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Interaction between Sec7p and Pik1p: The first clue for the regulation of a coincidence detection signal

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ABSTRACT

Sec7p, a guanine nucleotide exchange factor, regulates the activation of small Arf GTPases, which function in the formation of distinct classes of transport carriers from the Golgi. The recruitment of a subset of Arf effectors depends on the cooperation between these GTPases and phosphatidylinositol 4-phosphate. Here, we show that the catalytic domain of Sec7p interacts with a conserved region of the Golgi phosphatidylinositol 4-kinase Pik1p. We found that Sec7p and Pik1p as well as its product, colocalize at the late Golgi. Gea1p/Gea2p, an alternative pair of Arf activators, do not bind to Pik1p and function on a different Golgi sub-compartment. Sec7p and Pik1p interact with each other and cooperate in the formation of clathrin-coated vesicles. This interaction reveals a distinct role for Sec7p among the Golgi Arf-GEFs and provides a working model for the coordinated generation of Arf-GTP and phosphatiylinositol 4-phosphate as dual signal for specific recruitment of clathrin coats to the late Golgi.

Introduction

In all eukaryotes, compartmentalization of cellular function makes vesicular traffic an essential process that connects the different organelles with each other and supports the material flow between them. The Golgi apparatus is the central sorting station of this complicated network of transport pathways, and several different kinds of vesicles collaborate to support cargo transport from this organelle.

The activation of Arf GTPases at the Golgi promotes the formation of both clathrin and COPI-coated vesicles and is catalyzed by Arf guanine nucleotide exchange factors (Arf-GEFs) of the BIG/GBF subfamily (Jackson and Casanova, 2000). This conserved family of activators is characterized by a central Sec7 domain, which is both necessary and sufficient for the exchange activity. In yeast, two different Arfs and three Arf-GEFs have been identified at the Golgi. Interference with the function of either the essential BIG1/BIG2 homologue Sec7p or the closely related pair of GBF1 homologues Gea1p/Gea2p strongly impairs Golgi function and structure, resulting in aberrant glycosylation and delayed cargo delivery to the cell surface or vacuoles (Deitz et al., 2000; Peyroche et al., 2001; Spang et al., 2001). Despite their similar effect on transport, there are clear differences between the phenotypes of *sec7* and *gea* mutants. However the specific function of each of these proteins remains unclear.

In addition to Arf GTPases, the recruitment of clathrin adaptors is also regulated by phosphatidylinositol 4-phosphate (PI4P) (Krauss and Haucke, 2007). The Golgi PI4P pool is generated by Pik1p, a conserved type IIIβ PI4-kinase, whose activity is required for secretion and Golgi integrity (Audhya et al., 2000; Walch-Solimena and Novick, 1999). Phosphoinositides (PIs) control a broad range of cell functions and often collaborate with small GTPases to create a dual-key recognition signal for effector recruitment (Carlton and Cullen, 2005). However, the mechanisms regulating the simultaneous generation of two membrane-localization signals remain poorly understood.

In this study, we show that the yeast PI4-kinase Pik1p specifically interacts with Sec7p, one of the Golgi Arf-GEFs, and the two proteins share a common function in regulating the formation of clathrin-coated vesicles at the Golgi. We propose that the physical interaction of Pik1p and Sec7p coordinates Arf activation with PI4P production to generate a dual-key recognition system controlling the recruitment of specific effectors to the Golgi.

Materials and methods

Yeast strains, plasmids and growth conditions

Yeast strains and plasmids are listed in Tables 1 and 2, respectively. Genomic tagging was performed as described (Longtine

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Table 1
S. cerevisiae strain list.

Strain	Genotype	Source
NY10	MATα *ura3-52*	P. Novick
NY424	MATα *ura3-52 sec21-1*	P. Novick
NY737	MATα *ura3-52 leu2-3,112 sec23-1*	P. Novick
NY992	MATa *ura3-52 leu2-3,112 his3-Δ200*	P. Novick
NY1211	MATα *ura3-52 leu2-3,112 his3Δ200 GAL*$^+$	P. Novick
CSY712	MATα *ura3-52 leu2-3,112 pik1-101*	Ch. Walch-Solimena
CSY901	NY1211 *SEC7-DsRED-kanMX6*	L. Demmel
YGY84	NY10 *SEC7-GFP-kanMX6*	This study
YGY127	NY1211 *GEA2-GFP-HIS3MX6*	This study
YGY136	NY1211 *GEA1-GFP-HIS3MX6*	This study
YGY138	NY992 *GEA1-YFP-HIS3MX6*	This study
YGY139	NY992 *SEC7-YFP-HIS3MX6*	This study
YGY140	NY992 *GEA1-CFP-kanMX6*	This study
YGY141	NY992 *GEA2-YFP-HIS3MX6*	This study
YGY143	NY992 *GEA2-CFP-kanMX6*	This study
YGY152	MATα *ura3-52 leu2-3,112 his3Δ200 GEA1-CFP-kanMX6 GEA2-YFP-HIS3MX6*	This study
YGY162	MATα *ura3-52 leu2-3,112 his3Δ200 GEA1-CFP-kanMX6 SEC7-YFP-HIS3MX6*	This study
YGY163	MATα *ura3-52 leu2-3,112 his3Δ200 GEA2-CFP-kanMX6 SEC7-YFP-HIS3MX6*	This study
YGY166	MATα *ura3-52 leu2-3,112 his3Δ200 gea1-6 Δgea2::HIS3*	This study
YGY239	MATα *ura3-52 leu2-3,112 his3Δ200 pik1-101 CHC1-RFP-KanMX6*	This study
YGY240	MATa *ura3-52 leu2-3,112 his3Δ200 gea1-6 Δgea2::HIS3 CHC1-RFP-KanMX6*	This study
YGY247	MATα *ura3-52 leu2-3,112 trp1 his- sec7-4 SEC21-3xGFP*	This study
YGY248	MATα *ura3-52 leu2-3,112 trp1 (his- gea1-6 Δgea2::HIS3 SEC21-3xGFP*	This study
YGY250	MATα *ura3 leu2 his3- trp1-Δ901 lys2-801 sec7-4 CHC1-RFP-KanMX6*	This study
3100-20D	MATα *ura3-52 leu2-3,112 his3Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL*$^+$ *mel CHC1-RFP-KanMX6*	G. Payne
AFM69-1A	MATα *ura3-1 leu2 his3-11,15 sec7-4*	D. Gallwitz
BGY211	MATa *ura3-52 leu2-3,112 his4 trp1 rme1 HMLa SEC21-3xGFP*	B. Glick
YWZ361	MATa *ura3 leu2-3,112 his3-11,15 ade2-1 trp1-1 can1-100 CDC16-GFP-URA3*	W. Zachariae

Table 2
Plasmid list.

Construct	Vector	Insert	Source
pYG132	pGEX-6P-1	*GST-PIK1* aa 301–769	This study
pYG147	pGEX-6P-1	*GST-PIK1* aa 436–529	This study
pYG148	pGEX-6P-1	*GST-STT4* aa 1535–1625	This study
CSP8	pRS316	*PIK1* prom-*PIK1*	Ch. Walch-Solimena
pLD076	p426*ADH* prom	*hFAPP1-PH-YFP*	L. Demmel
pp1500-GP	pRS316	*PIK1* prom-*GFP-PIK1*	J. Thorner
pSKY5-RER1-0	pSKY5 (*CEN*, *URA3*)	*TDH3* prom-*GFP-RER1-CMK1* term	A. Nakano
pTL332	pRS406	*PHO5* prom-*GFP-PH*^{OSBP}	T. Levine

et al., 1998). Standard procedures were used for yeast cultures (Bonifacino, 2004; Bonifacino et al., 2001) and recombinant DNA manipulations (Sambrook and Russell, 2001). Temperature shifts were performed for 1 h prior to the experiment.

Live-cell microscopy

Early log phase cultures were observed with either a wide field fluorescent (Zeiss Axioplan 2 MOT) or a confocal (Zeiss Axioplan 2) microscope. All quantifications were done manually. For heat shock, the cells were incubated at 37 °C for 1 h.

Sequence alignments

PI4-kinase IIIα and IIIβ orthologues were assembled using reciprocal BLAST searches (Altschul et al., 1997) against the NCBI non-redundant protein database and aligned using ClustalW (Chenna et al., 2003) with manual refinement. The PI4-kinase similarity region as defined in Gehrmann and Heilmeyer (1998) was extracted from the multiple sequence alignment for display. For accession numbers and abbreviations see Table 3.

Immunoprecipitation

Yeast protein extracts were prepared from exponentially growing cells by glass bead lysis in 50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, protease inhibitors (Roche) and 0.5% Tween 20. The extracts were incubated for 2 h at 4 °C with 20 μl goat α-GFP antibody (3.5 mg/ml, MPI-CBG, Dresden) and 30 min with Protein

Table 3
Accession numbers and abbreviations.

	Species	Accession numbers
ScPik1	*Saccharomyces cerevisiae*	NP_014132
CaPik1	*Candida albicans*	XP_714748
SpPik1	*Schizosaccharomyces pombe*	CAA93903
SpurPI4Kβ	*Strongylocentrotus purpuratus*	XP_782151
DrPI4Kβ	*Danio rerio*	MP_00103149
HsPIK4β	*Homo sapiens*	NP_002642
XlPI4Kβ	*Xenopus laevis*	AAH73706
AmPI4Kβ	*Apis mellifera*	XP_391922
ScStt4	*Saccharomyces cerevisiae*	NP_013408
CaStt4	*Candida albicans*	XP_710429
SpStt4	*Schizosaccharomyces pombe*	CAB54814
HsPiKα	*Homo sapiens*	NP_477352
DrPiKα	*Danio rerio*	NP_001030144
XlPiKα	*Xenopus laevis*	AAH77604

G Sepharose (Pierce). After washing with lysis buffer containing 200 mM NaCl, Pik1p was detected with affinity purified rabbit α-Pik1 antibody (Walch-Solimena and Novick, 1999).

Pull-down assay

Recombinant proteins were produced in bacteria (BL21) as GST fusions. The expression of the constructs was induced overnight at 18 °C with 0.5 mM IPTG. Cells were lysed with a French press (Avestin Emulsiflex C5 high pressure homogenizer at 20,000 psi) in PBS, 1% Triton X-100 and protease inhibitors (Roche). The fusion proteins were immobilized on Glutathione Sepharose (Amersham) in buffer 1 (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT and protease inhibitors (Roche)). The beads were incubated for 2 h at 4 °C with yeast protein extracts prepared by glass bead lysis in buffer 1 and then washed with 20 mM Tris pH 7.5, 350 mM NaCl, 2 mM EDTA and 0.1% NP-40. Yeast protein extracts were prepared once for each strain and then distributed among the different GST samples. Samples were analyzed with mouse α-GFP antibody (Roche).

Subcellular fractionation

Yeast protein extracts were prepared from exponentially growing cells by glass bead lysis in 50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, protease inhibitors (Roche) and 1% NP-40. Lysates were cleared twice for 5 min at 800 × *g* and centrifuged at 100,000 × *g* for 45 min to generate the S100 and P100 fractions. The pellet fraction was resuspended in the original volume of lysis buffer and all samples were blotted with both α-ADH antibody (Chemicon International, Temecula, CA) and α-Chc1 antibody (from G. Payne).

Results

Pik1p specifically interacts with the Arf-GEF Sec7p in vitro

A yeast two-hybrid screen using full length Pik1p as bait identified Gea2p as potential interactor. The Gea2p fragment isolated in the screen corresponds to the N-terminal part of the highly conserved Sec7 domain of the GEF, and further yeast two-hybrid analysis showed that Pik1p interacts with the corresponding region (aa 819–942 of Sec7p) of the three yeast Golgi Arf-GEFs (data not shown). The interaction domain of Pik1p was mapped to aa 301–769 and contains the PI4-kinase β similarity region which, according to the literature, is conserved between the type IIIβ PI4-kinases only (Nakagawa et al., 1996). However, our sequence alignments (Fig. 1A) indicate that this domain is present in both the types IIIα and IIIβ PI4-kinase families and will therefore be referred to as PI4-kinase similarity domain. Although the PI4-kinase simi-

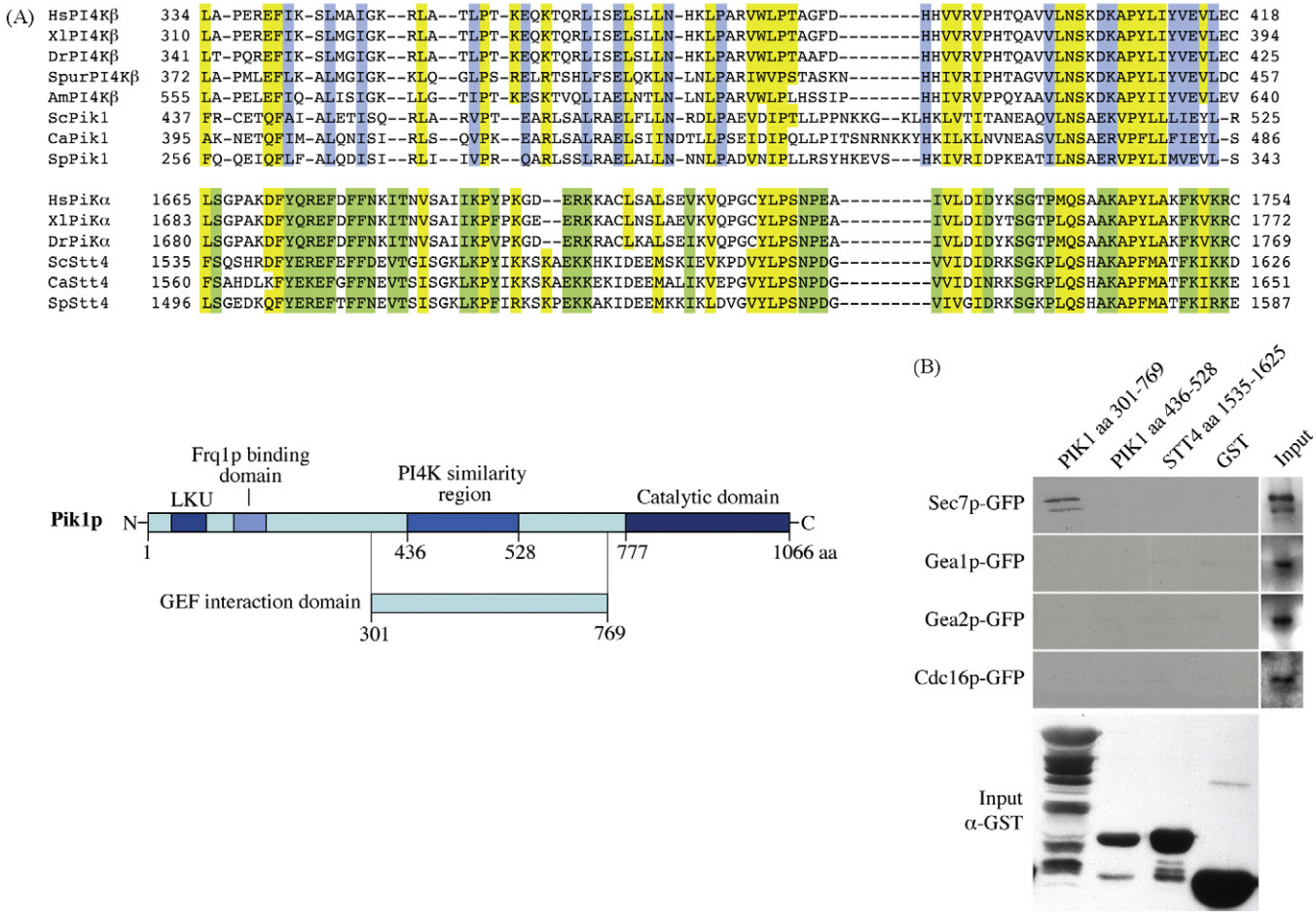


Fig. 1. Pik1p specifically interacts with the Arf-GEF Sec7p. (A) Yeast two-hybrid interaction domain of Pik1p and alignments of the PI4-kinase similarity region of the PI4-kinase III α and β proteins. Residues conserved among both families are highlighted in yellow, those conserved among either of the two subfamilies, in blue and green, respectively. The borders of the PI4 kinase similarity region are shown. (B) Pik1p interacts preferentially with Sec7p *in vitro*. The GST fusion proteins of Pik1p yeast two-hybrid interaction domain (Pik1 aa 301–769), the PI4K similarity region of Pik1p (aa 436–528) and Stt4p (aa 1535–1625) were immobilized on Glutathione sepharose columns and incubated with yeast lysate prepared from Sec7p-GFP, Gea1p-GFP, Gea2p-GFP or Cdc16p-GFP (negative control) strains. Samples were analyzed with α-GFP antibody. The second band in the Sec7-GFP sample as well as the fragments in the GST-Pik1p aa 301–769 purification are degradation products due to the instability of those proteins.

larity domain of the *A. thaliana* homologue of Pik1p interacts with a small Rab GTPase (Preuss et al., 2006), the function of this domain has not yet been elucidated.

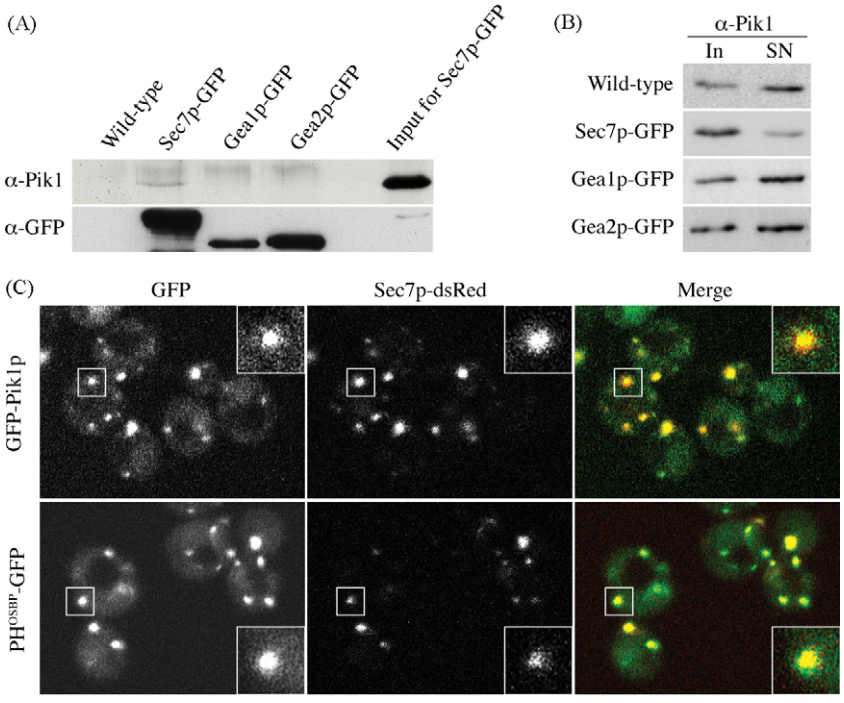
The relevance of the yeast two-hybrid data was then tested using *in vitro* pull-down experiments. For this, GST-Pik1p was incubated with yeast cytosol from cells expressing either one of the GFP-tagged Golgi Arf-GEFs. Since full-length Pik1p is not expressed in *E. coli*, we used the smallest fragment interacting in the yeast two-hybrid assay (aa 301–769). We found that this Pik1p fragment preferentially binds to Sec7-GFP (Fig. 1B). In parallel, we also tested whether the PI4-kinase similarity domain of Pik1p (aa 436–528) or of the type IIIα PI4-kinase Stt4p (aa 1535–1625) are sufficient for interaction. However, this does not seem to be the case in this assay (Fig. 1B).

Sec7p is the physiological interaction partner of Pik1p

To evaluate the physiological relevance of the interaction, we performed immunoprecipitation experiments with strains containing endogenously tagged GEF-GFP. Since the relatively low levels of endogenous Pik1p were insufficient to detect an interaction, this protein was overexpressed. The increase of Pik1p levels had no discernable effect on growth or the localization of the GEFs, as determined by live microscopy (data not shown). The results confirmed that Sec7p is the main physiological binding partner of Pik1p also *in vivo* (Fig. 2A). Under our experimental conditions, about half of the Pik1p (54%) remained bound to Sec7p-GFP after precipitation while no decrease in the amount of Pik1p was observed in the supernatant from either Gea1p-GFP or Gea2p-GFP samples (Fig. 2B). In addition, stronger exposure of the same blots showed no enrichment of Pik1p in the immunoprecipitates of Gea1p-GFP or Gea2p-GFP compared to the negative control (data not shown), indicating that a potential interaction between the Gea's and Pik1p is below the detection limit of our experiment. Hence our result shows that Sec7p is the main Arf-GEF partner of Pik1p.

Since Sec7p and Pik1p physically interact *in vivo*, we investigated their relative subcellular localization by live-cell microscopy. For this, we used endogenously tagged Sec7-dsRed strains and a plasmid encoded *GFP-PIK1* allele (Strahl et al., 2005). Our results (Fig. 2C upper panel, 2D) show that GFP-Pik1p is found on 79 ± 8% of all Sec7-DsRed labeled-structures and as much as 97 ± 3% of the GFP-Pik1p positive compartments also contain Sec7-DsRed (n = 288 cells). Hence, the two proteins colocalize extensively, supporting the biochemical data and suggesting that Pik1p and Sec7p function together *in vivo*.

The Golgi PI4P pool can be specifically detected using a GFP tagged pleckstrin homology (PH) domain construct derived from the mammalian OSBP (Levine and Munro, 2002). We used this construct to determine the activity of Pik1p at Sec7p containing compartments. The colocalization between Sec7-DsRed and PH^{OSBP}-GFP (Fig. 2C lower panel and D) shows that 94 ± 4% of the

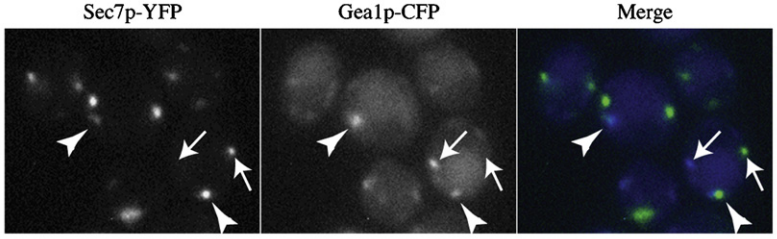


(D)

Strains	Total cells	Colocalization (%)	
		DsRed	GFP
Sec7p-DsRed GFP-Pik1p	288	79 ± 8	97 ± 3
Sec7p-DsRed PH^{OSBP}-GFP	129	94 ± 4	80 ± 7

Fig. 2. Sec7p is the *in vivo* interaction partner of Pik1p. (A) Pik1p specifically interacts with Sec7p *in vivo*. Endogenously tagged Sec7p-GFP, Gea1p-GFP, Gea2p-GFP strains containing 2μ*PIK1*, were precipitated with α-GFP antibodies and the presence of Pik1p detected with α-Pik1 antibody. (B) Amount of Pik1p in solution before and after immunoprecipitation. In = input, SN = supernatant. (C) The endogenously tagged *SEC7*-DsRed strains containing either GFP-Pik1p or PH^{OSBP}-GFP were observed by confocal microscopy. (D) Quantification of colocalization results expressed as % of dots exhibiting colocalization (±standard deviation).

(A)



(B)

GEF pairs	Total cells	Colocalization (%)	
		CFP	YFP
Sec7p-YFP Gea1p-CFP	338	19 ± 8	20 ± 10
Sec7p-YFP Gea2p-CFP	395	11 ± 2	17 ± 4
Gea1p-CFP Gea2p-YFP	301	62 ± 10	49 ± 9

(C)

GEF	Total cells	Average dots / cell
Sec7p	733	3.28 ± 0.87
Gea1p	639	3.38 ± 0.74
Gea2p	696	4.26 ± 0.88

(D)

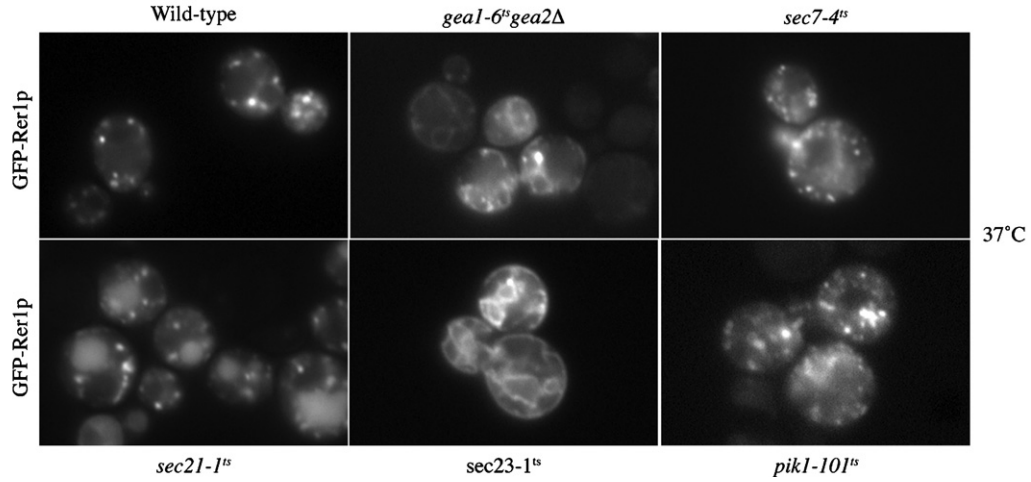


Fig. 3. Sec7p and Pik1p act at a different Golgi subcompartment than Gea1p and Gea2p. The colocalization between the Arf-GEFs was examined by live-cell microscopy using a combination of CFP/YFP tags introduced at the genomic locus. (A) Example of colocalization between Sec7p-YFP and Gea1p-CFP. Arrows = single labeled structures, arrowhead = double labeled compartment. (B) Quantification results expressed as % of dots exhibiting colocalization of labeled proteins (±standard deviation). (C) Average number of dots per cell (± standard deviation). (D) GFP-Rer1p accumulates in the ER of *gea1/gea2* but not *sec7* or *pik1* mutants. GFP-Rer1p was observed by live microscopy in wild-type, *sec7-4*^{ts}, *gea1-6*^{ts} Δ*gea2*, *pik1-101*^{ts}, *sec21-1*^{ts} and *sec23-1*^{ts} strains after 1 h at 37 °C.

Sec7-DsRed labeled compartments, and 80 ± 7% of the PH^{OSBP}-GFP ones colocalize with each other (n = 129) and confirms that PI4P production occurs on the same Sec7p-containing Golgi membranes.

Sec7p and Pik1p act on different Golgi sub-compartments than Gea1p/Gea2p

Studies in mammals suggest that Golgi Arf-GEFs of the GBF/BIG subfamilies localize to different sub-compartments and promote the recruitment of specific Arf effectors (Manolea et al., 2007; Shinotsuka et al., 2002; Zhao et al., 2002). In yeast, very few studies directly compare the function of Sec7p, Gea1p and Gea2p and although all three have been individually localized to the Golgi (Franzusoff et al., 1991; Peyroche et al., 2001; Spang et al., 2001), the available data are not sufficient to estimate the spatial overlap of these proteins *in vivo*. Therefore, individual GEFs where C-terminally tagged with CFP or YFP and their respective localization studied by live microscopy. To avoid artifacts due to overexpression, the GEFs were tagged at their genomic locus.

Analysis of over 300 cells per GEF pair indicates that while Gea1p and Gea2p do overlap to quite some extend, either colocalizes poorly with Sec7p (Fig. 3A–C). Despite their redundancy, Gea1p and Gea2p can be found on different compartments suggesting partially independent roles (Fig. 3B). In addition, we observe more Gea2p containing structures per cell than either Sec7p or Gea1p ones (P < 0.0001), and while the same amount of Sec7p-structures associates with Gea1p and Gea2p, a smaller proportion of Gea2p labeled compartments show Sec7p colocalization (Fig. 3B and C). Therefore, in addition to demonstrating a quite distinct localization pattern for Sec7p, our data argue against a model where Gea1p and Gea2p act consecutively and reveal the presence of a distinct Gea2p pool. This specific distribution of BIG and GBF a family member agrees well with localization studies in mammals (Zhao et al., 2002).

Direct colocalization studies between Pik1p or its product with either Gea1p or Gea2p was not possible due to the low expression levels of our constructs. To overcome this problem without disturbing the dynamic equilibrium of the Golgi by modifying

the expression levels of key regulatory enzymes of this compartment, we used a highly expressed exogenous PH^{FAPP}-YFP construct. However, while this construct could readily be visualized together with each Arf-GEF allowing us to quantify their respective overlap, it clearly affected Golgi morphology (reminding of the effect of *pik1*^{ts} mutants at permissive temperature). Despite this problem, the colocalization between this PH^{FAPP}-YFP construct and Sec7-DsRed (83 ± 5%) (Supplementary Fig. 1A and C) remains in the same range as the lower expressed PH^{OSBP}-GFP construct used in Fig. 2 (80 ± 7%), allowing us to get at least an indication of the overlap between the different GEFs with PI4P. As expected from our previous results, PH^{FAPP}-YFP colocalizes much better with Sec7-DsRed than with Gea1p-CFP (8 ± 3%) or Gea2p-GFP (16 ± 7%), confirming that most of the Sec7p and PI4P are located on different compartments than Gea1p and Gea2p (Supplementary Fig. 1B and C).

The differential localization of the Arf-GEFs suggests that these proteins could act at different Golgi domains, while the Pik1p-Sec7p interaction implies that these two factors might be required for a common transport step. We therefore investigated the transport phenotypes of several mutants, using a GFP-Rer1p reporter protein. In wild-type cells, GFP-Rer1p cycles between the ER and the cis-Golgi, and localizes to the Golgi at steady state. Disrupting either anterograde (*sec23-1*^{ts}) or retrograde (*sec21-1*^{ts}) transport between ER and Golgi results in accumulation of the reporter in the ER or the vacuole, respectively (Sato et al., 2001). For this and the following experiments, we selected the *sec7-4*^{ts} and *pik1-101*^{ts} alleles, two mutations that directly affect the catalytic activity of each protein and result in a tight secretion block upon shift to non-permissive temperature (Deitz et al., 2000; Walch-Solimena and Novick, 1999). For the Gea's, *gea1-6*^{ts}Δ*gea2* was preferred over *gea1-4*^{ts}Δ*gea2* mutants which, like *sec7-4*^{ts} carries mutation inside the Sec7 domain but is a relatively poor temperature sensitive allele (Peyroche et al., 2001). Note that due to their functional overlap the use of a *gea1/gea2* double mutant is compulsory and our results reflect the overall loss of Gea activity and do not allow us to discriminate between their individual roles. As expected from previous studies (Park et al., 2005), *gea1-6*^{ts}Δ*gea2* mutants accumulate this reporter in the ER. Loss of function of either *sec7* or *pik1* result in the accumulation of the marker in small structures spread throughout the cell (Fig. 3D). The lack of ER or vacuolar staining in these two mutants suggests that loss in Golgi integrity rather than a direct effect on Rer1p cycling causes the observed staining pattern. In addition, we could confirm that both *pik1-101*^{ts} and *sec7-4*^{ts} mutants exhibit delayed delivery of the endocytic dye FM4-64 to the vacuole while *gea1-6*^{ts}Δ*gea2* mutants do not directly influence the endosomal system (Peyroche et al., 2001; Walch-Solimena and Novick, 1999). Taken together, these results suggest that Gea1p and Gea2p function at a different Golgi compartment and affect an earlier transport step than Sec7p and Pik1p. The minor overlap between the two kinds of compartments suggested by our

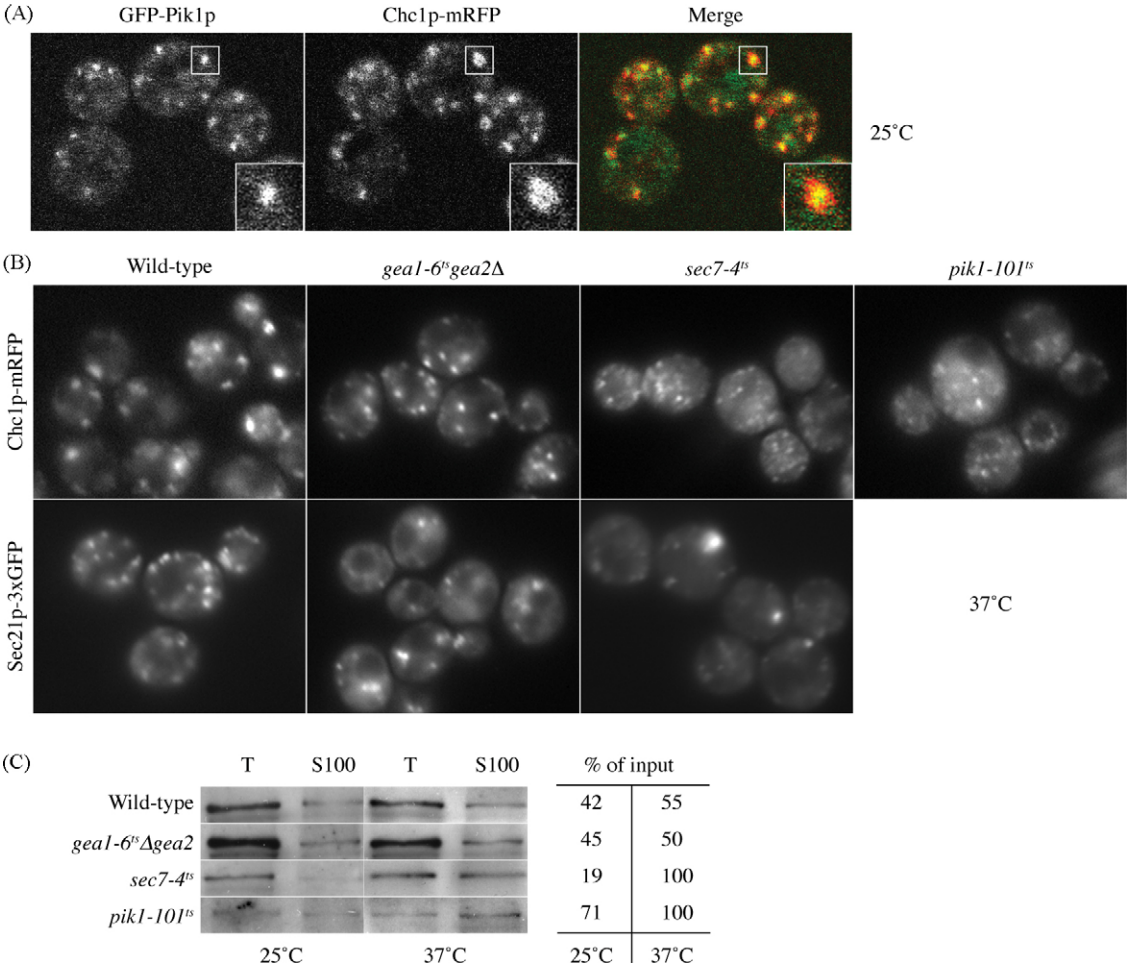


Fig. 4. Pik1p and Sec7p control clathrin recruitment. (A) Pik1p colocalizes with Chc1p. Endogenously tagged CHC1-mRFP strain containing GFP-PIK1 was observed by confocal microscopy. (B) Clathrin and COPI recruitment. Strains containing either endogenous CHC1-mRFP or SEC21-3xGFP were combined with *pik1-101*^{ts}, *sec7-4*^{ts} and *gea1-6*^{ts}Δ*gea2* mutations and observed by live microscopy after 1 h at 37 °C. (C) Membrane distribution of clathrin is affected in *sec7* and *pik1* mutants. Comparison of Chc1p-RFP in wild-type, *gea1-6*^{ts}Δ*gea2*, *sec7-4*^{ts} and *pik1-101*^{ts} mutants, before and after 1 h at 37 °C. T = Total input, S100 = supernatant after 100,000 × *g* fractionation. The table shows the percentage of the total clathrin recovered in the S100 fraction.

experiments could result from the maturation of yeast Golgi compartments and reflect the evolution from one stage to the next by the progressive replacement of proteins. Alternatively, both the Arf-GEFs and Pik1p participate in different processes simultaneously and small fractions of these enzymes could be located differentially and reflect a pool involved in a different activity.

Pik1p and Sec7p collaborate in clathrin recruitment to the Golgi

The interaction between Sec7p and Pik1p as well as the comparison between the localization and mutant phenotype of the three Arf-GEFs, suggest that these proteins might activate different pools of Arf at the Golgi. Since both Arf-GTP and PI4P are known to contribute to clathrin adaptor recruitment to the Golgi (Bonifacino, 2004; Carlton and Cullen, 2005; Demmel et al., 2008; Wang et al., 2007), we investigated the subcellular localization of clathrin heavy chain, Chc1p, with respect to Pik1p/Sec7p containing compartments. We also used the *sec7*, *gea1/gea2* an *pik1* mutants to compare the distribution of Chc1p and Sec21p, a subunit of COPI coats, which are Arf-GTP effectors but do not depend on PI4P for Golgi recruitment (D'Souza-Schorey and Chavrier, 2006).

By live-cell microscopy, Pik1p and Chc1p are found at the same compartments (Fig. 4A). The slight shift in the labeling of the two proteins can be explained by the association of Pik1p with Golgi membranes as opposed to the majority of clathrin being concentrated on small vesicles derived from this organelle.

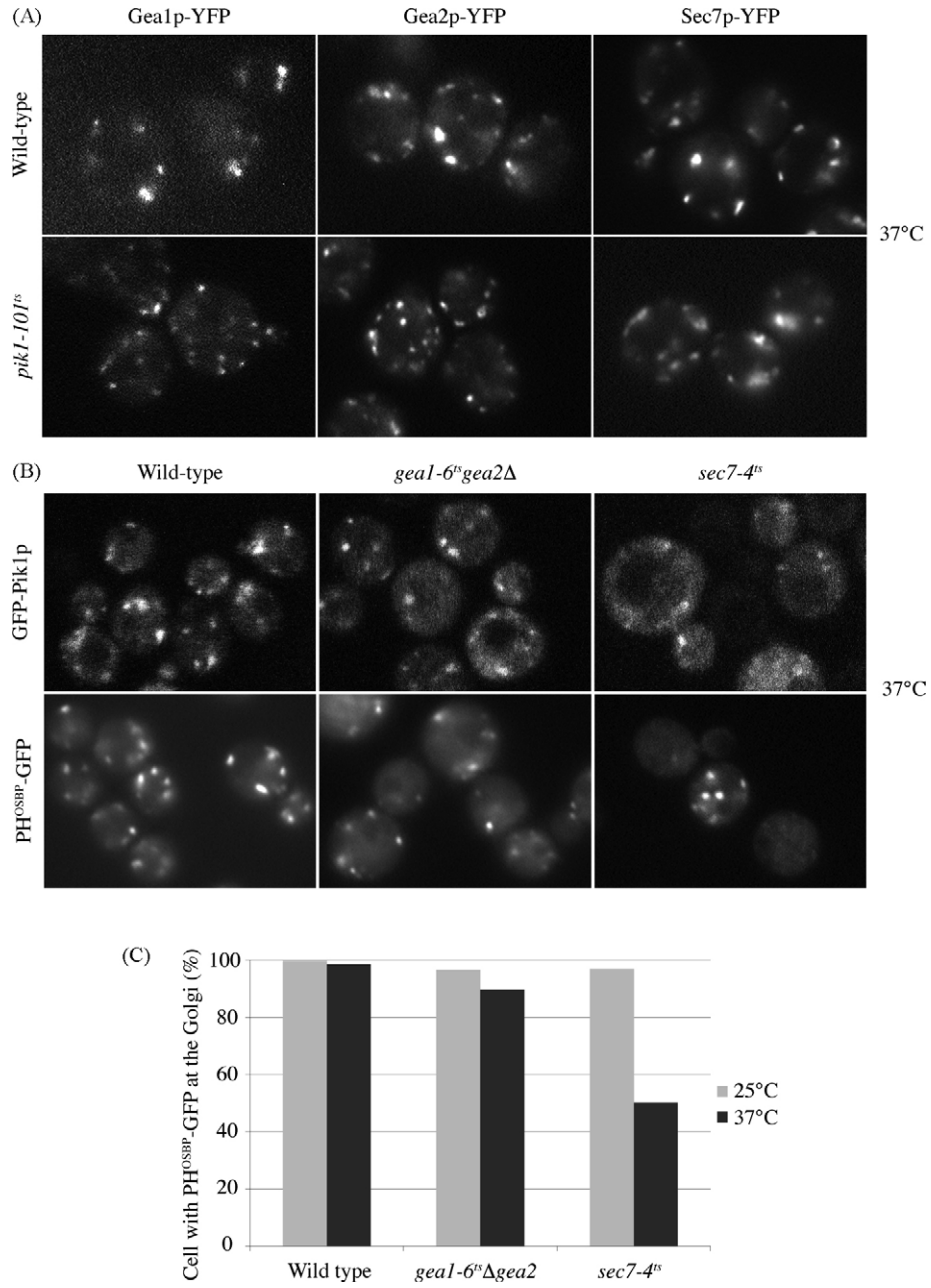


Fig. 5. Interdependence of Pik1p and Sec7p activities. (A) Pik1p function is not required for membrane localization of the Arf-GEFs. Sec7p-YFP, Gea1p-YFP and Gea2p-YFP were crossed into *pik1-101*^{ts} and observed by live microscopy after 1 h at 37°C. (B + C) Pik1p and PH^{OSBF}-GFP are still recruited to membranes in *gef* mutants. (B) Wild-type, *sec7-4*^{ts} and *gea1-6*^{ts}Δ*gea2* containing either GFP-Pik1p or the PH^{OSBP}-GFP reporter construct were observed by confocal microscopy. C. Percentages of cells with membrane bound PH^{OSBF}-GFP after 1 h at 37 °C.

When examining clathrin distribution in the different mutants by microscopy, we observe that its membrane association seems more affected in *sec7-4^{ts}* or *pik1-101^{ts}* than in *gea1-6^{ts}*Δ*gea2* mutants (Fig. 4B, upper panel). As expected from previous studies showing a direct interaction between Gea1p and Sec21p (Deng et al., 2009), we observe an increase in the cytosolic staining of Sec21p-3xGFP in *gea1-6^{ts}*Δ*gea2* mutants indicating that membrane recruitment of COPI coats is more affected by these mutations than by loss of Sec7p function (Fig. 4B, lower panel). The effect of *pik1* mutation on the membrane distribution of COPI coat remains unsolved, since despite our efforts, we were unable to create the required strain. The effect of *sec7-4^{ts}* and *pik1-101^{ts}* on membrane recruitment of clathrin is further confirmed by fractionation experiments showing a strong increase of the soluble pool of Chc1p-RFP in these two mutants compared to wild-type and *gea1-6^{ts}*Δ*gea2* mutants (Fig. 4C). These observations demonstrate a specific role for Sec7p in the recruitment of clathrin.

Pik1p and Sec7p influence each other directly

Previous studies addressing the cross-talk between Arf GTPases and PI-kinases have mainly focused on the direct or indirect influence of activated Arf on PI metabolism (e.g. PI4K IIIβ recruitment by ARF1 (Godi et al., 1999), PIP5Ks activation by ARF (Krauss and Haucke, 2007)) or modulation of the Arf cycle by PIPs (e.g. PH-domain mediated Arf-GEF localization (Casanova, 2007)). These studies have resulted in models implying that one component of the dual-key signal (GTP-bound Arf or PIPs) is directly responsible for the local amplification of the signal by acting on the upstream activator of its co-signal (PI-kinase or Arf-GEF). To know whether the Pik1p-Sec7p interaction serves a similar amplification mechanism or if those two activators regulate each other directly, we next considered the effect of loss of function mutants on the localization and enzymatic activity of these proteins.

We did not observe any reduction in membrane association of the Arf-GEFs in *pik1-101^{ts}* mutants (Fig. 5A) and although GFP-Pik1p is slightly more cytosolic in *sec7-4^{ts}* than *gea1-6^{ts}*Δ*gea2* mutants or wild-type cells, its ability to interact with membranes is not abolished (Fig. 5B, upper panel). Thus, PI4P and Arf1-GTP do not act as membrane receptors for Sec7p or Pik1p, respectively. We also evaluated Pik1p activity by using the same Golgi specific PH-domain reporter as for the colocalization studies (Fig. 2C and D). The membrane localization of this reporter in ∼50% of the *sec7-4^{ts}* cells indicates that Pik1p does not strictly require functional Sec7p for activity (Fig. 5B, lower panel and C). However, compared to *gea* mutants, the striking localization defect in the other half of *sec7-4^{ts}* mutants suggests a role of Sec7p in PI4P effector recruitment to the Golgi whether through a direct effect on Pik1p activity or through maintenance of compartment integrity. Note that the Golgi morphology defect of *sec7-4^{ts}* mutants alone cannot explain this phenotype since loss of function of the *gea1-6^{ts}*Δ*gea2* under our conditions strongly impair Golgi structure (Peyroche et al., 2001) and should therefore result in a similar phenotype.

The binding of Pik1p to the N-terminal half of the catalytic domain of Sec7p suggested by the yeast two-hybrid data, places this protein in an ideal position to positively or negatively control Arf activation. Unfortunately, the impact of Pik1p on the GEF activity of Sec7p cannot be directly tested *in vivo* due to the presence of Gea1p and Gea2p at the Golgi. Several attempts to establish an *in vitro* GEF activity assay failed since both Pik1p and Sec7p are too big for full-length expression in *E. coli* and our difficulty to stably reproduce the interaction data with truncated recombinant proteins. We also tried to create a dominant negative construct by using the Pik1p fragment corresponding to the positive yeast two-hybrid interaction domain (aa 301–769). However, overexpression of this construct *in vivo* did not interfere with the Sec7p-Pik1p interaction.

Discussion

The generation of transport vesicles from the Golgi apparatus is a dynamic process that requires tight coordination between both spatial and temporal signals (Carlton and Cullen, 2005). The association of the Arf-GEF Sec7p with the PI4-kinase Pik1p presented here directly couples Arf activation with PI4P production and highlights two new aspects of Arf-GEF function: (i) localized generation of an activated Arf pool and (ii) coordination of the generation of a dual-key signal for selective effector recruitment.

The comparison between the three Golgi Arf-GEFs together with the Sec7-Pik1p interaction demonstrates a particular role for Sec7p in the activation of a specific pool of Arf. Although previous studies in mammalian cells already suggested a functional specialization of different Arf-GEF families at the Golgi (Manolea et al., 2007; Shinotsuka et al., 2002; Zhao et al., 2006), the molecular mechanisms underlying this specificity remained unclear. Based on our results, we now propose that different members of the BIG/GBF Arf-GEF family control the timely activation of Arf by interacting with specific factors at the Golgi and thereby ensure the proper membrane composition required for efficient recruitment of distinct Arf effectors. This model places Arf-GEFs at the heart of the spatial and temporal regulation of vesicle formation at the Golgi and suggests a previously unrecognized impact of nucleotide exchange factors on regulation of the multiple functions of their target GTPase.

We propose that the physical interaction between Sec7p and Pik1p might serve to coordinate the timely and spatially restricted activation of a specific pool of Arf working together with PI4P providing the core machinery for generation of the dual-key signal pair Arf-GTP and PI4P at the late Golgi. This signal is then required for clathrin recruitment, in agreement with previous data showing the double Arf/PI4P dependency of AP1 and GGA localization to the TGN (Bonifacino, 2004; Carlton and Cullen, 2005; Demmel et al., 2008; Wang et al., 2007). The novelty of our study lies in the identification of a direct connection between the two key upstream activators of this dual-key signal. Although further work will be required to solve the exact molecular mechanism of this interaction, our results provide new insight in the regulation of coincidence detection signaling. Interestingly, the specific involvement of BIGs in the membrane recruitment of clathrin adaptors (Manolea et al., 2007; Shinotsuka et al., 2002) might suggest that a similar interaction could control vesicle formation at the mammalian TGN.

The physical interaction between Sec7p and Pik1p provides the first clue toward the regulation of the simultaneous production of two distinct membrane recruitment signals. Although many examples for coincidence detection signaling have been described (Carlton and Cullen, 2005), the underlying mechanisms controlling this process are not well understood. The idea that the regulation of phosphoinositide turnover could be intimately linked to the Arf activation cycle through a large family of clearly specialized Arf activators now opens the possibility to test whether similar mechanisms exist for GTPase/PI dual-key signals in other trafficking steps and signaling pathways. The high conservation of the domains involved in Sec7p-Pik1p binding suggests that this type of interaction might also occur between other GEFs and PI kinases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejcb.2010.02.004.

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