Human Bin3 complements the F-actin localization defects caused by loss of Hob3p, the fission yeast homolog of Rvs161p

Eric L. Routhier1, Timothy C. Burn2, Ilgar Abbaszade2,
Matthew Summers3, Charles F. Albright1 and George C. Prendergast1,3

1Cancer Research Group, Glenolden Laboratory, DuPont Pharmaceuticals Company, Glenolden PA 19036; 2Applied Biotechnology Group, DuPont Pharmaceuticals Company, Wilmington DE 19803; and 3The Wistar Institute, Philadelphia PA 19104

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*Corresponding author
The BAR adaptor proteins Rvs167p and Rvs161p from *Saccharomyces cerevisiae* form a complex that regulates actin, endocytosis, and viability following starvation or osmotic stress. In this study, we identified a mammalian homolog of Rvs161p, termed Bin3 (Bridging INtegrator-3), and a *Schizosaccharomyces pombe* homolog of Rvs161p, termed Hob3p (Homolog Of Bin3). In mouse tissues, the Bin3 gene was expressed ubiquitously except for brain. *S. pombe* cells lacking Hob3p were often multinucleate and characterized by increased amounts of Calcofluor-stained material and mislocalized F-actin. For example, while wild-type cells localized F-actin to cell ends during interphase, *hob3Δ* mutants had F-actin patches distributed randomly around the cell. In addition, medial F-actin rings were rarely found in *hob3Δ* mutants. Notably, in contrast to *S. cerevisiae rvs161Δ* mutants, *hob3Δ* mutants showed no measurable defects in endocytosis or response to osmotic stress, yet *hob3+* complemented the osmosensitivity of a *rvs161Δ* mutant. Bin3 failed to rescue the osmosensitivity of *rvs161Δ*, but the actin localization defects of *hob3Δ* mutants were completely rescued by Bin3 and partially rescued by RVS161. These findings suggest that Hob3p and Bin3 regulate F-actin localization, like Rvs161p, but that other roles for this gene have diverged somewhat during evolution.
Introduction

BAR (Bin/Amphiphysin/Rvs domain) adaptor proteins, which include proteins encoded by the mammalian genes Amphiphysin, BIN1, and BIN2 and the *Saccharomyces cerevisiae* genes RVS167 and RVS161, are characterized by a unique N-terminal region termed the BAR domain. While their exact functions are largely unknown, BAR adaptor proteins appear to integrate signal transduction pathways that regulate membrane dynamics, F-actin cytoskeleton, and nuclear processes, roles that are highlighted in the nomenclature of two recently identified members of the family (Bridging INtegrators or BIN proteins).

Both genes encoding BAR adaptor proteins in *S. cerevisiae* were initially identified in a genetic screen for mutants that lost viability upon nutrient starvation [1; 2]. Subsequent work revealed that Rvs167p and Rvs161p form a physiological complex that regulates F-actin localization, cell polarity, bud formation, and endocytosis [2-7]. Rvs161p is also important for karyogamy, the nuclear fusion process which follows mating [8]. A variety of Rvs-interacting proteins were identified that are consistent with Rvs161p and Rvs167p functions in F-actin regulation, lipid metabolism, cell cycle integration, and nuclear processes [9-13]. Despite the importance of Rvs161p and Rvs167p in these diverse functions, RVS161 and RVS167 genes are not required for viability.

Three genes encoding BAR adaptor proteins have been described in mammalian cells.
Two of these genes, amphiphysin and BIN1, encode structural orthologs of Rvs167p, whereas the third gene, BIN2, encodes a structurally unique protein. The expression patterns of each gene suggest different physiological roles: BIN1 is widely expressed whereas amphiphysin and BIN2 are tissue-restricted in their expression. The product of the amphiphysin gene, which was identified by virtue of its biochemical properties [14], is a neuronal adaptor protein that regulates synaptic vesicle endocytosis [15]. The restricted pattern of amphiphysin expression argues that its physiological function is limited to the specialized processes of synaptic vesicle recovery. In a similar way, BIN2 expression is restricted to hematopoietic cells. BIN2 function is undefined but appears to be nonredundant with other mammalian BAR proteins [16]. The BIN1 gene has a complex function(s) suggested by its diverse patterns of alternate splicing. BIN1 splice isoforms have been identified by virtue of interaction with the c-Myc oncoprotein, structural similarity to amphiphysin, interaction with the nuclear tyrosine kinase c-Abl, and characterization of the BIN1 gene itself [17-23]. Brain-specific isoforms, alternately termed amphiphysin II or amphiphysin-like isoforms, are exclusively cytosolic and can influence endocytosis [15]. However, only brain isoforms include regions required for interaction with key components of the endocytosis machinery [24]. Thus, it is unclear whether Bin1 participates in endocytosis outside the brain. Nuclear functions are suggested by the ability of muscle-specific and ubiquitous isoforms to localize to the nucleus and to functionally associate with the c-Myc and c-Abl proteins [17; 19; 25]. In particular, c-Myc-interacting isoforms have tumor suppressor and transcriptional properties that impact cell differentiation and cell death decisions [17; 25-31].
To further investigate the function of BAR adaptor proteins, we identified a mammalian homolog of Rvs161p, termed Bin3 (Bridging INtegrator-3), and a *Schizosaccharomyces pombe* homolog of Rvs161p, termed Hob3p (Homolog Of Bin3). Analysis of *hob3Δ* mutants revealed an important role for Hob3p in regulation of F-actin localization, as was found for Rvs161p. The F-actin localization defect of *hob3Δ* mutants was completely rescued by human BIN3 and partially rescued by RVS161, raising the possibility that Bin3 regulates F-actin localization in mammalian cells.
Materials and Methods

Cloning. The *S. pombe* *hob3*+ gene was identified through BLAST [32] searches of the *S. pombe* genome using the *S. cerevisiae* RVS161 gene as query. The *hob3*+ gene was cloned by PCR from a stationary-phase, *S. pombe* single-stranded cDNA library (Library-In-A-Tube”, QBiogen), using oligonucleotide primers derived from the *hob3*+ locus. Sequences encoding the human Bin3 protein were similarly identified by TBLASTN [32] searches of the translated EST database using the *S. cerevisiae* Rvs161p as a query. Human BIN3 cDNA was cloned by PCR from single-stranded Library-In-A-Tube” cDNA libraries (QBiogen). Information obtained from the EST databases was used to construct a full-length cDNA clone. Sequence determinations included the full-length IMAGE EST clones obtained from Research Genetics (Huntsville AL). Human BIN3 cDNA was subcloned as an untagged or hemagglutinin (HA)-tagged insert into pcDNA3/neo (Invitrogen) and these plasmids were used for PCR to amplify BIN3 cDNA for insertion into yeast vectors. cDNAs were digested with NdeI and PspAI or BamHI and cloned into the *S. pombe* expression plasmid pREP2 [33]. Gene deletions in *S. pombe* were performed as described [34] using plasmid pFA6a-kanMX6-HA as a template for the construction of a disrupted allele. BAR protein-encoding cDNAs were cloned between the XbaI and BamHI or Smal sites of the 2μ-based budding yeast expression vector YEpl95-ACN (courtesy J. Toyn). Yeast transformations were performed by standard methods [35; 36]. Oligonucleotide sequences are available upon request.
Strains and media. S. pombe strains FY71 (h-, ade6-M216, leu1-32, ura4-D18) and FY72 (h+, ade6-M210, leu1-32, ura4-D18) were obtained courtesy of S. Henry, Mellon College. Strain ELR6 (ade6-M210, leu1-32, ura4-D18, hob3Δ::kanMX6) is a derivative of diploid strain KGY246/249 [37], obtained by one-step gene disruption. Integration of the altered allele by homologous recombination was verified by Southern blotting using a 32P-labelled PCR product derived from the 5’ untranslated region of the hob3+ locus, extending from 280 bp to 986 bp from the putative start codon of hob3+ [38]. The probe was labelled with the High Prime DNA Labelling Kit (Roche Molecular Biochemicals) and α-32P-dCTP (NEN). S. cerevisiae strains BY4741 (MATa, ura3, leu2, his3, met15) and BY4741-3489 (MATa, ura3, leu2, his3, met15, rvs161Δ::kanR) were obtained from J. Toyn, Applied Biotechnology Group, DuPont Pharmaceuticals Company. S. pombe strains were grown in YE medium or EMM2 containing appropriate nutritional supplements when necessary [39]. Expression from pREP2 plasmids was achieved by growing cells to early log phase in medium containing 0.06 mM thiamine, washing the cells 3x in thiamine-free medium, and resuspending the cells in the same medium. Budding yeast were grown in YPAD or SC medium lacking the appropriate nutritional supplements, in some cases with the addition of 6% (w/v) NaCl [40].

Immunofluorescence. Exponential phase S. pombe cultures were stained for F-actin as described [41] using AlexaFluor 488-conjugated phalloidin (Molecular Probes). Nuclei were stained with DAPI. Images were captured on a Nikon Eclipse TE300 microscope fitted with a Nikon Plan Fluor 100X objective using a Toshiba 3CCD camera. Images were manipulated using Image Pro Plus version 4.0 software (Media Cybernetics).
**Endocytosis.** Exponential phase cultures of *S. pombe* cells were assayed for uptake of the lipophilic styryl dye FM4-64 (Molecular Probes) as described [42].

**Northern Analysis.** MTNI and MTNII human multiple tissue Northern blots obtained from Clontech (Palo Alto CA) were hybridized to $^{32}$P-labelled probes for Bin3, Bin1, and amphiphysin I generated by the random priming method as per the vendor’s instructions. The Bin1 and amphiphysin I probes have been described [14; 27]. The Bin3 probe was a $^{32}$P-labelled 600 bp BamHI-BgIII fragment of the human BIN3 cDNA. Hybridization of a Northern blot of RNA isolated from a panel of tumor cell lines, cultured and processed as described previously [17; 31], was performed using the BIN3 probe and a β-tubulin probe to normalize the blot.
Results

**Bin3 encodes a widely expressed BAR adaptor protein related to Rvs161p.** Sequences encoding Bin3, a novel human BAR adaptor protein, were identified using Rvs161p to search the EST database with the TBLASTN algorithm. Sequence analysis of full-length cDNA clones identified in this manner revealed that Bin3 was a protein of 253 residues in length and was comprised solely of a BAR domain, like Rvs161p (Fig. 1a, 1b). The Bin3 BAR domain was 27% identical to Rvs161p but less than 24% identical to other BAR domains (Table 1). Northern analysis of mouse tissue RNAs was performed to compare the Bin3 expression pattern to that of other mammalian BAR adaptor genes. A single mRNA species of ~2.2 kb was detected at similar levels in all embryonic and adult tissues examined, except for brain where Bin3 mRNA was undetectable (Fig. 2a). This wide expression pattern of Bin3 was similar to Bin1, which was widely expressed, but contrasted with amphiphysin, which was expressed primarily in brain, and Bin2, which was expressed primarily in hematopoietic cells. Since Bin1 expression was frequently decreased in malignant cells, Bin3 expression was determined in a panel of human tumor cell lines. All cell lines tested expressed Bin3 (Fig. 2b). We concluded that Bin3 was a widely expressed, BAR adaptor protein that was structurally most similar to *S. cerevisiae* Rvs161p.

**S. pombe hob3+ encodes a BAR adaptor protein related to Rvs161p and Bin3.** Sequences encoding Hob3p (Homolog of Bin3) were identified using Rvs161p to search the *S.
pombe genome (Fig. 1a). Hob3p was 264 residues in length, 56% identical to S. cerevisiae Rvs161p, and 29% identical to Bin3 throughout its entire sequence (Fig. 1b). In contrast, the Hob3p BAR domain sequences were less than 26% identical to the BAR domain sequences in Bin1, Bin2, and amphiphysin (Table 1). Like Rvs161p and Bin3, Hob3p was comprised solely of a BAR domain, without the additional C-terminal sequences found in Rvs167p or known mammalian BAR adaptor proteins (Fig. 1b). The similarity of BAR sequences and lack of non-BAR sequences suggest that Rvs161p, Bin3, and Hob3 comprise a subfamily within the family of BAR adaptor proteins. The other BAR adaptor protein encoded by the S. pombe genome was identified. The structure and characterization of this predicted protein, which was most similar to Rvs167p, will be described elsewhere.

**hob3Δ mutants have a cell division defect.** We began by studying S. pombe hob3+ since fission yeast genetics allowed us to rapidly characterize Hob3p function. Using standard methods, haploid S. pombe strains were made where the entire coding region of hob3+ was replaced with the kanMX6 cassette, conferring resistance to G418 [34]. Southern analysis confirmed construction of a strain with the hob3Δ allele (Fig. 3a). Examination of hob3Δ mutants revealed a fraction of cells that were longer than hob3+ cells and contained more than two nuclei (Fig. 3b). In particular, about 9% of hob3Δ cells from an actively-growing culture contained more than two nuclei, with most of these elongated cells containing four nuclei (n = 108). In contrast, no cells with more than two nuclei were observed in a parallel culture of hob3+ cells (n = 125). Calcofluor staining showed that septal material separated most of the nuclei in hob3Δ cells (Fig. 3b). Furthermore, the hob3Δ cells contained increased amounts of Calcofluor-
stained material, relative to \( \text{hob3}^+ \) cells (Fig. 3b). Consistent with their multinucleated phenotype, exponentially growing \( \text{hob3}\Delta \) cells exhibited a continuum of >4N ploidies when analyzed by flow cytometry (data not shown). The division of cell populations based on nuclear content by flow cytometry of \( \text{hob3}\Delta \) and \( \text{hob3}^+ \) cells mirrored results obtained by microscopic observation. Both \( \text{hob3}^+ \) and \( \text{hob3}\Delta \) null cells grew and mated with kinetics indistinguishable from wild type cells; however, \( \text{hob3}\Delta \) cells tended to grow in clumps of 5-15 cells (data not shown) and stopped dividing at lower cell density than \( \text{hob3}^+ \) cells (Fig. 3c). Overexpression of \( \text{hob3}^+ \) had no effect on \( \text{hob3}^+ \) cells (data not shown). Hence, \( \text{hob3}^+ \) had a role in cell division in fission yeast at the level of septation, with some proportion of cells failing to separate following septum formation and accumulating increased levels of septal material.

\textbf{hob3}\Delta  \textbf{mutants frequently mislocalize F-actin.} \textit{S. cerevisiae rvs161}\Delta mutants were defective in F-actin localization [3; 6; 43]. Since \textit{S. pombe} mutants with F-actin localization defects disrupt septation, cytokinesis, and cell separation [44-46], F-actin localization in \( \text{hob3}\Delta \) mutants was determined using fluorescent phalloidin. In \( \text{hob3}^+ \) \textit{S. pombe}, F-actin was normally localized to cortical patches during interphase and medial contractile rings during mitosis (Fig. 4) [41]. In contrast, \( \text{hob3}\Delta \) mutants displayed two significant F-actin localization defects (Fig. 4, Table 2). First, F-actin patches were frequently mislocalized in \( \text{hob3}\Delta \) cells with one nucleus. In particular, F-actin patches were found equally distributed along the entire length of mononuclear cells, with 86% of such mononuclear cells (\( n = 88 \)) exhibiting this staining pattern versus 4% of cells from a \( \text{hob3}^+ \) strain (\( n = 115 \)). Second, medial F-actin rings and patches were rarely observed in \( \text{hob3}\Delta \) mutants with two nuclei. In \( \text{hob3}\Delta \) mutant cells with 2 nuclei,
41% had medial F-actin patches in both compartments with an F-actin ring, 32% had delocalized F-actin patches in both compartments, and 27% had medial F-actin patches in one compartment with delocalized F-actin patches in the other compartment (n = 22). These findings contrast with hob3+ cells where 100% of cells containing two nuclei exhibited medial F-actin staining (n = 29). Hence, Hob3p plays an important role in the localization of F-actin in interphase and mitotic cells. Consistent with loss of cell polarity and consequent abnormally shaped cells observed in mutants of the S. pombe F-actin-encoding act1+ gene [47], we observed such misshapen cells in hob3Δ cultures (Fig. 4). Specifically, 13% of cells (n = 116) in Fig. 4 lost their cylindrical appearance and took on a rounded appearance, versus 0% of cells in a matched hob3+ culture (n = 163). Loss of shape was not limited to mononuclear cells; 48% of misshapen cells possessed two or more nuclei. F-actin delocalization was observed in all misshapen cells. We concluded that hob3+ was necessary for F-actin regulation and completion of septation, the process of cytokinesis in fission yeast cells.

**hob3Δ mutants respond normally to nutrient and osmotic stress.** RVS161 was discovered in a screen for mutants that had reduced viability upon starvation for glucose, nitrogen or sulfur [1]. In these experiments, rvs161Δ mutants had a 35% reduction in cell viability after 48 hours in N005 low nitrogen medium. Further analysis revealed that rvs161Δ mutants showed dramatic morphologic changes in response to high salt and low nitrogen media, and more significant reductions in cell growth when shifted to media with high salt [48]. Based on these results, the response of hob3Δ mutants to nutrient and osmotic stress was tested. This analysis revealed that hob3Δ mutant cells were relatively insensitive to lack of nitrogen or elevated/decreased
temperature, as assayed by growth on plates (Fig. 5). Furthermore, microscopic inspection of
hob3Δ mutant cells following temperature, osmotic, or nutrient shift did not reveal detectable
differences in cell morphology (data not shown). Based on these results, we concluded that
hob3Δ mutants, unlike rvs161Δ mutants, respond like hob3+ cells to changes in temperature,
osmolarity, and nutrients.

**hob3Δ mutants undergo normal fluid-phase endocytosis.** rvs161Δ mutants were
defective in fluid-phase and receptor-mediated endocytosis [2; 8]. To measure the rate of fluid-
phase endocytosis in hob3Δ mutants, the ability of cells to accumulate FM4-64 was quantified.
FM4-64 is a fluorescent lipophilic styryl dye which specifically accumulates in vacuolar
membranes of both budding and fission yeasts [42; 49]. When added to cells, FM4-64 initially
stained the plasma membrane (Fig. 6). Within 15 minutes, FM4-64 internalized at the cell ends
in presumed endocytic vesicles (Fig. 6). During the next 60 minutes, the number of FM4-64
staining structures decreased to 2-3 per cell and their size increased. Based on published data,
these final structures were vacuoles. A comparison of hob3+ and hob3Δ mutants at several times
did not reveal any detectable differences in the kinetics or morphology of the FM4-64 staining
structures. Based on these results, we conclude that fluid-phase endocytosis was normal in
hob3Δ mutants.

**F-actin localization defects in hob3Δ mutants are completely rescued by Bin3 and
partially rescued by RVS161.** The structural similarities between Hob3p, Bin3, and Rvs161p as
well as the functional similarities between rvs161Δ mutants and hob3Δ mutants, suggested that
Hob3p, Bin3, and Rvs161p share common functions. To test this hypothesis, we determined whether ectopic expression of Bin3 and Rvs161p could rescue the defects of hob3Δ mutants. For this purpose, plasmids were constructed where Hob3p, Bin3, Rvs161p, and Rvs167p were expressed using the thiamine-repressible nmt1 promoter of S. pombe [33]. These plasmids or a control plasmid were then introduced into hob3Δ mutants and the fraction of elongated cells and F-actin staining patterns were quantified (Table 2, Fig. 7). As expected, ectopic expression of hob3+ complemented the cell elongation and F-actin defects in hob3Δ cells whereas the control vector had no effect. Interestingly, Bin3 expression also corrected the cell elongation and F-actin defects of hob3Δ mutants while Rvs161 expression partially corrected the defects of hob3Δ mutants. In particular, hob3Δ mutant cells expressing Rvs161p were not elongated and contained easily detectable medial F-actin in mitotic cells. Rvs161p expression failed, however, to correct the mislocalization of F-actin patches in hob3Δ mutants. Rescue of hob3Δ mutants by Bin3 and Rvs161p was specific for these BAR adaptor proteins since Rvs167p expression did not correct the defects of hob3Δ mutants. We conclude that Bin3 and Rvs161p, but not Rvs167p, at least partially rescue the F-actin localization defects of hob3Δ mutants arguing that these proteins can perform similar functions.

**hob3+, but not Bin3, complements the osmotic sensitivity of S. cerevisiae rvs161Δ mutants.** Since both RVS161 and BIN3 complemented the F-actin localization defects of hob3Δ mutants, we tested whether expression of BAR-containing proteins could rescue a S. cerevisiae rvs161Δ mutant. Complementation was tested by the ability of a rvs161Δ strain to grow on synthetic dropout medium containing 6% NaCl. As expected, rvs161Δ cells lacking a plasmid or
containing a control plasmid failed to grow on media with 6% NaCl (Fig. 8). In contrast, rvs161Δ mutants expressing Rvs161p or Hob3p, but not Bin3 or Bin1, grew similarly to RVS161 cells (Fig 8). All strains which received a plasmid grew on synthetic dropout medium lacking 6% NaCl (data not shown). We conclude that, due to the lesser divergence between the Rvs161p and Hob3p proteins, Hob3p was able to complement the osmolarity defect of rvs161Δ null cells, but that the greater extent to which Rvs161p and Bin3 have diverged precluded complementation by Bin3.

Discussion

While the exact role of the N-terminal fold of the BAR family proteins is unknown, it is apparent that BAR family proteins are nonredundant in function. However, the BAR family may be subdivided based on structural considerations. Thus, a subset of BAR family members contain a C-terminal SH3 domain. In some cases, as for the Bin1-binding c-Abl oncoprotein, the protein partner responsible for interaction is known [19]. Another subset of BAR family members contain domains known to be involved in binding components of the endocytotic machinery and vesiculation [14; 22; 50]. Other domains, such as the c-Myc binding domain of Bin1 [17], are unique within the BAR family. The proteins described in the present study are characterized by a lack of identifiable functional domains outside of the BAR N-terminal fold. In combination with the greater homology exhibited between members of this subset and their ability to cross-complement, this suggests to us that they form a bona fide subfamily within the BAR family of proteins. The inability of other BAR-containing proteins to complement defects
in the expression of these proteins, even in the case of proteins native to the same organism, supports this notion\(^2\) [48]. While it is known that some members of the BAR family are able to interact, such as the yeast Rvs161p and Rvs167p proteins [7; 51], and amphiphysin and the brain isoform of Bin1 [22], the possibility of homo- or heterotypic interactions between other BAR family members remains to be determined.

As the case with Rvs161 in budding yeast, the phenotype caused by \(\text{hob3}^+\) deletion in fission yeast was linked to cytoskeletal actin regulation. The presence of multiple Calcofluor-reactive primary septa in \(\text{hob3}\Delta\) mutant cells coupled with the observation of an actin localization defect is reminiscent of the phenotypes exhibited by known cell separation and actin regulatory mutants of \(S.\) \(\text{pombe}\). Thus, inactivating mutations of the \(\text{sep2}^+, \text{sep12}^+, \text{spn1}^+\) and \(\text{rlc1}^+\) genes of \(S.\) \(\text{pombe}\) result in linear, multiseptated cells, in most cases with increased deposition of septal material [52-55]. \(\text{sep2}^+\) was identified in a screen for mutants with increased resistance to lysing enzymes, while \(\text{sep12}^+\) was isolated by application of the diploid enrichment screen of Chang \textit{et al} [56]. It is worth noting that a fraction of \(\text{sep2}\) cells contained double septa, which yielded two daughter cells and an anucleate minicell upon cleavage. Neither double septa nor anucleate cells were observed in cultures of \(\text{hob3}\Delta\). It was shown that cultures of \(\text{sep12}\) mutant cells contained 64% hyphae, a much larger percentage than the typical 10-15% observed in \(\text{hob3}\Delta\) cultures. In addition, \(\text{sep12}\) cells were sterile, while \(\text{hob3}\Delta\) mated with normal kinetics. \(\text{spn1}^+\) is a member of the \(S.\) \(\text{pombe}\) septin family of proteins. As such, it has a role in promoting septation of fission yeast. However, a role in actin patch movement has not been predicted. \(\text{rlc1}^+\) encodes a myosin regulatory light chain which associates with the yeast
Myo2p and Myo3p gene products. Although the morphology of \textit{hob3}\textsuperscript{Δ} cells closely resembled that of \textit{rlc1} mutant cells, the latter were found to be cold-sensitive for growth, a condition not seen in \textit{hob3}\textsuperscript{Δ} cultures. Interestingly, Rvs161p, Hob3p and Bin3 are homologous to unconventional myosins; Rvs161p is 25\% identical to Myo1p, the sole type II unconventional myosin of budding yeast; Hob3p is 20\% identical to Myo2p, one of two myosins in \textit{S. pombe}, and Bin3 is 24\% identical to human type VI unconventional myosin. BLAST analysis of the Bin3 protein assigns unconventional type VI myosins as the most highly homologous non-BAR polypeptides.

The mislocalization of F-actin patches by \textit{hob3}\textsuperscript{Δ} null cells has been previously observed in mutants of the Arp2/3 complex of \textit{S. pombe}, as well as in mutants of other, known actin-interacting proteins such as the products of the \textit{cdc3}\textsuperscript{+} and \textit{cdc8}\textsuperscript{+} genes, which encode profilin and tropomyosin, respectively [44; 45; 57; 58]. However, these mutants do not display the linear, multisepitated morphology characteristic of \textit{hob3}\textsuperscript{Δ} cells. In addition, loss of Cdc3p, Cdc8p or Arp3p function is lethal, whereas loss of Hob3p is not. Given that the major defect in these mutants is probably actin-related, and that perturbations of actin organization generally result in gross morphological defects throughout the cell cycle, it is not surprising that the \textit{hob3}\textsuperscript{+} gene is not essential, nor do \textit{hob3}\textsuperscript{Δ} cells exhibit the profound morphological abnormalities observed in more severe cases of loss of actin organization, such as in the \textit{cdc3} mutant [58]. Nevertheless, a defect in F-actin patch movement is apparent in \textit{hob3}\textsuperscript{Δ} cells. It remains to be determined whether Hob3p is directly associated with actin or with actin-binding proteins such as profilin, tropomyosin or the Arp2/3 complex.
We observed cross-species complementation of the hob3Δ F-actin defect by the budding yeast homolog Rvs161p, and by the mammalian homolog Bin3. A partial rescue of the F-actin localization defect by Rvs161p was observed insofar as the majority of dividing cells regained medial F-actin staining, but failed to correctly localize F-actin patches during interphase. On the other hand, Bin3 was able to rescue both loss of medial F-actin and localization of F-actin to cortical patches during interphase. It was noted that budding yeast Rvs167p, which is not a member of the subfamily of BAR proteins defined by Rvs161p, Hob3p and Bin3, was unable to rescue the F-actin defect of hob3Δ cells. An alternative explanation for the partial complementation observed with Rvs161p and the lack of complementation seen in the case of Rvs167p could be due to decreased steady-state levels of these proteins in S. pombe. However, we have confirmed the presence of either Bin1 or Bin3 polypeptides in hob3+ and hob3Δ S. pombe strains transformed with pREP2-based expression vectors. Furthermore, we were able to ascertain that the resulting Bin1 polypeptide failed to correct the F-actin defect observed in hob3Δ cells (data not shown). We thus favor the interpretation that Bin3 is the mammalian homolog of Rvs161p and Hob3p, but that there exists a degree of divergence in this gene during evolution. For example, Bin3 and Hob3p share important roles with Rvs161p in the control of the actin cytoskeleton, but only Rvs161p exhibits a role in endocytosis, and only Hob3p exhibits a role in cell division. Despite the lack of any role in cell division in budding yeast, RVS161 complemented the defects in this process caused by hob3Δ gene deletion as well as Bin3. In support of the notion of some evolutionary drift in the function of this gene during evolution, Bin3 was found to exhibit a unique localization in human cells to mitochondria and Golgi rather than to sites of actin polymerization as in the case of Rvs161p and Hob3p in budding and fission.
yeasts\textsuperscript{3}. Thus, a major finding of our study is that while Bin3 is clearly homologous to yeast Rvs161p and Hob3p at some levels, it is also clear that the function of this BAR adaptor protein has diverged to some extent and/or is being utilized differently in cells during evolution. Further insights into the exact mechanistic role of Bin3 in cell division processes will require studies in mouse cells in which the Bin3 gene has been targeted for homozygous deletion.
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Footnotes

1Abbreviations used are: EST, expressed sequence tag; PCR, polymerase chain reaction; YE, yeast extract medium; EMM2, Edinburgh minimal medium; EMM2-N, Edinburgh minimal medium without NH₄Cl; EMMG, Edinburgh minimal medium without NH₄Cl, with glutamate; YPAD, yeast complete medium with adenine; SC, synthetic complete yeast medium; DAPI, 4′,6′-diamidino-2-phenylindole.

2E.L.R., C.F.A. and G.C.P., manuscript in preparation.

3J.B. DuHadaway and G.C.P., unpublished observations

4The nucleotide sequences reported in this paper have been deposited in the GenBank® database under GenBank Accession Number AF2717232 (human BIN3 cDNA), AA418871 (human BIN3 EST), AAF76218 (human Bin3 protein), AF271733 (murine Bin3 cDNA), AF275638 (S. pombe hob3+ cDNA) and AAF86459 (S. pombe Hob3p). The sequence alignment in Fig. 1 included the budding yeast protein Rvs161p, available from the Swiss Protein Database as Swiss-Prot # 25343.

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Figure Legends

Figure 1. Bin3 and Hob3p are members of the BAR family. (a) Alignment of Bin3 with S. pombe Hob3p and S. cerevisiae Rvs161p. Identical residues in all sequences are contained in black boxes, while residues conserved between at least 50% of sequences are shown enclosed by gray boxes. (b) Cartoon depicting known BAR family proteins. The N-terminal BAR fold is shown in blue. Domains known to interact with c-Myc are in gray, while those implicated in endocytosis are in red. SH3 domains are depicted in yellow.

Figure 2. Distribution of mammalian BAR mRNAs in normal tissues and tumors. (a) Multiple tissue northern blots probed for Bin1 mRNA (top panel), Bin3 mRNA (middle panel), and amphiphysin mRNA (lower panel). (b) Bin3 mRNA expression in tumor cell lines (upper panel). The membrane was stripped and reprobed for β-tubulin mRNA as a loading control (lower panel). WM164 and WM1341D were derived from metastatic melanoma, while C33A, MS751, SiHa and HeLa were isolated from cervical carcinomas. A549 is a lung carcinoma line, while HepG2 originates from hepatocellular carcinoma. The C2C12 cell line is an undifferentiated mouse myoblast line.

Figure 3. Generation of a fission yeast strain harboring a deletion of the mammalian BIN3 homolog. (a) Southern blot of BamHI-digested genomic DNA from haploid hob3+ (right lane) and hob3Δ (left lane). (b) Morphology of the hob3Δ strain. Exponentially growing cells were stained with Calcofluor and DAPI. (c) Growth kinetics and viability of hob3Δ null cells. Graph
of cell density and viability versus time for exponential phase cells inoculated into YE medium. Cell density was determined by counting appropriate dilutions of cells with the aid of a hemocytometer. Viability was determined by a colony formation assay on solid YE medium. All measurements were obtained in triplicate.

**Figure 4. Deletion of hob3+ results in F-actin localization and septation defects.** Exponentially growing *S. pombe* cells were fixed and stained with AlexaFluor 488-phalloidin to visualize polymerized actin (top panels). DNA was stained with DAPI (bottom panels). *hob3*+ cells are depicted in the left panels, while *hob3*Δ cells are shown in the right panels. Hazy shading observed in some areas of the figures are due to the presence of cells in a different focal plane with respect to the majority of cells. Note that this effect is more pronounced in the *hob3*Δ strain due to the propensity of these cells to grow in clumps. *Insets*, smaller fields were magnified to highlight differences in F-actin staining. Arrows point to medial F-actin patches and rings. Asterisks indicate mislocalized F-actin patches.

**Figure 5. hob3Δ null cells do not display reduced viability upon starvation.** Effect of temperature and lack of nitrogen (EMMG) on growth on solid medium. Exponentially growing cells of each strain from liquid YE cultures were counted in triplicate. 1X10^4 to 1X10^1 cells of each strain were inoculated onto the indicated medium in tenfold dilutions from left to right, and the plates were allowed to grow at the indicated temperature until colonies formed (3-12 days).

**Figure 6. hob3Δ null cells lack gross endocytotic defects.** FM4-64 uptake assay of
exponentially growing cells. Cells were incubated at room temperature in the presence of FM4-64 and photographed at the indicated times.

**Figure 7. Ectopic expression of Bin3 and Rvs161p complements the F-actin defect of a \textit{hob3}\Delta null mutant.** A \textit{hob3}\Delta null strain was transformed with pREP2-based vectors expressing BAR family proteins. Exponentially growing transformants were stained with AlexaFluor 488-phalloidin to visualize polymerized actin. pREP2 is the empty expression vector. pREP2-\textit{hob3}+, pREP2-RVS161, pREP2-BIN3 and pREP2-RVS167 encode the \textit{hob3}+, RVS161, BIN3 and RVSD167 cDNAs, respectively. Contractile rings are indicated by arrows. Note delocalized cortical actin patches aligned along the length of the cells denoted by asterisks.

**Figure 8. The high salt intolerance of \textit{rvs161}\Delta can be complemented by ectopic expression of the fission yeast homolog \textit{hob3}+.** RVS161 strain BY4741 and \textit{rvs161}\Delta strain BY4741-3489 were transformed with the indicated plasmids. Transformants were streaked onto synthetic uracil dropout medium containing 6% NaCl and incubated at 30 °C for 7 days. Designations following YEp195-ACN indicate the identity of the encoded BAR protein.
Fig. 2

(a)

| Tissue          | Heart | Brain | Placenta | Lung | Liver | Kidney | Skeletal Muscle | Pancreas | Spleen | Thymus | Prostate | Testis | Ovary | Small Intestine | Colon | PBL |
|-----------------|-------|-------|----------|------|-------|--------|----------------|----------|--------|--------|----------|-------|-------|-----------------|-------|-----|
| Bin3            |       |       |          |      |       |        |                |          |        |        |          |       |       |                 |       |     |
|                 |       |       |          |      |       |        |                |          |        |        |          |       |       |                 |       |     |
| Bin1            |       |       |          |      |       |        |                |          |        |        |          |       |       |                 |       |     |
|                 |       |       |          |      |       |        |                |          |        |        |          |       |       |                 |       |     |
| Amphiphysin     |       |       |          |      |       |        |                |          |        |        |          |       |       |                 |       |     |

(b)

| Cell Line       | WM164 | WM1341D | C33A | MS751 | HeLa | SiHa | A549 | HepG2 | C2C12 |
|-----------------|-------|---------|------|-------|------|------|------|-------|-------|
| Bin3            |       |         |      |       |      |      |      |       |       |
| β-tubulin       |       |         |      |       |      |      |      |       |       |
Fig. 3

(a) $hob3^+\hspace{1cm}hob3\Delta$

(b) $hob3^+$ $hob3\Delta$

(c) Cell Density (cells/ml)

- % Viability $hob3^+$
- % Viability $hob3\Delta$
- Cell Density $hob3^+$
- Cell Density $hob3\Delta$

Time (hours)
Fig. 6

| Time  | hob3+ |          |          |          |          |
|-------|-------|----------|----------|----------|----------|
| 0 min | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| 15 min| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| 30 min| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| 45 min| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| 60 min| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |

| Time  | hob3Δ  |          |          |          |          |
|-------|--------|----------|----------|----------|----------|
| 0 min | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| 15 min| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| 30 min| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| 45 min| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| 60 min| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
Fig. 7

- $hob3\Delta/pREP2$
- $hob3\Delta/pREP2-hob3^+$
- $hob3\Delta/pREP2-RVS161$
- $hob3\Delta/pREP2-BIN3$
- $hob3\Delta/pREP2-RVS167$
Table 1. Relationships between known BAR-encoding polypeptides. The BAR domains of known BAR-containing polypeptides were aligned using the GCG program GAP [59] and the percentage of identical residues were calculated for each pairwise combination. Polypeptides were truncated to 253 N-terminal amino acids for comparison.

| % Similarity | Hob3p | Rvs161p | Bin3 | Bin1 | Rvs167p | Amphi1 | Bin2 |
|--------------|-------|---------|------|------|---------|--------|------|
| Hob3p        | -     | 56      | 29   | 24   | 31      | 27     | 26   |
| Rvs161p      | -     | 28      | 23   | 28   | 26      | 24     |      |
| Bin3         | -     | 27      | 20   | 22   | 24      |        |      |
| Bin1         | -     | 25      | 65   | 61   |         |        |      |
| Rvs167p      | -     | 25      | 29   |      |         |        |      |
| Amphi1       | -     | 53      |      |      |         |        |      |
| Bin2         | -     |         |      |      |         |        |      |
Table 2. Quantification of F-actin distribution in hob3Δ mutants expressing BAR-containing polypeptides. Cells were grown to exponential phase in the medium indicated, fixed, and stained with AlexaFluor 488-phalloidin and DAPI. Cells were counted and distributed according to their nuclear content and F-actin staining pattern. The number (n) of cells analyzed is indicated in parentheses. N/A, not applicable.
Human Bin3 complements the F-actin localization defects caused by loss of Hob3p, the fission yeast homolog of Rvs161p
Eric L. Routhier, Timothy C. Burn, Ilgar Abbaszade, Matthew Summers, Charles F. Albright and George C. Prendergast

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