6$ by incubation in extracts from yeast expressing this protein or incubated in control yeast lysates (control). They were then incubated with fluorescent Alexa-568/actin and wild-type (SPU) cell extract (WT), wild-type extract in the presence of 10 μM LatA (+LatA) or XB-200 buffer alone (no extract). The ability of the different extracts to stimulate Wsp1-mediated F-actin formation was observed by the presence of fluorescent F-actin halos around the beads. A proportion of the NTA-agarose beads were boiled and immunoblotted with the anti-HA (12CA5) antibody to detect Wsp1-HAHis$_6$ bound to the beads. (B) NTA-agarose beads were similarly incubated in control extracts or extracts from yeast expressing Wsp1-HAHis$_6$ (Wsp1), Wsp1-HAHis$_6$ and myc-Hob1 (+Hob1), or Wsp1-HAHis$_6$ and myc-Hob1$^{1-281}$ (+Hob1$^{1-281}$), and were assayed for the formation of F-actin halos in Alexa-568/actin in the presence of wild-type extract. The presence of Wsp1-HAHis$_6$ (shown on the right) and myc-Hob1 or myc-Hob1$^{1-281}$ (not shown) were confirmed by western blots. (C) Wsp1-HAHis$_6$-coated beads were assayed for F-actin formation in the presence of cell extracts derived from *nak1*-repressed (TYH1) cells containing a control vector (–Nak1) or expressing either Nak1 (+Nak1) or Nak1^{T171A} (+Nak1^{T171A}) grown in the presence of 100 μg ml^{-1} thiamine. No F-actin formation was observed in beads coated using control extracts (derived from the wild-type SPU strain, not shown).

Table 4. Wsp1-directed F-actin formation

Bead coating	Cell extract	F-Actin formation
Control	Wild type	–
Wsp1-HAHis$_6$	Wild type	+
Wsp1-HAHis$_6$	Wild type +LatA	–
Wsp1-HAHis$_6$	No extract	–
Wsp1-HAHis$_6$+Hob1	Wild type	–
Wsp1-HAHis$_6$+Hob1$^{1-281}$	Wild type	+
Wsp1-HAHis$_6$	*nak1* repressed	–
Wsp1-HAHis$_6$	*nak1* repressed+Nak1	+
Wsp1-HAHis$_6$	*nak1* repressed+Nak1^{T171A}	–

Furthermore, our observation that Hob1 overexpression partially rescued growth and morphological defects associated with *nak1* repression indicates that Hob1 can promote polarized growth and morphology. However, as Hob1 overexpression partially inhibits cell growth in wild-type cells in both budding and fission yeast, it appears that an excess of Hob1 might also negatively regulate cell growth. We note that the growth of wild-type cells overexpressing Hob1 and of *nak1*-repressed cells overexpressing Hob1 appeared to be comparable. Thus, Hob1 might have dichotomous functions that regulate cell growth.

Nak1 is required for the proper localization of Hob1 and Wsp1

The cytoplasmic localization of Nak1 suggests that the mechanism by which it directs cell polarity is dependent on its ability to regulate other polarity components at growing cell ends and sites of division. Our observation that Hob1 and Wsp1 localized to random sites upon *nak1* repression suggests that Nak1 mediates polarized growth, at least in part, by regulating the localization of Hob1 and Wsp1 at the growing cell ends.

Although Hob1 localization is dependent on the integrity of actin patches, Wsp1 localization appears to be independent of F-actin. We also found that, whereas Wsp1 localized properly to patches at cell ends in *hob1*Δ strains (data not shown), Hob1 localization to cortical patches is dependent on Wsp1. Thus, Wsp1 might localize to cortical patches independently of actin and facilitate recruitment of Hob1 to actin patches at the cell ends.

Nak1 and Hob1 regulate Wsp1-dependent F-actin formation

Our observation that Nak1 is required for Wsp1-mediated F-actin formation in vitro suggests that it might be required for Wsp1-mediated assembly of actin patches in vivo. Moreover, our results suggest that Nak1 kinase activity is important for Wsp1-dependent F-actin formation, suggesting that Wsp1-dependent F-actin formation might depend on phosphorylation of Nak1 targets.

Hob1 overexpression also inhibited Wsp1-directed F-actin formation in vitro, suggesting that Hob1 might regulate growth, in part, by restraining F-actin formation at cell ends. Our observation that co-overexpression of Hob1 remedied the growth and morphological effects of Wsp1 overexpression is also consistent with the idea that Hob1 overexpression inhibits Wsp1 activity. However, it is not yet clear whether these results reflect a dominant negative effect caused by Hob1 overexpression or a normal role for Hob1 as a negative regulator of Wsp1 and cell growth. WASP-family proteins that lack CRIB domains, such as WAVE1 and Bee1/Las17, appear to be constitutively active and are kept in an inactive state by their association with other factors (Eden et al., 2002; Rodal et al., 2003). Because Wsp1 also lacks a CRIB motif, its activity might be similarly regulated. Thus, it is plausible that Hob1 might negatively regulate Wsp1 activity under normal conditions in order to mediate polarized growth properly. However, our observation that Hob1 overexpression promotes polarized growth in the absence of Nak1 suggests that Hob1 has a distinct role in mediating growth and morphology.

Nak1 functions redundantly with Hob1 and Wsp1 to mediate growth and polarity

Deletion of *wsp1* or *hob1* fail to produce polarity or growth defects, thus other Nak1-mediated mechanisms might be functionally redundant with Wsp1 and Hob1. Indeed, our observation that *hob1* deletion results in synthetic growth defects when combined with *nak1*-repression suggests that Hob1 and Nak1 have overlapping functions that regulate cell growth. Similarly, the combination of *nak1* repression in the absence of *wsp1* leads to a severe growth defect, indicating that Nak1 and Wsp1 have distinct overlapping roles that cooperate to maintain normal growth. Also, whereas Nak1 overexpression had little effect on wild-type cells, overexpression of Nak1 in the absence of Wsp1 resulted in growth inhibition and loss of cell polarity, suggesting that Nak1 overexpression leads to dominant-negative effects that are masked by Wsp1. This could reflect that in addition to regulating Wsp1, Nak1 regulates another pathway that overlaps with Wsp1 function and Nak1 overexpression affects polarity through this Wsp1-independent mechanism.

Nak1 and other polarity pathways

It is yet unclear how Nak1-regulated growth and polarity processes fit within the context of existing pathways in *S. pombe*. Nak1 is most closely related to the *S. cerevisiae* GC kinase Kic1/Nrk1, a component of the RAM pathway that regulates polarized morphogenesis and cell separation (Nelson et al., 2003). However, these kinases are only similar within their catalytic domains, and expression of Kic1/Nrk1 is unable to suppress morphological defects associated with *nak1* repression (data not shown). Thus, it seems that Nak1 and Kic1 are not functionally conserved. Nevertheless, it is possible that Nak1 is in a pathway with conserved RAM components such as Orb6, Mob2 and Mor2 (Cbk1, Mob2 and Tao3 orthologs, respectively), or other polarity factors.

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